

**Translational regulation of plant arginine decarboxylases
by an ancient cis element and development of tools to study
its role in bacterial wilt disease.**

Dissertation

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Erin Samantha Ritchie
aus Christchurch, Neuseeland

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1. Berichterstatter:	Prof. Dr. Thomas Lahaye
2. Berichterstatter:	Prof. Dr. Ulrike Zentgraf

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Tübingen, den 03.06.2024

Erin Samantha Ritchie

*Science and technology are the keys to both our longevity and our demise.
Our entire existence on this planet is a double-edged sword.*
~ Rhys Darby, New Zealand comedian ~

Always blow on the pie.
~ Peter and Graham Ritchie ~

Abstract

Polyamines (PAs) are small cationic metabolites, essential for eukaryotic organisms and many prokaryotes. In plants, PAs are important for growth, development, gene expression, and abiotic and biotic stress responses, and their levels are finetuned through transcriptional and translational regulation of PA synthesis and catabolic enzymes (Chen et al., 2019). Synthesis of the most abundant PAs in plants, putrescine, spermidine, and spermine, predominantly starts with decarboxylation of arginine to agmatine by arginine decarboxylase (ADC, typically encoded by two genes, *ADC1* and *ADC2*). Interestingly, all examined land plant *ADC* transcripts contain a highly conserved GC-rich ~50 bp sequence in their 5' untranslated region (5'UTRs), termed the “*ADC-box*” (Wu et al., 2019). Using transient reporter assays, the *ADC-box* was found to inhibit translation of the downstream coding sequence, possibly by forming an RNA secondary structure that prevents ribosomal scanning. In addition to the *ADC-box*, *ADC* 5'UTRs contain a small uORF ~150 bp 5' to the *ADC-box*; however, no clear role for this uORF was found (Wu et al., 2019).

This work aimed to determine if the *ADC-box* and uORF regulate ADC translation *in planta* and investigate how the *ADC-box* translational regulatory mechanism functions. CRISPR/Cas9 mutagenesis of *ADC-boxes* in tomato, *Arabidopsis thaliana*, and *Marchantia polymorpha* (relative of the first land plants) was conducted. As a proxy for measuring *in planta* ADC protein levels, a previously established liquid chromatography-mass spectrometry (LCMS)-based ADC activity assay (Wu et al., 2019) was further optimised, improving sensitivity ~600-fold and number of measured PA-related metabolites. Increased ADC activity and levels of some PA-related metabolites were found in tomato, *A. thaliana*, and *M. polymorpha* *adc-box* mutants, demonstrating that the *ADC-box* is a repressor of ADC translation *in planta* and that its role has likely been conserved throughout land plant evolution. The tomato *ADC-box* RNA secondary structures were experimentally and computationally determined using SHAPE analysis, finding a triple hairpin *ADC-box* structure. Through analysing ADC activity levels across *adc-box* mutants, it seems that the central hairpin, which is conserved across vascular and early non-vascular land plants, is essential for *ADC-box* functionality. CRISPR/Cas9 mutagenesis of the tomato *ADC* uORFs resulted in reduced ADC activity compared to wildtype (WT) and a shorter plant phenotype with fewer leaves and flowers, suggesting that this uORF not only positively regulates ADC translation but is also essential for plant development. Overall, these findings suggest that plant ADC translation is both negatively and positively regulated by two highly conserved 5'UTR elements that finetune ADC protein levels and maintain PA homeostasis under various conditions.

In addition to influencing PA levels, ADC activity also correlated with levels of phenolamides, compounds made from PA conjugation to hydroxycinnamic acids (HCAs), which are both antimicrobial compounds and substrates for the formation of physical barriers in roots (i.e., lignin and suberin). Interestingly, the broad-host bacterial pathogen *Ralstonia solanacearum*, which infects plants via their roots, injects a conserved effector protein (RipTAL) into host cells to transcriptionally activate plant *ADC* genes and boost PA levels (Wu et al., 2019). Through this work, we hypothesise that *R. solanacearum* uses RipTALs to manipulate host phenolamide levels to either alter the plant microbiome or production of physical barriers in the roots. In brief, through expanding our capabilities of PA measurement by LCMS and our understanding of how ADCs influence the PA network, new experimental approaches and hypotheses as to how *R. solanacearum* benefits from a RipTAL-induced PA boost have been developed.

Zusammenfassung

Polyamine (PAs) sind kleine kationische Stoffwechselprodukte, die für pro- und eukaryontische Organismen von wesentlicher Bedeutung sind. In Pflanzen sind sie wichtig für Wachstum, Entwicklung, Genexpression und abiotische und biotische Stressreaktionen (Chen et al., 2019). Der PA-Gehalt wird durch die transkriptionelle und translationale Regulierung der PA-Synthese und der katabolen Enzyme fein abgestimmt. Die Synthese der in Pflanzen am häufigsten vorkommenden PAs, Putrescin, Spermidin und Spermin, beginnt in erster Linie mit der Decarboxylierung von Arginin zu Agmatin durch Arginin-Decarboxylase (ADC, typischerweise kodiert von zwei Genen, *ADC1* und *ADC2*). Interessanterweise wurde eine hochkonservierte GC-reiche ~50 bp Sequenz in der 5' untranslatierten Region (5'UTR) aller untersuchten *ADC*-Transkripte von Landpflanzen entdeckt, die als „*ADC-Box*“ bezeichnet wird (Wu et al., 2019). Mithilfe von transienten Reporter-Assays wurde festgestellt, dass die *ADC-Box* die Translation der stromabwärts gelegenen kodierenden Sequenz hemmt, möglicherweise durch Bildung einer RNA-Sekundärstruktur, die das ribosomale Scanning verhindert. Zusätzlich zur *ADC-Box* enthalten *ADC* 5'UTRs einen kleinen uORF ~150 bp 5' zur *ADC-Box*; es wurde jedoch keine klare Rolle für diesen uORF gefunden (Wu et al., 2019).

Mit dieser Arbeit sollte festgestellt werden, ob die *ADC-Box* und der uORF die *ADC*-Translation in Pflanzen regulieren und wie der *ADC-Box*-Translationsregulationsmechanismus funktioniert. Es wurde eine CRISPR/Cas9-Mutagenese der *ADC-Boxen* in Tomate, *Arabidopsis thaliana* und *Marchantia polymorpha* (Verwandte der ersten Landpflanzen) durchgeführt. Als Proxy für die Messung der *ADC*-Proteinspiegel in Pflanzen wurde ein zuvor etablierter Flüssigchromatographie-Massenspektrometrie (LCMS)-basierter *ADC*-Aktivitätsassay (Wu et al., 2019) weiter optimiert, wodurch die Empfindlichkeit um das 600-fache und die Anzahl der gemessenen PA-bezogenen Metaboliten verbessert wurde. In den *adc-box*-Mutanten von Tomate, *A. thaliana* und *M. polymorpha* wurden eine erhöhte *ADC*-Aktivität und ein erhöhter Gehalt an einigen PA-verwandten Metaboliten festgestellt, was zeigt, dass die *ADC-Box* ein Repressor der *ADC*-Translation in Pflanzen ist und dass ihre Rolle wahrscheinlich während der gesamten Evolution der Landpflanzen konserviert wurde. Die Sekundärstrukturen der *ADC-Box*-RNA der Tomate wurden experimentell und rechnerisch mit Hilfe der SHAPE-Analyse bestimmt, wobei eine dreifache *ADC-Box*-Haarnadelstruktur gefunden wurde. Die Analyse der *ADC*-Aktivität in verschiedenen *adc-box*-Mutanten ergab, dass die zentrale Haarnadel, die in vaskulären und frühen nicht-vaskulären Landpflanzen konserviert ist, für die Funktionalität der *ADC-Box* wesentlich ist. Die CRISPR/Cas9-Mutagenese des *ADC* uORFs der Tomate führte zu einer verringerten *ADC*-Aktivität im

Vergleich zum Wildtyp (WT) und einem kürzeren Pflanzenphänotyp mit weniger Blättern und Blüten, was darauf hindeutet, dass dieser uORF nicht nur die ADC-Translation positiv reguliert, sondern auch für die Pflanzenentwicklung wesentlich ist. Insgesamt deuten diese Ergebnisse darauf hin, dass die pflanzliche ADC-Translation durch zwei hoch konservierte 5'UTR-Elemente sowohl negativ als auch positiv reguliert wird, um die ADC-Proteinspiegel fein abzustimmen und die PA-Homöostase unter verschiedenen Bedingungen aufrechtzuerhalten.

Neben der Beeinflussung des PA-Gehalts korrelierte die ADC-Aktivität auch mit dem Gehalt an Phenolamiden, Verbindungen, die aus der Konjugation von PA zu Hydroxyzimtsäuren (HCA) entstehen, die sowohl antimikrobielle Verbindungen als auch Substrate für die Bildung physikalischer Barrieren in Wurzeln (d. h. Lignin und Suberin) sind. Interessanterweise injiziert das bakterielle Pathogen *Ralstonia solanacearum*, das Pflanzen über ihre Wurzeln infiziert, ein konserviertes Effektorprotein (RipTAL) in die Wirtszellen, um pflanzliche *ADC* Genen transkriptionell zu aktivieren und den PA-Gehalt zu erhöhen (Wu et al., 2019). Durch diese Arbeit stellen wir die Hypothese auf, dass *R. solanacearum* RipTALs verwendet, um den Phenolamid-Spiegel des Wirts zu manipulieren, um entweder das Pflanzenmikrobiom oder die Produktion von physischen Barrieren in den Wurzeln zu verändern. Kurz gesagt, durch die Erweiterung unserer Möglichkeiten der PA-Messung mittels LCMS und unseres Verständnisses, wie ADCs das PA-Netzwerk beeinflussen, wurden neue experimentelle Ansätze und Hypothesen darüber entwickelt, wie *R. solanacearum* von einem RipTAL-induzierten PA-Boost profitiert.

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List of publications:

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Manipulation of plant metabolism by pathogen effectors: more than just food.

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List of abbreviations

<i>35Sp</i>	Cauliflower mosaic virus 35S promoter
<i>5'UTR</i>	5' untranslated region
<i>85-10</i>	Xanthomonas euvesicatoria strain 85-10
α	Arbitrary threshold of significance
<i>AAD</i>	Acidic activation domain
<i>ABA</i>	Abscissic acid
<i>ADC</i>	Arginine decarboxylase
<i>ANOVA</i>	Analysis of variance
<i>At</i>	Arabidopsis thaliana
<i>ATG</i>	Start codon
<i>bp</i>	Base pair
<i>BSA</i>	Bovine serum albumin
<i>cDNA</i>	Complementary DNA
<i>CDS</i>	Coding sequence
<i>CFP</i>	Cerulean fluorescent protein
<i>cfu</i>	Colony forming units
<i>CO₂</i>	Carbon dioxide
<i>CRISPR</i>	Clustered Regulatory Interspersed Short Palindromic Repeats
<i>Cas9</i>	CRISPR associated protein 9
<i>DAO</i>	diamine oxidase
<i>DLR</i>	Dual luciferase reaction
<i>DNA</i>	Deoxyribose nucleic acid
<i>dNTPs</i>	Deoxyribonucleic acid triphosphates
<i>dpi</i>	Days post infection
<i>dTALE</i>	Designer Transcription Activator Like Effector
<i>EBE</i>	Effector binding element
<i>EDTA</i>	Ethylenediaminetetraacetic acid
<i>eEF</i>	Eukaryotic translation elongation factor
<i>eIF</i>	Eukaryotic translation initiation factor
<i>EtOH</i>	Ethanol
<i>EV</i>	Empty vector
<i>FLAG</i>	Epitope tag with a peptide sequence of DYKDDDDK
<i>FW</i>	Fresh weight (of tissue)
<i>GABA</i>	γ -aminobutyric acid
<i>GCN2</i>	General Control Nonderepressible 2 kinase
<i>gDNA</i>	Genomic DNA
<i>GC1-4</i>	GC-rich regions in ADC-box

<i>GFP</i>	Green florescent protein
<i>GG</i>	Golden gate
<i>gRNA</i>	Guide RNA
<i>GUS</i>	β-glucuronidase
<i>H₂O</i>	Water
<i>H₂O₂</i>	Hydrogen peroxide
<i>HA</i>	Human influenza hemagglutinin (amino acids 98-106)
<i>HCA</i>	hydroxycinnamic acid
<i>HCT</i>	N-hydroxycinnamoyl transferase
<i>HFBA</i>	Heptafluorobutyric acid
<i>His</i>	Histidine
<i>hpi</i>	Hours post infection
<i>HR</i>	Hypersensitive response
<i>HRP</i>	Horse radish peroxidase
<i>INDEL</i>	Insertion or deletion
<i>IRE</i>	Iron response element
<i>IRES</i>	Internal ribosome entry site
<i>LB</i>	Luria-Bertani media
<i>LC</i>	Liquid chromatography
<i>LCMS</i>	Liquid chromatography coupled to tandem mass spectrometry
<i>LDC</i>	Lysine decarboxylase
<i>LjUbip</i>	Lotus japonicus ubiquitin promoter
<i>LTi6b</i>	Low temperature induced protein 6b
<i>LUC</i>	Firefly luciferase
<i>KO</i>	Knock out
<i>MAS</i>	Mannopine synthase
<i>MES</i>	2-Morpholinoethanesulphonic acid
<i>MM</i>	Moneymaker tomato variety
<i>mORF</i>	Main open reading frame
<i>Mp</i>	Marchantia polymorpha
<i>MRM</i>	Multiple reaction monitoring
<i>MS</i>	Mass spectrometry
<i>MeOH</i>	Methanol
<i>mRNA</i>	Messenger RNA
<i>myc</i>	Epitope tag derived from c-myc
<i>m/z</i>	Mass to charge ratio
<i>n</i>	Sample size
<i>N₂</i>	Liquid nitrogen
<i>NAI</i>	2-methylnicotinic acid
<i>NATA</i>	N-acetyltransferase activity

<i>NLS</i>	Nuclear localization signal
<i>NOS</i>	Nopaline synthase
<i>NYG</i>	Nutrient yeast glycerol media
<i>OD₆₀₀</i>	Optical density measured at a wavelength of 600 nm
<i>ODC</i>	Ornithine decarboxylase
<i>ORF</i>	Open reading frame
<i>P-value</i>	Probability value
<i>P5P</i>	Pyridoxyl-5 phosphate
<i>PA</i>	Polyamine
<i>PAM</i>	Protospacer adjacent motif
<i>PAO</i>	Polyamine oxidase
<i>PBCV1-DC</i>	Paramecium bursaria chlorella virus-1 (PBCV-1) decarboxylase
<i>PCR</i>	Polymerase chain reaction
<i>pGrDL_SPb</i>	pGreenII dual luciferase vector from Moyle et al., 2017
<i>pGrDL_SPb (GG)</i>	Golden gate compatible pGrDL_SPb plasmid
<i>PI</i>	Propidium iodide
<i>PIC</i>	Preinitiation complex
<i>Q1-4</i>	Quadropoles 1-4
<i>qPCR</i>	Quantitative polymerase chain reaction
<i>REN</i>	Renilla luciferase
<i>RFP</i>	Red fluorescent protein
<i>RGQ</i>	RNA G quadruplex
<i>RipTAL</i>	Ralstonia injected protein with similarity to TAL effectors
<i>RNA</i>	Ribonucleic acid
<i>ROS</i>	Reactive oxygen species
<i>RPL18b</i>	Ribosomal protein L18b
<i>Rpm</i>	Revolutions per minute
<i>Rssc</i>	Ralstonia solanacearum species complex
<i>RT</i>	Room temperature
<i>RT-qPCR</i>	Reverse transcription quantitative polymerase chain reaction
<i>RVD</i>	Repeat variable diresidue
<i>S/N</i>	Signal to noise ratio
<i>SAMDC</i>	S-adenosylmethionine decarboxylase (also known as AdoMetDC)
<i>SDS</i>	Sodium dodecyl-sulfate
<i>SDS-PAGE</i>	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
<i>SHAPE</i>	selective 2'-hydroxyl acylation analysed by primer extension
<i>SNP</i>	Single nucleotide polymorphism
<i>Sl</i>	Solanum lycopersicum
<i>T-DNA</i>	Transfer DNA
<i>T2A</i>	Thosea asigna viral protein 2A

<i>T3SS</i>	Type 3 secretion system
<i>TALE</i>	Transcription activator like effector
<i>TAK1</i>	Wild type male Marchantia polymorpha
<i>TF</i>	Transcription factor
<i>THT</i>	Tyramine N-hydroxycinnamoyl transferase
<i>TRAP</i>	Tandem ribosome affinity purification
<i>uORF</i>	Upstream open reading frame
<i>WT</i>	Wild type
<i>YEB</i>	Yeast extract beef media
°C	Degrees Celsius
<i>g</i>	gravity
<i>V</i>	Volts
<i>Hz</i>	Hertz
<i>UV</i>	Ultraviolet
<i>v/v</i>	Volume per volume
<i>w/v</i>	Weight per volume
<i>g</i>	grams
<i>mg</i>	Miligram
<i>kDa</i>	Kilodalton
<i>M</i>	Molar
<i>mM</i>	Millimolar
<i>nM</i>	Nanomolar
<i>μM</i>	Micromolar
<i>cm</i>	Centimeter
<i>mm</i>	Millimeter
<i>L</i>	Liter
<i>mL</i>	Milliliter
<i>μL</i>	Microliter

1. Introduction

Plants were one of the first multicellular forms of life to conquer land, as barren and unforgiving as it was ~500 million years ago (Rensing, 2018). From this point onwards, plants have been exposed to very unpredictable and changing environments and, as they are unable to move to more favourable conditions, have evolved many methods to defend themselves and thrive. All animals, including humans, now totally rely on plants and their ability to provide sustenance. Yet, in the face of the climate change crisis, plants, particularly our crops, must withstand even more unpredictable environmental changes to survive and maintain yields (Seppelt et al., 2022). To improve crop resilience and therein food security in the future, development of durable lines, resistant to multiple stresses is essential (Kopec, 2024). To generate such lines, understanding of how plants detect stress, activate responses to cope with it, and how these responses are regulated is imperative.

One ancient mechanism that plants have used for mitigating stress since their arrival on land is through increasing production of secondary metabolites to protect cellular components and alleviate the stressor (Rensing, 2018). Polyamines (PAs) are one group of protective metabolites that have increased synthesis in response to both biotic and abiotic stress and can mitigate these stresses through a variety of mechanisms (Alcazar et al., 2010a; Mustafavi et al., 2018; Chen et al., 2019; Gonzalez et al., 2021). Understanding how metabolites such as PAs can protect plants in the face of multiple stresses may help to generate more resilient crops for the future.

In this work, an ancient regulatory mechanism that controls expression levels of a key PA-synthesising enzyme, arginine decarboxylase (ADC) is investigated, alongside its impact on the wider PA network. Although plants typically induce PA production to resist pathogen attack, *Ralstonia solanacearum*, the causal agent of the devastating plant bacterial wilt disease, injects an effector protein into host cells to boost their PA production. Therefore, to understand this seemingly counterintuitive action by *R. solanacearum*, and to better understand the role that PAs have in progression of bacterial wilt disease, various experimental approaches were developed in this work.

1.1 Polyamines are essential metabolites that have a multitude of cellular functions in plants.

PAs are found ubiquitously in all living organisms and are essential for eukaryotic life (Michael, 2016). They are low molecular weight metabolites that are positively charged at physiological pH and contain at least two amino groups (Kusano et al., 2008). In plants, the most abundant PAs are putrescine, spermidine, and spermine, which are predominantly found in free forms, but are also commonly bound to other molecules (Chen et al., 2019). PAs can be covalently bound via their positive charges to negatively charged macromolecules such as nucleic acids, proteins, lipids, and cell wall components, or conjugated to hydroxycinnamic acids (HCAs) to form phenolamides (also known as phenylamides or hydroxycinnamic acid amides [HCAAs]). Due to their cationic binding capabilities and abundance, PAs have a plethora of cellular interactors and functions. Through genetic and metabolic studies, PAs have been implicated in many processes such as gene expression (i.e., transcription and translation; Childs et al., 2003; Dever and Ivanov, 2018; Poidevin et al., 2019; Jimenez-Bremont et al., 2022), development and growth (i.e., embryogenesis and organogenesis; Chen et al., 2019), flower and fruit development (Applewhite et al., 2000; Anwar et al., 2019; Gao et al., 2021), senescence (Sobieszczuk-Nowicka, 2017), abiotic stress responses (i.e., drought, heat, salt, and cold and freezing tolerances; Alcazar et al., 2010a; Chen et al., 2019), and responses to biotic stresses (i.e., bacterial, fungal, and viral; Gonzalez et al., 2021). In addition to these benefits for the plants themselves, humans also require PAs. Although we can synthesise PAs, dietary intake of PAs, including from plant-based sources, provides antioxidant and other health promoting benefits, demonstrating the importance of PAs in crops for human consumption (Muñoz-Esparza et al., 2019).

Due to their many roles and interactions, it is challenging to determine the exact mode of action PAs have in each of these aforementioned processes as they most likely operate simultaneously on many different levels (Michael, 2016; Chen et al., 2019). PAs bind nucleic acids, influencing chromatin packing, DNA and RNA conformation and stability, gene expression, and translation (Childs et al., 2003; Dever and Ivanov, 2018). Yet PAs are also considered regulators of redox homeostasis as they protect cellular entities by increasing antioxidant enzyme activity, scavenge reactive oxygen species (ROS), and stabilising membranes during periods of oxidative stress (Chen et al., 2019). While on the other hand, during pathogen attack, PA catabolism is induced, resulting in increased ROS production, contributing to an oxidative burst that reduces pathogen proliferation (Gonzalez et al., 2021). PAs can also inhibit ion

channels, helping to regulate levels of calcium and ions during stresses to limit their damaging effects on cells (Napieraj et al., 2023). Additionally, PA levels and expression of PA synthesising enzymes have also been correlated with changes in levels of various phytohormones, growth regulators, and signalling molecules, such as auxin, cytokinin, jasmonates, gibberellic acid, abscisic acid (ABA), ethylene, salicylic acid, and nitric oxide, further confounding their direct and indirect effects (Chen et al., 2019; Napieraj et al., 2023; Tyagi et al., 2023). Covalently bound or conjugated PAs have additional molecular interactors and roles, further diversifying the impacts and functions of PAs in plants. And finally, differential localisation, regulation, and synthesis of PAs and their conjugated forms means that their roles may change depending on the developmental time, tissue, environment, and stress status of the plant (Chen et al., 2019; Tyagi et al., 2023). Therefore, although PAs are rather small and abundant metabolites they have many complex roles.

1.2 Polyamine synthesis and catabolism in plants.

In plants, there are two main reported pathways of PA synthesis, starting with either arginine or ornithine (Chen et al., 2019). The main PA synthesis pathway in plants uses arginine to generate agmatine via arginine decarboxylase (ADC) enzymes; ADC represents an essential point in PA synthesis in plants as without this enzyme, insufficient PA levels result in no flower and/or seed production (Figure 1; Urano et al., 2005; Alcazar et al., 2010a; Winter et al., 2015; Alcazar and Tiburcio, 2018; Chen et al., 2019). Plant ADCs belong to group IV pyridoxal 5-phosphate (P5P)-dependent decarboxylases (Sandmeier et al., 1994) and typically exist as two paralogues (i.e., ADC1 and ADC2; Maruri-Lopez and Jimenez-Bremont, 2017). ADCs form head-to-tail homo- and hetero-dimers, with two PLP co-factor and substrate binding sites between the dimer interfaces (Hanfrey et al., 2001). After decarboxylation by ADC, agmatine is then converted into *N*-carbamoyl putrescine, which is further hydrolysed into putrescine by *N*-carbamoyl putrescine amidohydrolase (AIH). Putrescine acts as a central point in the PA synthesis pathway (Mattoo et al., 2015; Gonzalez-Hernandez et al., 2022a), as it can either be further metabolised into spermidine and then spermine, or used to generate various spermidine derivatives, GABA, H₂O₂, or other secondary metabolites such as alkaloids (Tiburcio and Galston, 1986; Docimo et al., 2012; Gonzalez et al., 2021). Spermidine and spermine are produced by addition of decarboxylated S-adenosylmethionine, which is derived from methionine and also used for ethylene synthesis.

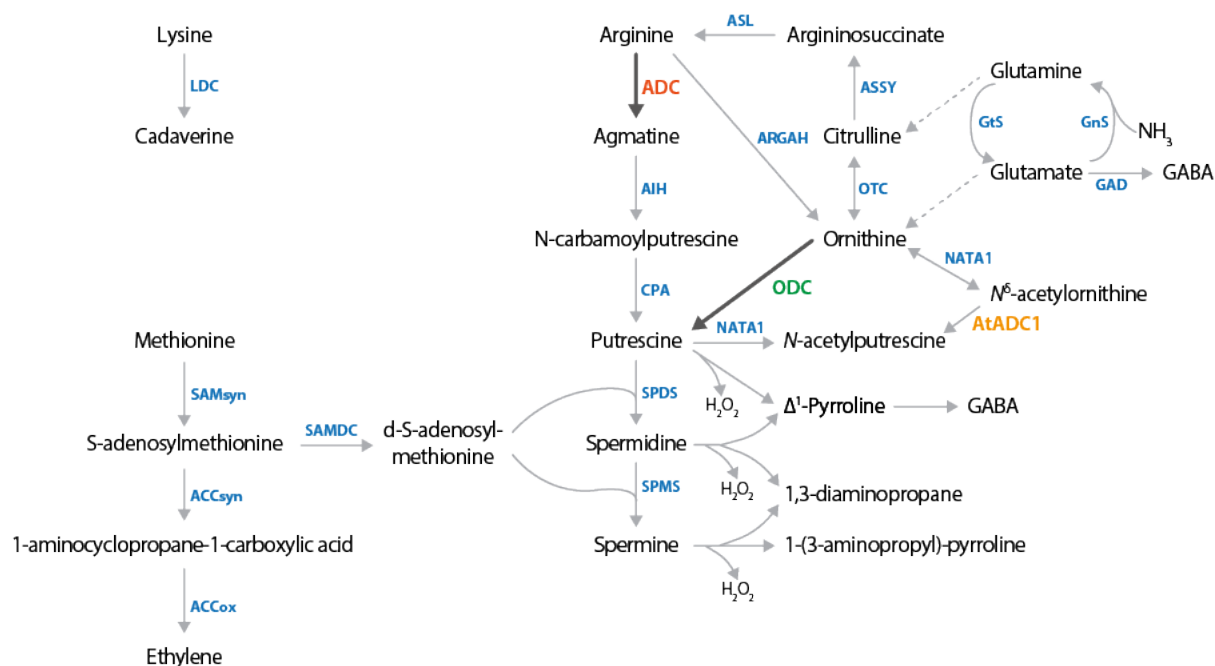


Figure 1: Plant polyamine (PA) metabolic pathways and enzymes.

The main PAs in higher plants are putrescine, spermidine, and spermine, which are catalysed from arginine and ornithine via the arginine decarboxylase (ADC)- and ornithine decarboxylase (ODC)-dependent pathways (bolded arrows). Putrescine is converted to the higher PAs through addition of dc-AdoMet, which is derived from methionine and also used for ethylene synthesis. Cadaverine is another PA catalysed from lysine via a separate pathway. ADC (orange); *A. thaliana* ADC1 (light orange), representing its neofunctionalised role as an *N*^δ-acetylornithine decarboxylase. All other enzyme abbreviations are shown in blue: glutamine synthase (GnS); glutamate synthase (GtS); glutamate decarboxylase (GAD); ornithine-carbamoyl transferase (OTC); arginosuccinate synthase (ASSY); arginosuccinate lyase (ASL); arginase (ARGAH); ornithine decarboxylase (ODC; green); *N*-acetyltransferase activity 1 (NATA1); S-adenosylmethionine synthase (SAMsyn); 1-aminocyclopropane-1-carboxylate synthase (ACCsyn); 1-aminocyclopropane-1-carboxylate oxidase (ACCoX); S-adenosylmethionine decarboxylase (SAMDC, also called AdoMetDC); and, lysine decarboxylase (LDC).

The second pathway is dependent on ornithine, which is directly decarboxylated into putrescine by ornithine decarboxylase (ODC; Winter et al., 2015; Alcazar and Tiburcio, 2018; Chen et al., 2019) and is also a member of the group IV P5P-dependent decarboxylases (Sandmeier et al., 1994). Interestingly, many Brassicaceae species, including the common model plant *Arabidopsis thaliana* have lost their *ODC*, resulting in total dependence on ADC for PA production (Hanfrey et al., 2001). To compensate for this loss, *A. thaliana* ADC1 has neofunctionalised into an *N*^δ-acetylornithine decarboxylase to convert *N*^δ-acetylornithine to acetylputrescine, thereby linking ornithine to putrescine production without an ODC (Lou et al., 2020). A third PA synthesis pathway has also been reported in sesame, which converts citrulline to putrescine via citrulline decarboxylase (CDC), yet this has not been observed in other plants (Chen et al., 2019).

Another PA, cadaverine, is produced via decarboxylation of lysine by lysine decarboxylase (LDC), and while its roles in plants have not been as well characterised as the other PAs, it still contributes to plant development and stress responses (Jancewicz et al., 2016). Interestingly, many LDC and ODC enzymes are dual functioning and can either produce cadaverine or putrescine from lysine or ornithine, respectively (Bunsupa et al., 2012).

Catabolism of putrescine and cadaverine is conducted by diamine oxidase (DAO) enzymes, which produce H_2O_2 and NH_3 in the process (Moschou et al., 2008). Subsequent catabolism leads to production of GABA (γ -aminobutyrate), a signalling molecule important for development and during plant stress responses (Shelp et al., 2012; Sebastia et al., 2021). Spermidine and spermine are catabolised by polyamine oxidase (PAO) enzymes, also producing H_2O_2 in the process (Moschou et al., 2008). Putrescine, spermidine, and spermine can also be acetylated by *N*-acetyltransferase activity (NATA) enzymes to fine tune the available PA pool and regulate H_2O_2 production during periods of stress (Lou et al., 2016; Shahul Hammed et al., 2024 *bioRxiv*).

As mentioned above, agmatine, putrescine, spermidine, and spermine can all be conjugated to different hydroxycinnamic acids (HCAs) to generate various phenolamides (Figure 2; Bassard et al., 2010; Roumani et al., 2021; Xu et al., 2023). This conjugation is catalysed by *N*-hydroxycinnamoyl transferase (HCT) enzymes, which are part of the BAHD acetyltransferase super family (Xu et al., 2023). Phenolamides have diverse roles in plants and are commonly

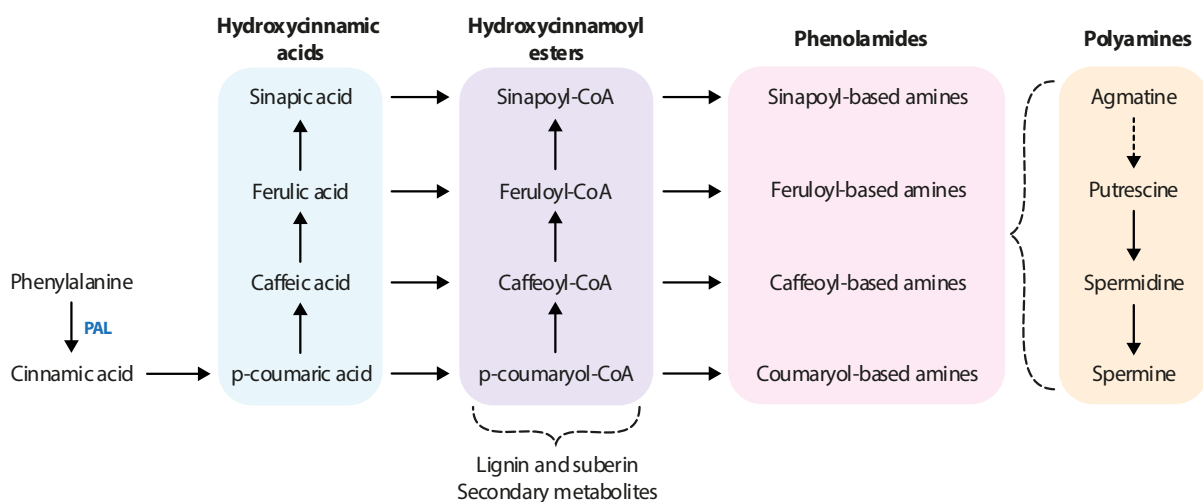


Figure 2: Phenolamide synthesis in plants.

The PAL pathway, named after the enzyme phenylalanine ammonia-lyase (PAL), is responsible for synthesis of phenylpropanoids (i.e., HCA derivatives). Hydroxycinnamic acids (HCAs; blue) can have CoA moieties added by lyases to form hydroxycinnamoyl-CoA esters (purple); these are substrates for synthesis of lignin and suberin, and secondary metabolites. Additionally, they can be conjugated to polyamines (yellow) by *N*-hydroxycinnamoyl transferases (HCT) to form phenolamides (pink).

found to play protective roles against abiotic and biotic stresses. Various enzymes or transcription factors producing or regulating phenolamide levels have been implicated in resistance against bacterial, viral, fungal, and insect pathogens in a variety of different plants as either antimicrobial compounds or by reinforcing the secondary cell wall (Bassard et al., 2010; Roumani et al., 2021). HCAs are also required for synthesis of lignin and suberin, important protective cell wall polymers, as well as other secondary metabolites such as flavanols and anthocyanins.

1.3 Regulation of polyamines in plants.

As PAs have so many influential roles in plants, their pools must be tightly controlled through regulation of enzymes involved in their synthesis and catabolism. Overall, their levels are maintained based on cellular needs by fine tuning their synthesis, catabolism, conjugation, and transport, enabling rapid and dynamic changes to PA pathway flux and their abundance. As this thesis focuses on plant ADCs, the following sections highlight their regulation transcriptionally and translationally, yet many components of the PA pathway are regulated by similar means to adjust PA levels.

1.3.1 *Transcriptional regulation of PA-related genes.*

While there is an abundance of reports regarding changes to expression of PA-related genes in response to different stressors or throughout development in different tissues at different time points, a detailed knowledge on the upstream transcription factors (TFs) controlling PA levels is somewhat lacking (Chen et al., 2019). Some *cis*-regulatory elements have been identified in multiple PA-related gene promoters, which may explain how PA synthesis and catabolism are regulated at the transcriptional level; these elements are typically light-, hormone-, stress-, and various developmental-responsive elements (Alcazar et al., 2006; Sun et al., 2014; Wu et al., 2016; Liu et al., 2018; Ebeed, 2022). For example, ODC promoters across multiple plant species were found to contain *cis* elements related to ethylene responses, light-dependent pathways, plant architecture, and alkaloid and carotenoid biosynthesis (Sivakumar et al., 2022). Specific TFs, such as MYB, ABF, and WRKY TFs, have been identified to either bind to *cis*-elements in promoters of PA-related genes and/or influence PA levels (Liu et al., 2015). For example, an abiotic stress-induced MYB TF from orange (*Citrus senensis*) binds to *ADC* promoters, increasing transcription and PA levels (Sun et al., 2014). Similarly, over expression of a cotton MYB in transgenic tobacco also increased *ADC* and *S-adenosylmethionine decarboxylase* (*SAMDC*) expression (Chen et al., 2015). Expression of PA biosynthetic genes,

particularly *ADCs*, are also increased following up regulation of ABF TFs, which are activated following ABA signalling under abiotic stresses such as drought and osmotic stress (Huang et al., 2010; Yoshida et al., 2010; Liu et al., 2015; Zhang et al., 2015). Interestingly, *ADC* expression is also positively regulated by at least one member of the large WRKY TF family, WRKY70 from Kumquat (*Fortunella creassifolia*), which is induced following drought and ABA treatment (Gong et al., 2015). Interestingly, the NAC72 TF from orange was found to act as a repressor of *ADC* expression and in tobacco lines overexpressing NAC72, *ADC* expression and putrescine levels were reduced. Through these studies, *ADC* genes have been shown to be regulated by multiple TFs and over expression of these TFs influences *ADC* expression, PA levels, and plant's ability to respond to abiotic stresses. Therefore, transcriptional control of *ADCs* may be central for fine tuning PA levels to the plant's current needs.

Expression of plant *ADCs* is typically upregulated in response to many abiotic and biotic stresses, such as cold, drought, osmotic, salt stress, wounding, and pathogen attack (Hussain et al., 2011; Chen et al., 2019; Gonzalez et al., 2021; Gonzalez-Hernandez et al., 2022b; Nandy et al., 2022). In line with this, over expression of *ADCs* increases resistance to drought, salt, cold, freezing, wounding, and pathogen stress, while *adc* knock out or silenced lines are more susceptible to such stresses (Roy and Wu, 2001; Perez-Amador et al., 2002; Urano et al., 2004; Alcazar et al., 2010b; Wang et al., 2011; Kim et al., 2013; Rossi et al., 2015; Espasandin et al., 2018; Kou et al., 2018; Hidalgo-Castellanos et al., 2019; Liu et al., 2020; Chavez-Martinez et al., 2020; Ran et al., 2022). Interestingly, addition of exogenous putrescine in *adc* knock out or silenced lines often at least partially restores stress responses. Overall, *ADCs* are transcriptionally activated in response to both abiotic and biotic stress responses and play an important role in regulating PA pathway flux.

1.3.2 Translational regulation of PA-related proteins

While transcriptional regulation of the PA pathway is important, PA levels and enzyme activities do not always correlate with transcript abundance, supporting the idea that PA pools are also controlled at the post transcriptional level (Paschalidis and Roubelakis-Angelakis, 2005; Wu et al., 2019; Jimenez-Bremont et al., 2022). In fact, many PA-related proteins are regulated by translational regulatory mechanisms, often involving upstream open reading frames (uORFs) in both plants and animals (Dever and Ivanov, 2018), as well as multiple examples of prokaryotic riboswitches controlled by PA-related metabolites (Kavita and Breaker, 2023; Salvail et al., 2023). Translational regulation allows for rapid and dynamic changes to protein levels and can be influenced by many cellular signals and stimuli to alter

protein abundance. In the case of PA-related proteins in plants, uORFs have been identified and at least partially characterised in *ADC*, *ODC*, *SAMDC* (also known as *AdoMetDC*), and *PAO-2*, *-3*, and *-4* mRNAs (Zhang et al., 2020a; Jimenez-Bremont et al., 2022). These uORFs, as is the case for most described uORFs, have an inhibitory effect on translation of their downstream coding sequences (von Arnim et al., 2014; Hellens et al., 2016; Zhang et al., 2020a). Typically, the presence of a uORF start codon in a transcript's 5' untranslated region (5'UTR) will cause scanning ribosomes to initiate translation. After completing uORF translation, translation termination occurs, and majority of the ribosome subunits disassociate from each other and the transcript. Therefore, very few of these ribosomes continue scanning, resulting in much lower translation initiation at the main ORF (mORF) start codon, thereby reducing mORF translation. The mORF can also be translated if scanning ribosomes do not recognise the uORF start codon, either due to a poor Kozak context or through external (i.e., trans) regulation influencing initiation factor activities; this process is called 'leaky scanning' (von Arnim et al., 2014; Hellens et al., 2016; Zhang et al., 2020a).

While these are general inhibitory mechanisms of uORFs, many can also regulate mORF translation depending on cellular conditions or environmental stimuli. Some uORFs in 5'UTRs of PA-related genes can regulate translation of their mORF in a PA level-dependent manner. For example, in the 5'UTR of *SAMDC*, two highly conserved overlapping uORFs (called 'tiny' and 'small') regulate levels of SAMDC translation in response to PA levels. Tiny, the most 5' uORF is responsible for allowing SAMDC translation under low PA levels, while the small uORF inhibits SAMDC translation; differential start codon initiation at either tiny or small in response to PA levels therefore fine-tunes SAMDC levels (Hanfrey et al., 2005). On the other hand, the PA catabolic enzyme POA2 in *A. thaliana* has a uORF that inhibits POA2 translation with low PA levels, thereby reducing PA catabolism (Guerrero-Gonzalez et al., 2014; Guerrero-Gonzalez et al., 2016). Although the mechanisms of the *ADC* and *ODC* uORFs have not yet been thoroughly investigated, they have both been reported to either inhibit their respective mORFs (Chang et al., 2000; Kwak and Lee, 2001). Overall, it seems that controlling PA-related enzyme expression via uORFs could be a common PA regulatory mechanism. However, it is not currently understood exactly how these uORFs sense cellular PA levels beyond the suggestion that these uORFs regulate translation of their mORF simply by amplifying the impact that PA levels have on global translation (Hanfrey et al., 2005).

1.3.3 Translational regulation of plant ADCs by highly conserved 5'UTR elements.

A recent publication identified a highly conserved and ancient *cis* element that regulates translation of plant ADCs, termed the *ADC-box* (Wu et al., 2019). Remarkably, the *ADC-box* was discovered through studying an effector protein from the plant pathogen *Ralstonia solanacearum*, the causal agent of bacterial wilt disease; this is further introduced in more detail below (section 1.5).

The *ADC-box* is a ~50 bp, GC-rich sequence that is found in the *ADC* 5'UTR of all investigated higher plants, with a primitive form of this sequence also found in *Marchantia polymorpha* (Figure 3A, Supplementary Figure 1; Wu et al., 2019). As *M. polymorpha* is a relative of the first land plants that evolved ~450 million years ago (Bowman et al., 2017) and that so far, all plant *ADCs* have an *ADC-box*, it is likely that this is a very ancient plant element. Using *in vivo* and *in vitro* reporter assays in *Nicotiana benthamiana* leaves and cell free lysates respectively, removal of the *ADC-box* increased downstream translation (Wu et al., 2019). Due to the high GC-content of the *ADC-box* sequence, it was hypothesised that it forms an RNA secondary structure, and through computational analysis, a double stem loop structure was predicted (Figure 3B). All together, these findings suggested that the *ADC-box* forms an RNA secondary

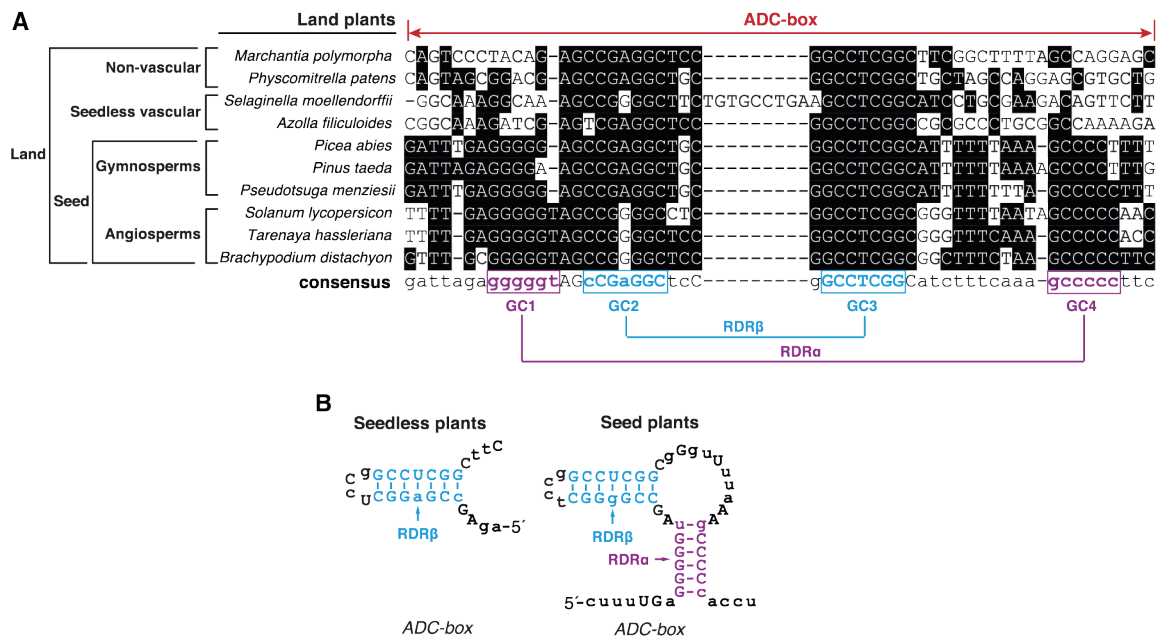


Figure 3: The *ADC-box* is a highly conserved GC-rich sequence that is predicted to form an RNA secondary structure.

Alignment of *ADC-box* sequences, including tomato and *M. polymorpha* with the conserved GC-rich regions highlighted in purple and blue (A). These GC-rich regions were hypothesised to join, forming the predicted RNA secondary structures; due to only having one set of GC-rich regions, seedless plants have a smaller single stem loop predicted structure. (B) Figure 4F and G from Wu et al., 2019.

structure that inhibits ADC translation, possibly by preventing movement of scanning ribosomes.

Interestingly, Wu et al., 2019 found that almost all investigated *ADC* 5'UTR sequences also contained a uORF that, at least in angiosperms, was typically found ~150 bp upstream of the *ADC-box* (Supplementary Figure 2). Despite this intriguing level of conservation, the *ADC* uORF was found to have no dramatic impact on downstream translation using transient dual reporter assays (Wu et al., 2019).

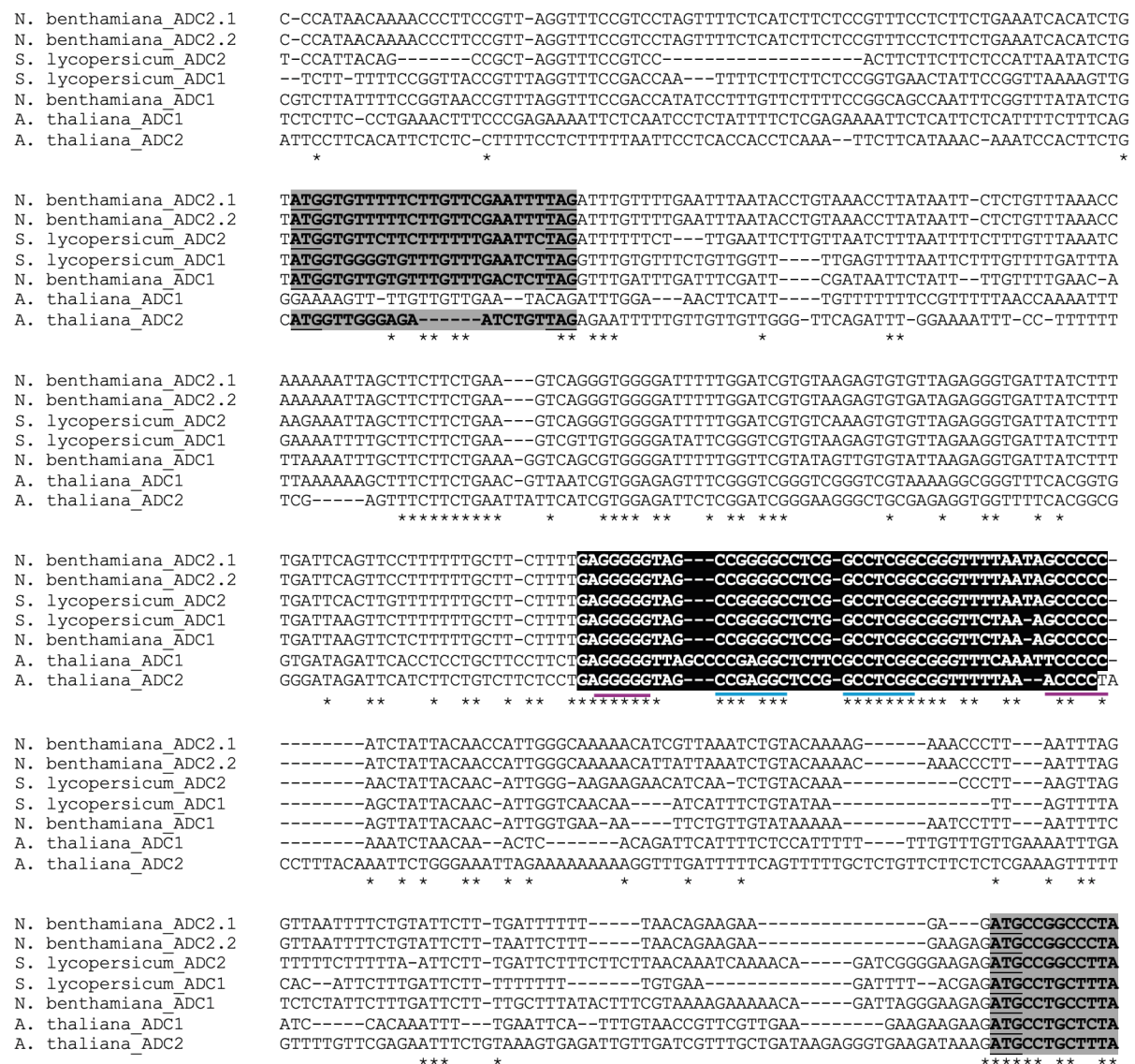


Figure 4: Alignment of *A. thaliana*, tomato, and *N. benthamiana* *ADC* 5'UTR sequences.

A. thaliana ADC1 5'UTR has sequence differences as well as having neofunctionalised to be an *N*^δ-acetylornithine decarboxylase; there is no clear *AtADC1* uORF in the same location as other *ADC* 5'UTRs and the *ADC-box* contains additional nucleotides. The uORF and start of *ADC* coding sequence is highlighted in grey with start and stop codons underlines; *ADC-box* sequence has white text with a black background and conserved GC-rich sequences underlined in blue and purple; an asterisk under the alignment indicates identical nucleotides at that position.

While there are very high levels of conservation regarding the location and sequence of the *ADC* uORF and *ADC-box*, particularly in angiosperms, *A. thaliana ADC1* seems to have alterations to its 5'UTR sequence (Figure 4), as well as evolving a new N^{δ} -acetylornithine decarboxylase function (Lou et al., 2020). Specifically, the *A. thaliana ADC1 ADC-box* (*AtADC1-box*) has additional sequences in the 5' regions of the *ADC-box*, while *AtADC1* 5'UTRs also appear to lack a uORF (i.e., no identified start codon 5' to the predicted transcriptional start site). Therefore, *AtADC1* may have not only evolved to have a new catalytic function, but also may have altered translational regulation through to these *ADC-box* and uORF changes.

Overall, identification of the *ADC-box* indicates translational regulation of PA synthesis may not be limited to uORFs, and that this ancient element may control flux through the entire pathway. However, whether it functions *in planta* and how it can control ADC translation remains unclear.

1.4 RNA secondary structures regulate translation through a variety of mechanisms.

The composition of 5'UTRs can massively influence translational efficiency. In general, higher GC content and longer 5'UTRs often correlate with reduced translational efficiency, as ribosomes must continuously scan the 5'UTR whilst their helicases unwind regions with strong pairing (Leppek et al., 2018). For example, mammalian *ODC* transcripts have a long and highly structured 5'UTR that represses *ODC* translation (Manzella and Blackshear, 1990). However, there are many cases of local RNA secondary structures that regulate translation of the downstream coding sequence. One of the most well studied class of RNA structures that regulate translation are riboswitches, which are predominantly found in prokaryotes (Garst et al., 2011; Kavita and Breaker, 2023). Such RNA structures are made up of multiple stem loops (i.e., hairpins) to form highly structured arrangements that, upon binding their ligand (i.e., ions, vitamins, and metabolites) undergo a conformational change to regulate translation rates of the downstream coding sequence. While this is a common and likely very ancient mechanism of translational regulation in prokaryotes, only one riboswitch class has been properly characterised in eukaryotes (i.e., TPP riboswitches regulating Thiazol Biosynthesis 1 (THI1) translation; Bocobza et al., 2007; Wachter et al., 2007). Another couple of other potential riboswitches have been found in fungi regulating translation of arginine and spermidine synthesis enzymes, yet further confirmational experimental evidence is required (Borsuk et al.,

2007; Sun et al., 2020; Kavita and Breaker, 2023). Although simpler in their structure, another type of well characterised RNA structures are hairpins, which can be specifically bound by RNA binding proteins that then prevent ribosome binding to or scanning along the 5'UTR. One example of these hairpins are the iron response elements (IREs) that form hairpin structures near the 5' cap, which, upon binding of an iron regulatory protein, prevent ribosome access and recognition of the 5' cap, thereby reducing translation of the downstream CDS (Leppek et al., 2018). RNA G quadruplexes (RGQs) are another type of RNA structure that can regulate translation either positively or negatively (Varshney et al., 2020). These are highly stable G-rich structures made up of four sets of repeating guanine nucleotides (i.e., $G_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}N_xG_{\geq 3}N_x$) that form a stacked tetrad, typically stabilised with a positive ion in the centre or a protein. RGQs have predominantly been characterised *in vitro* but have recently been identified and found to regulate translation *in vivo* in plants, also influencing their growth (Yang et al., 2020). Interestingly, multiple RGQs have been found to regulate expression of mammalian PA biosynthetic enzymes and proposed to maintain PA homeostasis (Lightfoot et al., 2018). Another types of RNA secondary structure that regulates translation are internal ribosome entry sites (IRES), which typically promote translation of the downstream coding sequence by recruiting ribosomes in a 5' cap-independent manner (Hellen and Sarnow, 2001). Interestingly, the highly structured and repressive mammalian *ODC* 5'UTR contains an IRES that promotes translation at specific points during the cell cycle (Pyrone et al., 2000). Overall, there are a multitude of RNA structures that can regulate translation both positively and negatively through various mechanisms and interestingly, many of these are found in transcripts encoding PA-related proteins. However, the mechanism by which the *ADC-box* RNA secondary structure regulates translation of ADCs and if this is PA level-responsive has not yet been determined.

1.5 *Ralstonia solanacearum* boosts host PA levels via a highly conserved T3SS effector by inducing ADC over expression.

1.5.1 *Ralstonia solanacearum* is the causal agent of bacterial wilt disease.

Bacterial wilt disease is one of the most devastating plant diseases, infecting approximately 450 plant species, many of which are economically important crops, such as members of the Solanaceous family (i.e., tomato, potato, and eggplant), as well as banana, ginger, flowers and ornamentals (e.g., geraniums), forest trees, and some spices (Paudel et al., 2020; Sharma et al., 2021). Bacterial wilt disease is caused by pathogenic bacteria of the *Ralstonia solanacearum*

species complex (*Rssc*), made up of four globally distributed phylotypes residing in tropical and subtropical regions (Genin, 2010; Wicker et al., 2012; Vailleau and Genin, 2023). Phylotype I and Phylotype III found in Asia and Africa respectively, and belong to the *R. pseudosolanacearum* species, infecting *Solanaceous* plant species. Phylotype II is found in the Americas, and although all strains belong to the *R. solanacearum* species, this phylotype is broken into two groups (i.e., IIA and IIB), which have strains infecting bananas (*Musa acuminata*), as well as *Solanaceous* species such as potatoes (*Solanacearum tuberosum*). Phylotype IV strains, coming from the *R. syzygii* species, are typically found in Indonesia and in northern Australia infecting *Solanaceous* species, bananas, and cloves. Through phylogenetic analyses, phylotypes II and IV are thought to have evolved first and then Phylotypes I and III emerging more recently (Vailleau and Genin, 2023).

These strains (commonly referred to as *R. solanacearum* in this work) reside in the soil and following migration and attachment to host roots, enter roots through small wounds, natural openings (i.e., emerging lateral roots), or root tips (Xue et al., 2020; Kashyap et al., 2021; Planas-Marques et al., 2020). For a successful infection, *R. solanacearum* must then travel through the root to the vasculature, where upon entering the xylem, it travels up through the plant proliferating greatly. Due to the amount of *R. solanacearum*, it eventually clogs the vasculature, causing wilting and death of its host plant.

1.5.2 *R. solanacearum* has a TALE-like effector, called a RipTAL.

To facilitate a successful infection, *R. solanacearum*, like many plant pathogens, injects effector proteins into the plant cells via a type III secretion system (T3SS) to both dampen the host's immune system and manipulate the plant into creating a favourable infection environment (Genin, 2010; Macho et al., 2010). One of these effectors shares functional and structural homology to Transcriptional Activator Like Effectors (TALEs or TAL effectors) from the *Xanthomonas* genus and are termed RipTALs (*Ralstonia* injected protein with similarity to TAL effectors; de Lange et al., 2013; Schandry et al., 2016).

These effectors have a remarkable structure, enabling them to manipulate expression of specific host genes. Both TALEs and RipTALs have an N-terminal T3SS signal and C-terminal nuclear localisation signal (NLS) and acidic activation domains (AAD, also known as the transcription factor binding [TFB] domain; Boch and Bonas, 2010; Bogdanove et al., 2010). Between these two terminal domains, TALEs and RipTALs have a central DNA binding domain, consisting of 34-35 amino acid near identical repeats. Each of these repeats only differ at their 12th and 13th residues, which are collectively called the repeat-variable diresidue (RVD). Following

injection, TALEs and RipTALs are translocated to the host nucleus where they bind to a specific sequence in the host genome called an effector binding element (*EBE*). It is the RVDs that bind to the host DNA bases in an *EBE*, with each RVD having a unique base preference, thereby giving the unique binding specificity of TALE and RipTALs (Boch et al., 2009; de Lange et al., 2013). Following this binding, the TALE or RipTAL recruits the host RNA polymerase complex through the TFB domain and induces transcription approximately 50 bp downstream of the *EBE*, generating a non-native transcript with a shorter 5'UTR. Through the specificity of the RVD-*EBE* interaction, TALEs and RipTALs can specifically activate certain host genes to promote infection.

1.5.3 *RipTALs activate host ADC transcription to boost PA levels.*

The first characterised RipTAL was from the *R. solanacearum* reference strain GMI1000 (phylotype I) and called Brg11 (Salanoubat et al., 2002; Macho et al., 2010; Wu et al., 2019). This RipTAL was found to bind an *EBE* in host *ADC* promoters, activating transcription, leading to increased ADC activity and putrescine levels, but not spermidine or spermine levels (Wu et al., 2019). ADC activity and levels of PAs and some associated metabolites were measured from the same plant extract sample using a liquid chromatography-mass spectrometry (LCMS)-based method established by Wu et al., 2019.

Interestingly, RipTALs from the other *Rssc* phylotypes were also found to activate host *ADC* genes, despite their global distribution, different host plant species, and having diverged approximately 180 million years ago (Gallas et al., 2023). This suggests that since this time, RipTALs have maintained *ADCs* as their target; a feat that is vastly different from the rapidly evolving *Xanthomonas* TALEs (Hutin et al., 2015). This conservation has possibly been due to the *EBE* being part of the genomic *ADC-box* sequence; the highly conserved translational regulatory element found in the *ADC* 5'UTR of all land plants (section 1.3.3; Wu et al., 2019). Yet despite the remarkable conservation of RipTALs targeting host *ADCs*, no growth promoting benefit of *R. solanacearum* or *Xanthomonas* strains containing an *ADC*-targeting RipTAL or TALE were found (Wu et al., 2019). It was then hypothesised that *R. solanacearum* may boost host PA levels to inhibit growth of other niche microbes, giving *R. solanacearum* a competitive advantage. This hypothesis was supported by a reduction of *Pseudomonas syringae* growth in tomato leaves when co-infecting with a *Xanthomonas* strain delivering Brg11 but not with the negative control *Xanthomonas* strain (i.e., delivering the defective RipTAL, Hpx17; Wu et al., 2019). Previous works have shown that *ADC* over expression and increased putrescine levels can inhibit *Pseudomonas* growth; however, both *R. solanacearum*

and *P. syringae* excrete putrescine during infection in response to an oxidative environment (Lowe-Power et al., 2018; Vilas et al., 2018; Liu et al., 2020; Solmi et al., 2023). Therefore, it remains unclear as to how exactly *R. solanacearum* benefits from boosting host PA levels via ADC induction and how these metabolic changes may influence growth of other microbes.

1.5.4 Experimental methods of *R. solanacearum* infection may have negated the role of RipTALs, leaving their impact on infection progression yet undiscovered.

One reason for why a RipTAL-dependent growth advantage has not been observed so far may be due to differences between a natural infection scenario and the artificial infection methods and highly controlled growth conditions used in laboratory experiments. If *R. solanacearum* uses RipTALs to boost PAs to inhibit growth of potential competitors, infections in a laboratory setting with ideal conditions and comparatively clean soil would not result in a dramatic growth promoting benefit for strains containing an ADC-targeting RipTAL. For many experimental infection procedures, all plants are raised in ideal greenhouse conditions, ensuring that the plants are perfectly healthy and experience little biotic or abiotic stressor prior to experimental infections. Fresh commercially bought soil is also used for growing the plants, which is unlikely to contain the diverse microbial community found in agricultural or natural soils. Therefore, if the role of RipTALs is to inhibit growth of competitive niche microbes, this is unlikely to be observed in this experimental setting.

The methods used to infect plants with *R. solanacearum* may have also eluded the biological role of RipTALs, particularly if this role is important during the early stage of infection. For example, in soil drench assays, liquid *R. solanacearum* culture with an OD₆₀₀ of 0.01 (10⁷ CFU/mL) is tipped over the soil in each pot, which is a much higher bacterial load than found in a natural infection scenario (i.e., 10³-10⁶ in infected soils; Shi et al., 2023). Additionally, in most soil drench infection protocols, the roots are wounded to increase the chance of *R. solanacearum* infection; this is typically done with a knife or a large pipette tip (Wu et al., 2019; Xu et al., 2022a). Another common infection methods include blunt end syringe infiltration into leaves, infection of plants on sterile agar plates, and fine needle injection of *R. solanacearum* directly into the lower plant stem (Macho et al., 2010; Khokhani et al., 2018; Wu et al., 2019, Morcillo et al., 2020; Xue et al., 2020; Yu and Macho, 2021; Xu et al., 2022a; Gallas et al., 2023; Yu et al., 2023). Overall, these experimental infection methods aim to infect plants in a regularised and methodical manner, ensuring all plants are infected with the same bacterial load at the same time to minimise experimental error. However, these methods generally do not mimic the natural infection scenario, where a low amount of *R. solanacearum*

is present in the soil, and only a small number of bacterial cells enter the host roots via small, natural openings caused by lateral root emergence or wounds (Xue et al., 2020). These cells then need to successfully travel through the root, passing the plants defensive barriers, to the xylem tissue, where they rapidly multiply to establish an infection. The methods described above bypass this initial infection stage or the roots entirely, leaving the possibility that the role of many effectors, including RipTALs, may not be observed if they are important for root entry, bypassing root barriers and defences, or for establishment of the xylem infection.

Furthermore, if RipTAL activity is important during the very early stages of infection when bacterial load is relatively low, prior analyses on a whole plant or tissue level may not have been suitable for uncovering the role of RipTALs. During the early stage of infection (i.e., from adhesion to host roots and entry into the roots, but prior to penetration of the vasculature), few *R. solanacearum* cells are present, meaning that only a small number of host cells would likely be injected with effectors. Therefore, sampling bulk infected root tissue early in the infection is highly unlikely to show any RipTAL-induced effects on tissue morphology or metabolite levels, as the number of effector-injected cells is substantially lower than non-injected cells, resulting in a massive dilution effect. This was previously seen when roots of tomato plants and seedlings were infected with either WT GMI1000 or $\Delta brg11$ *R. solanacearum* strains using either soil drench assays of 4-week-old plants or vacuum infiltration of tomato seedlings (Wu et al., 2019). The characteristic short Brg11-induced *SLADC1/2* transcripts were clearly observed from WT infected roots, but not $\Delta brg11$ -infected roots using all infection protocols, demonstrating that Brg11 had been delivered in all cases. However, no differences in overall *SLADC1/2* transcript levels were observed using any infection method, and induction of ADC activity and PA levels was only seen when *R. solanacearum* was vacuum infiltrated into tomato seedlings (Wu et al., 2019). Therefore, clear RipTAL induction of *SLADC1/2* transcription, ADC activity, and PA levels was likely missed due to massive dilution of cells that had been injected with RipTALs compared to the large number of root cells that did not. Consequently, to investigate the pathological role of ADC induction by RipTALs, especially during the early stages of *R. solanacearum* infection, alternative approaches and biological tools are required to specifically visualise, isolate, and study root cells that receive Brg11 at this infection stage. Overall, while manipulation of host metabolism via effectors is not uncommon (Cai et al., 2023), RipTALs are particularly extraordinary as they have maintained their *ADC* targets for ~180 million years. Therefore, understanding the role of RipTALs on a molecular level in infected host roots may provide insight into *R. solanacearum* infection.

1.6 Aims of this project

The overall goal of this project was to improve the understanding of ADC translational regulation *in planta* and to use this knowledge to better understand how RipTAL activation of host ADCs may benefit *R. solanacearum*.

Previously, Wu et al., 2019 discovered the ancient *ADC-box* and how conserved the *ADC* uORF was. Through preliminary investigations into the impact of the tomato *ADC-box* and uORF on ADC translation, they found that the *ADC-box* inhibits downstream translation in a *cis* manner. However, it remained unclear if and how these elements functioned to regulate ADC translation *in planta*. Additionally, it was not known if the *ADC-box* function was as highly conserved as its sequence. Therefore, the primary aims of this work were to:

- Establish whether the *ADC-box* and uORF regulate ADC translation *in planta*,
- Determine if the primitive *ADC-box* found in *M. polymorpha* was also able to repress ADC translation *in planta*, and
- Elucidate the molecular mechanism used by the *ADC-box* to repress downstream translation and if this can be altered to the cellular need for PAs.

To better understand how *R. solanacearum* may benefit from inducing host ADCs and boosting PA metabolism, investigation into how ADC levels influence the greater PA metabolic network was required. To do this, as well as investigate levels of ADC translation *in planta*, the previously published ADC activity and PA measurement method using LCMS (Wu et al., 2019) required optimisation.

Finally, to further investigate the impact of RipTAL activity in host plants, new experimental approaches needed to be developed. These approaches would aim to:

- Identify and monitor host cells that receive RipTALs during *R. solanacearum* infection and to specifically isolate their nuclei for transcriptomic experiments,
- Determine the metabolic and morphological changes induced by RipTALs and how these changes may impact the microbiome.

2. Materials and Methods

2.1 Materials

2.1.1 Plant genotypes:

Plant lines that were used for this work were wildtype (WT) *Nicotiana benthamiana*, *Arabidopsis thaliana* Col-0 (referred to here as *A. thaliana*), *Solanum lycopersicum* Moneymaker (referred to here as tomato), and male WT *Marchantia polymorpha* line TAK1. Previously established mutants of these backgrounds used in this work described in Table 1.

Table 1: Previously established mutant plant lines used in this work.			
Mutant name:	Background:	Generated by:	Mutant details:
<i>bs4</i>	Tomato WT	Schenstnyi et al., 2022	CRISPR/Cas9-induced knock out mutation of <i>Bs4</i> , the TALE-detecting nucleotide-binding leucine-rich repeat (NLR) resistance protein.
<i>adc1</i>	Tomato WT	Wu et al., 2019	CRISPR/Cas9-induced 4 bp deletion causing premature stop codon
<i>adc2</i>	Tomato WT	Wu et al., 2019	CRISPR/Cas9-induced 52 bp deletion causing premature stop codon
<i>adc1/2</i>	Tomato WT	Wu et al., 2019	Generated through genotyping progeny of a homozygous <i>adc1</i> and heterozygous <i>adc2ADC2</i> parent
<i>odc1</i>	Tomato WT	Dousheng Wu, unpublished [#]	CRISPR/Cas9-induced 5 bp deletion causing premature stop codon
<i>Mpadc-box</i> ⁺	<i>M. polymorpha</i> TAK1	Dr. Ezgi Süheyla Karaaslan*	CRISPR/Cas9-induced mutations described in detail in section 3.1.3
Footnotes:			
[#] Generated while in Lahaye Laboratory, ZMBP			
⁺ Multiple <i>ADC-box</i> and control mutants described in detail in section 3.1.3			
* In Prof. Chang Liu's Laboratory when they were at ZMBP			

2.1.2 Bacterial strains used:

For general cloning, *Escherichia coli* Top10 (Invitrogen™) cells were used. For *in planta* delivery of T-DNAs or effector proteins, *Agrobacterium tumefaciens* strain GV3101 (Holsters et al., 1980) and *Xanthomonas euvesicatoria* pathovar *vesicatoria* strain 85-10 (Adlung et al., 2016) were used, respectively. *Ralstonia solanacearum* phylotype I strain GMI1000 (Salanoubat et al., 2002) and phylotype IIA strain K60 (Remenant et al., 2012) were used for *N. benthamiana* infections, alongside the GMI1000 mutant GRS473, lacking two T3SS effectors (AvrA and PopP1) that are recognised in *N. benthamiana* (Poueymiro et al., 2009).

Both *R. solanacearum* K60 and GRS473 were gifted to Lahaye Laboratory by Stéphane Genin's Laboratory (UMR CNRS-INRAE, Castanet Tolosan, France).

2.1.3 Plant and bacterial media and antibiotics:

Table 2: Bacterial and plant media composition		
Media name:	Media composition:	Organism grown on media:
LB	1% (w/v) tryptone 1% (w/v) NaCl 0.5% (w/v) yeast extract	<i>E. coli</i>
YEB	0.5% (w/v) beef extract 0.5% (w/v) peptone 0.5% (w/v) saccharose 0.1% (w/v) yeast extract 2 mM MgSO ₄	<i>A. tumefaciens</i>
NYG	0.5% (w/v) peptone 0.3% (w/v) yeast extract 0.2% glycerine	<i>X. euvesicatoria</i>
φ	1% (w/v) peptone 0.1% (w/v) yeast extract 0.1% (w/v) tryptone 0.5% (w/v) glucose 0.005 % TTC [#]	<i>R. solanacearum</i>
½ MS	0.44% (w/v) Murashige & Skoog (MS) + vitamins 0.05% (w/v) MES 1% sucrose* pH to 5.8 with KOH Add 0.8% PhytoAgar for solid media	<i>A. thaliana</i>
Tomato growth media	0.44% (w/v) Murashige & Skoog (MS) + vitamins 0.01% (w/v) myo-inositol pH to 5.8 with KOH Add 0.8% PhytoAgar for solid media	Tomatoes
½ B5	0.15% Gamborg B5 0.05% MES 0.0005% Myo-inositol pH to 5.6 with KOH Add 1.5% PhytoAgar for solid media	<i>M. polymorpha</i>
Footnotes: For solid bacterial media, 3% (w/v) bactoagar [#] 1% sterile 2, 3, 5-triphenyl tetrazolium chloride (TTC) solution (stored at 4 °C) added to melted φ agar media prior to pouring plates *No sucrose was added to plant media in final LCMS experiments as sucrose induces <i>ADC</i> transcription.		

Table 3: Antibiotic and herbicide stock and working concentrations		
Antibiotic:	Stock concentration:	Working dilutions:
Ampicillin	100 mg/mL (water)	1:1000 (solid) for <i>E. coli</i> 1:2000 (liquid) for <i>E. coli</i>
Gentamycin	15 mg/mL (water)	1:1500 for <i>R. solanacearum</i> * 1:1000 for <i>E. coli</i> and <i>X. euvesicatoria</i> 1:300 for <i>A. tumefaciens</i>
Spectinomycin	100 mg/mL (water)	1:2500 for <i>R. solanacearum</i> 1:1000 for all other bacteria
Rifampicin	100 mg/mL (DMF)	1:500 for <i>R. solanacearum</i> 1:1000 for <i>A. tumefaciens</i> and <i>X. euvesicatoria</i>
Tetracycline	10 mg/mL (70% EtOH)	1:200 for <i>R. solanacearum</i> *
Kanamycin	25 mg/mL (water)	1:1000 for <i>E. coli</i> 1:250 for <i>A. tumefaciens</i> and <i>X. euvesicatoria</i> 1:500 for <i>A. thaliana</i>
Cefotaxime	200 mg/mL (water)	1:1000 for <i>A. thaliana</i>

		1:2000 for <i>M. polymorpha</i>
Hygromycin	50 mg/mL	1:5000 for <i>M. polymorpha</i>
BASTA®	200 g/L	1:500 for spraying <i>A. thaliana</i>
Footnotes: *When growing on plates, ½ strength used		

2.1.4 Antibodies:

Table 4: Antibodies used for this work.			
Antibody:	Details:	Raised in:	Working dilution:
Anti-HA-HRP	Monoclonal; Roche	Rat	1:5000
Anti-GFP-HRP	Monoclonal; SantaCruz Biotechnology	Mouse	1:5000
Anti-c myc	Monoclonal; Sigma	Mouse	1:5000
Anti-mouse-HRP	Whole molecule; Sigma	Rabbit	1:10000
Footnotes: Working dilutions of antibodies were stored at -20 °C and reused no more than 3 times within 2 months.			

2.2 Methods

2.2.1 Plant methods:

2.2.1.1 Plant growth conditions:

Table 5: Plant growth conditions.			
Plant:	Temperature:	Humidity:	Day length:
<i>A. thaliana</i>	20 °C in light; 18 °C in dark	30-40%	16 hrs light; 8 hrs dark
<i>M. polymorpha</i>	20 °C in light; 18 °C in dark	30-40%	16 hrs light; 8 hrs dark
Tomato	20 °C-22 °C	40-50%	16 hrs light; 8 hrs dark
<i>N. benthamiana</i>	20 °C-22 °C	30-35%	16 hrs light; 8 hrs dark

2.2.1.2 Seed propagation and harvesting:

A. thaliana plants with yellowing siliques were bagged (in paper) and left to dry; seeds were then separated from fully dried plant material using a fine sieve. *N. benthamiana* seeds were harvested by picking opened seed pods from plants and letting them dry in an open paper bag. Tomato fruits were collected once they turned fully red and seeds were harvested from the cut fruit into a sieve, then washed with cool water and left to dry on a plastic plate.

2.2.1.3 *M. polymorpha* propagation of adult plants and from gemmae:

To maintain *M. polymorpha* lines, pieces of adult plants were cut off and placed onto fresh, deep, solid ½ B5 media with an additional petri dish bottom on top to provide extra growth space. This was repeated when plants overgrew, or the media began to dry (~every 4 months). *M. polymorpha* were also propagated through their asexual reproductive gemmae. Due to issues with long term storage of *M. polymorpha* mutants containing large amounts of *A. tumefaciens*, gemmae had to be sterilised before plating on fresh ½ B5 plates. For this, a lot of gemmae were scooped out from the gemmae cups with tweezer tips and put into 1.5 mL Eppendorf tubes containing 500 µL sterile Milli-Q water. 500 µL of 2x sterilisation solution

was added (1% Triton and ~1% sodium hypochlorite solution in water) and incubated for exactly 2 minutes with ~3-4 inversions. This solution was removed and the gemmae washed with sterile Milli-Q water 6-8 times before being spread onto fresh ½ B5 plates.

2.2.1.4 Stable transformation of *N. benthamiana* and tomato:

Stable transformations of *N. benthamiana* and tomatoes was conducted under sterile conditions by the transformation unit in the central facilities of ZMBP based on the publication of Wittmann et al., 2016 and protocols published on their website:

<https://uni-tuebingen.de/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/zentren/zentrum-fuer-molekularbiologie-der-pflanzen/central-facilities/plant-transformation/>.

For preparation of *A. tumefaciens* cultures for transformation, *A. tumefaciens* carrying final constructs in plant expression plasmids were grown on solid and then in liquid YEB media containing appropriate antibiotics. Subcultures were used to make a 250 mL culture, which was then centrifuged at 4000-5500 rpm for 10 minutes and the OD₆₀₀ adjusted to ~1.0 in 10 mM MgCl₂ (for *N. benthamiana*) or 10 mM MgSO₄ with 0.1-0.2 mM Acetosyringone (for tomato) without antibiotics.

For preparation of *N. benthamiana* plants for transformation, leaves from plants grown in sterile jars were cut and incubated with the *A. tumefaciens* solution for 2 days. The leaf pieces are then transferred to media containing the selective antibiotic (i.e., kanamycin) and left to develop into a callus for some weeks. Any shoots were transferred to root inducing media. Once sufficient roots have grown, the transformant/plantlet can be transferred to soil with high humidity for ~5 days and darkness for the first 2 days.

For preparation of tomato plants for transformation, sterilised seeds were germinated on media and grown in the dark for ~12 days. Opened cotyledons were cut into pieces and moved to conditioning media for 2 days, before 2 drops of *A. tumefaciens* solution was added to each piece and incubated for another 2 days. The infected cotyledon pieces were then moved to selective media to form callus tissue. Once regenerated shoots appeared, they were cut and grown on rooting media. Again, once they had sufficient root tissue, they were transferred to soil in a high humidity environment.

All plantlets were then genotyped for the presence of the transgene using diagnostic PCRs.

2.2.1.5 Transient transformation in *N. benthamiana*:

A. tumefaciens containing desired plasmids are grown in liquid YEB media containing appropriate antibiotics overnight. Cultures were centrifuged at max speed for 5 minutes at room temperature (RT) before being resuspended in infiltration buffer (Table 6) and left to shake at 28°C for at least 1 hour. Cultures were then diluted to a final OD₆₀₀ of 0.4 in infiltration buffer; if two strains are co-infiltrated, then they are diluted to OD₆₀₀ of 0.8 and mixed 1:1. *A. tumefaciens* solutions were then infiltrated into the underside of *N. benthamiana* leaves using a blunt end 5 mL syringe; this process has been referred to as ‘agroinfiltration’ throughout this work. Unless otherwise specified, samples were taken 2 days post infiltration (DPI).

Table 6: Infiltration buffer for <i>A. tumefaciens</i> infiltration into <i>N. benthamiana</i> .		
Component:	Stock concentration:	Final concentration:
MgCl ₂	0.5 M	10 mM
MES	100 mM	5 mM
Acetosyringone	250 mM	150 µM
<u>Footnotes:</u> Sterile stock solutions are mixed freshly just before use		

2.2.1.6 Leaf infiltrations of *X. euvesicatoria* and *R. solanacearum*:

X. euvesicatoria and *R. solanacearum* were also infiltrated into *N. benthamiana* leaves using the same method but were suspended in water rather than buffer. *R. solanacearum* was diluted to different OD₆₀₀, but this is specified in appropriate results sections.

2.2.1.7 Stable transformation of *A. thaliana*:

To generate stable *A. thaliana* transgenic lines, *A. tumefaciens* containing a plasmid the desired T-DNA were grown on YEB plates with appropriate antibiotics for 2 days before being grown in liquid YEB media overnight. This culture was used to make a 25 mL culture in YEB with antibiotics, which was used to make a 300 mL overnight culture. The next day, the culture was centrifuged at 4600 rpm for 10 mins at RT. The supernatant was removed, pellet resuspended in a 50 g/L sucrose solution to an OD₆₀₀ of 0.8 to 500 mL, and 250 µL of Sylwet L-77 was added. Then, flowering *A. thaliana* plants were dipped into this solution for 5-10 seconds before being laid down on damp paper towels in a tray and then kept covered/dark overnight. The next day, the pots were stood up and plants kept for seed production.

To identify transgenic progeny selected with antibiotics, seeds were sterilised, spread onto ½ MS plates containing appropriate selective antibiotics (i.e., kanamycin) vernalised at 4°C for 2-3 days, and then placed into the growth chamber. For transformants with a hygromycin selection gene, seeds were sown to soil and after ~2 weeks, were sprayed with BASTA®

solution at least twice with ~4 days interval between sprayings. Any seedlings surviving antibiotic or BASTA[®] treatment were transferred to individual pots with soil. In addition to the T1 seeds, WT and antibiotic-resistant or BASTA-resistant seeds were always sown as negative and positive controls, respectively, to ensure selection worked as expected.

2.2.1.8 *A. thaliana* seed sterilisation:

In a 2 mL Eppendorf tube *A. thaliana* seeds were rinsed with 1 mL of 80% EtOH with 0.05% Triton X-100 and mixed thoroughly before the supernatant removed. Another 1 mL of the same solution was added and incubated for up to 15 minutes on a rotation wheel. Under a sterile bench, the supernatant was removed and 1 mL of 100% EtOH was added, mixed, and then removed again. This was repeated twice, but on the last time, seeds in 100% EtOH were tipped onto sterile filter paper to dry.

2.2.1.9 Sterilising tomato seeds and growing seedlings *in vitro*:

Tomato seeds were surface sterilised by shaking for 3 minutes in 200 mL 70% ethanol, then 10 minutes in 200 mL 1.5% hypochlorite solution containing 50 µL 0.001% Triton W-100. The seeds were then washed three times in 200 mL of sterile MilliQ H₂O. Note that it is easiest to use autoclaved tea strainers to hold tomato seeds and transfer them to the next solution.

Sterile seeds were then kept on damp filter paper in a sealed petri dish in the fridge for 2 days, before being transferred to a growth chamber. After 3 days, germinated seeds could be transferred agar plate containing solid tomato growth media and allowed to grow vertically for up to 10 days.

2.2.1.10 Tomato phenotyping:

Adult tomato plants were phenotyped in this work by measuring their height, leaf number, internode length, and number of flowers. Seeds of each genotype were sown to soil and once big enough, the seedlings were transferred to larger pots (n = 20). Two plants per genotype were kept on the same tray to reduce any bias of position in the greenhouse on their growth and the trays were also frequently swapped around. On day 30 after sowing the seed, the measurements began and were repeated every 3 days another 4 times (i.e., day 30, 33, 36, 39, and 42). To measure plant height, a ruler was placed with 0 cm at the cotyledons (to reduce bias of the depth each seedling was planted when transferred to new pots) and the height at which the new leaves emerged was measured (explanatory photographs in Supplementary Figure 3). Photos of each replicate plant were taken on day 36 using a black background.

2.2.2 Bacterial methods:

2.2.2.1 Growth of bacteria:

Table 7: Bacterial growth conditions.			
Bacteria:	Media type:	Temperature:	Shaking speed:
<i>E. coli</i>	LB	37 °C	200 rpm
<i>A. tumefaciens</i>	YEB	28 °C	180 rpm
<i>R. solanacearum</i>	φ	28 °C	200 rpm
<i>X. euvesicatoria</i>	NYG	28 °C	180 rpm

2.2.2.2 Transformation of bacteria:

To transform *E. coli*, 25 µL aliquot of chemically competent Top10 cells were removed from -80 °C onto ice and left for 10 minutes before 4 µL of reaction mix or 100 ng of plasmid was added. After another 10 minutes on ice, the tubes were incubated in a water bath set at 42 °C for 1 minute, before being immediately returned to ice and incubated for 2 minutes. 250 µL of sterile LB media was added and the tubes incubated at 37 °C shaking at 300 rpm for 1 hour. 80 µL was then spread onto fresh LB agar plates containing appropriate antibiotics and incubated at 37°C overnight.

To transform *A. tumefaciens*, 50 µL aliquot of electro-competent GV3101 cells were removed from -80 °C onto ice and left for 10 minutes before 100 ng of plasmid was added. After another 10 minutes on ice, the mix was transferred to MicroPulser Electroporator (BioRad) tubes, which were then electro-pulsed using the “Agr” setting and quickly returned to ice. 500 µL of sterile YEB media was mixed in and the entire solution transferred to a fresh 1.5 mL Eppendorf tube to be incubated at 28 °C, shaking at 300 rpm for 1 hour. Then 100 µL was spread onto fresh YEB agar plates containing appropriate antibiotics and incubated at 28 °C for 2 days.

2.2.2.3 *A. tumefaciens* co-transformation of *pGreenII* and *pSoup* plasmids

It is important to note that for *pGreenII*-based plasmids used for dual luciferase assays, co-transformation of a *pSoup* helper plasmid is also required. *A. tumefaciens* is unable to replicate the *pGreenII* plasmid (including the kanamycin resistance gene) without *pSoup*, which contains a tetracycline resistance gene. However, *A. tumefaciens* co-transformed with both *pGreenII* and *pSoup* plasmids will not initially grow on YEB media containing all appropriate antibiotics (i.e., rifampicin, gentamycin, kanamycin, and tetracycline). To circumvent this, initial selection of freshly transformed *A. tumefaciens* on YEB plates containing only rifampicin and kanamycin was conducted, followed by a colony PCR and re-streaking onto YEB plates containing all four antibiotics.

2.2.2.4 Long term storage of bacterial stocks:

Long term stocks of all bacteria used in this work were generated by combining equal volumes of bacterial culture and 60% sterile glycerol in cryogenic tubes and immediately frozen in liquid N₂; these were stored at -80 °C.

2.2.3 Molecular methods:

2.2.3.1 General notes on cloning:

In general, golden gate cloning, based on the toolkit created by Binder et al., 2014, was used to generate all plasmids in this work. Any element that was not present already in this toolkit was generated in this work. Any plasmid systems (i.e., *pGrDL_SPb* dual luciferase plasmid system; Moyle et al., 2017) or components (i.e., promoters or 5'UTR sequences from gDNA) that were not golden gate compatible were modified to make them compatible. This was typically done by adding restriction enzyme (i.e., BsaI or BpiI) recognition sites and overhang sequences. Sometimes elements from gDNA or plasmids from other cloning systems contained BsaI or BpiI sites within the element. Such sites had to be removed to prevent them interfering with the desired golden gate cloning sites; this was done through site directed mutagenesis of the unwanted restriction site. All plasmids that were generated were Sanger sequenced to verify their sequence. For a full list of primers and final plasmids used in this work, refer to Supplementary Table 1 and Supplementary Table 2, respectively. General methods used for cloning and construct assembly are presented below.

2.2.3.2 Polymerase chain reaction (PCR) methods:

Polymerase chain reactions (PCRs) were set up using the reaction scheme shown in Table 8 and then run in a thermocycler using the program settings in Table 9.

Table 8: PCR reaction scheme.	
Component:	Volume:
MilliQ H ₂ O	5.2 µL
HF buffer (5x) NEB	4 µL
PRECES	4 µL
dNTPs (2 mM)	2 µL
Forward primer (1 pmol/µL)	2 µL
Reverse primer (1 pmol/µL)	2 µL
Phusion polymerase	0.2 µL
Taq polymerase	0.1 µL
Template	0.5 µL
Total volume:	20 µL

Table 9: Standard PCR cycle settings.		
Temperature:	Time:	Repeat:
95 °C	5 minutes	
95 °C	20 seconds	x 30
x °C [#]	20 seconds	
72 °C	*	
72 °C	10 minutes	
Footnotes:		
# - dependent on primer annealing temperature		
* - dependent on PCR product length (~30-45 seconds per 1 kbp)		

2.2.3.3 DNA digestion, ligations, and cut ligations:

Restriction enzyme digestions were set up using the reaction scheme in Table 10 and then run for 1 hour at 37 °C (or 30 minutes for fast digest enzymes) and stopped by incubation at 65 °C or 80 °C for 5 minutes.

Table 10: Restriction enzyme reaction scheme.	
Component:	Volume:
Appropriate buffer (10x)	2 µL
Enzyme (ThermoScientific)	0.5 µL
DNA (~200-500 ng)	x µL
Milli Q H ₂ O	Up to 20 µL
Total volume:	20 µL

Ligation reactions were set up using the reaction scheme in Table 11 and then either incubated at 20 °C for 1 hour or left at RT overnight. Typically, the vector backbone was linearised using a blunt end cutting restriction enzyme (i.e., SmaI) and a PCR

Table 11: Ligation reaction scheme.	
Component:	Volume:
Ligase buffer (10x)	1 µL
T4 DNA Ligase (ThermoScientific)	1 µL
PEG4000	1 µL
Vector backbone	x µL
DNA insert	x µL
Milli Q H ₂ O	Up to 10 µL
Total volume:	10 µL

product was ligated; the vector-to-insert was estimated at a ratio of 1:5-10.

Cut ligation reactions were used for golden gate-based assembly of higher order plasmids (Binder et al., 2014). Here, lower-level plasmids (i.e., LI or LII plasmids) were combined to ensure all restriction enzyme-cut overhangs could be connected, generating a higher-level plasmid (i.e., LII or LIII). The reaction scheme is shown in Table 12 and the thermocycler program shown in Table 13.

Table 12: Cut-ligation reaction scheme.	
Component:	Amount:
Final vector	40 fmol/µL
Lower-level plasmids	Each 40 fmol/µL
Ligase buffer (10x)	2 µL
Bovine serum albumin (10 mg/mL)	2 µL
T4 DNA Ligase (ThermoScientific)	0.5 µL
Restriction enzyme	1 µL
Milli Q H ₂ O	Up to 20 µL
Total volume:	20 µL

Table 13: Standard cut ligation program.		
Temperature:	Time:	Repeat:
37 °C	2 minutes	x 25-40 [#]
16 °C	5 minutes	
37 °C	10 minutes	
50 °C	10 minutes	
80 °C	10 minutes	
Footnotes: # - depends on efficiency of cut ligation (i.e., number of components to combine).		

2.2.3.4 Plasmid isolation:

To isolate plasmids from bacteria, ~6 mL of culture grown overnight was centrifuged at max speed for 5 minutes and the supernatant discarded. Plasmids were then isolated using the NucleoSpin® Plasmid DNA Purification Kit by Machery-Nagel GmbH & Co. KG following the kit's instructions. The quality and concentration of each plasmid preparation was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (ThermoFischer Scientific).

2.2.3.5 Genomic DNA (gDNA) isolation:

To isolate gDNA from tomato, *A. thaliana*, *N. benthamiana*, and *M. polymorpha*, a fingernail sized piece of plant tissue was removed and put into a 2 mL Eppendorf tube with a ceramic ball and immediately frozen in liquid N₂. All samples were ground using the TissueLyser II (Qiagen®) for 30 seconds at 22 Hz in adapters pre-cooled to -20 °C and then rapidly transferred back to liquid N₂. 500 µL of gDNA extraction buffer (Table 14) was added to each sample individually and then vortexed until homogenised and fully thawed. All samples were then centrifuged for 5 minutes at 15,000g, after which 350 µL of supernatant was taken to a new 1.5 mL tube already containing 350 µL of isopropanol and mixed gently. Samples were centrifuged at 15,000g for 10 minutes. Supernatant was removed and 500 µL of 70% EtOH was added, mixed gently, and the samples centrifuged at 15,000g for 5 minutes. The supernatant was removed and then once the pellet was dry, 150 µL of MilliQ H₂O was added. Tubes were vortexed, then left at 4 °C overnight and vortexed again before use to fully dissolve the gDNA.

Table 14: gDNA extraction buffer.		
Component:	Stock concentration:	Final concentration:
Tris HCl pH 9	0.5 M	200 mM
LiCl	1 M	400 mM
EDTA	0.5 M	25 mM
SDS	10 %	1%
Footnotes: Stock components and final buffer made up in MilliQ H ₂ O 500 µL buffer was used per sample, except for <i>M. polymorpha</i> , where 150 µL was used.		

2.2.3.6 RNA isolation from tomato leaf tissue:

To isolate RNA from tomato leaf tissue, 8 leaf disks (6 mm) were added to a 2 mL Eppendorf tube containing a ceramic ball and immediately frozen in liquid N₂. Samples were ground using the TissueLyser II (Qiagen®) for 30 seconds at 22 Hz in adapters pre-cooled to -20 °C, then transferred back to liquid N₂. A Universal RNA Purification Kit from Roboklon GmbH was used to extract RNA, mostly following the manufactures instructions, with a few amendments. In brief, 400 µL of RL buffer (containing 1 % β-mercaptoethanol) was added to each sample and vortexed until completely homogenised and thawed. Samples were centrifuged for 3 minutes at max speed before the supernatant was added to a homogenisation spin column and centrifuged again at max speed for 2 minutes. 350 µL of 70% ethanol was added to the flowthrough, mixed, and pipetted onto an RNA binding spin column. Samples were centrifuged for 1 minute at 11,000 g and the flow through discarded. 400 µL of wash buffer D1 was added, and samples centrifuged again 1 minute at 11,000 g. To degrade all DNA, an ‘on-column’ DNase treatment was done by adding 50 µL of DNR buffer with 2 µL of DNaseI

(ThermoFischer Scientific) directly onto the column membrane and incubated for 15 minutes. To wash the column membrane, 400 μL of Wash Buffer RB1, 650 μL of Wash Buffer RBW, and then 350 μL of Wash Buffer RBW were added sequentially with a centrifugation step of 1 minute at 11,000 g between each wash. An additional centrifugation was done to dry the column and then 40 μL of RNase-free water was added to each column membrane and samples centrifuged again for 1 minute at 11,000 g.

The quality and concentration of extracted RNA was determined using the NanoDrop® ND-1000 UV-Vis Spectrophotometer from ThermoFischer Scientific. As *SLADC1* and *SLADC2* do not contain introns, meaning their cDNA and gDNA cannot be distinguished, an additional DNase treatment was conducted to ensure no remaining gDNA had contaminated the sample. 2000 ng of RNA was used (reaction scheme in Table 15), then incubated at 37 °C for 45 minutes, then at 65 °C for 10 minutes. Samples were once more analysed on the NanoDrop® and stored at -80 °C.

Table 15: Post RNA extraction DNase treatment scheme.	
Component:	Amount:
DNase buffer (10x)	1 μL
DNase (ThermoScientific)	0.5 μL
RNA	2000 ng
Milli Q H ₂ O	Up to 10 μL
Total volume:	10 μL

2.2.3.7 Complementary DNA (cDNA) synthesis via reverse transcription:

To make cDNA, the ThermoScientific RevertAid First Strand cDNA Synthesis Kit was used. The reaction scheme shown in Table 16 was used and samples were incubated at 42 °C for 1 hour and then at 70 °C for 5 minutes. cDNA samples were finally diluted 1:10 with RNase-free water and stored at -20 °C.

Table 16: cDNA synthesis reaction scheme.	
Component:	Amount:
Reaction buffer (5x)	2 μL
RiboLock RNase inhibitor	0.5 μL
Oligo (dT) ₁₈	0.5 μL
dNTP (10 mM)	1 μL
RevertAid Reverse Transcriptase	0.5 μL
RNA	850 ng
Milli Q H ₂ O	Up to 10 μL
Total volume:	10 μL

2.2.3.8 Real Time-quantitative PCR (RT-qPCR):

RT-qPCRs were used to quantify relative *SLADC* transcript abundance based on the method used by Wu et al., 2019. 8 μL reactions were set up as per Table 17, MESA Blue qPCR™ Mastermix Plus for SYBR® Assay (Eurogentec). Three technical replicates were used for each biological replicate, which were all loaded into a 384 well plate, and an RT-qPCR run using a CFX384™ Real-Time System from BioRad with the program outlined in Table 18. Reference gene primers were always included for all replicates; here, tomato *TIP41* primers were used. The threshold cycle (Ct; where the amplified signal is detected) for each biological replicate was calculated by averaging the Ct of each technical replicate. To calculate the relative

transcript abundance, the Ct value of each gene was divided by the Ct value of the reference gene in that biological sample.

Table 17: RT-qPCR reaction scheme.	
Component:	Amount:
MESA	4 μ L
Forward primer (1 pmol/ μ L)	1 μ L
Reverse primer (1 pmol/ μ L)	1 μ L
cDNA (1:10 diluted)	2 μ L
Total volume:	8 μ L

Table 18: RT-qPCR cycle settings.		
Temperature:	Time:	Repeat:
95 °C	5 minutes	
95 °C	15 seconds	x 39
59 °C	20 seconds	
59-95 °C	0.5 °C increments for melt curve	

2.2.4 Protein based methods:

2.2.4.1 Immunoblotting:

To specifically detect proteins expressed transiently in *N. benthamiana* leaves or in transgenic lines, plant protein extracts were run on denaturing SDS gels and then a western blot conducted using specific antibodies. In more detail, all glass

Table 19: Protein gel composition.		
Component:	Volume for 10% stacking gel:	Volume for 4% stacking gel:
1.5 M Tris pH 8.8	25 mL	-
0.5 M Tris pH 6.8	-	7.5 mL
10 % SDS	1mL	300 μ L
10% APS	1 mL	300 μ L
30% Acrylamide	33 mL	4 mL
TEMED	100 μ L	30 μ L
MilliQ H ₂ O	40 mL	18 mL
Total volume:	100 mL	30 mL

plates were cleaned with 0.5 M NaOH, rinsed with MilliQ water, and then again with 70% ethanol. Then, protein gels were made with 10% separating and 4% stacking gels as per Table 19, and left to fully dry before use (stored at 4 °C if not used immediately for max. 3 days).

Leaf disks (2-4 at 6 mm) were harvested into a 2mL Eppendorf tube containing a ceramic bead, frozen immediately in liquid N₂, then the tissue ground using a TissueLyser II (Qiagen®) for 30 seconds at 22 Hz in adapters pre-cooled to -20 °C. 100 μ L of protein extraction buffer (Table 20) was added to each sample, then vortexed until homogenised and thawed. Then, 40 μ L of 4x loading dye (20 mM Tris-HCl pH 8.6, 40% (v/v) glycerol, 10% (v/v) β -mercaptoethanol, and 0.1% (w/v) bromophenol blue) was added, tubes vortexed again, and finally, the samples were incubated at 90 °C for 10 minutes.

Table 20: Protein extraction buffer for protein gels.	
Component:	Final concentration:
100 mM Tris pH 7.5 (+ 3% SDS)	80 mM (+ 2.4% SDS)
500 mM EDTA pH 6.8	50 mM
25x protease inhibitor solution (cOmplete ULTRA Tablets, Roche)	2.5x strength

Samples were loaded onto protein gels with a marker in one lane (i.e., PageRuler™, ThermoScientific) and run at 170 V for 1 hour 15 minutes or until the dye front ran off the gel. Proteins were transferred to a PVDF membrane (Immobilon®-P, 0.45 μ m pore size) with a

Trans-Blot® Turbo Transfer System (BioRad) using the mixed molecular weight pre-programmed settings. The membrane was then blocked with 5 % milk (in 1x TBST; 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for minimum of 2 hours. A primary antibody to specifically detected proteins of interest in the sample was added to the milk (final dilution of 1:5000) and the blot left to rock gently at 4 °C overnight. The next day, the primary antibody-containing milk solution was removed, and the membrane washed three times with TBST for 10 minutes and once in TBS (TBST with no Tween-20) for 10 minutes, all with gentle rocking. If a secondary antibody was required, this was added at a final dilution of 1:10,000, also in 5 % milk in TBST for at least 2 hours, before the membrane was washed as previously described. The membrane was then ready to be imaged. As all antibodies used in this work were conjugated with a Horseradish Peroxidase (HRP), 500 µL of ECL Select™ Western Blotting Detection Reagent (Amersham™, cytiva) was incubated with the membrane to label the antibodies and an Amersham™ Imager 600 (GE Healthcare Life Sciences) was used to detect and image the membrane. After imaging, membranes were stained for total protein with Ponceau stain (0.1% (w/v) Ponceau S, and 5% (v/v) glacial acetic acid) for ~30 minutes, washed with water and left to dry.

2.2.4.2 Extraction of bands from protein gels for mass spectrometry:

To detect proteins using a mass western, protein gels were cast, proteins samples generated, and gels run as in the previous section (2.2.4.2). To detect proteins within the gel, the gels were incubated with InstantBlue® Coomassie Protein Stain (Abcam) for at least 1 hour and then scanned with some water to obtain an image. Using a light box, specific protein bands or regions of the gel where a protein was expected were cut using a sterile razor blade or scalpel and transferred to an Eppendorf tube, which was then given to the Central Facilities, ZMBP for digestion and analysis.

2.2.4.3 Dual luciferase assays from *N. benthamiana* leaf tissue:

To measure luciferase and renilla luminescence for a dual luciferase assay, a Dual-Luciferase® Reporter Assay System Kit (DLR™, Promega) was used. Initially, 4 or 5 leaf (6 mm) from *N. benthamiana* agroinfiltrated tissue were harvested 2 dpi into 2 mL Eppendorf tubes containing a ceramic ball and immediately frozen in liquid N₂. Samples were ground using a TissueLyser II (Qiagen®) for 30 seconds at 22 Hz in adapters pre-cooled to -20 °C. While keeping tubes on ice as much as possible, 1x Passive Lysis Buffer was added (50 µL per leaf disk) and vortexed until thawed and homogenised. All samples were centrifuged at 11,000 g for 10 minutes at 4

°C to give a clear supernatant. 5 µL of supernatant from each sample was pipetted into 1 well of a white 96 well microplate (Greiner Bio-One International GmbH) and the plate kept cold and covered with foil until ready to measure, which should be done at RT. 40 µL of firefly Luciferase Assay Buffer II (containing the Luciferase Assay Substrate, luciferin) was added to each well and the plate inserted into a TriStar² LB 942 Multimode Reader (Berthold Technologies GmbH & Co.KG) to measure luciferase luminescence using the settings shown in Table 21. After saving the luciferase luminescence data, the 96 well plate was removed at 40 µL of Stop & Glo[®] Reagent (containing the Stop & Glo[®] Substrate) was added to each well. This reagent not only contains the renilla substrate (coelenterazine), but also an agent that quenches all luciferase luminescence. Therefore, only renilla luminescence is detected when the same procedure and settings (Table 21) are used as for luciferase luminescence. For each sample, the luciferase luminescence was divided by the renilla luminescence for normalisation.

Table 21: Luminescence detection settings for the Tristar ² LB 942 Multimode Reader.	
Setting name:	Setting:
Shake	0.5 sec, normal speed, between each well measurement
Measurement mode	Measure by well, moving linearly across plate, 0.1 second count time
Detection settings	Photon counter, no emission filter, endpoint measurement

2.2.4.4 GUS staining of leaf tissue:

GUS stain solution was prepared as per Table 22 and the GUS substrate, X-Gluc, added just before use. Typically, 2-3 leaf disks (6 mm) per biological replicate were taken for GUS staining and placed in a tube containing 250-500 µL GUS staining solution and vacuum infiltrated until all samples were soaked with stain. Samples were wrapped in tin foil and incubated overnight at 37 °C. The next day, the GUS stain solution was removed, 80% ethanol was added, and the samples incubated at 60 °C. The solution was replaced until the tissue became white, and then each replicate was transferred to a plastic sleeve and scanned.

Table 22: GUS staining buffer.		
Component:	Stock concentration:	Final concentration:
Sodium phosphate pH 7 [#]	1 M	100 mM
EDTA	0.5 M	5 mM
Potassium ferrocyanide (II) K ₄ [Fe(CN) ₆] [*]	0.1 M	1 mM
Potassium ferricyanide (III) K ₃ [Fe(CN) ₆] [*]	0.1 M	1 mM
Triton-X-100	10%	0.1%
Notes: [#] Made by mixing 1 M NaH ₂ PO ₄ and 1 M Na ₂ HPO ₄ buffers together; this should be pH 7. [*] Made fresh when making GUS staining buffer. Use MilliQ H ₂ O to top up to final volume. Filter sterilise solution and wrap glass bottle in tin foil and store at 4 °C.		
X-Gluc	2% (0.5 mg/mL in DMSO)	0.05%
Notes: Add X-Gluc into GUS staining solution just before use (i.e., 25 µL of 2% stock to 1 mL GUS stain solution).		

2.2.4.5 Fluorescence microscopy:

To detect fluorophore fluorescence from transiently transformed or transgenic plant material, leaf disks (6 mm) were cut and placed upside on a microscopy slides with water. Typically, samples were analysed using a Laser Scanning Microscope (LSM) 880 confocal microscope (Zeiss Microscopy) using a x40 objective. GFP was detected with an excitation/emission of 488 nm/500-530nm, mCerulean3 with 458 nm/461-516 nm, RFP with 561 nm/579-634 nm, and chlorophyll autofluorescence with 561 nm/653-693nm. An epifluorescent Leica DMI3000 B microscope was also used to detect fluorescence of GFP and RFP using appropriate filters.

2.2.4.6 Fluorescence detection using Typhoon:

To detect GFP fluorescence from whole agroinfiltrated *N. benthamiana* leaves, leaves were placed flat into a Typhoon™ FLA 9500 (GE Healthcare Life Sciences). GFP was excited with the 473 nm laser at 700V and the emission filter BPB1/DBR530DF20 used.

2.2.5 Liquid Chromatography, Mass Spectrometry (LCMS) to detect and quantify proteins and metabolites:

LCMS was used in this work in an attempt to detect ADC peptides, as well as to measure ADC activity and PA-related metabolites based on a method published by Wu et al., 2019. This LCMS method to measure PA-related metabolites was optimised in this work in section 3.2. Below is an outline of the principal of LCMS (section 2.2.5.1; Pitt, 2009), the ADC activity assay and PA measurement method published by Wu et al., 2019 (section 2.2.5.2), and then the optimised processes for sample preparations (sections 2.2.5.3 and 2.2.5.4) and the LCMS method (section 2.2.5.5) used for assays from section 3.3 onwards.

2.2.5.1 An overview of LCMS:

LCMS uses liquid chromatography (LC) to specifically separate molecules, such as metabolites or peptides, based on certain characteristics like molecular weight or polarity, and then quantify their abundance using mass spectrometry (MS). Initially, samples are injected onto a column to separate molecules based on their specific characteristics (i.e., LC), after which the molecules are ionised and vapourised in the ion source of the mass spectrometer (Figure 5). These ionised analytes are then specifically selected in the first quadrupole (Q1) based on their mass to charge ratio (m/z) and only those pre-programmed selected “parent” compounds allowed to pass into the collision cell (Q2) to be fragmented into “daughter ions”. These fragmented ions are selected once more based on their m/z in the third quadrupole (Q3). Their

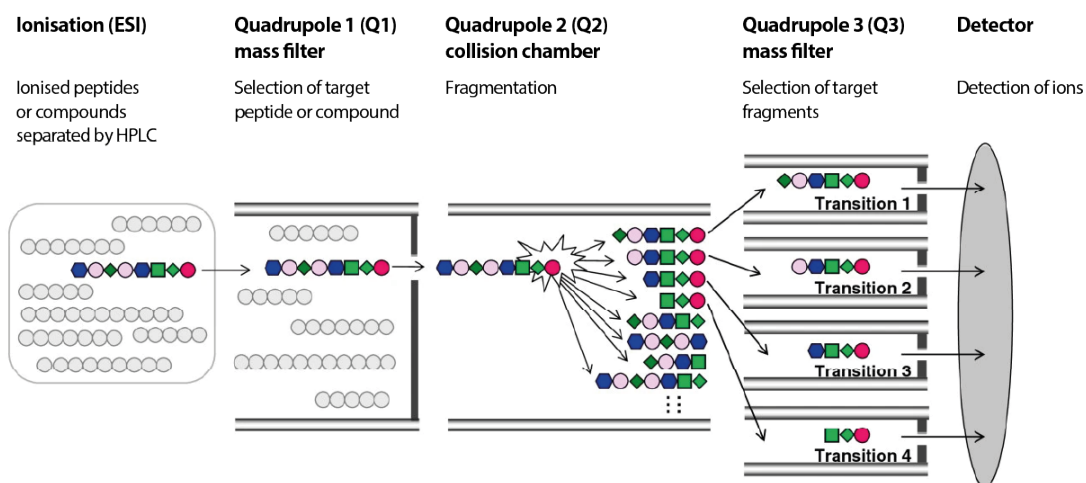


Figure 5: Principal for ionisation, fragmentation, selection, and detection of compounds using LCMS.

Following injection, compounds are ionised, then selected for in the first quadrupole (Q1) based on their molecular mass. Compounds are then fragmented in Q2 and then the fragmented daughter ions are then selected in Q3 before being detected and quantified by the detector. Figure taken from Uchida et al., 2013 and adjusted for this work.

abundance is then detected and displayed as peaks of m/z intensity across LC retention time (minutes).

Using LCMS to specifically detect and quantify peptides (i.e., a bottom-up MS proteomics, also known as a mass western) requires some preparation of the samples prior to injection into the LCMS. Initially, protein samples are digested with a protease, commonly trypsin, to produce peptides that are then separated by size and polarity using LC coupled to MS, capable of MSMS function. As trypsin digests polypeptides at known cleavage sites, the resulting cleaved peptide masses and their m/z ratios can be predicted, even to include common post-translational modifications. Therefore, each m/z peak in an MS mass western can be allocated to specific trypsin-digested peptides, and subsequently used to assess amounts of known proteins within the sample.

In the case of using a targeted LCMS approach to detect and measure levels of certain metabolites, fragmentation transitions of these compounds must be predetermined and programmed into the LCMS. Here, the molar mass of each desired compound is used to for selection in Q1, then the mass of the fragmented daughter ions is used to select which ions are allowed to pass into Q3 and then to be quantified by the detector; such molar masses are termed “transitions”.

A calibration curve is simultaneously run alongside LCMS samples using a series of known concentrations of the certain metabolites or peptides that are aimed to be measured in the assay samples. The calibration curve is used for two purposes. Firstly, to confirm that the predicted transitions of fragmented daughter ions are correct and eluting at the expected retention time.

Secondly, to calculate the concentration of each compound by comparing the m/z peak areas from the samples to those of the calibration curve. Therefore, by using a calibration curve and standards, LCMS can be used to measure the exact concentration of a selection of metabolites or peptides from a complex sample matrix.

2.2.5.2 Overview of the ADC activity assay protocol published by Wu et al., 2019.

The method for LCMS measurement of ADC activity and PAs within the same sample was originally published by Wu et al., 2019 and subsequently used by Gallas et al., 2023. ADC activity is determined as the amount of agmatine produced by ADC within a given time frame.

To differentiate the agmatine already in the plant extract to the agmatine produced within the assay incubation, a stable heavy isotopic variant of arginine is added to the assay buffer and LCMS is used to measure the accumulation of heavy agmatine. Heavy isotopic variants then can be distinguished from the normal variants found in biological tissues as their heavier weight can be selected for in the LCMS Q1 and Q3. Therefore, both ADC activity and PA-related metabolites can be measured within the same plant extract sample.

The Wu et al., 2019 sample preparation and LCMS method is summarised in Figure 6 and is as follows: bacterial-infected plant tissue was frozen in liquid N₂, homogenised in an ADC activity isolation buffer (Table 23; 3 μ L buffer/mg tissue), centrifuged at max speed, and 90 μ L of the supernatant was transferred to a new tube. The supernatant was then mixed with 5 μ L of 1 mM heavy arginine (final concentration of 50 μ M) and incubated for 1 hour at 37 °C. The reaction was stopped via acidification of all proteins with 5 μ L of heptafluorobutyric acid (HFBA), centrifuged again, before being diluted in solvent (water, 0.1% aqueous formic acid, 0.05% HFBA) for LCMS profiling using an Eksptert Micro-LC 200 and QTRAP4000 (ABSciex, USA). Multiple Reaction Monitoring (MRM) response peaks were analysed using

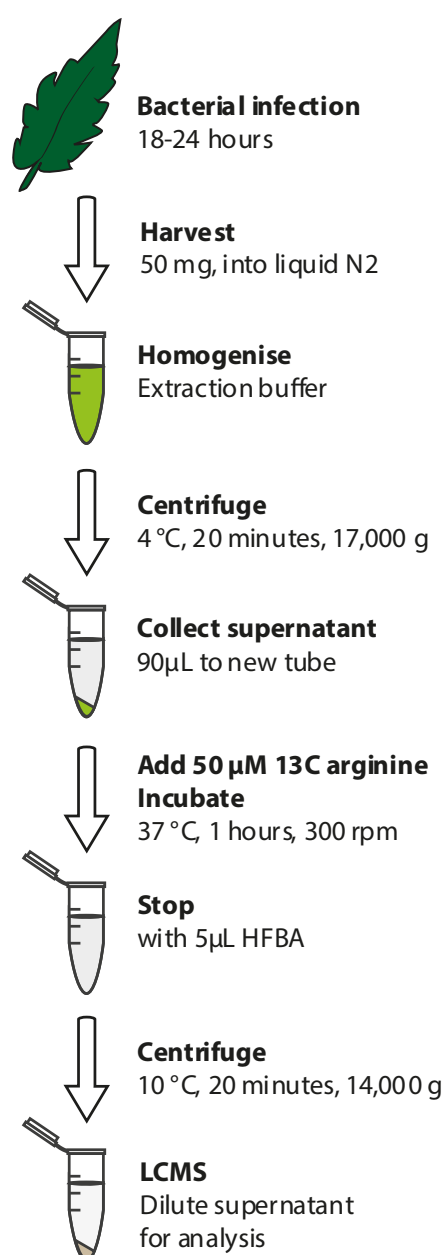


Figure 6: Previously published ADC activity assay protocol by Wu *et al.*, (2019).

Table 23: ADC activity assay buffer components used by Wu *et al.*, 2019

Component	Concentration	Role in ADC extraction buffer
Tris (pH 7.5)	5 mM	Buffers to physiological pH
Ascorbate	1 mM	Antioxidant
Pyridoxal 5'-phosphate (P5P)	50 mM	ADC co-factor
Polyvinylpyrrolidone (PVPP)	5%	Binds polyphenols
Heavy arginine*	50 μ M	Heavy substrate for ADC activity

* After extraction and centrifugation, 90 μ L of the sample supernatant was mixed with heavy arginine.

the MultiQuant software to measure the area under each peak. This area was then used to calculate the concentration of each metabolite by comparing the area to that of the calibration curve, which was run in conjunction to each LCMS experiment. Metabolite concentrations were typically presented as femtomoles per milligram of sample fresh weight (fmol/mg FW).

2.2.5.3 General ADC activity assay and sample preparation for LCMS used in this work:

During this work, the extraction buffer, sample preparation, and LCMS method was optimised from the Wu *et al.*, 2019 method. It is important to note that the extraction buffer (Table 24) used for these assays contains heavy isotopic variants of multiple metabolites, depending on the purpose of the experiment or plant species examined. Specifically, different isotopic variants of arginine were used during this work and are shown in Supplementary Figure 4. D₇ ¹⁵N₄ arginine (+11, 187 g/mol; Eurisotop) was used for tomato, *N. benthamiana*, and *A. thaliana* samples due to its affordability and that it produces heavy putrescine at least 3 g/mol heavier (i.e., D₇ ¹⁵N₂ putrescine (+9), 98.1 g/mol) than the putrescine isotopic variant produced by ODC with the ornithine isotopic variant (¹³C₇ ornithine (+7), g/mol from [Eurisotop] producing ¹³C₆ putrescine (+6) g/mol). Using this D₇ ¹⁵N₄ arginine isotope enables clear distinction between these putrescine variants and allows simultaneous measurement of ADC and ODC activity within the same sample. Additionally, ¹³C₆ arginine (+6, 182 g/mol; Carl Roth®) was used, but only for *M. polymorpha* samples due to a contaminating peak from this plant confounding the D₇ ¹⁵N₄ agmatine (+11) peak. Other isotopic variants used in this buffer were D₅ tryptophan (Sigma-Aldrich®) and ¹³C₁ D₂ citrulline (Eurisotop) as internal standards. For the sample preparation method, 50 mg of fresh weight (FW) tissue was weighed (exact weight recorded), transferred to a 2 mL Eppendorf tube with a ceramic ball, and immediately frozen in liquid N₂. Samples were ground using a TissueLyser II (Qiagen®) for 2x 30 seconds at 22 Hz in adapters pre-cooled to -20 °C. While keeping tubes on ice as much as possible, 300

Table 24: ADC activity assay buffer.			
Component:	Stock concentration:	Final concentration:	Role in ADC extraction buffer
Tris (pH 7.5) + PVPP*	20 mM + 3%	5 mM	Buffers to physiological pH PVPP binds polyphenols
Ascorbate	100 mM	1 mM	Antioxidant
Pyridoxal 5'-phosphate (P5P)	1 mM	50 µM	ADC co-factor
Heavy arginine [#]	10 mM	500 µM	Heavy substrate for ADC activity
¹³ C ₁ D ₂ citrulline	100 µM	2 µM	Allow for quantification of citrulline
D ₅ tryptophan	100 µM	2.5 µM	Internal normalisation control
Protease inhibitor	1 tablet	1x solution	Reduce protein degradation during assay
Footnotes: Stocks and final buffer made up to final volume in sterile MilliQ H ₂ O. 300 µL of buffer is used per sample unless specified otherwise. If ODC activity was also measured, a final concentration of 500 µM ¹³ C ₇ ornithine (+7) was also included in this buffer. * PVPP = Polyvinylpyrrolidone [#] D ₇ ¹⁵ N ₄ arginine (+11) was used for tomato, <i>N. benthamiana</i> , and <i>A. thaliana</i> samples; ¹³ C ₆ arginine (+6) was only used for <i>M. polymorpha</i> samples due to a contaminating peak confounding the D ₇ ¹⁵ N ₄ agmatine (+11) peak.			

µL of extraction buffer (Table 24) was added to each sample, which was then vortexed until thawed and completely homogenised with regular breaks and re-cooling on ice.

To determine the metabolite levels prior to the ADC assay, so-called 'stop samples' were taken, where 100 µL of this extraction was taken and mixed immediately with 5 µL of HFBA to denature any proteins and stop further metabolism occurring; these samples were kept on ice. The remaining 200 µL of sample was incubated at 37 °C for 1 hour with 350 rpm shaking for the ADC activity assay and then transferred back to ice. 5 µL of the assay sample was then transferred to a new tube containing 45 µL of water and used to roughly measure total protein content using the A280 absorbance on the NanoDrop® ND-1000 UV-Vis Spectrophotometer (ThermoFischer Scientific). Then 5 µL of HFBA was mixed into the assay samples to stop any further reactions occurring in these assay samples.

All samples (assay and stop samples) were then centrifuged at maximum speed for 30 minutes at 4 °C. The supernatant could then be diluted in 0.1% formic acid with 0.1% HFBA and used for LCMS analysis. The dilution factor depends on plant species and treatment and must be checked prior to running a whole experiment (i.e., metabolite levels of interest in the sample must be within the linear section of the calibration curve).

2.2.5.4 Additional clean up extraction required for *N. benthamiana* samples.

N. benthamiana tissue requires an additional clean up (section 3.2.12), whereby 80 µL of stopped assay sample (prior to centrifugation step) is taken to a new tube. 200 µL of methanol

and 100 μ L of chloroform was added to each sample, vortexed, then incubated at 25 °C for 10 minutes at 950 rpm. 120 μ L of water was added and samples incubated again for another 10 minutes at 25 °C with 950 rpm shaking. The samples were then centrifuged at maximum speed at RT for 10 minutes. 100 μ L of the supernatant was then taken to a new tube and speed vacuumed for 1 hour to dry. 80 μ L of 0.1% formic acid with 0.1% HFBA was used to dissolve the pellet, before samples were centrifuged again at max speed for 10 minutes at 4 °C. The supernatant could then be used directly or diluted for LCMS analysis.

2.2.5.5 LCMS method for ADC activity and PA-related metabolite detection.

LCMS profiling was conducted using a QTRAP6500+ with an LC M5-TE (Trap-Elute, AB Sciex, USA) as per Gallas et al., 2023, which was based on the Wu et al., 2019 method. 50 μ L were injected from each sample first onto a Trap column (Luna 5 μ m C18(2) 100 Å; 20 x 0.5 mm; Phenomenex) then onto a HaloFused C18 column (150 x 0.5 mm, 2.7 μ m, 90Å; AB Sciex, USA) for chromatographic separation with a binary gradient at a flow rate of 16 μ L/min at 50°C. Gradient: solvent A 0.1% formic acid, 0.05% HFBA; solvent B acetonitrile, 0.1% formic acid, 0.05% HFBA; timing shown in Table 25.

Analytes were ionised using an Optiflow Turbo V ion source with a SteadySpray T microelectrode (10-15 μ L/min) in positive ion mode. The following LCMS instrument settings were applied: nebuliser and heater gas were nitrogen at 25 and 45 psi; curtain gas: nitrogen, 30 psi; collision gas: nitrogen, medium; source temperature: 200 °C; ion spray voltage: +4800 V; entrance potential $\pm$ 10 V; collision cell exit potential $\pm$ 10V; scan time: 10 milliseconds. The transitions monitored for each metabolite presented in this work are shown in Supplementary Table 3.

Table 25: LCMS gradients.		
Time (min):	Solvent A:	Solvent B:
0	85	15
0.2	85	15
1.5	30	70
2.5	5	95
3	5	95
4	85	15
5	85	15

2.2.6 Computational methods and programs used:

2.2.6.1 Sequence analysis:

NCBI BLAST was used to identify and download sequences. CLC Main Workbench (Qiagen) was used for *in silico* cloning, primer design, and sequence analyses. To generate sequence alignment figures used in this work, the Clustal Omega Multiple Sequence Alignment server was used with the ClustalW parameter output selected.

2.2.6.2 Data analysis and figure generation:

Microsoft® Excel for Mac and R Studio (version 12) were used to analyse data. An ANOVA was used with a Tukey's post hoc test to determine statistical significance (to $\alpha = 0.05$) with 3 or more compared groups. When comparing only two groups, a two-way Student's T test was used to determine statistical significance. Typically, in this work boxplots are displayed with letters above each group, where different letters indicate a statistically significant difference. See Supplementary Figure 5 for exemplar R studio script for statistical analysis and generation for boxplots, including all packages used for such analysis. FIJI (ImageJ2, version 2.14.0; Schindelin et al., 2012) was used for processing of confocal microscopy images. Adobe Illustrator and R studio were used to generate figures for this work.

2.2.6.3 Writing and referencing tools:

For writing this work, Microsoft® Word for Mac was used, alongside Clarivate™ EndNote™ 20 for referencing.

3. Results

3.1 CRISPR mutagenesis of conserved *ADC* 5'UTR elements

Previously, Wu et al., 2019 identified the *ADC-box* as a highly conserved GC-rich element in the 5'UTR of plant *ADC* transcripts. Using transient dual reporter assays in *N. benthamiana*, they found that removing part, or the whole *SlADC1-box* increased translation from the *SlADC1* 5'UTR, demonstrating its repressive role on translation of the downstream coding sequence (CDS). However, it remained unclear if this repression also occurred in a native plant context, rather than transient expression, and to what extent each of the conserved GC-rich regions were required for this regulation. Therefore, CRISPR/Cas9 mutagenesis was used in tomato, *A. thaliana*, and *M. polymorpha* to generate a range of stable, inheritable mutations in the *ADC-box* to examine its role on *ADC* translation *in planta* and to determine which conserved regions were most vital for this regulatory mechanism.

Tomato was selected for this work as it is a model Solanaceous species and as various experimental systems (i.e., LCMS-based *ADC* activity assays) and mutant genotypes (*adc1*, *adc2*, *adc1adc2*, *odc1*, and *ebe* mutants) had already been established for tomato in the Lahaye Laboratory (Wu et al., 2019). Additionally, both *SlADC1*- and *SlADC2*-boxes were targeted for mutagenesis as previous transient work had only investigated the *SlADC1-box*.

A. thaliana ADC1- and *ADC2*-boxes were also targeted for CRISPR/Cas9 mutagenesis, as *A. thaliana* is a unique species in terms of PA synthesis. *A. thaliana* does not contain an *ODC*, so all PA precursors must be generated via *ADC* enzymes. However, *AtADC1* has evolved a new enzymatic function, converting N^δ-acetylornithine to N-acetylputrescine, enabling ornithine to once more be used to generate putrescine and compensating for the loss of *ODC* (Lou et al., 2020). *AtADC1* continues in its exceptionality as the 5'UTR structure differs in terms of the sequence variation in the *AtADC1-box* compared to that of *AtADC2* or other closely related species and lack of uORF (Figure 4).

Finally, the primitive *ADC-box* of *M. polymorpha*, a relative of the first land plants, was also mutagenised by CRISPR/Cas9 to determine if its repressive role on *ADC* translation was likely present in the early periods of plant evolution (collaboration with Dr. Ezgi Süheyla Karaaslan in Prof. Chang Liu's Laboratory). *M. polymorpha* was also selected as it only contains a single

ADC, possibly amplifying any impacts of CRISPR/Cas9-induced *MpADC-box* mutations and subsequent PA synthesis (Bowman et al., 2017).

In addition to mutagenesis of the *ADC-box* the tomato *ADC* uORFs were also targeted. The impact of the *SlADC1* uORF had also been previously analysed in transient reporter assays (Wu et al., 2019). Here, it was found that the translational efficiency was only marginally increased when mutating the uORF ATG, indicating that the uORF does not greatly contribute to regulating *ADC* translation. Contrastingly, the carnation (*Dianthus caryophyllus* L.) *DcADC* uORF was suggested to produce a peptide that inhibited downstream translation of *DcADC* in *in vitro* experiments (Chang et al., 2000). It is therefore possible that the role, if any, of the *ADC* uORFs may be better elucidated in the native *in planta* context. Therefore, the of *SlADC1* and *SlADC2* uORFs were targeted by CRISPR/Cas9 mutagenesis to assess their role in *SlADC* translation *in planta*.

The sections below present the mutagenesis and genotyping of CRISPR/Cas9 generated mutants in tomato (section 3.1.1), *A. thaliana* (section 3.1.2), and *M. polymorpha* (section 3.1.3).

3.1.1 CRISPR mutagenesis of tomato *ADC* uORFs and *ADC-boxes*.

To assess the impact of the conserved elements on *SlADC* translation *in planta*, CRISPR/Cas9 was used to generate mutations in the *SlADC1* and *SlADC2* uORFs and *ADC-boxes*. Single gRNAs were designed to individually target each of these elements in each gene, aiming to generate small INDELs in the targeted element through a single Cas9 cleavage event (Figure 7A). CRISPRdirect (crispr.dbcls.jp; Naito et al., 2015) was used to design gRNAs based on the tomato genome (Sl3.00 from 2017) and an NGG PAM recognition site. gRNAs were selected based on their predicted cleavage site and if they had only one predicted target site in the selected genome. Each gRNA was then cloned in duplicate into a modified version of the *p201N Cas9* vector, using the CRISPR/Cas9 golden-gate toolkit (Jacobs *et al.*, 2015; modified by Morbitzer, unpublished; Figure 7; Supplementary Figure 6; Supplementary Table 4) and subsequently transformed into *A. tumefaciens* GV3101. Single colonies were selected, infiltrated into *N. benthamiana* leaves at OD₆₀₀ = 0.4 (a process termed ‘agroinfiltrated’; section 2.2.1.5), and at 3 dpi, tested for nuclear fluorescence via Typhoon imaging (section 2.2.4.6). Positive GFP expression indicated that the T-DNA was successfully transferred (Supplementary Figure 7) and the *CRISPR/Cas9* construct in that *A. tumefaciens* strain was suitable for stable tomato transformation (section 2.2.1.4).

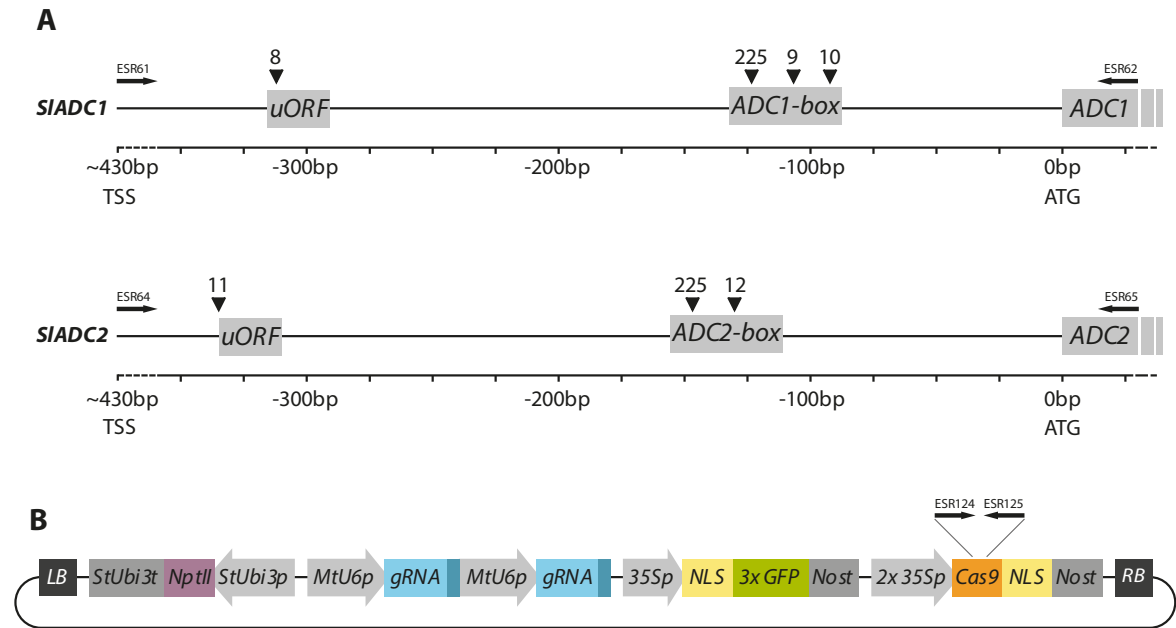


Figure 7: CRISPR/Cas9 mutagenesis of *SLADC* 5'UTR conserved elements.

(A) Schematic of *SLADC1* (top) and *SLADC2* (bottom) 5'UTR genomic sequence highlighting the *uORF*, *ADC-boxes*, and start of the *SLADC* coding sequences (grey boxes), and transcriptional start site (TSS) determined by Wu *et al.*, 2019. Scale below indicates base pairs (bp) relevant to each *ADC* start codon. Expected CRISPR/Cas9 cleavage site determined by each gRNA is shown above the 5'UTR elements (▼); note that gRNA225 targets Brg11 binding site in both *SLADC1* and *SLADC2* *ADC-boxes* (Wu *et al.*, 2019). Black arrows show primers used for genotyping. (B) CRISPR/Cas9 plasmid (p201N Cas9; Jacobs *et al.*, 2015, modified by Morbitzer, unpublished) used for WT tomato transformation, including: neomycin *in planta* resistance gene (*NptII*, mauve box) with *Solanum tuberosum* Ubiquitin 3 (*StUbi3*) promoter and terminator (grey arrow and box, respectively); *Medicago truncatula* U6 (*MtU6*) promoter driving identical gRNAs (blue box) in duplicate with scaffold spacer sequence (mid-grey box); Cauliflower mosaic virus 35S promoter (*35Sp*) driving triple *GFP* (green box) with SV40 nuclear localisation signal (*NLS*) and *Nos* terminator (*Nost*); double *35Sp* driven *Cas9* with *NLS* and *Nost*; and, left (*LB*) and right borders (*RB*) (dark grey box). Black arrows show primers used for genotyping presence of *Cas9* transgene.

One exception to this workflow was the *gRNA225* CRISPR/Cas9 construct, which was previously generated to target the conserved Brg11 *EBE* sequence in both *SLADC1* and *SLADC2*, generating the *ebe* mutant (Figure 7; Supplementary Table 4; Wu *et al.*, 2019). This construct was re-transformed into tomato to generate more mutants in the 5' end of both *SLADC-boxes*.

Following selection on kanamycin-containing media, all T0 surviving tomatoes were transferred to soil and gDNA was extracted (section 2.2.3.5). To verify that these surviving plants contained the CRISPR/Cas9 transgene, a diagnostic PCR amplifying a section of *Cas9* was conducted using primers ESR124 and ESR125 on extracted gDNA (Figure 8A; Supplementary Figure 6). Almost all plants contained the transgene; any that did not were discarded.

To determine if a mutation had occurred in the gRNA-targeted region, fragments of the genomic *SLADC1* and/or *SLADC2* 5'UTR sequence were PCR amplified using primers ESR61 and ESR62 and/or ESR64 and ESR65, respectively. PCR products were run on a 1% agarose gel to detect larger INDELs and to verify their quality prior to Sanger sequencing with primers ESR63 or ESR66 for *SLADC1* or *SLADC2*, respectively (Figure 8B). If a conflict occurred in a gRNA-targeted region when aligning the sequenced T0 line to the WT reference, this plant was

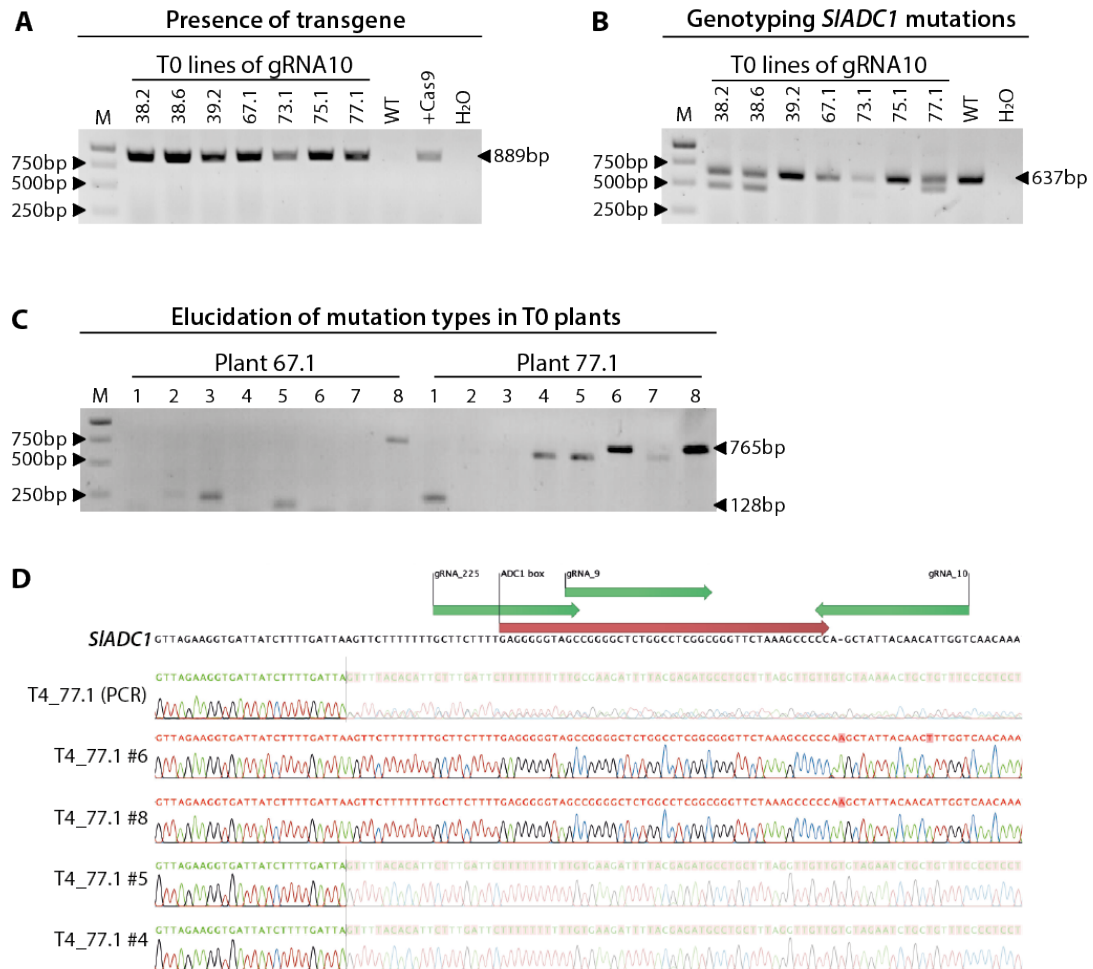


Figure 8: *SLADC1*-box mutant with large 108bp deletion identified by genotyping T0 tomatoes for CRISPR/Cas9-mediated mutations.

Plant T4_77.1 from cali transformed with gRNA10 CRISPR/Cas9 construct is shown here as an exemplar of the T0 genotyping process. (A) gDNA from T0 neomycin-resistant tomatoes was checked for the presence of the transgene (expected size 899bp); a previously confirmed transgene containing tomato was used as positive control (+Cas9), while WT and H₂O serve as negative controls. (B) As gRNA10 targets the *SLADC1*-box, part of the *SLADC1* promoter and coding sequence was amplified and run on a 1% agarose gel; WT gDNA shows the expected unmutated fragment size of 637bp; H₂O serves as negative control. (C) PCR fragments amplified from gDNA (B) were cloned into a pUC57 backbone and transformed into *E. coli*, after which a colony PCR was used to identify clones containing different mutations; insertion of WT fragment produces 765bp product and no insert produces 128bp product. (D) The initial gDNA PCR fragment (B) and a selection of clones (C) were sent for Sanger sequencing to determine the exact mutations and confirm the zygosity. Sanger sequencing reads were aligned to *SLADC1* WT reference, here annotated with the *SLADC1*-box (red arrow) and the gRNA target sequences (green arrow). Conflicts are highlighted in red or faded out when the sequence does not match the reference.

considered mutagenised; plants with no mutation were discarded. Based on this sequence information, the zygosity of the mutations could be predicted; a Sanger sequencing trace with clear individual peaks that do not match to the WT indicate a homozygous genotype, while heterozygous mutants show a single peak trace matching to the WT reference until the start of the mutation, after which double peak traces are seen, indicating a mix of two different sequences. To verify the zygosity and exactly which mutations occurred, both alleles needed to be individually sequenced. This was done by cloning PCR products into a pUC57 vector and selecting colonies containing different alleles to then be sent for Sanger sequencing (Figure 8C). In Figure 8, the genotyping process of an exemplar plant (T4_77.1) has been shown. In this case, the initial gDNA PCR showed two bands, one around the same as WT and the other ~100 bp smaller (Figure 8B), indicating that two alleles exist. This was then confirmed via Sanger sequencing of four cloned PCR fragments, whereby two different mutated genotypes were found: firstly, a 1bp insertion after the *SIADC1-box* (clones #6 and #8); and a 108bp deletion (clones #4 and #5; Figure 8C and D). This type of analysis was carried out for majority of the mutated plants, at least until a range of mutant genotypes were resolved. gRNA9 and gRNA12 did not produce any mutations, despite the T0 surviving plants containing the transgene; all transformants were discarded.

A full list of all analysed T0 mutants are shown in Supplementary Table 5 (gRNA8), Supplementary Table 6 (gRNA10), Supplementary Table 7 (gRNA11), and Supplementary Table 8 (gRNA 225). Table 26 shows the number of WT and mutant plants of each CRISPR/Cas9 transformation with a single gRNA target, including the predicted mutant zygosity based on the gDNA PCR sequencing and/or subsequent sequencing of clones.

Table 26: Zygosity of T0 tomato plants containing the CRISPR/Cas9 transgene with a single gRNA target.					
	gRNA8	gRNA9	gRNA10	gRNA11	gRNA12
<i>SIADC1 5'UTR</i>					
Homozygous	2	0	13		
Heterozygous	18	0	68		
Unsure	1	0	11		
Total	21	0	92		
<i>SIADC2 5'UTR</i>					
Homozygous				9	0
Heterozygous				23	0
Unsure				6	0
Total				38	0
WT	43	34	30	15	21
Total plants genotyped	64	34	122	38	21

Table 27: Zygosity of T0 tomato plants containing the CRISPR/Cas9 transgene where both <i>SIADC</i> are targeted by gRNA225.				
	Only <i>SIADC1</i> mutated	Only <i>SIADC2</i> mutated	Both <i>SIADC</i> mutated	Total mutations
<i>SIADC1</i> 5'UTR				
Homozygous	0		7	7
Heterozygous	7		22	29
Unsure	1		5	6
Total	8		34	42
<i>SIADC2</i> 5'UTR				
Homozygous		5	0	5
Heterozygous		18	29	47
Unsure		1	5	6
Total		24	34	58
WT				102
Total plants genotyped				168

Table 27 shows the same information for the gRNA225 CRISPR/Cas9 transformation with both *SIADC1* and *SIADC2* genotypes. Interestingly, several homozygous mutant genotypes were observed, which may have occurred if one mutant allele was used as a template for homology-directed repair of the other allele. It is also interesting to note that the same mutations were frequently observed from plants originating from the same cali; for example, cali number 22 from the gRNA10 CRISPR/Cas9 transformation (denoted as: T5_22) produced three T0 plants that all had the same homozygous 3bp deletion. This indicates that multiple plantlets may have developed from the same CRISPR/Cas9-mutated cali tissue. However, this was not always the case; for example, T5_15 cali produced three T0 plants with different mutations and zygositys (Supplementary Table 7).

Overall, a wide range of mutations were obtained in each of the targeted *SIADC* elements, including both large deletions and small SNPs in the *SIADC-boxes*, as well as mutations spanning the uORF start codons in either *SIADC1* or *SIADC2* (e.g., T3_13.1 and T5_27.1, respectively). All T0 plants that contained a mutation were kept for seed production, except those where the exact same mutation had already been identified in 2-3 other plants. The vast majority of T0 plants were able to produce seed; however, a small number either did not produce fruits or the fruits were always small and contained no seeds.

To study the impact of the 5'UTR mutations on *SIADC* translation, mutations should ideally be homozygous to fully elucidate the impact on subsequent translation of *SIADC* and to ensure that the mutation cannot be segregated out in subsequent generations. It is also preferable that the mutant plants no longer contain the T-DNA transgene for multiple reasons. Firstly, T-DNA insertion into important loci may result in disruption of developmental or physiological

processes, resulting in adverse effects that are not caused by the mutation in *SLADC*; this may have been why some T0 CRISPR/Cas9-mutagenised lines were unable to produce fruits and/or seeds. Secondly, the presence of an active CRISPR/Cas9 may induce further mutations in any un-mutated sequence that the gRNA targets; for example, a WT allele is still able to be mutated in any cell expressing the gRNA-CRISPR/Cas9 T-DNA. This is particularly problematic when crossing a CRISPR/Cas9-containing plant with another un-mutated line. Finally, removal of the transgene is also critical to ensure that the CRISPR-induced mutation is an inheritable mutation in the germline, rather than a somatic mutation.

As the CRISPR/Cas9 transgene and induced mutation are typically at distinct genomic loci, mendelian genetics should enable the identification of homozygous mutant plants that do not contain the transgene via genotyping of T1 plants. Based on the mutation type and zygosity information from T0 genotyping, a collection of mutants was selected for subsequent genotyping in the T1 generation. Figure 9 presents an example of the genotyping process for a T1 generation mutant: T4_77.1, which had a 108bp deletion and a 1bp insertion in the T0 generation. T1 progeny of selected T0 plants were initially genotyped for the presence of the transgene via PCR amplification of a section of the *Cas9* gene using primers ESR124 and ESR125 from extracted T1 gDNA; a known transgene containing T0 line was used as a positive control (Figure 9A). Initially, 60 T1 progeny plants from each selected T0 parent were genotyped for the presence of the transgene. For subsequent rounds of T1 genotyping, 20 plants were genotyped, and if no transgene-free plants were identified, a following 20 T1 progeny plants were tested.

During cali transformation, the transgene is inserted into one chromosome copy in somatic cells, which then produce a T0 transformant. If a single transgene insertion event has occurred in the T0 parent, T1 progeny are expected to segregate for the transgene in a 1:2:1 fashion, according to Mendelian genetics (i.e., one quarter of the progeny would lack the transgene). The T1 plants lacking a transgene were used for subsequent analysis, while those containing the transgene were discarded. However, some T0 plants had presumably more than one transgene insertion, as an unexpectedly high frequency (>75%) of transgene-containing T1 plants observed, or in some cases, none were identified. If two transgene insertions occur during cali transformation, this reduces the frequency of transgene-free T1 progeny to 1 in 16 plants; for three insertions, only 1 in 64 plants should lack the transgene. For example, this was the case for T1 progeny of T10_39.2, where no transgene-free progeny was found from 60 tested individuals; in cases such, all progenies were discarded, and another similar mutant was chosen instead.

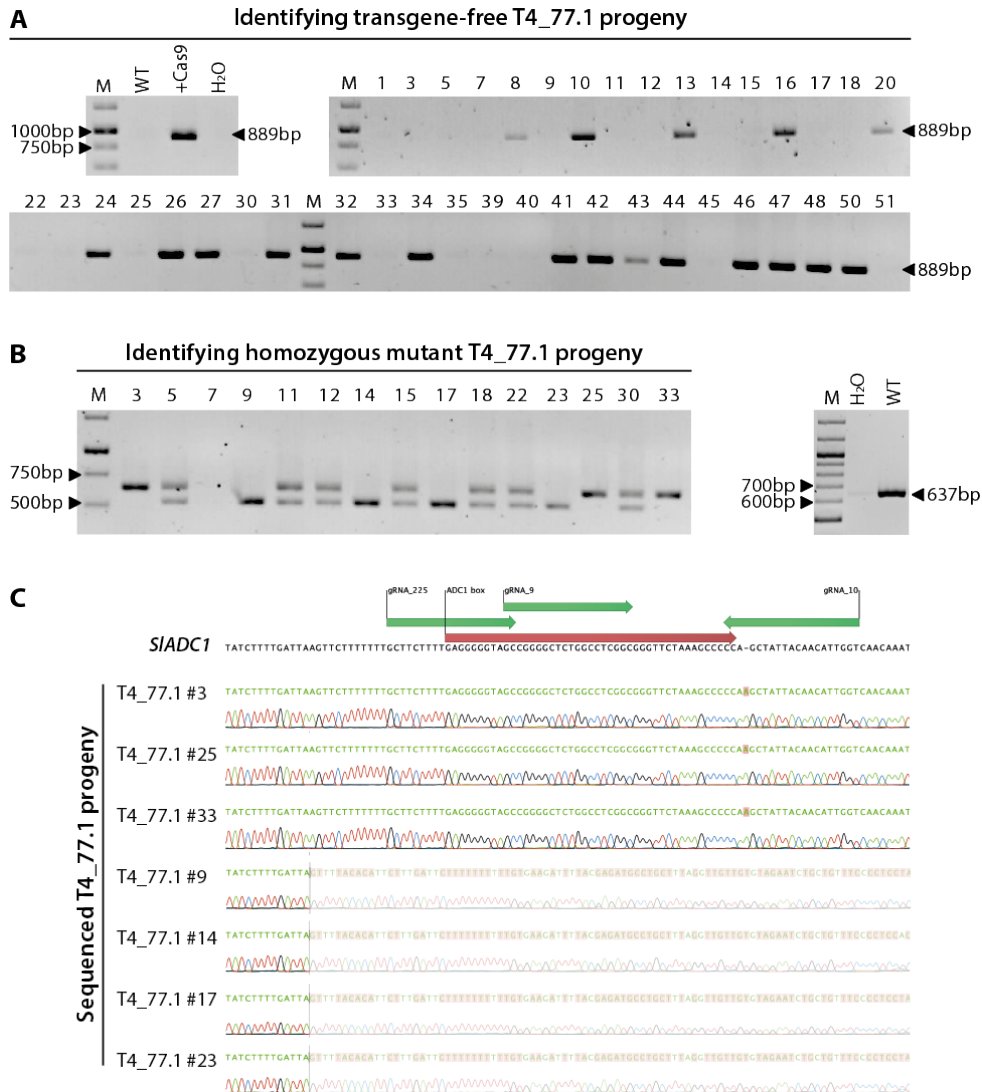


Figure 9: Identification of transgene-free, homozygous mutant plants in the T1 generation.

Plant T4_77.1 is shown here as an exemplar of the T1 genotyping process. (A) The presence of the transgene detected via diagnostic PCR using gDNA of T4_77.1 T1 progeny (expected size 899bp); a previously confirmed transgene containing tomato was used as positive control (+Cas9), while WT and H₂O serve as negative controls. (B) To identify homozygous mutants, gDNA of T1 progeny that did not produce a transgene-positive PCR product was used to amplify part of the *SIADC1* promoter and coding sequence and products were run on a 1% agarose gel (expected WT unmutated fragment size 637bp); H₂O serves as negative control. (C) PCR fragments were sent for Sanger sequencing to determine the exact mutations and confirm the zygosity; reads were aligned to *SIADC1* WT reference, here annotated with the *SIADC1*-box (red arrow) and the gRNA target sequences (green arrow). Conflicts are highlighted in red or faded out when the sequence does not match the reference at that location.

For all T1 progeny lacking the transgene, the CRISPR/Cas9-induced mutation in the genomic 5'UTR sequence of *SIADC1/2* was genotyped in a similar manner to that for the T0 plants. Again, due to Mendelian segregation, one quarter of the transgene-free T1 progeny should be homozygous for mutations that were heterozygous in the T0 generation. The gRNA targeted *SIADC1* or *SIADC2* 5'UTR region was amplified from extracted gDNA, run on an agarose gel (Figure 9B), and sent for Sanger sequencing to confirm the mutation zygosity (Figure 9C).

Identification of such transgene-free T1 progeny containing the CRISPR-induced mutation also shows that this mutation is present in the germline, and not a somatic mutation; all such T1 progeny were kept for seed production.

Wu *et al.*, 2019 generated a tomato line with CRISPR/Cas9-induced mutations in the *Brg11-EBE* sequence of both *SIADC1* and *SIADC2* to prevent Brg11 binding and transcriptional activation of *SIADC1/2*. This plant has a 1bp insertion in the *SIADC1-box* and a 4bp deletion in the *SIADC2-box* and did not contain the transgene. To elucidate the impact of each of these mutations separately, this line was crossed to WT and the F1 generation was genotyped to ensure the crossing was successful (Dousheng Wu, unpublished). In this work, the F2 generation was then genotyped to identify plants with either of the mutations in a homozygous configuration using the same method described above: PCR amplification of *SIADC1* and *SIADC2* 5'UTRs using primers ESR61 and ESR62, and ESR64 and ESR65, respectively, followed by Sanger sequencing of the PCR product (Figure 10). From this analysis, plant F2a55 was identified to be homozygous for only the 1bp insertion in *SIADC1-box*, while F2a14 was identified to be homozygous for the 4bp deletion in the *SIADC2-box*; these plants were kept for seed production and the progeny used for subsequent experiments.

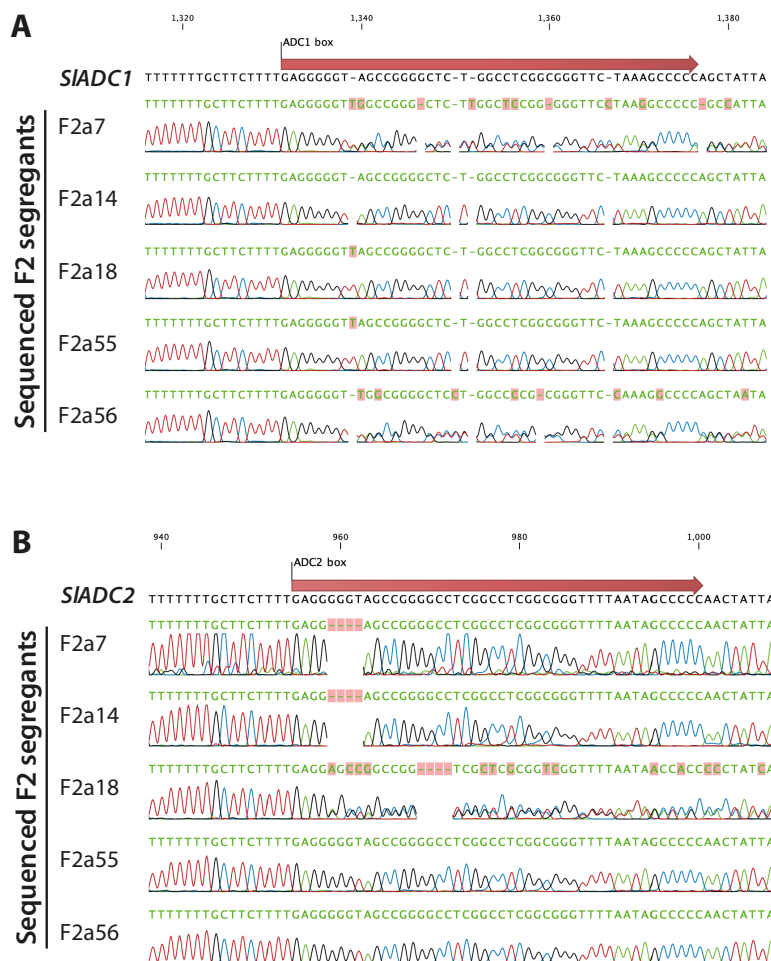


Figure 10: Identification of homozygous single *ebe* mutants.

gDNA from F2 progeny of the WT to *ebe* mutant crossing was used to amplify both *SIADC1* and *SIADC2* promoters to be sent for Sanger sequencing. Reads were aligned to WT *SIADC1* (A) and *SIADC2* (B) sequences to identify homozygous single mutants (i.e., one WT allele and one homozygous mutated allele); F2a14 and F2a55 were selected as such mutants.

Table 28: Tomato ADC-box and uORF CRISPR/Cas9 mutant names used in this work

Original name	Origin of plant	Name used in this thesis	Mutation
T4_2	gRNA10 CRISPR/Cas9 transformation (this work)	SI1C_-6	6 bp deletion 3' to <i>SIADC1</i> -box
F2a55	<i>ebe</i> mutant from Wu <i>et al.</i> , 2019 was segregated (this work)	SI1M_+1	1 bp insertion in <i>SIADC1</i> -box
T4_77.1	gRNA10 CRISPR/Cas9 transformation (this work)	SI1M_-108	108 bp deletion of entire <i>SIADC1</i> -box
T3_35.1	gRNA8 CRISPR/Cas9 transformation (this work)	SI1M_-7	7 bp deletion spanning <i>SIADC1</i> uORF ATG
T3_13.1	gRNA8 CRISPR/Cas9 transformation (this work)	SI1M_-127	127 bp deletion of <i>SIADC1</i> uORF
F2a14	<i>ebe</i> mutant from Wu <i>et al.</i> , 2019 was segregated (this work)	SI2M_-4	4 bp deletion in <i>SIADC2</i> -box
T10_26.2	gRNA225 CRISPR/Cas9 transformation (this work)	SI2M_-13	13 bp deletion in <i>SIADC2</i> -box
T5_27.1	gRNA11 CRISPR/Cas9 transformation (this work)	SI2M_-14	1 bp SNP and 14 bp deletion spanning <i>SIADC2</i> uORF ATG
T5_33.2	gRNA11 CRISPR/Cas9 transformation (this work)	SI2M_-172	172 bp deletion of <i>SIADC2</i> uORF
Footnotes: The CRISPR/Cas9 mutant names used in this thesis take the form: 1. “C”, where the mutation occurs outside of a conserved 5'UTR element (i.e., control), and “M”, where a mutation affects a conserved element (i.e., mutant) 2. Either “SI1” or “SI2”, corresponding to <i>SIADC1</i> or <i>SIADC2</i> 3. The number of base pairs that have been affected by this mutation are indicated			

A summary of *SIADC1* and *SIADC2* CRISPR/Cas9 homozygous mutants, lacking the *Cas9* transgene, that were used for subsequent experiments in this thesis is shown in Table 28. Here, these mutants were renamed to reflect their mutation, rather than the transformed plantlet number used up until this point. An *SIADC1*-box mutant was identified lacking the complete *SIADC1*-box due to a 108 bp deletion; this mutant is now called “SI1M_-108” (originally T4_77.1). A corresponding transgene-free mutant lacking the complete *SIADC2*-box was not identified; instead, a 13 bp deletion of the 5' half of the *SIADC2*-box was used (originally plant T10_26.2, now called “SI2M_-13”). Smaller single mutants in the *SIADC1*- and *SIADC2*-boxes were generated by segregating the *ebe* mutant from Wu *et al.*, 2019 (“SI1M_+1” and “SI2M_-4”, respectively). Finally, a mutant with a small 6 bp deletion, 3' to the *ADC1*-box was also used as a CRISPR/Cas9-induced mutation outside of the conserved *ADC*-box region (originally plant T4_2, now called “SI1C_-6). For both *SIADC1* and *SIADC2*, small and large deletion mutants of their uORFs were identified, all lacking the uORF start codon (i.e., “SI1M_-7” and


```

WT      TCATTTTCGCAGTTGTTTACAGTTTTCCTAAACGGAACCTACCATCTCTTTTCCGGTT
S11M_-7 TCATTTTCGCAGTTGTTTACAGTTTTCCTAAACGGAACCTACCATCTCTTTTCCGGTT
S11M_-127 TCATTTTCGCAGTTGTTTACAGTTTTCCTAAACGGAACCTACCATCTCTTTTCCGGTT
S11C_-6   TCATTTTCGCAGTTGTTTACAGTTTTCCTAAACGGAACCTACCATCTCTTTTCCGGTT
S11M_-108 TCATTTTCGCAGTTGTTTACAGTTTTCCTAAACGGAACCTACCATCTCTTTTCCGGTT
S11M_+1   TCATTTTCGCAGTTGTTTACAGTTTTCCTAAACGGAACCTACCATCTCTTTTCCGGTT
*****

WT      ACCGTTTAGGTTTCCGACCAATTTCTTCTTCCGGTGAACATTCGGTTAAAGTTG
S11M_-7  ACCGTTTAGGTTTCCGACCAATTTCTTCTTCCGGTGAACATTCGGTTAAAGTTG
S11M_-127 ACCGTTTAGGTTTCCGACCAATTTCTTCTTCCGGTGAACATTCGGTTAAAGTTG
S11C_-6   ACCGTTTAGGTTTCCGACCAATTTCTTCTTCCGGTGAACATTCGGTTAAAGTTG
S11M_-108 ACCGTTTAGGTTTCCGACCAATTTCTTCTTCCGGTGAACATTCGGTTAAAGTTG
S11M_+1   ACCGTTTAGGTTTCCGACCAATTTCTTCTTCCGGTGAACATTCGGTTAAAGTTG
*****

WT      TATGGTGGGGTGTTTGTTTGAATCTTAGGT TTGTGTTTCTGTGGTTTGTAGTTTAAATT
S11M_-7  GTGGGGTGTTTGTTTGAATCTTAGGT TTGTGTTTCTGTGGTTTGTAGTTTAAATT
S11M_-127 GTGGGGTGTTTGTTTGAATCTTAGGT TTGTGTTTCTGTGGTTTGTAGTTTAAATT
S11C_-6   TATGGTGGGGTGTTTGTTTGAATCTTAGGT TTGTGTTTCTGTGGTTTGTAGTTTAAATT
S11M_-108 TATGGTGGGGTGTTTGTTTGAATCTTAGGT TTGTGTTTCTGTGGTTTGTAGTTTAAATT
S11M_+1   TATGGTGGGGTGTTTGTTTGAATCTTAGGT TTGTGTTTCTGTGGTTTGTAGTTTAAATT

WT      CTTTGTGTTGATTTAGAAAATTTGCTCTTTCGAAAGTCGTTGTGGGGATATTCGGGTCG
S11M_-7  CTTTGTGTTGATTTAGAAAATTTGCTCTTTCGAAAGTCGTTGTGGGGATATTCGGGTCG
S11M_-127 CTTTGTGTTGATTTAGAAAATTTGCTCTTTCGAAAGTCGTTGTGGGGATATTCGGGTCG
S11C_-6   CTTTGTGTTGATTTAGAAAATTTGCTCTTTCGAAAGTCGTTGTGGGGATATTCGGGTCG
S11M_-108 CTTTGTGTTGATTTAGAAAATTTGCTCTTTCGAAAGTCGTTGTGGGGATATTCGGGTCG
S11M_+1   CTTTGTGTTGATTTAGAAAATTTGCTCTTTCGAAAGTCGTTGTGGGGATATTCGGGTCG
*****

WT      TGTAAAGAGTGTGTAGAAAGGTGATTAATCTTTTGATTAGTTCTTTTTCGCTCTTTTGA
S11M_-7  TGTAAAGAGTGTGTAGAAAGGTGATTAATCTTTTGATTAGTTCTTTTTCGCTCTTTTGA
S11M_-127 TGTAAAGAGTGTGTAGAAAGGTGATTAATCTTTTGATTAGTTCTTTTTCGCTCTTTTGA
S11C_-6   TGTAAAGAGTGTGTAGAAAGGTGATTAATCTTTTGATTAGTTCTTTTTCGCTCTTTTGA
S11M_-108 TGTAAAGAGTGTGTAGAAAGGTGATTAATCTTTTGATTAGTTCTTTTTCGCTCTTTTGA
S11M_+1   TGTAAAGAGTGTGTAGAAAGGTGATTAATCTTTTGATTAGTTCTTTTTCGCTCTTTTGA
*****

WT      GGGGGT-AGCCGGGCTCTGGCCTCGCGGGTTC-TAAAGCCCCAGCTATTACAACATG
S11M_-7  GGGGGT-AGCCGGGCTCTGGCCTCGCGGGTTC-TAAAGCCCCAGCTATTACAACATG
S11M_-127 GGGGGT-AGCCGGGCTCTGGCCTCGCGGGTTC-TAAAGCCCCAGCTATTACAACATG
S11C_-6   GGGGGT-AGCCGGGCTCTGGCCTCGCGGGTTC-TAAAGCCCCA-----ACAACATG
S11M_-108 GGGGGT-AGCCGGGCTCTGGCCTCGCGGGTTC-TAAAGCCCCA-----ACAACATG
S11M_+1   GGGGGT-AGCCGGGCTCTGGCCTCGCGGGTTC-TAAAGCCCCAGCTATTACAACATG

WT      GTCACAAATCATTTCTGTATAATTAGTTTACACATCTTTGATTCCTTTTGTG
S11M_-7  GTCACAAATCATTTCTGTATAATTAGTTTACACATCTTTGATTCCTTTTGTG
S11M_-127 GTCACAAATCATTTCTGTATAATTAGTTTACACATCTTTGATTCCTTTTGTG
S11C_-6   GTCACAAATCATTTCTGTATAATTAGTTTACACATCTTTGATTCCTTTTGTG
S11M_-108 GTCACAAATCATTTCTGTATAATTAGTTTACACATCTTTGATTCCTTTTGTG
S11M_+1   GTCACAAATCATTTCTGTATAATTAGTTTACACATCTTTGATTCCTTTTGTG
*****

WT      AAGATTTACGAGATGCCGCTTAGGTGTGTGTAGAACTGCTGTTCCC...
S11M_-7  AAGATTTACGAGATGCCGCTTAGGTGTGTGTAGAACTGCTGTTCCC...
S11M_-127 AAGATTTACGAGATGCCGCTTAGGTGTGTGTAGAACTGCTGTTCCC...
S11C_-6   AAGATTTACGAGATGCCGCTTAGGTGTGTGTAGAACTGCTGTTCCC...
S11M_-108 AAGATTTACGAGATGCCGCTTAGGTGTGTGTAGAACTGCTGTTCCC...
S11M_+1   AAGATTTACGAGATGCCGCTTAGGTGTGTGTAGAACTGCTGTTCCC...
*****

```

Figure 11: Alignment of CRISPR/Cas9-induced mutations in tomato *ADC1* 5'UTR sequence.

Tomato *ADC1* 5'UTR sequences and part of the CDS were aligned using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) to display the CRISPR/Cas9-induced mutations. Deletions are shown as red dashes; insertions or substitutions shown in bolded red text; *ADC-box* is shaded black with bolded white text; *uORF* and start of *ADC CDS* are shaded grey with bolded black text; start and stop codons are underlined. An asterisk under the alignment indicates that all sequences match.

“S11M_-127” for *SlADC1* uORF, and “S12M_-14” and “S12M_-172 for *SlADC2* uORF). Prior to any further experiments, T2 generation plants were genotyped again to ensure the mutation is as expected for that line and homozygous. The exact mutations are shown in alignments in Figure 11 and Figure 12 for *SlADC1* and *SlADC2* mutants, respectively.

To determine the overall impact of the uORFs and *ADC-boxes* on ADC translation and PA metabolism in tomato, crosses of various mutants were generated. It is possible that a

```

WT      GCCACGGATTTTAGCCCTATATAAAGCGCTTCGTCTCATGCAATCTCGCTCACCTCAT
S12M_-14 GCCACGGATTTTAGCCCTATAAAGCGTCTCGCTCATGCAATCTCGCTCACTCAT
S12M_-172 GCCACGGATTTAGCCCTATAAAGCGTCTCGCTCATGCAATCTCGCTCACTCAT
S12M_-13  GCCACGGATTTTAGCCCTATAAAGCGTCTCGCTCATGCAATCTCGCTCACTCAT
S12M_-4   GCCACGGATTTTAGCCCTATAAAGCGTCTCGCTCATGCAATCTCGCTCACTCAT
*****

WT      AAGCTGTAGTTTCAAGTTCCTTCGCAATTTCCATTACAGCGCTAGGTTCCGTCAC
S12M_-14 AAGCTGTAGTTTCAAGTTCCTTCGCAATTTCCATTACAGCGCTAGGTTCCGTCAC
S12M_-172 AAGCTGTAGTTTCAAGTTCCTTCGCAATTTCCATTACAGCGCTAGGTTCCGTCAC
S12M_-13  AAGCTGTAGTTTCAAGTTCCTTCGCAATTTCCATTACAGCGCTAGGTTCCGTCAC
S12M_-4   AAGCTGTAGTTTCAAGTTCCTTCGCAATTTCCATTACAGCGCTAGGTTCCGTCAC

WT      TTCTTCTCTCCATTAAATCTGTATGGTGTCTCTCTTTTGAATCTAGATTTTCT
S12M_-14 TTCTTCTCTCCATTAAATCTGTATGGTGTCTCTCTTTTGAATCTAGATTTTCT
S12M_-172 TTCTTCTCTCCATTAAATCTGTATGGTGTCTCTCTTTTGAATCTAGATTTTCT
S12M_-13  TTCTTCTCTCCATTAAATCTGTATGGTGTCTCTCTTTTGAATCTAGATTTTCT
S12M_-4   TTCTTCTCTCCATTAAATCTGTATGGTGTCTCTCTTTTGAATCTAGATTTTCT

WT      TTGAATTCTTGTAAATCTTAAATTTTCTTGTTTAAATCAAGAAATAGCTTCTCTGAA
S12M_-14 TTGAATTCTTGTAAATCTTAAATTTTCTTGTTTAAATCAAGAAATAGCTTCTCTGAA
S12M_-172 TTGAATTCTTGTAAATCTTAAATTTTCTTGTTTAAATCAAGAAATAGCTTCTCTGAA
S12M_-13  TTGAATTCTTGTAAATCTTAAATTTTCTTGTTTAAATCAAGAAATAGCTTCTCTGAA
S12M_-4   TTGAATTCTTGTAAATCTTAAATTTTCTTGTTTAAATCAAGAAATAGCTTCTCTGAA
*****

WT      GTGAGGGTGGGATTTTGGATCGTGTCAAAGTGTGTAGAGGGTGAATATCTTTTGAT
S12M_-14 GTGAGGGTGGGATTTTGGATCGTGTCAAAGTGTGTAGAGGGTGAATATCTTTTGAT
S12M_-172 GTGAGGGTGGGATTTTGGATCGTGTCAAAGTGTGTAGAGGGTGAATATCTTTTGAT
S12M_-13  GTGAGGGTGGGATTTTGGATCGTGTCAAAGTGTGTAGAGGGTGAATATCTTTTGAT
S12M_-4   GTGAGGGTGGGATTTTGGATCGTGTCAAAGTGTGTAGAGGGTGAATATCTTTTGAT
*****

WT      CACTTGTTTTTCGCTTCTTTGAGGGGTAGCCGGGGCTCGGCTCGGCGGGTTTAA
S12M_-14 CACTTGTTTTTCGCTTCTTTGAGGGGTAGCCGGGGCTCGGCTCGGCGGGTTTAA
S12M_-172 CACTTGTTTTTCGCTTCTTTGAGGGGTAGCCGGGGCTCGGCTCGGCGGGTTTAA
S12M_-13  CACTTGTTTTTCGCTTCTTTGAGGGGTAGCCGGGGCTCGGCTCGGCGGGTTTAA
S12M_-4   CACTTGTTTTTCGCTTCTTTGAGGGGTAGCCGGGGCTCGGCTCGGCGGGTTTAA
*****

WT      TAGCCCCCACTATTACAACATGGGAAGAAGAATCAATCTGTACAAACCTTAAGTT
S12M_-14 TAGCCCCCACTATTACAACATGGGAAGAAGAATCAATCTGTACAAACCTTAAGTT
S12M_-172 TAGCCCCCACTATTACAACATGGGAAGAAGAATCAATCTGTACAAACCTTAAGTT
S12M_-13  TAGCCCCCACTATTACAACATGGGAAGAAGAATCAATCTGTACAAACCTTAAGTT
S12M_-4   TAGCCCCCACTATTACAACATGGGAAGAAGAATCAATCTGTACAAACCTTAAGTT
*****

WT      AGTTTCTCTTTTAAATCTTGTATTCTTCTTCTTAACAAATCAAAACAGATCGGGGAAG
S12M_-14 AGTTTCTCTTTTAAATCTTGTATTCTTCTTCTTAACAAATCAAAACAGATCGGGGAAG
S12M_-172 AGTTTCTCTTTTAAATCTTGTATTCTTCTTCTTAACAAATCAAAACAGATCGGGGAAG
S12M_-13  AGTTTCTCTTTTAAATCTTGTATTCTTCTTCTTAACAAATCAAAACAGATCGGGGAAG
S12M_-4   AGTTTCTCTTTTAAATCTTGTATTCTTCTTCTTAACAAATCAAAACAGATCGGGGAAG
*****

WT      AGATGCCGGCCTTAGGTTGTTGTAGACG...
S12M_-14 AGATGCCGGCCTTAGGTTGTTGTAGACG...
S12M_-172 AGATGCCGGCCTTAGGTTGTTGTAGACG...
S12M_-13  AGATGCCGGCCTTAGGTTGTTGTAGACG...
S12M_-4   AGATGCCGGCCTTAGGTTGTTGTAGACG...
*****

```

Figure 12: Alignment of CRISPR/Cas9-induced mutations in tomato *ADC2* 5'UTR sequence.

Tomato *ADC2* 5'UTR sequences and part of the CDS were aligned using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) to display the CRISPR/Cas9-induced mutations. Deletions are shown as red dashes; insertions or substitutions shown in bolded red text; *ADC-box* is shaded black with bolded white text; *uORF* and start of *ADC* CDS are shaded grey with bolded black text; start and stop codons are underlined. An asterisk under the alignment indicates that all sequences match here.

cumulative affect may occur when both *SLADC1* and *SLADC2* elements are mutated *in planta*.

This may be especially the case for the uORFs, as only a very mild increase in translational efficiency was observed in transient assays (Wu *et al.*, 2019). A double *adc1/2 uorf* knock out ($\Delta sluorf1/2$) mutant was generated by crossing T1 plants of S11M_-127 and S1M1_-172bp. The *ebe* mutant generated by Wu *et al.*, 2019 was already a double *adc1/2-box* mutant; however, by specific selection, these mutations are small as this mutant was generated to prevent Brg11

binding without dramatically altering the native *ADC-box* sequence and function. Therefore, to generate an *adc1/2-box* mutant, lacking majority of the *SLADC1/2-box* sequences, a crossing of T1 plants of SI1M_-108 and SI2M_-13 was conducted. It was hypothesised that a plant lacking any ability to inhibit translation of ADC would have higher ADC enzymatic activity, leading to a greater impact on PA metabolism compared to single *adc-box* mutants. Finally, to determine the individual role of each *SLADC-box* in *planta*, without any contribution from other SLADC protein or *SLADC-box*, each *ADC-box* mutant was crossed to the opposite *adc* coding sequence mutant. Here, SI1M_-108 was crossed to *adc2* and SI2M_-13 was crossed to *adc1*. These crosses would result in the single remaining WT *ADC* coding sequence to have a mutated *ADC-box*, enabling analysis of the impact of only this *ADC-box* mutation.

For all crossings, both genotyped parental mutants were used as both pollen donors and acceptors, as PAs are involved in many developmental processes which could impact crossing efficiencies. Seeds from all crosses that produced fruit (F1 generation) were sown to soil and gDNA from typically 6-12 plants were genotyped via PCR amplification of the *SLADC* 5'UTRs (using primers mentioned above) and/or the *SLADC* CDS (DW406 and DW651 for *SLADC1*, and DW182 and DW183 for *SLADC2*). If the mutations were too small to see via a high percentage agarose gel, PCR products were sent for Sanger sequencing and compared to the wildtype and parental mutant reference sequence. In the F1 generation, the cross was considered successful if parental mutations were heterozygous; 2-3 F1 plants were kept for seed production. In the F2 generation, the mutations should segregate following Mendelian genetics (i.e., one quarter should be homozygous for both mutations). Again, the same genotyping method was used and 2-3 plants with both mutations in a homozygous configuration were kept for seed production. Supplementary Figure 8 shows an example of this genotyping procedure for the *sladc1/2-box* mutant and all tomato crosses generated in this work are summarised in Table 29.

Table 29: Summary of tomato CRISPR/Cas9 mutant crosses generated.		
Mutant name:	Parental genotypes	
	<i>SLADC1</i> genotype	<i>SLADC2</i> genotype
<i>ebe</i> mutant*	SI1M_+1	SI2M_-4
Δ <i>sladc1/2-box</i>	SI1M_-108	SI2M_-13bp
Δ <i>sluorf1/2</i>	SI1M_-127	SI2M_-172bp
Δ <i>adc1-box adc2</i>	SI1M_-108	<i>adc2</i> *
Δ <i>adc2-box adc1</i> [#]	<i>adc1</i> *	SI2M_-13bp
Footnotes:		
* Generated by Wu <i>et al.</i> , 2019		

3.1.2 CRISPR mutagenesis of *A. thaliana* ADC-boxes.

As explained above (section 1.2 and 1.3.3), *A. thaliana*, while being a model plant, is rather unique in its PA synthesis pathway as it lacks an ODC and its AtADC1 has neofunctionalised (Lou et al. (2020)). Sequence deviations of the *AtADC1-box* compared to *AtADC2-box* and *ADC-boxes* from other related species (Figure 4), also makes this an interesting case to study translational control of *ADC*.

Similar to the tomato CRISPR/Cas9 mutagenesis, both the *ADC-boxes* of *AtADC1* and *AtADC2* were targeted for mutagenesis. The gRNAs were designed using CRISPRdirect (crispr.dbcls.jp; Naito et al., 2015) with the *A. thaliana* reference genome from “TAIR10 (Nov, 2010)”, and an “NGG” PAM recognition sequence. Only gRNAs with a single predicted binding site in the reference genome that targeted distinct regions of the *ADC-box* were chosen (Figure 13A). The gRNAs were cloned into pDGE347 CRISPR/Cas9 plasmid using from Ordon *et al.*, 2020 (Figure 13B; Supplementary Table 4; Supplementary Figure 9). Unlike the

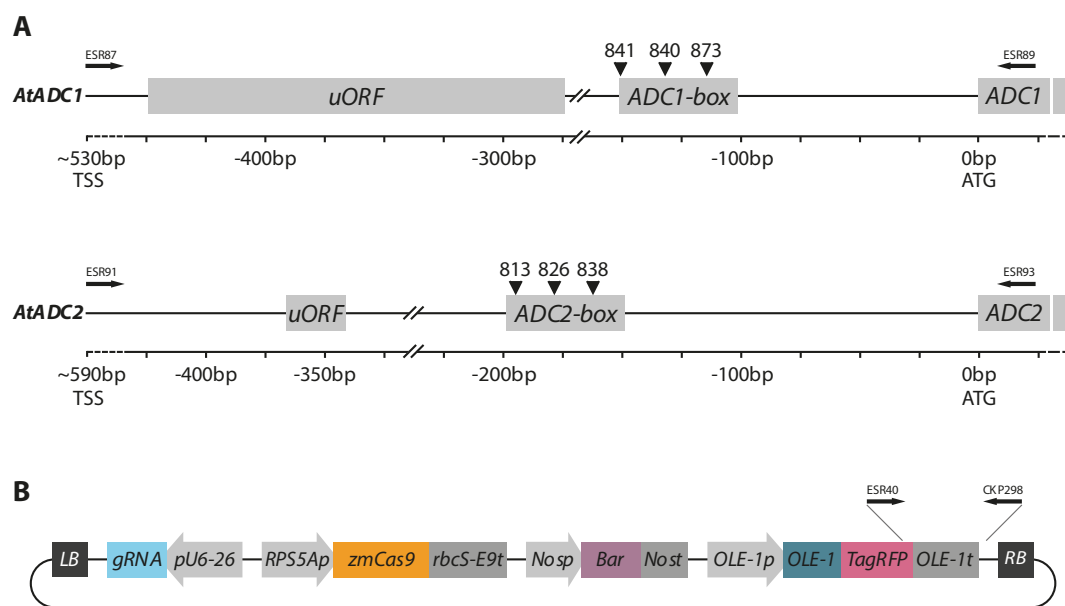


Figure 13: CRISPR/Cas9 mutagenesis of *AtADC1/2* 5'UTR conserved elements.

(A) Schematic of *AtADC1* (top) and *AtADC2* (bottom) 5'UTR genomic sequence highlighting the uORF, *ADC-boxes*, and start of the *AtADC1/2* coding sequences (grey boxes), and transcriptional start site (TSS) based on transcript information on TAIR (<https://www.arabidopsis.org>). Scale below indicates base pairs (bp) relevant to each *ADC* start codon. Expected CRISPR/Cas9 cleavage site determined by each gRNA is shown above the 5'UTR elements (▼). Black arrows show primers used for genotyping. (B) CRISPR/Cas9 plasmid (pDGE347; Ordon *et al.*, 2020) used for dip transformation of wildtype Arabidopsis, including: gRNA scaffold, including a *U6-26* promoter (*U6-26 pro*; light grey box) from *A. thaliana*, driving gRNA (light blue box); *A. thaliana* Ribosomal Protein *S5A* (*RPS5A*) promoter driving a *Zea mays*-codon optimised *Cas9* (*zmCas9*; orange box) containing 12 inserted introns and *Pisum sativum* *rbcS-E9* terminator (dark grey box); BASTA *in planta* resistance gene (*Bar*; phosphinothricin *N*-acetyltransferase; purple box) with *Nos* promoter (*Nos pro*) and terminator (*Nos term*); FAST marker of TagRFP (pink box) fused to *A. thaliana* Oleosin 1 (*OLE-1*; teal box) with its native promoter, 5'UTR, and terminator); and, left and right borders (black boxes). Black arrows show primers used for genotyping presence of *Cas9* transgene.

tomato mutagenesis, only one copy of each gRNA was cloned into the plasmid. However, this system was reportedly a highly efficient CRISPR/Cas9 system, specifically optimised for mutagenesis in *A. thaliana* using the *RPS5a* promoter to drive a *Zea mays* optimised *Cas9* variant with inserted 12 introns (Ordon *et al.*, 2020). Once the final plasmids were cloned and verified by Sanger sequencing, they were transformed into *A. tumefaciens*, which were then

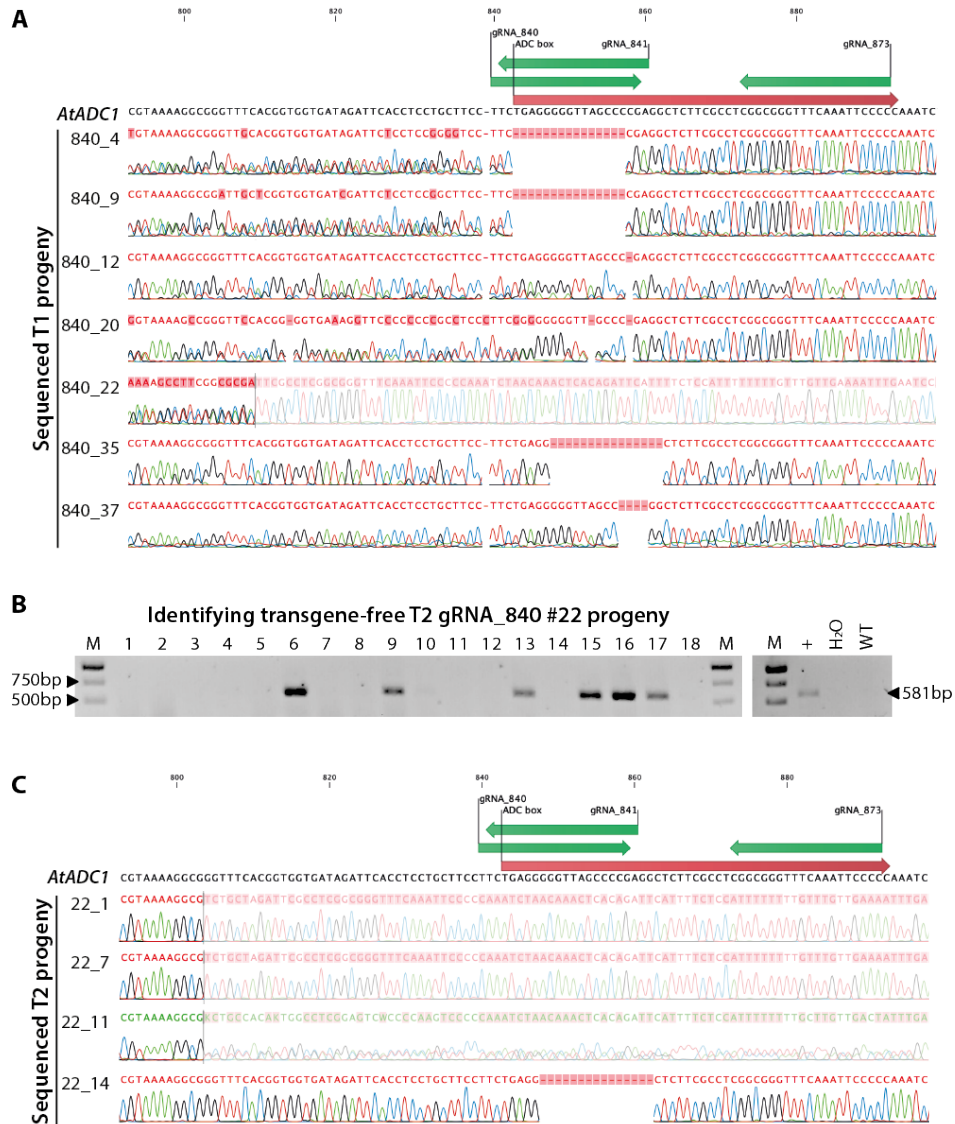


Figure 14: Genotyping process to identify transgene-free, homozygous mutant *A. thaliana* plants.

An exemplar of the T1 and T2 genotyping process shown with the T1 progeny of the gRNA840 CRISPR/Cas9 transformation and T2 progeny of gRNA840_22. (A) Following confirmation that each BASTA-surviving plant contained the CRISPR/Cas9 transgene (not shown), FTA card gDNA samples were used to amplify the *AtADC1* 5'UTR region via PCR. Products were sent for Sanger sequencing with ESR90 and aligned to the WT *AtADC1* reference sequence. Conflicts are highlighted in red or faded out when the sequence does not match the reference at that location. (B) The T1 plant gRNA840_22 was selected for T2 genotyping. Firstly, the presence of transgene-free T2 progeny was detected using the diagnostic PCR; this was repeated at least twice. The plasmid used for Arabidopsis transformation was used as a positive control; WT and H₂O serve as negative controls. (C) T2 progeny lacking a positive transgene product had the *AtADC1* 5'UTR region amplified and sent for Sanger sequencing to determine the mutation zygosity. Here, gRNA840_22_1 and 7 and gRNA840_22_14 were selected as homozygous, transgene-free mutants for subsequent analysis.

used to transform wildtype *A. thaliana* via floral dipping (section 2.2.1.7). All seeds were collected from these T0 plants.

To select for T1 progeny that contained the transgene, seeds were sown on soil alongside a WT and a BASTA resistant line (Danalyn Holmes, Lahaye Laboratory) after 3 days of stratification and grown in the greenhouse for ~1.5 weeks. All seedlings were sprayed with BASTA solution 2-3 times, each with 4 days recovery between sprayings. From the surviving seedlings, 40 plants from each gRNA-CRISPR/Cas9 transformation were transplanted to individual pots. To verify the presence of the transgene in these transplanted BASTA-survivors, gDNA was extracted and used for a diagnostic PCR to amplify a section of the *RFP* gene in the T-DNA using primers ESR40 and CKP298; almost all plants produced a positive PCR product here. To determine if CRISPR/Cas9 mutagenesis had taken place, the targeted *AtADC1* or *AtADC2* 5'UTR was amplified using primers ESR87 and ESR89 or ESR91 and ESR93, respectively. The PCR product quality was verified on a 1% agarose gel before being sent for Sanger sequencing using the nested primers ESR88 or ESR90, or ESR92 or ESR94 for *AtADC1* or *AtADC2*, respectively. Sequences were then mapped to a WT reference to determine if a mutation had occurred; all plants that had a mutation were kept for seed production and the rest were discarded.

Unlike the tomato CRISPR/Cas9 mutant genotyping, exact determination of the mutations and zygosity was not carried out in the T1 generation. Instead, a collection of T1 mutants were selected based on the sequencing data (Figure 14A) and their T2 seeds were sown to soil (Laura Schmaltz, Lahaye Laboratory). In the T2 generation, the transgene is expected to segregate based on Mendelian genetics, as well as any CRISPR/Cas9-induced mutations that were heterozygous in the T1 generation, allowing for selection of transgene-free homozygous mutants via genotyping. Firstly, the absence of the transgene was determined using the diagnostic PCR described above (primers ESR40 and CKP298), which was repeated at least a second time to verify the absence. *AtADC1* or *AtADC2* 5'UTRs of T2 plants that did not show a positive transgene product were PCR amplified and sent for Sanger sequencing. An example of this genotyping process is shown for plant gRNA840_22 in Figure 14. All resulting transgene-free, homozygous mutants that were obtained are shown in Table 30, as well as alignments of their *ADC-box* mutations in Figure 15 and entire 5'UTR sequences in Supplementary Figure 10 and Supplementary Figure 11. However, despite repeating the diagnostic CRISPR/Cas9 PCR multiple times in the T2 generation, the expected segregation ratio of 1:3 was not always observed (Figure 14B) and there were sometimes discrepancies between PCR replicates. Therefore, a method to confirm transgene absence other than PCR

Table 30: <i>A. thaliana</i> ADC-box CRISPR/Cas9 mutant names used in this work.			
Original name	Origin of plant	Name in this thesis	Mutation
gRNA840_35 #4/6 gRNA840_22 #14	gRNA840 CRISPR/Cas9 transformation (this work)	At1M_-15	15 bp deletion inside <i>AtADC1-box</i>
gRNA840_22 #1/7	gRNA840 CRISPR/Cas9 transformation (this work)	At1M_-53	42 bp deletion of 3' half of <i>AtADC1-box</i> and 11 bp deletion 3' to <i>AtADC1-box</i>
gRNA840_37 #18	gRNA840 CRISPR/Cas9 transformation (this work)	At1M_+1	1 bp insertion in <i>AtADC1-box</i>
gRNA840_37 #12/13/20	gRNA840 CRISPR/Cas9 transformation (this work)	At1M_-4	4 bp deletion in <i>AtADC1-box</i>
gRNA826_10 #9	gRNA826 CRISPR/Cas9 transformation (this work)	At2M_-18	18 bp deletion of <i>AtADC2-box</i>
gRNA826_19 #11/19	gRNA826 CRISPR/Cas9 transformation (this work)	At2M_-6	6 bp deletion in <i>AtADC2-box</i>
gRNA838_21 #4/8	gRNA838 CRISPR/Cas9 transformation (this work)	At2M_+1	1 bp insertion in <i>AtADC2-box</i>
Footnotes: The CRISPR/Cas9 mutant names used in this thesis take the form: 1. “M”, where a mutation affects a conserved element (i.e., mutant) 2. Either “At1” or “At2”, corresponding to <i>AtADC1</i> or <i>AtADC2</i> 3. The number of base pairs that have been affected by this mutation are indicated			

was required. T3 seeds of all mutants, WT, and a BASTA resistant line were sown on soil and after 1-2 weeks, seedlings were sprayed with BASTA. Apart from the BASTA-resistant control, none of the mutant lines survived (data not shown), demonstrating that their T2 parent do not contain the CRISPR/Cas9 transgene. Several plants from each T3 line that were not sprayed with BASTA had their *AtADC* 5'UTR genotype verified via PCR and Sanger sequencing and were kept for bulking seeds.

```

AtADC1_WT      TCTGAGGGGGTTAGCC-CCGAGGCTCTTCGCCTCGGCGGGTTTCAAATTC CCCAAA
At1M_-15      TCTGAGG-----CTCTTCGCC TCGCGGGTTTCAAATTC CCCAAA
At1M_-53      <-----GCCTCGGCGGGTTTCAAATTC CCCAAA
At1M_+1      TCTGAGGGGGTTAGCCACCGAGGCTCTTCGCCTCGGCGGGTTTCAAATTC CCCAAA
At1M_-4      TCTGAGGGGGTTAGCC-----GGCTCTTCGCCCTCGGCGGGTTTCAAATTC CCCAAA
                *****

AtADC2_WT      TCTCCTGAGGGGGTAGCCGAGGCTCCGGCCTCGG-CGGT'TTTTAAACCCCTACCTTT
At2M_-18      TCTCCTGAGGGGGTAGCCGAG-----TTTAAACCCCTACCTTT
At2M_+1      TCTCCTGAGGGGGTAGCCGAGGCTCCGGCCTCGGACGGT'TTTTAAACCCCTACCTTT
At2M_-6      TCTCCTGAGGGGGTAGCCGAGG-----CTCGG-CGGT'TTTTAAACCCCTACCTTT
                *****

```

Figure 15: Alignment of *A. thaliana* CRISPR generated *adc-box* mutations.

AtADC1 (top) and *AtADC2* (bottom) WT and mutated *ADC-box* sequences were aligned using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) to display the CRISPR/Cas9-induced mutations. Deletions are shown as red dashes; insertions or substitutions shown in bolded red text; *ADC-box* is shaded black with bolded white text; an asterisk under the alignment indicates that all sequences match here.

3.1.3 CRISPR mutagenesis of the *M. polymorpha* ADC-box.

The liverwort *M. polymorpha* is a relative of the first land plants (Bowman et al., 2017), which is often used to delve into how biological functions may have evolved. *M. polymorpha* has a single *ADC* gene that has a smaller *ADC-box* in the 5'UTR, with only the central GC-rich regions present (Figure 3; Wu *et al.*, 2019). However, this indicates that at least a primitive *ADC-box* has existed since the evolution of the first land plants, (i.e., ~450 million years ago). As *M. polymorpha* can be genetically manipulated and transformed via tissue culture, CRISPR/Cas9 mutagenesis of the *MpADC-box* was conducted in the male WT line, TAK1. Design of gRNA, CRISPR/Cas9 construct cloning, and *M. polymorpha* transformation selection and some genotyping was done by Dr. Ezgi Süheyla Karaaslan (Prof. Chang Liu's laboratory). A master's student Pia Ellissen (Lahaye laboratory, 2020) continued with genotyping, conducted initial LCMS ADC activity assay analysis of some lines, and generated

Table 31: *M. polymorpha* ADC-box CRISPR/Cas9 mutant names used in this work.

Original name	Origin of plant	Name used in this thesis	Mutation
#1-4	Generated by E. Süheyla Karaaslan	MpC_-30	30 bp deletion ~280 bp 3' to <i>MpADC</i> ATG
#1-6	Generated by E. Süheyla Karaaslan	MpC_-10	10 bp deletion ~280 bp 3' to <i>MpADC</i> ATG
#5-3	Generated by E. Süheyla Karaaslan	MpC_-4	4 bp deletion ~280 bp 3' to <i>MpADC</i> ATG
#5-4	Generated by E. Süheyla Karaaslan	MpC_-1	1 bp deletion ~280 bp 3' to <i>MpADC</i> ATG
#3-1	Generated by E. Süheyla Karaaslan;	MpM_-8	8 bp deletion in <i>MpADC-box</i>
#3-5	Generated by E. Süheyla Karaaslan;	MpM_+1 ^A	1 bp insertion in <i>MpADC-box</i>
#3-6	Generated by E. Süheyla Karaaslan;	MpM_-14	14 bp deletion in <i>MpADC-box</i>
#14-6	Generated by E. Süheyla Karaaslan	MpM_+1 ^B	1 bp insertion in <i>MpADC-box</i>
#15-2	Generated by E. Süheyla Karaaslan;	MpM_-6	6 bp deletion in <i>MpADC-box</i>
#16-7	Generated by E. Süheyla Karaaslan	MpM_+1 ^C	1 bp insertion in <i>MpADC-box</i>

Footnotes:

The CRISPR/Cas9 mutant names used in this thesis take the form:

1. "C", where the mutation occurs outside of a conserved 5'UTR element (i.e., control), and "M", where a mutation affects a conserved element (i.e., mutant),
2. The number of base pairs that have been affected by this mutation are indicated,
3. MpM_+1^{A/B/C} all have a 1bp insertion at different locations or new nucleotides within the *MpADC-box*

long term -80 °C storage stocks of all mutant plants. From this work, 6 *M. polymorpha* *adc-box* mutants were recovered with a range of mutations, as well as 4 control mutant plants, which had various deletions approximately 200 bp 5' to the *ADC-box* (Figure 16). Gemmae (asexually produced clonal *M. polymorpha* pro-plants) from each mutated line that had been frozen at -80°C as long term stocks for ~2 years, were all successfully defrosted and regenerated on ½ B5 media (Natalie Faiss, Laboratory of Dr. Julien Gronnier; section 2.2.1.3).

To ensure the correct genotypes were being used for further experiments, gDNA was extracted and primers PL69 and PL70 were used to amplify the *MpADC-box* 5'UTR (section 2.2.3.5). After verifying the PCR product quality via gel electrophoresis, all were Sanger sequenced with primer PL69 and compared to the wildtype *MpADC* sequence. It is important to note that as *M. polymorpha* is haploid and is typically asexually propagated, out segregation of the CRISPR/Cas9 transgene is not common practice and was not done in this work. Also, due to contamination issues, propagation of these *M. polymorpha* lines required gemmae sterilisation with a 10% Triton and 15% sodium hypochlorite solution prior to streaking onto fresh plates (section 2.2.1.3). The mutants used in this work are shown in Table 31 and an alignment of the *MpADC-box* mutations is shown in Figure 16; an alignment of the full *M. polymorpha* 5'UTR with all mutations is shown in Supplementary Figure 12.

MpADC_WT	AAATTGATTCAGTCCCTACAGAGCCGAG-GCTCCGGCCTCGG-C	TTCGGCTTTTAGCCAG
MpM_+1 ^C	AAATTGATTCAGTCCCTACAGAGCCGAG-GCTCCGGCCTCGG	C TTCGGCTTTTAGCCAG
MpM_+1 ^B	AAATTGATTCAGTCCCTACAGAGCCGAG-GCTCCGGCCTCGG	C TTCGGCTTTTAGCCAG
MpM_-8	AAATTGATTCAGTCCCTACAGAGCCG-----GCCTCGG-C	TTCGGCTTTTAGCCAG
MpM_-14	AAATTGATTCAGTCCCT-----CCGGCCTCGG-C	TTCGGCTTTTAGCCAG
MpM_-6	AAATTGATTCAGTCCCTACAGAGCCGAG-----GCCTCGG-C	TTCGGCTTTTAGCCAG
MpM_+1 ^A	AAATTGATTCAGTCCCTACAGAGCCGAG G GCTCCGGCCTCGG-C	TTCGGCTTTTAGCCAG
	*****	*****

Figure 16: Alignment of *M. polymorpha* *ADC-box* mutants.

WT and mutated *ADC-box* sequences were aligned using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) to display CRISPR/Cas9-induced mutations. Deletions are shown as red dashes; insertions or substitutions shown in bolded red text; *ADC-box* is shaded black with bolded white text; an asterisk under the alignment indicates that all sequences match here.

3.2 Optimisation of LCMS-based ADC assays

3.2.1 *Determining methods for quantifying ADC protein levels*

One major aim of this work was to assess the role of the *ADC-box* as a negative regulatory cis-element of ADC translation *in planta*. As the direct output of translation is the protein itself, an accurate and sensitive experimental assay for quantifying ADC protein levels in WT and *adc-box* mutant tissue from tomato, *A. thaliana* and *M. polymorpha* was required. There are three common methods used for determining protein amounts: immunoblotting, mass spectrometry (MS), or enzymatic assays developed for specific proteins. The following sections cover the efforts to decide which method is most suitable for native ADC protein quantification from plant tissue and subsequent optimisation steps conducted to improve this method.

3.2.2 *Immunoblotting of native ADC proteins from plant tissue*

Immunoblotting uses protein- or epitope tag-specific antibodies to directly detect specific proteins, and comparative analysis of western blot band intensities can then be used to indicate relative protein abundance between samples. However, a functional antibody distinguishing ADC1 and ADC2 proteins is currently not held in the Lahaye laboratory. Given the high sequence homology between tomato ADC1 and ADC2 homologs, antibodies would unlikely be specific; therefore, immuno-detection of native SIADC proteins is not straightforward. This method is also unlikely to be suitable for quantification of ADC proteins from multiple species (i.e., tomato, *A. thaliana*, and *M. polymorpha*) without generating specific antibodies for each species and each ADC homolog. Furthermore, comparative analysis of immunoblot bands is unlikely to be sufficiently quantitative for elucidation of smaller differences in ADC protein amounts between *ADC* transcripts of WT and genotypes with smaller *adc-box* mutations. Therefore, an immunoblotting-based method of ADC protein quantification was not favourable.

3.2.3 *Efforts to identify ADC peptides via MS for quantification.*

Another common approach for quantification of protein amounts is to use a mass western. This is a targeted proteomics approach, where the abundance of each protein within a sample can be quantified (Lyon et al., 2014; summarised in section 2.2.5.1). MS-based quantification of ADC proteins was initially attempted using WT tomato, untreated leaf tissue (personal communication with Dousheng Wu, Lahaye Lab, and Edda von Roepenack-Lahaye, Central Facilities, ZMBP). To verify the prediction of SIADC trypsin digested peptides for the

optimisation of the mass western, recombinant SIADC1 expressed from *E. coli* was used. Despite identifying SIADC1 m/z peaks from the recombinant SIADC1, no corresponding peaks were seen from the WT tomato leaf tissue. It was hypothesised that either the SIADC1 protein levels were too low to be detected from untreated tomato leaves or that plant-specific post-translational modifications of SIADC1 meant that the m/z ratios of trypsin-digested peptides were different to those predicted or produced by the recombinant SIADC1.

During this thesis work, it was hypothesised that transient overexpression of SIADC1/2 epitope-tagged proteins in *N. benthamiana* leaves may produce higher levels of SIADC1/2 proteins than from untreated tomato leaves and would also be subjected to any plant-specific post-translational modifications. To test this, constitutively expressed HA-SIADC1, MYC-SIADC2, and GUS (negative control) were agroinfiltrated into *N. benthamiana* leaves (section 2.2.1.5). To verify which time point had the highest expression, samples were taken at 1, 2, and 3 dpi for analysis of protein expression via western blots (Supplementary Figure 13A; section 2.2.4.1). As the western blot bands were most intense in the 2 dpi samples for both HA-SIADC1 and MYC-SIADC2 at the expected sizes (i.e., 79 and 80 kDa, respectively), these samples and the GUS negative control were run in duplicate on a new SDS-PAGE gel and stained with Coomassie (Supplementary Figure 13B). Although no additional or more intense bands at approximately 80 kDa were observed in the HA-SIADC1 or MYC-SIADC2 lanes compared to the GUS lanes, sections of gel were cut from each lane for further MS analysis (section 2.2.4.2). No SIADC peptide peaks were identified by MS, suggesting that even constitutive expression of SIADC1/2 proteins in *N. benthamiana* either remain below the threshold of MS detection or that *in planta* post-translational modifications generate unexpected peptide masses, which do not match to the predicted trypsin digested peptide m/z ratios. Therefore, at present, quantification of ADC protein abundance using MS-detection of ADC peptides is not feasible and an alternative method for quantification of ADC translation is needed.

3.2.4 LCMS-based approach for quantification of ADC activity

Finally, another common method for evaluating protein levels – if that protein is an enzyme – is to measure its specific enzymatic activity as a proxy for the abundance of that protein. Accumulation of the enzyme's product or by-product, or the reduction in the substrate concentration can be measured and the enzyme activity inferred. Typically, enzymatic assays must be conducted in an environment where nothing is limiting the rate or reaction (e.g., substrate or co-factor limitation or presence of inhibitors) to ensure that the enzyme activity is directly proportional to protein levels.

Previously, a targeted liquid-chromatography, mass-spectrometry (LCMS)-based assay was established to quantify ADC activity as a proxy for ADC protein abundance (described in section 2.2.5.2; Wu et al., 2019). As ADC proteins catalyse the conversion of arginine to agmatine, LCMS could be used to measure accumulation of agmatine to infer ADC activity. As one agmatine molecule is produced by one ADC enzyme from one arginine molecule at a time, the amount of agmatine produced is therefore directly proportional to the ADC protein abundance (assuming there are no *in planta* factors regulating ADC activity at the enzymatic level). Therefore, LCMS measurement of agmatine levels can be used to analyse the overall ADC protein content from plant tissues. However, measuring the total agmatine content in a sample does not directly inform on the rate of conversion from arginine to agmatine by ADC in a given timeframe, rather just the total pool of agmatine. Therefore, to determine the ADC activity, an alternative product to agmatine that is not present in the native sample at the start of the assay is needed to measure ADC activity. For this, stable heavy isotope variants of arginine were used that are physically distinct from normal arginine by having additional neutrons, while being recognised and metabolised in the same way as normal arginine. Specifically, a heavy arginine variant with $^{13}\text{C}_6$ instead of the normal $^{12}\text{C}_6$ atoms has a +6 molecular weight and following decarboxylation by ADC, produces a $^{13}\text{C}_5$ agmatine. These heavy isotope variants can be differentiated to their normal counterparts using LCMS as they have different m/z properties, enabling them to be distinctly isolated within the LCMS Q3, despite having essentially the same LC retention time (Figure 17; differential transitions for each compound presented in Supplementary Table 3). As these heavy isotopes are not naturally found in biological tissues, external addition of heavy arginine into the assay buffer and subsequent measurement of synthesised heavy agmatine at the end of the experiment can be interpreted as the ADC activity occurring for this assay time period.

Using LCMS to measure ADC activity, thereby inferring on ADC protein abundance, has a substantial benefit to other protein detection methods, as LCMS enables the simultaneous quantification of PAs and related metabolites within the same assay sample. Measurement of arginine (ADC substrate) and agmatine (ADC product) pools, as well as the wider PA metabolic network, provides a valuable and comprehensive snapshot of the whole PA metabolic profile. It is hypothesised that while the *ADC-box* mutants are expected to have higher ADC activity than the WT due to higher rates of ADC translation, it is also possible these mutations may also cause changes to levels of PAs and related metabolites. Therefore,

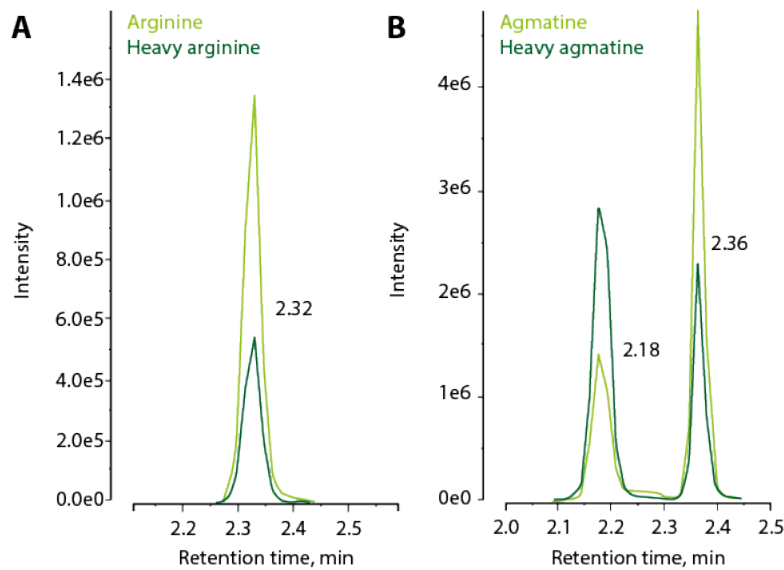


Figure 17: Detection and measurement of heavy stable arginine and agmatine isotopes compared to their normal counterparts. Heavy stable isotopes have the same LC retention time but can be distinctly selected within the LCMS based on their altered m/z ratio, allowing for measurement of both normal (light green) and heavy isotopes (dark green) from the same sample. Arginine (**A**) has a retention time of 2.32, while agmatine (**B**) has a retention time of 2.36. Also shown in **B** is the m/z peak of acetyl putrescine (retention time 2.18), which has similar m/z properties as agmatine so is co-selected in Q1.

by measuring not only the ADC activity via LCMS, but also quantifying metabolites in the PA network, the overall role of the *ADC-box* on PA homeostasis can be investigated. Based on these reasons, the LCMS-based ADC activity assay was used in this work to determine the *in planta* impact of the 5'UTR conserved elements on translational efficiency of ADC and PA metabolism.

3.2.5 Preliminary ADC activity assay using segregated *ebe* mutants.

To clarify if the LCMS-based ADC activity assay protocol established by Wu et al., 2019 (section 2.2.5.2) was appropriate for detecting differences in ADC activity between WT and *adc-box* mutants, a preliminary experiment was conducted on segregated *ebe* mutants (Table 28). Previously, this method had been used for quantifying ADC activity following transcriptional induction of ADCs by RipTALs, rather than native regulation of ADC translation. Here, 50 mg of untreated leaf samples were taken from WT and various segregating *ebe* mutants (including the homozygous single *SlADC1-box* mutant S11M_-4bp and *SlADC2-box* mutant S12M_+1bp) for an ADC activity assay, following the published Wu et al., 2019 protocol (section 2.2.5.2). Very low MRM signal peaks of PAs were observed, and no ADC activity was found for the WT or any segregated *ebe* mutant. Wu et al., 2019 always conducted ADC activity assays using infiltrated or infected tissue, meaning that all samples had been exposed to a biotic stress prior to LCMS measurements. As transcription of *ADCs* is known to be induced upon many biotic and abiotic stresses (Hussain et al., 2011, Chen et al., 2019,

Gonzalez et al., 2021), it is plausible that induction of *ADC* transcription by some form of stressor is required to elevate enzyme levels and metabolite concentrations to enable clear measurement of signal peaks above background level (i.e., improving the signal to noise [S/N] ratio). Additionally, optimisation of the extraction and assay protocol could also increase the relative abundance of measured metabolites, reduce the amount of technical error between replicates, and improve the S/N ratio, thereby improving the resolution between samples from different genotypes or treatments.

3.2.6 Preliminary work done on the *ADC* activity assay and LCMS protocol.

Between the Wu et al., 2019 publication and the following optimisation experiments, Edda von Roepenack-Lahaye (Central Facilities, ZMBP) had conducted some additional experiments to alter the extraction protocol. She had also expanded the scope of PA-related metabolites that could be measured within one LCMS-analysis. We hypothesised that as *ADC* proteins are localised to both the cytoplasm and plastids (Bortolotti et al., 2004), and a large proportion of PAs and related metabolites are bound to other cellular entities, such as the cell wall (Sobieszczuk-Nowicka and Legocka, 2014, Chen et al., 2019), using only the supernatant during the assay may remove a large proportion of the *ADC* protein and metabolites from the sample. Upon individually measuring the *ADC* activity of the pellet and supernatant fractions from extracted WT tomato leaf tissue (section 2.2.5.2), the pellet had 6-fold higher *ADC* activity compared to the supernatant (data not shown). Therefore, this centrifugation step was omitted from the protocol. Secondly, as the initial centrifugation step was no longer required, heavy arginine was added directly to the extraction buffer, thereby reducing possible technical pipetting errors. Thirdly, a heavy tryptophan isotope (i.e., D₅ tryptophan), was included to the extraction buffer to be used as an internal normalisation control. Like the heavy arginine and agmatine isotopes, D₅ tryptophan also has a distinct *m/z* ratio compared to cellular tryptophan. D₅ tryptophan was selected as a normalisation agent due to its lower polarity compared to PAs, thereby reducing possible chromatographic interference and/or ion suppression. Finally, to enable correct identification of citrulline and subsequent calculation of its concentration in each sample, a heavy isotope counterpart was also added to the extraction buffer (¹³C₁ D² citrulline, 100µM). In addition, a newly implemented LCMS machine was also used for later experiments: a QTRAP6500+ with an LC M5-TE (Trap-Elute, ABSciex, USA), which has a higher sensitivity than the previous LCMS platform. The higher sensitivity is based on the more advanced technology of the hybrid quadrupole-linear ion trap mass spectrometer, as well as the

possibility to run larger sample volumes through the trap/analytical column 2D-combination of the LC.

Using these new protocol adjustments, the following sections discuss the further ADC activity assay optimisation experiments for tomato (section 3.2.7), *M. polymorpha* (sections 3.2.8 to 3.2.10), and *A. thaliana* (section 3.2.11).

3.2.7 Optimisation of the tomato ADC activity assay and LCMS protocol

To improve sensitivity of the ADC activity assay, a reproducible stimulus to activate *ADC* transcription was sought after, with the intention to improve LCMS analysis accuracy, even at near low metabolite levels. Ideally, the level of transcriptional activation and resulting PA metabolic changes would be similar for both *SIADC1* and *SIADC2*, allowing the same “stress treatment” to be used for mutants of both genotypes. The genes of many PA-related genes are upregulated in response to a plethora of biotic and abiotic stressors, including *ADC* genes (Hussain et al., 2011, Chen et al., 2019, Gonzalez et al., 2021). As *SIADC1/2* genes have both been shown to be up regulated upon cold stress, this was an appealing stress treatment due to its simplicity and efficiency to administer in a uniform manner across all samples. Upadhyay et al., 2020 showed that *SIADC1* and *SIADC2* transcription is induced by cold stress, peaking at 24 hours at 4 °C, and importantly, levels of free, bound, and conjugated PAs also increased with this treatment. Controversially, Tsaniklidis et al., 2016 found that 16 hours of 5 °C cold stress induced PA levels compared to non-cold stressed controls, but only *SIADC1* expression was increased, not *SIADC2*. As a preliminary experiment to determine if cold treatment would induce ADC activity levels, three WT tomato leaflets were detached, inserted into open 2 mL Eppendorf tubes containing 1.5 mL sterile water, treated at 4 °C for ~16 hours overnight; three control WT leaflets were treated the same way, but kept at room temperature (RT) in the dark. All harvested samples were extracted in the updated ADC activity buffer (Table 24), incubated at 37 °C for 3 hours, centrifuged, and samples diluted for LCMS profiling (section 2.2.5.3). Cold-treated samples had ~1 nmol/g FW of heavy agmatine, a 25-fold increase compared to the RT controls (0.04 nmol/g FW). Putrescine levels were elevated 6-fold higher in cold-treated samples to ~17 nmol/g FW (Figure 18A). It is noteworthy that only the heavy agmatine and putrescine concentrations from cold-treated samples were in the linear range of the calibration curve, whereas the non-treated samples were outside the lower bounds of the calibration curve. Therefore, cold treatment dramatically improved the ability to accurately determine metabolite concentrations.

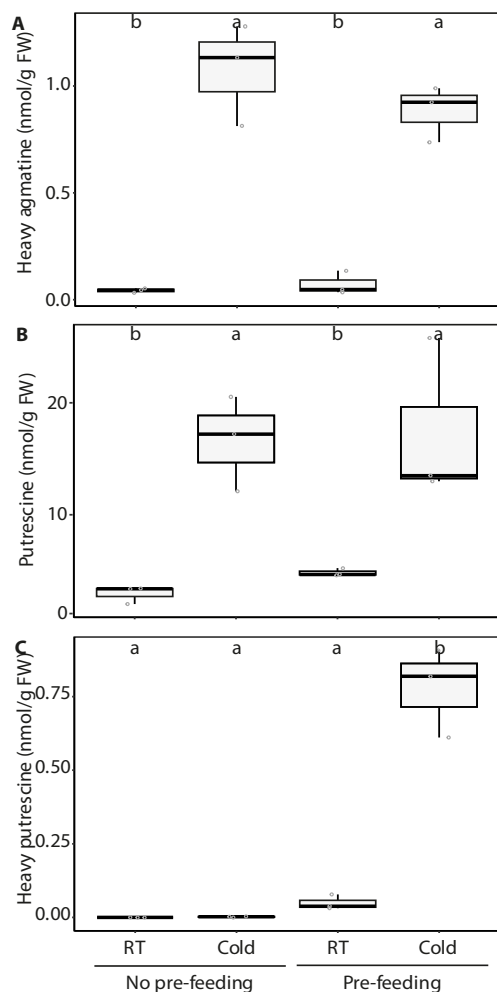


Figure 18: Cold treatment, but not pre-feeding with heavy arginine, increases ADC activity and putrescine levels in detached tomato leaflets.

WT tomato leaflets were detached, put into tubes with water or 250 μ M heavy arginine and kept in the dark at RT or 4 $^{\circ}$ C for 16 hours. 50mg of tissue was harvested, frozen, and homogenised in ADC activity buffer (3 hours, 37 $^{\circ}$ C, 300 rpm). Reactions were stopped with 5 μ L HFBA, centrifuged (4 $^{\circ}$ C, 14,000 g, 30 min), diluted supernatant was used for LCMS analysis. Accumulation of heavy agmatine (A), putrescine (B), and heavy putrescine (C, produced only from pre-fed heavy arginine) levels (nmol/g FW) with either RT- or cold- treatment and with and without pre-feeding of heavy arginine. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different ($\alpha = 0.05$).

Within this same experiment, a second set of RT- or cold-treated tomato leaflets were used to test if higher ADC activity levels could be achieved when pre-feeding the leaflets overnight with heavy arginine. In this experiment, 250 μ M heavy arginine was added to the 2mL tubes instead of sterile water (Figure 18A). Pre-feeding with heavy arginine did not significantly increase heavy agmatine levels during the 3-hour assay (Figure 18B), suggesting that increasing substrate availability overnight did not stimulate higher ADC activity. However, only heavy arginine pre-fed samples produced heavy putrescine (Figure 18C), indicating that at least some of the heavy arginine was taken up during the overnight treatment and metabolised by ADC. As demonstrated in the previous experiment, cold treatment increased WT tomato ADC activity levels into a detectable range. A preliminary ADC activity assay was conducted using WT and two tomato T1 CRISPR/Cas9 *adc-box* mutants to determine if the *ADC-box* mutations influenced ADC activity and if these differences could be easily observed using this assay protocol. The *adc-box* mutants selected had homozygous deletions removing either all or part of one of the *ADC-boxes* as these were hypothesised to have the greatest impairment to *ADC-box* translational regulation and therefore, the largest difference in ADC activity compared to

WT. An *sladc1-box* mutant was selected with a 108 bp deletion of the *SLADC1-box* (S11M_-108), as well as an *sladc2-box* mutant with 13 bp of the GC1 and GC2 regions deleted (S12M_-13) (Figure 11, Figure 12).

Samples from detached, cold-treated WT, S11M_-108, and S12M_-13 leaves were extracted, assayed, and analysed by LCMS. The *sladc1-box* mutant S11M_-108 had ~6-fold higher heavy agmatine (18.5 nmol/g FW) level compared to WT (3.2 nmol/g FW; ANOVA and Tukey's post hoc test, P -value < 0.001; Figure 19A). The *sladc2-box* mutant S12M_-13 also had higher heavy agmatine (7.6 nmol/g FW) compared to WT, but not significantly (P -value = 0.257). No significant differences in putrescine levels were found between any genotypes (Figure 19B). However, S11M_-108 had 8-fold and 2-fold higher heavy putrescine, generated from heavy arginine and subsequent heavy agmatine during the 3-hour assay compared to WT and S12M_-13, respectively (P -values < 0.01; Figure 19C). This suggested that the higher ADC activity of S11M_-108 led to higher putrescine production during the assay, but this difference was not able to be clearly seen in the total putrescine pool, which is typically the most abundant PA pool in plants (Gonzalez-Hernandez et al., 2022b). Overall, these findings show that deletion of the whole *SLADC1-box* increased ADC activity, demonstrating the repressive functionality

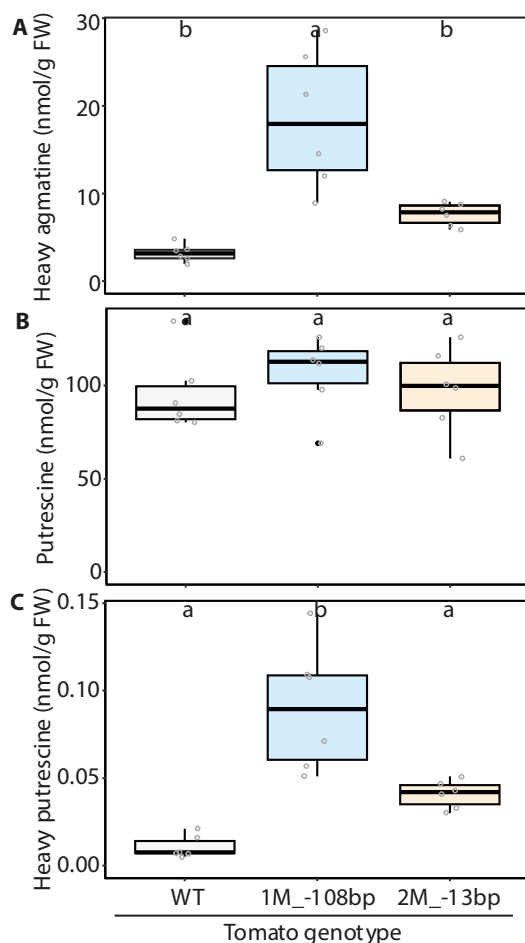


Figure 19: Deleting the *SLADC1-box*, but not mutating the *SLADC2-box* causes increased ADC activity compared to WT. WT (grey), S11M_-108 (blue), and S12M_-13 (yellow) tomato leaflets were detached and cold-treated for 16 hours ($n = 6$). 50mg samples were harvested, frozen, homogenised in ADC activity buffer, incubated (37°C for 3 hours), centrifuged and diluted for LCMS analysis. Concentrations of heavy agmatine (ADC activity) (A), putrescine (B), and heavy putrescine (C) are normalised to D₅ tryptophan (internal standard). Statistical significance was determined via an ANOVA and a Tukey HSD test; letters above each box indicate significance; groups with different letters are significantly different ($\alpha = 0.05$).

of the *SLADC1-box* in *planta* and that this increase may also translate into alterations to levels of PAs.

Despite finding a significant difference in ADC activity between S11M_-108, which lacks the entire *SLADC1-box*, and WT following cold-treatment, the smaller mutation in S12M_-13 did not have significantly higher ADC activity than WT. Based on this result, it was thought that mutants with ADC activities and PA levels similar to that of the WT would not be able to be significantly differentiated using the current stress treatment and/or ADC activity assay protocol. Additionally, any mutants with lower ADC activity and PA levels than WT would be at risk of falling out of the linear range of the calibration curves, resulting in inaccurately determined values. Therefore, further optimisation experiments were conducted using again WT and the *sladc-box* mutant lines S11M_-108 and S12M_-13, with the aim to improve the stress parameters and increase the sensitivity of the ADC activity assay, thereby resulting in clearer differentiation in metabolite levels between mutants and WT.

The first experiment tested two aspects of the current cold-treated tomato leaf ADC activity assay protocol: heavy arginine substrate availability and cold-treatment duration. Firstly, it was hypothesised that if the concentration of the ADC activity assay substrate (i.e., heavy arginine) was not sufficient, a “reaction bottleneck” in the assay would occur. This may result in lower heavy agmatine levels than what could theoretically be produced by the amount of available ADC proteins. Therefore, to alleviate this potential reaction bottleneck, a higher heavy arginine concentration in the assay buffer could result in higher ADC activity levels in an ADC protein concentration-dependent manner. To test this, two concentrations of heavy arginine were used in the assay extraction buffer: 40µM and 400µM, whereas 50µM had been used previously. Secondly, the overnight cold-treatment duration was tested to determine if extending from 16 hours to 24 hours would increase ADC activity, as induction of *SLADC* transcription was reportedly highest after 24 hours of cold treatment (Upadhyay *et al.*, 2020).

For this experiment, leaves from WT, S11M_-108, and S12M_-13 were cold treated overnight for either 16 or 24 hours and extracted in ADC assay buffer with either 40µM or 400 µM heavy arginine; the rest of the assay was carried out and diluted for LCMS profiling (section 2.2.5.3). On average, ADC activity was ~22-fold higher in samples extracted with 400 µM heavy arginine compared to 40 µM (Figure 20A). Following 24 hours of cold treatment, the *SLADC-box* mutant S11M_-108 had 8.3 nmol/g FW heavy agmatine when extracted with 40 µM heavy arginine, but this rose to 159.1 nmol/g FW when extracted with 400 µM (p-value <0.0001, ANOVA and Tukey post hoc test). Similarly, the heavy agmatine content of the *sladc2-box* mutant S12M_-13 rose from 3.7 nmol/g FW to 95.2 nmol/g FW after 24 hours of cold treatment

(Figure 20A). This dramatic ~22-fold increase in ADC activity demonstrates that the heavy arginine substrate availability was previously much lower than the ADC enzymatic capabilities for this 3-hour assay. With the higher heavy arginine substrate concentration, significant differences in ADC activity were able to be seen between the *sladc-box* mutants and compared to WT. It is also important to note that the higher heavy agmatine levels increased MRM signal peaks well above background levels, reducing the S/N ratio and pushing the peak areas well into the range of the calibration curve, thereby increasing the accuracy of calculating metabolite concentrations.

Putrescine levels were also increased on average ~2.8 fold with the higher heavy arginine substrate concentration (Figure 19B). The *sladc-box* mutants SI1M_-108 and SI2M_-13 both had significantly higher putrescine levels compared to WT with 400 μ M heavy arginine and

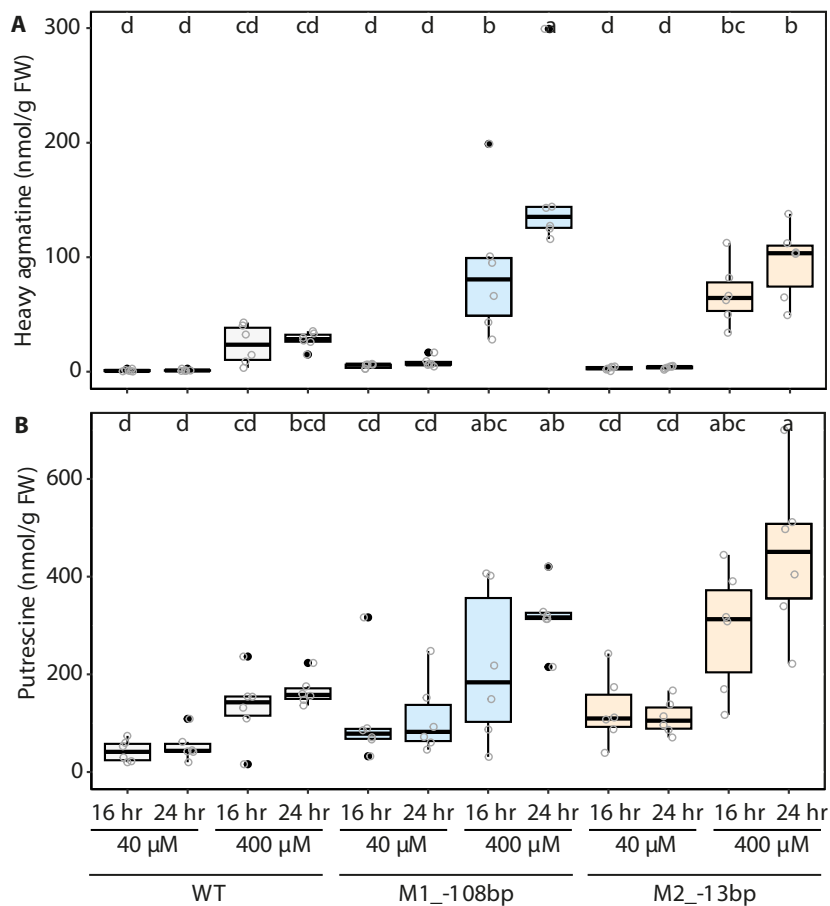


Figure 20: Higher heavy arginine substrate concentration increases ADC activity but does not change putrescine levels.

WT (grey), SI1M_-108 (blue), and SI2M_-13 (yellow) tomato leaflets were detached and cold-treated overnight at 4 °C for either 16 or 24 hours (n = 6). Duplicate 50mg samples were harvested from each leaflet and frozen. One of each duplicate sample was homogenised in ADC activity buffer containing either 40 or 400 μ M heavy arginine and incubated (37 °C, 3 hours, 300 rpm), stopped with HFBA, centrifuged, and diluted for LCMS analysis. The concentration of heavy agmatine (ADC activity) (A) and putrescine (B) are shown in nmol/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different (α = 0.05).

24 hours of cold treatment (318 nmol/g FW and 446 nmol/g FW, respectively compared to 166 nmol/g FW of WT). Overall, these results demonstrate that a higher ADC substrate concentration escalates the ADC activity and putrescine content differences between WT and each *sladc-box* mutant, thereby improving the assay sensitivity.

In general, the prolonged cold treatment of 24 hours compared to 16 hours only marginally increased the ADC activity levels by ~1.6-fold. However, significant differences between each *sladc-box* mutant were only seen after 24 hours of cold treatment and 400 μ M heavy arginine, but not after 16 hours (Figure 20A). A significantly higher putrescine content was also observed in the SI2M_-13 mutant compared to WT after 24 hours of cold treatment and 400 μ M heavy arginine (Figure 20B). Therefore, 24 hours of overnight cold treatment was selected as the most suitable stress treatment for future assays. In summary, the substrate concentrations and ADC-stimuli conditions were adjusted to have a higher heavy arginine concentration (500 μ M) in the assay buffer (Table 24) and a 24 hour overnight cold treatment, respectively.

The second tomato optimisation assay experiment tested if the assay duration would influence the amount of heavy agmatine produced during the assay period and/or reduce the amount of variation between replicates. Previously, samples were incubated for 3 hours at 37 °C, yet this duration may not optimally show differences in ADC activity between genotypes. For example, larger differences in ADC activities may be observed with shorter incubation times before the ADC activity plateaus due to reduced substrate availability, protein degradation or deterioration, or possible inhibition of ADC activity by increasing agmatine levels or an unknown feedback mechanism. On the other hand, it is possible that extending the ADC activity assay incubation time may allow for even more agmatine to be produced. Therefore, an experiment in which assay incubation times of 0.5, 1, 3, and 6 hours were tested. Here, 24 replicate WT, SI1M_-108, and SI2M_-13 detached tomato leaflets were cold treated for 24 hours. All replicate samples were extracted in the same buffer (Table 24), with a final heavy arginine concentration of 500 μ M. All samples were incubated at 37 °C at the same time, but after 0.5, 1, 3, and 6 hours, 6 replicates from each genotype were removed, the reaction stopped, and samples prepared for LCMS analysis (section 2.2.5.3).

Unexpectedly, the levels of D₅ tryptophan, which was meant to serve as an internal technical standard, decreased in all genotypes with ongoing assay time (Figure 21A). As metabolite concentrations in each sample are typically normalised to the level of D₅ tryptophan, this assay-duration-dependent decrease would impact the ability to compare metabolite concentrations between assay durations. Therefore, for this experiment, the D₅ tryptophan normalisation was omitted from the calculations.

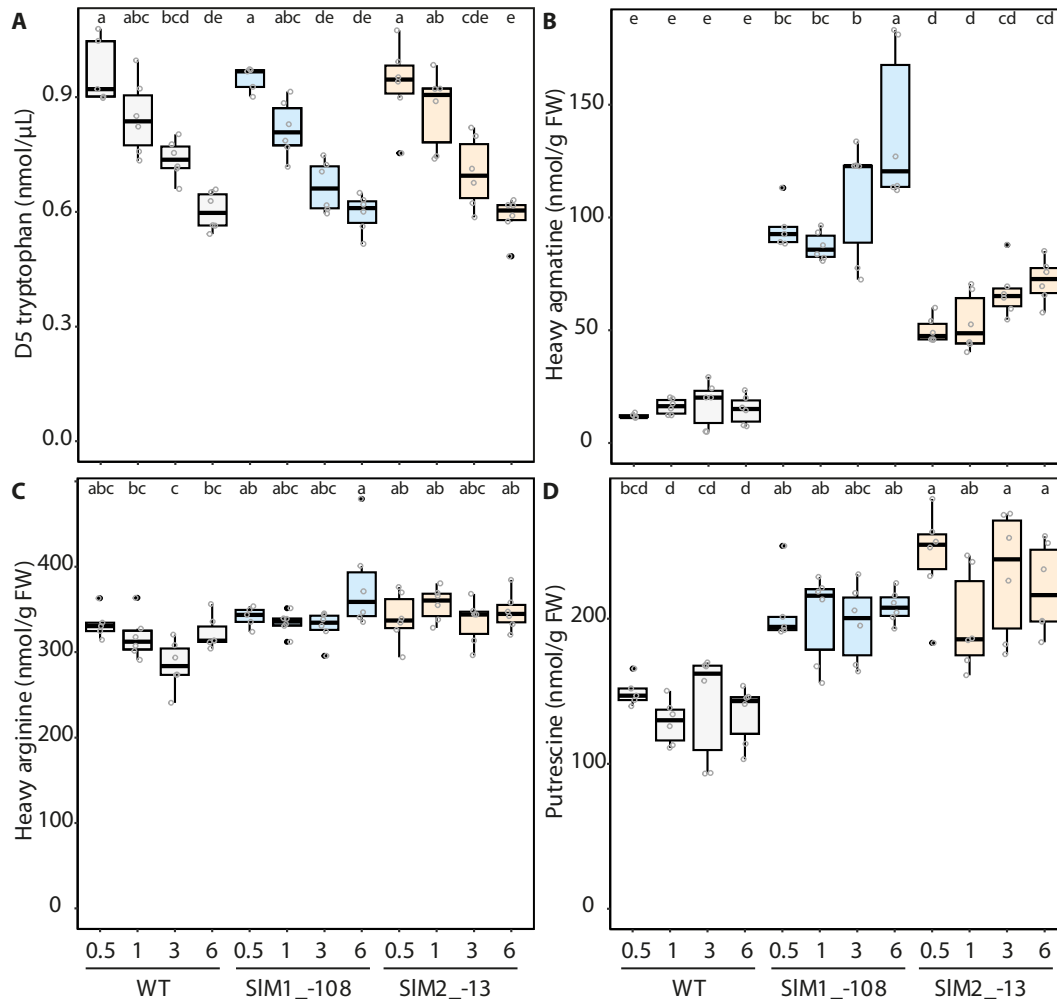


Figure 21: Duration of the 37 °C ADC assay incubation does not dramatically alter ADC activity levels.

WT (grey), SIM1_-108 (blue), and SIM2_-13 (yellow) tomato leaflets were detached and cold-treated for 24 hours (n = 6). Harvested samples were frozen, homogenised in ADC activity buffer containing 500 μM heavy arginine and incubated at 37 °C for either 0.5, 1, 3, or 6 hours, then prepared for LCMS analysis. D₅ tryptophan (A) was not used to normalise heavy agmatine (ADC activity) (B), putrescine (C), and heavy arginine (D) levels for this experiment. An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters above each box indicate significance, where groups with different letters are significantly different ($\alpha = 0.05$).

Heavy agmatine levels in SIM1_-108 and SIM2_-13 generally increased with longer assay durations, but this was only significant for the SIM1_-108 mutant after a 6-hour incubation compared to the other incubation times (Figure 21B). When comparing heavy agmatine levels between genotypes with the same incubation duration, significant differences were always observed (i.e., WT always had the lowest heavy agmatine levels, while SIM1_-108 the highest, and SIM2_-13 in the middle). This indicates that significant differences in ADC activity can be observed irrespective of the assay duration. For example, after a 1-hour incubation duration, SIM1_-108 had 5.4-fold higher ADC activity, while SIM2_-13 had 3.3-fold higher ADC activity. It is also important to note that the concentration of heavy arginine, the ADC activity

assay substrate, did not change over the course of the assay (Figure 21C), indicating that it is both stable and consistently available for decarboxylation by ADC for at least 6 hours.

Putrescine levels were not impacted by the duration of the assay for any genotype (Figure 21C). However, both *SLADC-box* mutants had significantly higher putrescine levels compared to WT resulting from the 1- and 6-hour assay durations, with both mutants having approximately 1.6-fold higher putrescine levels compared to WT after both assay durations.

Overall, based on these results, a 1-hour assay duration was decided on for future assays for multiple reasons. Firstly, the variation in ADC activity and putrescine levels between replicated was relatively small at this time point, enabling clearer differences to be seen between the *sladc-box* mutants and WT. The deterioration of D₅ tryptophan worsened as the assay duration increased, so a shorter assay duration would limit this, as well as possible deterioration of other metabolites not measured in this assay. Finally, a shorter incubation time of 1 hour, compared to the previous 3 hours, also reduces the experimental time pressure, allowing for larger experiments to be run within one day.

As a final optimisation experiment, the method used for stopping the ADC activity was tested. Typically, ADC activity assays were stopped by adding 5 μ L of HFBA to the samples, causing rapid acidification and protein denaturation, preventing subsequent proteinaceous reactions. Alongside the ADC activity assay samples, so called “stop samples” were also made by taking 100 μ L from the extracted sample before the 37 °C incubation step, and immediately stopping the reaction with 5 μ L HFBA. These stop samples were used to determine the original PA profile of plant extracts prior to the 37 °C assay incubation. This enables the measurement of compounds before they can be further metabolised or degraded during the 37 °C incubation due to heat sensitivity. This is especially the case for various hydroxycinnamic acid (HCA)-PA conjugates (i.e., phenolamides), as many of which had significant decreases in concentrations or all but disappeared after the 37 °C incubation. It was in these stop samples that a small amount of heavy agmatine was found, indicating that some ADC activity was already occurring, despite the extremely short storage of samples on ice prior to acidification. While this highlighted the necessity to keep all samples as cold as possible outside of the 37 °C incubation step, it also introduced the idea that a more efficient method for stopping the reactions may reduce the error between replicates, which may have been introduced with low levels of activity occurring while the remaining samples are extracted. Therefore, as an alternative “stopping” method, heat inactivation of proteins in the samples was considered, as it is possible that heat may denature proteins and stop enzymatic reactions faster than acidification by HFBA.

Three replicates of 24-hour cold-treated WT and SI2M₋13 tomato leaflets were extracted in 400 μ L of ADC extraction buffer (Table 24) and divided into 4 tubes, each with 100 μ L. One set of replicates were incubated at 37 °C for an hour as for a normal ADC activity assay, while the second set was directly stopped by adding 5 μ L HFBA to generate stop samples. The final two sets were stopped with either 1 or 5 minutes of 90 °C heat treatment. All samples were then analysed via LCMS. Both acidification and heat inactivation equally prevented ADC activity in the stop samples, with hardly any heavy agmatine found above background levels (Figure 22A). The ADC activity assayed samples showed significantly higher heavy agmatine levels compared to all stopped samples, with ADC activity was 5.6-fold higher in the SI2M₋

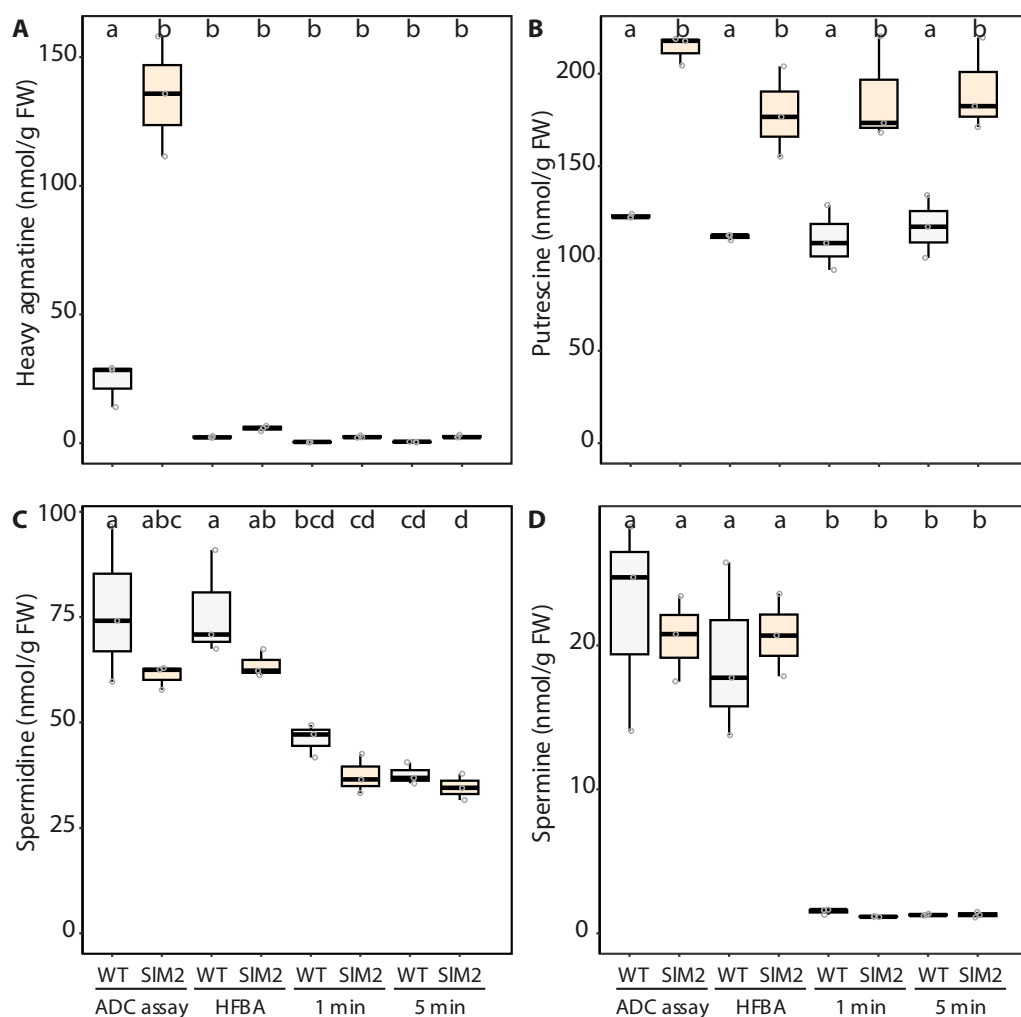


Figure 22: Acidification with HFBA is the best method for stopping ADC activity assays.

WT (grey) and SI2M₋13 (yellow, “SIM2”) tomato leaflets were detached and cold-treated overnight at 4 °C for 24 hours ($n = 3$). Samples for LCMS analysis were prepared with 400 μ L of ADC activity extraction buffer containing 500 μ M heavy arginine. 100 μ L of each sample was transferred into 4 new tubes and either incubated at 37 °C for 1 hour (ADC assay), directly stopped with 5 μ L HFBA (HFBA), or 90 °C heat treated for 1 or 5 minutes. The concentration heavy agmatine (ADC activity) (A) putrescine (B), spermidine (C), and spermine (D) were measured in nmol/g FW, normalised to D₅ tryptophan (internal standard). An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters displayed above each box indicate significance; groups with different letters are significantly different ($\alpha = 0.05$).

13 mutant compared to WT (135 nmol/g FW compared to 24 nmol/g FW; P -value < 0.000000; Figure 22A).

No difference in putrescine levels was observed between the ADC assay samples or any of the stopped samples between samples from the same genotype (Figure 22B); however, *Sl2M_13* had consistently ~1.6-fold higher putrescine levels compared to WT across all sample treatments. This demonstrated that putrescine levels were not altered during the 37 °C assay incubation compared to levels in the raw extracts. On the other hand, both spermidine and spermine levels were dramatically lower in heat inactivated samples compared to the HFBA-stopped samples and normal assay samples (Figure 22C and D, respectively). While spermidine levels were not significantly different between WT and *Sl2M_13* with any treatment, in almost all cases, heat inactivation significantly reduced spermidine levels compared to the assay or HFBA-stopped samples (Figure 22C). This pattern of results was even more striking for spermine, where almost no spermine could be observed above background levels after heat inactivation for both 1 and 5 minutes. These results reveal that a high heat treatment (i.e., 95 °C) can cause rapid deterioration of some metabolites, while the lower 37 °C assay incubation did not affect the level of these metabolites (i.e., when comparing assay samples to HFBA-stopped samples). In summary, while heat inactivation was an efficient stopping method for ADC activity, HFBA acidification remained the optimal stopping method as some of the measured metabolites seemed to be heat-sensitive but not affected by the acid treatment.

In summary, the ADC activity assay procedure was greatly optimised from the Wu *et al.*, 2019 published method; an overview of the updated tomato ADC activity assay procedure is shown in Figure 23. Initial ADC activity in WT un-treated tomatoes was hardly detectable (0.04 nmol/g FW), but with the additional cold-treatment step, ADC activity increased 25-fold to 1 nmol/g FW (Figure 18A). In the final optimisation assays (Figure 21B and Figure 22A), WT cold-treated tomato leaves had ~24 nmol/g FW heavy agmatine levels, equating to approximately 600-fold increase compared to the initial 0.04 nmol/g FW in Figure 18A.

This substantial increase in ADC activity levels was mostly due to the following changes: use of the entire extraction rather than just the supernatant, the addition of an abiotic stress treatment to induce *ADC* transcription (i.e., cold treatment of detached tomato leaflets), use of a higher ADC activity assay substrate concentration, and the new, more sensitive LCMS machine with a Trap-Elute system. These optimisations and changes now enable clear distinction between different *sladc-box* mutants and to WT.

Similarly, concentrations of other measured metabolites also increased due to the ADC activity assay optimisation experiments. Initially, WT untreated putrescine levels were 2.8 nmol/g FW,

which increased almost 6-fold to 16.6 nmol/g FW with 16-hour cold treatment (Figure 18B). In the final optimisation assays (Figure 21D and Figure 22B), putrescine levels were ~123 nmol/g FW in cold-treated WT tomato leaves, which is a ~44-fold increase compared to the non-treated leaves in Figure 18B. Similar to induced *SLADC* expression by Brg11 (Wu et al., 2019), putrescine levels, but not spermidine or spermine, were significantly higher in the *sladc-box* mutants S11M_-108 and S12M_-13 compared to WT (Figure 21 and Figure 22). This result suggests that mutating the *SLADC-box* may also influence PA levels, which was not observed in experiments prior to assay optimisation (Figure 19B).

Overall, using the optimised method, ADC activity, PAs, and related metabolites can now be measured accurately in tomato leaves. The S/N ratios have also been improved as metabolite levels fall within the linear range of the calibration curve and the *m/z* peaks are large and clearly distinct from background levels. These optimisations have increased assay sensitivity, enabling clear and significant differences between WT and *sladc1-box* and *sladc2-box* mutants (Figure 19A and Figure 21B). Finally, these results demonstrate that this ADC activity assay is a suitable method for analysing ADC protein abundance and is sensitive enough to analyse impact of different *ADC* 5'UTR mutations on overall ADC protein levels from plant extracts.

3.2.8 Preliminary work on *M. polymorpha* ADC activity assays

To determine if the repressive role of the *ADC-box* on ADC translation has been maintained across plant evolution, Pia Ellissen (Lahaye laboratory Masters student, 2020) conducted ADC

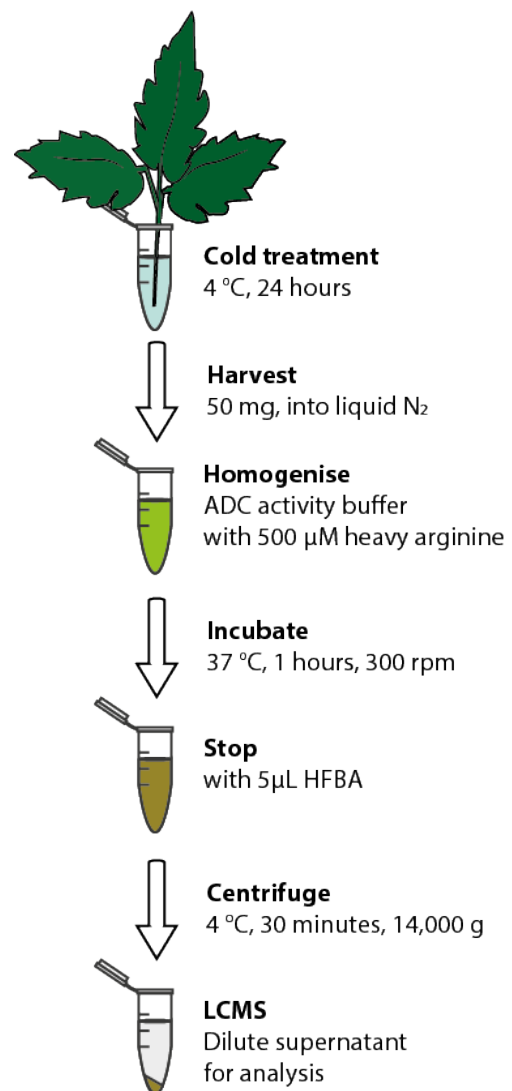


Figure 23: Schematic of optimised ADC activity assay protocol for tomato leaf tissue.

Detached tomato leaves are cold treated for 24 hours, before 50 mg samples are frozen in liquid N₂. Ground samples are homogenised in 300 µL of ADC activity buffer, containing 500 µM heavy arginine, and incubated for 1 hour at 37 °C. To stop the reactions, 5 µL of HFBA is added and samples are vortexed, before centrifugation at 4 °C for 30 minutes. Supernatant is the diluted in solvent for LCMS analysis.

activity assays on WT *M. polymorpha* (TAK1), four *adc-box* mutants (MpM_-8, MpM_+1^A, MpM_-14, and MpM_-6), and one control mutant (called #2-1, lacking 10 bp ~190bp 3' to the *MpADC-box*). After verifying that cold treatment also induced MpADC activity, an ADC activity was conducted with these six *M. polymorpha* genotypes following 16 hours of cold treatment. All four *MpADC-box* mutants had dramatically higher ADC activity levels, as well as agmatine and putrescine concentrations compared to TAK1 or the negative control mutant #2-1 (Pia Ellissen, Master thesis, 2020). These results demonstrated that the *MpADC-box* is a functional repressive element of MpADC and that mutating it also alters the greater PA metabolism. Overall, this work revealed that the *ADC-box* has likely remained a functional cis-element since the first land plants.

However, since this work, a further two *Mpadc-box* mutants (M_Mp_+1^B, and M_Mp_+1^C) and four additional control mutants (MpC_-30, MpC_-10, MpC_-4, and MpC_-1) with mutations outside of the conserved *MpADC-box* region had been generated. It was hypothesised that more *Mpadc-box* mutants would improve the elucidation of which specific *ADC-box* regions were important for its function. Therefore, to better understand the role of the *MpADC-box* on ADC translation and PA metabolism, and to interrogate the different GC-rich elements of the *MpADC-box*, further LCMS analysis was to be conducted.

Additionally, since the previous *M. polymorpha* ADC activity assays, the new, more sensitive LCMS machine was installed (QTRAP6500+ with an LC M5-TE (Trap-Elute, ABSciex, USA)) and various aspects of the ADC activity assay procedure had been optimised (section 3.2.7). As these improvements had dramatically increased ADC activity and PA levels in tomato, and therefore the overall assay sensitivity, it was vital to check that these protocol alterations were still suitable for *M. polymorpha* tissue, determine an optimal dilution for the LCMS samples, and if anything could be further optimised for this species.

3.2.9 Initial ADC activity assay test using *M. polymorpha* TAK1.

An initial experiment was conducted to test if the changes to the ADC activity assay protocol and new LCMS machine still enabled clear and sensitive measurement of ADC activity, PAs, and other related metabolites from *M. polymorpha* tissue. For this experiment, TAK1 plants were grown from gemmae on ½ B5 agar plates (1% sucrose; Table 2; section 2.1.3 2.2.1.3) for 4.5 weeks, were either cold treated for ~18 hours in the dark overnight or covered by tin foil and left in the climate chamber as a room temperature (RT) control. Samples were frozen and extracted in ADC activity buffer (Table 24) using 500 µM heavy arginine and then an ADC

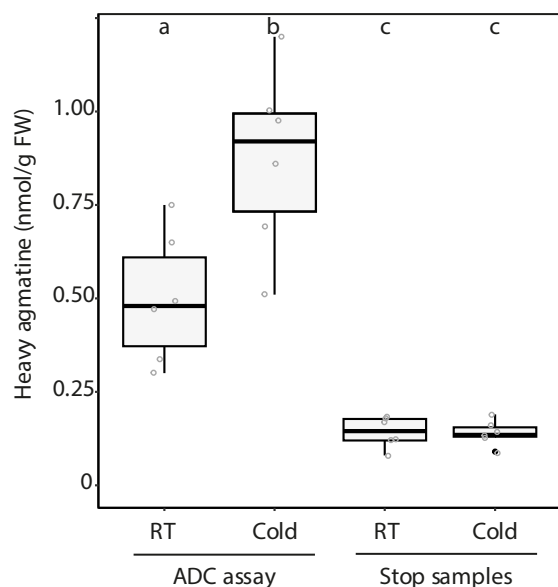


Figure 24: Cold treatment only minorly increased *M. polymorpha* ADC activity.

WT TAK1 plants were kept in the dark for 18 hours either in the cold (4 °C) or at room temperature (RT) (n = 6). Samples for ADC activity assays were harvested, homogenised in buffer containing heavy arginine (+11), and both normal assay and stop samples were generated for LCMS analysis. heavy agmatine concentration is normalised to the internal D₅ tryptophan standard. An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters displayed above each box indicate significance; groups with different letters are significantly different ($\alpha = 0.05$).

activity assay was conducted and samples were prepared for LCMS analysis (section 2.2.5.3). A range of sample supernatant dilutions in solvent were initially checked, but a final dilution of 1:25 as almost all measured metabolite levels fell within the linear range of the calibration curves. In contrast to previous findings, where cold treated TAK1 heavy agmatine levels were ~5-fold higher than the RT controls (Figure 13 of Pia Ellissen's Master Thesis, 2020), only a 1.7-fold increase in heavy agmatine levels was observed compared to the RT control (Figure 24; P -value = 0.002). Almost no heavy agmatine was seen in the stop samples, demonstrating that while the TAK1 RT ADC activity level was low, it was still significantly higher than background levels by 3.5-fold (P -values < 0.004). There were also no significant differences in agmatine, or putrescine levels seen between cold- or RT-treated samples in this experiment, implying that the cold treatment and/or the current experimental parameters did not induce ADC activity.

Upon closer inspection of the LCMS signal peaks, there was another compound that had a similar m/z ratio and retention time to heavy agmatine. Therefore, this other compound had a slightly overlapping peak with heavy agmatine, obscuring it in samples with low amounts of heavy agmatine and preventing clear isolation of the peak area for subsequent calculations. This other overlapping peak has been previously seen in tomato samples with very low ADC activity when using the D₇ ¹⁵N₄ arginine (+11) isotope variant, but this peak was much more prominent in *M. polymorpha* extracts. It was therefore hypothesised that by using the ¹³C₆ arginine (+6) isotope variant, instead of the D₇ ¹⁵N₄ arginine (+11) variant, unobscured ¹³C₅ agmatine peaks would be produced, enabling more accurate ADC activity measurement. Therefore, a second ADC activity assay experiment with the 6 *M. polymorpha* *adc-box*

mutants, 4 control mutant lines, and TAK1 was conducted to test if using the alternative $^{13}\text{C}_6$ arginine isotope would provide clearer ADC activity results. All plants were grown on $\frac{1}{2}$ B5 agar plates (containing 1% sucrose) and cold treated for 16 hours, before samples were harvested and prepared for an ADC activity assay with 500 μM $^{13}\text{C}_6$ arginine (Table 24; section 2.2.5.3).

Use of the alternative $^{13}\text{C}_6$ arginine produced unobscured $^{13}\text{C}_5$ agmatine peaks, allowing for clearer isolation of the peak area for subsequent calculations, confirming that this $^{13}\text{C}_6$ arginine isotope variant should be used for all future *M. polymorpha* ADC activity assays. Despite using $^{13}\text{C}_6$ arginine, no significant difference between the TAK1 cold-treated and RT control was observed (Figure 25A; P -value = 1.0), once more indicating that the 16-hour cold treatment

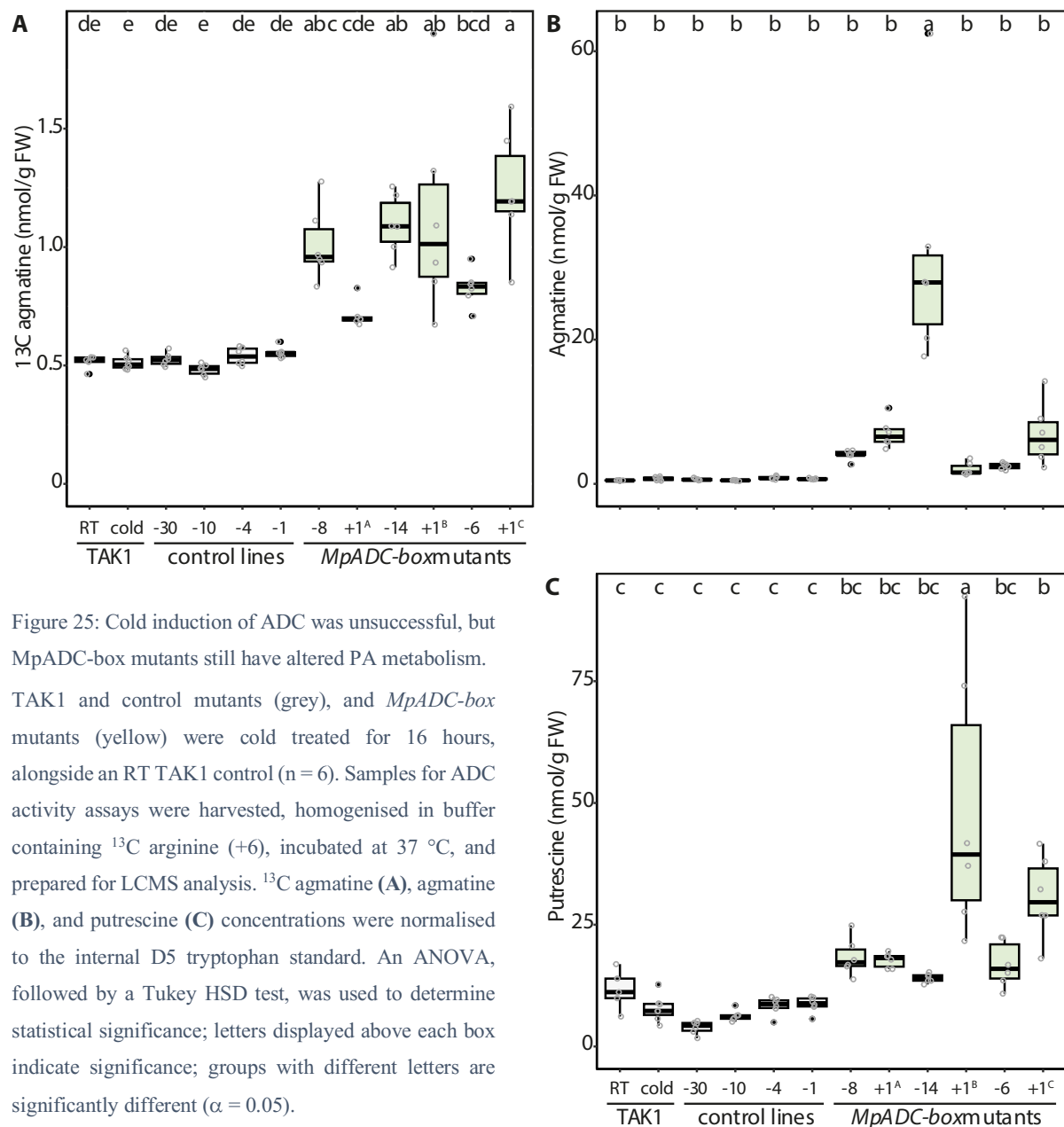


Figure 25: Cold induction of ADC was unsuccessful, but MpADC-box mutants still have altered PA metabolism. TAK1 and control mutants (grey), and *MpADC-box* mutants (yellow) were cold treated for 16 hours, alongside an RT TAK1 control ($n = 6$). Samples for ADC activity assays were harvested, homogenised in buffer containing ^{13}C arginine (+6), incubated at 37 °C, and prepared for LCMS analysis. ^{13}C agmatine (**A**), agmatine (**B**), and putrescine (**C**) concentrations were normalised to the internal D5 tryptophan standard. An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters displayed above each box indicate significance; groups with different letters are significantly different ($\alpha = 0.05$).

did not induce ADC activity levels as it did previously (Pia Ellissen, Master's thesis). However, despite the lack of cold-induction, all *Mpadc-box* mutants except for MpM₊1^A had significantly higher ADC activity compared to the cold-treated TAK1, albeit with an average increase of only ~2-fold. Previously, *adc-box* mutant MpM₋14 had 19-fold higher ADC activity compared to TAK1 following cold-treatment (Figure 14, Pia Ellissen Master's thesis), which is vastly greater to the 2-fold increase seen here. This confirmed that the current cold treatment and/or experimental set up did not stimulate ADC levels to enable clear differentiation of ADC activities between TAK1 and the *Mpadc-box* mutants.

However, the *Mpadc-box* mutants had much greater agmatine levels compared to the TAK1 and the control mutants (Figure 25B), with MpM₋14 having 44-fold more agmatine compared to TAK1 (*P*-value = 0.0000). Putrescine levels of the *Mpadc-box* mutants were also significantly higher than TAK1 and control mutant lines (Figure 25C). It is noteworthy that no significant differences in agmatine or putrescine levels were found between RT or cold-treated TAK1.

Overall, agmatine and putrescine levels were dramatically higher in the *MpADC-box* mutants, despite only a ~2-fold increase in ADC activity levels. This finding suggested that the *MpADC-box* mutations had altered the PA metabolism, but this was not clearly observed in the ADC activity assay results as it had previously (Pia Ellissen, Master thesis, 2020) and not due to cold-treatment. Therefore, some aspect of the experimental procedure was causing MpADC to not be induced as it had previously by cold treatment and optimisation was required.

3.2.10 Optimisation of *M. polymorpha* ADC activity assays

Firstly, to determine if the shortened 37 °C incubation duration (i.e., 1 hour) was enough time for MpADC to produce sufficiently large heavy agmatine signal peaks, a second experiment was conducted to compare assay durations of 1 hour and 3 hours. Here, both TAK1 and the *Mpadc-box* mutant MpM₋14 (with a 14 bp deletion spanning the first GC-rich motif of *MpADC-box*; Figure 16) were used for this experiment and grown on ½ B5 agar plates (1% sucrose; Table 2). It was thought that any differences in ADC activity caused by the extended assay duration would be more pronounced in the MpM₋14 mutant due to its almost complete loss of the *MpADC-box*. Here, no cold treatment was used, as the primary aim of this experiment was to solely investigate the assay duration, and previous experiments had not showed any difference in ADC activity with the current protocol (Figure 25). Samples were harvested from these genotypes, extracted in ADC activity assay buffer (Table 24), incubated for either 1 or 3 hours at 37 °C, stopped, and prepared for LCMS analysis. No significant

differences in heavy agmatine levels were found between each genotype after a 1- or 3-hour incubation (Figure 26A). However, ADC activity of MpM_-14 was only significantly higher than TAK1 after the 1-hour incubation (P -value = 0.043; Figure 26A), but not after 3 hours (P -value = 0.056). Putrescine levels were ~9-fold higher in MpM_-14 compared to TAK1 but were not significantly altered by extending the 1-hour assay duration to 3 hours (Figure 26B). Interestingly, spermidine levels were reduced in the 3-hour incubated samples compared to the 1-hour incubated samples for both TAK1 and MpM_-14, although not significantly (Figure 26C). As previous tomato assays have indicated that PA levels may be influenced by heat (Figure 22), keeping to a 1 hour 37 °C incubation may maintain levels of various measured metabolites. Overall, similarly to the tomato ADC activity assay procedure, a 1-hour, 37 °C incubation was upheld for future experiments.

Despite finding that a 1 hour 37 °C assay incubation duration is optimal, it did not explain the lack of a dramatic increase in heavy agmatine accumulation in the MpM-14 mutant compared

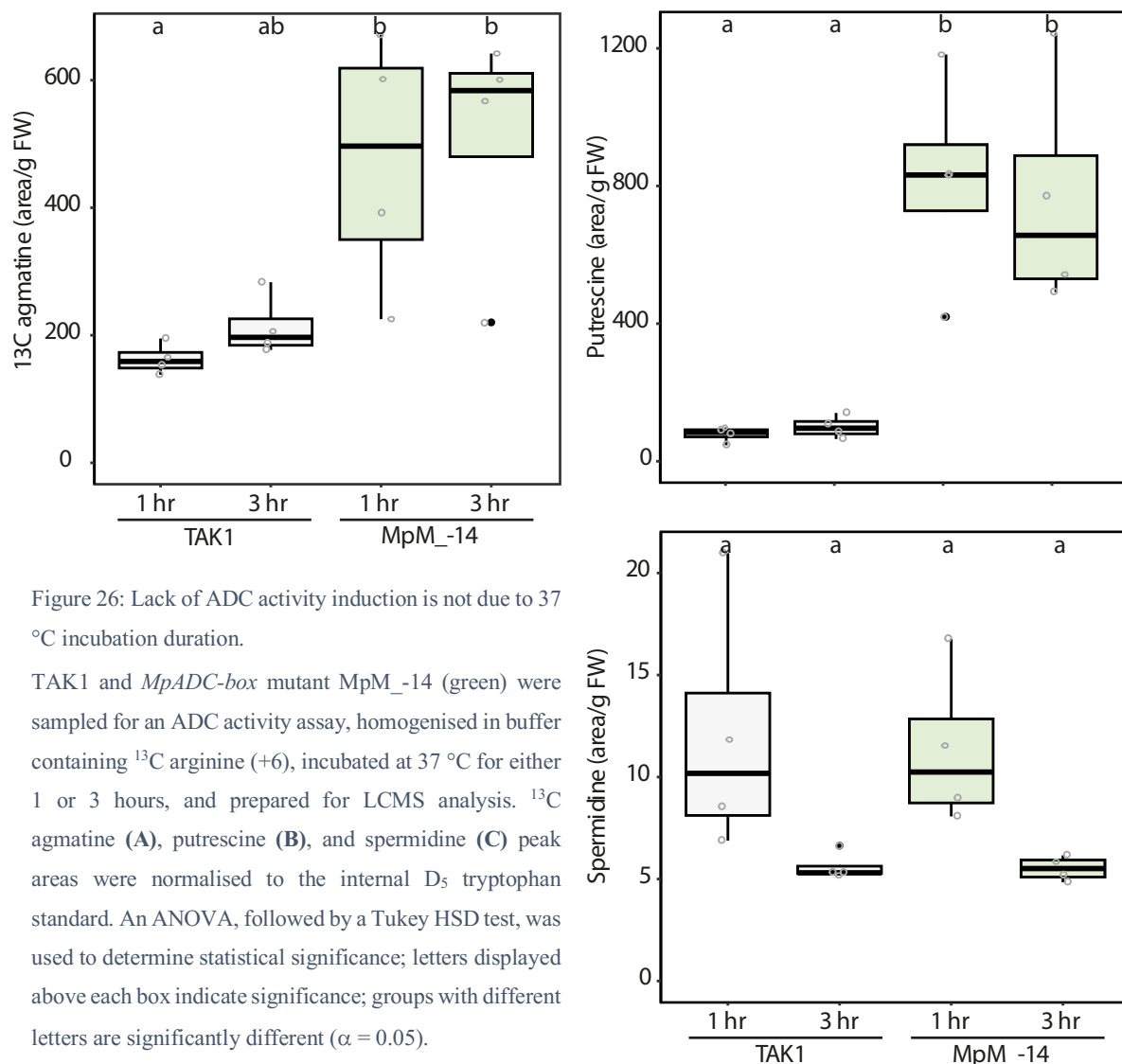


Figure 26: Lack of ADC activity induction is not due to 37 °C incubation duration.

TAK1 and *MpADC-box* mutant MpM_-14 (green) were sampled for an ADC activity assay, homogenised in buffer containing ^{13}C arginine (+6), incubated at 37 °C for either 1 or 3 hours, and prepared for LCMS analysis. ^{13}C agmatine (A), putrescine (B), and spermidine (C) peak areas were normalised to the internal D₅ tryptophan standard. An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters displayed above each box indicate significance; groups with different letters are significantly different ($\alpha = 0.05$).

to TAK1, which was previously observed. Therefore, two other aspects of the experimental procedure were interrogated. Firstly, the growth conditions of the *M. polymorpha* were scrutinised. So far, *M. polymorpha* were grown on ½ B5 agar containing 1% sucrose, yet although addition of sucrose is common for *M. polymorpha* media, is not required for growth. Importantly, sucrose has been found to induce *ADC* transcription and accumulation of putrescine, spermine, and spermidine in both *A. thaliana* and maize (Hummel et al., 2004, El Amrani et al., 2019, Altuntas et al., 2019). This led to the hypothesis that the sucrose in the ½ B5 media may be inducing *MpADC* transcription for the whole growth period, resulting in no observed additional *MpADC* activity with cold treatment. In conjunction with this, agmatine and putrescine levels were significantly higher in the *Mpadc-box* mutants compared to controls or TAK1, despite only minor increases in *ADC* activity (Figure 25), suggesting that high amounts of agmatine had been produced prior to the *ADC* assay incubation. The second aspect of the *ADC* assay procedure that was interrogated was the cold treatment duration. Previously, 16 hours of cold treatment was used, but either a shorter or longer duration may induce *MpADC* activity more. Therefore, an *ADC* activity experiment was conducted using TAK1 and *MpM*_-14 grown in the absence of sucrose, and cold treated for 12, 16, or 24 hours. Cold treatment did not significantly increase TAK1 *ADC* activity levels compared to the RT control (Figure 27). However, cold treatment for 12 and 16 hours significantly increased *ADC* activity in the *MpM*_-14 mutant by 2.1- and 2.7-fold, respectively (*P*-values = 0.003 and <0.0001, respectively). *ADC* activity began to decrease again with 24 hours of cold treatment to levels

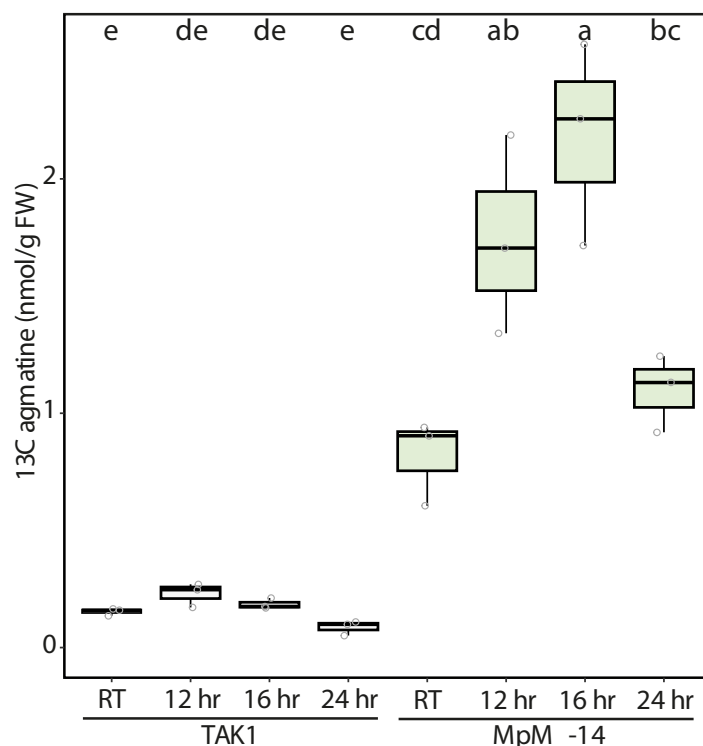


Figure 27: 16 hours of cold treatment produces the highest *ADC* activity in an *M. polymorpha* *adc-box* mutant.

TAK1 and *MpADC-box* mutant *MpM*_-14 (green) were cold treated for either 12, 16, or 24 hours and sampled for an *ADC* activity assay, alongside a room temperature (RT) control. Samples were homogenised in buffer containing ¹³C arginine (+6), incubated at 37 °C for either 1 or 3 hours, and prepared for LCMS analysis. ¹³C agmatine (A), putrescine (B), and spermidine (C) peak areas were normalised to the internal D₅ tryptophan standard. An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters displayed above each box indicate significance; groups with different letters are significantly different to $\alpha = 0.05$.

similar to that of the RT control for both TAK1 and MpM_-14. This experiment confirmed that 16 hours of cold treatment is the optimal duration for inducing MpADC activity.

In this experiment, the MpM_-14 mutant had 11.8-fold higher ADC activity compared to TAK1 after 16 hours of cold treatment (P -value < 0.000001), which is much greater than previous assays that had 2.1-fold (Figure 25A) and 2.9-fold (Figure 26A) higher ADC activity. This dramatic improvement in difference between the *Mpadc-box* mutant and TAK1, is likely attributed to the lack of sucrose in the media, and therefore, no additional *MpADC* induction occurring prior to the cold treatment.

In summary, the procedure for conducting ADC activity assays with *M. polymorpha* tissue was interrogated and optimised. Initially, cold treatment only induced MpADC activity 1.7-fold (Figure 24) and the ADC activity of *Mpadc-box* mutants was only ~2-fold greater than that of TAK1 (Figure 25), which was greatly lower than the 19-fold difference previously reported (Pia Ellissen, Master's thesis, 2020). However, this initial experiment highlighted that contaminating metabolites with similar retention times and m/z ratios can produce signal peaks that overlap with measured heavy D₇ ¹⁵N₄ agmatine. To avoid this contaminating peak, the alternative ¹³C₆ arginine (+6) was used for further *M. polymorpha* ADC activity assays. After ensuring that shortening the assay duration to 1 hour from 3 hours was not the cause of these reduced fold changes (Figure 26), other aspects of the *M. polymorpha* growth conditions and cold treatment were interrogated. A cold treatment time course experiment verified that a 16-hour cold treatment produced the highest induction of MpADC activity (Figure 27). However, removing the sucrose from the ½ B5 media resulted in 11.8-fold higher ADC activity in MpM_-14 compared to TAK1 (Figure 27), which in previous experiments was only 2-fold higher. Overall, the protocol for *M. polymorpha* ADC activity assays was verified and fine-tuned during this work, enabling clear differentiation of ADC activities and PA levels between TAK1 and a *M. polymorpha adc-box* mutant.

3.2.11 Optimisation of *A. thaliana* ADC activity assays

Unlike tomato, *A. thaliana ADC1* and *ADC2* genes are not stimulated by the same stressor. *AtADC1* is primarily induced by abiotic stressors such as cold, heat, and drought, while *AtADC2* is induced by biotic stressors such as *P. syringae* infection, elicitor treatment, or wounding, as well as abiotic stressors such as salt and osmotic stress (Perez-Amador et al., 2002, Urano et al., 2004, Kilian et al., 2007, Cuevas et al., 2008, Hussain et al., 2011, Chen et al., 2019). Therefore, to induce ADC activity levels of WT and *A. thaliana adc-box* mutants

into a range that can be analysed by LCMS, different inducers of *AtADC1* and *AtADC2* transcription are required.

To individually induce *AtADC1* or *AtADC2*, cold treatment or infection with *P. syringae*, respectively, had previously been tested by Laura Schmalz (Master thesis, 2022). While cold treatment specifically increased ADC activity in an *AtADC1*-dependent manner, infection with *P. syringae* induced both *AtADC* genes, with high variation between replicates (Figure 16 and 17 of Laura Schmalz, Master thesis, 2022). Significant increases in ADC activity were observed following a 12-hour cold treatment of some *Atadc1-box* mutant plants grown in soil, with the highest having 6-fold higher ADC activity compared to WT (Figure 21 of Laura Schmalz, Master thesis, 2022). Only one *Atadc2-box* mutant showed significantly higher ADC activity following *P. syringae* infection compared to WT, but with only a 2-fold increase (Figure 25 of Laura Schmalz, Master thesis, 2022). In conclusion, while some *Atadc-box* mutants had statistically significant increases in ADC activity compared to WT, the reproducibility and effectiveness of the stimuli, as well as clear difference between the *adc-box* mutants and the WT, were not as pronounced as for tomato and *M. polymorpha*. Therefore, alternative stressors or experimental conditions were proposed to improve reproducible and homogeneous induction of *AtADC1/2* expression.

Firstly, it was hypothesised that growing plants vertically on agar and cold treating whole plates would provide a more pronounced and homogeneous cold stress to the whole plant, thereby increasing *AtADC1* expression more than what was previously observed (Figure 16 of Laura Schmalz, Master thesis, 2022). To test this, seeds of WT and two *Atadc1-box* mutants with either a 53 bp deletion (*At1M_-53*) or a 15 bp deletion (*At1M_-15*) spanning the *Atadc1-box* were sterilised, sown on ½ MS agar plates (without sucrose; Table 2), and grown vertically for 2 weeks, before being cold treated for 12 hours. Three seedlings had to be pooled together to create a sample of ~10 mg FW (i.e., five times lower than the typical 50 mg used). A control group of plates were kept in the climate chamber wrapped in tinfoil as non-treated RT controls; note that only one control replicate for each genotype, each made up of three seedlings, was used in this experiment. Despite the much lower amount of FW tissue used for the samples, a dilution of 1:40 was still found to be optimal for the LCMS samples (i.e., same dilution as for 50 mg FW tomato samples).

Cold-treated WT seedlings had 16-fold higher ADC activity compared to the RT control (*P*-value = 0.66), while the *At1M_-53* and *At1M_-15* mutants had an average of ~27-fold higher ADC activity compared to their RT controls (*P*-values = 0.013 and 0.04, respectively; Figure 28A). Cold-treated *At1M_-53* had 3.3-fold higher ADC activity compared to WT, while

At1M₋₁₅ had levels 2.7-fold higher (P -values = 0.009 and 0.04, respectively). These results demonstrate that cold treatment for 12 hours of whole seedlings induced ADC activity dramatically, and enabled differences to be seen between the *Atadc1-box* mutants and WT. Additionally, the replicate variation was rather low, despite only 3-4 replicates being used here. Despite the substantial induction of ADC activity by cold treatment, no other measured metabolites were significantly altered compared to the RT controls (Figure 28 and Supplementary Figure 14). Additionally, acetylputrescine was the only metabolite to be

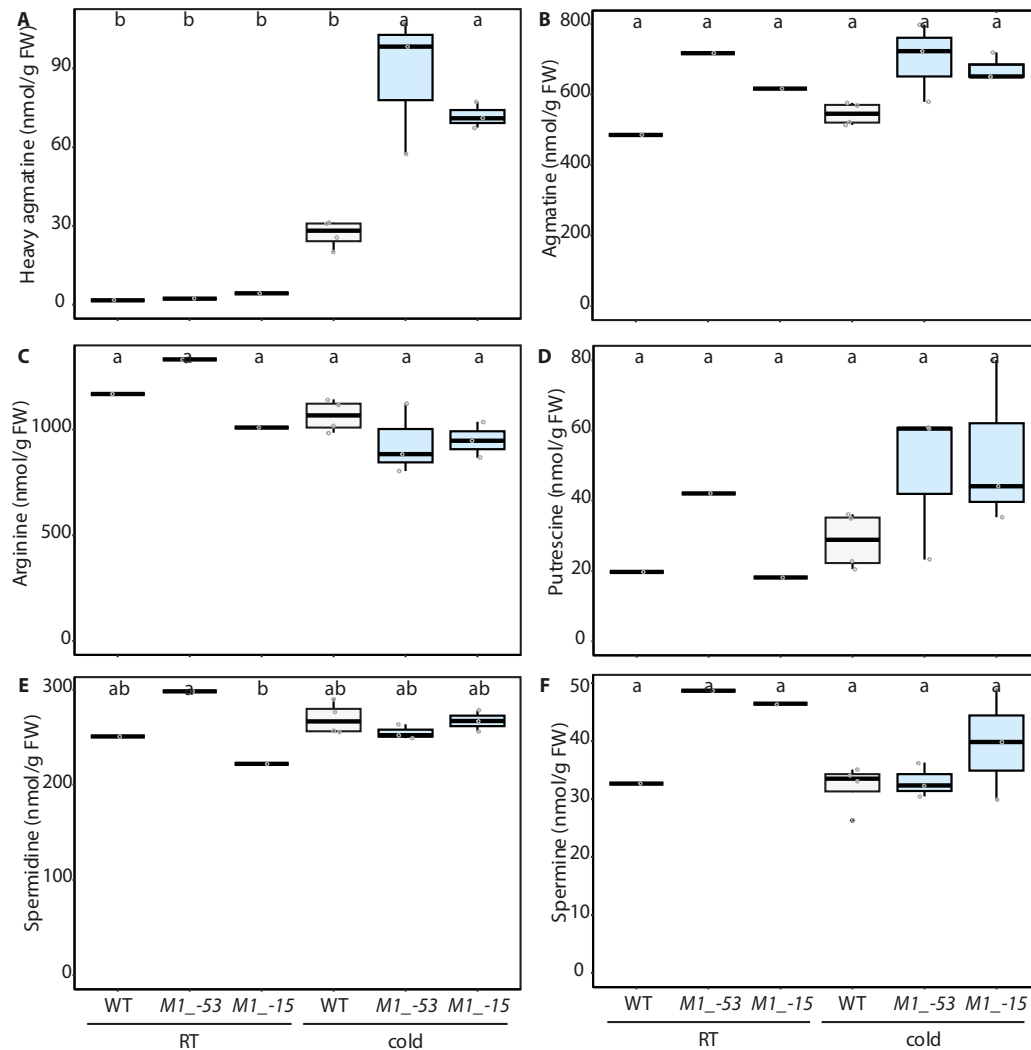


Figure 28: Cold treatment induces ADC activity but does not alter levels of other metabolites.

WT (grey) and *Atadc1-box* mutants AtM1₋₅₃ and AtM1₋₁₅ (blue) were grown vertically on ½ MS plates for 2 weeks before being cold treated for 12 hours at ~5°C and sampled for an ADC activity assay and LCMS analysis of the PA-related metabolite profile. Three seedlings were pooled to generate a sample of ~10 mg ($n = 3-4$). One replicate plate was kept in the climate chamber, wrapped in tinfoil as a room temperature (RT) negative control ($n = 1$). Samples were homogenised in buffer containing heavy arginine, incubated at 37 °C for 1 hour, and prepared for LCMS analysis. heavy agmatine (A), agmatine (B), arginine (C), putrescine (D), spermidine (E), and spermine (F) levels are shown in nmol/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

significantly increased in the *Atadc1-box* mutants compared to the WT (Supplementary Figure 14A). D₅ tryptophan levels were equal across treatments and genotypes (Supplementary Figure 14F).

Overall, it seems that although ADC activity increases upon cold treatment, this does not resonate to agmatine levels or any other ADC-influenced metabolite, signifying that further experiments to identify the best cold treatment duration are required. However, this experiment has shown that by using whole seedlings, commonly measured metabolite levels can clearly be detected, which was not always the case with leaf samples of *A. thaliana* grown on soil. Additionally, variation between samples is greatly reduced when using whole seedlings, rather than leaves; however, this is most likely due to pooling 3 seedlings into one sample, and future experiments should focus on using a single biological replicate.

To induce *AtADC2* expression, *P. syringe* infections were previously attempted but led to high variation between replicates and did not induce ADC activity in an *AtADC2*-dependent manner (Laura Schmaltz, Master thesis, 2022). Therefore, another treatment to specifically induce *AtADC2* transcription that was more homogeneously and reproducibly applied to all plants was sort after. Salt stress was found to stimulate *AtADC2* expression (Hummel et al., 2004, Urano et al., 2004) and thought to be a simpler and more homogeneous treatment to administer compared to *P. syringae* infection. Growing seedlings on media containing salt would affect whole seedlings to an equal degree, possibly reducing variation between replicates.

Therefore, to test whether salt stress would induce ADC activity, seeds of WT and two *Atadc2-box* mutants with either an 18 bp deletion (*At2M_-18*) or a 6 bp deletion (*At2M_-6*; Figure 15) in the *AtADC2-box* were sterilised and then grown vertically on ½ MS agar (without sucrose; Table 2). After 7 days, seedlings were transferred to either fresh ½ MS agar with or without 150 mM NaCl; this concentration and method was based on that used by Urano et al., 2004. After another 7 days, control and salt-treated seedlings were harvested for ADC activity assays and LCMS analysis of PAs and related metabolites. Again, 3 seedlings were pooled together to generate a single sample, with a total of 4-6 replicate samples produced.

ADC activity was not affected by salt treatment as no significant differences were observed between genotypes grown on 0 mM or 150 mM NaCl-containing media (Figure 29A). ADC activity levels here were in a similar range to the RT controls in the previous experiment testing cold treatment induction on ADC activity (i.e., ~1 nmol/g FW; Figure 28A). The *Atadc2-box* mutants had significantly higher ADC activity compared to the WT following both control and salt-treated conditions. *At2M_-18* and *At2M_-6* had 3.7- and 2.9-fold higher ADC activity compared to WT when grown on control media (*P*-values = 0.006 and 0.065, respectively),

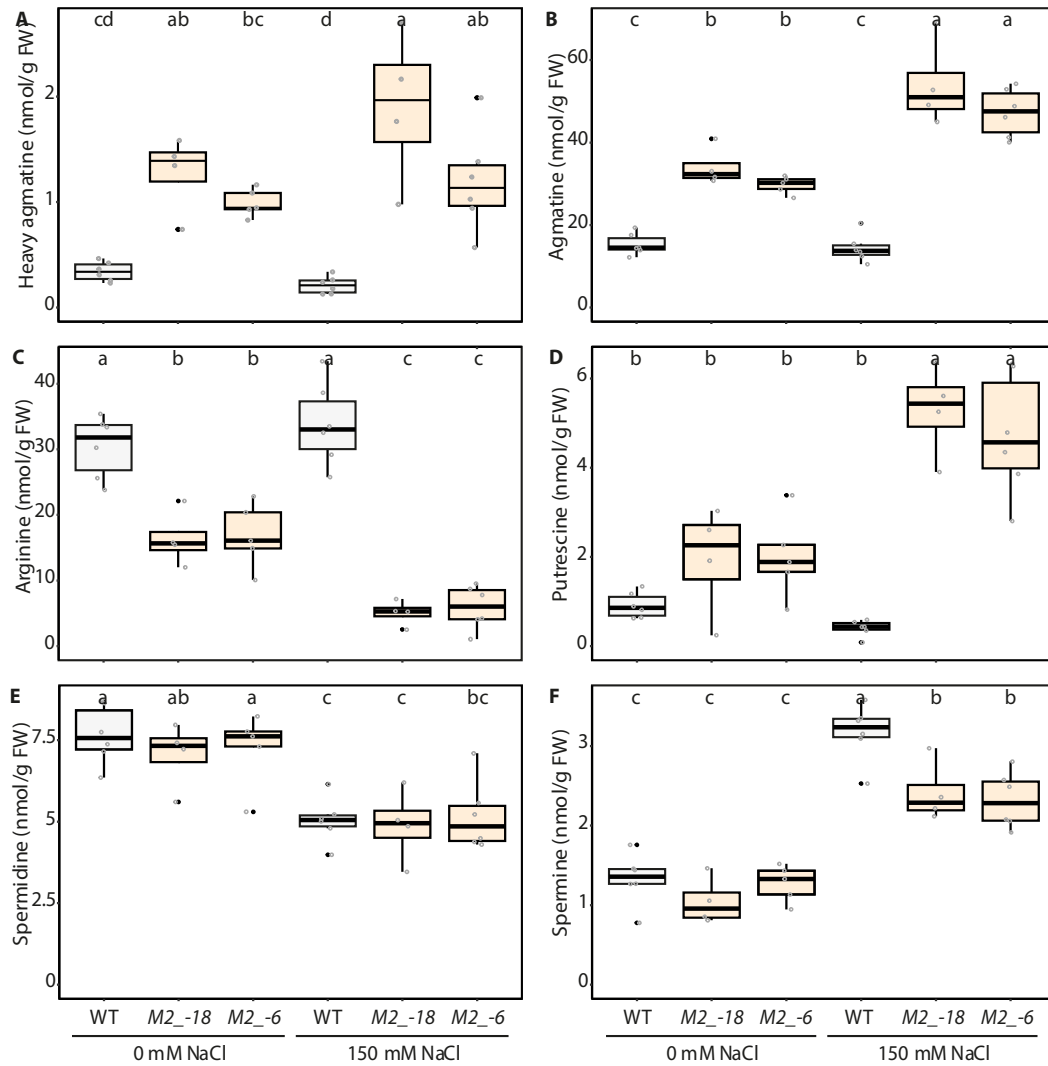


Figure 29: Salt treatment does not induce ADC activity but amplifies the effect of mutating the *AtADC2-box*.

WT (grey) and *Atadc2-box* mutants AtM2_-18 and AtM2_-6 (yellow) were grown vertically on $\frac{1}{2}$ MS plates for 7 days before being transferred to fresh $\frac{1}{2}$ MS plates with or without 150 mM NaCl. After another 7 days of growth, samples for an ADC activity assay and LCMS analysis of the PA-related metabolite profile were taken. Three seedlings were pooled to generate a sample of ~10 mg ($n = 4-6$). Samples were homogenised in buffer containing heavy arginine, incubated at 37 °C for 1 hour, and prepared for LCMS analysis. Heavy agmatine (A), agmatine (B), arginine (C), putrescine (D), spermidine (E), and spermine (F) levels are shown in nmol/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

while salt treatment increased these differences to 8.9- and 5.6-fold higher than WT (P -values = 0.000002 and 0.0011, respectively, Figure 29A). This indicated that although there were no significant differences between the same genotype grown on different media, salt treatment increased the extent to which the *Atadc2-box* mutants differed from WT.

Similarly, salt treatment did not affect levels of agmatine, arginine, or putrescine levels in the WT (Figure 29B, C, and D). However, the *Atadc2-box* mutants had significantly higher agmatine and putrescine levels following salt treatment compared to the control treatment. Yet

on the other hand, salt treatment decreased arginine levels more in the *Atadc2-box* mutants, compared to their non-treated controls. Salt treatment also reduced spermidine levels by approximately 1.4-fold for all genotypes (Figure 29E). Spermine levels were significantly increased upon salt treatment, even in the WT by 2.4-fold (P -value < 0.00000), with the *Atadc2-box* mutants having lower spermine levels compared to WT (P -values = 0.017 and 0.002; Figure 29F). Acetylputrescine levels were also increased only in the salt-treated mutants (Supplementary Figure 15A), while ornithine and especially citrulline levels tended to be higher following salt treatment (Supplementary Figure 15B and C). Interestingly, glutamine and GABA levels were reduced upon salt treatment (Supplementary Figure 15D and E). D₅ tryptophan levels were not influenced by salt treatment (Supplementary Figure 15F).

Overall, it seemed that salt treatment did not have a large impact on metabolite levels in the WT, but its effect seemed to be amplified in the *Atadc2-box* mutants. ADC activity was not induced at all by this salt treatment, suggesting that either a higher salt concentration or a different stimulus is required. Use of pooled whole seedlings also resulted in lower variation between replicates, allowing for finer differences to be observed compared to previous results using *P. syringae* as an *AtADC2*-inducer (Laura Schmaltz, Master thesis, 2022).

In summary, these initial experiments testing cold and salt treatment on WT and *Atadc-box* mutant seedlings demonstrated that these stimuli influence ADC activity and metabolite levels, but there is still room for improvement. Interestingly, by using whole seedlings rather than adult leaves, not only did the variance between replicates reduce, but it was also possible to measure ADC activity and metabolite levels in non-induced plants, something that was not the case for adult leaf tissue.

3.2.12 Optimisation of *N. benthamiana* ADC activity assays

Previously, ADC activity assay samples, prepared following the Wu et al., 2019 protocol (section 2.2.5.2) clogged the LCMS column, preventing further measurement of the samples. This suggested that *N. benthamiana* samples contain a different chemical profile to that of other plant species tested in this work, which interfered with the LCMS column. As transient expression of T-DNA constructs via agroinfiltration is simple and effective in *N. benthamiana*, it would be valuable to be able to measure ADC activity and the PA profile from *N. benthamiana* leaf tissue. For example, different ADC homologues or mutant variants could be transiently expressed in *N. benthamiana* leaves to compare their activities or impact on PA metabolism. To facilitate this type of experiment, it was hypothesised that an additional purification step may be required to prevent *N. benthamiana* clogging the LCMS column. A

typical purification approach for this is to use methanol-chloroform-water extraction for phase separation, whereby the upper methanol-water phase contains polar molecules (i.e., PAs), the interphase contains proteins, and the lower chloroform phase contains non-polar, organic moieties such as lipids (Sapcariu et al., 2014). This additional purification method was therefore tested to determine if it would prevent *N. benthamiana* ADC activity assay samples clogging the LCMS column. For this experiment, *N. benthamiana* leaves were agroinfiltrated with constitutively expressed, epitope-tagged SIADC proteins (35S:HA-SIADC1 and 35S:MYC-SIADC2), as well as an empty *A. tumefaciens* GV3101 strain as a control for background ADC activity. After 2 days, samples were harvested for ADC activity assays, as well as for western blot analysis to ensure that the ADC proteins were expressed. Assay samples were extracted in ADC activity assay buffer (Table 24), incubated for 1 hour at 37 °C, before being stopped with HFBA and 80 µL moved to a new tube for methanol-chloroform extraction (section 2.2.5.4).

All samples ran through the LCMS column without any dramatic changes in pressure or errors, demonstrating that the additional methanol-chloroform extraction had purified the sample of impurities that had previously caused problems within the LCMS machine. Expression of either SIADC1 or SIADC2 produced 38- or 67-fold higher ADC activity compared to the empty strain control, respectively (Figure 30A). Interestingly, ADC activity was 1.8-fold higher with expression of SIADC2 compared to SIADC1 (P -value < 0.000001). However, as SIADC1 and SIADC2 had different epitope tags, direct comparison of their expression levels is not possible, yet both proteins were expressed well at the point of harvesting for LCMS analysis

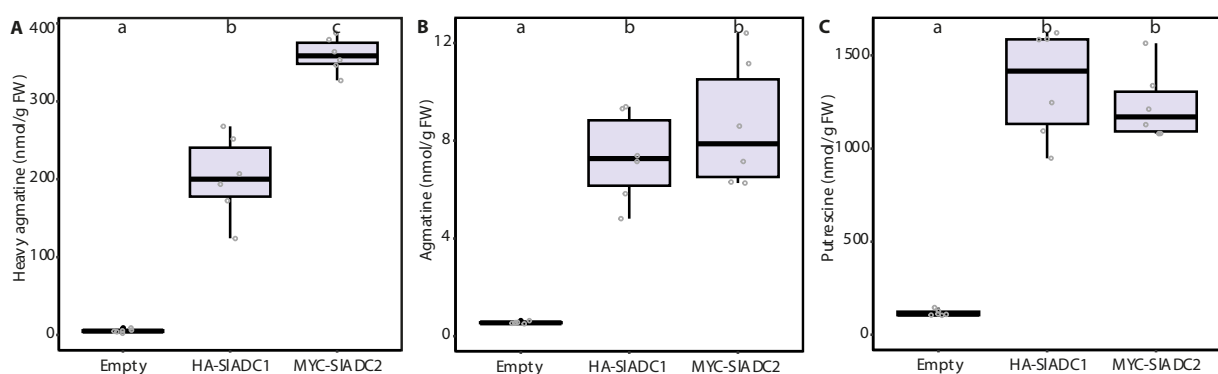


Figure 30: Additional methanol-chloroform purification step enables clear LCMS measurement of ADC activity from *N. benthamiana* leaf samples.

N. benthamiana leaves, agroinfiltrated with 35Sp:HA-SIADC1, 35Sp:MYC-SIADC2, or an empty strain, were sampled, homogenised in buffer containing heavy arginine, incubated at 37 °C for 1 hour, extracted in methanol-chloroform, and prepared for LCMS analysis (n = 6). Concentrations of heavy agmatine (A), agmatine (B), and putrescine (C) were normalised to the internal D5 tryptophan standard. An ANOVA, followed by a Tukey HSD test, was used to determine statistical significance; letters displayed above each box indicate significance; groups with different letters are significantly different ($\alpha = 0.05$).

(Supplementary Figure 16). Similarly, levels of agmatine and putrescine levels were 14- and 11-fold higher upon SlADC protein expression compared to the empty strain, respectively (Figure 30B and C). Overall, the additional methanol-chloroform extraction enabled ADC activity assays and PA analysis via LCMS, producing extremely clear results with low variation between replicates, as well as high metabolite signal peaks compared to the background and negative control. This additional step therefore enables analysis of ADC proteins and their metabolic consequences via transient expression in *N. benthamiana* leaves.

3.3 Characterising the impact of *ADC* 5'UTR mutations

Previously, the tomato *ADC1-box* was found to negatively regulate translation of the downstream CDS using transient reporter assays (Wu et al., 2019), yet its role within its native context *in planta* had not been elucidated. Therefore, *adc1/2-box* single and double mutants in tomato and *A. thaliana* were generated to determine the *in planta* role of the *ADC-box* on both ADC translation and the wider PA metabolic network (section 3.1.1 and 3.1.2). As *M. polymorpha* is a relative of the first land plants, *M. polymorpha adc-box* mutants were also generated to determine if the repressive role of the *ADC-box* has been conserved throughout plant evolution (section 3.1.3). The presence of a uORF in *ADC* transcripts is also highly conserved, yet in transient reporter assays, no clear impact on translation was found when its start codon was mutated in the *SlADC1* 5'UTR (Wu et al., 2019). To test if the *ADC* uORF has a role *in planta*, both single and double *SlADC1/2 uorf* mutants were generated (section 3.1.1). To understand how these CRISPR/Cas9-induced *ADC* 5'UTR mutations influence ADC translation, measurement of ADC protein abundance in plant tissues was required. As a proxy for ADC protein abundance, measurement of ADC activity in plant extracts via LCMS had previously been used (Wu et al., 2019). This LCMS-based assay and methods to stimulate *ADC* expression were further optimised for tomato, *A. thaliana*, and *M. polymorpha* to enable clear distinction between WT and *adc-box* mutants (section 3.2).

With all tools in hand, ADC activity assays, as well as measurement of PAs and related metabolite concentrations of *ADC* 5'UTR mutants could be conducted. In the following sections, LCMS-based metabolic analysis and phenotypic analysis of tomato (section 3.3.1) and *M. polymorpha* (section 3.3.2) mutants is presented.

3.3.1 *ADC* activity, PA profile, and phenotypes of tomato *ADC* 5'UTR mutants

3.3.1.1 ADC activity and PA measurement of single and double tomato *adc-box* mutants

To investigate the role of the tomato *ADC-box* on *in planta* ADC translation, an ADC activity assay and LCMS analysis of single and double *adc-box* mutants was conducted. The single mutants used were S11C_-6, S11M_-4, S11M_-108, S12M_+1, and S12M_-13, as well as the double *adc-box* mutants $\Delta brg11-ebe$ and $\Delta sladc1/2-box$, which have small or large mutations in both *SlADC-boxes*, respectively (Figure 11, Figure 12, Table 29). These mutants were selected as they have a range of mutation severities. For example, only 4bp of the *SlADC1-box* is mutated in S11M_-4 and expected to have a lesser impact on translation of *SlADC1*, while the entire *SlADC1-box* in S11M_-108 is deleted, and therefore expected to have a greater impact

on ADC translation. It was also hypothesised that the double *adc-box* mutants would have higher ADC activity compared to WT and to their respective single mutants, as all ADC transcripts would have perturbed or no *ADC-box* RNA secondary structures to regulate levels of ADC translation.

Detached leaves of WT tomato and the above-mentioned tomato *adc-box* mutants were cold treated, and an ADC activity assay was conducted, before preparing the samples for LCMS analysis (section 2.2.5). D₅ tryptophan, the internal normalisation control, had no significant differences between genotypes (Supplementary Figure 17). ADC activities of S11M_-108, S12M_-13, and the corresponding double mutant, Δ *sladc1/2-box*, were all significantly higher than WT, the S11C_-6 control line, and the less severe S11M_-4, S12M_+1, and Δ *brg11-ebe* mutants (Figure 31A). Specifically, ADC activities of S11M_-108 and S12M_-13 were 4.3- and 3.8-fold higher than WT, respectively (*P*-values = 0.0005 and 0.003), while the double mutant Δ *sladc1/2-box* had 10.3-fold higher ADC activity (*P*-value < 0.0000000); this finding indicates an additive effect on ADC activity when both *SLADC-boxes* are mutated. This showed that ADC activity, and therefore ADC protein abundance, is significantly increased when the *SLADC-box* is mutated, demonstrating that the *SLADC-box* is a repressive element of ADC protein accumulation *in planta*. These findings also reveal that only the mutants with larger *adc-box* mutations had increased ADC activity, while those with more minor mutations had largely unaffected ADC activity.

To verify these findings, this ADC activity assay and LCMS analysis was conducted a second time with all mutants, and then a third time with only WT, S11M_-108, S12M_-13, and Δ *sladc1/2-box*; these assays are presented in Supplementary Figure 18 and Supplementary Figure 19, respectively. In each assay, the larger *sladc-box* mutants S11M_-108 and S12M_-13 had significantly higher ADC activity compared to WT or the other smaller *sladc-box* mutants. However, the Δ *sladc1/2-box* double mutant always had the highest ADC activity with 6.7-fold and 6.3-fold higher ADC activity compared to WT (Supplementary Figure 18 and Supplementary Figure 19, respectively).

In general, agmatine and putrescine levels of the *sladc-box* mutants followed the same trend as their ADC activity levels. Here, mutants with more severe *adc-box* mutations had significantly higher agmatine and putrescine levels compared to WT, S11C_-6 control, and the mutants with more minor *adc-box* mutations (Figure 31B and C, Supplementary Figure 18, Supplementary Figure 19). The Δ *sladc1/2-box* double mutant had the highest agmatine and putrescine levels of any mutant, with 2.7-fold and 2.1-fold higher levels compared to WT, respectively (*P*-values = 0.000003 and 0.0002; Figure 31). Interestingly, despite having higher ADC activity,

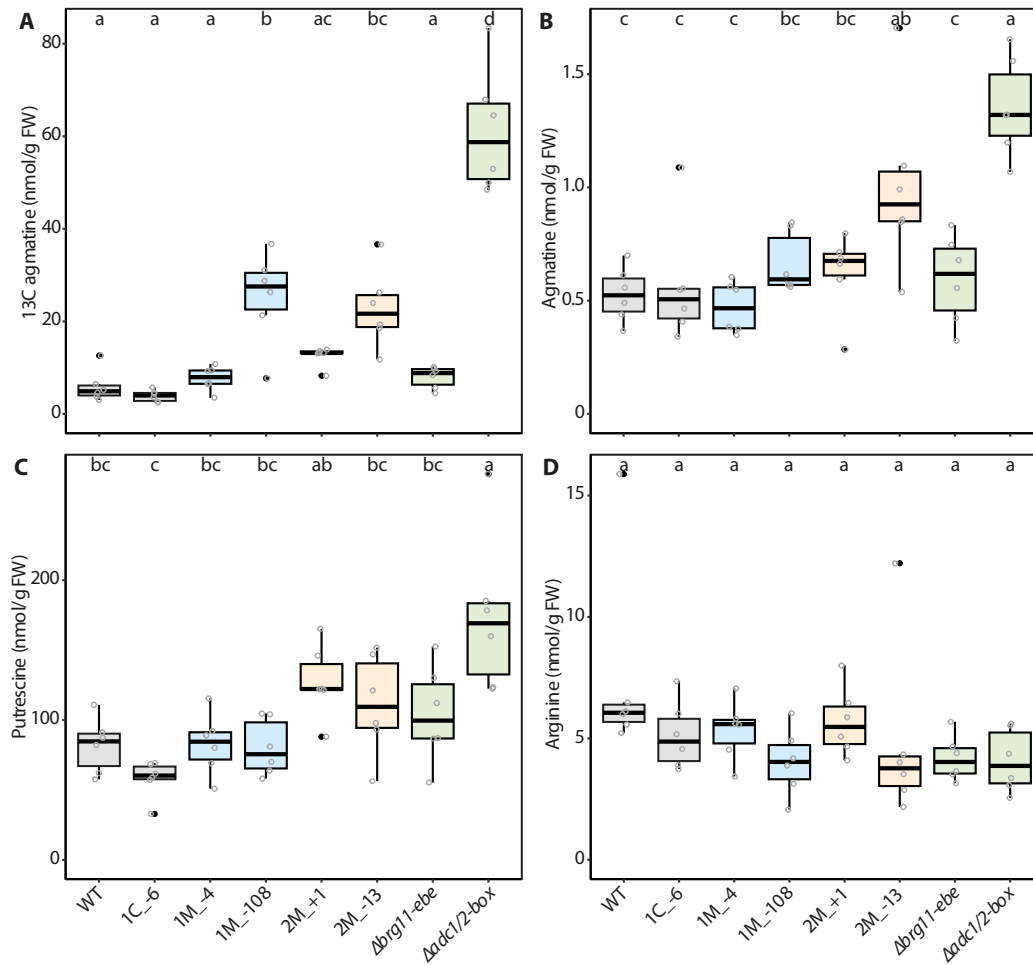


Figure 31: Tomato *ADC-box* single and double mutants have higher ADC activity compared to WT and altered PA metabolism. Samples from cold-treated detached leaves of single and double tomato *ADC-box* mutants were prepared for ADC activity assays and LCMS analysis of their polyamine profile ($n = 6$). ADC activity (heavy agmatine) (A), agmatine (B), putrescine (C), and arginine (D) levels are shown in nmol/g FW, normalised to D₅ tryptophan. WT and the control line (SI1C-6) are shown in grey, *SIADC1-box* mutants (SI1M-4 and SI1M-108) in blue, *SIADC2-box* mutants (SI2M-1 and SI2M-13) in yellow, and double *SIADC-box* mutants (*brg11-ebe* and *adc1/2-box*) in green. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

agmatine, and putrescine levels, SI1M-108, SI2M-13, and $\Delta sladc1/2-box$ did not have altered arginine levels compared to WT (Figure 31D; Supplementary Figure 19D). This indicated that either the ADC substrate pool was sufficiently large or was able to be replenished to counteract the higher ADC activity.

Putrescine, the most abundant PA, is the precursor to spermidine, which can then be metabolised into spermine (Chen et al., 2019). Despite some mutants having higher putrescine levels, no significant differences in spermidine or spermine levels were observed in any mutant compared to WT (Figure 32A and B; Supplementary Figure 19F and G). This suggests that the impact of mutating the *sladc-boxes* only resonates to putrescine, and not the subsequent PAs.

Levels of other PA-related metabolites in the tomato *adc-box* single and double mutants were also measured. Levels of ornithine, which is the precursor for the second route to PA synthesis via ODC, were 3.8-fold and 6.4-fold higher in the Sl2M₋13 and Δ *sladc1/2-box* mutants compared to WT, respectively (*P*-values = 0.00003 and <0.000000, respectively; Figure 32C). Similar findings were seen in a second assay, but here, Sl1M₋108 also had significantly higher ornithine levels compared to WT (Supplementary Figure 18H). Ornithine can also be converted into citrulline via ornithine transcarbamylase (OTC; Quesada et al., 1999, Majumdar et al., 2016) and then further metabolised into arginine (via arginosuccinate), generating a cyclic arginine metabolic pathway (Winter et al., 2015). Despite observing significantly higher ornithine levels in some *adc-box* mutants, citrulline levels were generally not altered by *SLADC-box* mutations, apart from the Sl2M₊1 mutant (Figure 32D; Supplementary Figure 19I). Putrescine can be acetylated by N-acetyltransferase activity 1 (NATA1) into acetylputrescine, which interestingly causes reduced H₂O₂-related defence responses to *P. syringae* infection (Lou et al., 2016). Levels of acetylputrescine in Sl2M₋13 and Δ *sladc1/2-box* were 1.6-fold and 2-fold higher than WT (*P*-value = 0.02 and 0.00002, respectively; Figure 32E; Supplementary Figure 19J). Interestingly, despite having 4.3-fold higher ADC activity compared to WT, no differences in acetylputrescine levels in the Sl1M₋108 mutant were observed. GABA (γ -aminobutyric acid) can be produced from putrescine via diamine oxidase (DOA) and is a key metabolite during for biotic and abiotic stress responses (Li et al., 2021, Xu et al., 2022b). However, no significant differences in GABA levels were observed compared to WT, despite some mutants having increased putrescine levels (Figure 32F; Supplementary Figure 19K).

PAs are not only found in free forms, but a large proportion are also covalently and noncovalently bound to other moieties in plant cells, greatly expanding and diversifying their physiological roles (section 1.2; Chen et al., 2019). Agmatine and PAs are covalently conjugated to HCAs, such as coumaric, caffeic, and ferulic acids, to form phenolamides (Bassard et al., 2010). Levels of phenolamides were also measured in the same LCMS run to determine if the increased ADC activity in the tomato *adc-box* mutants also increased conjugation to HCAs. Typically, in these LCMS experiments, the *m/z* ratio of an ionised analyte and its fragmented daughter ions are predicted and then confirmed using a

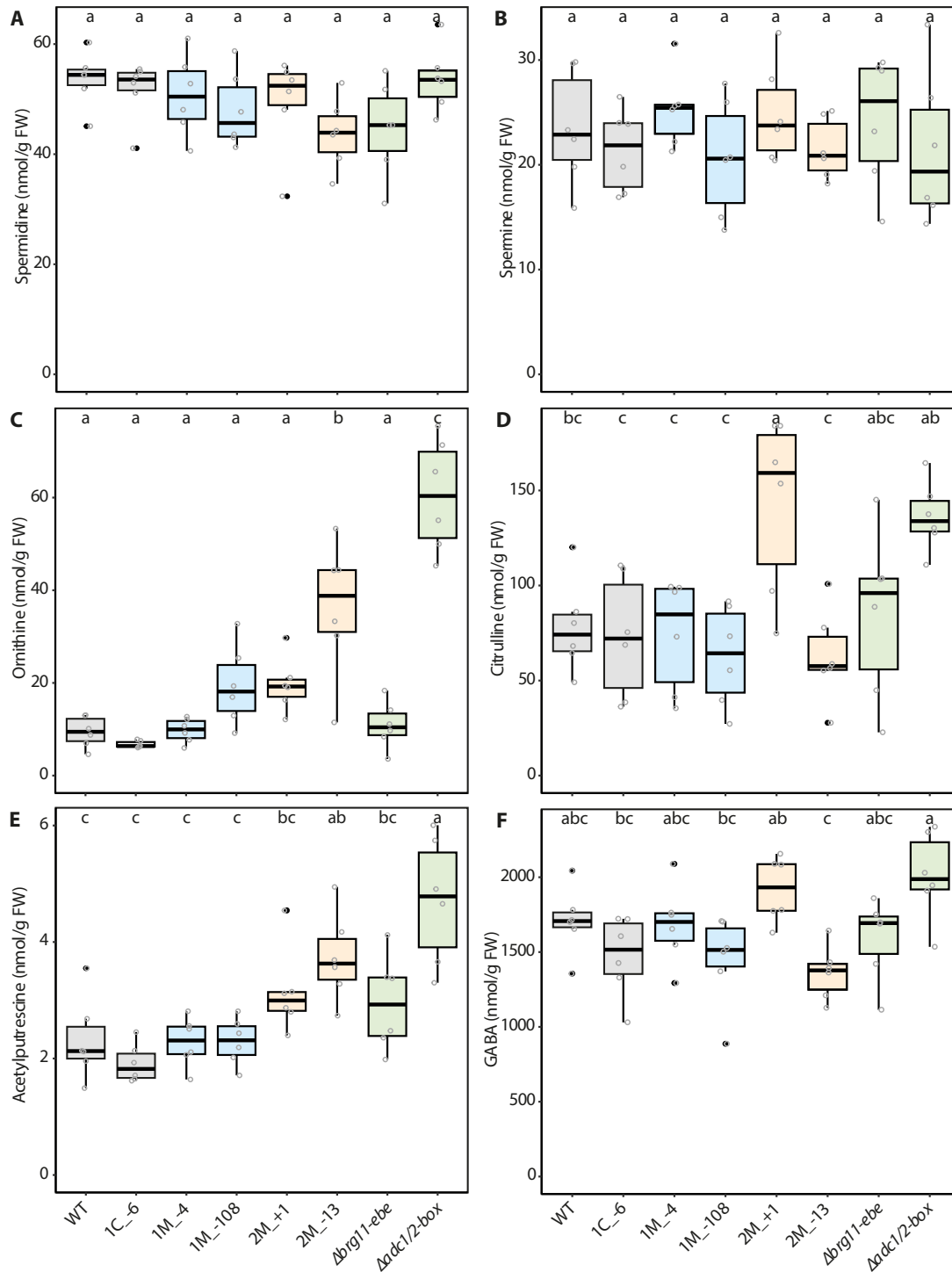


Figure 32: Tomato *ADC-box* single and double mutants do not have altered spermidine or spermine levels, but levels of some other PA-related metabolites are altered.

Samples from cold-treated detached leaves of single and double tomato *ADC-box* mutants were prepared for ADC activity assays and LCMS analysis of their polyamine profile ($n = 6$). Spermidine (**A**), spermine (**B**), ornithine (**C**), citrulline (**D**), acetylputrescine (**E**), and GABA (**F**) levels are shown in nmol/g FW, normalised to D_5 tryptophan. WT and the control line (SI1C_-6) are shown in grey, *SIADC1-box* mutants (SI1M_-4 and SI1M_-108) in blue, *SIADC2-box* mutants (SI2M_+1 and SI2M_-13) in yellow, and double *SIADC-box* mutants (*brg11-ebe* and *adcl/2-box*) in green. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

pure standard. However, as no synthetic PA-based phenolamide standards were commercially available during this work, it was not possible to confirm that the signal peak is the expected metabolite. It is also important to note, that without a standard, a calibration curve cannot be generated to calculate the exact *in planta* phenolamide concentration. Due to these reasons, the signal peak *area* per gram of fresh weight tissue (area/g FW), normalised to the internal standard (D₅ tryptophan) is presented. Levels of coumaroylagmatine and coumaroylputrescine, but not feruloylagmatine and feruloylputrescine, were increased in the *sladc2-box* mutants and the double *sladc-box* mutants (Figure 33). SI2M_+1, SI2M_-13, and Δ *sladc1/2-box* mutants had 3.6-, 2.8-, and 5.5-fold higher coumaroylagmatine levels compared to WT, respectively

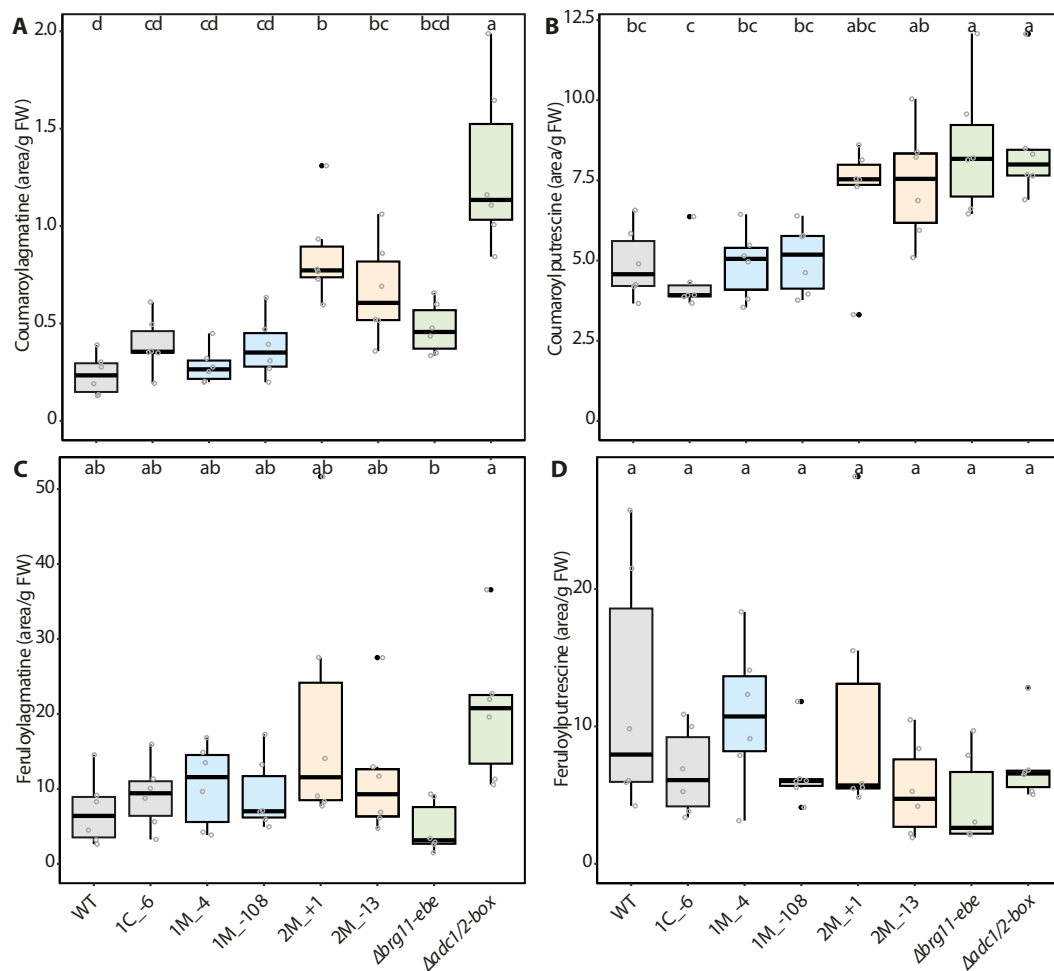


Figure 33: Levels of certain phenolamide are induced when the tomato *ADC2-box* is mutated, but not the *ADC1-box*.

Samples from cold-treated detached leaves of single and double tomato *ADC-box* mutants were prepared for LCMS analysis of their phenolamide profile (n = 6). Coumaroylagmatine (A), feruloylagmatine (B), coumaroylputrescine (C), and feruloylputrescine (D) levels are shown in area/g FW, normalised to D₅ tryptophan. WT and the control line (SI1C_-6) are shown in grey, *SLADC1-box* mutants (SI1M_-4 and SI1M_-108) in blue, *SLADC2-box* mutants (SI2M_+1 and SI2M_-13) in yellow, and double *SLADC-box* mutants (*brg11-ebe* and *adc1/2-box*) in green. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

(P -values = 0.005, 0.03, and <0.00000). Levels of coumaroylputrescine were 1.7-fold higher in both $\Delta brg11-ebe$ and $\Delta adc1/2-box$ double *SLADC-box* mutants (P -values = 0.0053 and 0.0054, respectively). These results demonstrated that the increase in ADC activity caused by *SLADC-box* mutations increased levels of certain phenolamides, but only in mutants with a mutated *SLADC2-box*.

To check that the observed differences in ADC activity and metabolite levels between the *sladc1/2-box* mutants and WT were not due to altered *SLADC1/2* transcription or transcript stability, an RT-qPCR was conducted. Samples from both non-treated tomato leaves and the cold-treated leaves used for LCMS analysis were taken for RNA extraction and relative *SLADC1/2* transcript levels were measured via RT-qPCR (sections 2.2.3.6 to 2.2.3.8).

No significant differences in *SLADC1* or *SLADC2* transcript levels of any mutant in either non-treated or cold-treated conditions compared to WT were found (Figure 34). Therefore, the

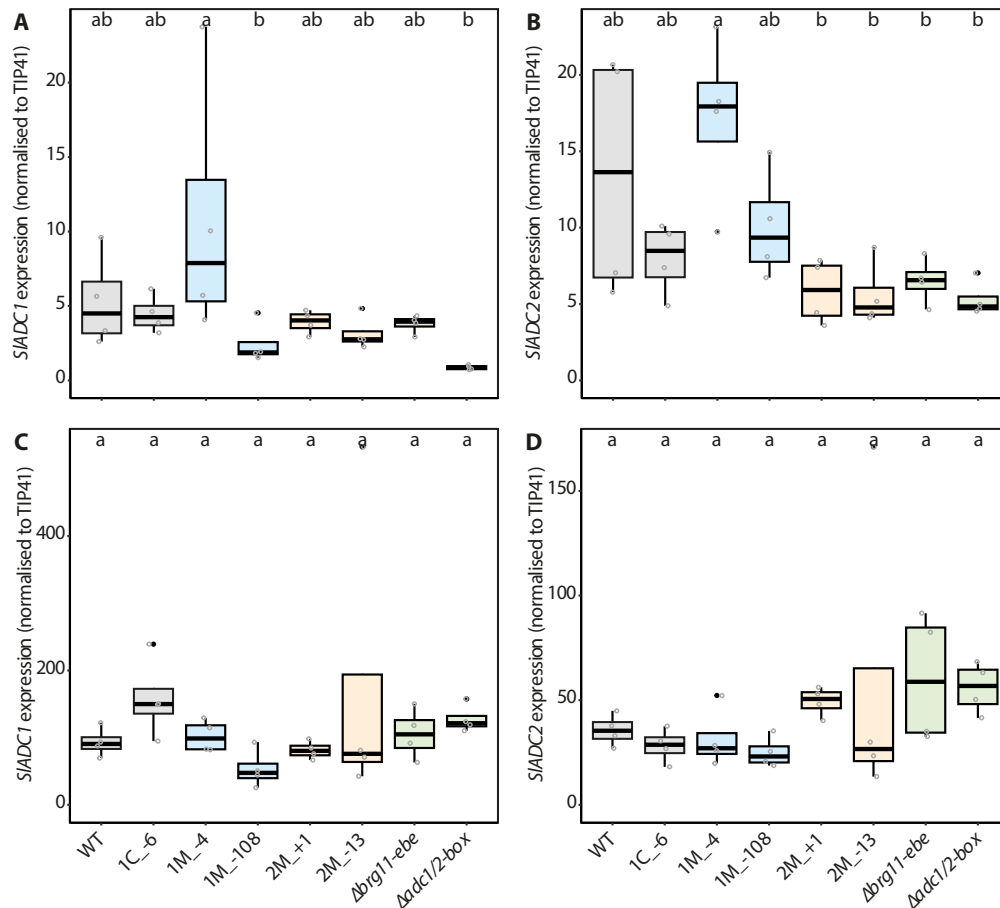


Figure 34: *SLADC-box* mutations do not significantly affect *SLADC* transcript abundance.

RNA was extracted from non-treated (A and B) and cold-treated (C and D) WT (grey), control (1C_-6, grey), *SLADC1-box* (blue), *SLADC2-box* (yellow), and *SLADC1/2-box* double (green) mutants ($n = 4$) for analysis of *SLADC1* (A and C) and *SLADC2* (B and D) transcript abundance. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

observed differences in metabolic and ADC activity levels of the *sladc-box* mutants were not due to altered *SLADC* transcript abundance, but due to impairment of translational inhibition by the native *ADC-box*. Interestingly, in all RT-qPCRs conducted on tomato *adc-box* mutants, their *SLADC1* and *SLADC2* transcript levels have tended to be lower than WT, but not significantly, suggesting that some form of negative feedback regulation on *SLADC1/2* transcription or transcript stability may be occurring due to higher ADC activity and/or PA levels.

3.3.1.2 ADC activity and LCMS analysis of single and double tomato *uorf* mutants

Alongside *SLADC-box* CRISPR mutagenesis, the uORF of *SLADC1* and *SLADC2* were also mutated to determine if they influenced *SLADC* translation *in planta*. Previously, no significant difference in transient reporter expression was observed between constructs with an *SLADC1* WT or $\Delta uorf$ 5'UTR sequence (Wu et al., 2019). A preliminary LCMS experiment was carried out to test whether *SLADC1/2* $\Delta uorf$ mutants had altered ADC activity or levels of measured PAs or related metabolites. Here, plants with large deletions spanning the *SLADC1/2* uORFs were used: S11M_-127, which has a 127 bp deletion spanning the *SLADC1* uORF (also called $\Delta uorf1$); S12M_-172, which has a 172 bp deletion spanning the *SLADC2* uORF (also called $\Delta uorf2$); and, a double mutant, $\Delta uorf1/2$, which was generated by crossing S11M_-127 and S12M_-172 (Figure 11, Figure 12, Table 29). Leaves from 6 replicate plants of each genotype were cold treated and samples harvested and processed for ADC activity assays and LCMS measurements (section 2.2.5.3). ADC activity from both single $\Delta uorf$ mutants and the double $\Delta uorf1/2$ mutant was approximately half that of the WT (P -values = 0.0045, 0.0133, and 0.0074, respectively; Figure 35A). Although this difference to WT ADC activity was not as dramatic as the $\Delta sladc1/2$ -*box* mutant (Figure 31, Supplementary Figure 18, and Supplementary Figure 19), this result showed that the uORF may also play a role in ADC translation, but as a positive regulatory element rather than a negative regulatory element like the *ADC-box*.

Interestingly, agmatine levels of the $\Delta uorf1/2$ mutant were 2.8-fold lower than the WT (P -value = 0.0003) and only the $\Delta uorf2$ single mutant had significantly lower agmatine levels compared to WT (1.8-fold; P -value = 0.012; Figure 35B). Despite the reduction in agmatine levels, arginine levels were consistent across all four genotypes, indicating that a reduced ADC substrate pool was not the cause for the reduction in ADC activity or agmatine levels (Figure 35C). Putrescine, acetylputrescine, and ornithine levels were also significantly reduced in the $\Delta uorf1/2$ mutant compared to WT by 1.6-, 2.0-, and 7.4-fold, respectively (P -values = 0.02,

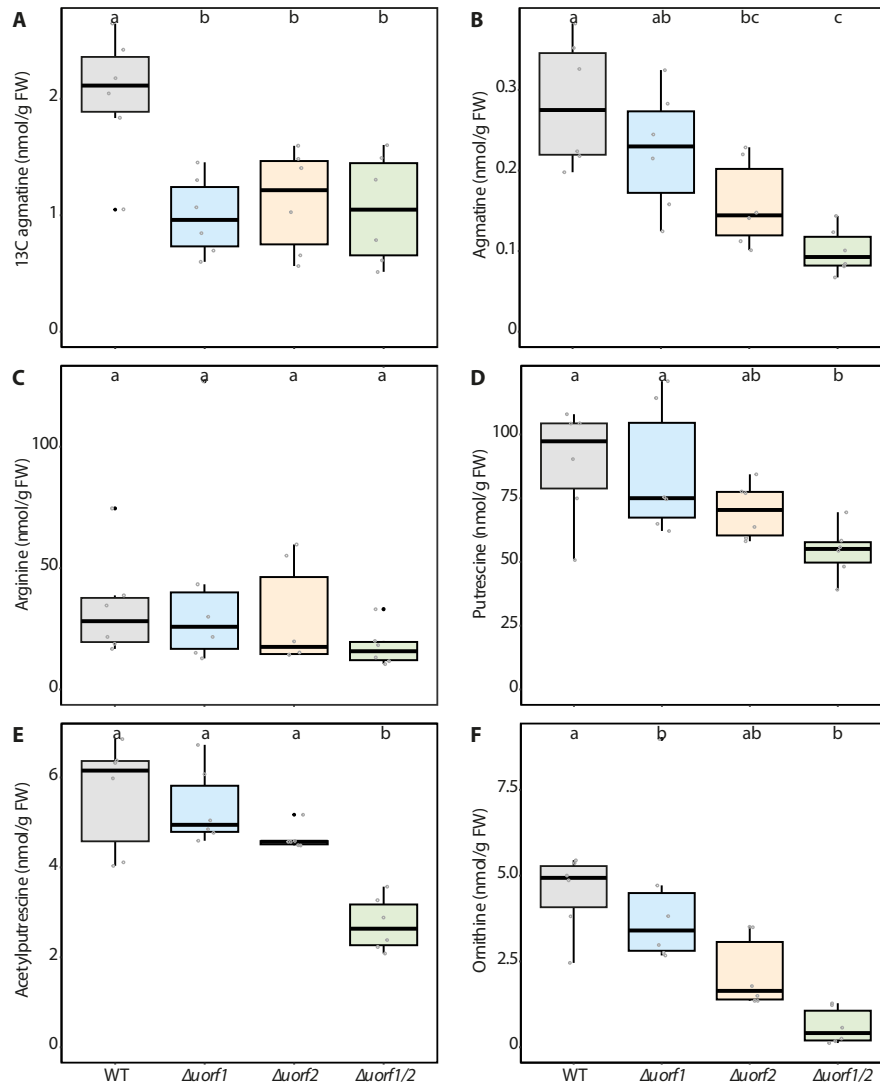


Figure 35: *SLADC* $\Delta uorf$ mutants have lower ADC activity and some metabolite levels compared to WT.

Samples from cold-treated detached leaves of WT (grey), $\Delta uorf1$ (blue), $\Delta uorf2$ (yellow) and the double tomato *uorf* mutant ($\Delta uorf1/2$, green) were prepared for ADC activity assays and LCMS analysis of their polyamine profile. ADC activity (A), agmatine (B), arginine (C), putrescine (D), acetylputrescine (E), and ornithine (F) levels are shown in nmol/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

0.00003, and 0.0009, respectively; Figure 35). Other metabolites were also measured, but no significant differences in their levels were found between the WT and any $\Delta uorf$ mutants (Supplementary Figure 20). Levels of D₅ tryptophan, the internal normalisation control, were unusually uneven in this assay, where $\Delta uorf1/2$ levels were 1.6-fold higher than WT (Supplementary Figure 20E). This experiment was only conducted once and requires further repetitions to ensure the results observed are consistent.

Overall, these results demonstrate that despite previous transient assays indicating no role for the *SLADC1* uORF, stable CRISPR/Cas9-induced mutagenesis of the *SLADC1/2* uORFs and

LCMS analysis indicate that the *SLADC* uORF plays a positive regulatory role in ADC translation and PA production.

3.3.1.3 Phenotypic analysis of tomato $\Delta uorf1/2$ and $\Delta adc1/2$ -box mutants

PAs are essential metabolites involved in a plethora of processes, including plant development and growth. As ADC is a key regulatory enzyme of PA synthesis, the conserved uORF and *ADC-box* are responsible for regulating ADC translation, they may also be responsible for fine-tuning PA levels at different stages of development. To test whether the uORF and *ADC-box* impact growth and development, a phenotypic evaluation of the $\Delta sluorf1/2$ and $\Delta sladc1/2$ -box mutants and WT tomatoes was conducted. Here, 20 plants from each genotype were sown to soil (July 2023) and approximately two weeks later, were transplanted to larger pots and their genotype confirmed by PCR-amplification (data not shown). After 30, 33, 36, 39, and 42 days after sowing seeds, plant height, leaf number, and the total number of flowers were measured or counted for each plant (details in section 2.2.1.10 and Supplementary Figure 3).

The $\Delta uorf1/2$ mutant was shorter and had fewer leaves and flowers compared to WT and the $\Delta adc1/2$ -box mutant, with no measured phenotypic differences observed between the WT and $\Delta sladc1/2$ -box mutant (Figure 36A, B, and C). The internode lengths between leaves were also measured and again, the $\Delta uorf1/2$ mutant had shorter internodes than the WT and the $\Delta adc1/2$ -box mutant (Supplementary Figure 21). On day 36, photographs of all plants were taken to show the shorter phenotype of the $\Delta uorf1/2$ mutant (two replicates shown in Figure 36D). To ensure that the mutations in the *SLADC* 5'UTRs did not dramatically alter *SLADC1* and *SLADC2* transcript abundance, RNA was harvested from leaflets of leaf number 2 on day 33 and an RT-qPCR was conducted (sections 2.2.3.6 to 2.2.3.8). Despite the marked phenotype, the $\Delta uorf1/2$ mutant did not have significantly different *SLADC1* or *SLADC2* transcript abundance, indicating that the ADC activity and phenotypic changes caused by deleting the uORF-encoding sequence altered *SLADC* translation and not *SLADC* transcription, at least in the harvested adult leaf tissue (Figure 36E). Interestingly, despite the $\Delta sladc1/2$ -box mutant having between 6- and 10-fold higher ADC activity compared to WT (Figure 31, Supplementary Figure 18, and Supplementary Figure 19), no significant differences in any measured phenotype were found. Overall, this phenotypic experiment showed that there is no developmental impact of the Δadc -box1/2 mutations, but deletion of the *SLADC1/2* uORFs resulted in smaller plants with fewer leaves and flowers. This work, alongside the ADC activity assay and LCMS measurement of PAs, indicates that the *SLADC1/2* uORFs may play a positive regulatory role in *SLADC* translation, influencing plant growth and development.

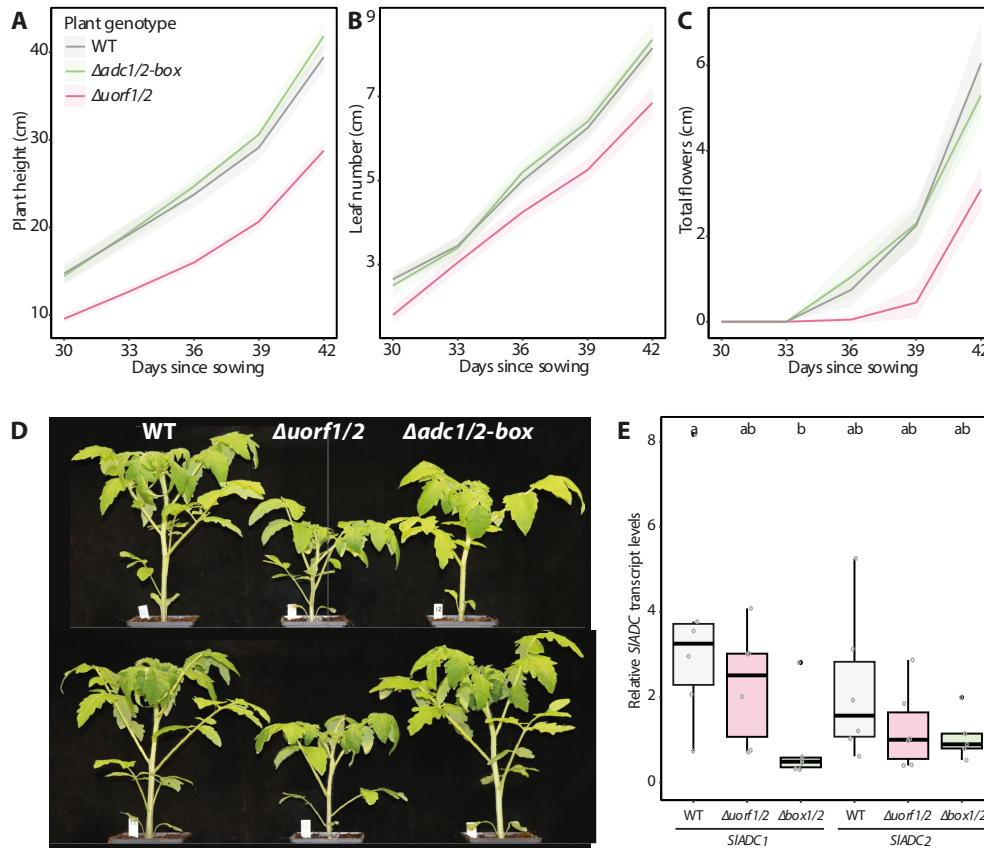


Figure 36: The $\Delta uorf1/2$ mutant is smaller and has fewer leaves and flowers compared to WT and the $\Delta adc1/2-box$ mutant. After 30, 33, 36, 39, and 42 days after sowing seeds, plant height (A), leaf number (B), and flower number (C) of WT (grey), $\Delta uorf1/2$ (pink), and $\Delta adc1/2-box$ (green) tomato plants were measured ($n = 20$). Shaded area shadowing each line represents a 95% confidence interval, where any point that the shaded areas of two lines are not covered by one another, they are statistically significantly different to the $\alpha = 0.05$ level; when the shaded area is lacking, less than 3 plants had those leaves at that measurement day. On day 36, photographs of all plants were taken; two representative replicate groups are shown (D). To verify that changes in *SIADC1/2* transcription were not the cause of the altered $\Delta uorf1/2$ phenotypes, an RT-qPCR of *SIADC1* (left) and *SIADC2* (right) was conducted from RNA extracted from leaf 2 of the first 6 plants on day 33; transcript levels were normalised to *SITIP41* (E).

3.3.2 ADC activity, PA profile, and phenotypes of *M. polymorpha* ADC-box mutants

The *ADC-box* has been conserved since the first land plants (Wu et al., 2019), with a primitive variant found in the liverwort model plant *Marchantia polymorpha*, which is considered as a relative of the first land plants. To determine if the *MpADC-box* is also a repressive element for regulating ADC translation, *Mpadc-box* mutants and control lines with mutations upstream of the *MpADC-box* were generated (section 3.1.3). The ADC activity assay and PA measurement method by LCMS was also optimised for *M. polymorpha* tissue (section 3.2.10). *M. polymorpha* TAK1, *Mpadc-box* mutants, and control lines were grown on $\frac{1}{2}$ B5 media for 3 weeks before being cold treated for 12 hours and harvested for ADC activity assays and

LCMS analysis (section 2.2.5.3). To ensure the cold treatment induced ADC activity, one TAK1 plate was wrapped in tin foil and kept in the climate chamber as a non-treated RT control. All metabolite levels were normalised to D₅ tryptophan, the internal buffer control; no differences were observed between genotypes (Supplementary Figure 22).

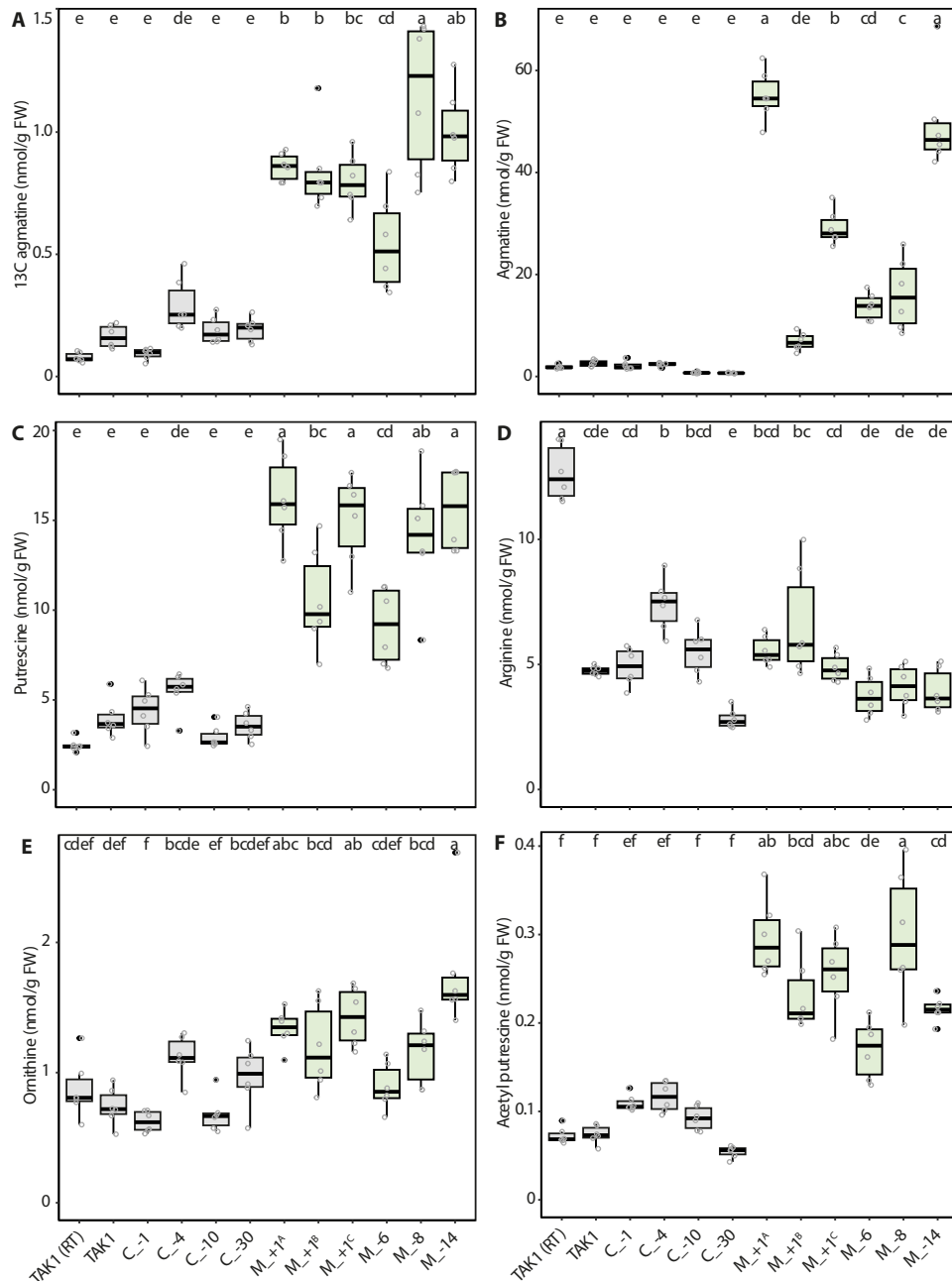


Figure 37: *M. polymorpha* ADC-box mutants have higher ADC activity and levels of some PAs and related metabolites.

M. polymorpha TAK1 (grey), control mutants (grey), and *Mpadc-box* mutants (green) were cold-treated for 16 hours, before samples were taken and prepared for ADC activity assays and LCMS analysis of the polyamine profile (n = 6). As a negative treatment control, an additional plate of TAK1 plants was kept at room temperature in the dark (RT; grey; n = 6). ADC activity (A), putrescine (B), arginine (C), agmatine (D), ornithine (E), and acetylputrescine (F) levels are shown in nmol/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

ADC activity of all *Mpadc-box* mutant plants was significantly higher than TAK1 and the control lines, of which none had different ADC activity levels to TAK1 (Figure 37A). MpM_-8 and MpM_-14 had the highest and second highest ADC activity, being 7.0- and 6.1-fold higher than TAK1, respectively (P -values < 0.0000000). MpM_-6 had the lowest ADC activity of all *Mpadc-box* mutants but was still 3.3-fold higher than TAK1 (P -value = 0.0007). Interestingly, cold treatment of TAK1 for 12 hours only increased ADC activity 2-fold compared to the non-treated TAK1, which was not statistically significant (P -value = 0.995), suggesting that the cold treatment did not influence ADC activity in this experiment. Regardless, these results demonstrate that mutating the *MpADC-box* increases ADC activity, indicating that this primitive *ADC-box* is also a functional repressive element of ADC translation.

While heavy agmatine is only produced during the 1-hour ADC assay, measurement of total agmatine includes what was already present in the plant prior to sampling. Here, although all *Mpadc-box* mutants have higher agmatine levels compared to TAK1, dramatic differences between the mutants were observed (Figure 37B). MpM_+1^A had the highest agmatine levels, followed by MpM_-14, and then MpM_+1^C, with 21-, 19-, and 11-fold higher levels than TAK1, respectively. MpM_+1^B had the lowest agmatine level of the *Mpadc-box* mutants, with only 2.7-fold higher levels compared to TAK1. Putrescine levels were increased in all *Mpadc-box* mutants, at an average of 3.4-fold higher than TAK1, while none of the control lines were significantly different to TAK1 (Figure 37C). Interestingly, despite the varied differences in agmatine levels between the *Mpadc-box* mutants, putrescine levels were more equal between the *Mpadc-box* mutants.

Similarly to the tomato *adc-box* mutants, no differences in arginine, the substrate of ADC, were observed (Figure 37D). This indicates that ADC activity and PA production was unlikely to be limited by insufficient ADC substrate levels. Also like the PA analysis of tomatoes, ornithine and acetylputrescine levels were higher in some *Mpadc-box* mutants compared to TAK1 and the control lines (Figure 37E and F). This suggests that mutating the *MpADC-box* influences the wider PA metabolic network, not just the immediate downstream pathway metabolites. Overall, these results demonstrate that the *MpADC-box* is a functional repressive element controlling ADC expression and impacts the levels of some metabolites downstream of the ADC-catalysed reaction.

3.3.2.1 Phenotypic analysis of *M. polymorpha* ADC-box mutants

Phenotypic analysis of tomato $\Delta adc\text{-}box1/2$ mutants showed that despite the higher ADC activity and levels of many PA-related metabolites, no obvious phenotypic differences were observed (Figure 36). While working with the *M. polymorpha* CRISPR/Cas9-induced mutant lines, no obvious and consistent phenotypic differences were observed between the *Mpadc-box* mutants and TAK1 and the control mutants (Figure 38). This is consistent with the lack of altered phenotype of the tomato $\Delta adc\text{-}box1/2$ mutant; however, no quantitative measurements were taken for these *M. polymorpha* plants.

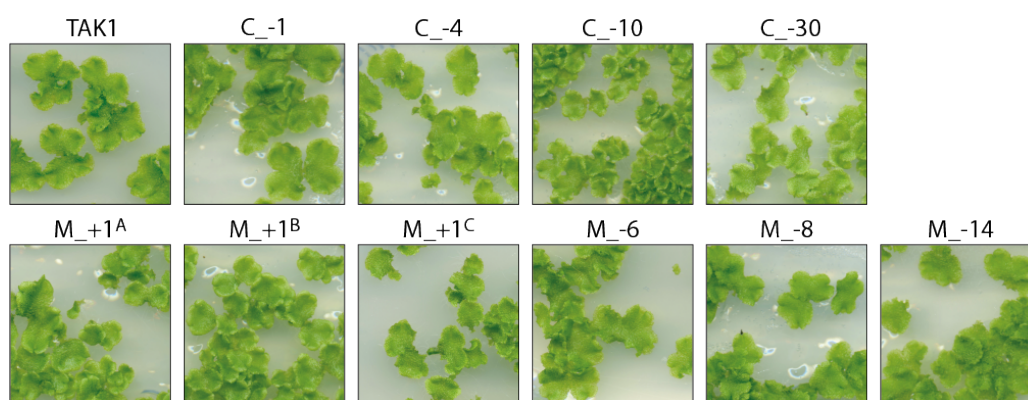


Figure 38: *M. polymorpha* *adc-box* mutants do not have any apparent phenotype.

M. polymorpha TAK1, control mutant lines (C_-, C_-4, C_-10, and C_-30), and *adc-box* mutants (MpM_+1^A, MpM_+1^B, MpM_+1^C, MpM_-6, MpM_-8, and MpM_-14) gemmae were sterilised, spread onto ½ B5 plates, grown for 3 weeks, and photographs were taken.

3.4 Metabolic analysis of tomato ADC and ODC knock out mutants.

Previously, tomato *adc1*, *adc2*, *adc1adc2*, and *odc1* mutants were generated by CRISPR mutagenesis (Table 1; Dousheng Wu, Lahaye lab; Wu et al., 2019), but their ADC activity and PA profile had not been analysed. Since these mutants were generated, the protocol for preparation of tomato leaf tissue via cold treatment and the ADC activity assay has been optimised (section 3.2.7), enabling clearer measurement of ADC activity and many other metabolites above background levels and between genotypes. This therefore provided an opportunity to measure the ADC activities and PA-related metabolite levels in plants with reduced ADC/ODC protein levels compared to WT. While the *adc-box* mutant plants have provided information on how increasing ADC expression influences the levels of PA-pathway metabolites, mutants of the *ADC* coding sequences would inform on what happens when one or both SIADC proteins are lacking. Analysis of the single *sladc-box* mutants also suggested

that SIADC1 and SIADC2 may have unique roles or influences on the PA pathway, as some metabolite levels were only induced in *sladc2-box* mutants and not *sladc1-box* mutants, such as ornithine, citrulline, and acetylputrescine, as well as the phenolamide, coumaroylagmatine (Figure 32 and Figure 33). Therefore, conducting an ADC activity assay and PA metabolite measurement under the same conditions as the Δ *sladc1/2-box* mutant assays would provide more insight into any potential unique roles of each SIADC protein. The double *sladc1sladc2* mutant was also included in this assay to give insight on how the PA metabolic pathway functions without ADC proteins; specifically, how the ODC-dependent pathway could compensate for the loss of ADC proteins. Additionally, the *slodc1* mutant was included to elucidate how the PA pathway flux was influenced by ODC enzymes.

All plants were genotyped prior to leaves being cut for the 24-hour cold treatment and harvested for an ADC activity assay and metabolite level measurement. It is important to note that *sladc1sladc2* mutant plants need to be genotyped from a parental line heterozygous for the *sladc2* mutation and homozygous for *sladc1*, as no flowers or fruits can develop from the double *sladc1sladc2* mutant (Wu et al., 2019). Typically, the double *sladc1sladc2* plants are much smaller than the single mutants, *slodc1* mutant, or WT, and struggle to grow past the size of a 3-week-old plant; no quantitative phenotypic measurements or photographs were taken. As with other ADC activity assays and LCMS measurements of PA-related metabolites, no differences in D₅ tryptophan were observed between any of the genotypes (Supplementary Figure 23). No ADC activity was detected from the *adc1adc2* double mutant, while the single mutants, *adc1* and *adc2*, had ~42% and ~76% ADC activity of the WT respectively, with only *adc1* being significantly lower (P -value = 0.002; Figure 39A). There was no difference in ADC activity between the *odc1* mutant and WT. Despite no ADC activity observed above background levels, there was a very small agmatine peak in the double *adc1adc2* mutant samples, suggesting that agmatine can be generated at very small amounts in the absence of ADC proteins. The *adc* single mutants also had lower agmatine levels than WT, but only *adc2* was significantly lower by 2-fold (P -values = 0.005; Figure 39B). Arginine, the ADC substrate, was significantly increased in the *adc1adc2* double mutant, but not in the single *adc* or *odc1* mutants, suggesting a build-up of the substrate in the absence of a functional ADC protein (3.3-fold higher with P -value = 0.0002; Figure 39C). This is the only assay where a difference in arginine levels has been observed. Like arginine, ornithine levels were also significantly higher in the *odc1* mutant, indicating a build-up of the ODC substrate in the absence of a functional ODC protein (1.4-fold higher with P -value = 0.014; Figure 39D). Interestingly, ornithine levels were significantly reduced in the single and double *adc1adc2*

mutants, suggesting higher flux through the ODC-dependent PA pathway in the absence of a fully functional ADC-dependent pathway. Here, the *adc1adc2* mutant had 5.5-fold lower ornithine than WT (P -value < 0.000001). Putrescine levels were also decreased in the *adc2* and

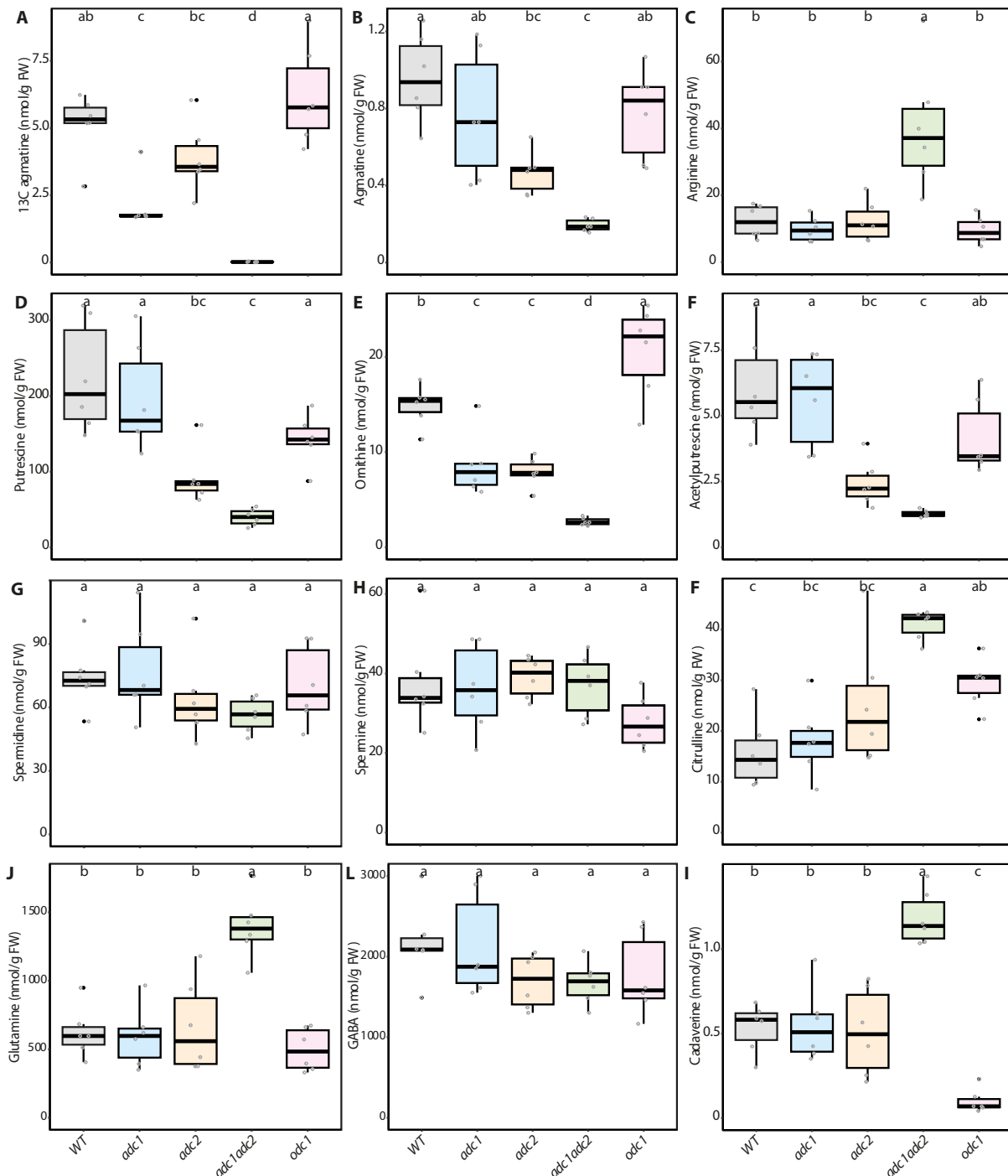


Figure 39: Tomatoes lacking both ADC proteins have dramatic reductions in ADC activity and altered PA-pathway metabolism.

Samples from cold-treated detached tomato leaves of WT (grey), single *adc1* (blue), *adc2* (yellow), and double *adc* (green) mutants, and the *odc* mutant (pink) were prepared for ADC activity assays and LCMS analysis of their polyamine profile ($n = 6$). ADC activity (heavy agmatine; **A**), agmatine (**B**), arginine (**C**), ornithine (**D**), putrescine (**E**), acetylputrescine (**F**), spermidine (**G**), spermine (**H**), citrulline (**I**), glutamine (**J**), GABA (**K**), and cadaverine (**L**) levels are shown in nmol/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance:

adc1adc2 mutants by 2.4- and 5.6-fold, respectively (P -values = 0.001 and 0.00001, respectively; Figure 39E). Interestingly, no significant difference decrease in putrescine levels were observed for the *odc1* mutant, despite ODC catalysing the decarboxylation of ornithine to putrescine. Acetylputrescine, which is produced from putrescine by NATA1, was also reduced in the *adc2* and *adc1adc2* mutants (P -values = 0.001 and 0.00003, respectively; Figure 39F). Despite changes to agmatine and putrescine levels, no differences in spermidine or spermine were observed in any of the mutants (Figure 39G and H). Citrulline levels were significantly higher in both *adc1adc2* and *odc1* mutants compared to WT by 2.6- and 1.8-fold, respectively (P -values = 0.00005 and 0.033, respectively; Figure 39F), possibly indicating some arrangements to the arginine-ornithine-citrulline cycle in these mutants. Glutamine, which also links into this cycle, was 2.2-fold higher in the *adc1adc2* mutant compared to WT (P -value = 0.00006; Figure 39J). No differences in GABA levels were observed between any mutant and WT (Figure 39K).

Surprisingly, cadaverine levels were 2.2-fold higher in *adc1adc2* compared to WT (P -value = 0.00002), while the *odc1* mutant had 5.4-fold lower levels compared to WT (P -value = 0.003; Figure 39L). Cadaverine is normally produced by lysine decarboxylase (LDC) from lysine, using the same co-factor as ADC and ODC, pyridoxyl-5-phosphate (P5P; Tomar et al., 2013). However, ODC is a promiscuous enzyme that can also decarboxylate lysine to cadaverine, meaning that the observed changes in cadaverine levels may be due to changes in ODC activity. In addition to ADC activity and measurement of PAs and PA-pathway related metabolites, some phenolamide levels were also measured in this experiment. Interestingly, in all identified phenolamides in this experiment, *adc2* and *adc1adc2* had reduced levels compared to WT, while *adc1* never showed statistically significantly different levels (Figure 40). The agmatine-based phenolamides, coumaroylagmatine and feruloylagmatine, were undetectable above background levels in the *adc1adc2* mutant, with even the single *adc2* mutant having 12-fold lower levels feruloylagmatine compared to WT (P -value = 0.003; Figure 40C). Compared to WT, the *adc1adc2* mutant had 8-, 13-, and 6-fold lower levels of coumaroylputrescine, feruloylputrescine, and cinnamoylputrescine, respectively (P -values = 0.000007, 0.0002, 0.03, and 0.0003; Figure 40B, D, and E). These results show that phenolamide levels are severely reduced in the absence of a functional ADC-dependent PA pathway and that SIADC2 activity seems to be the primary contributor to phenolamide production compared to SIADC1.

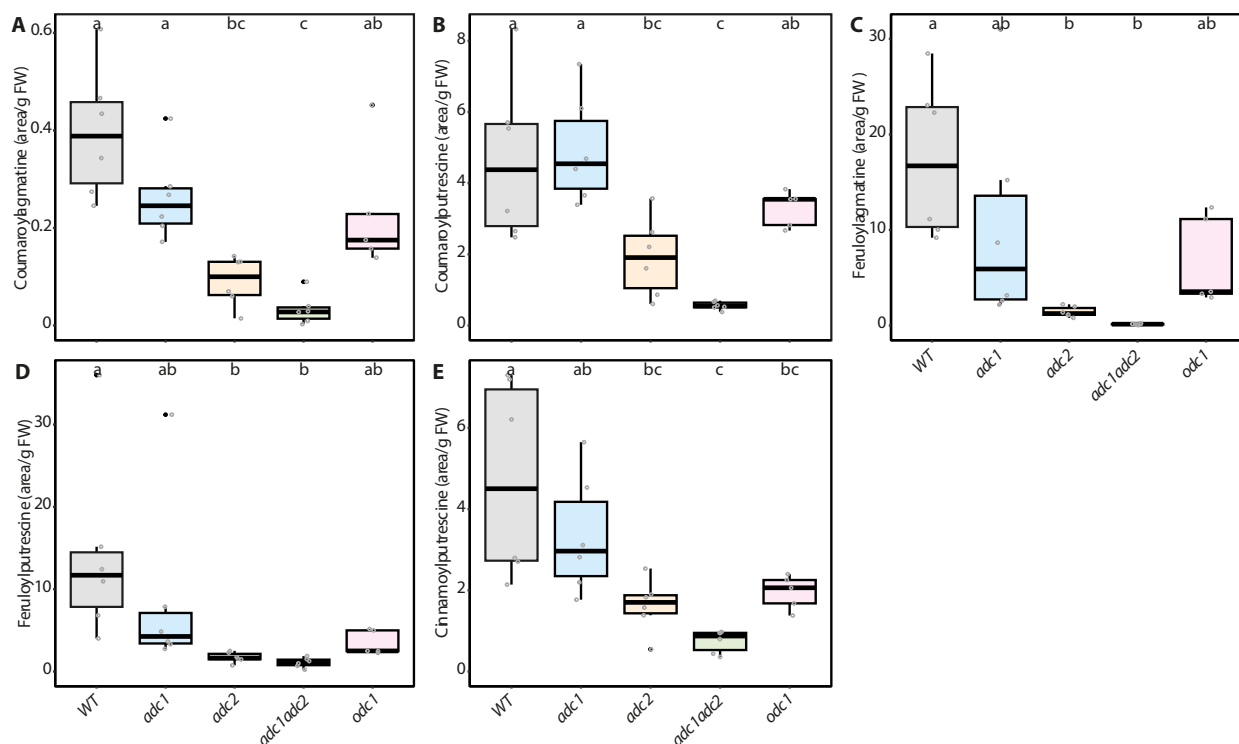


Figure 40: Levels of phenolamides are reduced in the absence of SIADC2, and even more reduced when both SIADC1 and SIADC2 are mutated.

Samples from cold-treated detached tomato leaves of WT (grey), single *adc1* (blue), *adc2* (yellow), and double *adc* (green) mutants, and the *odc* mutant (pink) were prepared for LCMS analysis of their phenolamide profile ($n = 6$). Coumaroylagmatine (A), coumaroylputrescine (B), feruloylagmatine (C), feruloylputrescine (D), and cinnamoylputrescine (E) levels are shown in area/g FW, normalised to D₅ tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

In summary, ADC activity of mutants lacking one SIADC protein showed decreased ADC activity, while no activity was detected in the *adc1adc2* mutant, indicating that ADC is the only enzyme capable of catalysing this reaction and one functional SIADC enzyme can somewhat compensate for the loss of the other. Agmatine, ornithine, putrescine, and acetylputrescine levels were reduced in the absence of SIADC proteins, while arginine levels were increased in the double *adc1adc2* mutant (Figure 39). Interestingly, reduction of these metabolites also resulted in lower levels of phenolamides (Figure 40). Overall, these results demonstrate the importance of ADC on the wider network of PA-related metabolites, with SIADC2 possibly having a greater impact on PA conjugation with HCAs to generate phenolamides.

3.5 How does the *ADC-box* fine-tune *ADC* translation to maintain PA homeostasis?

3.5.1 Background information and hypotheses

The *ADC-box* is a repressive *cis*-element, found to inhibit *ADC* translation in both transient reporter assays (Wu et al., 2019) and *in planta* via *ADC* activity assays of CRISPR/Cas9-induced *adc-box* mutants in tomato, *A. thaliana*, and *M. polymorpha* (section 3.3.1, section 3.2.11, and section 3.3.2, respectively). Yet, it remains unclear as to exactly *how* the *ADC-box* inhibits translation to regulate *ADC* expression. The *ADC-box* was predicted to form an RNA secondary structure, whereby the GC-rich regions pair to form a double stem loop structure, which obstructs scanning ribosomes (i.e., preinitiation complexes, PICs), causing stalling, and preventing access to the *ADC* start codon to initiate translation (Wu et al., 2019). However, it is logical that this translational repression is not permanent and can be alleviated during periods of low cellular PAs, increasing *ADC* translation and PA production to restore PA levels and maintain cellular homeostasis. Therefore, the *ADC-box* regulatory mechanism must be sensitive to cellular PA levels via some form of feedback signal. This signal could trigger the formation of the *ADC-box* RNA secondary structure, a conformational change, or increase its stability so that PICs cannot bypass the *ADC-box*.

Four feedback signals have been hypothesised and are shown in Figure 41. The first hypothesised signal is the *ADC* protein, as it is the direct downstream product of the *ADC-box* translational regulation. Some metabolic enzymes have “moonlighting” functions, a term given to enzymes that have additional roles outside of their primary catalytic function (Castello et al., 2015; Figure 41A). Some moonlighting enzymes bind specific mRNAs related to their catalytic function, thereby regulating translation of the encoded protein and fine-tuning flux through their metabolic pathway. One such example is the binding of iron regulator proteins (IRP), which are not only cytosolic aconitase enzymes, but also detect cellular iron levels and regulate translation of specific mRNAs that contain iron-response element (IRE) RNA secondary structures (Rouault, 2006; Castello et al., 2015). It is therefore possible that *ADC* may also be a moonlighting enzyme, binding to the *ADC-box* RNA sequence to regulate its own translation. The second type of hypothesised PA-sensing signal molecule are PAs themselves, or a related metabolite (i.e., arginine or agmatine) (Figure 41B). This type of translational regulatory mechanism, where a metabolite binds to an RNA secondary structure to regulate translation, is called a riboswitch. In the case of the *ADC-box*, a metabolite would bind to the RNA secondary structure during periods of high PA availability to either induce a conformational change or the

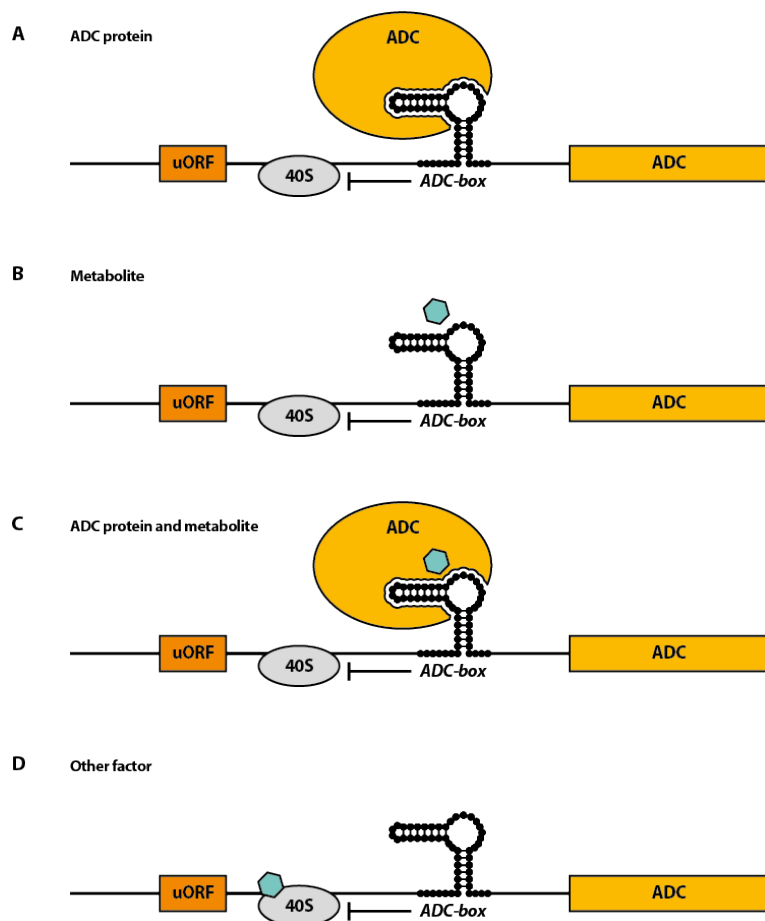


Figure 41: Hypothesised mechanisms for how the *ADC-box* inhibits ADC translation in a PA level-dependent manner.

To correctly regulate the levels of ADC translation according to the cellular needs for ADC activity (i.e., PA levels), there needs to be a feedback signal to indicate when ADC should be translated or not. The first hypothesised feedback signal is the ADC protein binding to the *ADC-box* as a moonlighting function (**A**). The second hypothesis is that the *ADC-box* functions as a riboswitch, whereby a PA-related metabolite binds to the *ADC-box* to prevent translation (**B**). A combination of these two hypotheses is shown in (**C**), whereby only when ADC binds to a metabolite can it bind to the *ADC-box* to inhibit translation (**C**). The final hypothesis is if part of the translational machinery is PA-sensitive and can only bypass the *ADC-box* when PA levels are low (**D**).

structure's stability to prevent PIC scanning to the *ADC* start codon. The third hypothesised feedback signal is a combination of the ADC protein and a related metabolite interacting with the *ADC-box* (Figure 41C). In this hypothesised mechanism, only when a specific metabolite is in high abundance and binds to the ADC protein, can the complex then interact with the *ADC-box* and regulate *ADC* translation. The final hypothesised PA-sensitive signal molecule is another protein that is unrelated to ADC or PA metabolism, such as a ribosomal subunit or an RNA processing protein (Figure 41D). PAs are known to be associated and/or bound to ribosomes, with specific PAs being required for functionality or regulation of certain translation factors (Dever and Ivanov, 2018). Therefore, the ability of the scanning PIC to bypass or unwind the *ADC-box* secondary structure may be influenced by PA-dependent translational machinery activities.

The following section investigates the mechanism of how the *ADC-box* inhibits ADC translation and to elucidate its feedback signal molecule that senses cellular PA levels.

3.5.2 Development of a suitable dual reporter assay to interrogate the *ADC-box* regulatory mechanism.

Dual reporter assays are commonly used to analyse translational regulatory mechanisms and are typically based on dual luciferase or fluorophore reporters. These assays rely on two reporters expressed from one construct. Expression of the first reporter is controlled by the translational regulatory element in question (e.g., a 5'UTR containing a uORF and/or RNA secondary structure). The second reporter is constitutively expressed from the same construct and should not be impacted by the translational *cis*-regulatory mechanism being analysed via the first reporter. Therefore, this second reporter is used as an internal control for the baseline expression capability in that system. Expression levels of the first reporter are normalised to those of the internal control, which accounts for some biological error between replicates and improves the sensitivity of the assay. An advantage of dual reporter assays is that co-expression of other proteins or co-delivery of chemicals is possible, enabling the assessment of these on the translational regulatory mechanism. To investigate the translational regulatory mechanism of the *ADC-box* and to possibly identify the PA level-sensitive feedback signal, a sensitive dual reporter system was required, where other proteins can be co-expressed.

3.5.2.1 Previous dual reporter assays used to study *ADC-box* regulation.

Previously, a dual fluorophore system was used to determine the impact of the *ADC-box* and uORF on downstream translation (Wu et al., 2019). Here, the *SLADC1* 5'UTR sequence was inserted between a 35S constitutive promoter (35Sp) and a GFP reporter, while a 35Sp-driven *dsRED* construct was used as an internal expression control. However, when this system was tested during this work, high background autofluorescence prevented the accurate reading of dsRED fluorescence (internal expression control) and the GFP reporter expression was not high enough to clearly detect differences between different reporter constructs. Therefore, an alternative, more sensitive dual reporter system was required for further analysis of the *ADC-box* regulatory mechanism.

As mentioned above, dual luciferase assays are commonly used for investigating translational regulatory mechanisms. Typically, firefly luciferase is used as the test reporter and renilla luciferase as the internal expression control. These luciferase enzymes convert luciferin to oxyluciferin in a light-producing reaction that can be detected via a luminometer plate reader (Gould and Subramani, 1988, Sherf et al., 1996). Using the golden gate toolkit (Binder et al., 2014), a dual luciferase reporter system was established using the LII α F 4-5 vector backbone. Using this vector backbone, 5'UTR sequences could be assembled between a 35Sp and a

luciferase reporter gene, with a *35Sp*-driven renilla luciferase control gene, all encoded on a single T-DNA construct. This dual luciferase reporter plasmid could then be agroinfiltrated into *N. benthamiana* leaves and 3 days later, the luminescence measured (section 2.2.4.3). However, high replicate variation from both reporters, very high renilla expression that overloaded the luminescence plate reader, and low reproducibility between experiments meant that this system was also not suitable for further work. Therefore, an alternative, more reliable dual reporter expression system was required.

3.5.2.2 Generating and testing a golden-gate-compatible *pGrDL_SPb* plasmid

Many dual reporter systems based on the *pGreenII* vector and helper plasmid *pSoup* (Hellens et al., 2000, Hellens et al., 2005) have been generated and repeatedly optimised to be robust. An optimised *pGreenII*-*pSoup* dual-luciferase vector system was generated after the discovery that some plant extracts, including those from *N. benthamiana*, can inhibit firefly luciferase activity (Moyle and Birch, 2013; Liu et al., 2014; Moyle et al., 2017). This vector, called *pGrDL_SPb*, was modified to have a tomato actin promoter (*SlActinp*) driving renilla luciferase and extra restriction sites added for classical cloning. This optimised plasmid was purchased from Addgene to be used for analysis of the *ADC-box* regulatory mechanism (reference number: 83205, www.addgene.org; Moyle et al., 2017).

To enable streamlined cloning with many pre-existing plasmids, the *pGrDL_SPb* plasmid was cloned to be golden-gate (GG) compatible and have a *ccdB* cassette in the empty backbone, meaning that no colonies containing an uncloned backbone following a cut-ligation would survive. This was completed using a mixture of classical cloning and site-directed mutagenesis, where the pre-existing *35Sp* was removed and replaced with *BsaI* restriction sites with A-B overhangs flanking a *ccdB* cassette; this vector was then called *pGrDL_SPb (GG)* (Figure 42A). To simply insert a single promoter without an additional 5'UTR construct, LI promoter plasmids with A-B overhangs are required (Binder et al., 2014; Figure 42B). To generate reporter constructs with a promoter and a 5'UTR driving luciferase expression, an additional *BsaI* overhang was created of “*TGGT*” to fit between the A-B overhangs (i.e., A [*GCGG*] – *TGGT* – B [*TCTG*]). Therefore, LI promoter constructs require A-*TGGT* overhangs, and 5'UTR LI plasmids require *TGGT*-B overhangs to be assembled into the *pGrDL_SPb (GG)* vector as a *35Sp:5'UTR:LUC* construct (Figure 42C).

To verify that the GG cloning works as expected and to test the functionality of the modified *pGrDL_SPb (GG)* plasmid, various reporter constructs were generated. The *35Sp* from Binder

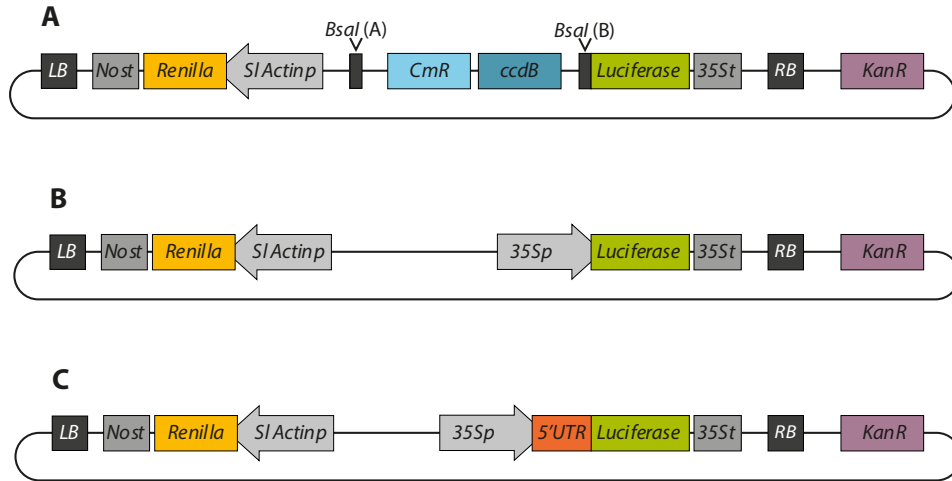


Figure 42: Schematic of Golden Gate-compatible *pGrDL_SPb* (GG) backbone and reporter construct examples.

(A) *pGrDL_SPb* plasmid (Moyle *et al.*, 2017) was made Golden Gate compatible with A (GCGG) and B (TCTG) BsaI overhangs (according to Binder *et al.*, 2014), and ccdB cassette and chloramphenicol resistance genes (*CmR*; blue) to improve efficiency of cloning. The B overhang allows for cloning desired promoters or upstream sequences to drive expression of luciferase (green) with a 35S terminator (dark grey). Constitutive expression of renilla (orange) via the tomato actin promoter (*SLActinp*; grey) and *Nos* terminator (*Nos*; dark grey) is encoded in the opposite direction to the luciferase gene. Kanamycin resistance (*KanR*; mauve) allows for bacterial selection. (B) *35Sp:LUC* construct made via a cut ligation with a 35S promoter (*35Sp*) with A-B overhangs and the *pGrDL_SPb* (GG) plasmid. (C) *35Sp:5'UTR:LUC* construct made via cut ligation with a 35Sp with A-X overhangs, a 5'UTR with X-B overhangs (red), and the *pGrDL_SPb* (GG) plasmid.

et al., 2014 was cloned into the *pGrDL_SPb* (GG) vector to generate *35Sp:LUC*, enabling a comparison to the *35Sp:LUC pGrDL_SPb* plasmid (Moyle *et al.*, 2017; Figure 42B). As a negative control for luciferase expression, the tight minimal *Bs3* promoter (*Bs3p*) was used to generate *Bs3p:LUC*. Finally, *SLADC* 5'UTR reporter constructs *35Sp:SLADC1_{WT} 5'UTR:LUC* and *35Sp:SLADC2_{WT} 5'UTR:LUC* were generated to test the functionality of the new “X” overhang during plasmid assembly and to eventually investigate translational regulation from *SLADC* 5'UTRs (Figure 42C). All cut-ligated *pGrDL_SPb* (GG) constructs were transformed into *E. coli* and correct colonies were selected via colony PCR using primers ESR147 and ESR215, and the plasmid was verified via Sanger sequencing, then co-transformed into *A. tumefaciens* GV3101 with the *pSoup* helper plasmid (section 2.2.2.3).

The newly generated reporter constructs in the GG-compatible plasmid, the original *pGrDL_SPb* plasmid (containing *35Sp:LUC*), and the previously made *35Sp:LUC* in the *LIIα F 4-5* expression vector (section 3.5.2.1) were each individually agroinfiltrated into *N. benthamiana* leaves (section 2.2.1.5). Samples were harvested at 2 dpi and 3dpi to determine the best day for luciferase and renilla expression and replicate homogeneity. Samples were extracted and prepared for luminescence measurement (section 2.2.4.3). For each replicate, the

raw luciferase luminescence was divided by the raw renilla luminescence (i.e., LUC/REN), and then mean luciferase to renilla calculated.

The newly made GG-compatible vector containing *35Sp:LUC* had significantly higher LUC/REN than the original *35Sp:LUC* plasmid (*pGrDL_SPb*) at both 2 and 3 dpi by 1.35- and 1.46-fold, respectively (Figure 43; *P*-value = 0.0328 and <0.0001, respectively). While both constructs have *35Sp:LUC* reporters, the *35S* promoter assembled into the *pGrDL_SPb* (GG) plasmid has an extra 486 bp 5' compared to that in the original plasmid, as well as a *BsaI* overhang near the *luciferase* start codon (Supplementary Figure 25); these sequence differences in the promoters are likely the cause for higher expression in the GG-compatible vector, rather than the cloning strategy used for the new vector. In contrast to the *pGrDL_SPb*-based plasmids, mean LUC/REN from the *35Sp:LUC LII* construct was significantly lower and had higher replicate variation in the individual luciferase and renilla measurements (Supplementary Figure 24). The negative control *Bs3p:LUC* construct had very low luciferase luminescence at

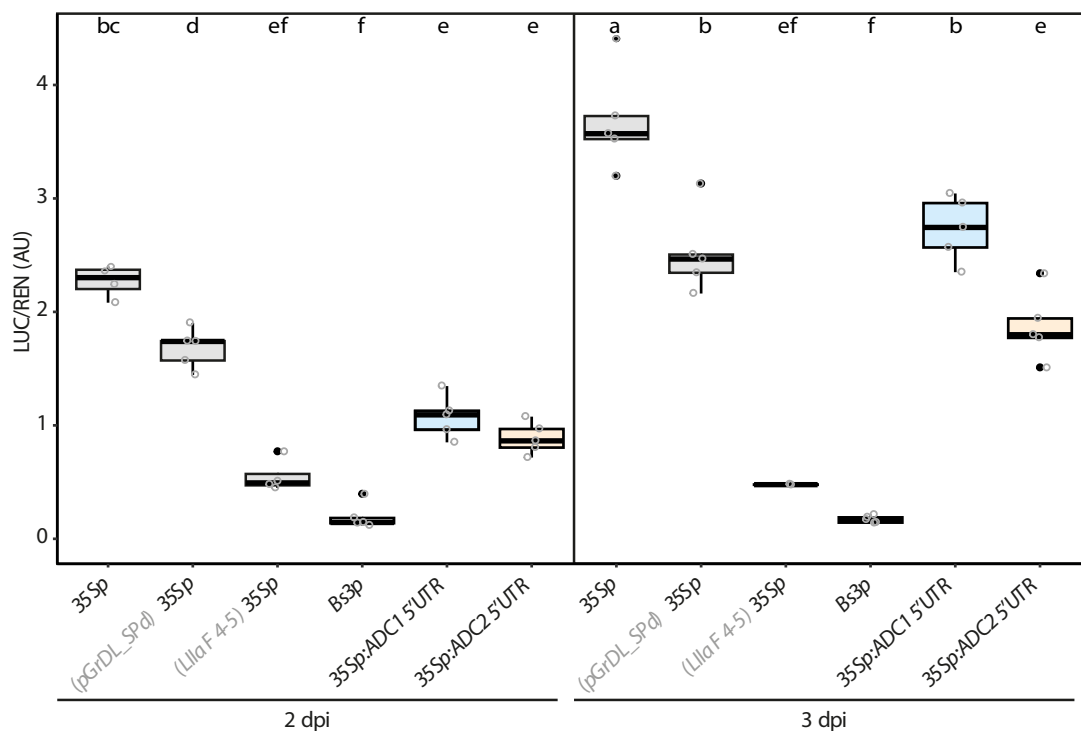


Figure 43: Golden gate compatible *pGrDL_SPb* (GG) plasmid is suitable for investigating translation regulatory mechanisms. Golden-gate compatible (*pGrDL_SPb* (GG)) luciferase reporter constructs with a *35S* promoter (*35Sp*) as a positive control, *Bs3* promoter (*Bs3p*) as a negative control, and *35Sp*-driven tomato *ADC* 5'UTRs (*SLADC1/2* 5'UTR) were generated and agroinfiltrated into WT *N. benthamiana* leaves (n = 5). The original *pGrDL_SPb* plasmid (Moyle *et al.*, 2017) with a *35Sp:LUC* reporter, as well as the previously generated *35Sp:LUC* construct with an *LIIα F 4-5* backbone were also agroinfiltrated in the same manner. After 2 dpi (left) and 3 dpi (right), samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

both 2 and 3 dpi, luciferase, while renilla expression was not significantly different from any other *pGrDL_SPb(GG)*-based construct (Supplementary Figure 24). Therefore, as the *35Sp:LUC* positive control and *Bs3p:LUC* negative control constructs produced high and low levels of luciferase, respectively, that could be easily detected, with low variation between replicates, the *pGrDL_SPb(GG)*-based system was deemed suitable for further experiments to investigate the *ADC-box* regulatory mechanism. In line with this, the *SIADC1* and *SIADC2* 5'UTR reporter constructs both had equal LUC/REN at 2 dpi, while at 3 dpi, *35Sp:SIADC1_{WT} 5'UTR:LUC* had 1.46-fold higher LUC/REN than the *SIADC2* 5'UTR construct (P -value = 0.00008; Figure 43). These results show that addition of either *SIADC1* or *SIADC2* 5'UTR sequence significantly reduces translation compared to the *35Sp:LUC* control, indicating that both *SIADC* homologs are indeed regulated translationally. Additionally, most LUC/REN values were higher at 3 dpi compared to 2 dpi; however, as both time points had luminescence levels to be easily measured and with low variability between replicates, either time point could be used for future experiments.

3.5.2.3 Generating and testing functional and catalytically dead SIADC constructs.

To narrow down which moiety is acting as the PA-sensor and feedback signal (Figure 41), catalytically functional or non-functional *SIADC1/2* protein constructs were generated. Here, it was hypothesised that if the ADC protein binds to the *ADC-box*, then co-expression of both catalytically functional and non-functional ADC proteins should repress downstream luciferase translation from *35Sp:SIADC1/2_{WT} 5'UTR:LUC* reporters. However, if an ADC-related metabolite is required for the *ADC-box* regulatory mechanism, then repression of luciferase translation would only occur when a catalytically functional ADC protein is co-expressed. Additionally, co-expression of other PA-producing enzymes could also help to narrow down which metabolite (if any) is required for this mechanism. A construct expressing the tomato ODC enzyme with a C-terminal MYC tag had already been generated (*35Sp:SIODC-MYC*; Niels Gallas, AG Lahaye) and could be used to produce PAs in an ADC-independent manner. Interestingly, the *Paramecium bursaria chlorella virus-1* (*PBCV-1*) genome encodes a decarboxylase (*PBCV-1 DC*) that has a distinct peptide sequence from plant ADCs but is metabolically similar (Shah et al., 2004). *PBCV-1 DC* is only 372 amino acids long, compared to the ~600 amino acids of tomato and *A. thaliana* ADCs, and shares ~40% sequence homology to eukaryotic ODCs (Shah et al., 2007). Due to its lack of sequence homology to eukaryotic decarboxylases and high arginine decarboxylation activity, a construct expressing *PBCV-1 DC* was also generated. It was hypothesised that if only the metabolic function of plant ADCs is

important for the *ADC-box* regulatory mechanism, then co-expression of PBCV-1 DC should repress luciferase translation in a similar manner to a plant ADC protein.

The following sections describe the cloning of catalytically functional and non-functional SlADC constructs and PBCV-1 DC (section 3.5.2.4), as well as testing their expression and catalytic functionality when transiently expressed in *N. benthamiana* leaves (section 3.5.2.5).

3.5.2.4 Cloning catalytically functional and non-functional enzyme constructs

Initially, *SlADC1* and *SlADC2* coding sequences were cloned from tomato gDNA into LI pUC57 vectors with C-D BsaI overhangs (Binder et al., 2014). These were used to generate *35Sp:HA-SlADC1* and *35Sp:MYC-SlADC2* constructs, where each SlADC protein is constitutively expressed and has an N-terminal epitope tag to monitor protein expression via immunoblotting. While no catalytically dead tomato ADC variants have been published, key residues essential for catalytic function of *A. thaliana* ADC1 have been identified based on mammalian ODC enzymes (Hanfrey et al., 2001). As eukaryotic ODC and ADC enzymes belong to the same P5P-dependent amino acid decarboxylase family (group IV; Sandmeier et al., 1994), the residues used for catalysis in eukaryotic ODC enzymes are likely to be the same for ADC enzymes. Enzymes in this family dimerise to form two active sites in the dimer interface (Maruri-Lopez and Jimenez-Bremont, 2017), with a key lysine residue forming a Schiff base with the enzyme co-factor P5P (K136 in AtADC1) and a cysteine residue involved in substrate binding (C524 in AtADC1; Hanfrey et al., 2001). The peptide sequences of AtADC1, AtADC2, SlADC1, and SlADC2 were aligned to identify which two SlADC1/2 and AtADC2 residues correspond to the K136 and C524 AtADC1 residues essential for catalysis (Supplementary Figure 26). An area of ~15 amino acids surrounding these two essential AtADC1 residues was identical across all four peptide sequences. The corresponding essential catalytic residues in SlADC1 were K149 and C540, K156 and C548 in SlADC1, and K147 and C537 in AtADC2. These residues were individually mutated in each *SlADC* to alanine to generate non-functional mutations; K149A and C540A in SlADC1 (i.e., SlADC1_{KA} and SlADC1_{CA}) and K156A and C548A SlADC2 (i.e., SlADC2_{KA} and SlADC2_{CA}). To ensure catalytic function was truly prevented, the mutations were combined to generate SlADC1 K149A C540A (i.e., SlADC1_{KA_CA}) and SlADC2 K156A C548A (i.e., SlADC2_{KA_CA}) double mutants. These mutant enzyme sequences were cloned into LII vectors with the same promoter and epitope tags as the WT constructs (i.e., an HA tag for SlADC1 variants and a MYC tag for SlADC2 variants; Figure 44).

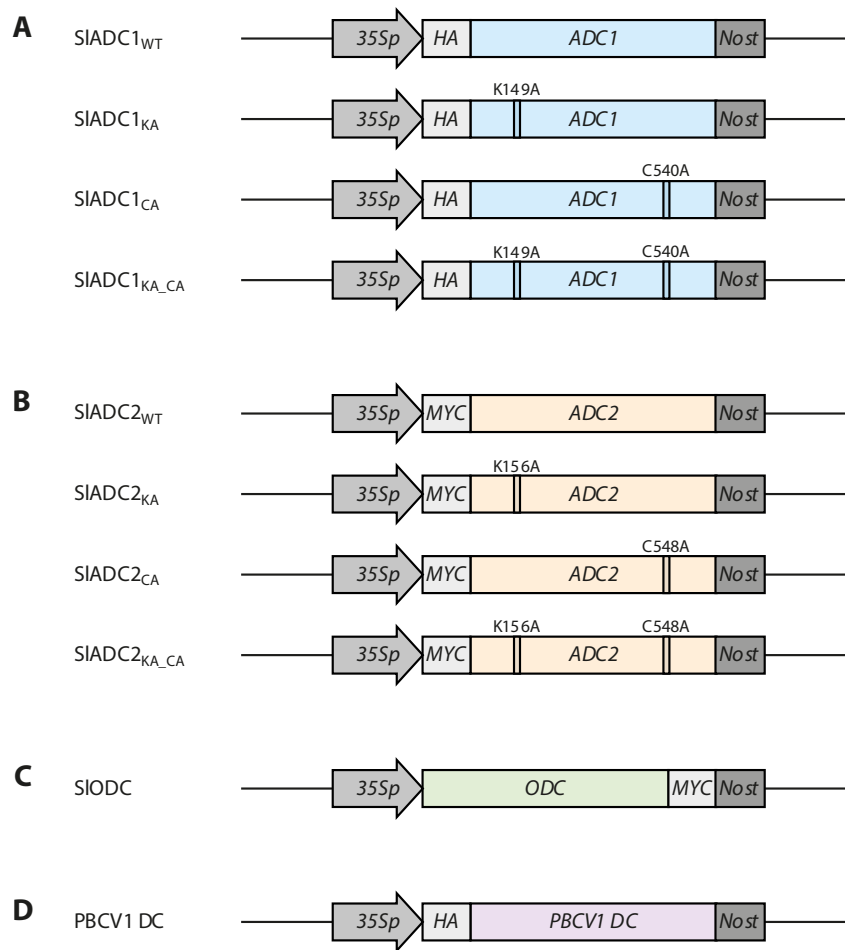


Figure 44: Construct design of epitope-tagged, constitutively expressed SIADC1/2 WT and mutant proteins, and SIODC and PBCV1-DC proteins.

Constructs for constitutive expression of WT and mutant SIADC1 (blue, **A**) and SIADC2 (yellow, **B**), WT SIODC (green, **C**), and WT PBCV1-DC (purple **D**) proteins. 35S promoters and Nos terminators (grey) were used for all constructs. An HA epitope tag was added to the N-terminus of SIADC1 and PBCV1-DC proteins, while a MYC tag was used for SIADC2 and SIODC.

As mentioned above, a decarboxylase enzyme encoded by *PBCV-1* (PBCV-1 DC) has efficient arginine decarboxylase activity, but a different peptide sequence to eukaryotic ODC and ADCs. The PBCV-1 DC peptide sequence was codon optimised for expression in solanaceous plants and synthesised by GeneScript with golden-gate compatible C-D BsaI overhangs (Binder et al., 2014). A constitutively expressed, epitope-tagged PBCV-1 DC construct was then generated (*35Sp:HA-PBCV-1 DC:35St*). Once all constructs had been verified via Sanger sequencing, they were transformed into *A. tumefaciens* GV3101.

3.5.2.5 Testing expression and enzymatic functionality of enzyme-expressing constructs

Prior to analysing the effect of the WT and mutant SIADC (i.e., SIADC1/2_{WT}, SIADC1/2_{K_A}, SIADC1/2_{C_A}, and SIADC1/2_{K_AC_A}), SIODC, and PBCV-1 DC proteins on the *ADC-box* translational regulatory mechanism, the *in planta* expression and expected catalytic activity

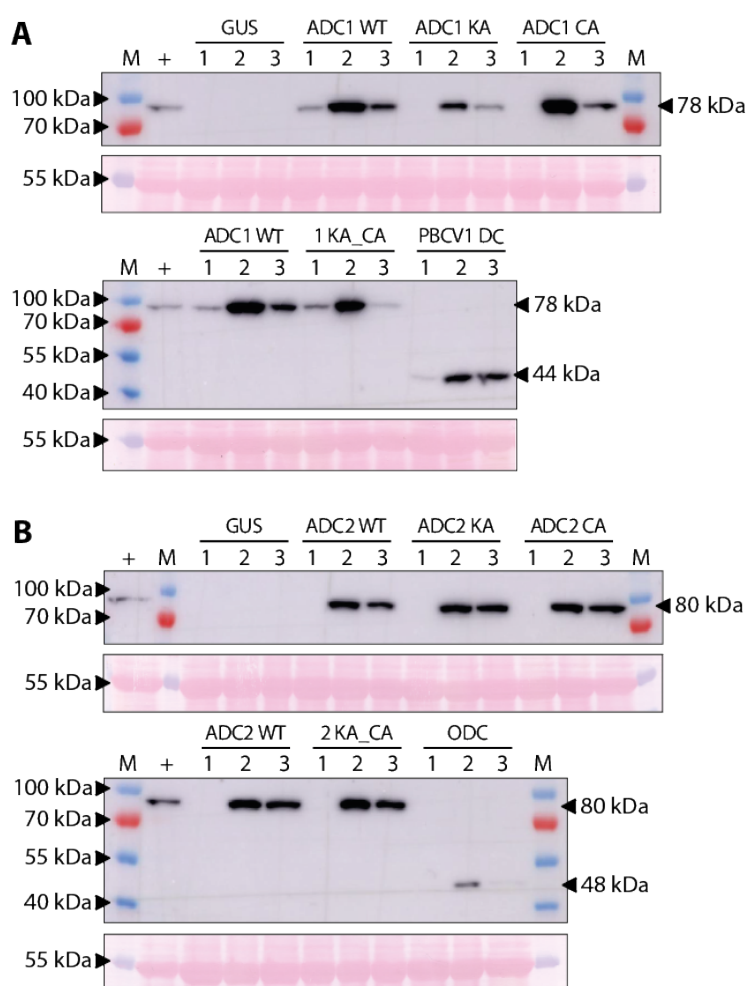


Figure 45: Expression of SIADC1, SIADC2, SIODC, and PBCV-1 DC proteins was highest after two days.

Epitope-tagged, WT and catalytic SIADC1/2 mutants, SIODC, and PBCV-1 DC were transiently expressed in WT *N. benthamiana* leaves for 1, 2, or 3 days; samples were taken each day for western blot analysis. HA-tagged SIADC1 proteins, HA-tagged PBCV-1 DC, and GUS (A), and MYC-tagged SIADC2 proteins, SIODC-MYC, and GUS (B) were run on a 10% SDS-PAGE gel; previously verified tagged protein was used as a positive control (+). Blots were incubated with anti-HA-HRP (A) or with anti-MYC primary and anti-mouse secondary (B) antibodies. Molecular weight marker (M) band sizes (left) and expected weights of expressed tagged proteins (right) are shown; SIADC1 78 kDa, PBCV-1 DC 44 kDa, SIADC2 80 kDa, and SIODC 48 kDa. Ponceau stained blot shown below each western blot.

was verified. To find the optimal timepoint for the highest abundance of transiently expressed proteins, all constructs, alongside a *35S::GUS* negative control, were agroinfiltrated into WT *N. benthamiana* leaves (section 2.2.1.5). At 1, 2, and 3 dpi, samples were taken for western blot analysis (section 2.2.4.1). All constructs had the most prominent band in the 2 dpi samples (Figure 45), indicating that at 2 dpi, expression of all proteins was highest and this timepoint should be used for future experiments.

All constructs were again agroinfiltrated into WT *N. benthamiana* leaves and after 2 dpi samples were taken for an LCMS analysis of ADC and ODC activity, as well as measurement of PAs and related metabolites (sections 2.2.5.3 and 2.2.5.4). As positive and negative controls for ADC activity, *35Sp::Brg11-mRFP* and *35Sp::GUS* constructs were included and protein expression of SIADC1/2 variants, SIODC, and PBCV-1 DC was verified via western blots (Supplementary Figure 27); however, myc-tagged SIODC could not be detected in this experiment.

ADC activity (heavy agmatine) from the SIADC1_{WT} and SIADC2_{WT} proteins was 189- and 168-fold higher than the GUS negative control (Figure 46A), demonstrating that the epitope-

tagged SIADC proteins were functional. No other infiltrated construct had significantly different ADC activity compared to the negative GUS control, including the Brg11- and PBCV1-DC-expressing constructs, which were expected to induce ADC activity. Importantly, none of the mutant SIADC1/2 protein variants had higher ADC activity than the negative GUS

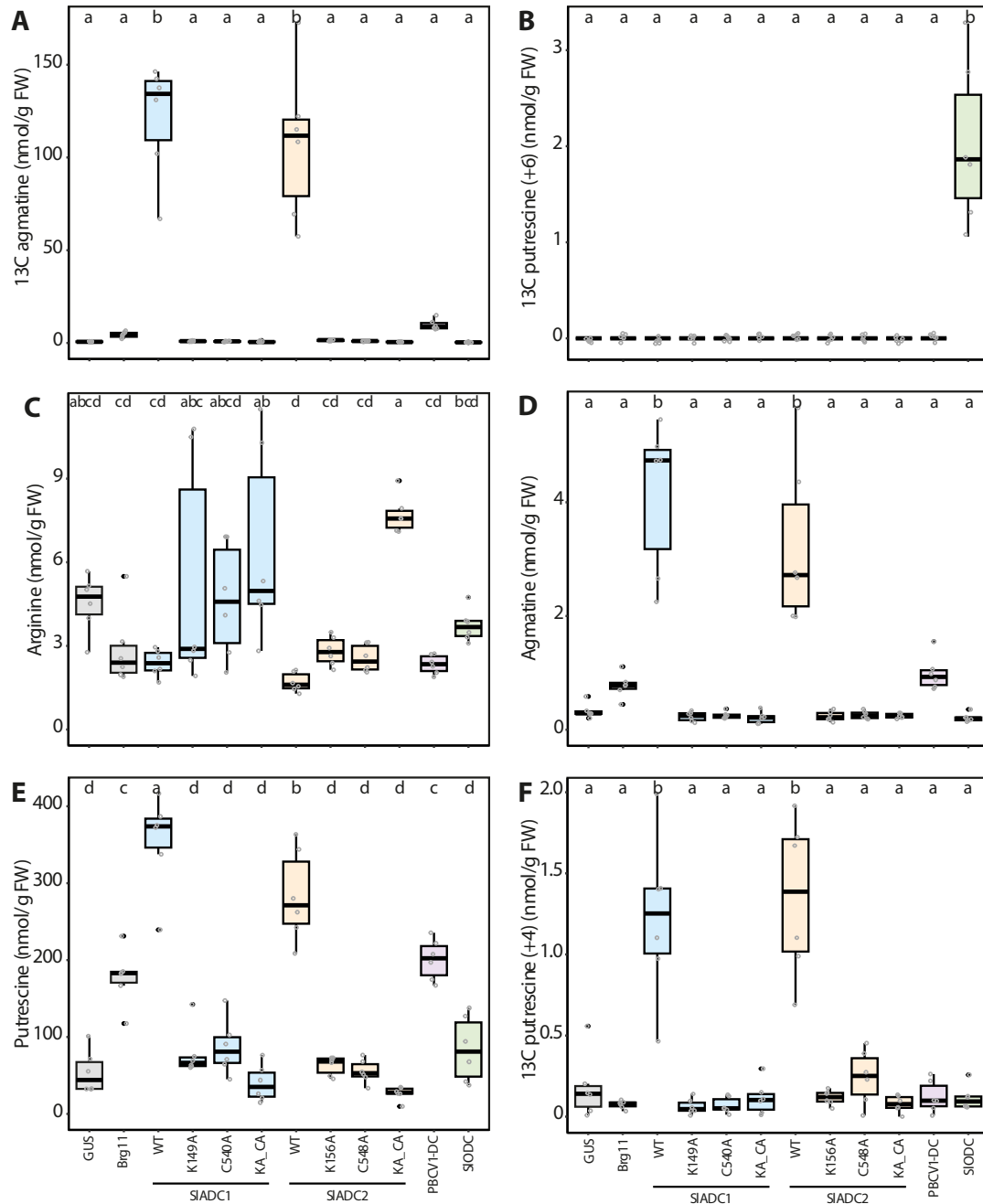


Figure 46: Analysis of PA levels from transiently expressed WT and catalytically dead ADCs, ODC, and PBCV1-DC proteins. After 2 dpi, samples of GUS (grey), Brg11 (grey), SIADC1 WT and mutant variants (blue), SIADC2 WT and mutant variants (yellow), PBCV1-DC (purple), and SIODC (green) constitutively expressed in *N. benthamiana* leaves were harvested for LCMS analysis (n = 6). ADC activity (heavy agmatine; **A**), ODC activity (heavy putrescine (+6); **B**), arginine (**C**), agmatine (**D**), putrescine (**E**), and putrescine generated via ADC during the 1-hour activity assay (heavy putrescine (+4); **F**) were measured and normalised to the internal standard D_5 tryptophan. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

control, indicating that both mutations were able to prevent normal ADC catalytic activity. ODC activity (i.e., $^{13}\text{C}_6$ putrescine) was only detected from the SIADC-expressing construct, demonstrating the expected catalytic activity of this protein (Figure 46B), despite no protein detected via western blot (Supplementary Figure 27).

PA and related metabolite levels were also measured during this experiment to gain an overview of how transient over expression of PA enzymes in *N. benthamiana* impacts the PA metabolic network. Interestingly, despite the dramatic increase in ADC activity from the SIADC1/2_{WT} proteins, no difference in arginine levels were observed compared to the negative GUS control (Figure 46C). However, the SIADC1/2_{WT} variants had significantly lower arginine levels compared to the SIADC1/2_{KA_CA} proteins (P -values = 0.00989 and 0.00002, respectively), suggesting that perhaps the catalytically active and inactive proteins differentially influence the cellular ADC substrate pool.

Agmatine levels were significantly increased upon expression of SIADC1/2_{WT} (Figure 46D), while putrescine levels were significantly increased upon over expression of Brg11, SIADC1/2_{WT}, and PBCV1-DC proteins (Figure 46E). This indicates that although no significant changes in ADC activity or agmatine levels were observed from over expression of Brg11 and PBCV1-DC proteins, their activity increased putrescine levels 3.3- and 3.7-fold compared to the GUS negative control (P -values = 0.000008 and 0.0000001, respectively). The heavy isotopic variant of putrescine (i.e., +4 variant) originating from the heavy arginine variant was detected also with SIADC1/2_{WT} expression (Figure 46F). This indicates that overexpression of functional SIADC1/2_{WT} proteins also induces rapid changes to the PA pathway.

Other metabolites that were increased upon overexpression of SIADC1/2_{WT} proteins were citrulline, ornithine, and acetylputrescine, while no influence on spermine, glutamine, or GABA was observed (Supplementary Figure 28). Surprisingly, spermidine levels were significantly reduced upon Brg11, SIADC1/2_{WT}, and PBCV1-DC expression by 2.6-, 4.1-, 4.5, and 4.8-fold, respectively (Supplementary Figure 28A). Previous experiments to over express ADC, either by $\Delta\text{adc-box}$ mutations (Figure 32) or induction by Brg11 (Wu et al., 2019), have never shown any difference in spermidine levels, indicating that something to do with either *N. benthamiana* or *A. tumefaciens* has influenced spermidine levels upon ADC over expression. Levels of some detected phenolamides were also measured and interestingly, upon SIADC1_{WT} expression, but not SIADC2_{WT} expression, coumaroylputrescine, feruloylputrescine, cinnamoylputrescine, and coumaroylspermidine levels were increased (Supplementary Figure 29).

In summary, transient overexpression of SIADC1/2_{WT} proteins produced high levels of ADC activity. On the other hand, none of the mutated SIADC1/2 constructs showed any ADC activity, not only indicating that mutating even one of the essential residues is enough to kill catalytic function, but that these catalytically vital residues are conserved with AtADC1 and the mouse ODC. ODC activity was also significantly increased upon expression of SIODC, indicating that this construct is also functional. Neither Brg11 nor PBCV1-DC expression increased ADC activity, but slightly higher agmatine and significantly higher putrescine levels were observed, suggesting that overexpression of these proteins may have increased ADC activity, but this was below the detectable limit of this assay. Overall, these experiments showed that the SIADC1/2 protein variants and SIODC proteins were expressed and function as expected.

3.5.3 Is the ADC-box translational regulatory mechanism dependent on a catalytically active ADC protein?

To determine if the catalytic function of an ADC protein is required for the *ADC-box* regulatory mechanism, *35Sp:SIADC1/2_{WT} 5'UTR:LUC* reporter constructs were co-infiltrated with either an empty vector (EV), or a corresponding active (i.e., SIADC1/2_{WT}) or catalytically non-functional (i.e., SIADC1/2_{KA_CA}) protein constructs (Figure 44, Figure 46). In the case of both

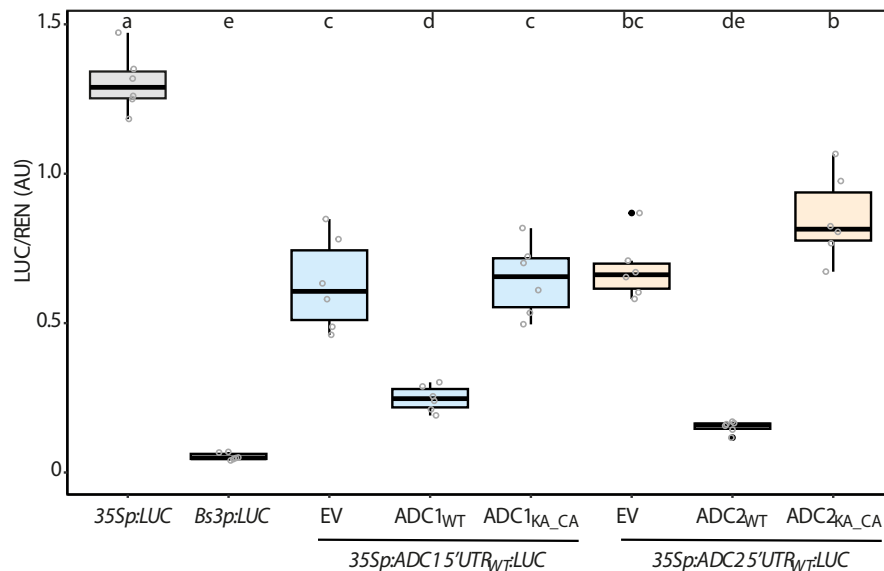


Figure 47: Translation from *SIADC* 5'UTR reporters is decreased upon co-expression with an active ADC protein, but not a catalytically dead ADC protein.

Wildtype *SIADC1* (blue) and *SIADC2* (yellow) 5'UTR reporter constructs were co-infiltrated into *N. benthamiana* leaves with either an empty vector (EV), functional SIADC1/2 (i.e., SIADC1/2_{WT}), or catalytically dead SIADC1/2 (i.e., SIADC1/2_{KA_CA}) proteins (n = 6). After 2 dpi, samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

SLADC1 and *SLADC2* 5'UTR reporters, co-expression with the corresponding *SLADC1/2_{WT}* protein reduced LUC/REN compared to co-expression with EV. Interestingly, no significant differences between co-expression with EV or the *SLADC1/2_{KA_CA}* proteins were observed (Figure 47). This finding indicated that the enzymatic function of an ADC protein is required to inhibit translation and therefore, a functional ADC protein is necessary as or to trigger the *ADC-box* regulatory mechanism feedback signal. As positive and negative reporter controls, *35Sp:LUC* and *Bs3p:LUC* constructs were also included and responded as expected, with high LUC/REN from the *35Sp:LUC* construct and low LUC/REN from the *Bs3p:LUC* construct (Figure 47). Interestingly, renilla luminescence was significantly lower from the *Bs3p:LUC* reporter compared to all other constructs (Supplementary Figure 30).

To confirm that ADC-dependent translational repression was due to the *SLADC-box* in the 5'UTR, another experiment was conducted to include an *SLADC1_{Δadc-box}* 5'UTR reporter construct. As expected, when co-expressed with an EV, the *SLADC1_{Δadc-box}* reporter had significantly higher LUC/REN compared to the *SLADC1_{WT}* reporter (P -value > 0.00002 ; Figure 48), demonstrating once more that deletion of the *ADC-box* increases translation of the downstream gene. However, upon co-expression of an active *SLADC1_{WT}* protein, LUC/REN from the *SLADC1_{Δadc-box}* reporter was significantly reduced compared to the EV control (P -value = 0.039), while there was no significant difference in LUC/REN upon co-expression of EV or the *SLADC1_{KA_CA}* protein (P -value = 0.94). This result suggests that the ADC protein-dependent repression of translation observed in Figure 47 is not due to the *ADC-box*.

To determine if the ADC protein-dependent reduction of translation may be due to the presence of the uORF, an *SLADC1_{Δuorf}* 5'UTR:*LUC* reporter construct was also included in this experiment. When co-delivered with the active *SLADC1_{WT}* protein, no significant difference in LUC/REN from the *SLADC1_{Δuorf}* 5'UTR reporter was observed compared to with EV (P -value = 0.95). Similarly, the *SLADC1_{WT}* 5'UTR reporter also had no significant change in LUC/REN upon co-delivery with *SLADC1_{WT}* compared to EV (P -value = 0.91); these result conflict with the previous observation of reduced translation from the *SLADC1_{WT}* 5'UTR reporter in the presence of an active *SLADC1_{WT}* protein (Figure 47). Interestingly, both *SLADC1_{WT}* and *SLADC1_{Δuorf}* 5'UTR reporters had increased LUC/REN upon co-delivery of *SLADC1_{KA_CA}* compared to *SLADC1_{WT}*, indicating that the active and non-functional *SLADC1* protein variants differentially affected luciferase reporter expression. However, co-expression of *SLADC1* proteins did not alter renilla expression, indicating that only luciferase expression was influenced by *SLADC1_{WT}* (Supplementary Figure 31).

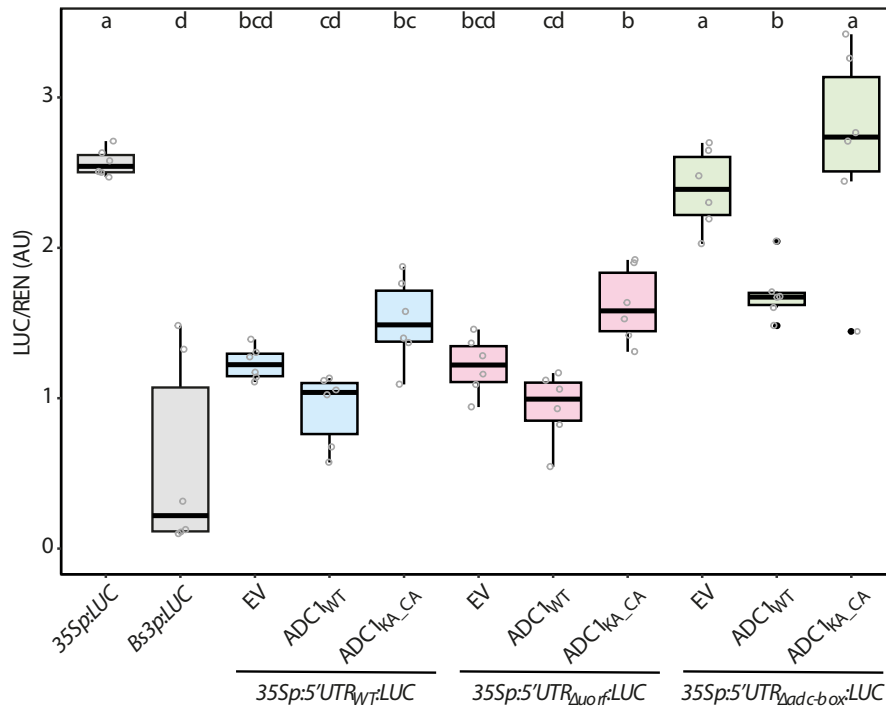


Figure 48: Repression of translation upon co-expression of a functional SIADC protein occurs in an *ADC* 5'UTR element-independent manner.

SIADC1 5'UTR wildtype (blue), $\Delta uorf$ (pink), and $\Delta adc\text{-}box$ (green) 5'UTR reporter constructs were co-infiltrated into *N. benthamiana* leaves with either an empty vector (EV), functional SIADC1 (i.e., SIADC1_{WT}), or catalytically dead SIADC1 (i.e., SIADC1_{KA_CA}) proteins (n = 6). After 2 dpi, samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

A western blot, detecting HA-tagged SIADC1_{WT/KA_CA} proteins was conducted to ensure even protein expression (Supplementary Figure 32). Here, the SIADC1_{WT} and SIADC1_{KA_CA} bands were of equal intensity when co-delivered with *SIADC1*_{WT} 5'UTR and *SIADC1* _{$\Delta uorf$} 5'UTR reporters. However, upon co-delivery with the *SIADC1* _{$\Delta adc\text{-}box$} 5'UTR reporter, the SIADC1_{WT} band was fainter than all others and the SIADC1_{KA_CA} band was much thicker than all others. It is possible that such differences in protein expression could differentially influence translational efficiency from the *SIADC1* _{$\Delta adc\text{-}box$} 5'UTR reporter in a manner that was not imposed on the *SIADC1*_{WT} and *SIADC1* _{$\Delta uorf$} 5'UTR reporters.

Due to these inconclusive results and to investigate if this influence on translational efficiency also occurs with an SIADC2 protein and *SIADC2* 5'UTR reporters, *SIADC2* WT, $\Delta uorf$, and $\Delta adc\text{-}box$ 5'UTR reporter variants were generated. In this experiment, WT, $\Delta uorf$, and $\Delta adc\text{-}box$ *SIADC1* and *SIADC2* 5'UTR reporter constructs were co-delivered with either their

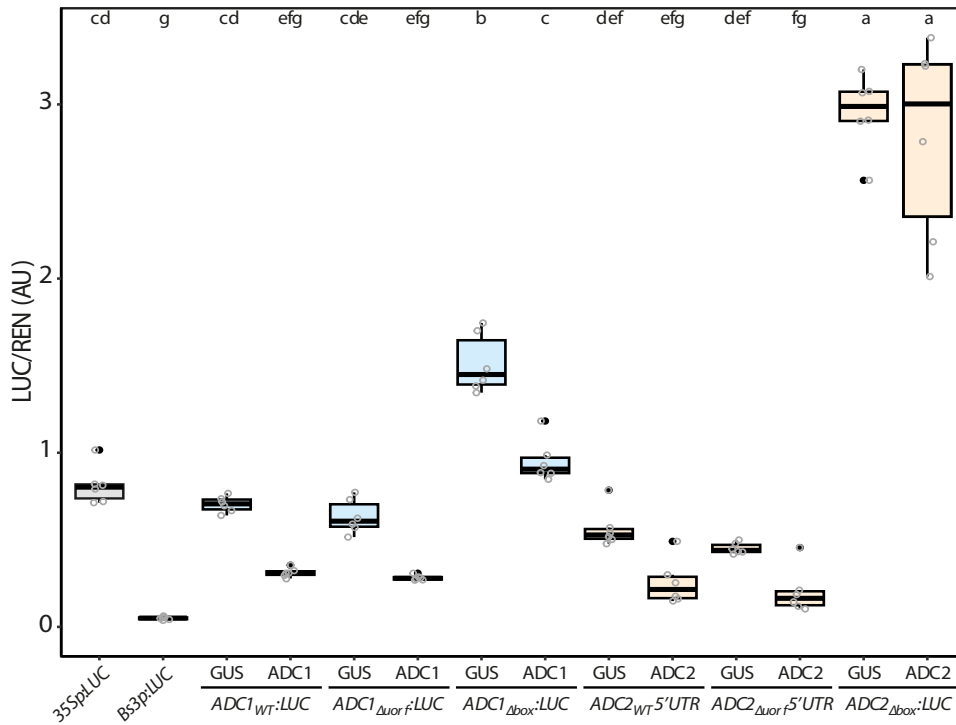


Figure 49: Functional SIADC1 and SIADC2 proteins reduce translation in a *uORF*- and *ADC-box*-independent manner.

SIADC1 (blue) and *SIADC2* 5'UTR (yellow) wildtype, $\Delta uorf$, and $\Delta adc-box$ 5'UTR reporter constructs were co-infiltrated into *N. benthamiana* leaves with either *35S::GUS* (GUS) as a negative control or with a functional SIADC1/2_{WT} protein (n = 6). After 2 dpi, samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

corresponding SIADC1/2_{WT} protein or a *35S::GUS* construct as a negative control. Although not always statistically significant, co-delivery of an *SIADC1/2* 5'UTR reporter construct with an active SIADC1/2_{WT} protein reduced reporter expression compared to the GUS control (Figure 49). The only exception to this pattern was expression from the *SIADC2* $\Delta adc-box$ 5'UTR reporter, which was not affected by co-expression of SIADC2_{WT}. However, the overall LUC/REN from this construct was unusually high, at approximately 3.5-fold higher than *35S::LUC* (co-expressed with *35S::GUS*); a high expression level was observed when testing the functionality of the *35S::SIADC2* $\Delta adc-box$ 5'UTR:LUC construct, but not to this extent (data not shown). Irrespective of this data point, overexpression of SIADC1/2_{WT} reduced translation from all other reporters, regardless of the presence or absence of a *uORF* or *SIADC-box*, further supporting the conclusion that this reduction in translation is not dependent on these elements. Once more, renilla luminescence from each reporter was not statistically different upon co-delivery of GUS or SIADC1/2_{WT} (Supplementary Figure 33), indicating that only the luciferase reporter is affected by SIADC1/2_{WT} expression.

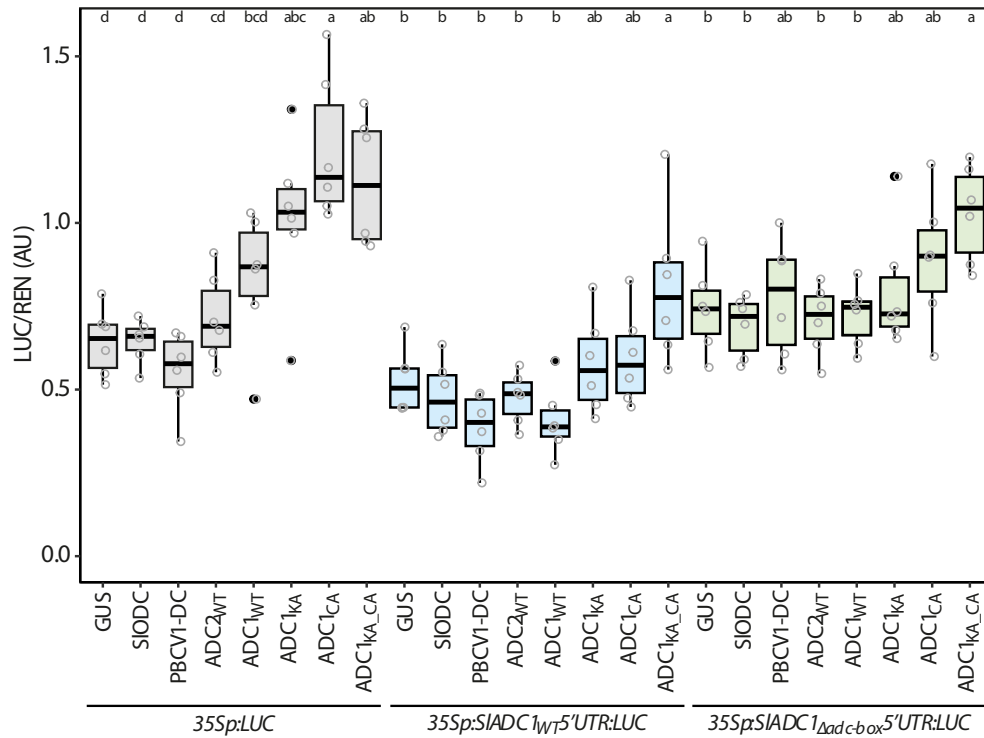


Figure 50: Co-expression of polyamine-related enzymes may influence global transcription or translation processes and influence read out from *35Sp:LUC*-style reporters.

35Sp:LUC (grey), *35Sp:SIADC1_{WT} 5'UTR:LUC* (blue), and *35Sp:SIADC1_{Δadc-box} 5'UTR:LUC* (green) reporter constructs were co-infiltrated into *N. benthamiana* leaves with GUS (negative control), SIODC, PBCV1-DC, SIADC2_{WT}, SIADC1_{WT}, SIADC1_{KA}, SIADC1_{CA}, or SIADC1_{KA_CA} (n = 6). After 2 dpi, samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted between samples from the same LUC reporter and not between all other samples; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

To determine if over expression of other PA-related enzymes impacts luciferase luminescence, the *35Sp:LUC*, *35Sp:SIADC1_{WT} 5'UTR:LUC*, and *35Sp:SIADC1_{Δadc-box} 5'UTR:LUC* reporters were co-expressed with GUS (as a negative control), SIODC, PBCV1-DC (i.e., the viral ADC-like protein), SIADC2_{WT}, SIADC1_{WT}, SIADC1_{KA}, SIADC1_{CA}, and SIADC1_{KA_CA} proteins. Interestingly, in this experiment, no reduction in LUC/REN was observed upon co-delivery if the *SIADC1_{WT}* and *SIADC1_{Δadc-box} 5'UTR* reporters with an active SIADC1/2_{WT} protein (Figure 50). However, co-delivery with the catalytically dead SIADC1/2 protein variants increased LUC/REN, not only from the *SIADC1 5'UTR* reporters, but also from the *35Sp:LUC* reporter. Renilla expression was not significantly different between any reporter-protein combination (Supplementary Figure 35), further demonstrating that any impact of SIADC protein variants on LUC/REN was due to influencing the luciferase reporter. To ensure that equal protein expression occurred between each reporter, a western blot was conducted detecting SIADC1/2 protein variants, PBCV1-DC, and SIODC (Supplementary Figure 34). Here, protein band

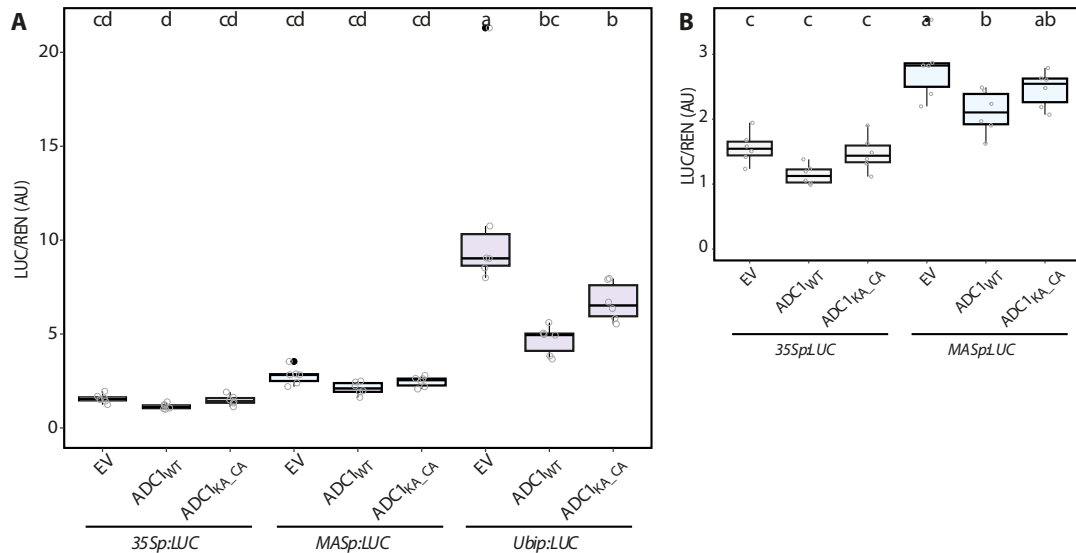


Figure 51: Co-expression of SIADC1WT reduces expression from MASp:LUC and Ubip:LUC.

35S:LUC, *MASp:LUC*, and *Ubip:LUC* reporters were co-expressed with either EV, SIADC1_{WT}, or SIADC1_{KA_CA} in *N. benthamiana* leaves (n = 6). After 2 dpi, samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance between all samples (A); letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level. *Ubip:LUC* initially produced luciferase luminescence that overloaded the plate reader, so sample volumes were reduced to enable measurement. Due to this difference in measurement, results for *35Sp:LUC* and *MASp:LUC* are displayed and statistically analysed separately as well (B).

densities were overall consistent upon co-delivery with SIADC1_{WT} and SIADC1 _{Δ adc-box} 5'UTR reporters. However, in some cases, co-delivery with *35Sp:LUC* produced fainter bands than the other reporters. Such differences in protein expression cannot be explained.

Overall, these results suggest that the SIADC protein variants may be influencing luciferase expression and/or luminescence in an *ADC* 5'UTR-independent manner, indicating that this reporter might not be suitable to study the translational regulatory mechanism of *ADC* transcripts in combination with co-expression of *ADC* proteins.

From the experiments presented in this section (3.5.3), it remains unclear how the active and catalytically dead SIADC1/2 proteins influence translation from *SIADC* 5'UTR reporters or support a model for how the *ADC-box* regulatory mechanism may function. In fact, the data indicate that expression of either active or catalytically inactive SIADC1/2 proteins impact luciferase expression, but not renilla expression. Thus far, luciferase has been expressed primarily with a 35S promoter, and renilla by the tomato actin promoter. To determine if the 35S promoter may be influenced by co-delivery with SIADC1 proteins, two promoter reporter constructs were generated with either the mannopine synthase (*MAS*) promoter or *Lotus japonicus* polyubiquitin (*LjUbi*) promoter. The reporter constructs *35Sp:LUC*, *MASp:LUC*, and

Ubip:LUC were then co-delivered with either EV, SIADC1_{WT}, or SIADC1_{KA_CA} for a dual luciferase assay. All three promoter reporter constructs showed lower LUC/REN when co-delivered with SIADC1_{WT} compared to EV (Figure 51); however, this was not significant in the case of the *35S* promoter. This indicates that regardless of the promoter, luciferase expression was affected by SIADC1_{WT} expression.

Interestingly, both the *MASp:LUC* and *Ubip:LUC* constructs had higher renilla expression upon co-delivery with SIADC1_{WT}, which was not the case for *35S:LUC* (Supplementary Figure 36), nor had this occurred in prior experiments. This is unexpected as all constructs have the same *SlActin:REN* reporter gene, suggesting that altered transcriptional activity on the T-DNA due to the different promoters, may influence expression of renilla. A western blot showed that expression of SIADC1_{WT} and SIADC1_{KA_CA} proteins were relatively even when co-delivered with each reporter and unlikely to be the cause of differential reporter expression (Supplementary Figure 37). Overall, these results show that out of the three tested promoters, the *35S* promoter was the least affected co-delivery of SIADC1_{WT} and SIADC1_{KA_CA} proteins. In summary, the *pGrDL_SPb (GG)* dual luciferase assay system has a straightforward cloning procedure and the error between biological replicates is low, providing clearly distinguishable differences between constructs. However, co-infiltration of *pGrDL_SPb (GG)* reporters with both catalytically active and inactive SIADC1/2 proteins altered general luciferase expression when driven by *35S*, *MAS*, or *Ubi* promoters, obscuring their effect on translation from *SIADC1/2* 5'UTRs. Due to this, no clear conclusions or working models could be made as to how SIADC1/2 proteins influence translation in an *ADC-box*-dependent manner to regulate downstream translation.

3.5.4 Preliminary analysis of tomato *ADC* 5'UTR secondary structures using SHAPE analysis.

Previously, the *ADC-box* was hypothesised to form an RNA secondary structure based on its high GC-content. A predicted structure was published for both seedless and seed plants, with either one or two hairpins, respectively (Figure 52A; Wu et al., 2019). However, this structure was only computationally predicted and not based on experimental evidence. Therefore, to support the hypothesis that the *ADC-box* forms an RNA secondary structure and to know exactly what conformation it forms, a structure based on experimental evidence was required. This would overall improve our understanding of how the *ADC-box* can regulate translation of *ADC* and possibly give insights into its mechanism.

One method of RNA secondary structure determination that combines both experimental data and computational models is selective 2'-hydroxyl acylation analysed by primer extension (SHAPE). This technique requires treatment of the living biological sample with an electrophile (e.g., 2-methylnicotinic acid, NAI), which acetylates 2'-hydroxyl groups of all four RNA nucleotides depending on their level of local conformational flexibility (i.e., being unpaired to or unrestricted by another nucleotide, or bound to another moiety). If the nucleotide has flexibility, it is more likely to act as a nucleophile and form a 2'-*O*-adduct upon acetylation with NAI (Deigan et al., 2009, Lucks et al., 2011). These 2'-*O*-adducts can then be detected by primer extension to a single nucleotide resolution with RNA sequencing, as RNA transcriptase is unable to pass such modified nucleotides. The reads are then analysed by a data analysis pipeline to determine a SHAPE reactivity score for each nucleotide, essentially quantifying how available that nucleotide was for acetylation and therefore its flexible or thermodynamic accessibility (Lucks et al., 2011). Based on these scores and neighbouring nucleotides, an RNA secondary structure can be computationally modelled with very high accuracy (i.e., 97%; Deigan et al., 2009).

As SHAPE analysis requires a substantial amount of technical and computational knowledge, a collaboration with Dr Yiliang Ding (John Innes Centre, UK), a specialist in SHAPE analysis of plant tissues, was established. The Ding laboratory were able to provide images of preliminary RNA secondary structures for *SlADC1* and *SlADC2* 5'UTRs using RNA extracted from tomato flowers and mature green fruits. It was hypothesised that although the *SlADC1/2* 5'UTRs only share 65% sequence identity (Supplementary Figure 38), the high sequence conservation of their *ADC-boxes* would enable a common structure to be elucidated. The *ADC-box* RNA secondary structures of both *SlADC1* and *SlADC2* from both tissues formed triple hairpin structures with the same GC-rich sequences forming the same stem structures (Figure 52B-E; original images shown in Supplementary Figure 39). Interestingly, the central stem loop of these SHAPE-derived *ADC-box* structures correspond to the same highly conserved GC2 and GC3 regions predicted to form a hairpin in both the seedless and seed plants by Wu et al., 2019; this sequence is highlighted in blue in all structures (Figure 52).

Overall, these results indicate that in tomato flowers and mature green fruits, the *SlADC1/2-boxes* form a triple stem loop structure, with the central hairpin corresponding to the most highly conserved GC-rich regions.

3.6 Approaches to study the impact of *R. solanacearum* RipTAL effectors

3.6.1 Background information for developing approaches to investigate the role of *R. solanacearum* RipTAL effectors.

During this work, we aimed to develop experimental approaches to investigate the functional relevance of RipTAL-induction of host ADCs during *R. solanacearum* infection. Previous work had found no growth-promoting benefit for strains containing the RipTAL Brg11 compared to a $\Delta brg11$ knock out strain using soil-drench or leaf infiltrated growth assays (section 1.5; Wu et al., 2019). It was hypothesised that perhaps *R. solanacearum* boosts host PA levels to inhibit growth of niche microbes in a ternary interaction (i.e., pathogen-plant-microbe; Wu et al., 2019). However, it is also possible that RipTALs play another role, but this was not observed due to the experimental procedures used (described in section 1.5.4). Therefore, alternative approaches and biological tools were developed during this work with the aim of specifically detecting, monitoring, and isolating transcripts from cells that receive Brg11 at an early infection stage. Through these tools, we aimed to specify into which cell types *R. solanacearum* injects Brg11 during the initial infection stages, and if these cell types had any Brg11-dependent morphological or transcriptional changes. Two approaches were initiated along this line. Firstly, a Brg11-inducible fluorophore and GUS reporter construct was generated to specifically visualise and isolate nuclei from host cells that receive Brg11. Secondly, a construct that produces epitope-tagged ribosomes in a Brg11-inducible manner to enable affinity-purification of ribosome-bound transcripts only from root cells that receive Brg11 was also used. These tools are explained and presented in section 3.6.2 below.

In addition to these cellular-focused approaches, alternative methodologies were needed to investigate the metabolic and microbiome-related consequences of overexpressing ADC. It is not feasible to harvest sufficient quantities of Brg11-receiving host cells during the early stages of infection to measure their PA metabolic profile due to the limitations of the LCMS (i.e., at least 25 mg of tissue is required to detect and accurately measure PAs and related metabolites with the current protocols). Similarly, it would be challenging to determine changes to the microbial community in only the local area surrounding root cells that were injected with Brg11. To circumvent these technical challenges, overexpression of Brg11/ADC in all root cells would enable both metabolic and microbiome analyses in root tissue. Experimental approaches that were developed for these purposes are presented in section 3.6.3.

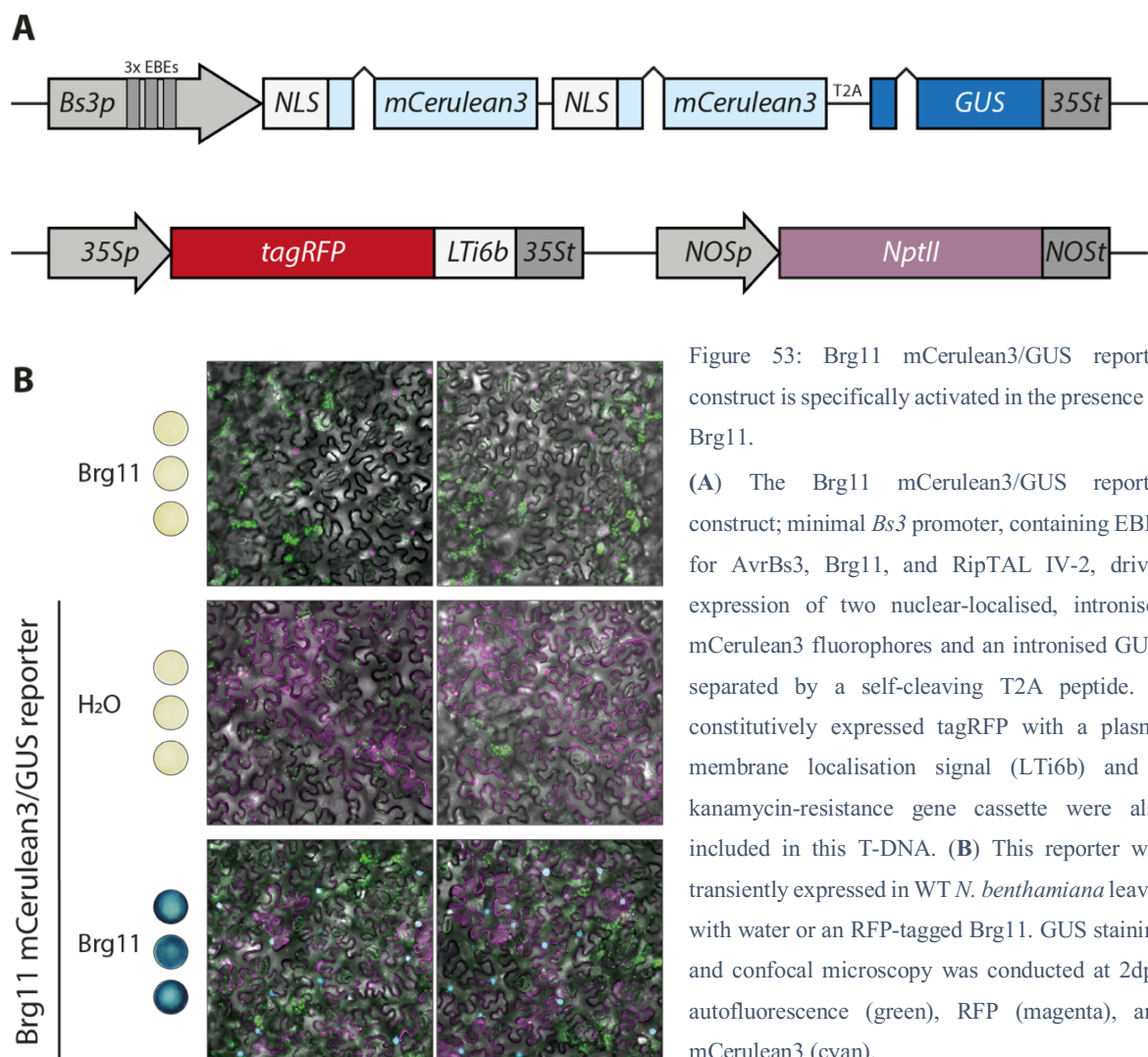
3.6.2 Tools to investigate the impact of Brg11 at the cellular level in roots.

Two transgenic approaches were established for the purpose of detecting and isolating transcripts from root cells that receive Brg11 during *R. solanacearum* infection. The first is a fluorophore-based approach, enabling the visualisation of cells that receive Brg11 and subsequent isolation of the nuclei of these cells to study their transcriptome; work on this approach is presented in section 3.6.2.1. The second approach is based on purification of ribosomes and their associated transcripts from cells that receive Brg11 via Translating Ribosome Affinity Purification (i.e., TRAP or RiboTRAP), presented in section 3.6.2.2.

3.6.2.1 Fluorophore-based reporter to visualise and capture root cells injected with Brg11.

To visualise and specifically capture root cells/nuclei that receive Brg11 during *R. solanacearum* infection, Brg11-inducible reporter construct was developed that encodes a nuclear-targeted fluorophore and GUS (Figure 53A). To ensure that only the cells injected with Brg11 express the reporter, the promoter containing a Brg11-compatible *EBE* (Brg11 *EBE*) must be very tight, with no transcription in the absence of Brg11. For this purpose, the minimal promoter of the pepper resistance gene *Bs3* was used (Romer et al., 2009). *Bs3* encodes the Bs3 protein, which causes cell death upon translation, and therefore, the *Bs3* promoter must be very tight when Bs3 protein is not required. Previously, the Brg11-*EBE* sequence was cloned into this promoter (de Lange et al., 2013, Schandry et al., 2016), so that transcription can be specifically activated only upon bacterial delivery of Brg11. The *Bs3* promoter naturally contains the *EBE* for AvrBs3, a TALE protein from *Xanthomonas*, but can also tolerate the introduction of multiple *EBEs* for other TALEs, whilst remaining a tight promoter (Romer et al., 2009). The *EBE* from RipTAL IV-2 was also introduced into this *Bs3* promoter, enabling transcriptional activation following delivery of RipTAL IV-2 (Figure 53A). Due to differences in the RipTAL IV-2 RVDs, this RipTAL cannot bind to the native Brg11 *EBE* and therefore does not activate the endogenous *ADC* genes of *Solanaceous* or *Brassica* species (de Lange et al., 2013; Gallas et al., 2023). Therefore, AvrBs3 and RipTAL IV-2 can be used to activate transcription of the reporter, but not the endogenous *ADC* genes, thereby acting as negative controls for the ADC-inducing activity of Brg11. However, as AvrBs3 has known endogenous targets (Kay et al., 2007) and RipTAL IV-2 may have unknown off targets (i.e., not the Brg11 *EBE* or reporter transgene), AvrBs3, RipTAL IV-2, and a dTALE targeting the *Bs3* promoter may all need to be used in combination as negative controls.

While a tight promoter that can be specifically activated upon Brg11 injection is important, it is also essential that the reporter output be very apparent and discernible from the surrounding cells that did not receive Brg11. As it is hypothesised that only a small number of cells are injected with Brg11 during the early stages of infection, the reporter must clearly highlight these cells for easy identification; for this purpose, both fluorophore and histological staining reporters were used. Specifically, two nuclear-localised mCerulean3 fluorophores, with distinct nucleotide sequences and introns, were designed and synthesised (GenScript). mCerulean3 was selected as it was reported to be 20% brighter than similar fluorophores (i.e., mTurquoise; Markwardt et al., 2011), and it emits at 475 nm, allowing for additional fluorophores emitting in the green, yellow, and red spectrums to be used as additional reporters if needed. Additionally, introns were included to improve expression and reduce the risk of silencing and avoid expression within *A. tumefaciens* during transient experiments in *N. benthamiana* leaves. The mCerulean3 fluorophores were targeted to the nucleus to increase the intensity of



fluorescence by localising all expressed fluorophore to a small cellular compartment. Additionally, nuclei from these cells can be specifically isolated using the fluorophore, via Fluorescence-Activated Nuclei Sorting (FANS) and RNA extracted from these nuclei can be sent for RNA sequencing to determine the transcriptional impacts of Brg11 in the effector-injected root cells.

Furthermore, to enable visualisation of root cells that receive Brg11 in whole root tissue, this reporter also contained an intronised GUS gene linked to the mCerulean3 fluorophores via a T2A self-cleaving peptide (Figure 53A). Staining whole *R. solanacearum*-infected roots for GUS activity would enable fast and sensitive identification of where in the root effectors are injected during infection. In subsequent experiments, these areas in the root could be more closely analysed via confocal microscopy. Additionally, the GUS reporter provides an easy and sensitive screening method for identification of good candidate transgenic lines.

Alongside the Brg11 reporter construct described above, a constitutively expressed, plasma membrane localised red fluorescent protein (tagRFP-LTi6b) was also included in this construct (Figure 53A). As *Solanaceous* roots are rather thick and less permissive to cell wall or plasma membrane stains such as propidium iodide (PI), labelling the plasma membrane may ease the visualisation and identification of the root cell borders during microscopy. It was also thought that as *R. solanacearum* infection causes cellular morphological changes and root tissue damage (Xue et al., 2020), the ability to use stains such as PI to visualise individual cells may be impeded.

Finally, a constitutively expressed neomycin resistance gene was included in the T-DNA to allow for *in planta* selection of transformed plants (Figure 53A). These constructs were all assembled to form a single T-DNA construct, termed the Brg11 mCerulean3/GUS reporter, and transformed into *A. tumefaciens* GV3101 (Supplementary Figure 40; Binder et al., 2014). To test if the Brg11 mCerulean3/GUS reporter construct functions as expected, it was agroinfiltrated into WT *N. benthamiana* leaves with either water or an *A. tumefaciens* strain carrying a constitutively expressed RFP-tagged Brg11 (made by Dousheng Wu, Lahaye Lab). As a negative control for reporter expression, the Brg11-containing *A. tumefaciens* was infiltrated alone. After 2 dpi, samples were taken for GUS staining and for confocal microscopy (sections 2.2.4.4 and 2.2.4.5; Figure 53B). Leaves infiltrated with Brg11-RFP alone showed no GUS staining, but nuclear localised RFP, indicating that the Brg11-RFP was expressed and correctly localised to the nucleus. The Brg11 mCerulean3/GUS reporter co-infiltrated with water showed no GUS staining and no nuclear localised mCerulean3 fluorescence, while having plasma membrane localised RFP. This demonstrated that the Brg11 mCerulean3/GUS

reporter was expressed, but due to the lack of Brg11, no mCerulean3 nor GUS transcription occurred from the tight *Bs3* promoter. Upon co-infiltration with Brg11, both strong GUS staining and bright nuclear-localised mCerulean3 fluorescence were observed. Therefore, based on these results and additional repetition experiments (data not shown), the Brg11 mCerulean3/GUS reporter was found to be a highly specific and intense reporter for the presence of Brg11, whilst clearly outlining each cell due to the RFP-labelled plasma membrane. The Brg11 mCerulean3/GUS reporter was then stably transformed into *A. thaliana*, *N. benthamiana*, and tomato, as all three species can be infected with *R. solanacearum*, but each provide their own distinct experimental benefits and limitations. Although *A. thaliana* is not a native *R. solanacearum* host, it can be infected in an experimental setting and comes with the advantage of having abundant genetic resources and published literatures that may be required for subsequent work. *N. benthamiana* was transformed with this reporter construct as it is a close relative of tobacco (a natural *R. solanacearum* host), can be transiently transformed with *A. tumefaciens*, and used for confocal microscopy with low levels of background autofluorescence. Tomato was also transformed with the Brg11 mCerulean3/GUS reporter construct as it is an important commercial crop and a common model species for *Ralstonia* research. However, due to issues with the transformation procedure, no transgenic tomatoes were identified during this work. The following sections describe identification of candidate *N. benthamiana* (section 3.6.2.1.1) and *A. thaliana* (section 3.6.2.1.3) lines.

3.6.2.1.1 Selection of candidate *N. benthamiana* containing the Brg11-inducible reporter.

N. benthamiana plantlets that had grown from calli on kanamycin containing media were transferred to soil and grown in the greenhouse (section 2.2.1.4). Once established, samples were taken from each plant for gDNA extraction and a PCR for the presence of the transgene was conducted using primers CKP46 and ESR41 (sections 2.2.3.5 and 2.2.3.2).

The plants with a positive PCR band for the Brg11 mCerulean3/GUS reporter transgene, were infiltrated with *A. tumefaciens* carrying an RFP-tagged Brg11 to induce activation of the transgene reporter. After 2 dpi, samples were taken for GUS staining from both the Brg11-infiltrated leaf area and a non-infiltrated area. Samples were also taken for confocal microscopy at 2 dpi to check for Brg11-activated nuclear-localised mCerulean3 fluorescence and the plasma membrane-localised RFP. GUS staining and confocal images from a selection of T0 generation plants have been shown in Figure 54. Many plants, such as plants 3.5 and 6.2, show very little, if any GUS staining, and no visible nuclear-localised mCerulean3 fluorescence, despite expression of Brg11 (observed by red nuclear fluorescence), and were not used for

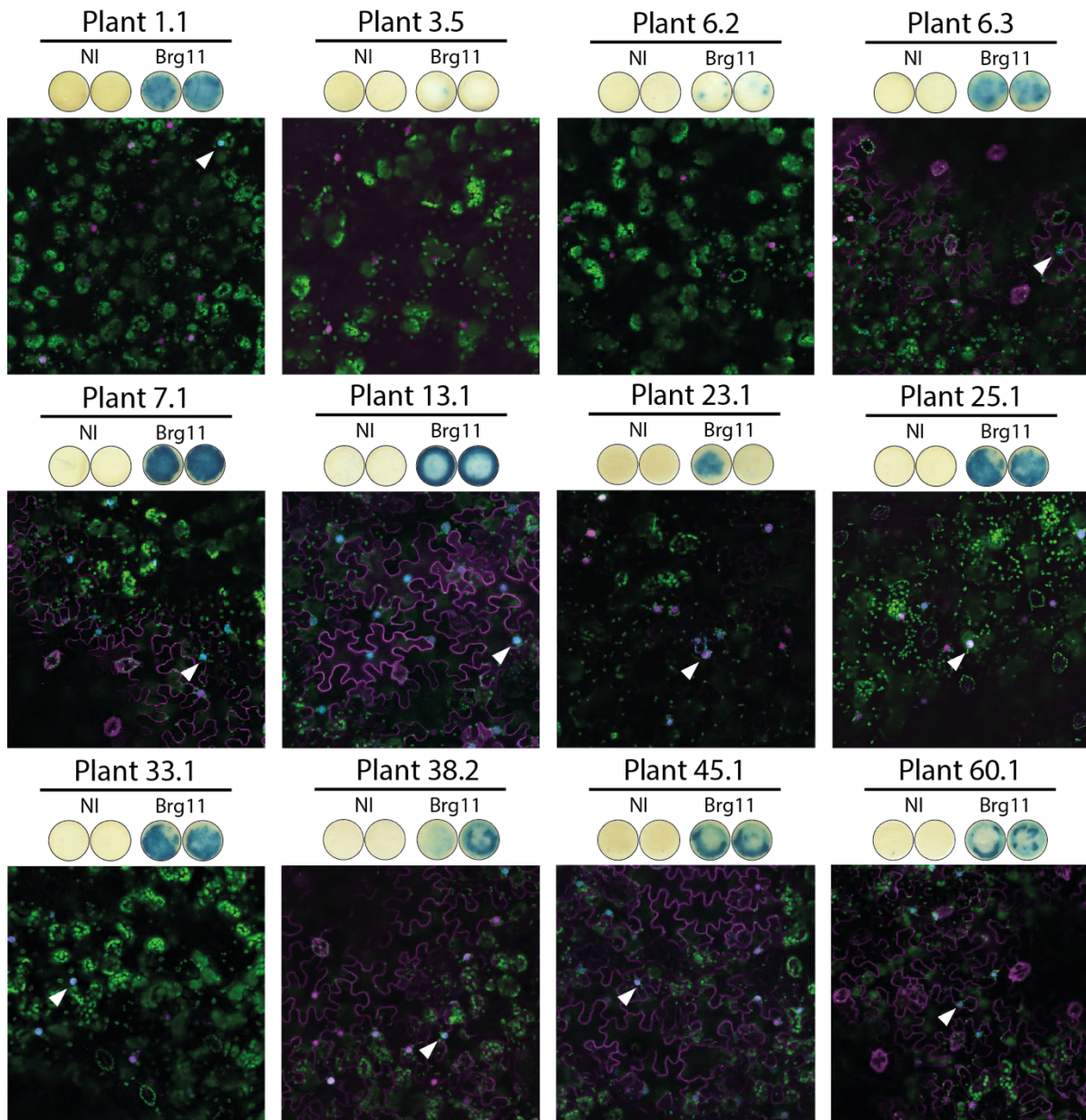


Figure 54: Selection of candidate T0 Brg11 mCerulean3/GUS reporter *N. benthamiana* lines via GUS staining and confocal microscopy.

T0 generation Brg11 mCerulean3/GUS reporter *N. benthamiana* lines were agroinfiltrated with an RFP-tagged Brg11. After 2 dpi, samples were taken from both Brg11-infiltrated and non-infiltrated (NI) leaves for GUS staining and confocal microscopy; autofluorescence (green), RFP (magenta), and mCerulean3 (cyan). A selection of 12 T0 plants have been shown here. White arrows highlight a representative nucleus with mCerulean3 fluorescence in the confocal images; if no arrow is shown, no mCerulean3 fluorescence was seen in this line.

further analysis. Alternatively, plants such as plant 1.1, 25.1, and 33.1 had clear GUS staining only in the Brg11-infiltrated samples, as well as nuclear-localised mCerulean3 fluorescence, but no plasma membrane localised RFP fluorescence was observed. However, some lines showed clear GUS and mCerulean3 expression in the Brg11-infiltrated samples as well as

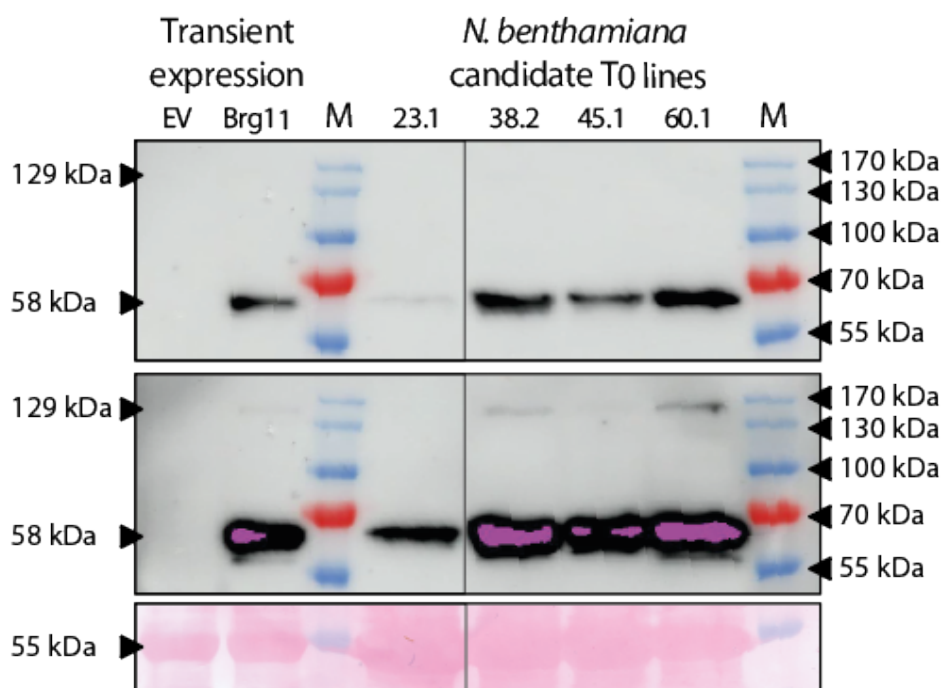


Figure 55: mCerulean3 detection via western blot was possible for selected T0 candidate Brg11 mCerulean3/GUS *N. benthamiana* lines.

2 dpi of RFP-tagged Brg11, samples from T0 Brg11 mCerulean3/GUS reporter *N. benthamiana* lines were taken for western blot, alongside controls of transiently expressed Brg11 mCerulean3/GUS reporter with either empty vector (EV) or Brg11. Samples were run on a 10% SDS-PAGE gel, transferred, and blots were incubated overnight with anti-GFP-HRP conjugated antibody. Top image was exposed for 1.5 minutes, and middle image exposed for 2.5 minutes. Bottom image shows ponceau stain of total protein. The double mCerulean3-GUS is expected to be 129 kDa, reduced to 58 kDa after T2A cleavage of GUS.

plasma membrane localised RFP (e.g., plants 6.3, 7.1, 13.1, 38.2, 45.1, and 60.1); these lines were considered ideal candidate lines for future experiments.

A western blot was also conducted on a selection of lines agroinfiltrated with Brg11-RFP to detect mCerulean3 expression and determine if the T2A peptide was efficiently cleaving off the GUS peptide (section 2.2.4.1). As mCerulean3 is a derivative of GFP, anti-GFP antibodies were used. As a positive control, the Brg11 mCerulean3/GUS reporter construct was co-delivered with Brg11-RFP. As a negative control, the Brg11 mCerulean3/GUS reporter was agroinfiltrated with an EV. Bands between 55 and 70 kDa could be detected in the positive control, as well the selected candidate *N. benthamiana* lines, but not the negative control when the blot was exposed for 1.5 minutes (Figure 55, upper blot). When exposing the blot for 2.5 minutes, faint bands between 130 – 170 kDa were observed (Figure 55, lower blot). The double mCerulean3-T2A-GUS polypeptide has an expected size of 128 kDa, while after T2A self-cleavage, the expected size of the double mCerulean3 polypeptide is expected to be 58 kDa. From these blots, the molecular weight of these bands seems slightly larger than expected.

Regardless, this blot indicates that the T2A peptide is functional and the majority of detected mCerulean3 is approximately the size of the GUS-cleaved double NLS-mCerulean3.

In total, from the 37 transformant plants tested, 26 lines had positive GUS staining following agroinfiltration with Brg11. Of these plants, 18 also had nuclear localised mCerulean3 fluorescence, with only 10 of these having plasma membrane localised RFP fluorescence (i.e., plants 1.1, 6.3, 7.1, 13.1, 23.1, 35.1, 38.2, 45.1, and 60.1). These good candidate lines, as well as some additional lines that had Brg11-dependent GUS staining and mCerulean3 fluorescence, but no plasma membrane localised RFP, were kept for seed production.

Seeds from 7 selected candidate lines were sown out for subsequent testing in the T1 generation (i.e., lines 1.1, 6.3, 13.1, 23.1, 38.2, 45.1, and 60.1); the work in this generation was done with the help of Sruthi Sunil. In this generation, the transgene is expected to segregate as T0 transformants are heterozygous. Therefore, ~20 plants from each line were transplanted to individual pots and their gDNA extracted for PCR detection of the transgene. Plants that had a positive PCR band, indicating the presence of the transgene (either heterozygous or homozygous), were kept, while the ~¼ of plants from each line lacking the transgene were discarded. The plants from these 7 lines were subsequently infiltrated with *A. tumefaciens* carrying RFP-tagged Brg11 and samples were taken for GUS staining and confocal microscopy at 2 dpi. All lines had positive GUS staining following agroinfiltration with Brg11 (Figure 56).

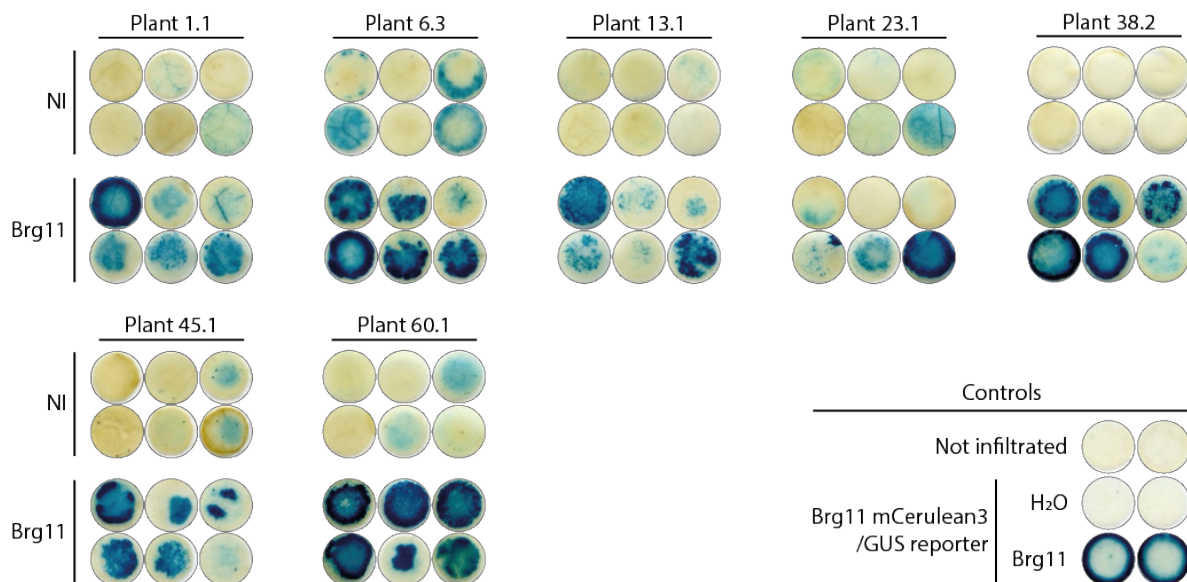


Figure 56: *N. benthamiana* Brg11 mCerulean3/GUS reporter line 38.2 is the most promising candidate line in the T1 generation.

T1 generation progeny of candidate T0 Brg11 mCerulean3/GUS reporter *N. benthamiana* lines were infiltrated with RFP-tagged Brg11. After 3 dpi, samples were taken for GUS staining, alongside non-infiltrated samples. Transient expression of the Brg11 mCerulean3/GUS reporter with either H₂O or Brg11 in WT *N. benthamiana* demonstrate Brg11 functionality and GUS staining.

However, many lines also showed GUS staining in samples that were not infiltrated (NI), indicating some leaky expression of the Brg11 mCerulean3/GUS reporter in the absence of Brg11. Only line 38.2 had no GUS staining in the non-infiltrated samples, whilst showing strong Brg11-dependent GUS staining and mCerulean3 fluorescence, and plasma membrane localised RFP expression. Therefore, line 38.2 was considered as the best candidate line for further analysis.

To identify which T1 generation plants of line 38.2 were homozygous for the transgene, seeds from T1 plants #3, #8, #14, and #15 were sown for segregation analysis. These plants had positive PCR bands for the transgene in the T1 generation, yet only by genotyping the T2 generation can the transgene zygosity of the T1 generation be determined. Therefore, 20 descendent plants from line 38.2, T1 generation plants #3, #8, #14, and #15 were genotyped for the transgene using the same method as for the T0 transformants. A positive PCR band was observed in all progenies of plants #8 and #15 (data not shown), indicating that these lines were most likely homozygous in the T1 generation and can be used for future experiments.

3.6.2.1.2 Activation of Brg11 mCerulean3/GUS reporter by *R. solanacearum*

Unlike tomato and *A. thaliana*, the commonly used *R. solanacearum* reference strain GMI1000 cannot grow in tobacco species, such as *N. tabacum* and *N. benthamiana*. Two type III secretion system effectors, *avrA* and *popP1*, are recognised by these host species, producing a hypersensitive response (HR) and prevent wilting (Poueymiro et al., 2009). Removing both *avrA* and *popP1* (strain GRS473) restores the ability of GMI1000 to grow in *N. tabacum* and *N. benthamiana* and cause the characteristic bacterial wilt disease symptoms. As this strain is a GMI1000 mutant, it contains Brg11, so can be used for activation of the Brg11 mCerulean3/GUS reporter in *N. benthamiana* without causing an HR. This strain was kindly donated to the Lahaye laboratory by Stéphane Genin (CNRS, Toulouse, France).

N. benthamiana leaf infections of *R. solanacearum* had not previously been carried out in the Lahaye laboratory conditions. Initial experiments were conducted, infiltrating WT *N. benthamiana* leaves with *R. solanacearum* strains GMI1000, GRS473, and K60 (another strain gifted by Stéphane Genin that can infect *N. benthamiana*, but does not contain a RipTAL; Poueymiro et al., 2009, Schandry et al., 2016). These experiments aimed to find an infection strategy and time point that produced healthy appearing leaf tissue, that would be able to express the Brg11 mCerulean3/GUS reporter.

Firstly, strains GRS473 and K60 were infiltrated at OD₆₀₀ 0.01 and 0.001, and GMI1000 was infiltrated at OD₆₀₀ 0.001. After 4 dpi, GMI1000 produced a strong HR phenotype, while no

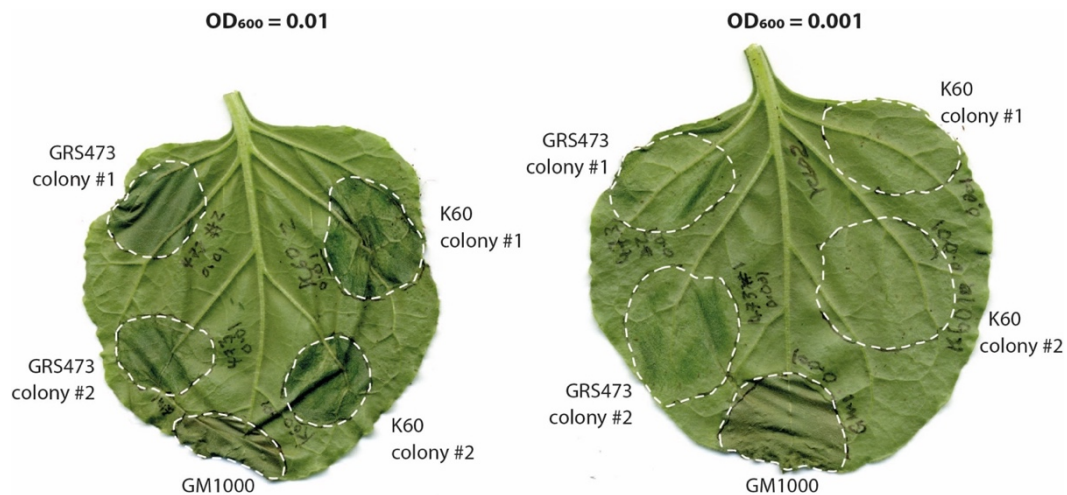


Figure 57: *R. solanacearum* strains GRS473 and K60 induce water soaking in WT *N. benthamiana* leaves, but do not produce an HR like GMI1000.

R. solanacearum strains GRS473 (GMI1000 lacking *avrA* and *popPI*), and K60 were infiltrated into WT *N. benthamiana* leaves at an OD₆₀₀ of either 0.01 (left leaf) or 0.001 (right leaf) (n = 4). GMI1000 was infiltrated at an OD₆₀₀ of 0.001 on all leaves. After 4 dpi, leaves were photographed; one representative replicate is shown here.

HR was seen for GRS473 or K60 strains at either OD₆₀₀ (Figure 57). However, water soaking was observed where GRS473 had been infiltrated, and to a lesser extent in the areas of K60 infiltration. In a second experiment a higher inoculum of OD₆₀₀ 0.1 of *R. solanacearum* strains GMI1000, GRS473, and K60 was used to infect WT *N. benthamiana* leaves. After only one day, an HR could be seen where GMI1000 was infiltrated, but areas where GRS473 and K60 were infiltrated appeared as the healthy, non-infiltrated tissue (data not shown). These results confirm previous findings that removal of *avrA* and *popPI* from GMI1000 prevents HR (Poueymiro et al., 2009). Additionally, these results show that a low inoculum of GRS473 leads to disease symptoms after 4 days, and a high GRS473 inoculum of OD₆₀₀ 0.1 can infiltrated without any apparent effects after 1 dpi.

To test whether delivery of Brg11 by *R. solanacearum* could activate expression of the Brg11 mCerulean3/GUS reporter, transient expression of the reporter by *A. tumefaciens* and co-inoculation with either GMI1000 or GRS473 was conducted. Recently, a method for co-inoculation of *N. benthamiana* with *A. tumefaciens* and *R. solanacearum* was published, whereby *A. tumefaciens* was infiltrated at OD₆₀₀ of 0.2-0.5 24 hours before infiltration of *R. solanacearum* (Yu and Macho, 2021). However, as this method aimed to measure *R. solanacearum* growth, the initial *R. solanacearum* inoculum was very low (i.e., 1 x 10⁵ cfu/mL, or OD₆₀₀ 0.0004). After some trial experiments, agroinfiltration of the Brg11 mCerulean3/GUS reporter at OD₆₀₀ 0.4 and then infiltrating *R. solanacearum* at OD₆₀₀ 0.4, 24 hours later was found to work best (Figure 58). *A. tumefaciens* delivery of Brg11 caused GUS staining at 2 dpi,

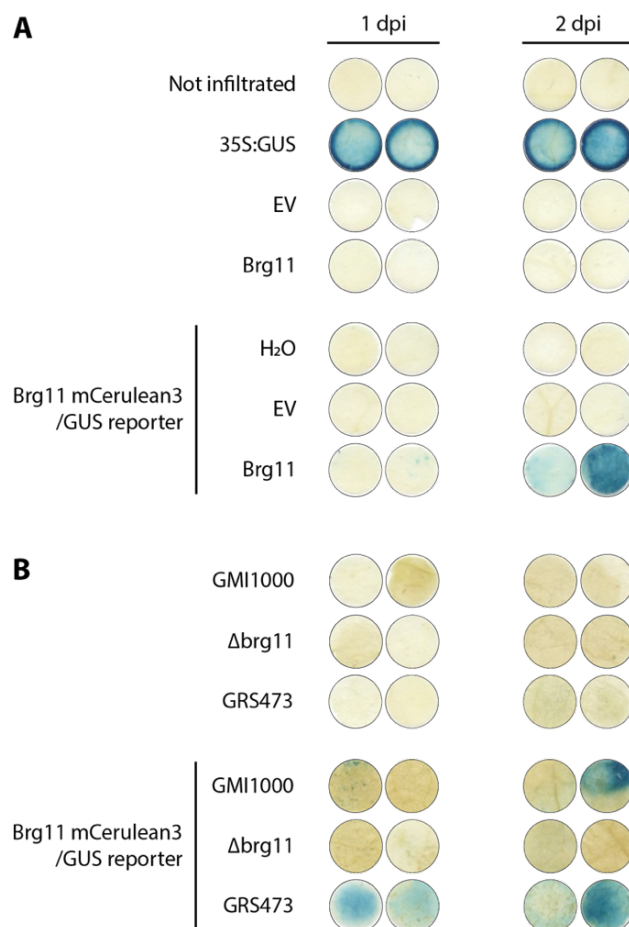


Figure 58: The Brg11 mCerulean3/GUS reporter, transiently expressed by *A. tumefaciens*, can be activated by *R. solanacearum* strain GRS473 after 2 and 4 dpi.

A. tumefaciens carrying the Brg11 mCerulean3/GUS reporter was infiltrated into WT *N. benthamiana* leaves at OD₆₀₀ 0.4. (A) After 24 hours, *A. tumefaciens* carrying either empty vector (EV) or RFP-tagged Brg11 were infiltrated into non-infiltrated areas or where the Brg11 mCerulean3/GUS reporter was infiltrated. 35S:GUS was also agroinfiltrated as a positive control. (B) *R. solanacearum* strains GMI1000 WT, Δ brg11, or GRS473 were also infiltrated in the same manner. 1 dpi and 2 dpi after the second infiltration, samples were taken for GUS staining.

while no GUS staining was observed when water or the empty vector control was infiltrated with the reporter (Figure 58A). When GRS473 was infiltrated where the Brg11 mCerulean3/GUS reporter was expressed, GUS staining was observed after 1 and 2 dpi (Figure 58B). GMI1000 WT also activated GUS expression, but to a lesser extent. Both GMI1000 WT and the Δ brg11 GMI1000 mutant strain caused HR (i.e., brown leaf tissue). No GUS staining was observed in the absence of the Brg11 mCerulean3/GUS reporter (Figure 58B). Overall, these results show that *R. solanacearum* GRS473 strain can activate expression of the Brg11 mCerulean3/GUS reporter when it is transiently expressed in WT *N. benthamiana* by *A. tumefaciens*.

Next, an experiment to test if *R. solanacearum* GRS473 could also activate the Brg11 mCerulean3/GUS reporter in stably transformed *N. benthamiana* plants was conducted. GRS473 was infiltrated at either OD₆₀₀ 0.1 or 0.4 into previously genotyped and phenotyped T2 generation progeny of the Brg11 mCerulean3/GUS reporter line 38.2 (section 3.6.2.1.1), alongside WT *N. benthamiana* as a negative control. Samples were taken for GUS staining at 1, 2, and 3 dpi to determine the optimal infection procedure for transgene activation. GUS staining was observed in samples infiltrated with OD₆₀₀ 0.4 after 1 dpi in all transgenic lines,

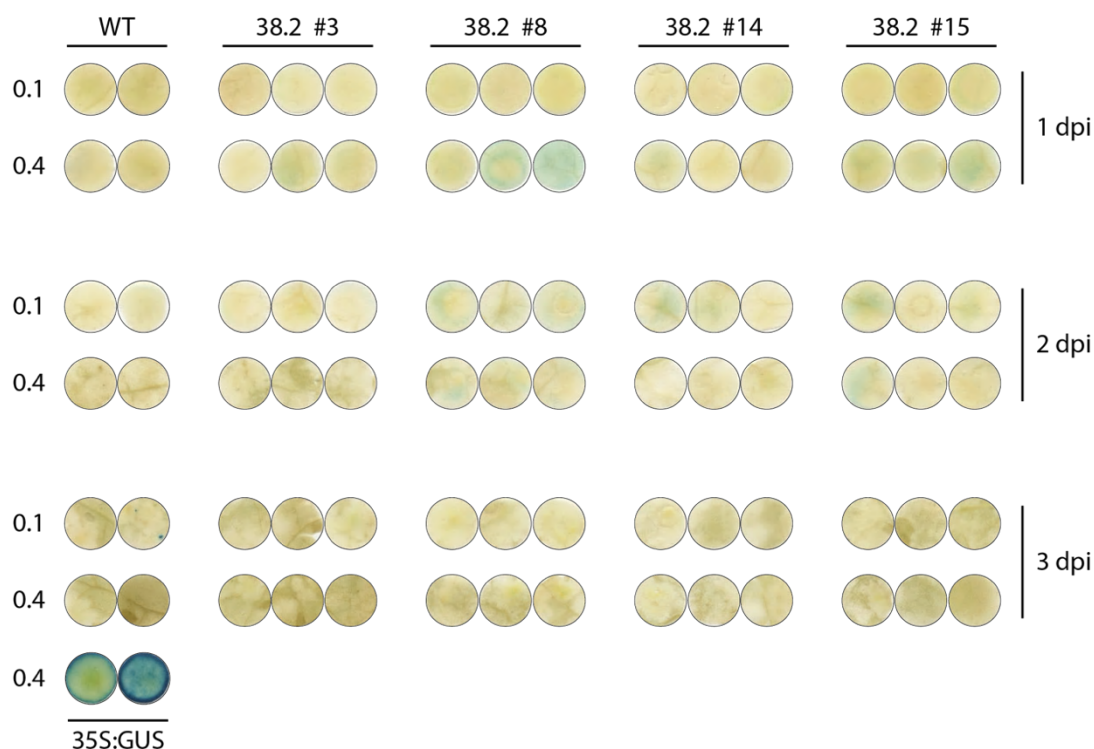


Figure 59: Activation of the Brg11 mCerulean3/GUS reporter in stable transgenic *N. benthamiana* lines by *R. solanacearum* GRS473.

R. solanacearum GRS473 was infiltrated into WT *N. benthamiana* and the T2 generation progeny of the Brg11 mCerulean3/GUS reporter line 38.2 leaves at an OD₆₀₀ of 0.1 and 0.4. After 1, 2, and 3 dpi, samples were taken for GUS staining. As a positive control for GUS staining, 35S:*GUS* was agroinfiltrated into WT leaves and harvested at 3 dpi.

but most prominently in line 38.2 #8 (Figure 59). After 2 dpi, faint GUS staining was observed when infiltrated with either an OD₆₀₀ 0.1 or 0.4 in lines #8, #14, and #15. However, during this infection, the leaves began to look necrotic at 2 dpi, and by 3dpi, it was challenging to cut clean leaf samples due to the water soaking and necrosis, and no GUS staining was observed in the transgenic lines at 3 dpi. Overall, the positive GUS staining seen at 1 dpi indicates that *R. solanacearum* GRS473 can activate the Brg11 mCerulean3/GUS reporter from stable transgenic lines. However, these results demonstrate that further efforts to standardise and improve *R. solanacearum* infection in *N. benthamiana* leaves may be required.

3.6.2.1.3 Selection of candidate *A. thaliana* containing the Brg11-inducible reporter.

WT *A. thaliana* plants were also transformed with the Brg11 mCerulean3/GUS reporter via floral dipping (section 2.2.1.7). Seeds from these plants were sterilised, vernalised in the fridge for 4 days, before being sown onto ½ MS media containing kanamycin to select for plants containing the transgene. WT *A. thaliana* and a transgenic line confirmed as kanamycin-resistant were also sown on this media in parallel as negative and positive controls, respectively. The 18 largest kanamycin-resistant T1 progeny from the Brg11 mCerulean3/GUS

reporter-dipped plants were transferred to soil and the presence of the transgene was confirmed by PCR of extracted gDNA (using primers CK46 and ESR41; sections 2.2.3.5 and 2.2.3.2). To test the functionality of the transgene in the *N. benthamiana* Brg11 reporter lines, *A. tumefaciens* was used to transiently deliver RFP-tagged Brg11. However, transient transformation of *A. thaliana* with *A. tumefaciens* is not efficient, and many methods require strict plant growth conditions that do not suit testing first generation transformants (i.e., plants grown in liquid media or long day conditions and specific *A. tumefaciens* strains; Boch and Bonas, 2010, Wu et al., 2014, Zhang et al., 2020b). Therefore, *R. solanacearum* GMI1000 was used to deliver Brg11 and test the functionality of the 18 candidate Brg11 mCerulean3/GUS reporter *A. thaliana* lines. Due to safety restrictions of working with *R. solanacearum*, whole *A. thaliana* plants could not be taken into the S2 laboratory, otherwise they could not be taken

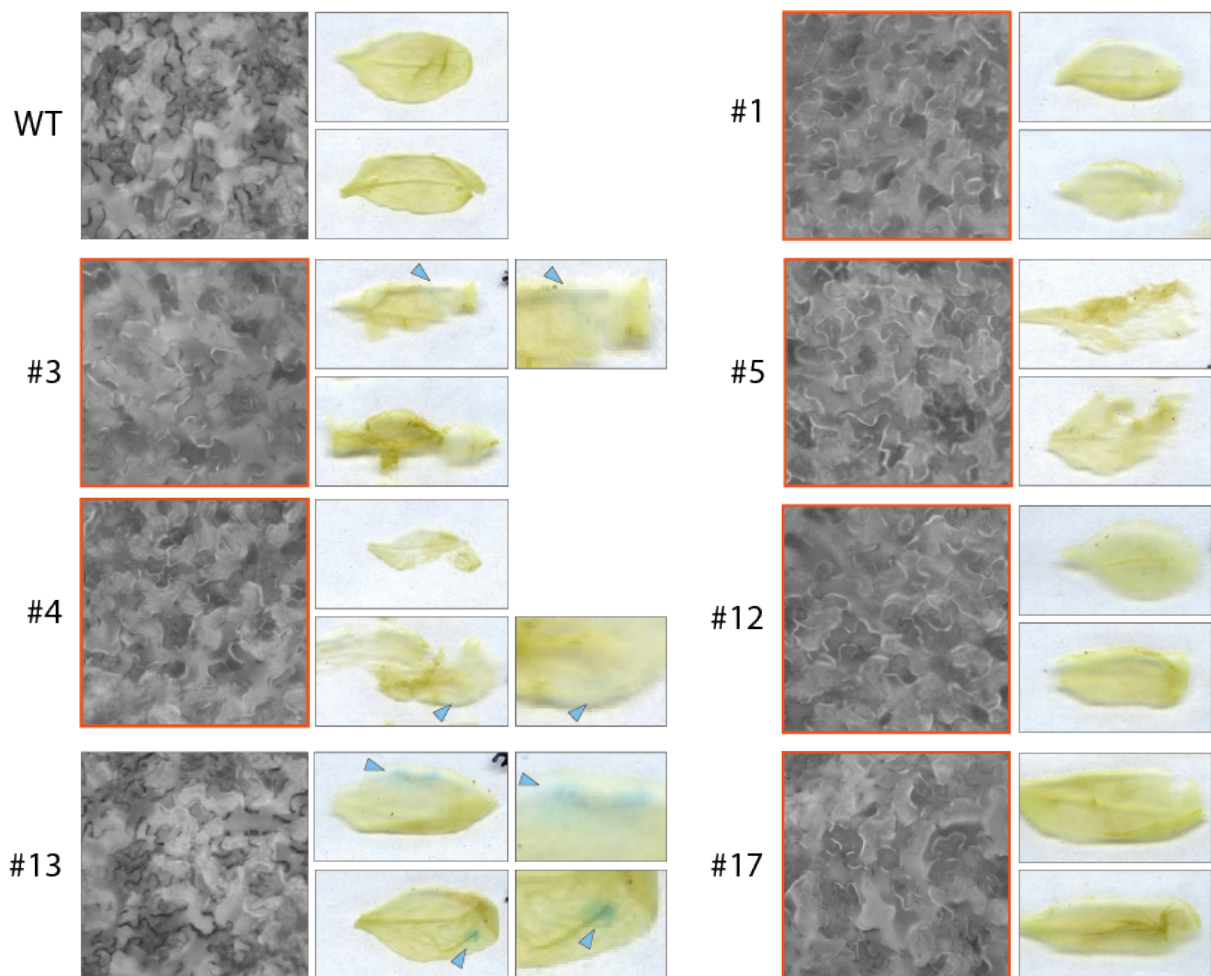


Figure 60: *A. thaliana* Brg11 mCerulean3/GUS reporter T1 generation lines were tested for GUS expression following *R. solanacearum* infiltration and for plasma membrane-localised RFP fluorescence.

Epifluorescent microscopy was conducted on leaves from WT and 18 T1 generation plants containing the Brg11 mCerulean3/GUS reporter transgene to detect plasma membrane RFP fluorescence (left images). Where RFP fluorescence was observed, the image was surrounded by a red box. Detached leaves from these plants were infiltrated with *R. solanacearum* OD₆₀₀ 0.1 and after 2 dpi, samples were taken for GUS staining (right images). A red arrow indicates where GUS staining was observed.

out again for seed production. To circumvent this, two leaves from each of the 18 lines were detached, taken into the S2 laboratory, infiltrated with *R. solanacearum* GMI1000 at an OD₆₀₀ of 0.1 and kept on damp paper towels in a sealed 6 well plate. After 2 dpi, the leaves were GUS stained. Many leaves were beginning to look necrotic at this stage, mostly in the areas where *R. solanacearum* was infiltrated. GUS staining was observed on both leaves of plant #13, only in the area where GMI1000 had been infiltrated, as well as small or faint areas of GUS staining in lines #3 and #4 (Figure 60). No GUS staining was observed in the WT control leaves. These results indicated that not only could Brg11 had been delivered by GMI1000 to activate the Brg11 mCerulean3/GUS transgene, but that this reporter is also functional in *A. thaliana*. In addition to the Brg11-activated GUS staining, the presence of plasma membrane-localised RFP was also checked for in each candidate *A. thaliana* Brg11 mCerulean3/GUS reporter line under the epifluorescent microscope. Plasma membrane localised RFP was observed in lines #1, #3, #4, #5, #12, and #17, but not in WT leaves. Therefore, as lines #3 and #4 had both plasma membrane-localised RFP and faint GUS staining with *R. solanacearum* infiltration, they are the best candidates for further work. Despite not showing RFP fluorescence, line #13 should also be considered as it showed the strongest GUS expression. All 18 candidate *A. thaliana* Brg11 mCerulean3/GUS lines were kept for seed production, but no further work was conducted on these lines during this work.

3.6.2.2 Development of Brg11-inducible Tandem Ribosome Affinity Purification (TRAP) lines.

In addition to the Brg11 mCerulean3/GUS reporter lines, another approach was developed to specifically isolate transcripts from only from root cells injected with Brg11 during *R. solanacearum* infection. This approach uses Translating Ribosome Affinity Purification (TRAP, also referred to as RiboTRAP) followed by RNA sequencing (TRAP-SEQ; Reynoso et al., 2015). Here, a gene encoding an epitope-tagged ribosomal subunit was driven by a minimal, tight promoter containing the Brg11-*EBE*. Following transcriptional activation by Brg11, these tagged subunits are then incorporated into ribosomes, which can subsequently be purified using the epitope tag, along with their associated transcripts. RNA sequencing of these isolated transcripts would then inform on the Brg11-induced transcriptomic changes in effector-injected root cells. In addition to total RNA sequencing, TRAP also provides an opportunity to conduct ribosome foot printing, giving an insight into the translation status of Brg11-injected cells. As PAs are known to regulate translation in both an mRNA-specific manner (Jimenez-Bremont et al., 2022) and in a global manner (Dever and Ivanov, 2018),

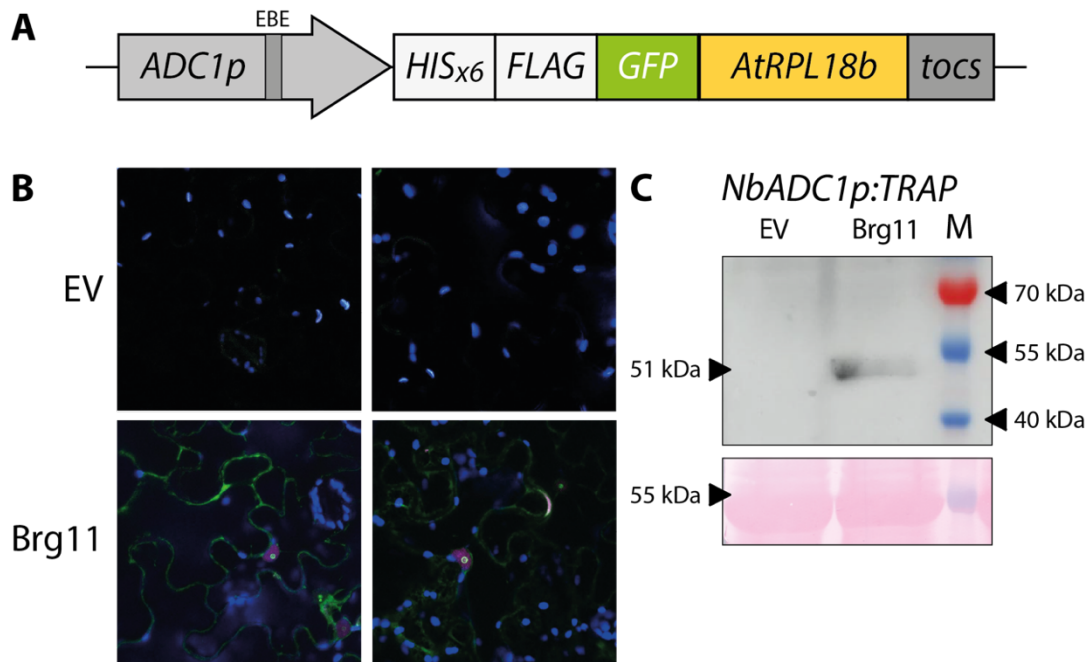


Figure 61: *NbADC1p:Hisx6-FLAG-GFP-AtRPL18b* construct produces weak GFP fluorescence with Brg11 co-expression. TRAP constructs consist of a histidine (His_{x6} ; light grey), FLAG (light grey), and GFP (green) tagged *A. thaliana* RLP18b (*AtRPL18b*) ribosomal subunit (yellow), driven by an *ADC1* minimal promoter (~350 bp) from either tomato or *N. benthamiana*, both containing the Brg11 EBE (A). The *NbADC1p*-driven TRAP construct was agroinfiltrated into WT *N. benthamiana* leaves with an RFP-tagged Brg11 or an empty vector control (EV). After 2 dpi, confocal microscopy was conducted. Chloroplast autofluorescence has been falsely coloured blue, while RFP-tagged Brg11 fluorescence was coloured magenta, and GFP coloured green (B). Western blot of samples transiently expressing *NbADC1p:TRAP* with either EV or RFP-tagged Brg11 using an anti GFP-HRP conjugated antibody (C). Expected TRAP construct size is 51 kDa.

understanding if and how Brg11 impacts the plant translome may further elucidate the impact of RipTAL-induced *ADC* expression during *R. solanacearum* infection.

A Brg11-inducible TRAP construct was previously generated by Dousheng Wu (Lahaye Lab, 2019) by introducing a Brg11-inducible promoter upstream of a previously generated TRAP construct that was used in tomato roots (Ron et al., 2014). This TRAP construct consists of the *A. thaliana* Ribosomal Protein L18b (*AtRPL18b*), a component of the large ribosomal subunit, that is N-terminally tagged with a histidine (x6) tag, FLAG tag, and a GFP (His_6 -FLAG-GFP-*AtRPL18b*) and localises to the nucleus and cytoplasm (Ron et al., 2014). This TRAP construct was cloned downstream of the *SLADC1* or *NbADC1* minimal promoters (~350 bp), both naturally containing the Brg11-*EBE* (Figure 61A). Here, the *SLADC1* minimal promoter-driven TRAP construct could be transformed into *N. benthamiana*, while the *NbADC1*-driven TRAP construct could be transformed into tomato. This would allow for each minimal promoter to be distinguished from the endogenous *ADC1* promoter, so that a dTALE targeting only the TRAP promoter could be designed and delivered by *R. solanacearum* strain lacking Brg11. Transcripts isolated from dTALE-activated TRAP cells would therefore act as a negative

control for transcriptional changes caused by *R. solanacearum* infection, but not Brg11 induction of host *ADCs*.

To test the functionality of these TRAP constructs, they were agroinfiltrated into WT *N. benthamiana* leaves in combination with either the RFP-tagged Brg11 or an EV control. Confocal microscopy was used to detect GFP fluorescence as a proxy for Brg11 activation of TRAP construct expression. Co-expression with Brg11-RFP produced weak GFP fluorescence in the cytoplasm, with slightly brighter nucleolar fluorescence (Figure 61B), possibly as ribosomes are assembled in the nucleolus (Fromont-Racine et al., 2003). Following repeated experiments, GFP fluorescence was typically observed most strongly in the nucleolus, faintly in the nucleus, and sometimes observed in the cytoplasm or endoplasmic reticulum, although no localisation markers were ever co-expressed with the TRAP constructs to verify these localisations. In addition to checking for TRAP expression via confocal microscopy, a western blot was also conducted to detect the epitope tagged AtRPL18 subunits. Here, an anti GFP-HRP conjugated antibody was used, and a faint band was detected at the expected size of 51 kDa only in the sample co-expressed with Brg11 (Figure 61C; Ron et al., 2014).

Below are sections describing the stable transformation of *bs4* tomatoes with the *NbADC1p:TRAP* construct (3.6.2.2.1) and WT *N. benthamiana* with the *SlADC1p:TRAP* construct (3.6.2.2.2). WT *A. thaliana* was also stably transformed via floral dipping with the *NbADC1p:TRAP* construct but were not analysed further in this work.

3.6.2.2.1 Selection of candidate tomato lines containing the Brg11-inducible TRAP construct.

The *NbADC1p:His_{x6}-FLAG-GFP-AtRPL18b* TRAP construct was transformed into the *bs4* mutant tomato background. Bs4 is a resistance protein found in tomatoes that recognises TALEs and dTALEs, but not RipTALs, and activates an HR-based immune response (Schenstnyi et al., 2022). Therefore, by using a *bs4* background tomato, dTALEs and TALEs can be used to activate transcription of the TRAP transgene or an additional endogene if ever required.

All plants that grew from cali on selective kanamycin media were transferred to soil in the greenhouse (section 2.2.1.4), and once established, samples were taken for gDNA extractions (section 2.2.3.5). The presence of the transgene was verified by PCR using ESR81 and ESR82 primers; 12 plants that lacked the transgene were discarded (from ~75 tested).

To identify candidate lines that specifically express the TRAP construct in a Brg11-dependent manner, *X. euvesicatoria* 85-10 strain carrying Brg11 was infiltrated into leaves of each transformant. Either an empty 85-10 or an 85-10 strain carrying Hpx17, a RipTAL lacking

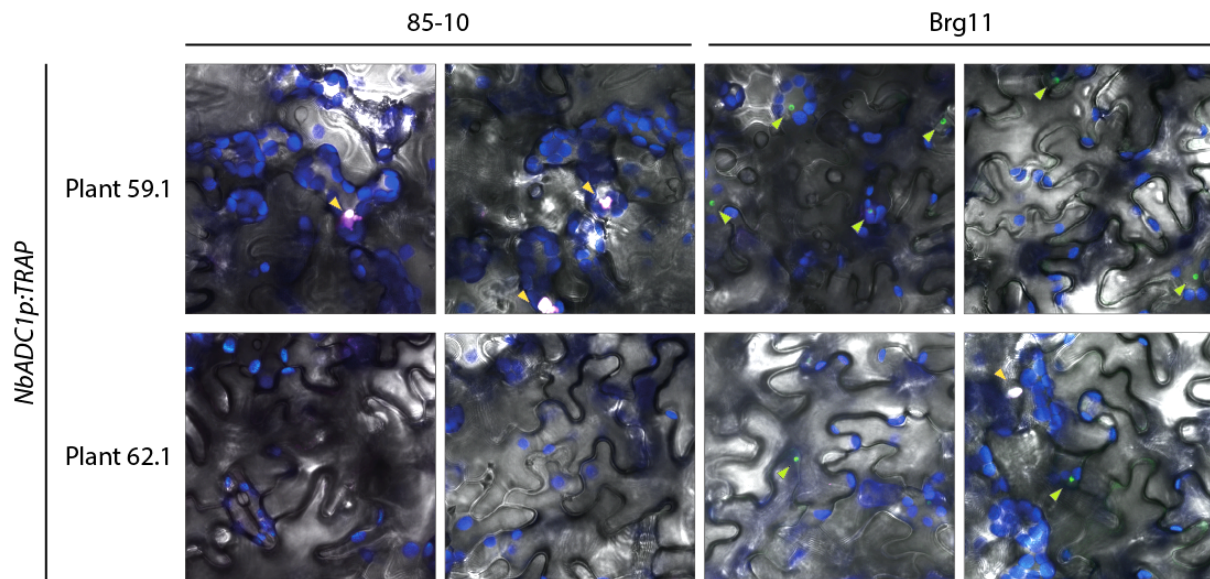


Figure 62: Expression of the *NbADC1p:TRAP* construct can be activated by *Xanthomonas* 85-10 delivery of Brg11 in transgenic tomato lines.

Transgenic tomatoes containing the *NbADC1p:TRAP* construct were infiltrated with an empty *Xanthomonas* 85-10 or a strain carrying Brg11 (modified to have the AvrBs3 T3SS signal). After 2 dpi, confocal microscopy was used to search for lines with GFP fluorescence only in samples infiltrated with Brg11; two such plants are shown here (59.1 and 62.1) with green arrows highlighting GFP-fluorescing nuclei. Images are merged autofluorescence (blue), GFP (green), and RFP channels. Orange arrows highlight highly autofluorescent moieties found in tomato leaves; this fluorescence is seen in all three channels.

most of the DNA binding domain (Mukaihara et al., 2004), was used as a negative control. Both RipTALs had the native T3SS signal replaced with that of AvrBs3, allowing them to be secreted by *X. euvesicatoria* (Wu et al., 2019). Confocal or epifluorescent microscopy was conducted to identify plants with GFP fluorescence only upon Brg11-delivery. Two plants (59.1 and 62.1) showed weak GFP fluorescence, appearing to localise to the nucleolus of only following delivery of Brg11, but not in the negative controls (Figure 62). This GFP fluorescence was very weak and no cytoplasmic GFP was observed, unlike the transiently expressed *NbADC1p:TRAP* construct (Figure 61).

In many tomato leaves, including WT, non-infiltrated leaves, bright autofluorescent moieties were often seen in CFP, GFP, and RFP channels (orange arrows; Figure 62). However, they are much larger than nuclei, not uniformly round, and sometimes move within the cell. These puncti can be distinguished from the GFP-fluorescing nuclei with a high enough magnification. Overall, lines 59.1 and 62.1 were identified as potential Brg11-inducible TRAP tomato lines; however, due to the weak GFP fluorescence, *N. benthamiana* was selected as an alternative plant species for transformation.

3.6.2.2.2 Selection of candidate *N. benthamiana* lines containing the *Brg11*-inducible *TRAP* construct.

The *SLADC1_p:His_{x6}-FLAG-GFP-AtRPL18b* TRAP construct was stably transformed into WT *N. benthamiana* plants. Screening for a good candidate line was hypothesised to be easier in this species as *A. tumefaciens* T-DNA delivery into *N. benthamiana* produces more effector protein than T3SS delivery of effector proteins via *Xanthomonas*, possibly increasing transgene activation. Additionally, *N. benthamiana* has less autofluorescence than tomato, enabling visualisation of even poorly expressed fluorophores (i.e., TRAP GFP) by confocal microscopy. Again, plantlets from cali, grown from kanamycin selective media were transferred to soil (section 2.2.1.4) and grown in the greenhouse and gDNA samples were taken (section 2.2.3.5) for PCR-based detection of the transgene using primers ESR81 and ESR82. Of the 59 transformant plants, 51 had a positive PCR band. To identify candidate lines that selectively express the TRAP transgene in the presence of Brg11, the *A. tumefaciens* strain carrying the RFP-tagged Brg11 was infiltrated into each of these 51 lines. Infiltrated and non-infiltrated

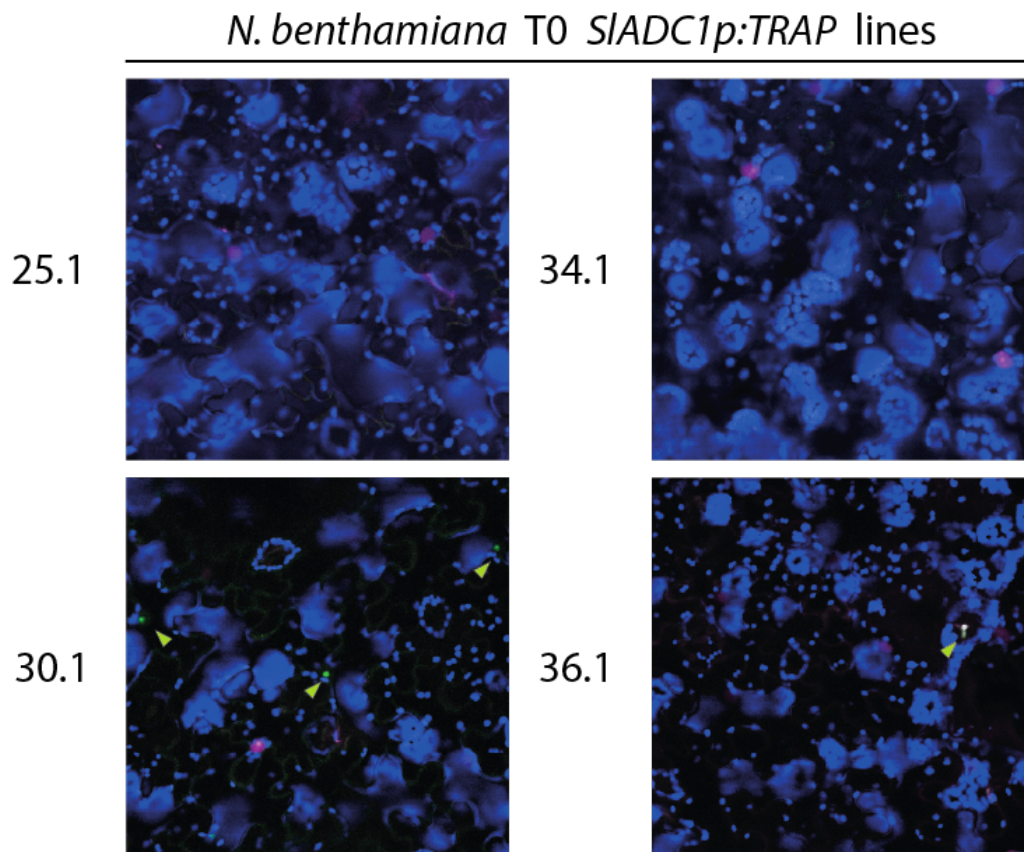


Figure 63: Expression of the *SLADC1_p:TRAP* construct can be activated by *A. tumefaciens* delivery of Brg11 in transgenic *N. benthamiana* lines.

Transgenic *N. benthamiana* containing the *SLADC1_p:TRAP* construct were infiltrated with *A. tumefaciens* carrying an RFP-tagged Brg11. After 2 dpi, confocal microscopy was used to search for lines with GFP fluorescence; indicated by green arrows. Images are merged autofluorescence (blue), GFP (green), and RFP channels.

samples of each line were taken for confocal or epifluorescent microscopy after 2 dpi to detect GFP fluorescence.

No line was identified that showed as bright GFP fluorescence as shown in Figure 61. However, lines 30.1 and 36.1 showed some nucleolar-localised GFP fluorescence that resembled what was also seen in the tomato *NbADC1p:TRAP* lines 59.1 and 62.1 (Figure 63). Additionally, lines 1.1, 1.2, and 3.1 showed very faint nucleolar fluorescence using the GFP filter on the epifluorescent microscope (data not shown). Western blots were also carried out on Brg11-infiltrated samples from some *N. benthamiana* lines to detect Brg11-induced TRAP construct expression, but no bands were ever detected at the expected size of 51 kDa (data not shown). Overall, the weak GFP fluorescence from the *ADC1p:TRAP* constructs meant that detection of a well-expressed transgene in the presence of Brg11 was challenging, resulting in only a couple of candidate tomato and *N. benthamiana* lines.

3.6.3 Deciphering the impact of ADC over expression in bulk root tissue

To understand the metabolic and microbiome changes that occur following Brg11-induced ADC over expression, it is not feasible to study only the root area injected by Brg11 during *R. solanacearum* infection or to harvest bulk infected tissue due to the dilution effect of RipTAL-receiving cells compared to all other root cells (described in section 1.5.4). Instead, over expression of ADCs in all root cells would enable harvesting of bulk root tissue for either metabolomic or microbiome analyses. Two approaches have been undertaken to achieve this and are presented in the sections below. Firstly, using *adc-box* mutants, which have increased translation of ADC protein in all cells (section 3.6.3.1), and secondly, to express Brg11 in all cells via an inducible promoter (section 3.6.3.2).

3.6.3.1 Tomato *adc-box* mutants as a tool for studying ADC over expression in roots.

During this work, *adc-box* mutants were found to have higher levels of ADC activity, some PAs, and related metabolites due to the lack of translational repression, normally exerted by the *ADC-box* RNA secondary structure; this was seen in tomato, *A. thaliana*, and *M. polymorpha* *adc-box* mutants (sections 3.3.1, 3.2.11, and 3.3.2, respectively). Many of these metabolomic experiments were conducted using cold-treated plants, as transcription of *ADC* genes are often stimulated by abiotic stressors, boosting PA levels into a range that can be easily measured by LCMS (section 3.2). This transcriptional induction of *ADCs*, in combination with the lack of translational regulation in the *adc-box* mutants, somewhat mimics the action of Brg11, as Brg11 induces transcription of host *ADCs*, but with a shorter 5'UTR lacking the *ADC-box*, thereby increasing both transcription and translation of *ADCs*. In both

cases, either Brg11-induction of *ADCs* or cold-treated *adc-box* mutants, there is transcription induction of *ADC* and a lack of translational repression by the *ADC-box*, leading to overexpression of *ADCs*. Therefore, the *adc-box* mutants can be used as a proxy tool for studying the downstream metabolic impacts of Brg11.

To confirm this line of thought and to investigate if *ADC* activity is also higher in the roots of tomato *adc-box* mutants compared to WT, a preliminary experiment was conducted. Seeds of both WT and the *sladc2-box* mutant (SI2M₋₁₃) were sterilised and then grown vertically on

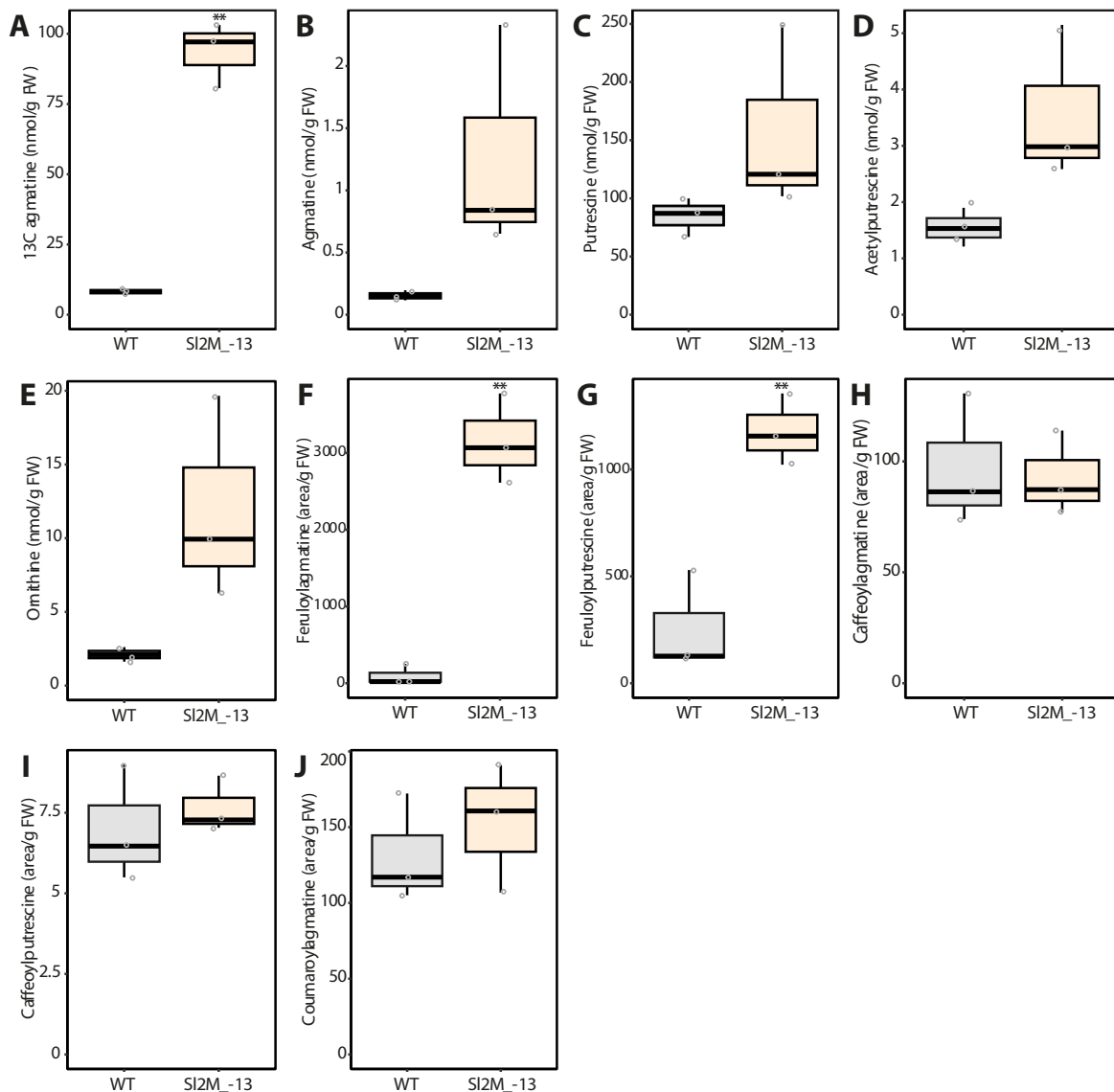


Figure 64: Cold treated *adc2-box* tomato roots have increased levels of *ADC* activity and some phenolamides.

WT (grey) and *adc2-box* mutant (SI2M₋₁₃; yellow) tomato seeds were sterilised, grow vertically for a week before being cold treated for 24 hours. The roots of 3-4 seedlings were combined to form one replicate sample (n = 3, total ~40 mg FW), which were then processed for LCMS measurement of *ADC* activity (A), and levels of agmatine (B), putrescine (C), acetylputrescine (D), and ornithine (E) in nmol/g fresh weight (FW), normalised to the internal standard D5 tryptophan. The phenolamides feruloyl agmatine (F), feruloyl putrescine (G), caffeoyl agmatine (H), caffeoyl putrescine (I), and coumaroyl agmatine (J) were also measured and reported as area per g FW, normalised to D₅ tryptophan.

agar plates for one week (section 2.1.3), before being cold treated for 24 hours. The plates were then scanned, and the roots cut off each seedling for LCMS analysis of ADC activity and PA measurement. There was no apparent phenotypic difference between the WT and SI2M₋₁₃ seedling roots (Supplementary Figure 41A), nor any statistically significant difference found in the root lengths or weights (P -values = 0.15 and 0.83, respectively, using a Welch T-test; Supplementary Figure 41B and C).

ADC activity of cold-treated SI2M₋₁₃ seedling roots was 11.4-fold higher than that of the WT (P -value = 0.006 using a Welch T-test; Figure 64A), which is substantially greater than the fold changes observed from cold-treated adult SI2M₋₁₃ tomato leaves of 3.8-fold (Figure 31). Agmatine, putrescine, acetylputrescine, and ornithine levels were also higher in the SI2M₋₁₃ roots compared to WT, but these differences were not statistically significant (Figure 64B, C, D, and E). Arginine and spermidine levels were slightly lower in the SI2M₋₁₃ mutant, but this was also not significant (Supplementary Figure 42). Interestingly, levels of feruloylagmatine were 32.4-fold higher in SI2M₋₁₃ compared to WT (P -value = 0.009 using a Welch T-test; Figure 64F), while feruloylputrescine levels were increased 4.6-fold (P -value = 0.007; Figure 64G). No significant differences in the levels of caffeoylagmatine, caffeoylputrescine, or coumaroylagmatine between WT and SI2M₋₁₃ were observed (Figure 64H, I, and J).

These results show that not only does the *adc2-box* mutant SI2M₋₁₃ have higher ADC activity compared to WT in seedling root tissue, but that the difference is considerably greater than in adult leaves. Additionally, the dramatically higher levels of feruloylagmatine and feruloylputrescine indicate that overexpression of ADCs in root tissue may have a more pronounced impact on PA metabolism compared in adult leaf tissue. Therefore, further analysis of PA metabolism in root tissue from *adc-box* mutant seedlings or older plants may provide valuable insight into the potential metabolic impacts of Brg11 in root tissue.

3.6.3.2 Tomato lines with an ethanol-inducible Brg11-GFP to induce ADC over expression in all root cells.

In addition to the *adc-box* mutants, another approach was implemented to investigate how overexpression of ADCs may contribute to *R. solanacearum* infection. Here, a GFP-tagged Brg11 was put under the control of an ethanol inducible expression system, so that upon activation with ethanol, all cells in the plant would express Brg11, and subsequently overexpress ADCs. This system could be used to study the metabolic changes caused by Brg11

induction of ADCs from harvesting bulk root tissue, as well as studying how these metabolic changes alter the root microbiome.

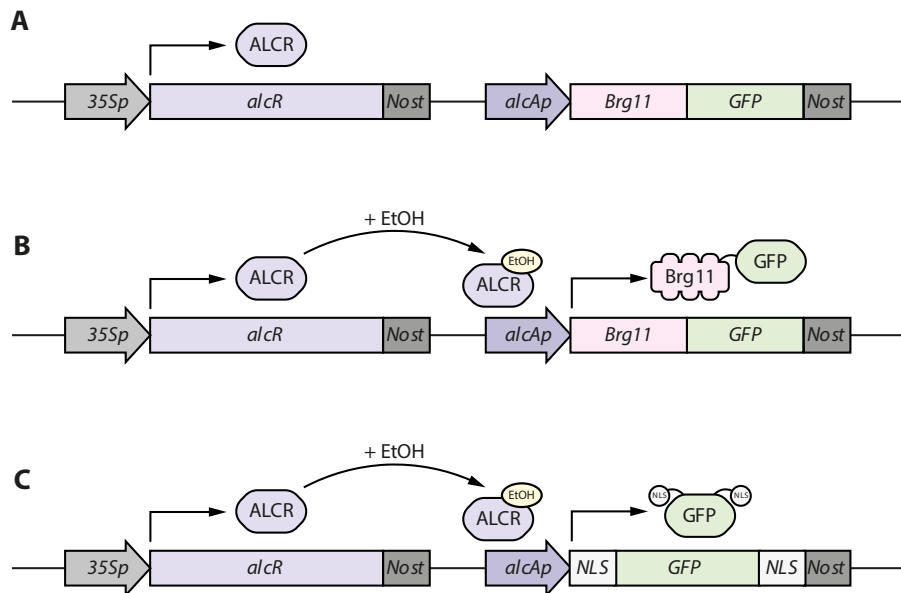


Figure 65: Schematic of ethanol inducible promoter system used to activate transcription of GFP-tagged Brg11 and the nuclear-localised GFP control construct.

Constructs contain a constitutively expressed ALCR transcription factor (purple) and the *alcA* promoter (purple) driving the gene of interest; here, Brg11 (pink) tagged with a GFP (green; **A**). Following ethanol treatment, ALCR binds ethanol and activates transcription from the *alcA* promoter and expressing the gene of interest (**B**). As a negative control, a GFP with two nuclear localised signals (NLS, grey) was also put under the control of this ethanol inducible system (**C**).

The ethanol inducible system relies on a constitutively expressed fungal transcription factor ALCR, which is responsible for activating alcohol metabolising enzymes, such as alcohol dehydrogenase I, encoded by the gene *alcA* (Li et al., 2005). ALCR is only active once it binds to ethanol, after which it binds to the *alcA* promoter and activates transcription. This system has been developed into an inducible system to activate transcription of a gene of interest by cloning it downstream of the *alcA* promoter. Here, a GFP-tagged Brg11 was put under the control of the *alcA* promoter, so that upon binding ethanol, ALCR will initiate its transcription (Figure 65A and B). As a negative control, a GFP with two nuclear localisation signals (NLS) was also put under the control of this inducible expression system (Figure 65C). Upon activation by ethanol, the NLS-GFP-NLS will be expressed and targeted to the nucleus like Brg11-GFP but will not activate expression of *ADC* genes. These constructs were generated by Robert Morbitzer (Lahaye laboratory).

To test the functionality of the two ethanol-inducible constructs, they were agroinfiltrated into WT *N. benthamiana* leaves and after 24 hours, the plants were either watered with 5% ethanol or water, or alternatively had 2% or 5% ethanol infiltrated into the previously agroinfiltrated

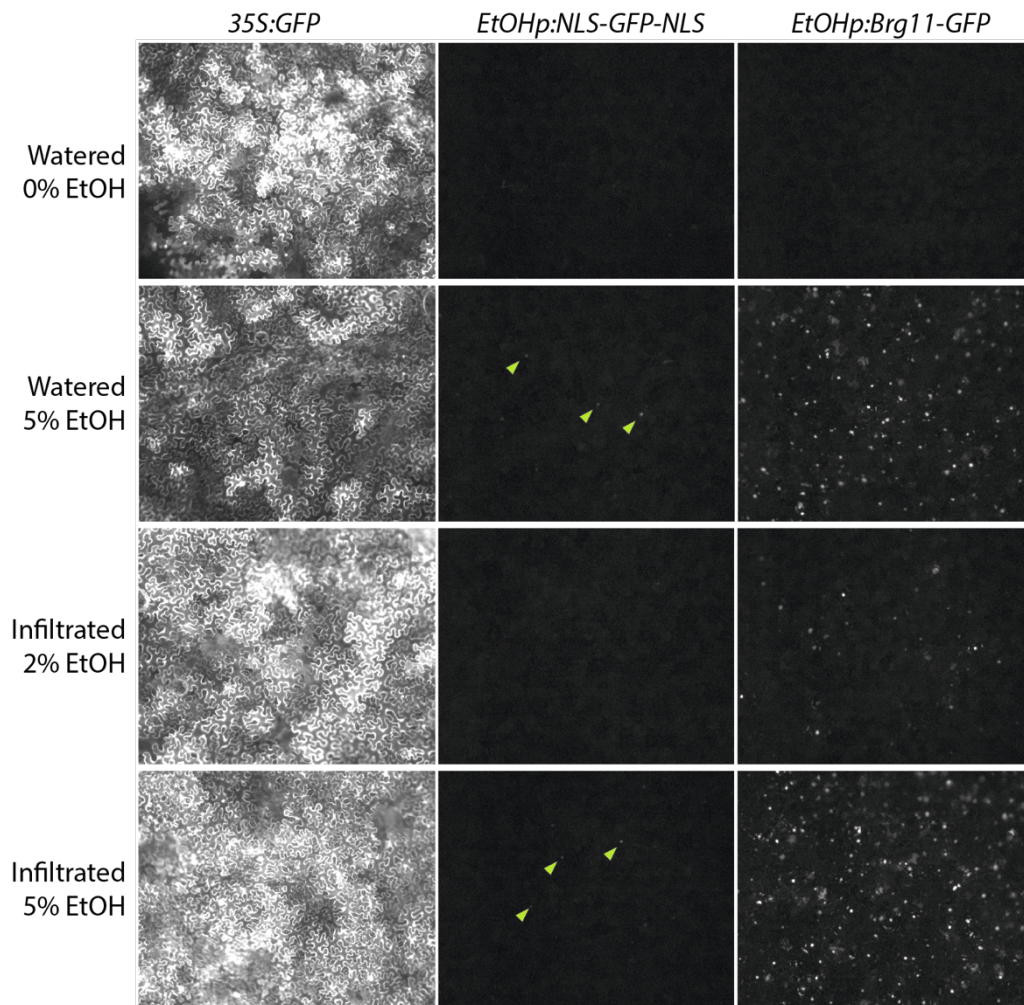


Figure 66: Transient transformation of the ethanol inducible Brg11-GFP and NLS-GFP-NLS constructs show nuclear localised GFP fluorescence only with ethanol treatment.

The ethanol inducible Brg11-GFP and NLS-GFP-NLS constructs were agroinfiltrated into WT *N. benthamiana* plants; a constitutively expressed GFP (*35S::GFP*) was also agroinfiltrated as a positive control for fluorescence. 24 hours later, these plants were either watered with 0% or 5% ethanol, or leaves were infiltrated with 2% or 5% ethanol. After another 24 hours, epifluorescent microscopy was used to detect GFP fluorescence using the GFP filter. Faint nuclear GFP fluorescence has been highlighted with green arrows.

leaf areas. As a control for GFP fluorescence, a *35S::GFP* reporter was also agroinfiltrated and treated with ethanol in the same way. After another 24 hours, GFP fluorescence was visualised using the epifluorescent microscope. Nuclear localised fluorescence could be seen in samples agroinfiltrated with the Brg11-GFP ethanol-inducible construct following watering with 5% ethanol, but not when watered only with water (Figure 66). Similar levels of GFP fluorescence were observed from the Brg11-GFP construct following infiltration of 5% ethanol but were slightly lower following infiltration with 2% ethanol. This was expected as the ethanol inducible system has been reported to be activated in a dose-dependent manner (Li et al., 2005). Nuclear localised GFP fluorescence from the NLS-GFP-NLS construct was also observed following watering or infiltration with 5% ethanol, but at a much lower level compared to the

Brg11-GFP construct. Very little to no GFP fluorescence was observed from the NLS-GFP-NLS construct following infiltration of 2% ethanol and no GFP fluorescence when watered with only water (Figure 66). These results indicate that activation of the Brg11-GFP inducible construct can be achieved by both watering and infiltrating with ethanol, while no observable activation occurs in the absence of ethanol. As fluorescence from the NLS-GFP-NLS control construct was observed, although much lower than that of the Brg11-GFP construct, this construct was also considered functional. Without detecting ALCR protein levels, the cause of the lower GFP fluorescence from the NLS-GFP-NLS control cannot be exactly determined.

Following these tests, both the Brg11-GFP and NLS-GFP-NLS ethanol-inducible constructs were stably transformed into *bs4* tomatoes, allowing for the use of TALEs or dTALEs in these plants if required. Plantlets that grew from calli on kanamycin containing media were transferred to soil and grown in the greenhouse until established enough to take samples for gDNA extractions. All plants were genotyped using the primers ESR81 and ESR82, amplifying part of the GFP on both constructs; any plants not producing a positive PCR band were discarded.

To identify lines which expressed the Brg11-GFP or NLS-GFP-NLS constructs only following ethanol treatment, genotyped lines were infiltrated with 5% ethanol and 24 hours later, samples were taken for confocal microscopy. However, no clear, differential GFP fluorescence was observed from samples treated with ethanol compared to their non-treated controls. Western blots were also conducted on samples from ethanol-infiltrated lines, but no bands were detected at molecular weights corresponding to Brg11-GFP or NLS-GFP-NLS using an anti-GFP antibody (data not shown).

An alternative method to confocal microscopy or protein detection via western blotting was therefore required to identify candidate lines that specifically express the Brg11-GFP or NLS-GFP-NLS constructs upon ethanol treatment. As Brg11 induces ADC transcription, and consequently induces ADC activity, an LCMS-based approach was employed to identify such Brg11-GFP-expressing candidate lines. Here, any plant that produced higher ADC activity following ethanol treatment compared to before ethanol treatment would be considered a good candidate line. Unfortunately, such an LCMS-based assay was not suitable to detect expression of NLS-GFP-NLS expression, and therefore, while a selection of plants was kept for seed production, no candidate line was identified within this work. It was thought that WT plants treated with ethanol would be a sufficient negative control instead of the NLS-GFP-NLS lines for future experiments.

An experimental workflow was planned to generate ethanol-induced replicate samples for ADC activity measurements from 46 transformants and one WT plant, alongside both positive and negative controls (Figure 67A). As a positive control for induced ADC activity, *Xanthomonas* 85-10 carrying Brg11 was infiltrated into one leaf of each plant. After 24 hours, a ~25 mg sample was taken from each of these infiltrated areas, as well as samples from non-infiltrated areas as negative controls for ADC induction. After these two samples were taken, all plants were watered with the same volume of 5% ethanol. After another 24 hours, 4 ~25 mg replicate leaf samples were taken from different adult leaves of each plant.

To avoid extracting and running all 282 samples at once, two experiments were conducted. The first experiment ran the positive and negative control samples from the WT control, alongside two ethanol-treated replicates from the WT and each of the 46 lines. ADC activity of the WT was highly induced following infiltration of *Xanthomonas* containing Brg11 compared to the non-treated control, while ADC activity of the ethanol-treated samples was similar to the non-treated control (initial data not shown). This indicated that the assay and experimental set up were functioning as expected, with no ADC activity induction occurring in the WT plant following ethanol treatment. ADC activity of the two ethanol-treated replicates from each transformant were then compared to the WT ethanol-treated control. Any transformant line that showed induced ADC activity were selected as a candidate line to be tested in the second experiment. In the second experiment, all 4 ethanol-treated replicates and the positive and negative controls from these 11 selected lines were run on the LCMS to determine their ADC activity and levels of some associated metabolites. The two additional ethanol-treated WT replicates were also included in this assay, and still the ADC activity of the WT control was similar to the non-treated control (Figure 67B). Specifically, the average ADC activity of the ethanol-treated WT samples was only 1.8-fold higher than the non-treated sample, while *Xanthomonas* delivery of Brg11 produced ADC activity levels 46-fold higher than the non-treated control. This indicates that watering with 5% ethanol does not induce ADC activity alone.

Plants such as 32.1, 40.1, 43.1, 46.1, 48.1, 50.1, 55.1, and 76.1 had much higher ADC activity following ethanol-treatment compared to the ethanol treated WT, while lines 53.1, 64.1, and 74.1 showed only minimal ADC induction with ethanol treatment (Figure 67B). Of the lines with induced ADC activity following ethanol-treatment, lines 55.1, 48.1, and the 32.1 showed the highest ADC activity with (in descending order), with the ethanol-treated samples from line 55.1 having similar ADC activity levels to its *Xanthomonas* Brg11-infiltrated positive control. While line 48.1 had the second highest ethanol-induced ADC activity, the non-treated control

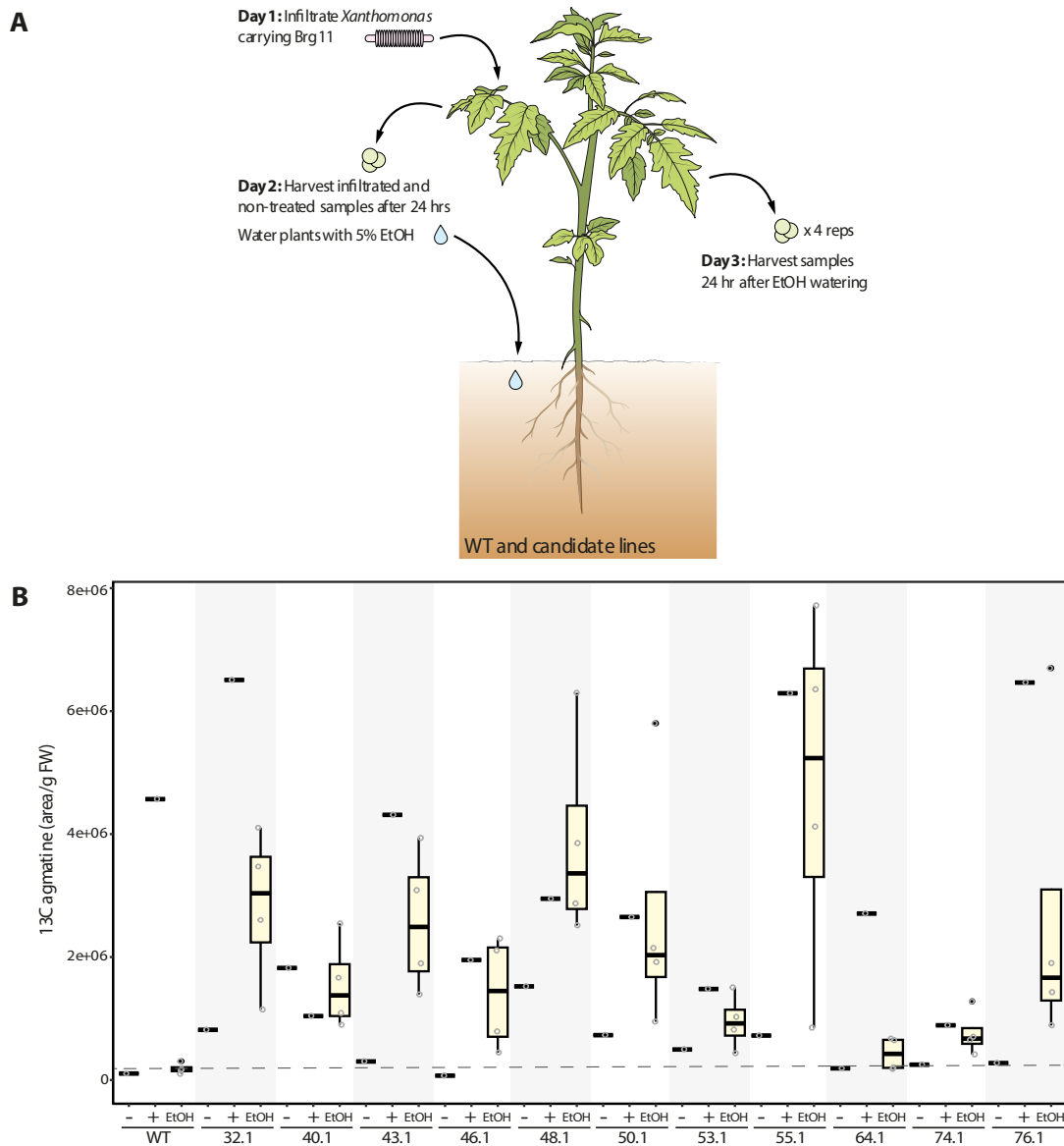


Figure 67: An LCMS ADC activity assay approach was used to identify candidate ethanol-inducible Brg11-GFP tomato lines. (A) WT and 46 stably transformed lines containing the ethanol-inducible Brg11-GFP construct were infiltrated with *Xanthomonas* 85-10 carrying Brg11 (with AvrBs3 T3SS signal). After 24 hours, one sample per plant was taken from this infiltrated area as a positive control for induced ADC activity and a non-treated sample as a negative control for induced ADC activity. All plants were then watered with 5% ethanol. After a following 24 hours, 4 replicate samples were taken from leaf tissue of all plants. (B) ADC activity of positive (+) and negative (-) controls, and ethanol-treated samples (EtOH) from WT and 11 selected lines was measured via LCMS. The dotted horizontal line represents the median ADC activity of the ethanol-treated WT.

also had the highest ADC activity of all non-treated control samples and was 14-fold higher than the WT non-treated sample. Although this was only a single replicate, this may suggest that the transgene is leaky and expressing Brg11 in the absence of ethanol treatment. Therefore, the top candidate lines from this experiment were selected based on high ADC activity following ethanol treatment (preferably in a similar range to the Brg11-treated sample) and low

ADC activity levels in the non-treated control; from this criteria, lines 32.1, 43.1, 46.1, 50.1, 55.1, and 76.1 qualified, with lines 32.1, 43.1, and 55.1 being the most promising.

In addition to measuring ADC activity, agmatine and putrescine levels were measured. Lines 32.1, 40.1, 43.1, 50.1, and 55.1 had higher agmatine levels compared to the ethanol-treated WT samples and their respective non-treated control samples (Supplementary Figure 43A). Putrescine levels of the ethanol treated samples from lines 32.1, 40.1, 50.1, and 55.1 were higher than the ethanol-treated WT samples, but of these lines, only 32.1, 40.1, and 50.1 had putrescine levels higher than their respective non-treated controls (Supplementary Figure 43B). These results suggest that 24 hours after watering with 5% ethanol, expression of Brg11-GFP can be activated, leading to higher levels of ADC activity, agmatine, and putrescine.

Previous LCMS experiments showed that over expression of ADC can produce higher levels of some phenolamides (Figure 64). Therefore, in the same LCMS experiment measuring ADC activity, agmatine, and putrescine levels of the ethanol-inducible Brg11-GFP lines, some phenolamides were also detected. While all positive control samples, infiltrated with *Xanthomonas* carrying Brg11, had higher feruloylagmatine levels compared to the negative control, only line 50.1 had higher feruloylagmatine levels following ethanol treatment (Supplementary Figure 44A). However, coumaroylagmatine levels of almost all selected lines following ethanol treatment were higher than the ethanol treated WT, as well as their respective negative controls (Supplementary Figure 44B).

Overall, some candidate ethanol-inducible Brg11-GFP tomato lines were successfully identified using the LCMS-based screening method. While additional experiments with more replicates need to be conducted on future generations of these lines, the results presented here indicate that ADC activity, agmatine, putrescine, and phenolamide levels can be induced by ethanol activation of Brg11-GFP expression. Therefore, these lines can be used to study the impacts of overexpressing ADC by Brg11 on the tomato root metabolism and the microbiome.

4. Discussion

This work aimed to understand how plant ADCs are translationally regulated by highly conserved 5'UTR elements, primarily the *ADC-box*, as well as the uORF. To do this, these elements were mutated by CRISPR/Cas9 mutagenesis in tomato, *A. thaliana*, and *M. polymorpha* (section 3.1). However, to study how these mutations influenced ADC protein levels, the previously published LCMS-based ADC activity assay (Wu et al., 2019) required optimisation (section 3.2). This optimised method was then used to determine the impact of the CRISPR/Cas9-induced mutations of the tomato and *M. polymorpha* *adc-box* and tomato *uorf* mutants on ADC activity and the greater PA metabolic network. Additionally, the impact of these mutations on plant phenotypes was also investigated (section 3.3). The ADC activity and PA network of the previously generated tomato *adc* coding sequence mutants (Wu et al., 2019) were also analysed (section 3.4). To understand how the *ADC-box* may function to regulate ADC translation, a dual luciferase reporter system and various ADC protein variants were generated, as well as elucidation of the *ADC-box* RNA secondary structures by SHAPE analysis (section 3.5). Finally, to investigate how *R. solanacearum* may benefit from RipTAL-induced ADC overexpression, various new approaches were developed during this work (section 3.6). The results of these sections are discussed below.

4.1 Optimisation of ADC activity assay and measurement of PAs levels using LCMS

4.1.1 *The LCMS-based ADC activity assay was improved ~600-fold during this work as well as measurement of PAs and related metabolites*

To efficiently study the role of *ADC* 5'UTR conserved elements and how Brg11-induced ADC overexpression contributes to *R. solanacearum* infection, the LCMS-based method to measure ADC activity and levels of PAs and related metabolites required optimisation. Initial LCMS experiments conducted on un-treated tomato leaves following the method published by Wu et al., 2019 found that ADC activity and many PAs were below the measurable threshold. During this work, the following changes to the Wu et al., 2019 method were implemented (section 3.2): incubation of heavy arginine with whole plant extracts, rather than just the supernatant; use of internal standards (i.e., D₅ tryptophan and ¹³C₁ D₂ citrulline); 10-fold higher concentration of assay substrates (Figure 20); induction of *ADC* transcription via a stress

treatment (i.e., cold; Figure 18); reduction of assay duration (Figure 21); addition of heavy ornithine for concurrent measurement of ODC activity (Figure 39); and, development of the LCMS protocol to enable measurement of more PA-related metabolites (i.e., Wu et al., 2019 measured 5 metabolites, which was increased to 11 here; Supplementary Table 3), as well as relative levels of multiple phenolamides. Overall, these improvements resulted in an ~600-fold increase in ADC activity from cold treated WT tomato leaves compared to the initial measurements of non-treated leaves (section 3.2). Using this method, ADC activity and PA levels were also successfully measured in leaves of plant species other than tomato (i.e., *A. thaliana*, *M. polymorpha*, and *N. benthamiana*), as well as roots of tomato seedlings, demonstrating the robustness of this method in other species and tissues.

Overall, optimising this LCMS method meant that smaller differences in ADC activity and metabolite levels could be measured, enabling the effects of different *adc-box* mutations to be distinguished. Additionally, this method provided a more complete picture of PA profile and subsequent conjugation to HCAs, providing insight into how overexpression of ADC, either via transcriptional activation from Brg11 or removal of translational control in *adc-box* mutations, impacts PA metabolism and downstream pathways.

4.1.2 *How does this optimised method compare with other ADC activity and PA measuring methods currently used in the field?*

To the best of our knowledge, no publications other than Wu et al., 2019 and Gallas et al., 2023 (both from Lahaye laboratory) simultaneously measure ADC enzyme activity alongside levels of related metabolites. Many others measure multiple arginine- or PA-related metabolites using LCMS-based methods, but do not measure enzyme activity within the same sample (Amatya et al., 2020; Shen et al., 2000; Tassoni et al., 2000; Alcázar et al., 2005; Urano et al., 2005; Liu and Moriguchi, 2007; Cuevas et al., 2008; Naka et al., 2010; Zhang et al., 2014; Rossi et al., 2015; Espasandin et al., 2018; Ding et al., 2021; Upadhyay et al., 2021; Song et al., 2023; Gonzalez-Hernandez et al., 2022a; Zhang et al., 2023). In our optimised LCMS-based ADC activity and PA measurement assay, the use of heavy isotope substrates adds valuable information on enzyme catalytic rates, in addition to measurement of exact *in planta* metabolite concentrations. In the past, ADC activity in plant extracts has often been measured using radioactive arginine substrates and subsequent the release of radioactive CO₂ produced during decarboxylation (Lee, 1997; Shen et al., 2000; Ziosi et al., 2003; Espasandin et al., 2014; Espasandin et al., 2018; Jang et al., 2012; Alcazar and Tiburcio, 2018) or by using an ELISA-based ADC activity assay (Song et al., 2023). While both LCMS and use of such radioactive

chemicals require specific training and facilities, heavy stable isotopes, such as those used in our method, are safer alternatives and can be used in normal laboratory conditions or in the greenhouse for pre-treatment of plants prior to sampling for LCMS (i.e., pre-feeding). Additionally, multiple different stable isotopes can be added to plant extracts for simultaneous measurement of multiple enzyme activities (i.e., heavy arginine and ornithine substrates for ADC and ODC activities, respectively). Measurement of multiple decarboxylase enzyme activities is not possible using the radioactive isotope method, as regardless of the radioactively labelled substrate, all decarboxylase enzymes will produce the same radioactively labelled CO₂ moiety.

Another benefit of using stable isotopes is that tracing of heavy moieties is not just limited to the enzyme of interest but can also be traced through the enzymatic pathway. For example, heavy putrescine was generated from pre-feeding of heavy arginine (Figure 18) and preliminary experiments (not presented here) have indicated that heavy isotopic variants of phenolamides could also be detected (e.g., heavy feruloylagmatine). Using this type of experiment, tracking heavy moieties is often used in untargeted metabolomics to identify pathway intermediates or to follow metabolite pathway flux; this has also recently been used to study lysine decarboxylase activity in *A. thaliana* (Shimizu et al., 2019). Therefore, while ADC activity was the focus of this work, it may also be possible to use heavy arginine isotope variants to track the flow on effect of increased ADC activity (either due to *adc-box* mutations or induction by Brg11) on the wider PA and phenolamide pathway.

Overall, using isotopic substrate variants as ADC and ODC substrates, and subsequent measurement of their isotopic products using LCMS enables measurement of enzyme activity in a biologically relevant context. Within the same LCMS sample, measurement of the *in planta* concentration of multiple PA-related metabolites means that this LCMS method is incredibly informative and a step above many other published methods.

4.1.3 Additional clean up step enables LCMS measurement of ADC activity, PAs, and PA-related metabolites from transiently transformed *N. benthamiana* leaves.

For some species, such as *N. benthamiana*, the previous Wu et al., 2019 extraction protocol and buffer resulted in compounds that clogged the LCMS TRAP column. However, with an additional phase separation step to isolate polar metabolites (i.e., PAs) from these compounds, transiently transformed *N. benthamiana* samples could then be used for ADC and ODC activity assays and measurement of PAs (section 3.2.12). LCMS of transiently transformed *N. benthamiana* leaves opens the door for PA-related enzyme activities to be verified or compared

in an *in planta* environment in a relatively a simple manner. In this work, the expected catalytic activities of epitope-tagged WT and catalytically non-functional SIADC1/2 mutant protein constructs were confirmed using this method (section 3.5.2.5; Figure 46). As the key catalytic residues of plant ADCs had only been verified for *A. thaliana* ADC proteins (Hanfrey et al., 2001), measurement of these WT and mutant SIADC1/2 variant activities confirmed that mutating one or both key residues in tomato ADCs prevented any higher catalytic activity compared to the negative control (Figure 46). Interestingly, mutating C524 in AtADC1 and the corresponding C360 in mouse ODC did not totally prevent enzyme activities, despite this residue being important for substrate binding (Hanfrey et al., 2001). However, in this work, no differences in the ADC activities and PA measurements between the SIADC1 or SIADC2 single mutants were found (Figure 46), indicating that this cystine residue is essential for tomato ADC activity. Therefore, transient expression of other enzyme variants in *N. benthamiana* enables investigation of other important residues, without the need for recombinant expression in *E. coli*, protein purification, and *in vitro* enzymatic analysis, such as the work done by Guo et al., 2023 to investigate SIADC1/2 activities.

In addition to ADC activity, measurements of the wider PA network in transiently transformed *N. benthamiana* leaves demonstrated that expression of WT SIADC1/2 caused increases in agmatine, putrescine, ornithine, citrulline, acetylputrescine, and various phenolamide levels, as well as reduction in arginine and spermidine levels (Figure 46, Supplementary Figure 28, Supplementary Figure 29). However, mutating either or both catalytically essential SIADC1/2 residues did not alter any of these metabolite levels compared to the negative GUS control, indicating that such catalytically non-functional enzymes do not influence the downstream PA network. Without optimisation of the *N. benthamiana* sample preparation for LCMS measurement of ADC activity and PA-related metabolites, obtaining this valuable information would not have been possible.

This method could be further extended to ADC proteins from other species, other PA pathway enzymes, or to possibly study additional or alternative enzyme activities of ADCs. For example, AtADC1 has been reported to have evolved a new catalytic function of decarboxylating N^δ-acetylornithine to N-acetylputrescine, so that with NATA1, the loss of ODC has been compensated for (Lou et al., 2020). Using this optimised *N. benthamiana* extraction protocol it would be possible to investigate AtADC1 catalytic activities (as both an ADC and an N^δ-acetylornithine decarboxylase) and compare these to other ADC1 proteins from other species. Here, both arginine and N^δ-acetylornithine heavy isotopic variants could be added and the production of their isotopic products measured. One would be expected that

only ADC1 homologs from Brassicaceae that have lost their ODC would have this additional N²-acetylornithine decarboxylase activity, while ADCs from species containing an ODC would exclusively have ADC activity. Through using transient transformation of *N. benthamiana*, all ADC1 enzymes can be equally compared, with the same expression level and in the same *in planta* background, removing the need for optimising the LCMS sample extraction protocol for each species of interest.

Overall, addition of a simple chloroform-methanol-based extraction step has opened the experimental doors for studying ADC and other PA-related enzyme activities and their impacts on the whole PA pathway in a relatively fast and reliable manner using transient transformation of *N. benthamiana*.

4.1.4 Remaining limitations of the LCMS-based ADC activity assay and measurement of PA-related metabolites.

Despite the vast improvements made to the LCMS-based ADC activity assay and measurement of PA-related metabolites, some limitations of this method remain. Firstly, verification that ADC activity and ADC protein levels directly correlate has not been done. Attempts to directly detect and quantify ADC proteins from plant extracts (i.e., from tomato leaf tissue or transient overexpression of SlADC proteins in *N. benthamiana*) using MS have so far been unsuccessful. However, due to the additional methanol-chloroform extraction step, transiently transformed *N. benthamiana* leaf material can now be used for ADC activity assays (section 3.2.12). Therefore, constitutively expressed epitope-tagged ADC proteins can be agroinfiltrated into *N. benthamiana* leaves in a range of inoculum levels (e.g., OD₆₀₀ from 0.01 to 1.0) and an ADC activity assay could be conducted to show that increasing ADC activity correlates with increasing ADC protein levels (shown via immunoblotting).

The second limitation of this method is that there is still high variation between technical replicates for some metabolites. This could be reduced further by using a higher sample size than 6 biological replicates, conducting an additional clean up step (i.e., methanol-chloroform extraction on samples from other tissue types), or by altering the initial extraction protocol. For example, some metabolites are heat instable (e.g., spermidine and spermine; Figure 22) or their levels reduced during the 1 hour, 37 °C ADC activity assay (e.g., some phenolamides). In this case, taking stop samples is essential to determine the impact of the assay on each metabolite and to enable their exact *in planta* concentrations to be measured.

A third limitation is that methods for specific and intense induction of *AtADC1* and *AtADC2* to attain high levels of ADC activity are still lacking. Initial experiments used seedlings grown

vertically on agar plates and produced more consistent results compared to prior experiments using whole, soil-grown plants either cold-treated or infected with *P. syringae* (Laura Schmaltz's Master thesis, 2022), yet further optimisation is needed. For induction of *AtADC1*, a cold-treatment time-course could be conducted to determine at which point ADC activity is highest, while induction of *AtADC2* by salt treatment could be optimised by testing increasing salt concentrations.

However, the most significant aspect of this ADC activity assay and measurement of PAs and related metabolites by LCMS that requires further development is the measurement of phenolamides. During this work, MRM transitions for phenolamides were predicted (Supplementary Table 3), and metabolic peaks matching these mass signals were assigned to these phenolamides. However, without known standards, these peaks cannot be validated. Additionally, without such standards, calibration curves cannot be made and the *in planta* concentrations of individual phenolamides cannot be calculated; in this work, only the peak areas per gram of fresh weight tissue was reported, rather than a concentration (i.e., nmol/g FW). At the time of this work, phenolamides derived from conjugation of agmatine or PAs to HCA-CoA thioesters (i.e., feruloyl-CoA, caffeoyl-CoA, and coumaroyl-CoA) could not be commercially purchased. Therefore, each phenolamide compound would need to be generated in house. This could be done by chemical synthesis using methods such as those published by our collaborators Prof. Atsushi Isihara from Tottori University, Japan and Dr. Naoki Ube from Toyama Prefectural University, Japan (Ube et al., 2019a). Alternatively, phenolamides could be generated *in vitro* by recombinantly expressing conjugating enzymes and adding in the amine and HCA-CoA thioester substrates to produce the desired product. Such an experimental method has previously been utilised, where tyramine (*N*-hydroxycinnamoyl) transferase (THT) was recombinantly expressed in *E. coli* and combined with tyramine and various HCA-CoA substrates to produce different tyramine-based phenolamides (Von Roepenack-Lahaye et al., 2003). As many of the BAHD transferase enzymes responsible for conjugating HCA-CoAs and PAs have been reported to have promiscuous enzymatic activities, especially for accepting various HCA-CoAs, recombinant expression of only 1-2 enzymes may be satisfactory to produce all required phenolamides (Roumani *et al.*, 2022 BioRxiv; Moghe et al., 2023). Using either of these approaches, sufficient quantities of various phenolamides could be routinely produced for LCMS standards and calibration curves, enabling accurate measurement of phenolamides within the same LCMS run as the ADC activity assay measurements.

Overall, the LCMS-based ADC activity assay and PA measurement method has been greatly optimised during this work. Although there are still some aspects that require further

development, it has been invaluable for analyses in this work discussed below and will likely benefit others researching PA metabolism.

4.2 Conserved *ADC* 5'UTR elements impact ADC translation *in planta*

4.2.1 *ADC* 5'UTR elements can be mutated using CRISPR-Cas9, stably inherited, and such plants can be used to investigate the role of these elements.

Previous investigations into the role of the *ADC* uORF and *ADC-box* on ADC translation were conducted using transient reporter assays (Wu et al., 2019). It was found that deleting the *ADC-box* increased translation by at least 2-fold, while deleting the uORF start codon only slightly influenced translation in a cell-free tobacco (BY2) lysate-based assay. However, many aspects of such reporter assays, either conducted *in vivo* via agrobacterium-mediated transient transformation of *N. benthamiana* or *in vitro* in cell-free extracts, do not fully represent the native regulatory scenario in its biological context. For example, infiltration of *A. tumefaciens* into *N. benthamiana* leaves induces stress (Pitzschke, 2013; Drapal et al., 2021), which will eventually result in elevated ADC activity. Thus, in this assay it might be difficult to identify *ADC-box* mutant constructs with mutations that reduce ADC activity. Additionally, BY2 lysates lack a nuclei and other complexities of plant tissues (Buntru et al., 2014). It is therefore important to study these regulatory elements in their native *ADC* context to understand their true biological role/s.

To study the impact of the *ADC-box* on translation *in planta*, CRISPR/Cas9-induced mutagenesis of the *ADC1/2-boxes* was conducted in tomato (section 3.1.1) and *A. thaliana* (section 3.1.2), and the *ADC-box* in *M. polymorpha* (collaboration with Ezgi Süheyla Karaaslan, Liu Lab, University Hohenheim; section 3.1.3). To determine if the *ADC* uORF plays a role in translational regulation *in planta*, CRISPR/Cas9 mutagenesis of the tomato *ADC* uORFs was also conducted (section 3.1.1). Stable, homozygous double $\Delta sladc1/2-box$ and $\Delta sluorf1/2$ mutants were also generated in tomato (Table 29). Plants with mutated *ADC* 5'UTR elements in either one or both *ADC* genes could produce seed and mutants identified in the first transformed generation produced progeny with the same mutations, despite lacking a Cas9 transgene. This demonstrated that these mutations could be stably inherited, and that homozygous mutant progeny was viable, which is unlike double *adc* coding sequence mutants in tomato (Wu et al., 2019) and *A. thaliana* (Urano et al., 2005) that do not produce flowers or viable seeds, respectively.

4.2.2 Tomato, *M. polymorpha*, and *A. thaliana* ADC-boxes all regulate ADC translation in planta, demonstrating the conserved role of this ancient element.

To determine how mutagenesis of the *ADC-box* impacts ADC translation, ADC activity assays and measurement of PA-related metabolites were conducted on pre-treated WT and mutant plant tissue using the optimised LCMS method (section 3.2.7). In all three plant species (i.e., tomato, *M. polymorpha*, and *A. thaliana*), *adc-box* mutants with higher ADC activities and altered ADC-related metabolite levels compared to the WT control were identified (Figure 28, Figure 29, Figure 31, Figure 32, Figure 37, Supplementary Figure 18, and Supplementary Figure 19). Additionally, the double $\Delta adc1/2$ -*box* tomato mutant had 10-fold higher ADC activity compared to WT following cold-treatment, while the single *sladc1/2-box* mutants SIM1_-108 and SIM2_-13 did not produce such high ADC activity levels. This demonstrates that the *adc-box* mutations have a dominant effect and by mutating both *ADC-boxes*, translational control of ADC expression by the *ADC-boxes* is lost. Overall, these increased ADC activity levels, without changes to *SLADC1/2* transcript abundance, demonstrate that mutating the *ADC-box* increases translation of ADC, resulting in higher ADC protein abundance. While the details of the exact mutants are discussed further below (section 4.2.3), the consistent finding in all three species that the *ADC-box* negatively regulates ADC translation *in planta* shows that this role has been extensively conserved. In fact, as *M. polymorpha* is a relative of the first land plants and the *ADC-box* is found in all plants, one can infer that its repressive role on ADC translation has been conserved throughout land plant evolution (i.e., ~450 million years; Bowman et al., 2017).

Interestingly, the impact of the *adc-box* mutations not only affected ADC activity in tomato, *M. polymorpha*, and *A. thaliana*, but also other measured metabolites. Levels of agmatine, putrescine, acetylputrescine, and ornithine were increased in tomato (Figure 31, Supplementary Figure 18, and Supplementary Figure 19) and *M. polymorpha* *adc-box* mutants (Figure 37), while preliminary data indicated that agmatine, putrescine, and acetylputrescine levels were also increased in *A. thaliana* *adc-box* mutants (Figure 28, Figure 29). Additionally, levels of some phenolamides were increased in cold treated adult tomato *adc-box* mutant leaves (Figure 33) and roots of SIM2-13 cold treated seedlings (Figure 64). Together, these results demonstrate that the *ADC-box* regulation of ADC translation not only impacts levels of ADC proteins, but influences flux through the PA pathway, cascading at least to phenolamide synthesis. Further untargeted metabolomic studies are required to identify how many

metabolites and pathways are influenced by the *ADC-box*, which have not been identified through our current targeted LCMS metabolomics approach.

While the role of such an ancient and influential element is striking, it is not totally surprising that plant ADCs would be translationally regulated. In fact, many PA-synthesis enzymes are translationally regulated in plants (Jimenez-Bremont et al., 2022) and other eukaryotes (Kahana and Nathans, 1985; Dever and Ivanov, 2018), as well as in some prokaryotes via riboswitches (Kavita and Breaker, 2023; Salvail et al., 2023). ADCs therefore represent another example of a translationally regulated PA-related enzyme, yet in plants, translation of the other PA-related enzymes is regulated by single or multiple uORFs (Kwak and Lee, 2001; Hanfrey et al., 2005; Jimenez-Bremont et al., 2022). Therefore, the *ADC-box* is both an ancient and unique type translational regulation for PA-related enzymes in plants.

4.2.3 *SHAPE-derived SlADC1/2-box RNA secondary structures indicate why some CRISPR/Cas9-induced adc-box mutants impact ADC translation more than others.*

To better understand how the *ADC-box* may function and regulate ADC translation, ADC activity and PA levels in the different CRISPR/Cas9-induced mutants were compared. Tomato single *adc1-box* or *adc2-box* mutants with 108 bp or 13 bp deletions, respectively (i.e., S11M_-108 and S12M-13), both had ~4-fold higher ADC activity compared to WT (Figure 31, Supplementary Figure 18, and Supplementary Figure 19). However, the smaller single *adc-box* mutants, S11M_-4 and S12M_+1 with either a 4 bp deletion or a 1 bp insertion in their respective *ADC-box*, did not have significantly different ADC activities compared to WT (Figure 31, Supplementary Figure 18). These results indicate that minor mutations within the tomato *ADC-box* sequences do not impact the ability of the *ADC-box* RNA secondary structure to repressing downstream translation, while these larger mutations impair translational inhibition to a similar extent. Interestingly, despite the S12M_-13 mutant only being a partial *sladc2-box* knock out, it still impacts ADC activity (i.e., ADC translation rates), to the same extent as the S11M_-108 mutant, which totally lacks the *ADC1-box*. Overall, these results suggest that there are some regions of the *SlADC-box* where mutations can be tolerated and translational inhibition still occurs, while larger deletions in the *SlADC1/2-boxes* result in loss of translational regulation. *SlADC1/2-box* RNA secondary structures generated both experimentally and computationally via SHAPE analysis (Yiliang Ding's laboratory, John Innes Centre, UK) from RNA extracted from tomato flowers and mature green tomato fruit. Interestingly, the same triple stem-loop structure was found for both *SlADC1*- and *SlADC2-boxes* from both tissues (Figure 52), which have the same central hairpin as the previously predicted *ADC-box* structures by Wu et al.,

2019. This central hairpin is made up of the GC-rich regions, designated as “GC2” and “GC3”, which are also conserved in seedless plants (Supplementary Figure 1; Wu et al., 2019). The CRISPR/Cas9-induced *SLADC1/2-box* mutations were overlaid onto these SHAPE-derived *SLADC1/2-box* RNA structures to enable investigation into how the mutations may differentially influence ADC translation and explain the differing ADC activities between the tomato *adc-box* mutants (Figure 68). Using these diagrams, one can determine that the mutations in the S11C_-6 control mutant and the S11M_+1 mutant do not dramatically interfere with a hairpin structure (Figure 68B and C) and are therefore unlikely to compromise the *SLADC1-box* structure. In line with this, these mutants had no significant difference in ADC activity or metabolite levels compared to the WT (Figure 31, Supplementary Figure 18). On the other hand, while the S11M_-108 lacks the entire triple stem loop structure (Figure 68D), it has similar levels of ADC activity as the S12M_-13 mutant (Figure 31, Supplementary Figure 18, and Supplementary Figure 19), which has a 13 bp deletion spanning two different hairpins (Figure 68G). This indicates that disruption of these hairpins by this 13 bp deletion has a similar impact on the RNA secondary structure’s ability to inhibit downstream translation as a

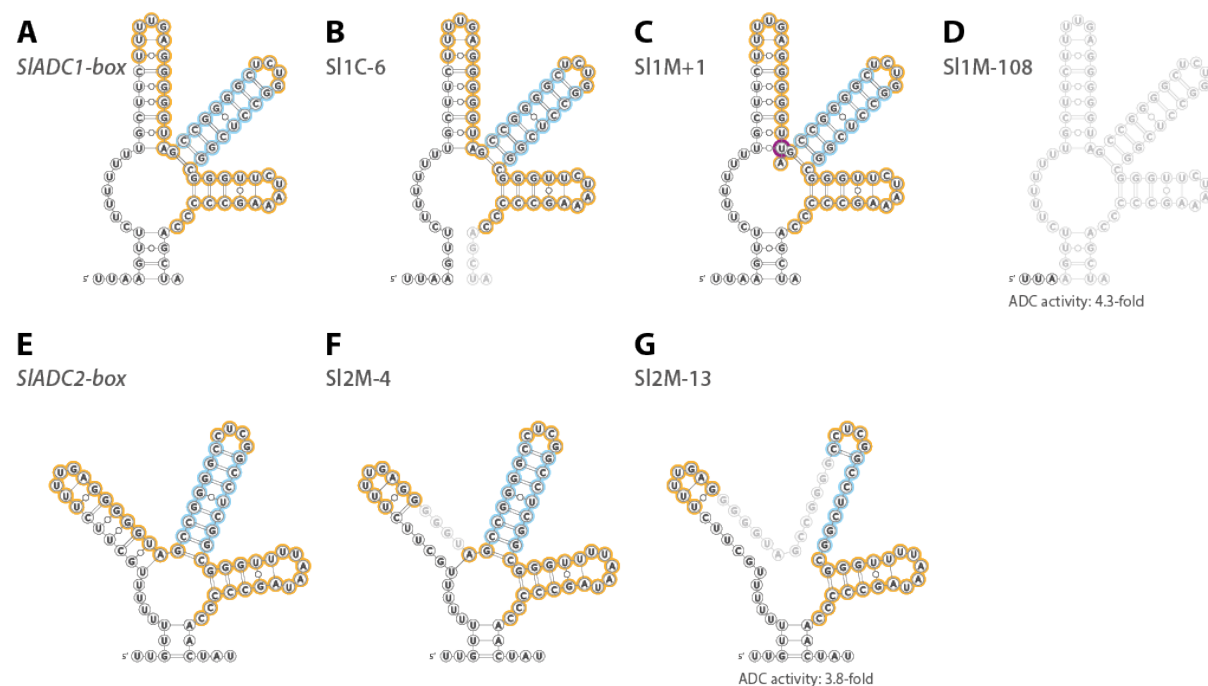


Figure 68: How the CRISPR/Cas9-induced mutations may affect tomato *ADC-boxes* secondary structures.

The WT *SLADC1/2-box* SHAPE-derived structures (generated by Yiliang Ding’s laboratory, John Innes Centre, UK from RNA extracted from mature green tomato fruit) were used to identify where the CRISPR/Cas9-induced *SLADC1/2-box* mutations are located; WT *SLADC1-box* (A), control mutant S11C-6 (B), *adc1-box* mutants S11M_+1 (C) and S11M-108 (D), WT *SLADC2-box* (E), and *adc2-box* mutants S12M-4 (F) and S12M-13 (G) are shown. The *ADC-box* sequence is highlighted in orange and central conserved hairpin, corresponding to sequences designated as GC2 and GC3 regions by Wu et al., 2019, highlighted in blue. Deletions are shown as faded out nucleotides and inserted nucleotides are highlighted in purple. Mutants with significantly higher ADC activity compared to WT (based on Figure 31) have the fold changes displayed.

complete loss of the *ADC-box*. Interestingly, the 4 bp deletion in the Sl2M₋₄ mutant also impacts the same first hairpin as the Sl2M₋₁₃ mutant (Figure 68F and G), but no significant difference in ADC activity or metabolite levels were found for this mutant compared to WT (Figure 31, Supplementary Figure 18). This indicates that disruption of the first hairpin either does not interfere with the formation of the *SlADC2-box* structure or that another compensatory hairpin or structure may form to fulfil the same role as a WT *SlADC2-box* structure. These findings are also supported by transient reporter assays conducted by Wu et al., 2019, where mutating GC1 had no significant impact on downstream translation levels, while deleting GC2, which is part of the central hairpin, significantly increased translation compared to a WT *SlADC1* 5'UTR reporter. Collectively, these findings show that disruption of the central hairpin disables the ability of the *ADC-box* to inhibit downstream translation (i.e., Sl2M₋₁₃), while alterations to the first hairpin (i.e., Sl1M₋₄) do not impede *ADC-box* functionality, nor does deletion of the third hairpin result in dramatically higher ADC translation rates (i.e., Sl1M₋₁₀₈).

Similar to the tomato *adc-box* mutants, *M. polymorpha adc-box* mutants also had different ADC activities and metabolite levels depending on their mutation. While all *M. polymorpha adc-box* mutants had significantly higher ADC activity compared to TAK1 or the control mutants (Figure 37), MpM₋₈ had the highest ADC activity (i.e., 7-fold higher than TAK1), while MpM₋₆ only had 3-fold higher ADC activity compared to TAK1. Additionally, agmatine levels were much more varied between different *M. polymorpha adc-box* mutant compared to the ADC activity levels; this contrasts to the tomato *adc-box* mutants, where the agmatine, putrescine, and acetylputrescine levels mimicked the ADC activity. What also contrasts to the tomato *adc-box* findings is that even CRISPR/Cas9-induced SNPs in the *MpADC-box* resulted in significantly higher ADC activity and metabolite levels compared to the controls (Figure 37). The impact of these small mutations and SNPs may be more pronounced in *M. polymorpha* compared to tomato as there is only one *ADC* homolog and *MpADC-box* sequence only contains the central GC2 and GC3 regions, predicted to only form a single hairpin RNA secondary structure (Figure 52; Wu et al., 2019). Therefore, any disruption to the *MpADC-box* structure may result in a more impactful change to the *MpADC-box* structure and translation rates of the only *ADC* homology. Alternatively, the CRISPR/Cas9-induced SNPs and small deletions may also result in the loss of important elements of the *MpADC-box* RNA secondary structure. For example, MpM_{+1^A} has just as high levels of ADC activity, agmatine, ornithine, putrescine, and acetylputrescine as the larger MpM₋₁₄ mutant, which were all significantly higher than TAK1 by factors of 6-, 20-, 2-, 4-,

and 3-fold, respectively (Figure 37). However, total agmatine levels of the other two mutants with 1 bp insertions (i.e., MpM₊1^B and MpM₊1^C) and putrescine levels of MpM₊1^B were significantly lower than MpM₊1^A, suggesting that the single bp insertion of the MpM₊1^A has a more pronounced impact on MpADC translation. However, it is important to note that the LCMS analysis of *M. polymorpha* ADC activity and PA metabolite levels was only conducted once and should be repeated to verify these results.

To understand how these mutations may differentially affect the ability of the *MpADC-box* RNA secondary structure to regulate MpADC translation, the *M. polymorpha adc-box* RNA secondary structure was overlaid onto the *ADC-box* hairpin structure of seedless plants previously predicted by Wu et al., 2019, which also corresponds to the central hairpin of the *SLADC1/2-box* structures (Figure 52). Compared to the WT *MpADC-box* (Figure 69A), the 1 bp G insertion of MpM₊1^A may result in an enlarged loop up to 5 nucleotides with a non-Watson-Crick pairing of two G nucleotides or a larger 7 bp loop if these G nucleotides do not pair (Figure 69B). In contrast, the MpM₊1^B and MpM₊1^C single bp insertions occur at the base of the hairpin (Figure 69C and D). As these mutants have higher ADC activity compared to TAK1 (Figure 37), they must still impact the *MpADC-box* structure or function; however, without SHAPE-derived structures of these *adc-box* mutants, this cannot currently be explained.

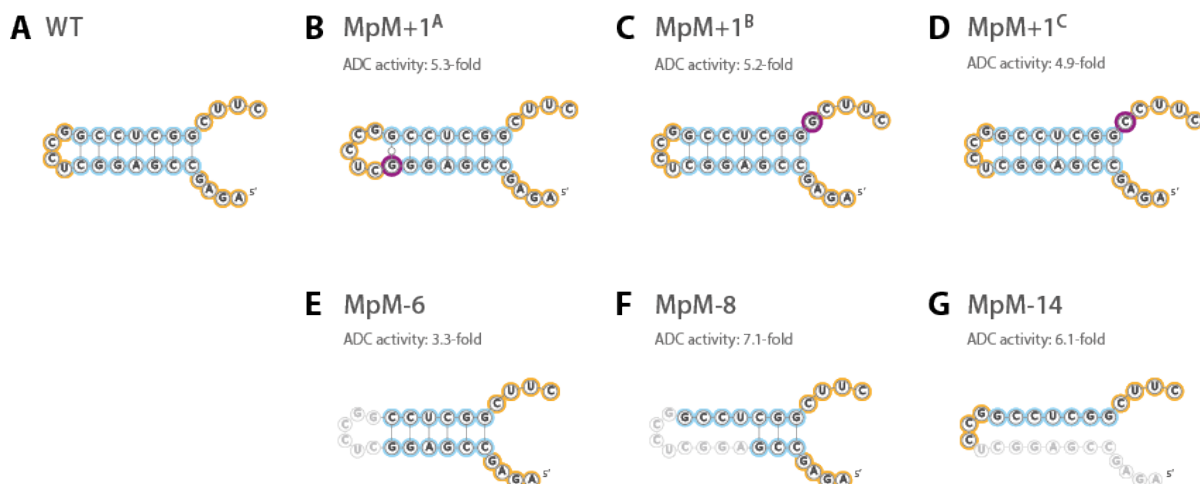


Figure 69: How the CRISPR/Cas9-induced mutations may affect *M. polymorpha* *ADC-boxes* secondary structures.

The predicted RNA secondary structure of seedless plants by Wu *et al.*, 2019 was used to identify where the CRISPR/Cas9-induced *MpADC-box* mutations are located; WT *MpADC-box* (A), MpM₊1^A (B), MpM₊1^B (C), MpM₊1^C (D), MpM-6 (E), MpM-8 (F), and MpM-14 (G) are shown. The *ADC-box* sequence is highlighted in orange and central conserved hairpin, corresponding to sequences designated as GC2 and GC3 regions by Wu *et al.*, 2019, highlighted in blue. Deletions are shown as faded out nucleotides and inserted nucleotides are highlighted in purple. Mutants with significantly higher ADC activity compared to WT (based on Figure 37) have the fold changes displayed.

Interestingly, the MpM₋₆ mutant had the lowest ADC activity compared to the other *M. polymorpha* *adc-box* mutants, as well as lower agmatine, putrescine, and acetylputrescine levels (Figure 37). The 6 deleted nucleotides in MpM₋₆ correspond exactly to the loop nucleotides and do not interfere with the stem sequence (Figure 69E). Therefore, while this mutation has impaired the *MpADC-box* structure, it has not affected ADC translation to the same extent as the single bp insertion of MpM₊₁^A or the larger mutations in MpM₋₈ and MpM₋₁₄, which impact the hairpin stem (Figure 69F and G).

Through using the SHAPE-derived and predicted *ADC-box* structures in combination with the LCMS ADC activity and metabolite data, insights into why some *adc-box* mutants had greater impact on ADC translation can be inferred. Specifically, in all cases where the *ADC-box* sequence encoding stem nucleotides of the central hairpin, ADC activity and some metabolite levels were significantly higher than WT or *adc-box* mutants where this hairpin stem was not impacted (i.e., S11M₋₄, S12M₊₁, MpM₊₁^B, MpM₊₁^C, and MpM₋₆). Therefore, the central hairpin stem, made up of the most highly conserved GC-rich regions (i.e., GC2 and GC3), seems to be essential for *ADC-box* functionality. Seedless plants such as *M. polymorpha* seem to only have GC2 and GC3 sequences, indicating that this essential hairpin evolved first to enable regulation of ADC translation, and angiosperms later evolved to have two additional flanking hairpins. However, as the S11M₋₄ mutant, which has a 4 bp deletion in the stem of the first hairpin, does not have altered ADC activity, the role of these two flanking hairpins remains unclear.

To further investigate the *ADC-box* structure, analysis of more tomato *adc-box* mutants with different mutations in only the first or third hairpins would provide further insight into whether these flanking hairpins play a role in *ADC-box* functionality. Alternatively, the triple hairpin *ADC-box* structure could be interrogated using transient dual reporter assays, where specific mutations in the stems or loops of the *ADC-box* could be generated and the impact on downstream translation analysed. Specifically, comparing the level of translational inhibition between a WT *SlADC-box* and one with the two flanking hairpins deleted would inform on their role in *ADC-box* functionality. Flipping the entire *ADC-box* sequence, or simply swapping the first and third hairpins could also indicate whether their sequence or order is important, or if the only role of the outer hairpins is to slow the PIC so that it can interact with the central hairpin. Additionally, to confirm that the central hairpin indeed forms *in planta*, the GC2 and GC3 sequences could be swapped so that the central hairpin could still form and inhibit downstream translation, but the *ADC-box* sequence would be altered. Furthermore, swapping the *SlADC-box* with the *MpADC-box* in the context of the *SlADC* 5'UTR would also inform

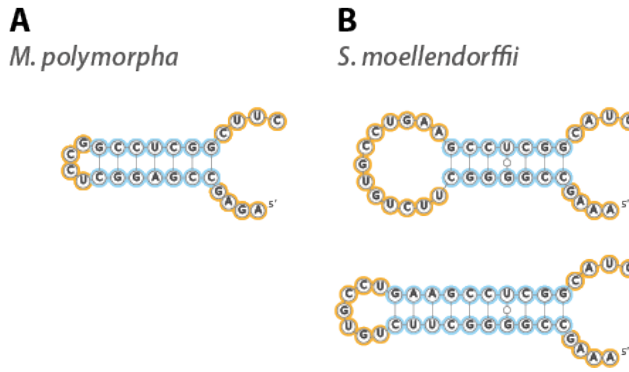


Figure 70: *Selaginella moellendorffii* has a rare insertion in its *ADC-box* sequence.

The predicted RNA secondary structure of seedless plants by Wu *et al.*, 2019 was overlaid with the *MpADC-box* (A) and *SmADC-box* (B) sequences; two possible *SmADC-box* structures are shown. The *ADC-box* sequence contains a conserved hairpin, corresponding to sequences designated as GC2 and GC3 regions by Wu *et al.*, 2019, highlighted in blue.

on how well this primitive *ADC-box* structure can regulated ADC translation compared to the more evolved angiosperm triple hairpin *ADC-box*. Similarly, insertion of other interesting *ADC-box* sequences from other species into an *SlADC* 5'UTR would also inform on how natural *ADC-box* sequence variation may influence translational efficiency. For example, the *A. thaliana ADC1-box* contains some additional nucleotides (Figure 4), while the *ADC-box* from *Selaginella moellendorffii* (i.e., *SmADC-box*) has ~9 nucleotides inserted between the GC2 and GC3 regions, possibly extending the loop or the stem of the central hairpin (Figure 70; Wu *et al.*, 2019). Such examples of alternative *ADC-boxes*, although rare, may shed light on where sequence variation may be tolerated to still enable regulation of ADC translation.

A final future perspective would be to definitively determine the effect of the CRISPR/Cas9-induced *adc-box* mutations on their *ADC-box* RNA secondary structures. Here, SHAPE-derived *ADC-box* structures of the Sl1M₋₄, Sl2M₊₁, and Sl2M₋₁₃ mutants would provide insight into how the structure may be altered due to the mutations or if an alternative structure may form due to these mutations. Additionally, determining the TAK1 *MpADC-box* structure would not only confirm that only a single hairpin forms, but if this is the same as the central hairpin in the *SlADC1/2-boxes*. SHAPE-derived *MpADC-box* structures of the *adc-box* mutants would also inform on how single bp insertions (i.e., MpM₊₁^A) can result in ADC activity levels comparable to a 14 bp deletion mutant (i.e., MpM₋₁₄).

In conclusion, SHAPE analysis has determined that the *SlADC1/2-box* RNA secondary structures form a triple hairpin structure, where the central stem is formed from the most highly conserved GC-rich regions in the *ADC-box* sequence. These SHAPE structures, alongside the LCMS metabolic data, have provided indications as to how the CRISPR/Cas9-induced *adc-box* mutations affect the ability of the *ADC-box* to form and thereby control ADC translation. Particularly, informing on how even small SNPs can affect the *ADC-box* structure to impair its ability to repress translation. This work has elucidated that the *ADC-box* central hairpin is the most essential element for *ADC-box* functionality, and it is likely that this hairpin evolved first

in early land plants and then expanded to become a triple hairpin structure in angiosperms. Overall, combining LCMS metabolic analysis of CRISPR/Cas9-induced mutants with SHAPE-derived *SLADC1/2-box* RNA secondary structures has not only validated that the *ADC-box* has regulated ADC translation since the first land plants, but also that the same central hairpin structure may have also been conserved since then too.

4.2.4 Impact of the tomato *ADC* uORFs on *ADC* translation and plant development.

Despite the presence of a small uORF in most plant *ADC* 5'UTRs, with a conserved distance of ~150 bp to the start of the *ADC-box* (Supplementary Figure 2), previous analysis of the *SLADC1* 5'UTR elements using transient assays found little to no impact of the uORF on ADC translation (Wu et al., 2019). This was also found in this work using the pGreenII-based dual luciferase assay system, where no difference in luciferase expression was found between WT and $\Delta uorf$ knock out *SLADC1* 5'UTR reporters (Figure 48). Interestingly, the *ADC* uORF in carnation (*Dianthus caryophyllus* L.) was previously found to inhibit ADC translation using *in vitro* methods (Chang et al., 2000). It was suggested that this was due to the *ADC* uORF peptide, yet this peptide also inhibited translation of the non-*ADC* control protein.

However, when analysing the CRISPR-induced single and double tomato *uorf* mutants in this work, they had approximately 2-fold lower ADC activity compared to WT following cold treatment (Figure 35), indicating a positive role of the uORF on ADC translation. Lower agmatine in the *uorf2* and *uorf1/2* mutants, and lower putrescine, acetylputrescine, ornithine, and glutamine in the $\Delta uorf1/2$ double mutant were also observed (Figure 35, Supplementary Figure 20), demonstrating that the effect of the uORF propagates through the PA pathway. When analysing the phenotypes of WT, $\Delta adc1/2-box$, and $\Delta uorf1/2$ tomato plants, the double $\Delta uorf1/2$ mutant was found to be shorter, with fewer leaves and flowers compared to the WT and $\Delta adc1/2-box$ mutant, without any differences in *SLADC1/2* transcript abundance between the $\Delta uorf1/2$ and WT (Figure 36). Together, these findings indicate that the uORF positively regulates ADC translation and is also important for normal plant development. Therefore, ADC translation may be regulated both positively and negatively by the uORF and *ADC-box*, respectively.

At first, it may seem somewhat unusual for the *ADC* uORF to have positive effect on translation of ADC, as uORFs typically have an inhibitory role on mORF translation (von Arnim et al., 2014). However, a key difference between the LCMS analysis of the tomato *uorf* mutants and other *ADC* uORF-investigating reporter assays is the addition of a cold stress treatment. During

periods of stress, uORFs have been found to promote translation of the downstream mORF, despite a suppression of global translation that is initiated to preserve energy during this period (Palam et al., 2011; Andreev et al., 2015; Wek, 2018). Various stresses, such as excess light, nutrient deprivation, cold, wounding, and pathogen attack, activate the General Control Nonderepressible 2 (GCN2) kinase that phosphorylates the translation initiation factor 2 alpha subunit (eIF2 α ; Lageix et al., 2008, Lokdarshi and von Arnim, 2022, Lokdarshi et al., 2022). This phosphorylation inhibits recycling of the eIF2-GDP to an active eIF2-GTP, which is required for assembly of a functional PIC and therefore, translation is globally repressed (Wek, 2018). Despite this global translation repression, some transcripts, which often encode proteins required for stress remediation, as well as a uORF in their 5'UTR, are still translated (Wek, 2018). Therefore, the role of the *ADC* uORF may be to mark/promote the *ADC* transcript for translation during periods of stress, thereby acting as a conditional positive regulator of ADC translation. In the case of the ADC activity assays conducted here, cutting the tomato leaves for cold treatment may have induced eIF2 α phosphorylation, yet due to the presence of the uORF, ADC could still be translated from the WT 5'UTR. However, the CRISPR/Cas9-induced *uorf* mutants lack the uORF, meaning translation of the downstream ADC was repressed like all other transcripts following cutting of the leaves and cold stress, resulting in the reduced ADC activity compared to WT (Figure 35).

In Figure 71, two proposed mechanisms for how the uORF may promote ADC translation during stress are proposed. During periods of no stress, PICs may either scan past the uORF via leaky scanning to initiate at the ADC start codon, or otherwise translate the uORF, and then following termination, recruit new initiation factors (i.e., eIF2-GTP and eIF3) to allow for reinitiation at the next downstream start codon (i.e., ADC). Reinitiation is more likely to occur following translation of a short uORF compared to a longer one (Gunisová et al., 2018), which may be why such a small *ADC* uORF has been conserved (i.e., 9 amino acids; Supplementary Figure 2). Following recruitment of all required factors for reinitiation, the scanning ribosome would then encounter the *ADC*-box, which would be responsible for allowing scanning to the ADC start codon in a PA-level dependent manner (Figure 71B). On the other hand, during periods of stress, the uORF bypass model (Figure 71C) states that low levels of eIF2-GTP and abundance of active PICs reduces uORF initiation, which may occur if the time/distance on the 5'UTR required to recruit an eIF2-GTP is longer. In this way, less uORF translation would occur during stress, meaning that more scanning PICs could reach the ADC start codon to initiate translation. This model is based on the stress-dependent translational regulatory

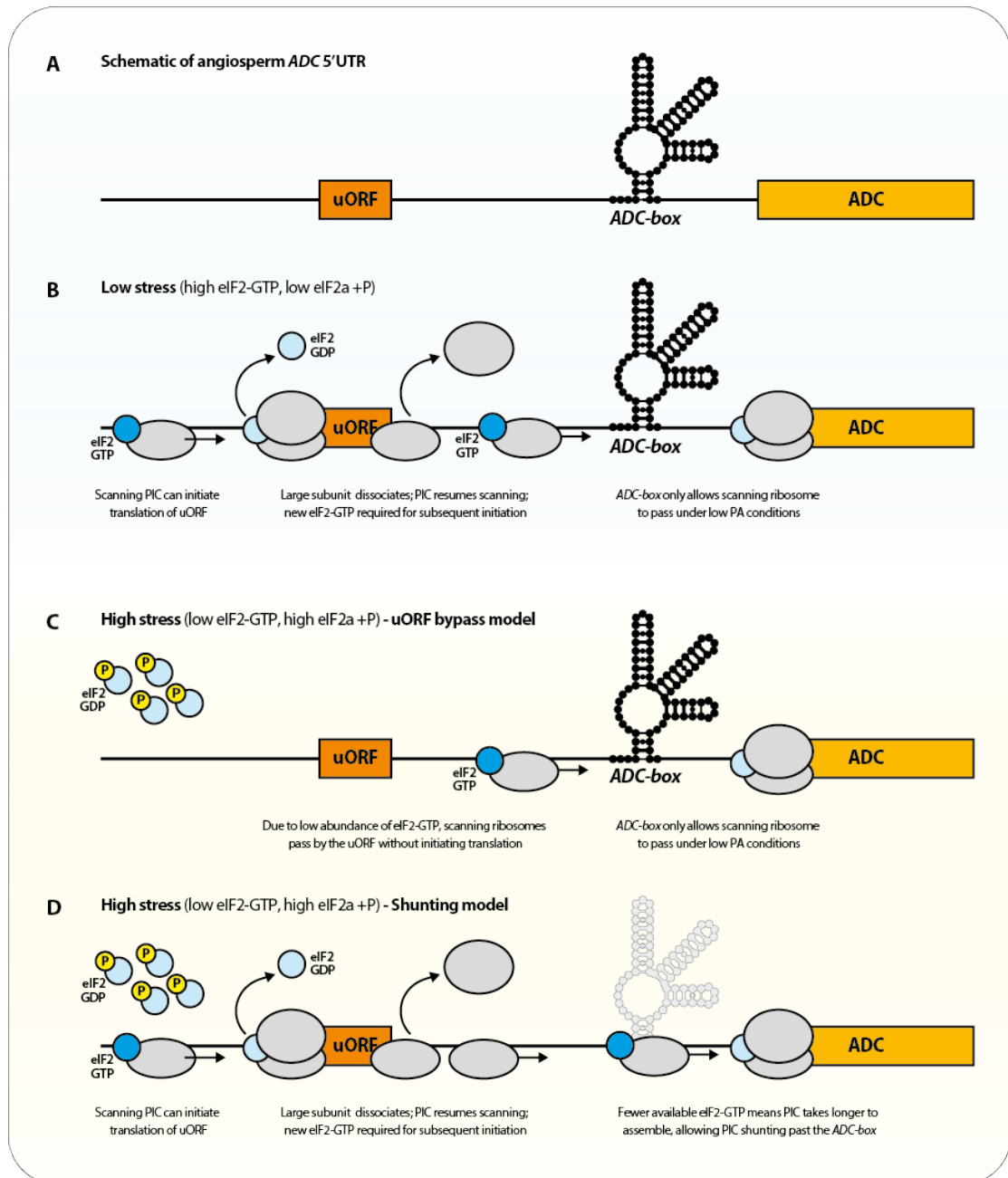


Figure 71: Proposed model of how the uORF positively regulates ADC translation during periods of stress.

(A) Most *ADC* 5'UTRs in angiosperms contain a uORF (dark orange) ~150 bp 5' to the *ADC-box* (depicted as the *SlADC1-box* triple hairpin structure; black), which sits ~100 bp upstream of the *ADC* coding sequence (light orange). (B) During periods of low stress, eukaryotic initiation factor 2 (eIF2) with a bound Met-tRNA and GTP (dark blue) is abundant and incorporated into the preinitiation complex (PIC, grey). Upon start codon recognition, such as the *ADC* uORF, hydrolysis of the eIF2 GTP to GDP (light blue) occurs, the eIF2α-GDP is released, and large ribosomal subunit recruited to initiate translation. Occasionally the PIC can resume scanning after termination but requires a new eIF2-GTP to initiate translation. (C) Stresses (i.e., nutrient deprivation, biotic or abiotic stress, etc.) induce phosphorylation (yellow) of the eIF2 α subunit, reducing available eIF2-GTP and thereby abundance of functional PICs, thus suppressing translation initiation and protein synthesis. In such situations, transcripts with uORFs often maintain mORF translation. In the *ADC* uORF bypass model, functional PICs bypass the uORF, maintaining the eIF2-GTP for translational initiation of *ADC*, only if the *ADC-box* also detects low PA levels and does not inhibit PIC scanning. (D) In the shunting model, PIC initiation at the uORF start codon still occurs, but due to low eIF2-GTP levels, a functional scanning PIC takes longer to assemble. With fewer eIFs, the *ADC-box* allows the PIC to pass or be shunted past to the *ADC* start codon, regardless of PA levels.

mechanism of CHOP, a protein required for stress-induced apoptosis. Here, phosphorylation of eIF2 α increases PIC scanning past a strong inhibitory uORF to increase CHOP translation (Palam et al., 2011). As the *ADC* uORF has not previously been observed to inhibit ADC translation *in planta*, this model is not fully supported by the current data.

Another model, called the shunting model, has also been proposed (Figure 71D). Here, under stressed conditions, the few active PICs with an eIF2-GTP can initiate translation of the *ADC* uORF, yet due to the reduced abundance of eIF2-GTP during the stress response, reassembly of an active PIC requires more time/distance on the transcript. This PIC, either due to lacking various initiation factors or due to stalling as it waits for an available eIF2-GTP, may be allowed to bypass the *ADC-box* regardless of PA levels, or be shunted past it to the ADC start codon. This would result in increased levels of ADC translation in a uORF-dependent, but *ADC-box*-independent manner. Similar mechanisms have previously been reported to occur with viral transcripts (including plant pararetroviruses, like the cauliflower mosaic virus), where a short uORF is translated, but following termination, the small ribosomal subunit is shunted forward past an RNA secondary structure to the start codon of the mORF (Pooggin and Ryabova, 2018). This hypothesised model is also supported by the conserved spacing of the uORF and *ADC-box* found in angiosperm *ADC* transcripts and the uORFs consistently being small (Supplementary Figure 2), both of which would need to be maintained evolutionarily for this reinitiation and shunting mechanism to function.

Before testing these hypothesised models, the idea that the *ADC* uORF is a stress-dependent positive regulator of ADC translation must first be verified. This could be confirmed by an ADC activity assay on non-treated and cold-treated WT and $\Delta uorf$ tomatoes, where only under stressed conditions would $\Delta uorf$ mutant have reduced ADC activity. Additionally, transient dual reporter assays in *N. benthamiana* leaves could be used to compare translation rates of WT and uORF start codon knock out *ADC* 5'UTRs under non-stressed and stressed (i.e., cold-treated) conditions. This would not only support the ADC activity assay data, but also indicate whether a transient reporter system in combination with cold stress would be suitable for further investigation into the *ADC* uORF models. To test the shunting model via transient dual reporter assays, the placement of the uORF stop codon on the transcript, and thereby the location of translation termination relative to the *ADC-box*, could be altered. As the spacing between the uORF and the *ADC-box* is conserved at ~150 bp (Supplementary Figure 2), this may be an important sequence feature for shunting to occur. Here, translation of the mORF would be expected to only be promoted following stress if the uORF stop codon is ~150 bp 5'

to the *ADC-box*. Additionally, shunting often requires an AT-rich sequence at the ribosome landing site, so further sequence analysis of the AT-content 3' to the *ADC-box* across angiosperms could be conducted. Finally, as only one LCMS experiment has been conducted on tomato WT, single, and double $\Delta uorf$ tomato mutants, more repetitions must be conducted, alongside mutagenesis of *ADC* uORFs in other species to confirm that the protective role of the uORF for maintaining ADC translation during periods of stress is a conserved mechanism. Overall, LCMS analysis of CRISPR/Cas9-induced tomato *uorf* mutants has resulted in a possible positive regulatory role for this conserved element, adding to the complexity of ADC translational regulation.

The hypothesis that the *ADC* uORF is a positive regulatory element for ADC translation is also supported by the phenotyping experiment conducted on WT, $\Delta adc1/2-box$, and $\Delta uorf1/2$ tomatoes. The $\Delta uorf1/2$ mutant was shorter, with fewer leaves and flowers compared to WT and the $\Delta adc1/2-box$ mutant (Figure 36), suggesting that the *ADC* uORF is important for maintaining normal plant development. While the phenotyping experiment was conducted in a controlled greenhouse, small periods of stress may still have occurred during development to influence ADC translation in a uORF-dependent manner (i.e., via a model proposed in Figure 71). Without the protective action by the uORF to maintain ADC translation, the $\Delta uorf1/2$ mutant may have had lower PAs during critical periods of development, resulting in the observed phenotype. In fact, RNA silencing both *A. thaliana* *ADCs* resulted in a stunted phenotype and delayed flowering (Sánchez-Rangel et al., 2016), suggesting that reduced ADC levels affect plant development. Further experiments investigating the $\Delta uorf1/2$ phenotype at earlier stages of development and under different stress conditions may elucidate when and where the uORF promotes ADC translation to ensure normal plant development. Specifically, conducting LCMS analysis on different tissues at different time points of development of WT and $\Delta uorf1/2$ mutant tomatoes could be conducted. Finally, to ensure that the phenotypic and LCMS metabolic analysis of the tomato *uorf* mutants is due to deletion of the uORF and not another element of the *ADC* 5'UTR, more mutants should be investigated. The CRISPR/Cas9-induced *uorf* mutants analysed in this work have large deletions of 127 bp and 172 bp spanning the *SLADC1* and *SLADC2* uORFs, respectively (Figure 11, Figure 12). Although no differences in *SLADC1/2* transcript abundances between WT and $\Delta uorf1/2$ plants were found in RNA from adult tomato leaves, it is possible that such large deletions have a secondary impact on ADC translation, rather than being a direct impact of knocking out the uORF. For example, a smaller leader sequence between the 5' cap and the *ADC-box* may impact its repressing action on PICs, resulting in lower ADC translation rates in these $\Delta uorf$ mutants. Therefore, to ensure that the

results observed in this work were due to knocking out the uORF, rather than impacting translation through another mechanism, $\Delta uorf1/2$ tomato mutants with smaller mutations spanning the uORF ATG need to be analysed. A double $\Delta uorf1/2$ with 7 bp and 14 bp deletions spanning the *SLADC1* and *SLADC2* uORFs respectively, has been initiated in this work, but not yet analysed (Table 29).

Overall, generation and analysis of tomato *uorf* mutants resulted in finding that perhaps the uORF is essential for maintaining ADC translation during periods of stress and development. This finding adds ADC to the list of plant PA-related enzymes that have uORFs in their 5'UTR, alongside ODC, SAMDC, the PA catabolic enzymes POA-2, -3, and -4, and proteins regulated by PAs, such as the transcription factor SAG51 (Jimenez-Bremont et al., 2022). uORFs may therefore be a common feature in these 5'UTRs to promote their translation throughout periods of stress, ensuring the production of PAs and related stress remediating metabolites.

4.3 Investigating the mechanism of plant ADC translational regulation.

After finding that the *ADC-box* has a repressive regulatory role on ADC translation *in planta*, and that this role has been conserved throughout land plant evolution, we sort to understand how the *ADC-box* mechanistically functions. To maintain PA homeostasis, it would be counterintuitive if the *ADC-box* could only repress ADC translation without alleviating this repression during periods of insufficient PA levels. It was therefore hypothesised that the *ADC-box* must somehow sense levels of PAs or the ADC protein itself to correctly control ADC translation to match the cellular PA need. For this sensing, some sort of feedback mechanism would be required to connect the cellular PA level to the *ADC-box* structure. Such feedback signals were hypothesised to be either: the ADC protein itself; PAs or a related metabolite; a combination of the ADC protein and a bound metabolite; or another biological factor completely unrelated to ADC or PA metabolites that senses the cellular need for PAs and then acts upon the *ADC-box*. To narrow down these 4 hypothesised feedback signals, a dual luciferase reporter assay system was established, and functional and catalytically non-functional ADC expression constructs were generated (section 3.5.2.3).

4.3.1 A dual luciferase reporter system was established to investigate how the *ADC-box* regulates translation.

Here, a luciferase-based dual reporter system was established using the pGreenII-based plasmids (*pGrDL_SPb* (GG); Hellens et al., 2000, Moyle et al., 2017), enabling golden-gate

assembly of promoters and/or 5'UTR constructs to be inserted upstream of the luciferase reporter gene (Figure 42, Figure 43, section 3.5.2.2). Using this cloning strategy, various promoter, as well as 35S-driven *SLADC1* and *SLADC2* WT, Δ *adc-box*, and Δ *uorf* 5'UTR luciferase reporter T-DNA constructs were generated and agroinfiltrated into *N. benthamiana* leaves. Similar to the findings of Wu et al., 2019, deletion of the *SLADC1 ADC-box* increased translation of the downstream reporter, while deletion of the *SLADC1* uORF start codon knock out had no effect in (Figure 48). This also showed that despite using a different dual reporter system, the impact of mutating these conserved elements on downstream translation in transient *N. benthamiana* reporter assays was consistent. Additionally, the error between technical replicates was relatively low using the *pGrDL_SPb (GG)* reporter system and the results were reproducible between experiments, demonstrating its efficiency and reliability for investigating translation rates of individually expressed reporters.

4.3.2 Transient overexpression of ADC for dual luciferase assays causes metabolic changes that influence translation in an ADC 5'UTR-independent manner.

To narrow down which feedback signal might be involved in the *ADC-box* regulatory mechanism, experiments co-delivering *SLADC* 5'UTR reporters with functional or catalytically non-functional PA enzymes were conducted. However, throughout all experiments that co-expressed an active *SLADC* protein, luciferase expression and/or activity was reduced compared to the negative control (i.e., EV or GUS), regardless of the type or presence of a 5'UTR or the promoter used (Figure 48, Figure 49, Figure 50, Figure 51).

It is possible that ADC-induced metabolic changes may have influenced general transcription or translation of the reporters, or activity of luciferase itself, causing this ADC-specific reduction in luciferase. When assessing ADC activity and metabolite levels of the transiently expressed WT *SLADC* proteins in *N. benthamiana*, not only were agmatine and putrescine levels increased, but also arginine or spermidine levels were significantly lower than the catalytically non-functional *SLADC* proteins (Figure 46). As spermidine has been implicated in general translational efficiency due through a variety of mechanisms (Dever and Ivanov, 2018; Jimenez-Bremont et al., 2022), it is possible that reduced levels caused by ADC overexpression may influence global translation. Spermidine, as well as other PAs, are known to bind to DNA, RNA, and ribosomes (Dever and Ivanov, 2018), with two spermidine binding sites identified within the peptidyl transferase centre (PTC) of bacterial ribosomes (Xaplanteri et al., 2005). Spermidine is also required for the function of translation factor eIF5A, which is involved in translation elongation, alleviating ribosome stalling at proline-rich repeat

polypeptides. In eIF5A, spermidine is required to convert a lysine residue into a hypusine residue, a process that has also been implicated in stress responses in higher plants (Belda-Palazon et al., 2016, Poidevin et al., 2019, Palfi et al., 2021). Therefore, reduction of spermidine upon overexpression of ADCs may impact general translational efficiency, either generally through nucleotide or ribosome binding, or via specific factors such as eIF5A, thereby resulting in reduced luciferase reporter expression.

Alternatively, the reduced arginine or spermidine levels, or the increase in other metabolites following transient *SlADC* overexpression in *N. benthamiana* may impact luciferase activity. Moyle and Birch, 2013 found that undiluted plant extracts could inhibit activity of firefly luciferase but did not affect renilla luciferase, while complex bodily fluids were also found to alter luciferase reporter activity (Neefjes et al., 2021). As no ADC overexpression-dependent impact on renilla luminescence was ever observed (Supplementary Figure 30, Supplementary Figure 31, Supplementary Figure 33, Supplementary Figure 35, Supplementary Figure 36), it is possible that some metabolic consequence of ADC overexpression may specifically impact luciferase activity. It is possible that the small volume of plant extract used for these assays (i.e., 5 μ L of *N. benthamiana* extract supernatant diluted in 40 μ L of each dual luciferase assay buffer) was sufficient to inhibit luciferase activity; diluting the sample further may alleviate this issue but this remains to be tested. Additionally, monitoring activity of purified luciferase proteins in the presence of ADC-related metabolites (i.e., arginine, agmatine, putrescine, spermidine, or some phenolamides) could be conducted to determine if one of these metabolites impact luciferase activity. Overall, the results presented in this work demonstrate that transient co-expression of ADC proteins is incompatible with the firefly luciferase reporter. Because of this, no conclusions could be made regarding the effect of active or inactive ADC proteins on *SlADC* 5'UTR translation using this reporter system. Therefore, another experimental method for investigating how the *ADC-box* can respond to cellular needs of PAs is required.

4.3.3 *Alternative experimental approaches could give indications into how the ADC-box secondary structure regulates ADC translation.*

To further investigate how the *ADC-box* may regulate ADC translation, including if and how it responds to cellular PA levels, could be conducted using more structural-focused approaches. In this work, SHAPE analysis of the *SlADC1/2-box* RNA secondary structures found that they form a triple hairpin structure, rather than the previously predicted double stem loop structure presented by Wu et al., 2019 (Figure 52). However, these structures were elucidated from mRNA sampled from tomato floral tissue and mature green fruit, rather than adult tomato

leaves, in which the ADC activity and PA levels were measured in the CRISPR/Cas9-induced tomato mutants. As it is possible that a different structure forms in these tissues due to differences in PA requirements or tissue development, SHAPE analysis of *SLADC1*- and *SLADC2* 5'UTRs in adult tomato leaf tissue is required. It is also conceivable that upon recognition of high PA levels, the *ADC-box* RNA structure undergoes a conformational change to prevent PICs from scanning past to the ADC start codon. However, this seems unlikely as the two sets of *SLADC1*- and *SLADC-box* structures that have been determined so far have come from two very different tissues with different levels of *SLADC1/2* transcription and PAs and still show similar triple hairpins structures. Tomato flowers have much higher PA levels compared than other plant organs (Tassoni et al., 2000; Applewhite et al., 2000; Chen et al., 2019), while *SLADC1/2* transcript levels and PAs are lower in mature green tomato fruit compared to other fruit stages (Tsaniklidis et al., 2016). However, to support the hypothesis that the *ADC-box* does not undergo a conformational change in response to PA levels, SHAPE-derived *ADC-box* structures in high and low PA conditions are needed. A “high PA state” could be generated by cold-treating single *adc-box* mutants, where the remaining WT *ADC-box* structure could be determined. On the other hand, a “too low PA state” could be generated by using *adc* coding sequence mutants, particularly the tomato *adc1/adc2* double mutant, which lacks any measurable ADC activity and has dramatically low PA levels (Figure 39). If all *ADC-box* RNA secondary structures remain the same in these conditions, then one could conclude that the *ADC-box* structure remains constant and only binding/recognition of a PA-level-dependent feedback signal increases the stability of the *ADC-box*, thereby preventing PIC scanning. However, it is important to note that SHAPE reactivity values are generated from all sequenced transcripts in a cell, rather than derived from a single transcript. Therefore, a subset of *ADC-box* structures could exist in an alternative conformation, but this is likely to be missed by SHAPE analysis.

While further SHAPE analysis would give insight into the structural and mechanistic conservation of the *ADC-box in planta*, another experimental technique may help to elucidate the feedback signal and therefore, *ADC-box* regulatory mechanism. As the *ADC-boxes* in tomato, *M. polymorpha*, and *A. thaliana* were all found to repress ADC translation, it is likely that they all function in the same manner and respond to the same PA level feedback signal. Therefore, if the *ADC-box* mechanism has been conserved since the first land plants, the PA level feedback signal must also be present and consistent throughout this entire evolutionary timeline. ADC protein was hypothesised to be the feedback signal (Figure 41); yet for this to be the case, an *ADC-box* binding site on ADC proteins must be conserved, despite divergence

in the peptide sequence over time and between ADC homologs. What seems more likely however, is that a metabolite would be the feedback signal, as metabolite structure and charges do not change throughout evolution. Arginine and PAs are ancient metabolites that are essential for all forms of life (Miller-Fleming et al., 2015) and are therefore, more likely candidates for being the *ADC-box* feedback signal compared to an evolving ADC protein.

To identify a feedback signal, an *in vitro* method whereby the *ADC-box* RNA secondary structure could be exposed to various metabolites or the ADC protein and tested for potential binding. Optical tweezers are a precision tool that can be used to study the forces restricting single molecule movement or motility. Optical tweezers have previously been used to catch either end of single a RNA strand and then, through gradually pulling on the RNA, the forces that hold RNA secondary structures together can be measured (Ghimire and Guo, 2021; Marinus et al., 2024). Such experiments could be conducted in collaboration with Erik Schäffer's laboratory (ZMBP, University Tübingen), who specialise in using optical tweezers. Here, pure *ADC* 5'UTR probes could be secured (either between two optical tweezers or with one RNA end tethered to a surface and the other end caught by the tweezer) and the force required to unwind the *ADC-box* RNA secondary structure could be determined. Such information would be informative for understanding how much energy ribosomal helicases must overcome to allow PICs scanning past the *ADC-box*. Additionally, the force required to unwind the *ADC-box* structure could also be measured in the presence of different metabolites or purified ADC proteins. Here, when the correct feedback signal binds to the *ADC-box*, the energy required by the optical tweezers to pull apart the *ADC-box* structure would be increased, thereby identifying the feedback signal. As a negative control for such experiments, the *ADC-box* sequence could be scrambled, preventing the same secondary structure from forming. This would enable classification of when a metabolite or protein binds in an *ADC-box* structure-dependent manner, rather than random binding. Overall, to understand how the *ADC-box* structure functions and what the feedback signal may be, further experiments using both SHAPE analysis and *in vitro* methods like optical tweezers are required.

4.4 Together, the *adc-box* and *adc* coding sequence mutants shed light on the role of ADCs in PA metabolism.

4.4.1 Tomato *adc-box* and *adc cds* mutants have mirroring impacts on PA metabolism.

While the *adc-box* mutants were primarily generated to study the role of the *ADC-box* in *planta*, they have also become a valuable tool for understanding the role of ADCs in PA metabolism. The *adc-box* mutants essentially act as mild ADC over expressing lines due to their lack of inhibitory control over ADC translation. Due to higher levels of ADC activity in the tomato Δ *adc1/2-box* mutant, increased levels of downstream metabolites were observed (i.e., agmatine, putrescine, acetylputrescine, and some phenolamides), while alterations to the metabolites in the urea cycle were also observed (i.e., ornithine and citrulline; Figure 31, Figure 32, Figure 33, Figure 64). Complementary to such lines are the ADC coding sequence single (i.e., *sladc1* and *sladc2*) and double (i.e., *sladc1/2*) knock out lines, which have lower or no ADC proteins. The *sladc1* mutant had only ~42% of the ADC activity in the WT, while the *sladc2* mutant had ~76% (Figure 39), indicating that in cold-treated adult tomato leaves, SLADC1 plays a bigger role than SLADC2. This may be due to higher levels of *SLADC1* transcription following cold treatment, which was observed in the *SLADC1* relative transcript abundance in cold-treated WT and *adc-box* tomatoes compared to *SLADC2* levels (Figure 34). No ADC activity was observed in the *sladc1/2* double mutant line, confirming that no other enzyme is present in tomatoes to decarboxylate arginine to agmatine and consequently, arginine levels build up (Figure 39). Possibly due to this build up in arginine or the plants attempt to increase flux through the PA pathway, citrulline and glutamine levels are also increased in the Δ *sladc1/2* mutant. Interestingly, ornithine levels in the single and double were reduced in the Δ *sladc1/2* mutants but increased in the Δ *sladc1/2-box* mutants, while arginine levels were never affected in these lines. As arginine is made from ornithine and the ADC-dependent pathway for PA synthesis is more dominant in plants (Winter et al., 2015; Chen et al., 2019), it seems that the ornithine pool is compromised in an effort to maintain ADC-dependent PA synthesis. Alternatively, the higher and lower ornithine levels in the Δ *sladc-box* and *sladc1/2* mutants may be due to lower and higher ODC activities in these lines, respectively; yet ODC activity remains to be measured in these mutants. Combining the information of the tomato *adc-box* and *adc cds* mutants demonstrates the connectedness of the ADC- and ODC-dependent PA pathways in plants through the substrate cycling pools.

Downstream of the ADC and ODC activities, the tomato *adc-box* and *adc cds* mutants appear to metabolically mirror each other. The *adc-box* mutants have increased agmatine, putrescine, acetylputrescine, and some phenolamides (i.e., coumaroylagmatine and coumaroylputrescine; Figure 33, Figure 64), while the *sladc1/2* mutant has severely reduced agmatine, putrescine, and acetylputrescine levels (Figure 39), as well as the phenolamides coumaroylagmatine, coumaroylputrescine, feruloylagmatine, feruloylputrescine, and cinnamoylputrescine (Figure 40). These striking results demonstrate that ADC plays a vital role in not only regulating agmatine and putrescine levels, but also levels of their conjugated products. However, despite such dramatic metabolic changes in these mutants, spermidine and spermine levels were never significantly different to WT in any mutant. Similar results have also been found in transgenic plants overexpressing ADCs, where spermidine and spermine levels were also unaffected, regardless of very high ADC activity levels (Burtin and Michael, 1997; Masgrau et al., 1997; Alcázar et al., 2005; Espasandin et al., 2014). Little to no changes in spermidine and spermine levels in transgenic plants overexpressing ODC were observed either (Bhatnagar et al., 2001; Roy and Wu, 2001). Overall, this suggests that there is a level of very tight regulation of spermidine and spermine levels in plants, possibly due to their extensive biological roles (Chen et al., 2019).

In summary, comparative analysis of the *adc-box* and *adc cds* mutants has provided valuable insight into the impact of ADCs on PA metabolism in tomato. Yet similar in-depth LCMS analysis of *adc-box* and *adc cds* mutants in other systems such as *A. thaliana* would help to determine if these metabolic patterns, and therefore regulatory circuits and breaks, are also present in other species.

4.4.2 adc-box mutants do not have as high ADC activity as published over expression lines but provide a unique opportunity for researching the role of ADCs in planta.

Interestingly, the tomato Δ *adc1/2-box* mutant and the *M. polymorpha* mutants had 10-fold and ~6-7-fold higher ADC activities compared to their WT counterparts, respectively. While these increases in ADC activity are impressive, other transgenic lines over expressing ADCs have achieved much higher increases in ADC activity. For example, tobacco lines transformed with an oat *ADC* gene driven by a 35S promoter showed between 10-20-fold higher ADC activity compared to a control line (Burtin and Michael, 1997), while tetracycline-inducible oat ADC construct transformed into tobacco produced up to 55-fold higher ADC activity levels compared to the WT control (Masgrau et al., 1997). Additionally, individual *A. thaliana* lines transformed with a 35S promoter-driven *AtADC2* construct showed between 2- to 32-fold

higher ADC activity compared to the WT control when grown under short day conditions (Alcázar et al., 2005). Finally, transgenic Lotus plants expressing oat ADC had ~ 14-fold and ~36-fold higher ADC activity than WT under non-stressed and drought stressed conditions, respectively (Espasandin et al., 2014). However, many of these ADC overexpressing lines were phenotypically compromised. For example, *AtADC2* over expression in *A. thaliana* causes dwarfism (Alcázar et al., 2005), induced expression of oat ADC caused chlorosis and reduced root growth in tobacco (Masgrau et al., 1997), and over expression of carnation ADC in tobacco caused so much chlorosis that no plants survived to the next generation (Chang et al., 2000). These findings indicate that very high levels of ADC over expression can be detrimental for normal plant development and physiology.

While the tomato and *M. polymorpha adc-box* mutants in this study did not have such dramatic increases in ADC-activity as these transgenic ADC-overexpressing lines, they may provide unique benefits to study the role of ADCs in plants. In both tomato and *M. polymorpha*, *adc-box* mutants had no discernible phenotype under non-stressed conditions (Figure 36, Figure 38), which vastly contrasts the phenotypes reported in the transgenic lines mentioned above. As the native genomic context and transcriptional control of the *ADC* genes have been maintained in the *adc-box* mutants, and as under both non- and cold-treated conditions no increase in *SLADC1/2* transcription was observed (Figure 34), native regulation of *ADC* transcription following developmental cues or during stress responses likely remain intact in this mutant. However, *adc-box* mutants produce exaggerated levels of ADC protein and subsequent metabolic outputs compared to WT due to lack of translational repression (shown through increased ADC activity; Figure 31, Supplementary Figure 18, Supplementary Figure 19). Therefore, such *adc-box* mutants could be used to investigate the protective role of ADCs during various stress responses, without the detrimental phenotypes typically observed in constitutive overexpression lines (i.e., dwarfed, or chlorotic plants).

It is also possible that while the *adc-box* mutants have no observed phenotypic difference to WT under non-stressed conditions, they may be more resistant to various stresses due to higher levels of ADC proteins and downstream stress-mitigating metabolites. If this is the case, *adc-box* mutants may be more reliable during turbulent environmental changes and therefore be agriculturally valuable lines. Yet, further phenotypic analysis is required to determine if the *adc-box* mutants are more resistant to persisting and possible multiple stresses (e.g., drought, temperature, or pathogen attack) or if stress-induced *ADC* transcription and excessive ADC protein levels in the *adc-box* mutants may negatively impact their long-term phenotype and agricultural output.

It is unlikely that abiotic or biotic stress resistant *adc-box* mutants would have been previously identified via traditional breeding or screening methods, such as crossings, mutagenic screens, or T-DNA insertion libraries (Page and Grossniklaus, 2002). So far, very little sequence variation has been found in *ADC-box* sequences of closely related species (e.g., Solanaceae species; Figure 4; Supplementary Figure 1; Wu et al., 2019), nor have any ADC sequences lacking an *ADC-box* been found, meaning there is likely little available germplasm with natural *ADC-box* variations. Additionally, chemical- or radiation-induced mutagenic screens for stress resistant plants are unlikely to identify *adc-box* mutants as these treatments typically generate SNPs, which are unlikely to disrupt the larger angiosperm *ADC-box* structure significantly to be identified in a screen. Finally, T-DNA insertion libraries are also unlikely to identify an *adc-box* mutant as it is likely translation of ADC would be massively compromised by insertion of a T-DNA into the 5'UTR, producing an ADC knock down or knock out line, rather than an ADC over expressor. Therefore, targeted approaches to mutate *ADC-boxes* in crop plants (i.e., CRISPR-based approaches) would be required to develop lines with mis-regulated ADC translation. Such *adc-box* mutant lines may become valuable agricultural tools for boosting PA-related plant stress responses, whilst maintaining control over exactly when and where this occurs due to the intact native *ADC* transcriptional regulation. In summary, *adc-box* mutants, both those generated here and in other species in the future, may improve the community's understanding of the role of ADCs on PA production, as well as be valuable agricultural assets.

4.5 Investigating the translational regulation of ADCs has shed new light on how *R. solanacearum* may benefit from RipTAL activation of *ADCs*.

4.5.1 RipTAL induction of host ADCs may lead to accumulation of phenolamides.

R. solanacearum injects RipTAL effectors into its host plants to induce *ADC* transcription, resulting in a boost in ADC activity and levels of agmatine and putrescine (Wu et al., 2019; Gallas et al., 2023). During this work, the method for measuring ADC activity and levels of PA-related metabolites with LCMS has been greatly optimised and expanded, enabling measurement of more metabolites than in these original publications. Through conducting LCMS experiments on tomato *adc-box* mutants, *adc cds* mutants, as well as *N. benthamiana* leaves transiently over expressing SIADC proteins, more knowledge of the impact of ADCs on

PA metabolism has been gained. Such knowledge can be transferred to understanding how RipTAL induction of *ADCs* may impact the wider PA network to benefit *R. solanacearum*. During this work it was consistently observed that relative levels of phenolamides were dramatically increased upon heightened ADC activity and reduced with lower ADC activity. In cold-treated adult leaves of $\Delta sladc1/2$ -*box* mutant, which had 10-fold higher ADC activity compared to WT, coumaroylagmatine and coumaroylputrescine levels increased 5.5- and 1.7-fold compared to WT, respectively (Figure 33). Cold-treated *adc2-box* mutant (SI2M_-13) seedling roots had 11.4-fold higher ADC activity than WT, as well as 32.4- and 4.6-fold increases in feruloylagmatine and feruloylputrescine, respectively (Figure 64). Transient over expression of SIADC1 in *N. benthamiana* leaves also led to increased relative levels of coumaroylputrescine, feruloylputrescine, cinnamoylputrescine, and coumaroylspermidine (Figure 46). Finally, reduction of ADC activity in the *adc cds* mutants led to significantly reduced relative levels of coumaroylagmatine, coumaroylputrescine, feruloylagmatine, feruloylputrescine, and cinnamoylputrescine by 12-, 8-, 13-, and 6-fold, respectively (Figure 40). Over expression of LDC in transgenic *A. thaliana* also showed increased levels of PAs and phenolamides (Shimizu et al., 2019), suggesting that boosting PA levels, regardless of the source, leads to increase phenolamide accumulation. Collectively these results demonstrate that phenolamide levels are greatly impacted by alterations to agmatine and putrescine levels caused by different ADC protein levels.

Based on these findings, it is likely that RipTAL induction of host *ADCs* also leads to increased phenolamides. In fact, preliminary data from pepper leaves infiltrated with *Xanthomonas* carrying Brg11 found increased relative levels of coumaroylagmatine and coumaroylputrescine (Niels Gallas, unpublished). It is therefore possible that manipulation of host phenolamide levels may be the purpose of RipTAL induction of *ADCs*. Phenolamides typically protect plants against biotic stresses through two main mechanisms: as antimicrobial compounds and reinforcement of the secondary cell wall (Roumani et al., 2021). Hypothesised mechanisms for how *R. solanacearum* may benefit from boosting phenolamides via RipTAL induction of host *ADCs* despite their role in biotic stress are outlined in the sections below.

4.5.2 RipTAL-induction of host ADCs to produce anti-microbial compounds that inhibit growth of niche competitors.

Release of HCAs and their conjugated phenolamides is an antimicrobial strategy used by plants to reduce growth of pathogens. Previously, phenolamides have been found to be upregulated during infection of various plant pathogens and cause direct inhibition of their growth *in vitro*

(Roumani et al., 2021). These include bacterial pathogens *Xanthomonas campestris*, *X. oryzae* and *Pseudomonas syringae* on both Solanaceae species and rice (Newman et al., 2001; Von Roepenack-Lahaye et al., 2003; Zacaes et al., 2007; Morimoto et al., 2018), as well as a vast number of fungal pathogens infecting monocot crops such as rice, wheat, and barley (Morimoto et al., 2018; Ube et al., 2019a; Ube et al., 2019b). Plants also release *de novo* synthesised HCAs into the rhizosphere in response to pathogen attack (Lanoue et al., 2010a; Lanoue et al., 2010b; Wallis and Chen, 2012). While it seems counterintuitive for *R. solanacearum* to induce host phenolamide levels when they have antimicrobial actions on a diverse range of plant pathogens, this boost may benefit *R. solanacearum* by either reducing its need to compete with other niche microbes or reducing levels of other more potent antimicrobial compounds.

For a successful infection, *R. solanacearum* must compete with various other microbes in the soil that associate with or near the host's roots, as well as overcome the plant's chemical and physical defences (Xue et al., 2020). If *R. solanacearum* were able to inhibit the growth of these other microbes by inducing host phenolamides, then it may achieve a growth advantage over its niche competitors. Such a ternary interaction was initially hypothesised by Wu et al., 2019, yet the antimicrobial compound they suggested was putrescine, rather than phenolamides, which have direct antimicrobial activities. However, such a strategy would only be viable if *R. solanacearum* were more resistant to the antimicrobial activity of phenolamides than its competitors. Lowe et al., 2015 found that many diverse *R. solanacearum* strains carry a gene cluster responsible for degrading plant derived HCAs (i.e., *p*-coumarate, caffeate, and ferulate), which is rarely found in soil microbes. Degradation of these HCAs was found to be important during the early stages of infection enabling *R. solanacearum* entry into the roots. Additionally, genes encoding multidrug efflux pumps that protect *R. solanacearum* from toxic compounds such as caffeate are also important for infection (Brown et al., 2007). It is therefore plausible that *R. solanacearum* has multiple strategies to protect itself from HCAs, strategies which may also work for related metabolites such as phenolamides, providing it with an advantage over its competing niche microbes.

To test this hypothesis in the future, the transgenic ethanol inducible lines expressing GFP-tagged Brg11 or the $\Delta sladc1/2$ -box mutants generated in this work (section 3.6.3), both of which lead to ADC over expression, could be used to analyse the impact of ADC over expression on the microbiome. To then determine which metabolites that are increased upon ADC over expression affect the microbiome and are responsible for giving *R. solanacearum* a competitive growth advantage, *R. solanacearum* and any soil microbes found to have ADC-dependent differential growth could be grown *in vitro* in media containing such metabolites.

As the LCMS method of measuring PAs enables quantification of *in planta* metabolite concentrations, microbes could be grown in physiologically relevant concentrations of each metabolite. Through this method, any metabolites that do not impact *R. solanacearum* growth, but impact growth of other microbes, could be identified as RipTAL-induced ternary interactor metabolites.

Alternatively, *R. solanacearum* may induce host ADCs by RipTALs to boost phenolamide levels, causing subsequent changes to other metabolic pathways. For example, RipTAL-induced increases mono- and polyamine levels may stimulate HCT enzyme activity due to a higher substrate availability, thereby increasing phenolamide levels. Yet, while PAs are highly abundant due to increased ADC activity, levels of HCAs, the other HCT substrate, may not be as high, causing a drain in the pool of available HCA substrates. This reduction in the plant's HCA pool could benefit *R. solanacearum* by reducing the energy required to degrade or export these toxic compounds. Previous assays analysing bacterial growth in leaves or disease progression following soil drenching and root wounding may not have observed this phenomenon if such toxic compounds are not present in high levels in the leaves or if wounding the roots alters the plants' ability to synthesise such compounds. This hypothesis could be tested using ADC over expressing plants (i.e., transgenic ethanol inducible lines expressing GFP-tagged Brg11 or the $\Delta sladc1/2$ -box mutants; section 3.6.3), where, in a non-wounding, infection scenario, *R. solanacearum* would be expected to more easily infect ADC over expressing plants compared to the WT lines.

4.5.3 *RipTAL-induction of host ADCs to alter physical barrier formation and facilitate entry into host vasculature.*

Physiochemical barriers are a vital element of plants' defence repertoire against pathogens. As soil-borne pathogens that aim to colonise the vasculature of their hosts, *R. solanacearum* must pass through these physiochemical barriers on their journey from the soil to their hosts xylem (Kashyap et al., 2021; Shi et al., 2023; Jian et al., 2024). Four major barriers for *R. solanacearum* have been reported by Planas-Marques et al., 2020, which must be overcome for a successful infection to occur: root colonisation, circular invasion of the vasculature bundle, radial spread through the cortex, and vertical movement up the plant vascular. To restrict pathogen entry and horizontal and radial movement in the root, plants deposit lignin and suberin physiochemical barriers, which have previously been found to be important in resistance against *R. solanacearum* infection (Ishihara et al., 2012; Ferreira et al., 2017; Kashyap et al., 2021; Kashyap et al., 2022; Shi et al., 2023). Both lignin and suberin are

partially made up of phenolic compounds derived from the phenylpropanoid pathway (i.e., hydroxycinnamoyl alcohols) and aliphatic polymers (Boerjan et al., 2003; Vishwanath et al., 2015; Xie et al., 2018; Serra and Geldner, 2022). Due to these physiochemical barriers being such an important hurdle to overcome in *R. solanacearum* infection, it is possible that RipTAL-induced ADC overexpression is used to divert HCA substrates away from lignin and suberin synthesis by sequestering them into phenolamides. If this is the case, the plant would have a reduced capacity to form these physiochemical barriers near cells that are injected with RipTALs during infection, easing the journey of *R. solanacearum* into the xylem.

Again, to determine if ADC over expression results in changes to suberin and/or lignin deposition, ADC over expression lines could be used (i.e., transgenic ethanol inducible lines expressing GFP-tagged Brg11 or the $\Delta sladc1/2$ -box mutants; section 3.6.3). Various histochemical stains exist that label suberin or lignin in plant tissue (Ursache et al., 2018) and enable visualisation by fluorescence microscopy. If ADC over expression in the root leads to reduced deposition of physical barriers, relatively lower staining would be observed compared to a WT plant.

In summary, advances in LCMS measurement of PAs and related metabolites have provided new hypothesised mechanisms for how *R. solanacearum* might benefit from a RipTAL-induced boost in host PAs. Transgenic and CRISPR/Cas9-mutated lines generated in this work will enable investigations into these hypotheses, hopefully leading to the elucidation of the role of RipTALs and why they have targeted host ADCs for the past ~180 million years.

4.6 Conclusion

This work aimed to investigate how plant ADC translation is regulated by highly conserved 5'UTR elements and to develop new approaches to investigate how *R. solanacearum* benefits from inducing ADC overexpression via a conserved RipTAL effector protein. To determine *in planta* levels of ADC proteins and concentrations of PA-related metabolites, an LCMS-based assay was employed. Optimisation of this assay increased measured ADC activity levels in WT tomato leaves by ~600-fold, as well as increasing the number of metabolites whose concentration could be measured from 5 up to 11. This, in addition to measuring relative levels of phenolamides, vastly improved our ability to study the PA metabolic network. Using this optimised LCMS method, CRISPR/Cas9-induced tomato, *A. thaliana*, and *M. polymorpha* *adc-box* mutants were analysed and mutants with increased ADC activity, agmatine, and putrescine levels compared to WT were found in all three species. This demonstrated the

repressive role of the *ADC-box* on ADC translation *in planta* and that this role has been conserved throughout evolution since the first land plants. RNA secondary structures of the *SLADC1* and *SLADC2* 5'UTRs, derived from SHAPE analysis, indicated that the *SLADC-boxes* form a triple stem loop structure. By combining information from both ADC activity and PA levels of different *SLADC1/2-box* mutants and these *SLADC1/2-box* RNA secondary structures, the central *ADC-box* hairpin was identified as an essential element for controlling ADC translation. Interestingly, the sequence encoding the central hairpin is conserved in gymnosperms and any CRISPR/Cas9-induced mutations in the *MpADC-box* disrupting the stem of this hairpin resulted in higher ADC levels compared to mutations elsewhere in the sequence. Overall, this indicates that the central hairpin possibly evolved first to regulate ADC translation, and angiosperms have later evolved a larger triple hairpin structure. However, exactly how these hairpin structures can control ADC translation rates, particularly how this mechanism may be sensitive to cellular PA needs, remains to be elucidated.

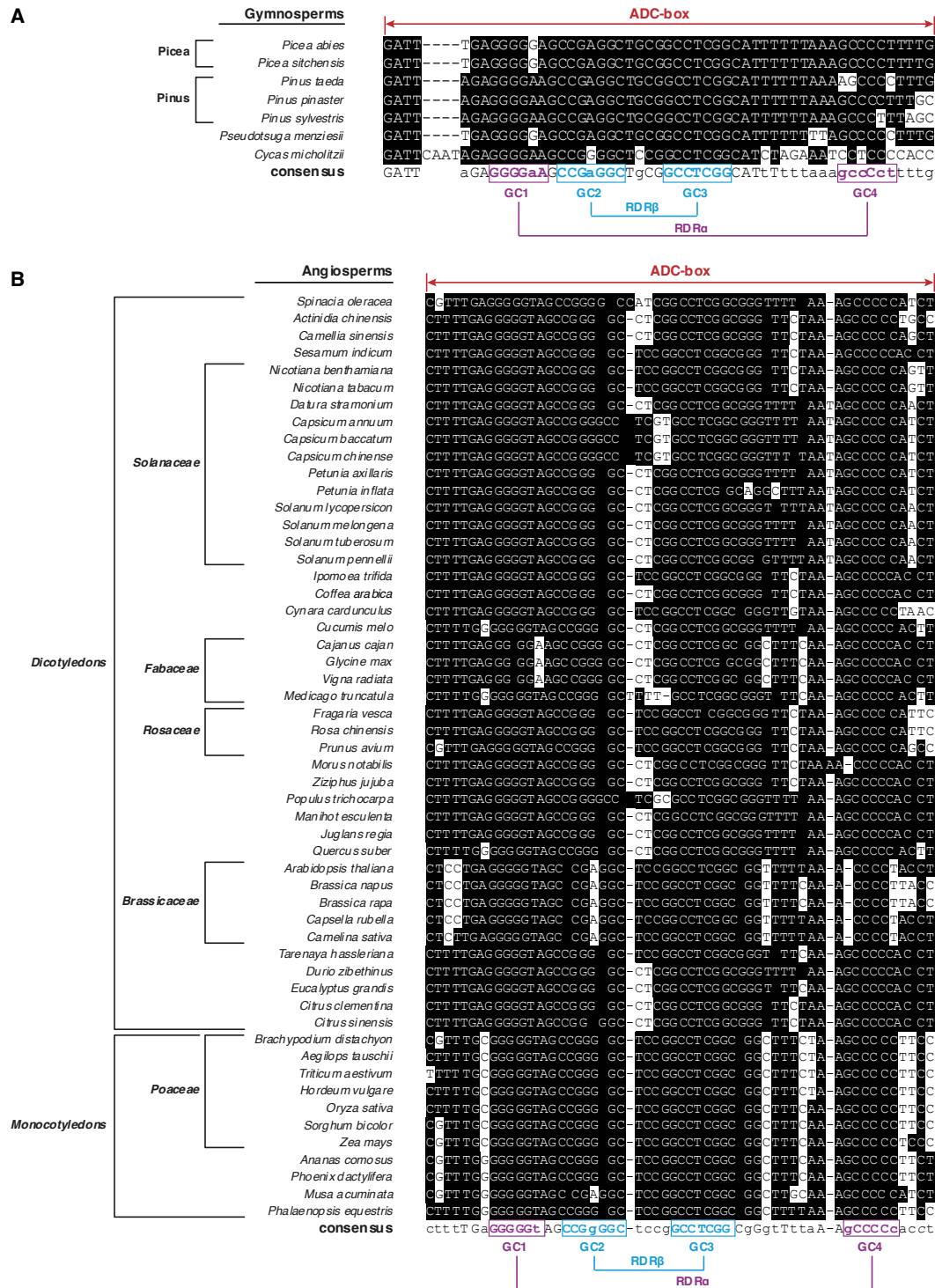
The *ADC* uORF, which is conserved in terms of its small size and position in the 5'UTR relative to the *ADC-box*, was also investigated in this work through CRISPR/Cas9 mutagenesis, LCMS measurements, and phenotypic analysis. Mutating the tomato *ADC* uORFs reduced ADC activity levels following cold treatment, while the non-treated $\Delta uorf1/2$ double mutant had a shorter phenotype with fewer flowers and leaves compared to the WT and $\Delta adc1/2-box$ mutant, which had no discernible phenotypic difference to WT. These findings, although preliminary, have suggested a mechanistic model whereby the *ADC* uORF may be important for maintaining ADC translation during periods of stress and/or development. Collectively, these results suggest that ADC translation in plants may be both positively and negatively regulated by the uORF and *ADC-box*, respectively, to respond to both cellular stresses and PA levels; however, this still needs to be confirmed.

Analysis of the tomato *adc cds* mutants alongside the *adc-box* mutants highlighted the role of ADCs in PA synthesis and the wider PA network, including phenolamides. Low or high ADC levels, generated from the *adc cds* or *adc-box* mutants respectively, resulted in lower or higher phenolamide levels. This finding not only demonstrated the impact of ADC as a key regulatory enzyme in PA pathway flux, but also gave new insights into how *R. solanacearum* may benefit from increasing ADC levels via RipTAL effectors. We hypothesise that RipTAL-induced ADC levels cause increased PA conjugation into phenolamides, which lowers the HCA pool, and consequently reduces available substrates to form anti-microbial physical barriers in the root. Alternatively, increased phenolamide levels may inhibit growth of niche microbes, giving *R. solanacearum* a competitive advantage to infect plants.

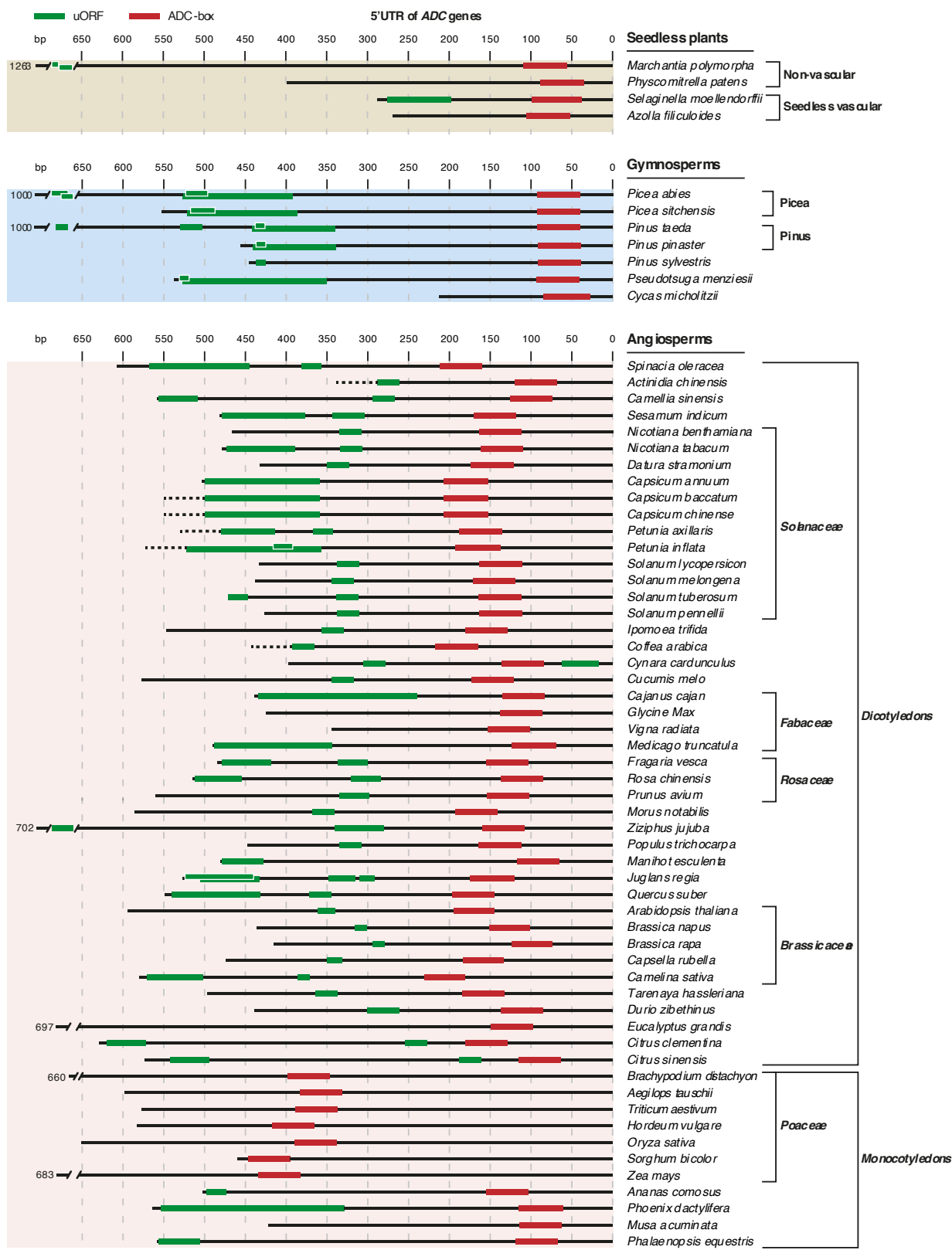
Overall, this work has expanded the current knowledge on plant ADC translational regulation, the impact of ADCs on the wider PA metabolic network, as well as providing hypotheses as to how *R. solanacearum* may benefit from a RipTAL-induced boost in ADC levels.

5. Supplementary figures and tables

5.1 Introduction



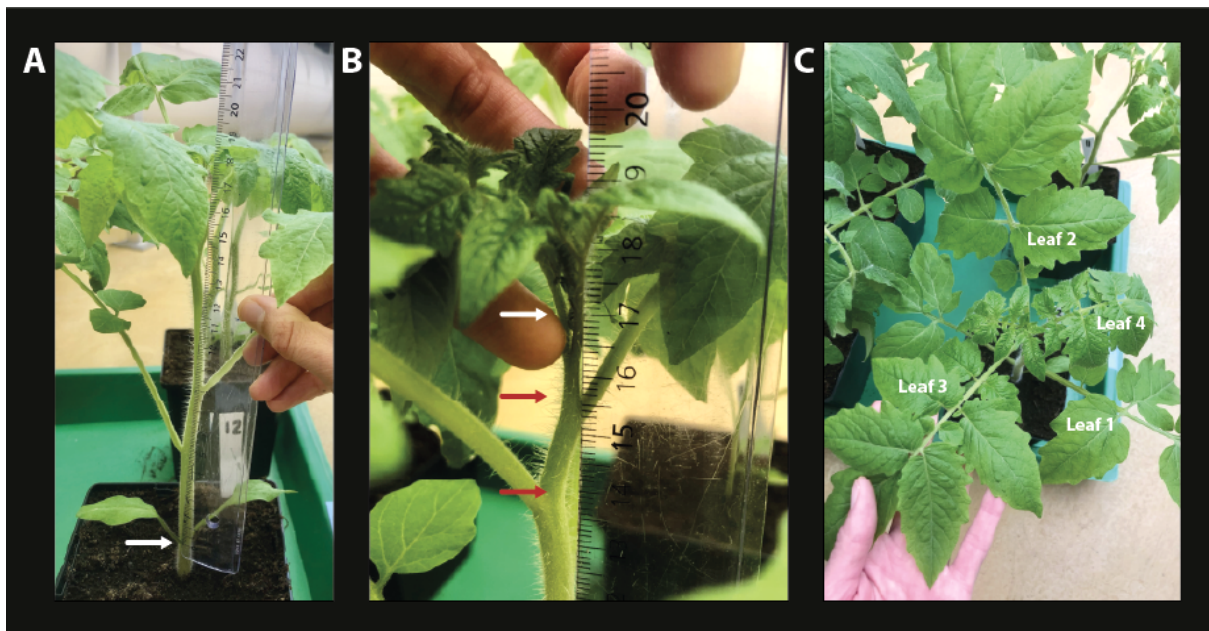
Supplementary Figure 1: ADC-box sequences from gymnosperms (A) and angiosperms (B) were aligned. Wu et al., 2019 supplemental figure 4.



Supplementary Figure 2: Schematic alignment of *ADC* 5'UTRs, highlighting the *ADC-box* and uORFs.

ADC 5'UTRs were aligned, and a schematic generated to show the conserved *ADC-box*, as well as the presence and placement of a uORF of the transcript relative to the *ADC-box* position. Figure taken from Wu *et al.*, 2019 supplemental figure 5.

5.2 Materials and Methods



Supplementary Figure 3: How to phenotype adult tomato plants.

30, 33, 36, 39, and 42 days after sowing seeds, plant height, internode length, leaf number, and flower number were determined for each biological replicate ($n = 20$). Plant height was measured by holding the 0 cm mark of a ruler to the cotyledons (white arrow; **A**) and then measuring the height until meristem where new leaves emerge, i.e., 17 cm in (white arrow; **B**). The internode length was measured as the length between emerging leaves (red arrows; **B**). Leaf number only included adult and mature leaves; this did not include the cotyledons or any immature leaves (e.g., first leaf in **A**). Tomato in figure **C** has four leaves.

Supplementary Table 1: Primers used in this work

Name	Sequence 5'-3'	Description	Project	Reference:
ADC1 gRNA_26-45	GCGGTTAAAGTTGTATGGTGGTTTTAGAGCTAGAAATAGCAAG	CRISPR mutagenesis of SIADC1 uORF gRNA	CRISPR mutagenesis uORF TOMATO	ESR (this work)
ADC1 gRNA_226-245	GCCGGGGCTCTGGCCTCGCGTTTTAGAGCTAGAAATAGCAAG	CRISPR mutagenesis of SIADC1 ADC box (GC3) gRNA	CRISPR mutagenesis ADCbox TOMATO	ESR (this work)
ADC1 gRNA_260-279	GACCAATGTTGTAATAGCTGGGTTTTAGAGCTAGAAATAGCAAG	CRISPR mutagenesis of SIADC1 ADC box (GC4) gRNA	CRISPR mutagenesis ADCbox TOMATO	ESR (this work)
ADC2 gRNA_52-71	GAACACCATACAGATATTAAGTTTTAGAGCTAGAAATAGCAAG	CRISPR mutagenesis of SIADC2 uORF gRNA	CRISPR mutagenesis uORF TOMATO	ESR (this work)
ADC2 gRNA_250-272	GAGCCGGGGCCTCGGCCTCGGGTTTTAGAGCTAGAAATAGCAAG	CRISPR mutagenesis of SIADC2 ADC box (GC3) gRNA	CRISPR mutagenesis ADCbox TOMATO	ESR (this work)
A.thal.ADC2_gRNA628 (F)	attgCAAATCCACTTCTGCATGGT	Mutate start codon of A1ADC2 uORF_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 uORF	ESR (this work)
A.thal.ADC2_gRNA628 (R)	aaacACCATGCAGAAAGTGGAATTTG	Mutate start codon of A1ADC2 uORF_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 uORF	ESR (this work)
A.thal.ADC2_gRNA826 (F)	attgAACC GCCGAGGCCGGAGCCT	Mutate start of A1ADC2 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 box	ESR (this work)
A.thal.ADC2_gRNA826 (R)	aaacAGGCTCCGGCCTCGGCGGTT	Mutate start of A1ADC2 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 box	ESR (this work)
A.thal.ADC2_gRNA838 (F)	attgTAGGGGTTTAAACC GCCG	Mutate middle of A1ADC2 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 box	ESR (this work)
A.thal.ADC2_gRNA838 (R)	aaacCGGCGGTTTTTAAACCCCTA	Mutate middle of A1ADC2 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 box	ESR (this work)
A.thal.ADC2_gRNA813 (F)	attgGGAGCCTCGGCTACCCCTC	Mutate 2nd half of A1ADC2 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 box	ESR (this work)
A.thal.ADC2_gRNA813 (R)	aaacGAGGGGTAGCCGAGGCTCC	Mutate 2nd half of A1ADC2 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 box	ESR (this work)
A.thal.ADC1_gRNA841 (F)	attgTCGGGGCTAACCCCTCAGA	Mutate start of A1ADC1 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 box	ESR (this work)
A.thal.ADC1_gRNA841 (R)	aaacTCTGAGGGGTTAGCCCCGA	Mutate start of A1ADC1 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 box	ESR (this work)
A.thal.ADC1_gRNA840 (F)	attgTTCTGAGGGGTTAGCCCCG	Mutate middle of A1ADC1 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 box	ESR (this work)
A.thal.ADC1_gRNA840 (R)	aaacCGGGGCTAACCCCTCAGAA	Mutate middle of A1ADC1 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 box	ESR (this work)
A.thal.ADC1_gRNA873 (F)	attgGGGGAATTTGAAACCCGCCG	Mutate 2nd half of A1ADC1 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 box	ESR (this work)
A.thal.ADC1_gRNA873 (R)	aaacCGGGGGTTCAAATTCGCC	Mutate 2nd half of A1ADC1 box_gRNA	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 box	ESR (this work)
A.thal.ADC2_gRNA673 (F)	attgGTTGTGTTGGGTTCA GATT	2x gRNA to cut out about 80 nt from spacer A1ADC2	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 spacer	ESR (this work)
A.thal.ADC2_gRNA751 (F)	attgTCGGGAAGGGCTGCGAGAGG	2x gRNA to cut out about 80 nt from spacer A1ADC2	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 spacer	ESR (this work)
A.thal.ADC2_gRNA751 (R)	gtttCCTCTCGCAGCCCTTCCCGA	2x gRNA to cut out about 80 nt from spacer A1ADC2	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 spacer	ESR (this work)
RFP (TCTG) FWD primer	aaGGTCTCaCTGTATGGTGTCTAAGGGCGAAGA	Create RFP overhang to TCTG (BsaI)	Cloning for Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RFP (TTAG) REV primer	aaGGTCTCaGATTGGTGATGATATAAGAGCGT	Create RFP overhang to TTG (BsaI)	Cloning for Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RFP_BsaI-mut (FWD) (ESR40)	GAaACCTACGTCGAGCAG	Remove internal BsaI site from RFP CDS	Cloning for Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RFP_BsaI-mut (REV) (ESR41)	TTTGTGCGCCTCCTTGAT	Remove internal BsaI site from RFP CDS +phosphate	Cloning for Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RFP_Bpil-mut (FWD)	GAgGACGGGGCGTGCTG	Remove internal Bpil site from RFP CDS	Cloning for Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RFP_Bpil-mut (REV)	GTATGTGGTGACTCTCTCC	Remove internal Bpil site from RFP CDS +phosphate	Cloning for Brg11 mCerulean3/GUS reporter construct	ESR (this work)
A.thal.ADC2_gRNA673 (R)#2	aaacAATCTGAACCCAACAACAAC	2x gRNA to cut out about 80 nt from spacer A1ADC2	CRISPR mutagenesis in <i>A. thaliana</i> - ADC2 spacer	ESR (this work)
RipTAL IV-2 EBE_downstream_F	gtagccgggTCATCCCTTTCTCTTTTC	Add RipTALIV-2 EBE into Bs3 promoter (downstream of Brg11 EBE)	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
A.thal.ADC1_gRNA553 (F)	attgAGATGGTGAGAAATGCATGA	Mutate start codon of uORF A1ADC1	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 uORF	ESR (this work)
NEW_RipTALIV-2_EBE_dwn_R	cccccctcTAACCTGAAGTTGTGAGGAT	Add RipTALIV-2 EBE Bs3 promoter (downstream of Brg11 EBE) +phosphate	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
SIADC1_F (ESR61)	ATATACCCCTCTTATCTCTCGC	Amplify region 637bp of SIADC1 (incl. start of CDS)	Genotyping CRISPR tomato mutants	ESR (this work)
SIADC1_R (ESR62)	GTGGAAAGGAAGGAATAGCC	Amplify region 637bp of SIADC1 (incl. start of CDS)	Genotyping CRISPR tomato mutants	ESR (this work)
SIADC1_F_seq (ESR63)	ACTTTGTTAGATAAGGCCCA	Sequencing primer for PCR SIADC1 (FWD)	Genotyping CRISPR tomato mutants	ESR (this work)
SIADC2_F (ESR64)	ACTCCAAGAAAGAAGCAT	Amplify region 621bp of SIADC2 (incl. start of CDS)	Genotyping CRISPR tomato mutants	ESR (this work)

SIADC2_R (ESR65)	TCCCAAGAAAAGGCATAGC	Amplify region 621bp of SIADC2 (incl. start of CDS)	Genotyping CRISPR tomato mutants	ESR (this work)
SIADC2_F_seq (ESR66)	AAAGTGAGAGAGTTTCGATG	Sequencing primer for PCR SIADC2 (FWD)	Genotyping CRISPR tomato mutants	ESR (this work)
A.thal.ADC1_gRNA553 (R) (correct)	aaacTCATGCATTTCTCACCATCT	Mutate start codon of uORF (corrected rev primer)	CRISPR mutagenesis in <i>A. thaliana</i> - ADC1 uORF	ESR (this work)
SIADC1_R_seq (ESR72)	CAGATTCTACACAACAACC	Sequencing primer for PCR SIADC1 (REV)	Genotyping CRISPR tomato mutants	ESR (this work)
SIADC2_R_seq (ESR73)	AAGCGTCTACACAACAAC	Sequencing primer for PCR SIADC2 (REV)	Genotyping CRISPR tomato mutants	ESR (this work)
T2A-GUSi_F	ACATGCCGGTGACGTGCGAGGAGAATCCTGGACCTATGGTAGATCTGAGGGTAAA	SDM T2A peptide 5' to GUS ATG	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
T2A-GUSi_R+Bsal (aagg)	TAGCAGACTTCTCTGCCCTCTCCGCTTCCcttTGAGACCATCCTCTGCAGTC	SDM T2A peptide 5' to GUS ATG & Bsal overhang (AAGG) +phosphate	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RiboTrap_genotyping_F	GACCACATGAAGCAGCAC	Genotyping RiboTrap transgenic tomatoes (Basic internal GFP primer)	Ribotrap	ESR (this work)
RiboTrap_genotyping_R	GCTCAGGTAGTGTTGTC	Genotyping RiboTrap transgenic tomatoes (Basic internal GFP primer)	Ribotrap	ESR (this work)
mCerulean3 #2_internal(#2)_F	GGAACCTCTCTTTTGTGTC	Sequence from internal mCerulean3 construct #2 - SECOND PRIMER	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
RFP (TTAG) REV primer - New	aaGGTCTCaGATTTCAAAAGGTGATGATATAAAGAGCGT	Create RFP overhang to TTGC (Bsal) and insert RFP stop codon	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
At.ADC1_F (ESR87)	GAATGAGCAGGAAGCTTGA	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC1_F_seq (ESR88)	AATCACAAATAGGGCCCA	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC1_R (ESR89)	CAGACGACGGTGAAAGAT	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC1_R_seq (ESR90)	AGGTATCGATGGGAGTATCA	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC2_F (ESR91)	CGCCTTCTCTTCTTCTCT	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC2_F_seq (ESR92)	AAAGTGAGAGAGTTTCGATG	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC2_R (ESR93)	GATAAAACATCACCGGCG	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
At.ADC2_R_seq (ESR94)	TAGCGAGGAGGTACAAAGGA	Genotyping CRISPR <i>A. thaliana</i> mutants	Genotyping CRISPR <i>A. thaliana</i> mutants	ESR (this work)
mCerulean3 #3_F_seq	TATGGTGCTCCTGGAATTTG	LIII construct sequencing	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
35Sp_R_seq (ESR96)	CATCTGTGGTTAGCATTCT	LIII construct sequencing	Brg11 mCerulean3/GUS reporter construct	ESR (this work)
SI.ADC1_F_Bsal(B)	GGGTCTCTTCTGCCTAAACGGAACCTACC	LUC/REN - SI.ADC1_5'UTR (B-ATGG)	LUC/REN Assay	ESR (this work)
SI.ADC1_R_Bsal(ATGG)	CGGTCTCACCATCTCGTAAAATCTTCACAA	LUC/REN - SI.ADC1_5'UTR (B-ATGG)	LUC/REN Assay	ESR (this work)
SI.ADC2_F_Bsal(B)	GGGTCTCTTCTGAGCTGTAGTTTCACGTTCTC	LUC/REN - SI.ADC2_5'UTR (B-ATGG)	LUC/REN Assay	ESR (this work)
SI.ADC2_R_Bsal(ATGG)	CGGTCTCACCATCTCTTCCCCGATCTGT	LUC/REN - SI.ADC2_5'UTR (B-ATGG)	LUC/REN Assay	ESR (this work)
SI.ADC1_uORF.KO_F	GTGGGGGTGTTGTTTGAAT	LUC/REN - SI.ADC1_uORF knockout	LUC/REN Assay	ESR (this work)
SI.ADC1_uORF.KO_R	gtTACAACTTTTAACCGGAATAG	LUC/REN - SI.ADC1_uORF knockout + Phos	LUC/REN Assay	ESR (this work)
SI.ADC2_uORF.KO_F	GTGTTCTCTTTTTGAATTCTAG	LUC/REN - SI.ADC2_uORF knockout	LUC/REN Assay	ESR (this work)
SI.ADC2_uORF.KO_R	gtTACAGATATTAATGGAGAAGAAG	LUC/REN - SI.ADC2_uORF knockout + Phos	LUC/REN Assay	ESR (this work)
SI.ADC1_uORF_F	TCCGACCAATTTCTTCTTCTC	Tomato CRISPR T1 genotyping	General Tomato ADC 5'UTR PCRs	ESR (this work)
SI.ADC1_uORF_R	TCCCCACAACGACTTCAG	Tomato CRISPR T1 genotyping	General Tomato ADC 5'UTR PCRs	ESR (this work)
SI.ADC1_ADC1box_F	AAGTCGTTGTGGGATATTC	Tomato CRISPR T1 genotyping	General Tomato ADC 5'UTR PCRs	ESR (this work)
SI.ADC2_uORF_F	TTACAGCCGCTAGGTTTC	Tomato CRISPR T1 genotyping	General Tomato ADC 5'UTR PCRs	ESR (this work)
SI.ADC2_uORF_R	CAAAAAATCCCACCCTGA	Tomato CRISPR T1 genotyping	General Tomato ADC 5'UTR PCRs	ESR (this work)
SI.ADC2_ADC2box_F	GTGTTAGAGGGTGATTATCT	Tomato CRISPR T1 genotyping	General Tomato ADC 5'UTR PCRs	ESR (this work)
SI.ADC1_boxKO_F	AGCTATTACAACATTGGTCA	LUC/REN cloning for SI.ADC1 5'UTR LI (SDM ADC box KO)	LUC/REN Assay	ESR (this work)
SI.ADC1_boxKO_R_+P	AAAAAGAAGCAAAAAAGAAGCTTAAT	LUC/REN cloning for SI.ADC1 5'UTR LI (SDM ADC box KO)	LUC/REN Assay	ESR (this work)
SI.ADC2_boxKO_F	AACTATTACAACATTGGGAAG	LUC/REN cloning for SI.ADC2 5'UTR LI (SDM ADC box KO)	LUC/REN Assay	ESR (this work)
SI.ADC2boxKO_R_+P	AAAAAGAAGCAAAAAACAAGTGA	LUC/REN cloning for SI.ADC2 5'UTR LI (SDM ADC box KO)	LUC/REN Assay	ESR (this work)
SI.ADC1_C540A_F	TGACAGTGATGGAAAGATCG	Catalytically dead SI.ADC1 protein (C540A)	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC1_C540A_R	gcTGTCAAGTCGGATAGAATTCC	Catalytically dead SI.ADC1 protein (C540A) +Phos	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)

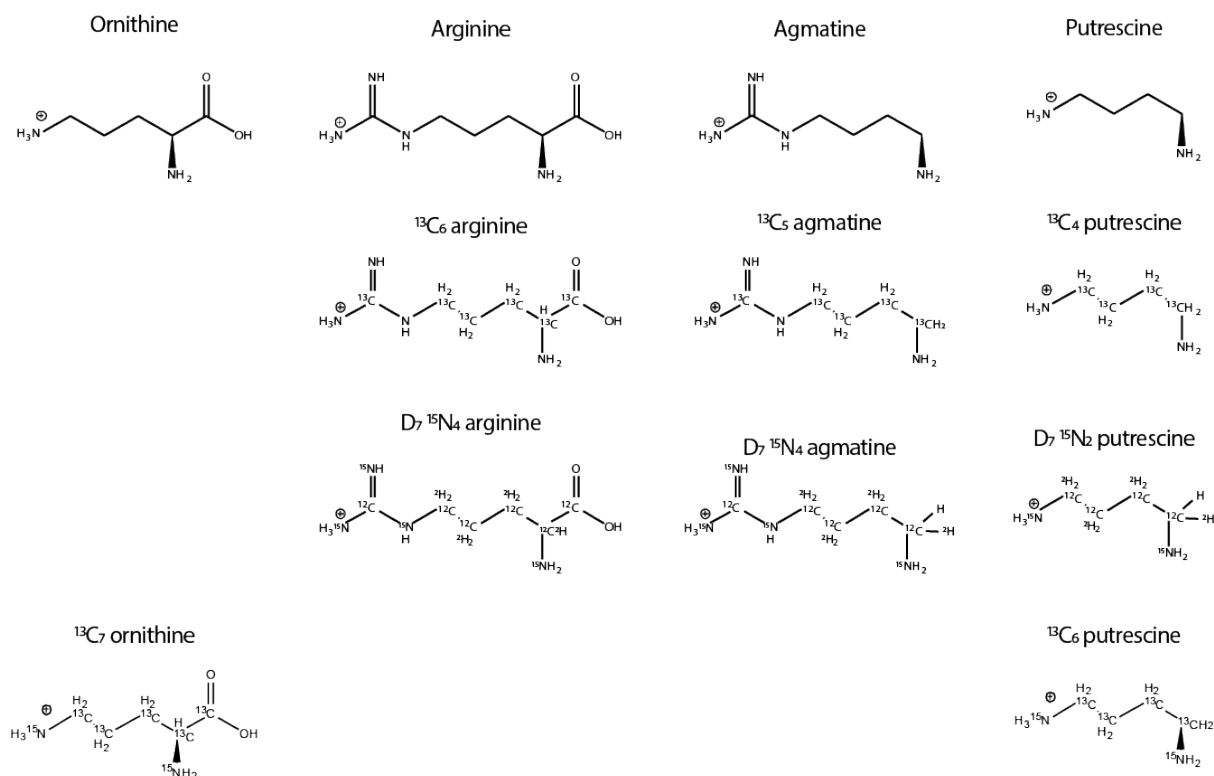
SI.ADC2_C548A_F	TGACAGTGATGAAAGGTTG	Catalytically dead SI.ADC2 protein (C548A)	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC2_C548A_R	gcCGTCAGGTCAGACAGTATT	Catalytically dead SI.ADC2 protein (C548A) +Phos	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC1_K149A_F	ATGCAATCAGGACAGGTTTC	Catalytically dead SI.ADC1 protein (K149A)	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC1_K149A_R	GCTACAGGATAAACACCTTGGTA	Catalytically dead SI.ADC1 protein (K149A) +Phos	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC2_K156A_F	ATGCAATCAAGATAGGTTCTGT	Catalytically dead SI.ADC2 protein (K156A)	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC2_K156A_R	GCCACCGGATAAACACCTTGG	Catalytically dead SI.ADC2 protein (K156A) +Phos	Overexpressing SIADC proteins (catalytically dead)	ESR (this work)
SI.ADC1_BsalC_F	gggtctcacaccATGCCTGCTTTAGGTTGT	Cloning tomato ADC proteins for LII GG cloning	Overexpressing SIADC proteins (WT)	ESR (this work)
SI.ADC1_BsalD_R	gggtctcacCTTCAAGCAACGCAGTAT	Cloning tomato ADC proteins for LII GG cloning	Overexpressing SIADC proteins (WT)	ESR (this work)
SI.ADC2_BsalC_F	gggtctcacaccATGCCGGCCTTAGGTTG	Cloning tomato ADC proteins for LII GG cloning	Overexpressing SIADC proteins (WT)	ESR (this work)
SI.ADC2_BsalD_R	gggtctcacCTTAGTAGGACCAATCTCATCTT	Cloning tomato ADC proteins for LII GG cloning	Overexpressing SIADC proteins (WT)	ESR (this work)
Add Bsal (B) for pGrDL_F	gaaggtctcatctgCTCGAGCTTTTGAAGACAA	Cloning pGreenII_DL_SPd (golden gate)	LUC/REN Assay	ESR (this work)
Add Bsal (B) for pGrDL_R	gaaggtctcacgcTAGCCCTTTGGTCTTCTG	Cloning pGreenII_DL_SPd (golden gate) +phosphate	LUC/REN Assay	ESR (this work)
SI.ADC1_F_Bsal (TGGT)	GGGTCTCTTggTCCTAAACGGAACCTCTACC	For pGrII A-x-B 35Sp-5'UTR-LUC - ADC1 400bp 5'UTR	LUC/REN Assay	ESR (this work)
SI.ADC1_R_Bsal (B)	CGGTCTCAGACTCGTAAATCTTCACAA	For pGrII A-x-B 35Sp-5'UTR-LUC - ADC1 400bp 5'UTR	LUC/REN Assay	ESR (this work)
SI.ADC1_F_Bsal (A) long	GGGTCTCTCGGACCCCTTCTATCTTCTCGC	For pGrII A-B 5'UTR-LUC - ADC1 581bp 5'UTR	LUC/REN Assay	ESR (this work)
SI.ADC2_F_Bsal (TGGT)	GGGTCTCTTggTAGCTGTTAGTTTCACGTTCTC	For pGrII A-x-B 35Sp-5'UTR-LUC - ADC2 400bp 5'UTR	LUC/REN Assay	ESR (this work)
SI.ADC2_R_Bsal (B)	CGGTCTCAGACTCTTCCCCGATCTGT	For pGrII A-x-B 35Sp-5'UTR-LUC - ADC2 400bp 5'UTR	LUC/REN Assay	ESR (this work)
35S SDM (TGGT)_F	ggTAGAGACCCcATCTAGATG	For mutating 35S pro to go into pGreenII with GCCG-TGGT overhangs	LUC/REN Assay	ESR (this work)
35S SDM (TGGT)_R	ACGTGTTTCTCTCCAATGA	For mutating 35S pro to go into pGreenII with GCCG-TGGT overhangs	LUC/REN Assay	ESR (this work)
pGrDL_Col PCR	TTGCGCAGATAATAGTTCGT	Cloning pGreenII_DL_SPd (golden gate)	LUC/REN Assay	ESR (this work)
LII_vector backbone_F	GAGTGGTGATTTTGTGCC	General cloning with Binder GG toolkit	General cloning and genotyping	Christine Krönauer (AG Lahaye)
LII_vector backbone_R	GATAAACCTTTTACGCC	General cloning with Binder GG toolkit	General cloning and genotyping	Christine Krönauer (AG Lahaye)
35S promoter_F	CATCGTGGAAAAAGAACAC	General cloning with Binder GG toolkit	General cloning and genotyping	Christine Krönauer (AG Lahaye)
CKP298	GACGCTTAGACAACCTTAATAACAC	General cloning with Binder GG toolkit	General cloning and genotyping	Christine Krönauer (AG Lahaye)
Cas9_F (ESR124)	GGCGGAGCAAGCCAGGAGGAA	Tomato CRISPR Cas9-free genotyping	Tomato CRISPR	Schenstnyi et al., 2022 Kyrylo Schenstnyi
Cas9_R (ESR125)	CTTGACAGCCGCCCCCATCCT	Tomato CRISPR Cas9-free genotyping	Tomato CRISPR	Schenstnyi et al., 2022 Kyrylo Schenstnyi
ScmADC2-F1	AATCGGCTTTTGACATGGCG	Genotype tomato adc2 knock out_F	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye) Wu et al., 2019
ScmADC2-R1	AAGGAGCTTTCTTGGGACCA	Genotype tomato adc2 knock out_R	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye) Wu et al., 2019
ScmODC1-F1	CCGTCTCAAAGGGAACAGAGA	Genotype tomato odc1 knock out_F	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye)
ScmODC1-R1	CTTGAGCTGTACGGAGGAGC	Genotype tomato odc1 knock out_R	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye)
Kana seq R1	GCCAAGCTCTTCAGCAATAT	Plant Kanamycin selection marker_FWD	General cloning and genotyping	Dousheng Wu (AG_Lahaye)
Nos P seq R1	GGATTGAGAGTGAATATGAG	Nos promoter_REV	General cloning and genotyping	Dousheng Wu (AG_Lahaye)
Kana seq F1	GCTTGCCGAATATCATGG	Plant Kanamycin selection marker_REV	General cloning and genotyping	Dousheng Wu (AG_Lahaye)
GUS R1	ATCAATTCACAGTTTTCGC	5' end of GUS gene_rev	General cloning and genotyping	Dousheng Wu (AG_Lahaye)
ScmADC1-F1	TACAGATACCCTTCTCACCA	Genotype tomato adc1 knock out_F	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye) Wu et al., 2019
ScmADC1-R1	TGTCATACCAAAATCAAGC	Genotype tomato adc1 knock out_R	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye) Wu et al., 2019
adc1 M F	GGCTTTTGATTATGCTGTTTC	Genotype tomato adc1 knock out_F	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye)
adc1 M R	CATACAAGAAGACCTCCGA	Genotype tomato adc1 knock out_R	LCMS analysis of PA coding sequence mutants	Dousheng Wu (AG_Lahaye)
GUS_F (DW696)	CTGATAGCGCGTGACAAAAA	GUS_FWD	General cloning and genotyping	Dousheng Wu (AG_Lahaye)
GUS_R (DW697)	TGAGCGTCGCAGAACATTAC	GUS_REV	General cloning and genotyping	Dousheng Wu (AG_Lahaye)
TIP41_2_F	GCTTAGGGTTGATGAGTGC	Tomato qPCR housekeeping primer	qPCR	Wu et al., 2019

TIP41_2_R	CTCTCCAGCAGCTTTCACG	Tomato qPCR housekeeping primer	qPCR	Wu et al., 2019
Sl.ADC1_F_qPCR	TAGCGAAAGTGGCAGAGCAA	Tomato qPCR of Sl.ADC1	qPCR	Wu et al., 2019 AG_Lahaye
Sl.ADC1_R_qPCR	AGGTATCGTACTCTCCGCGA	Tomato qPCR of Sl.ADC1	qPCR	Wu et al., 2019 AG_Lahaye
Sl.ADC2_F_qPCR	TTGGTCGCAAGAAAGCTCCT	Tomato qPCR of Sl.ADC2	qPCR	Wu et al., 2019 AG_Lahaye
Sl.ADC2_R_qPCR	TGGCCAGAATGCTTTGTCCT	Tomato qPCR of Sl.ADC2	qPCR	Wu et al., 2019 AG_Lahaye
LucNrev	CCTTATGCAGTTGCTCTCC	LUC/REN - seq for overhangs LII	LUC/REN Assay	Moyle et al., 2017
FWD_Sl.Actin pro	TGACAGATTGCGCCCTCCA	Tomato actin promoter (for pGrII colony PCR and sequencing)	LUC/REN	Moyle et al., 2017
JS1132 (REV)	AACGCTCTTTTCTCTTAGGT	Sequencing <i>A. thaliana</i> CRISPR destination vectors (pDGE)	CRISPR mutagenesis in <i>A. thaliana</i>	Ordon et al., 2020
M13_F	GTTGTAACGACGGCCAG	General cloning	General cloning	
M13_R	CAGGAAACAGCTATGACCATG	General cloning	General cloning	
PL69	TATCTCTCAGGCTCTCGAGG	Genotyping <i>M. polymorpha</i> ADC 5'UTR	Genotyping CRISPR <i>M. polymorpha</i> mutants	Pia Ellison (Masters thesis, 2020)
PL70	GGGCATGTTTGCTTCTGGG	Genotyping <i>M. polymorpha</i> ADC 5'UTR	Genotyping CRISPR <i>M. polymorpha</i> mutants	Pia Ellison (Masters thesis, 2020)

Supplementary Table 2: Final plasmids used in this work

Plasmid backbone	Insert:	Resistance:	Results section used in:	Generated by:
LIII p201N Cas9	Ubip:NeoR:Ubit_(U6p:gRNA8:U6t)x2_35Sp:NLS:3xGFP:35St_35Spx2:Cas9-NLS:Nost	Kan	CRISPR mutagenesis	ESR (this work)
LIII p201N Cas9	Ubip:NeoR:Ubit_(U6p:gRNA9:U6t)x2_35Sp:NLS:3xGFP:35St_35Spx2:Cas9-NLS:Nost	Kan	CRISPR mutagenesis	ESR (this work)
LIII p201N Cas9	Ubip:NeoR:Ubit_(U6p:gRNA10:U6t)x2_35Sp:NLS:3xGFP:35St_35Spx2:Cas9-NLS:Nost	Kan	CRISPR mutagenesis	ESR (this work)
LIII p201N Cas9	Ubip:NeoR:Ubit_(U6p:gRNA11:U6t)x2_35Sp:NLS:3xGFP:35St_35Spx2:Cas9-NLS:Nost	Kan	CRISPR mutagenesis	ESR (this work)
LIII p201N Cas9	Ubip:NeoR:Ubit_(U6p:gRNA12:U6t)x2_35Sp:NLS:3xGFP:35St_35Spx2:Cas9-NLS:Nost	Kan	CRISPR mutagenesis	ESR (this work)
LIII p201N Cas9	Ubip:NeoR:Ubit_U6p:gRNA225:U6t_35Sp:NLS:3xGFP:35St_35Spx2:Cas9-NLS:Nost	Kan	CRISPR mutagenesis	Dousheng Wu (AG_Lahaye)
pDGE347	U6p:gRNA628:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
pDGE347	U6p:gRNA826:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
pDGE347	U6p:gRNA838:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
pDGE347	U6p:gRNA841:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
pDGE347	U6p:gRNA840:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
pDGE347	U6p:gRNA873:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
pDGE347	U6p:gRNA553:U6t_RPS5Ap:zmCas9(12xintrons):RPS5At_Nosp:bastaR:Nost_OLE-1p:OLE-1-tagRFP:OLE1t	Spec	CRISPR mutagenesis	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:HA-SIADC1 (WT):35St	Spec	<i>LCMS optimisation; ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:MYC-SIADC2 (WT):35St	Spec	<i>LCMS optimisation; ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:HA-SIADC1 (K149A):35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:HA-SIADC1 (C540A):35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:HA-SIADC1 (K149A_C540A):35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:MYC-SIADC2 (K156A):35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:MYC-SIADC2 (C548A):35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 5-6 (AB120)	35Sp:MYC-SIADC2 (K156A_C548A):35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 4-5 (AB118)	35Sp:SIODC1-MYC:35St	Spec	<i>ADC-box mechanism</i>	Niels Gallas (AG Lahaye)
LIIa F 5-6 (AB120)	35Sp:HA-PBCV-1 DC:35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
LIIa F 4-5 (AB118)	35Sp:LUC:Nost_35Sp:REN:35St	Spec	<i>ADC-box mechanism</i>	ESR (this work)
pALICE01 (GG compatible)	T7p:HA-SIADC1 (WT):T7t	Amp	<i>ADC-box mechanism</i>	ESR (this work)
pALICE01 (GG compatible)	T7p:HA-SIADC1 (K149A_C540A):T7t	Amp	<i>ADC-box mechanism</i>	ESR (this work)
pALICE01 (GG compatible)	T7p:MYC-SIADC2 (WT):T7t	Amp	<i>ADC-box mechanism</i>	ESR (this work)
pALICE01 (GG compatible)	T7p:MYC-SIADC2 (K156A_C548A):T7t	Amp	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb	35Sp:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	Moyle <i>et al.</i> , 2017
pGrDL_SPb_GG	GG (A-B)_codb cassette CmR_LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pSoup		Tet	<i>ADC-box mechanism</i>	Hellens <i>et al.</i> , 2000
pGrDL_SPb_GG	35Sp:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb_GG	Bs3p:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb_GG	MASp:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb_GG	Lj Ubip:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb_GG	35Sp:SIADC1 5'UTR:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb_GG	35Sp:SIADC1 5'UTR ΔuORF:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)
pGrDL_SPb_GG	35Sp:SIADC1 5'UTR ΔADCbox:LUC:35St_Slactinp:REN:Nost	Kan	<i>ADC-box mechanism</i>	ESR (this work)

pGrDL_SPb_GG	35Sp:SIADC2 5'UTR:LUC:35St_Slactinp:REN:Nost	Kan	ADC-box mechanism	ESR (this work)
pGrDL_SPb_GG	35Sp:SIADC2 5'UTR ΔuORF:LUC:35St_Slactinp:REN:Nost	Kan	ADC-box mechanism	ESR (this work)
pGrDL_SPb_GG	35Sp:SIADC2 5'UTR ΔADCbox:LUC:35St_Slactinp:REN:Nost	Kan	ADC-box mechanism	ESR (this work)
LIIa F 4-5 (AB118)	35Sp:GUS (+inton):35St	Spec	ADC-box mechanism; Tools to study <i>Ralstonia</i>	Dousheng Wu (AG_Lahaye)
pGWB654	35Sp:Brg11-RFP:Nost	Spec	ADC-box mechanism; Tools to study <i>Ralstonia</i>	Dousheng Wu (AG_lahaye)
pGWB654	35Sp:RFP:Nost	Spec	Tools to study <i>Ralstonia</i>	Christine Krönauer (AG_Lahaye)
LIIa F 4-5 (AB118)	35Sp:RFP-LT16b-35St	Spec	Tools to study <i>Ralstonia</i>	Dousheng Wu (AG_lahaye)
pUC57	(Bsal_B)_NLS-mCerulean3 #1 (+intron)-linker_(Bsal_C)	Amp	Tools to study <i>Ralstonia</i>	ESR (this work)
pUC57	(Bsal_C)_NLS-mCerulean3 #2 (+intron)-linker_(Bsal_D)	Amp	Tools to study <i>Ralstonia</i>	ESR (this work)
pUC57	(Bsal_D)_NLS-(+intron)-mCerulean3 #3-NLS_(Bsal_E)	Amp	Tools to study <i>Ralstonia</i>	ESR (this work)
LIIa F 2-3 (AB114)	35Sp:mCerulean3 #123:35St	Spec	Tools to study <i>Ralstonia</i>	ESR (this work)
LIIa F 4-5 (AB118)	Bs3p(EBEs: RipTAL IV-2, Brg11, AvrBs3):mCerulean3x3:35St	Spec	Tools to study <i>Ralstonia</i>	ESR (this work)
LIIa F 4-5 (AB118)	Bs3p(EBEs: RipTAL IV-2, Brg11, AvrBs3):mCerulean3x2-T2A-GUSi:35St	Spec	Tools to study <i>Ralstonia</i>	ESR (this work)
LIIa R A-B (AB122)	Bs3p(EBEs: RipTAL IV-2, Brg11, AvrBs3):mCerulean3x2-T2A-GUSi:35St_35Sp:RFP-LTi6b:35St_Nosp:Neo:Nost	Kan	Tools to study <i>Ralstonia</i>	Tomato transformation
LIIa R A-B (AB122)	Bs3p(EBEs: RipTAL IV-2, Brg11, AvrBs3):mCerulean3x3:35St_35Sp:RFP-LTi6b:35St_Nosp:Neo:Nost	Kan	Tools to study <i>Ralstonia</i>	Tomato transformation
pBin	35Sp:alcR:Nost_alcAp:NLS-GFP-NLS:Nost_Nosp:NeoR:Nost	Kan	Tools to study <i>Ralstonia</i>	Robert Morbitzer (AG_Lahaye)
pBin	35Sp:alcR:Nost_alcAp:Brg11-GFP:Nost_Nosp:NeoR:Nost	Kan	Tools to study <i>Ralstonia</i>	Robert Morbitzer (AG_Lahaye)
pK7WG Trap	NbADC1p(350bp):His(x6)-FLAG-GFP-AtRPL18:tocst_Nosp:NeoR:Nost	Spec	Tools to study <i>Ralstonia</i>	Dousheng Wu (AG_lahaye)
pK7WG Trap	SIADC1p(350bp):His(x6)-FLAG-GFP-AtRPL18:tocst_Nosp:NeoR:Nost	Spec	Tools to study <i>Ralstonia</i>	Dousheng Wu (AG_lahaye)
pDSK602	Lacp:AvrBs3 T3SS-Hpx17-FLAG	Spec	Tools to study <i>Ralstonia</i>	Niklas Schandry (AG_lahaye)
pDSK602	Lacp:AvrBs3 T3SS-Brg11-FLAG	Spec	Tools to study <i>Ralstonia</i>	Niklas Schandry (AG_lahaye)
LIIa F 4-5 (AB118)	35Sp:GFP:35St	Spec	General use	Dousheng Wu (AG_lahaye)



Supplementary Figure 4: Isotopic variants used for ADC and ODC activity assays.

Normal ornithine, arginine, agmatine, and putrescine isotopes found in biological tissues (top) and heavy isotopic variants of each compound (bottom rows) are presented. Two heavy arginine isotopes were used in this work; their metabolised agmatine and putrescine variants are shown. One heavy ornithine was used; its heavy putrescine variant is shown (bottom right).

Supplementary Table 3: MRM transitions monitored for each compound.				
Compound:	Q1 mass (Da)	Q3 mass (Da)	Declustering potential (V)	Collision energy (V)
Arginine 1	175.1	158.0	50	20
Arginine 2	175.1	116.1	50	20
Agmatine 1	131.1	114.1	22	15
Agmatine 2	131.1	72.1	22	20
Putrescine 1	89.1	72.0	22	14
Putrescine 2	89.1	72.0	22	25
Acetylputrescine 1	131.1	114.1	22	15
Acetylputrescine 2	131.1	72.0	22	20
Spermidine 1	146.2	129.0	20	20
Spermidine 2	146.2	72.0	20	20
Spermine 1	203.2	112.1	48	15
Spermine 2	203.2	129.1	48	24
Cadaverine 1	103.1	86.0	40	15
Cadaverine 2	103.1	69.1	40	25
Ornithine 1	133.0	116.0	50	10
Ornithine 2	133.0	70.0	50	20
Citrulline 1	176.1	159.1	22	15
Citrulline 2	176.1	113.1	22	25
Glutamine 1	147.1	130.0	40	10
Glutamine 2	147.1	84.0	40	30
GABA 1	104.1	87.0	90	17
GABA 2	104.1	59.1	90	20
Isotopic variant transitions:				
D ₅ tryptophan (+5) 1	210.1	192.0	60	22
D ₅ tryptophan (+5) 2	192.1	150.1	100	22
¹³ C ₁ D ₂ citrulline (+3) 1	179.1	162.1	22	15
¹³ C ₁ D ₂ citrulline (+3) 2	179.1	116.1	22	20
¹³ C ₆ arginine (+6) 1	182.1	165.1	50	20
¹³ C ₆ arginine (+6) 2	182.1	122.1	50	20
¹³ C ₅ agmatine (+5) 1	136.1	119.1	22	15
¹³ C ₅ agmatine (+5) 2	136.1	76.1	22	20
¹³ C ₄ putrescine (+4)	93.1	76.1	22	14
D ₇ ¹⁵ N ₄ arginine (+11) 1	187.1	169.0	50	20
D ₇ ¹⁵ N ₄ arginine (+11) 2	187.1	125.0	50	20
D ₇ ¹⁵ N ₄ agmatine (+11) 1	142.1	124.1	22	20
D ₇ ¹⁵ N ₄ agmatine (+11) 2	142.1	80.1	22	20
D ₇ ¹⁵ N ₂ putrescine (+9)	98.1	80.1	22	14
¹³ C ₇ ornithine (+7) 1	141.1	123.1	50	10
¹³ C ₇ ornithine (+7) 2	141.1	76.1	50	20
¹³ C ₆ putrescine (+6)	95.1	77.1	22	14
Phenolamides*:				
Coumaroylagmatine 1	277.2	147.0	40	30
Coumaroylagmatine 2	277.2	114.1	40	25
Coumaroylputrescine 1	235.1	147.0	40	20
Coumaroylputrescine 2	235.1	89.0	40	20
Caffeoylagmatine	293.2	163.0	40	25
Caffeoylagmatine	293.2	114.0	40	30

Caffeoylputrescine 1	251.2	163.0	40	20
Caffeoylputrescine 2	251.2	89.0	40	20
Feruloylagmatine 1	307.2	177.0	100	25
Feruloylagmatine 2	307.2	114.0	40	30
Feruloylputrescine 1	265.2	177.0	40	20
Feruloylputrescine 2	265.2	89.0	40	20
Cinnamoylputrescine	219.2	131	40	20

Footnotes:

A dwell time of 5 milliseconds applies to all compounds.

*No standards for phenolamides to confirm retention time and MRM transitions.

Norspermidine, norspermine, 1,3-diaminopropane, 2-aminobutanoic acid (AABA), and 3-aminobutanoic acid (BABA) can also be measured within the same LCMS analysis as the metabolites presented here; however, these metabolites were not presented in this thesis. Additionally, MRM transitions of phenolamide combinations made from the mono/polyamines agmatine, putrescine, spermidine, spermine, tyramine, noradrenaline, octopamine, dopamine, norspermidine, and norspermine conjugated to *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, sinapoyl-CoA, and cinnamoyl-Co have been established, but not yet measured from plant extracts.

```

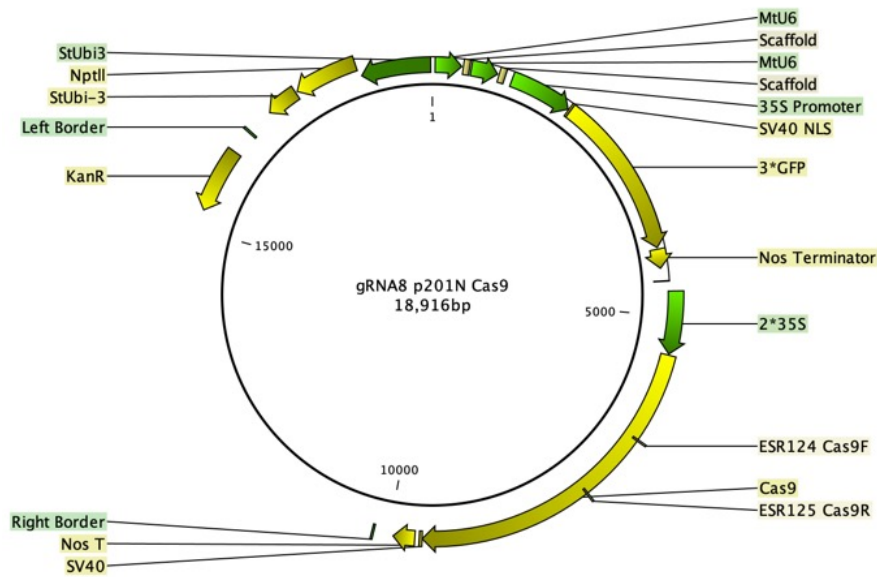
###_ADC activity tomato cds mutants (July 2023)_###
# Importing and fixing data -----
# Clear all previous lists
rm(list = ls())
# Create working directory
setwd("/Users/erinritchie/Documents/THESIS/4. Characterising ADC-box mutants/Tomato ADC assays/CDS mutants")
# Importing csv file
RawData <- read.csv("Rdata_Final Copy of CDS mutants May 2022 05102022 results.csv")
# Open view of raw dataframe
View(RawData)
# See structure of dataframe and vector types
str(RawData)
# Activate the packages that you will need for the script
install.packages(c("dplyr", "ggplot2", "ggpubr", "tidyverse", "broom", "AICcmodavg", "agricolae", "multcompView"))
library(dplyr)
library(ggplot2)
library(ggpubr)
library(tidyverse)
library(broom)
library(AICcmodavg)
library(agricolae)
library(multcompView)
# Formatting dataframe -----
# Change Sample name from character to factor vector
RawData$Sample.name <- as.factor(RawData$Sample.name)
str(RawData)
# Reorder the "Sample name" factor - otherwise it will be alphabetical order
Sample.name_ordered <- factor(RawData$Sample.name, levels = c("WT", "adc1", "adc2", "adcladc2", "odc1"))
Sample.name_groups <- group_by(RawData, Sample.name)
Sample.name_groups_ordered <- factor(Sample.name_groups$Sample.name, levels = c("WT", "adc1", "adc2", "adcladc2", "odc1"))
# ADC activity (C13 agmatine) -----
# Plot exact 13C agmatine levels as boxplot
ggplot(RawData, aes(x = Sample.name_ordered, y = C13.Agmatine.nmol_gFW.D5Trp.)) + #make background plot.
  geom_boxplot(colour = "black") + #add boxplot
  geom_jitter(width = 0.1, color = "dark gray", shape = 21) + #add data points, jittered.
  theme(axis.text.x = element_text (angle = 90, vjust = 0.5, hjust = 1)) + #change position and angle of x-labels.
  ggtitle("ADC activity of tomato cds mutants") + # add graph title.
  xlab("Tomato genotype") + #x-axis title.
  expand_limits(y = 0) + #force y axis to start at 0.
  ylab("13C agmatine (nmol/g FW)") + #y-axis title.
  theme(panel.background = element_rect(fill = "white")) + #remove graph background (white).
  theme(axis.line = element_line(colour = "black"))
#Calculate WT mean
C13agmatine_sum <- summarise(Sample.name_groups,
                           C13agm.mean = mean(C13.Agmatine.nmol_gFW.D5Trp.),
                           C13agm.sd = sd(C13.Agmatine.nmol_gFW.D5Trp.))
C13agmatine_sum #View summary
#Conduct ANOVA
anova.ADCActivity <- aov(C13.Agmatine.nmol_gFW.D5Trp. ~ Sample.name_groups_ordered, data = RawData)
summary(anova.ADCActivity)
#If ANOVA is not significant, then there are no differences between the groups
#If ANOVA is significant, then continue to Tukey's post hoc test to find out which groups are different from each other
# Use Tukey's post-hoc test to find differences between each group (corrects for multiple testing)
TukeyHSD_ADCActivity <-TukeyHSD(anova.ADCActivity)
TukeyHSD_ADCActivity
# Significance letters for plot display
multcompLetters4(anova.ADCActivity, TukeyHSD_ADCActivity)

```

Supplementary Figure 5: Example of R script used for generating box plot figures and statistical analysis of LCMS data.

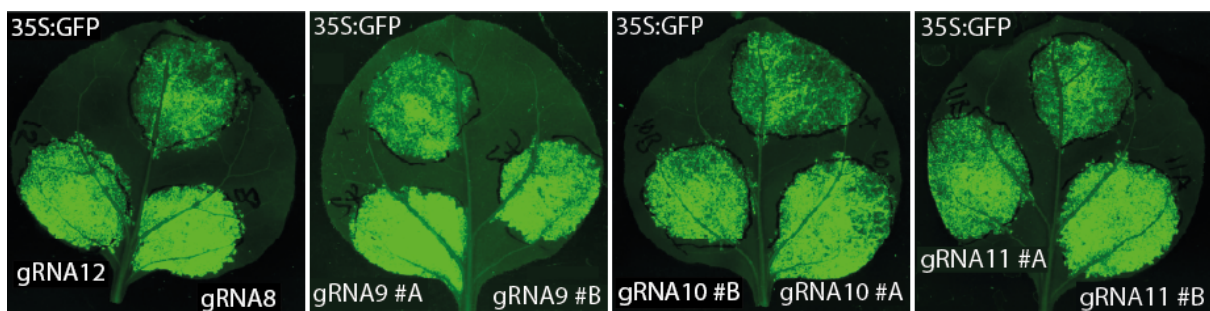
Comments are written in green text; libraries are in blue text. When only two groups were compared, a Student's T-test was used rather than an ANOVA.

5.3 CRISPR mutagenesis



Supplementary Figure 6: Exemplar plasmid map of p201N Cas9 with gRNA8 in duplicate used for stable tomato transformation.

gRNA9, gRNA10, gRNA11, and gRNA12 were also cloned in the same manner into p201N Cas9 (modified from Jacobs *et al.*, 2015). Plasmid contains: bacterial kanamycin resistance (*KanR*); neomycin *in planta* resistance gene (*NptII*) with *Solanum tuberosum Ubiquitin 3* (*StUbi3*) promoter and terminator; *Medicago truncatula U6* (*MtU6*) promoter driving identical gRNAs in duplicate with scaffold spacer sequence; Cauliflower mosaic virus 35S promoter (*35Sp*) driving triple *GFP* with SV40 nuclear localisation signal (NLS) and *Nos* terminator (*Nost*); double *35Sp* driven *Cas9* with NLS and *Nost*; and, left and right borders. Binding sequences of genotyping primers ESR124 and ESR125 in *Cas9* sequence are also shown.



Supplementary Figure 7: Transient expression of gRNA-p201N Cas9 constructs in *N. benthamiana* leaves demonstrates T-DNA functionality through GFP expression.

Each gRNA-p201N Cas9 plasmid was transformed into *A. tumefaciens* GV3101 and single colonies were selected and further tested via blunt-end syringe infiltration into *N. benthamiana* leaves at OD₆₀₀ 0.4, alongside 35S:GFP as a positive control. For some constructs, two colonies were tested (#A and #B). After 2 days, fluorescent images of whole leaves were taken using the Typhoon with eGFP filter (DBR530DF20; 700V).

Supplementary Table 4: gRNA target sequences used for tomato and <i>A. thaliana</i> CRISPR/Cas9 mutagenesis				
Species	Targeted ADC 5'UTR element	gRNA name	gRNA sequence	CRISPR/Cas9 vector used
Tomato	<i>SLADC1</i> uORF	gRNA8 ^a	GAACACCATACAGATATTAA	p201N Cas9 ^b
Tomato	<i>SLADC1</i> -box (middle)	gRNA9 ^a	GCCGGGGCTCTGGCCTCGGC	p201N Cas9 ^b
Tomato	<i>SLADC1</i> -box (3' end)	gRNA10 ^a	ACCAATGTTGTAATAGCTGG	p201N Cas9 ^b
Tomato	<i>SLADC2</i> uORF	gRNA11 ^a	GAACACCATACAGATATTAA	p201N Cas9 ^b
Tomato	<i>SLADC2</i> -box (middle)	gRNA12 ^a	ACCCGCCGAGGCCGAGGCC	p201N Cas9 ^b
Tomato	<i>SLADC1</i> and <i>SLADC2</i> (Brg11 <i>EBE</i>)	gRNA225 ^c	GCTTCTTTTGAGGGGGTAGC	p201N Cas9 ^b
<i>A. thaliana</i>	<i>AtADC1</i> -box (5' end)	gRNA841	TCGGGGCTAACCCCTCAGA	pDGE347_FAST_Bar_pRPS5a Cas9 ^d
<i>A. thaliana</i>	<i>AtADC1</i> -box (middle)	gRNA840	TTCTGAGGGGGTTAGCCCG	pDGE347_FAST_Bar_pRPS5a Cas9 ^d
<i>A. thaliana</i>	<i>AtADC1</i> -box (3' end)	gRNA873	GGGGAATTGAAACCCGCCG	pDGE347_FAST_Bar_pRPS5a Cas9 ^d
<i>A. thaliana</i>	<i>AtADC2</i> -box (5' end)	gRNA813	GGAGCCTCGGCTACCCCTC	pDGE347_FAST_Bar_pRPS5a Cas9 ^d
<i>A. thaliana</i>	<i>AtADC2</i> -box (middle)	gRNA826	AACCGCCGAGGCCGAGCCT	pDGE347_FAST_Bar_pRPS5a Cas9 ^d
<i>A. thaliana</i>	<i>AtADC2</i> -box (3' end)	gRNA838	TAGGGGTTAAAAACCGCCG	pDGE347_FAST_Bar_pRPS5a Cas9 ^d
Footnotes:				
^a two copies of same gRNA cloned into p201N Cas9 vector				
^b Originally from Jacobs <i>et al.</i> , 2015, but modified by Morbitzer, unpublished				
^c Cloned by Wu <i>et al.</i> , 2019 and gRNA				
^d Ordon <i>et al.</i> , 2020				

Supplementary Table 5: Genotypic information of mutant T0 transformants of gRNA8 p201N Cas9			
Plant code	ADC1 or ADC2 targeted by which gRNA?	Zygosity *	Cloning (or final PCR result) sequencing - result
T3 25.3	ADC1 - gRNA8	?	1bp deletion of +4 + ?
T3 10	ADC1 - gRNA8	Heterozygous	WT + mutant (4 bp across uORF ATG)
T3 13.1	ADC1 - gRNA8	Heterozygous	WT + mutant (127 bp across uORF and 5'UTR)
T3 15.1	ADC1 - gRNA8	Heterozygous	1bp deletion of +4 (frameshift) + 4bp deletion of -1 and ATG
T3 18.1	ADC1 - gRNA8	Heterozygous	WT + mutant (1bp of +4 nucleotide = frame shift)
T3 18.2	ADC1 - gRNA8	Heterozygous	WT + mutant (1bp of +4 nucleotide = frame shift)
T3 20.1	ADC1 - gRNA8	Heterozygous	WT + 2bp deletion of TG of ATG
T3 21.1	ADC1 - gRNA8	Heterozygous	WT + mutant (9 bp across uORF ATG)
T3 26.1	ADC1 - gRNA8	Heterozygous	1bp deletion of +4 (frameshift) + 4bp deletion of -1 and ATG
T3 30.1	ADC1 - gRNA8	Heterozygous	WT + mutant (2 bp deletion of 'TG' of uORF ATG)
T3 30.2	ADC1 - gRNA8	Heterozygous	uORF ATG KO (4bp deletion) + 1bp deletion of +4 nucleotide (frameshift)
T3 31.2	ADC1 - gRNA8	Heterozygous	36bp deletion across uORF start codon and 5' + (?)
T3 33.1	ADC1 - gRNA8	Heterozygous	1bp deletion of +4 + (?)
T3 33.2	ADC1 - gRNA8	Heterozygous	1bp deletion of +4bp (frameshift) + 18bp deletion of uORF ATG
T3 33.3	ADC1 - gRNA8	Heterozygous	WT + 1 bp deletion of +4 nucleotide (frameshift)
T3 35.1	ADC1 - gRNA8	Heterozygous	1bp SNP (ATG-->ATT) + 7p deletion over ATG
T3 36.1	ADC1 - gRNA8	Heterozygous	WT + mutant (1 bp deletion of +4 nucleotide = frameshift)
T3 43.1	ADC1 - gRNA8	Heterozygous	WT + mutant (5 bp deletion of +1 to +5 nucleotides = ATG KO)
T3 50.1	ADC1 - gRNA8	Heterozygous	1bp insertion (ATGG --> ATGAG = change frame and kozak)
T3 12.2	ADC1 - gRNA8	Homozygous	137bp deletion incl. uORF (4x clones all mutant)
T3 25.2	ADC1 - gRNA8	Homozygous	1bp deletion of +4 nucleotide (frameshift)
Footnotes:			
* Based on initial gDNA PCR and subsequent cloning (if conducted)			

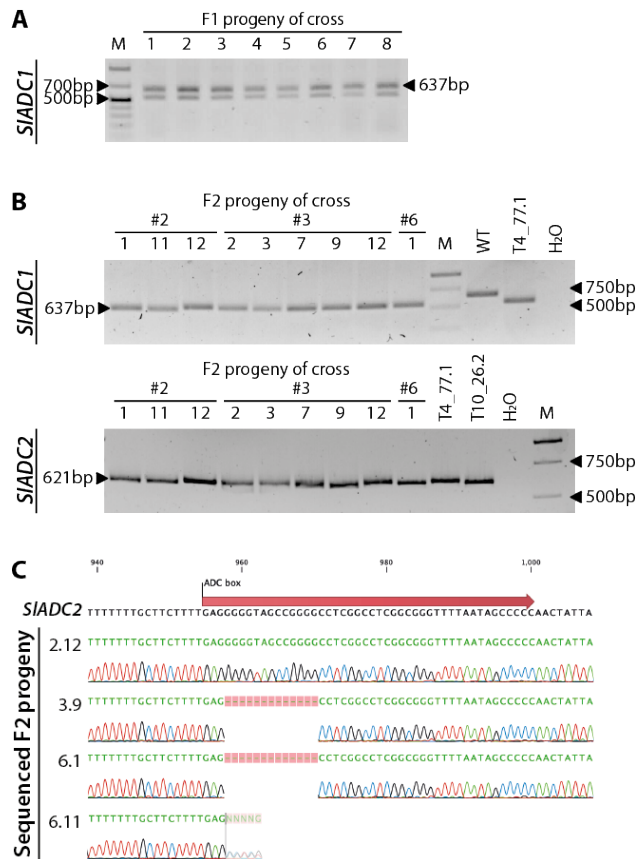
Supplementary Table 6: Genotypic information of mutant T0 transformants of gRNA10 p201N Cas9			
Plant code	ADC1 or ADC2 targeted by which gRNA?	Zygosity *	Cloning (or final PCR result) sequencing - result
T4 12.1	ADC1 - gRNA10	?	4bp deletion after ADC1 box + (?)
T4 17.8	ADC1 - gRNA10	?	
T4 3.2	ADC1 - gRNA10	?	
T4 33.4	ADC1 - gRNA10	?	
T4 38.1	ADC1 - gRNA10	?	
T4 38.5	ADC1 - gRNA10	?	
T4 50.3	ADC1 - gRNA10	?	
T4 70.1	ADC1 - gRNA10	?	
T4 73.1	ADC1 - gRNA10	?	
T4 89.2	ADC1 - gRNA10	?	
T4 98.1	ADC1 - gRNA10	?	
T4 1.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 12.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 12.4	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 13.3	ADC1 - gRNA10	Heterozygous	4bp deletion after box and possibly 2 x SNP after box
T4 17.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 17.1 #2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 17.3	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 17.5	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 17.6	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 17.7	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 17.9	ADC1 - gRNA10	Heterozygous	14 bp deletion after ADC1 box + ?
T4 19.1	ADC1 - gRNA10	Heterozygous	1bp insertion after ADC1-box and probably 4 bp deletion after box
T4 21	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 26.3	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 27	ADC1 - gRNA10	Heterozygous	
T4 28.1	ADC1 - gRNA10	Heterozygous	4 or 5 bp deletion after ADC1 box + (?)
T4 3.4	ADC1 - gRNA10	Heterozygous	Single bp SNP after box
T4 30.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 30.3	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 35.2	ADC1 - gRNA10	Heterozygous	1bp deletion after ADC1-box and also possible deletion (?)
T4 37.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 39.4	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 39.5	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 4.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 40.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 46.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 50.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 50.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 51.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 51.3	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 52.1	ADC1 - gRNA10	Heterozygous	WT and possible deletion after box
T4 52.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 54.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 55.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 56.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 58.1	ADC1 - gRNA10	Heterozygous	Single bp SNP after box
T4 58.2	ADC1 - gRNA10	Heterozygous	1bp SNP after box + ?
T4 6.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 6.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 63.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 63.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 64.1	ADC1 - gRNA10	Heterozygous	Single bp SNP after box
T4 64.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 64.3	ADC1 - gRNA10	Heterozygous	Hetero - 7bp deletion after box
T4 66.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 7.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 7.2	ADC1 - gRNA10	Heterozygous	Hetero - 4 bp deletion after box
T4 71.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 72.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 73.3	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 75.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 77.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 77.3	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 79.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 80.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 83.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 86.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 87.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 91.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 92.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box

T4 92.2	ADC1 - gRNA10	Heterozygous	Possible large deletion
T4 93.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 94.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 95.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 95.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 96.1	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 96.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 98.2	ADC1 - gRNA10	Heterozygous	Mutations occurring after ADC1-box
T4 12.3	ADC1 - gRNA10	Homozygous	1 bp insertion after box
T4_2	ADC1 - gRNA10	Homozygous	6 bp deletion after box
T4 24.2	ADC1 - gRNA10	Homozygous	3bp deletion after box
T4 33.1	ADC1 - gRNA10	Homozygous	1bp insertion after ADC1 box and large deletion of 5' of ADC box
T4 34.1	ADC1 - gRNA10	Homozygous	2 bp insertion after box
T4 38.2	ADC1 - gRNA10	Homozygous	Truncated??
T4 38.3	ADC1 - gRNA10	Homozygous	4 bp deletion after box
T4 38.4	ADC1 - gRNA10	Homozygous	7 bp deletion after box
T4 38.6	ADC1 - gRNA10	Homozygous	Truncated??
T4 43.1	ADC1 - gRNA10	Homozygous	5 bp deletion after box
T4 43.2	ADC1 - gRNA10	Homozygous	8 bp deletion after box
T4 62.2	ADC1 - gRNA10	Homozygous	3 bp insertion after box
T4 68.1	ADC1 - gRNA10	Homozygous	6 bp deletion after box
T4 78.1	ADC1 - gRNA10	Homozygous	4 bp insertion after box
Footnotes:			
* Based on initial gDNA PCR and subsequent cloning (if conducted)			

Supplementary Table 7: Genotypic information of mutant T0 transformants of gRNA11 p201N Cas9			
Plant code	ADC1 or ADC2 targeted by which gRNA?	Zygosity *	Cloning (or final PCR result) sequencing - result
T5 32.2	ADC2 - gRNA11	?	
T5 1.1	ADC2 - gRNA11	Heterozygous	9bp deletion (Δ kozak) + 2bp deletion
T5 12.1	ADC2 - gRNA11	Heterozygous	8bp deletion (ATG KO) + 5bp deletion
T5 14.1	ADC2 - gRNA11	Heterozygous	WT + 3bp deletion 5' to ATG
T5 14.2	ADC2 - gRNA11	Heterozygous	3x mutations - 1bp deletion, 3bp deletion, + 7bp deletion 5' from ATG
T5 15.2	ADC2 - gRNA11	Heterozygous	5bp deletion + 12bp deletion and 2bp SNP
T5 15.3	ADC2 - gRNA11	Heterozygous	2bp deletion + 3bp deletion (both 5' to ATG)
T5 16.1	ADC2 - gRNA11	Heterozygous	2bp deletion + 3bp deletion (both 5' to ATG)
T5 16.2	ADC2 - gRNA11	Heterozygous	2bp deletion + 5bp deletion + 6bp deletion (3x mutations?)
T5 16.4	ADC2 - gRNA11	Heterozygous	2bp deletion + 3bp deletion (both 5' to ATG)
T5 18.2	ADC2 - gRNA11	Heterozygous	3bp deletion + 3bp deletion
T5 19.1	ADC2 - gRNA11	Heterozygous	WT + 8bp deletion (Δ kozak)
T5 23.1	ADC2 - gRNA11	Heterozygous	5bp deletion of -5 and -8 nucleotides + (?)
T5 23.2	ADC2 - gRNA11	Heterozygous	1bp deletion + approx. 230bp deletion
T5 23.3	ADC2 - gRNA11	Heterozygous	WT + 8bp deletion (Δ kozak)
T5 23.4	ADC2 - gRNA11	Heterozygous	3bp deletion + 4bp deletion
T5 24.1	ADC2 - gRNA11	Heterozygous	WT + 1bp deletion + 3bp SNP (3x mutation)
T5 25.1	ADC2 - gRNA11	Heterozygous	WT + 2bp deletion 5' to ATG
T5 25.2	ADC2 - gRNA11	Heterozygous	WT + 2bp deletion 5' to ATG
T5 26.3	ADC2 - gRNA11	Heterozygous	WT + 3bp deletion 5' to ATG
T5 27.1	ADC2 - gRNA11	Heterozygous	1bp deletion + 14bp deletion (ATG KO)
T5 3.1	ADC2 - gRNA11	Heterozygous	3bp deletion + 1bp insertion + 5bp deletion (3x mutations?)
T5 30.2	ADC2 - gRNA11	Heterozygous	5bp deletion + 173bp deletion over uORF
T5 31.1	ADC2 - gRNA11	Heterozygous	6bp deletion (Δ kozak) + ?
T5 33.1	ADC2 - gRNA11	Heterozygous	172bp deletion over uORF + 4bp deletion 5' to ATG
T5 33.2	ADC2 - gRNA11	Heterozygous	4bp deletion + 172bp deletion
T5 7.1	ADC2 - gRNA11	Heterozygous	3bp deletion + 1bp deletion 5' to ATG
T5 7.2	ADC2 - gRNA11	Heterozygous	6bp deletion + ?
T5 15.1	ADC2 - gRNA11	Homozygous	1bp deletion, 7bp 5' to ATG
T5 18.1	ADC2 - gRNA11	Homozygous	3bp deletion of -5, -6, and -7
T5 22.1	ADC2 - gRNA11	Homozygous	3bp deletion 5' to ATG
T5 22.3	ADC2 - gRNA11	Homozygous	3bp deletion 5' to ATG
T5 22.4	ADC2 - gRNA11	Homozygous	3bp deletion 5' to ATG
T5 26.1	ADC2 - gRNA11	Homozygous	3bp deletion 5' to ATG
T5 26.2	ADC2 - gRNA11	Homozygous	3bp deletion 5' to ATG
T5 37.1	ADC2 - gRNA11	Homozygous	3bp deletion 5' to ATG
T5 4.1	ADC2 - gRNA11	Homozygous	1bp insertion at -8bp
T5 4.1	ADC2 - gRNA11	Homozygous	
Footnotes:			
* Based on initial gDNA PCR and subsequent cloning (if conducted)			

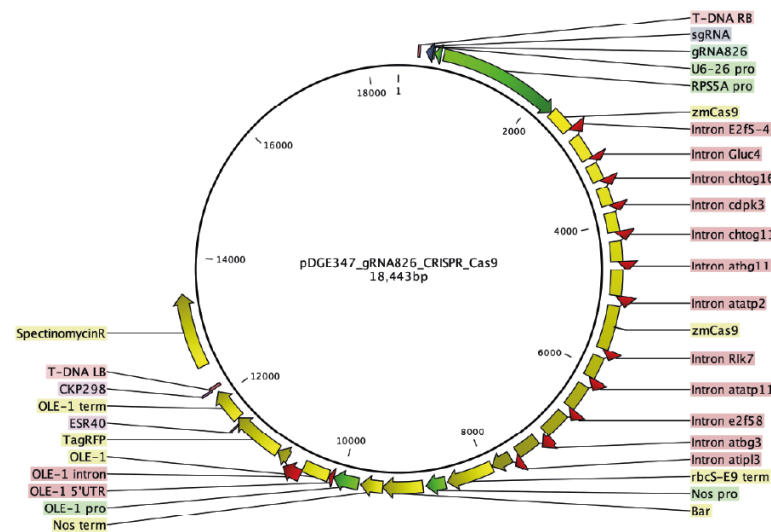
Supplementary Table 8: Genotypic information of mutant T0 transformants of gRNA225 p201N Cas9						
Plant code	<i>SIADC1</i> PCR sequencing	<i>SIADC2</i> PCR sequencing	<i>SIADC1</i> zygosity*	<i>SIADC1</i> mutations	<i>SIADC2</i> zygosity*	<i>SIADC2</i> mutations
T10_62.6	Mutant?	WT	Heterozygous	WT + 1bp substitution	WT	WT
T10_27.3	Mutant?	WT	Heterozygous	WT + 5bp deletion of GC1 region (1bp of loop too)	WT	WT
T10_7.4	Mutant?	WT	Heterozygous	WT + 3bp deletion of GGT	WT	WT
T10_56.1	Mutant?	WT	Heterozygous	WT + 1bp insertion (G) in loop (TGAG)	WT	WT
T10_45.1	Mutant?	WT	Heterozygous	WT + 1bp insertion (A in loop)	WT	WT
T10_99.1	Mutant?	WT	Heterozygous	WT + 1bp insertion of Loop between GC1-2	WT	WT
T10_67.2	Mutant?	WT	Heterozygous	WT + 2bp deletion of GT	WT	WT
T10_72.3	Mutant?					
T10_62.4	WT	Mutant?	WT	WT	Heterozygous	WT, 13bp deletion, 8bp deletion
T10_65.8	WT	Mutant?	WT	WT	Heterozygous	WT + 13bp deletion??
T10_77.2	WT	Mutant?	WT	WT	Heterozygous	WT + large deletion... Sequence partially matches to
T10_1.3	WT	Mutant?	WT	WT	Homozygous	2bp deletion (GT)
T10_64.1	WT - ?	Mutant?	WT	WT	Heterozygous	WT + 6bp deletion of GC1 region
T10_1.7	WT	Mutant?	WT	WT	Heterozygous	WT + 3bp deletion (GGT)
T10_98.2	WT	Mutant?	WT	WT	Heterozygous	1bp deletion (A in loop) + 9bp deletion of loop and GC2
T10_26.2	WT	Mutant?	WT	WT	Heterozygous	WT + 13bp deletion from (+4 to +16)
T10_52.1	WT	Mutant?	WT	WT	Homozygous	278bp deletion of ADC-box and 34bp of ADC2 CDS
T10_1.8	WT	Mutant?	WT	WT	Heterozygous	WT + 3bp deletion (GGT)
T10_60.2	WT	Mutant?	WT	WT	Heterozygous	287bp deletion of 5'UTR and 68bp of ADC2 CDS + 3bp
T10_26.3	WT	Mutant?	WT	WT	Heterozygous	WT + 13bp deletion from (+4 to +16)
T10_26.4	WT	Mutant?	WT	WT	Heterozygous	WT + 13bp deletion from (+4 to +16)
T10_41.1	WT	Mutant?	WT	WT	Heterozygous	4bp deletion (GGGT) + 1bp insertion (A - loop)
T10_52.2	WT	Mutant?	WT	WT	Homozygous	278bp deletion of ADC-box and 34bp of ADC2 CDS
T10_53.1	WT	Mutant?	WT	WT	Heterozygous	1bp insertion (T - loop region) + deletion of T
T10_76.3	WT	Mutant?	WT	WT	Homozygous	278bp deletion of ADC-box and 34bp of ADC2 CDS
T10_78.2	WT	Mutant?	WT	WT	Homozygous	278bp deletion of ADC-box and 34bp of ADC2 CDS
T10_82.2	WT	Mutant?	WT	WT	Heterozygous	1bp insertion (T - loop region) + 6bp deletion of GC1
T10_96.1	WT	Mutant?	WT	WT	Heterozygous	WT + possible 278bp deletion (weird sequences)
T10_1.2	WT	Mutant?	WT	WT	Heterozygous	3bp deletion of GGT + 2bp deletion of GT
T10_102.2	WT	Mutant?	?	WT??	Heterozygous	WT + 2bp deletion
T10_29.3	WT	Mutant?	?	WT + ??	Heterozygous	WT + 3bp deletion of loop (TAG missing)
T10_89.2		Mutant?				
T10_72.1	Mutant?	Mutant?	Homozygous	1bp deletion of loop	Heterozygous	WT + 1bp deletion (T)
T10_86.2	Mutant?	Mutant?	Homozygous	11bp deletion of ADC1-box (GGTAGCCGGGG)	Heterozygous	3bp deletion of loop (TAG missing) + 32bp deletion of
T10_66.1	Mutant?	Mutant?	Heterozygous	7bp deletion of GC1 and loop + 16bp deletion of GC1 and	Heterozygous	4bp deletion (GGGT) + 5bp deletion (GGTAG)
T10_37.2	Mutant?	Mutant?	Heterozygous	WT + 2bp deletion of GT	Heterozygous	WT + 4bp deletion of GGGT
T10_12.3	Mutant?	Mutant?	Heterozygous	WT + 1bp deletion in GC2 region	Heterozygous	WT + 1x 13bp deletion
T10_65.2	Mutant?	Mutant?	Heterozygous	1bp deletion of T + 8bp deletion of GC1 (GAGGGGGT)	Heterozygous	WT + 103bp deletion (GC4 intact, but deletion goes 5')
T10_39.2	Mutant?	Mutant?	Heterozygous	WT + 8bp deletion of GC1	Heterozygous	1bp insertion (A - loop region) + 1bp deletion T in loop
T10_66.2	Mutant?	Mutant?	Heterozygous	7bp deletion of GC1 and loop + 16bp deletion of GC1 and	Heterozygous	WT + 5bp deletion of GGTAG
T10_10.1	Mutant?	Mutant?	Heterozygous	25bp deletion of GC1 and GC2 + 47bp deletion (+4bp	Heterozygous	WT + 1bp deletion (G in GC1)
T10_15.2	Mutant?	Mutant?	Heterozygous	WT + 4bp deletion in GC1	Heterozygous	WT + 1bp insertion in loop (TTAG)
T10_34.1	Mutant?	Mutant?	Heterozygous	WT + 6bp deletion of GC1 (GGGGGT)	Heterozygous	5bp deletion + large deletion (GC4 still intact, but 144bp
T10_38.3	Mutant?	Mutant?	Heterozygous	83bp deletion of 5'UTR (1/2 of ADC1-box and 5'into	Heterozygous	1bp deletion (G in GC1) + 5bp deletion GC1

T10_38.4	Mutant?	Mutant?	Heterozygous	1bp deletion in loop + 83bp deletion (1/2 of ADC1-box	Heterozygous	1bp deletion (G in GC1) + 5bp deletion of GC1
T10_38.5	Mutant?	Mutant?	Homozygous	83bp deletion of 5'UTR (1/2 of ADC1-box and 5'into	Heterozygous	1bp deletion (G in GC1) + 5bp deletion GGGGT
T10_38.6	Mutant?	Mutant?	Homozygous	13bp deletion of 1/2 GC1 and most of GC2 (incl. Loop)	Heterozygous	1bp deletion of T in loop + 12bp deletion of GC1 + loop
T10_39.1	Mutant?	Mutant?	Homozygous	292bp deletion of 5'UTR (incl. ADC1-box, but excl.	Heterozygous	3xtypes (2x large deletion (GC4 and last loop region
T10_60.1	Mutant?	Mutant?	Homozygous	230bp deletion of 5'UTR (incl. uORF until half of ADC1-	Heterozygous	3bp deletion of GGT + 177 bp deletion of ADC2-box
T10_67.1	Mutant?	Mutant?	Heterozygous	WT + 1bp insertion in loop (TAAG)	Heterozygous	2bp deletion (TA) + 3bp deletion (AGC)
T10_83.2	Mutant?	Mutant?	Heterozygous	WT + 2bp deletion of GT	Heterozygous	5bp deletion (GGGGT) + 1bp insertion in loop (TAAG)
T10_86.3	Mutant?	Mutant?	Homozygous	11bp deletion of ADC1-box (GGTAGCCGGGG)	Heterozygous	3bp deletion of loop (TAG missing) + possible deletion
T10_87.1	Mutant?	Mutant?	Heterozygous	WT + 3bp deletion (GGT)	Heterozygous	WT + 1bp insertion (A - loop region)
T10_31.2	Mutant?	Mutant?	?	Loop deletions (TAG between GC1-2 and 1bp deletion of	Heterozygous	WT + 4bp deletion of GGGT
T10_69.1	Mutant?	Mutant?			Heterozygous	2bp deletion (GT) + 6bp deletion of 1/2 GC2 (AGCCGG)
T10_103.1	Mutant?	Mutant?		?	Heterozygous	WT + 1bp deletion (T).....?
T10_37.3	Mutant?	Mutant?	Heterozygous	WT + 1bp deletion of T in loop (between GC1-2)	Heterozygous	WT + large deletion (?)
T10_52.0	Mutant?	Mutant?	Heterozygous	WT + 4bp deletion of loop (GTAG)	?	4xdifferent mutations/degraded PCR
T10_52.3	Mutant?	Mutant?	Heterozygous?	8bp deletion of loop and GC2 + ??	Heterozygous	WT + 5bp deletion of GC1
T10_58.2	Mutant?	Mutant?	Heterozygous	7bp deletion of GC1 and loop + 4bp deletion of loop	Homozygous?	2bp deletion (AG)
T10_62.3	Mutant?	Mutant?	?	2bp deletion of GT	Homozygous?	1bp insertion (TTAG)
T10_71.1	Mutant?	Mutant?	WT?	WT?	Heterozygous	WT + 1bp deletion (T)
T10_75.1	Mutant?	Mutant?	Heterozygous	WT + 1bp insertion in loop (GC1-2)		
T10_83.1	Mutant?	Mutant?	Heterozygous	WT + 2bp deletion of GT	Heterozygous	5bp deletion (GGGGT) + 1bp deletion (T)
T10_1.1	Mutant?	Mutant?	Heterozygous	WT + 1bp insertion (A in loop)	Heterozygous	WT + 6bp deletion of GC1 region
T10_102.1	Mutant?	Mutant?	Heterozygous	4bp deletion (GGGT) + 1bp substitution (TAAG)		2xWT + shorter seq??
Footnotes: * based on initial gDNA PCR and subsequent cloning (if conducted)						



Supplementary Figure 8: Exemplar of genotyping crosses of tomato CRISPR mutants.

Flowers from *SIADC1-box* mutant T4_77.1 (#14) and *SIADC2-box* mutant T10_26.2 (#48) were crossed and F1 seeds from the resulting tomato were sown and progeny plants tested via PCR of *SIADC1* 5'UTR; two bands indicate heterozygosity, with the expected WT product size is 637bp and the T4_77.1 parental mutation would produce a 529bp product. F1 plants #2, #3, and #6 were kept for seed production. Approximately 20 F2 progeny from these three plants were genotyped; all showing a homozygous *SIADC1* mutant product size (529bp) were confirmed in second PCR (**B, top**), and a PCR of *SIADC2* 5'UTR was conducted, run on a 3% agarose gel (**B, bottom**), and sent for Sanger sequencing (**C**). Plants 3.9 and 6.1 were both homozygous for parental T10_26.2 the *SIADC2-box* mutation (13bp deletion); plant 3.9 was kept for seed and used for subsequent experiments.



Supplementary Figure 9: Exemplar plasmid map of pDGE347 CRISPR/Cas9 plasmid with gRNA826 used for stable *A. thaliana* transformation.

gRNA841, gRNA840, gRNA873, gRNA813, and gRNA838 were also cloned in the same manner into pDGE347; cloning method and vectors from Ordon *et al.*, 2020. Plasmid contains: gRNA scaffold, including a U6-26 promoter (*U6-26 pro*) from *A. thaliana*, driving gRNA transcription; *A. thaliana* Ribosomal Protein S5A (*RPS5A*) promoter driving a *Zea mays*-codon optimised *Cas9* (*zmCas9*) containing 12 inserted introns and *Pisum sativum* *rbcS-E9* terminator; BASTA *in planta* resistance gene (*Bar*; phosphinothricin *N*-acetyltransferase) with *Nos* promoter (*Nos pro*) and terminator (*Nos term*); FAST marker for seed coat expressed TagRFP (*TagRFP* fused to *A. thaliana* Oleosin 1 (*OLE-1*) with its native promoter, 5'UTR, and terminator); bacterial spectinomycin resistance (*SpectinomycinR*); and, left and right borders. Binding sequences of genotyping primers CKP298 and ESR40 in *TagRFP* are also shown.


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AtADC1_WT      CTCCTTCATGCATTCTCACCATCTCTCTCTTTTCACGTCTCTCTTCC
At1M-15      CTCCTTCATGCATTCTCACCATCTCTCTCTTTTCACGTCTCTCTTCC
At1M-53      CTCCTTCATGCATTCTCACCATCTCTCTCTTTTCACGTCTCTCTTCC
At1M+1       CTCCTTCATGCATTCTCACCATCTCTCTCTTTTCACGTCTCTCTTCC
At1M-4       CTCCTTCATGCATTCTCACCATCTCTCTCTTTTCACGTCTCTCTTCC
*****

AtADC1_WT      CTGAAACTTTCCCGAGAAAATTCTCAATCCTCTATTTTCTCGAGAAAATT
At1M-15      CTGAAACTTTCCCGAGAAAATTCTCAATCCTCTATTTTCTCGAGAAAATT
At1M-53      CTGAAACTTTCCCGAGAAAATTCTCAATCCTCTATTTTCTCGAGAAAATT
At1M+1       CTGAAACTTTCCCGAGAAAATTCTCAATCCTCTATTTTCTCGAGAAAATT
At1M-4       CTGAAACTTTCCCGAGAAAATTCTCAATCCTCTATTTTCTCGAGAAAATT
*****

AtADC1_WT      CTCATTCTCATTTTCTTTTCAGGGAAAAGTTTGTGTGAATACAGATT
At1M-15      CTCATTCTCATTTTCTTTTCAGGGAAAAGTTTGTGTGAATACAGATT
At1M-53      CTCATTCTCATTTTCTTTTCAGGGAAAAGTTTGTGTGAATACAGATT
At1M+1       CTCATTCTCATTTTCTTTTCAGGGAAAAGTTTGTGTGAATACAGATT
At1M-4       CTCATTCTCATTTTCTTTTCAGGGAAAAGTTTGTGTGAATACAGATT
*****

AtADC1_WT      GGAAACTTCATTTGTTTTTTTCCGTTTTTAAACCAAAATTTTAAAAAAGC
At1M-15      GGAAACTTCATTTGTTTTTTTCCGTTTTTAAACCAAAATTTTAAAAAAGC
At1M-53      GGAAACTTCATTTGTTTTTTTCCGTTTTTAAACCAAAATTTTAAAAAAGC
At1M+1       GGAAACTTCATTTGTTTTTTTCCGTTTTTAAACCAAAATTTTAAAAAAGC
At1M-4       GGAAACTTCATTTGTTTTTTTCCGTTTTTAAACCAAAATTTTAAAAAAGC
*****

AtADC1_WT      TTTCTTCTGAACGTTAATCGTGGAGAGTTTCGGGTCGGGTCGGGTCGTAA
At1M-15      TTTCTTCTGAACGTTAATCGTGGAGAGTTTCGGGTCGGGTCGGGTCGTAA
At1M-53      TTTCTTCTGAACGTTAATCGTGGAGAGTTTCGGGTCGGGTCGGGTCGTAA
At1M+1       TTTCTTCTGAACGTTAATCGTGGAGAGTTTCGGGTCGGGTCGGGTCGTAA
At1M-4       TTTCTTCTGAACGTTAATCGTGGAGAGTTTCGGGTCGGGTCGGGTCGTAA
*****

AtADC1_WT      AAGGCGGGTTTCACGGTGGTGATAGATTACCTCCTGCTTCTCTCTGAGG
At1M-15      AAGGCGGGTTTCACGGTGGTGATAGATTACCTCCTGCTTCTCTCTGAGG
At1M-53      AAGGCG-----TC-----TGCTAGATTCTC-----GAGG
At1M+1       AAGGCGGGTTTCACGGTGGTGATAGATTACCTCCTGCTTCTCTCTGAGG
At1M-4       AAGGCGGGTTTCACGGTGGTGATAGATTACCTCCTGCTTCTCTCTGAGG
*****      **      **      *****

AtADC1_WT      GGGTTAGCC-CCGAGGCTCTTCGCCTCGGCGGGTTTCAAATCCCCCAA
At1M-15      -----CTCTTCGCCTCGGCGGGTTTCAAATCCCCCAA
At1M-53      -----GCCTCGGCGGGTTTCAAATCCCCCAA
At1M+1       GGGTTAGCC-CCGAGGCTCTTCGCCTCGGCGGGTTTCAAATCCCCCAA
At1M-4       GGGTTAGCC-----GGCTCTTCGCCTCGGCGGGTTTCAAATCCCCCAA
*****

AtADC1_WT      TCTAACAAACTCACAGATTCATTTTCTCCATTTTTTTTGTGTGTGAAAA
At1M-15      TCTAACAAACTCACAGATTCATTTTCTCCATTTTTTTTGTGTGTGAAAA
At1M-53      TCTAACAAACTCACAGATTCATTTTCTCCATTTTTTTTGTGTGTGAAAA
At1M+1       TCTAACAAACTCACAGATTCATTTTCTCCATTTTTTTTGTGTGTGAAAA
At1M-4       TCTAACAAACTCACAGATTCATTTTCTCCATTTTTTTTGTGTGTGAAAA
*****

AtADC1_WT      TTTGAATCCACAAATTTTGAATTCATTGTAAACCGTTCGTTGAAGAAGAA
At1M-15      TTTGAATCCACAAATTTTGAATTCATTGTAAACCGTTCGTTGAAGAAGAA
At1M-53      TTTGAATCCACAAATTTTGAATTCATTGTAAACCGTTCGTTGAAGAAGAA
At1M+1       TTTGAATCCACAAATTTTGAATTCATTGTAAACCGTTCGTTGAAGAAGAA
At1M-4       TTTGAATCCACAAATTTTGAATTCATTGTAAACCGTTCGTTGAAGAAGAA
*****

AtADC1_WT      GAAGATGCCTGCTCTAGCTTTTGT
At1M-15      GAAGATGCCTGCTCTAGCTTTTGT
At1M-53      GAAGATGCCTGCTCTAGCTTTTGT
At1M+1       GAAGATGCCTGCTCTAGCTTTTGT
At1M-4       GAAGATGCCTGCTCTAGCTTTTGT
*****

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Supplementary Figure 10: Alignment of *AtADC1-box* CRISPR/Cas9 mutants used in subsequent experiments in this work.

AtADC1 WT and CRISPR mutant promoter and part of the CDS were aligned using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) to demonstrate CRISPR/Cas9-induced mutations, where deletions are shown as red dashes and insertions or substitutions shown in bolded red text and the absence of an asterisk under the alignment. The *ADC-box* is shaded black with bolded white text. The *uORF* and start of *ADC* CDS are shaded grey with bolded black text; start and stop codons are underlined.


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AtADC2_WT      CCAC TTCTGC ATGGTTGGGAGAATCTGTTAGAGAATTTTGTGTTGTTG
At2M_-18      CCAC TTCTGC ATGGTTGGGAGAATCTGTTAGAGAATTTTGTGTTGTTG
At2M_+1       CCAC TTCTGC ATGGTTGGGAGAATCTGTTAGAGAATTTTGTGTTGTTG
At2M_-6       CCAC TTCTGC ATGGTTGGGAGAATCTGTTAGAGAATTTTGTGTTGTTG
*****

AtADC2_WT      GGTT CAGATTTGGAAAATTCCTTTTTTCGAGTTTCTTCTGAATTATTC
At2M_-18      GGTT CAGATTTGGAAAATTCCTTTTTTCGAGTTTCTTCTGAATTATTC
At2M_+1       GGTT CAGATTTGGAAAATTCCTTTTTTCGAGTTTCTTCTGAATTATTC
At2M_-6       GGTT CAGATTTGGAAAATTCCTTTTTTCGAGTTTCTTCTGAATTATTC
*****

AtADC2_WT      ATCGTGGAGATTCTCGGATCGGGAAGGGCTGCGAGAGGTGGTTTTTCACGG
At2M_-18      ATCGTGGAGATTCTCGGATCGGGAAGGGCTGCGAGAGGTGGTTTTTCACGG
At2M_+1       ATCGTGGAGATTCTCGGATCGGGAAGGGCTGCGAGAGGTGGTTTTTCACGG
At2M_-6       ATCGTGGAGATTCTCGGATCGGGAAGGGCTGCGAGAGGTGGTTTTTCACGG
*****

AtADC2_WT      CGGGGATAGATTCATCTTCTGTCTTCTCCTGAGGGGGTAGCCGAGGCTCC
At2M_-18      CGGGGATAGATTCATCTTCTGTCTTCTCCTGAGGGGGTAGCCGAG-----
At2M_+1       CGGGGATAGATTCATCTTCTGTCTTCTCCTGAGGGGGTAGCCGAGGCTCC
At2M_-6       CGGGGATAGATTCATCTTCTGTCTTCTCCTGAGGGGGTAGCCGAGGC----
*****

AtADC2_WT      GGCCTCGG-CGGTTTTTTAAACCCCTTACCTTTACAAATCTGGGAAATTAG
At2M_-18      -----TTTAAACCCCTTACCTTTACAAATCTGGGAAATTAG
At2M_+1       GGCCTCGGCGGTTTTTTAAACCCCTTACCTTTACAAATCTGGGAAATTAG
At2M_-6       ---CTCGG-CGGTTTTTTAAACCCCTTACCTTTACAAATCTGGGAAATTAG
*****

AtADC2_WT      AAAAAAAAAAGGTTTGATTTTTTCAGTTTTTGCTCTGTTCTTCTCTCGAAAG
At2M_-18      AAAAAAAAAAGGTTTGATTTTTTCAGTTTTTGCTCTGTTCTTCTCTCGAAAG
At2M_+1       AAAAAAAAAAGGTTTGATTTTTTCAGTTTTTGCTCTGTTCTTCTCTCGAAAG
At2M_-6      AAAAAAAAAAGGTTTGATTTTTTCAGTTTTTGCTCTGTTCTTCTCTCGAAAG
*****

AtADC2_WT      TTTTGTGTTTGTTTCGAGAATTCTGTAAAGTGAGATTGTTGATCGTTTGC
At2M_-18      TTTTGTGTTTGTTTCGAGAATTCTGTAAAGTGAGATTGTTGATCGTTTGC
At2M_+1       TTTTGTGTTTGTTTCGAGAATTCTGTAAAGTGAGATTGTTGATCGTTTGC
At2M_-6      TTTTGTGTTTGTTTCGAGAATTCTGTAAAGTGAGATTGTTGATCGTTTGC
*****

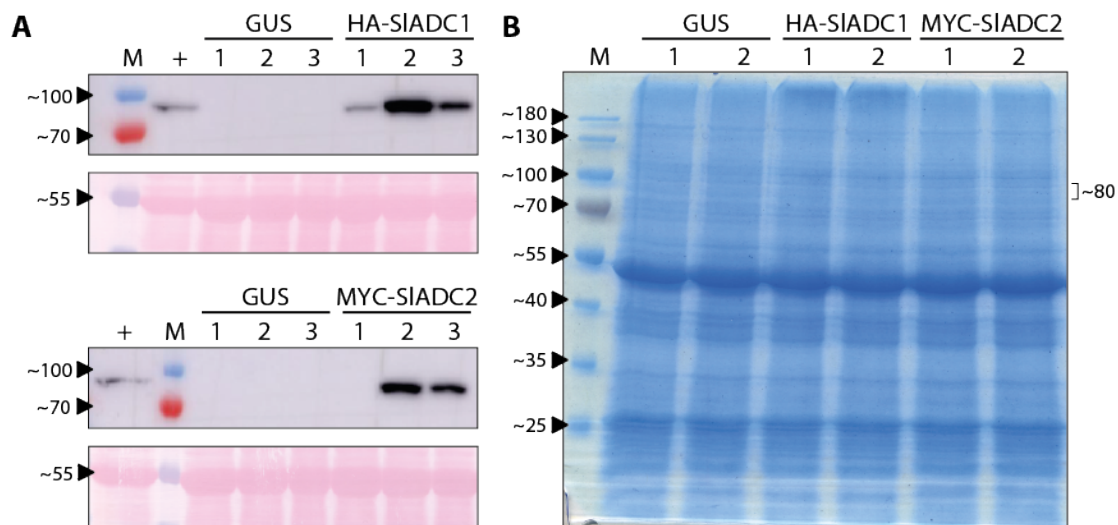
AtADC2_WT      TGATAAGAGGGTGAAGATAAAGATGCCTGCTTTAGCTTGCGT
At2M_-18      TGATAAGAGGGTGAAGATAAAGATGCCTGCTTTAGCTTGCGT
At2M_+1       TGATAAGAGGGTGAAGATAAAGATGCCTGCTTTAGCTTGCGT
At2M_-6      TGATAAGAGGGTGAAGATAAAGATGCCTGCTTTAGCTTGCGT
*****

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Supplementary Figure 11: Alignment of *AtADC2-box* CRISPR/Cas9 mutants used in subsequent experiments in this work.

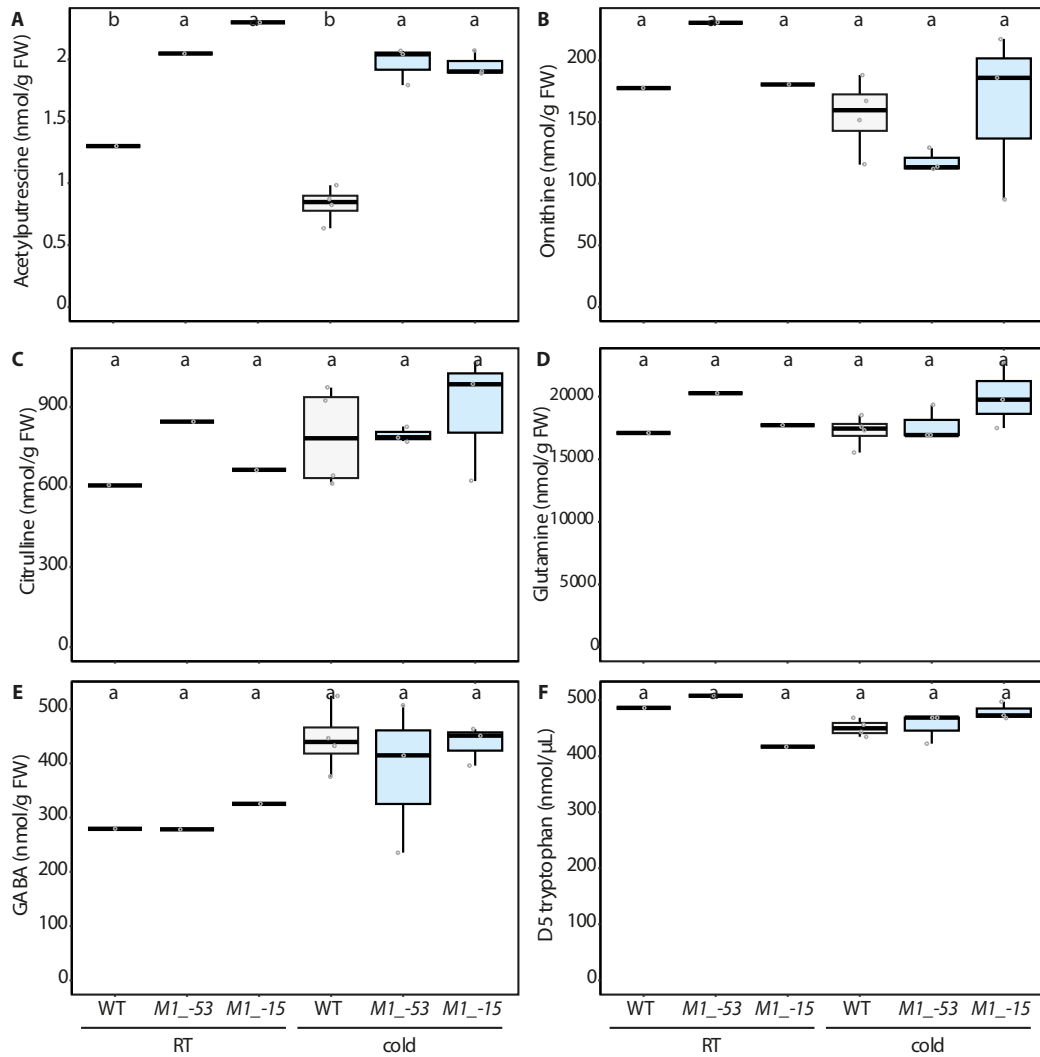
AtADC2 WT and CRISPR mutant promoter and part of the CDS were aligned using CLUSTALW (<https://www.genome.jp/tools-bin/clustalw>) to demonstrate CRISPR/Cas9-induced mutations, where deletions are shown as red dashes and insertions or substitutions shown in bolded red text and the absence of an asterisk under the alignment. The *ADC-box* is shaded black with bolded white text. The *uORF* and start of *ADC* CDS are shaded grey with bolded black text; start and stop codons are underlined.

5.4 LCMS optimisation



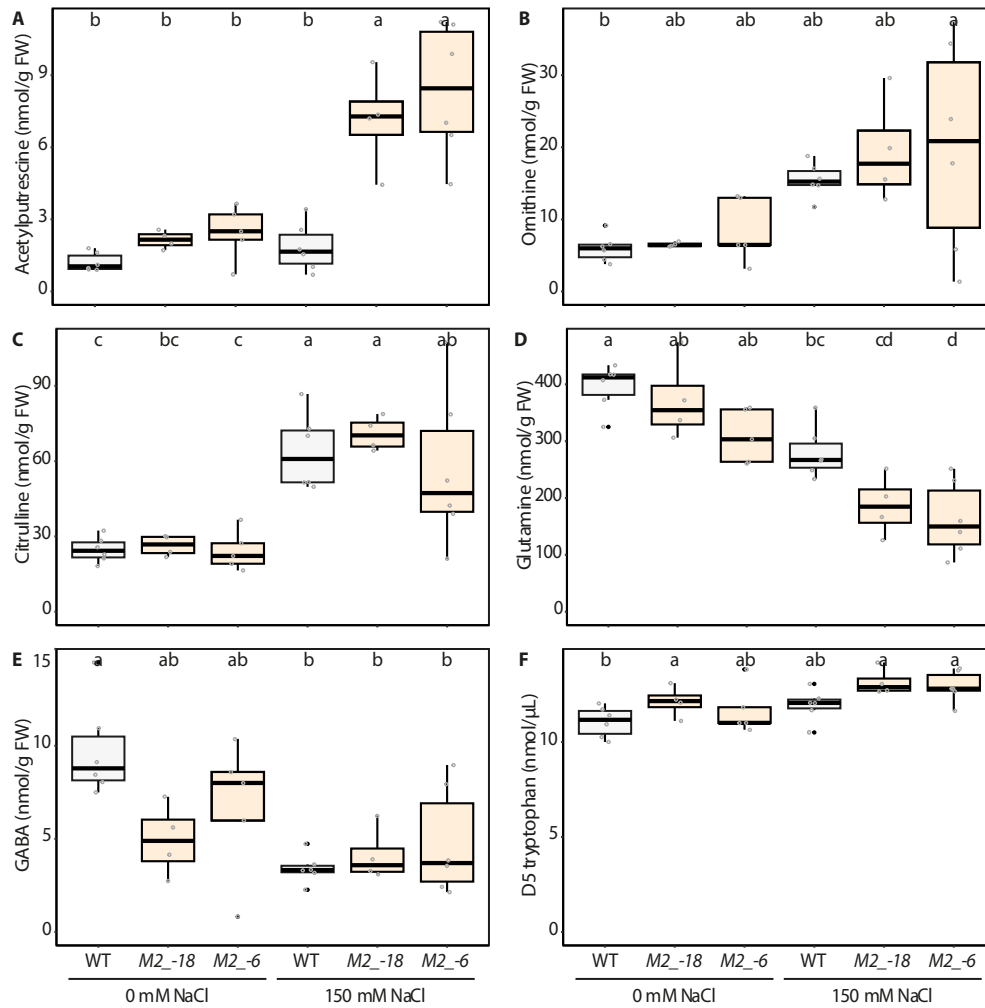
Supplementary Figure 13: Over expression of epitope-tagged tomato ADC proteins in *N. benthamiana* leaves for protein identification and quantification via MS.

N. benthamiana leaves were agroinfiltrated with constitutively expressed *HA-SIADC1*, *MYC-SIADC2*, and *GUS* (negative control) constructs. Samples were harvested for protein extraction at 1, 2, and 3 dpi, and protein expression was analysed via western blotting against the HA (**A, top**) or MYC epitope tags (**A, bottom**); expected sizes are 78.7 kDa and 79.9 for HA-SIADC1 and MYC-SIADC2, respectively. As positive controls (“+”), samples from a previously successful western blot were used, while GUS, with no epitope tag, was used as a negative control; 1, 2, or 3 dpi is displayed above each blot. Ponceau stains of each blot are shown below the western to verify even protein loading. (**B**) As the 2 dpi samples had the strongest western blot bands, these were run on an additional SDS-PAGE gel in duplicate (1 and 2, above gel) and stained with Coomassie before gel sections were cut at approximately 80 kDa for MS analysis.



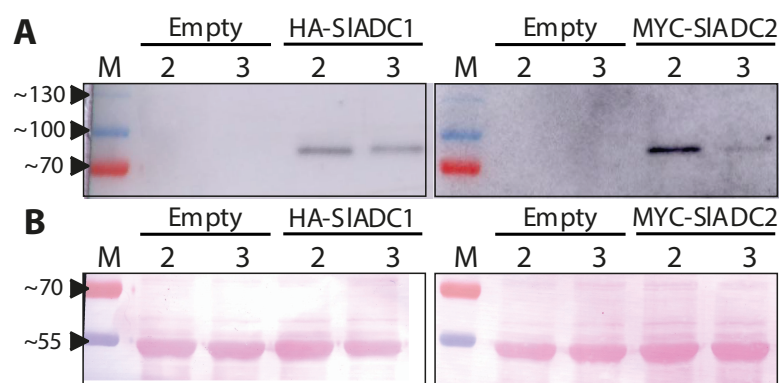
Supplementary Figure 14: Cold treatment did not influence levels of metabolites other than ADC activity.

WT (grey) and *Atadc1-box* mutants AtM1_-53 and AtM1_-15 (blue) were grown vertically on $\frac{1}{2}$ MS plates for 2 weeks before being cold treated for 12 hours at $\sim 5^{\circ}\text{C}$ and sampled for an ADC activity assay and LCMS analysis of the PA-related metabolite profile. Three seedlings were pooled to generate a sample of ~ 10 mg ($n = 3-4$). One replicate plate was kept in the climate chamber, wrapped in tinfoil as a room temperature (RT) negative control ($n = 1$). Samples were homogenised in buffer containing heavy arginine, incubated at 37°C for 1 hour, and prepared for LCMS analysis. Acetylputrescine (A), ornithine (B), citrulline (C), glutamine (D), and GABA (E) levels are shown in nmol/g FW, normalised to D5 tryptophan; D5 tryptophan (F) shown as nmol/ μL of buffer. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.



Supplementary Figure 15: Salt treatment amplified the effects of the *Atadc2-box* mutations on PA-related metabolite levels.

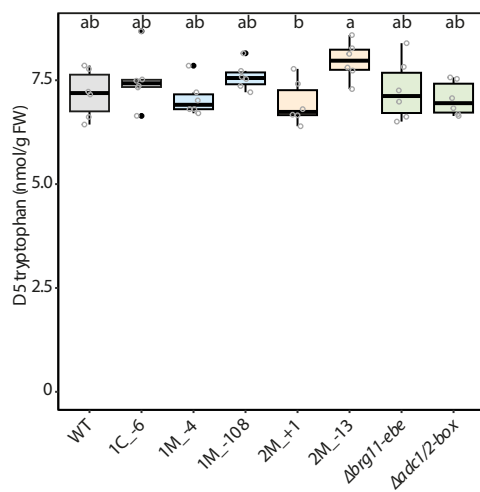
WT (grey) and *Atadc2-box* mutants AtM2_-18 and AtM2_-6 (yellow) were grown vertically on ½ MS plates for 7 days before being transferred to fresh ½ MS plates with or without 150 mM NaCl. After another 7 days of growth, samples for an ADC activity assay and LCMS analysis of the PA-related metabolite profile were taken. Three seedlings were pooled to generate a sample of ~10 mg (n = 4-6). Samples were homogenised in buffer containing heavy arginine, incubated at 37 °C for 1 hour, and prepared for LCMS analysis. Acetylputrescine (A), ornithine (B), citrulline (C), glutamine (D), and GABA (E) levels are shown in nmol/g FW, normalised to D5 tryptophan; D5 tryptophan (F) shown as nmol/μL of buffer. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.



Supplementary Figure 16: Transient expression of epitope-tagged tomato ADC proteins in *N. benthamiana* leaves for ADC activity and PA measurement via LCMS.

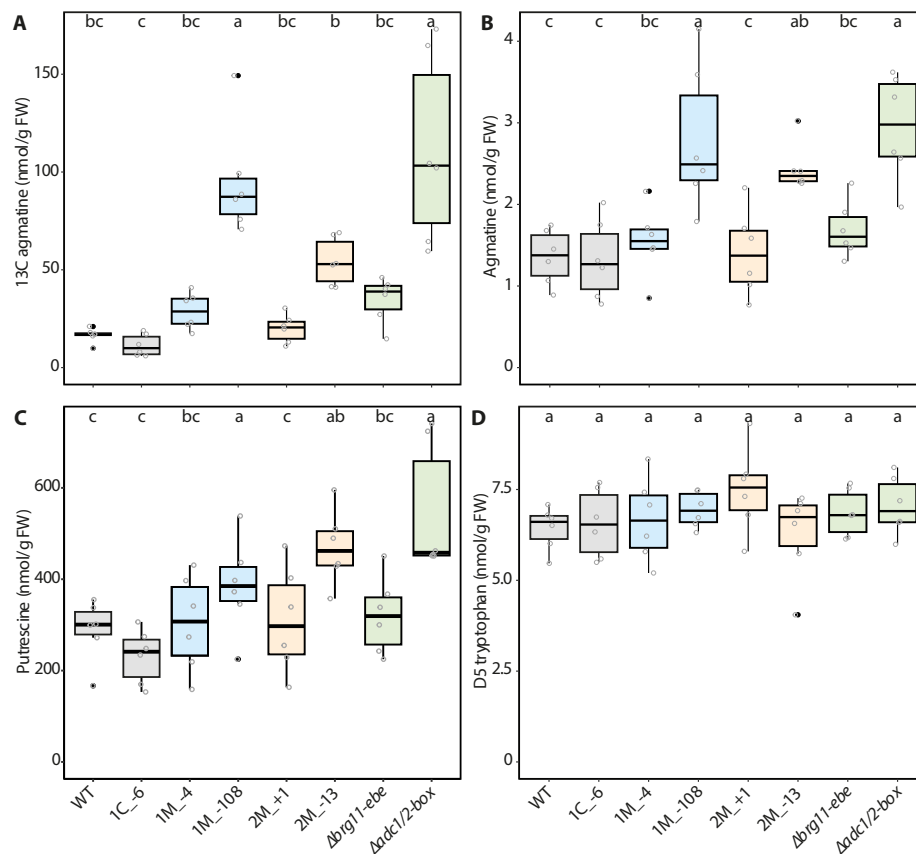
N. benthamiana leaves were agroinfiltrated with *35S:HA-SIADC1*, *35S:MYC-SIADC2*, or an empty strain (negative control). Samples were harvested for LCMS analysis after 2 days, but at both 2 and 3 days for protein extraction. Protein expression was analysed via western blotting against the HA (**A, left**) or MYC epitope tags (**A, right**); expected sizes are 78.7 kDa and 79.9 for HA-SIADC1 and MYC-SIADC2, respectively. Ponceau stains of each blot are shown below the western to verify even protein loading (**B**).

5.5 Characterisation of CRISPR mutants



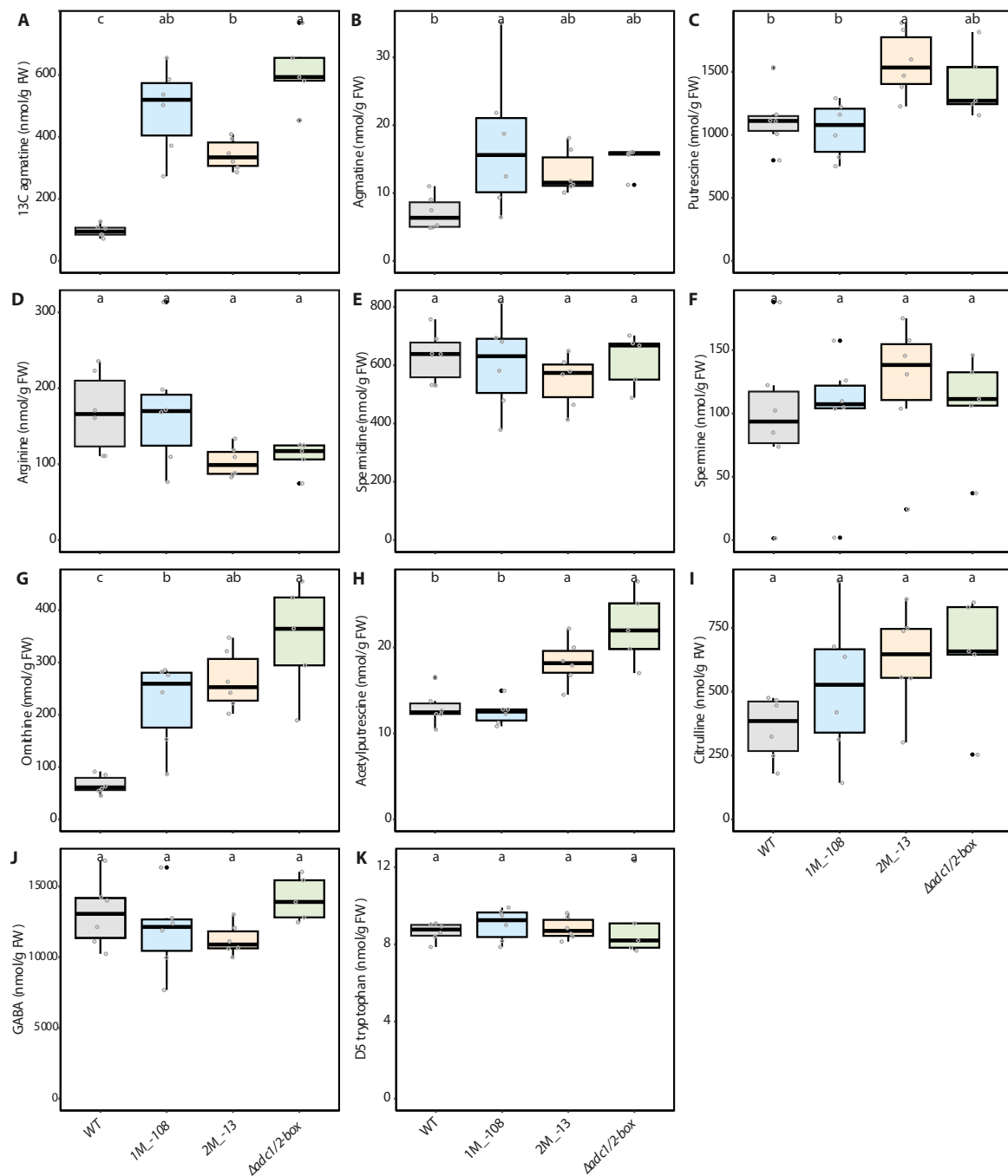
Supplementary Figure 17: D5 tryptophan levels are consistent across cold-treated *adc-box* mutant tomato genotypes.

Samples from cold-treated detached leaves of single and double tomato *ADC-box* mutants were prepared for ADC activity assays and LCMS analysis of their polyamine profile ($n = 6$). D5 tryptophan is used as an internal control in the ADC activity assay buffer and its levels are presented here in nmol/g FW. WT and the control line (S11C_-6) are shown in grey, *SLADC1-box* mutants (S11M_-4 and S11M_-108) in blue, *SLADC2-box* mutants (S12M_+1 and S12M_-13) in yellow, and double *SLADC-box* mutants (*brg11-ebe* and *adc1/2-box*) in green. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.



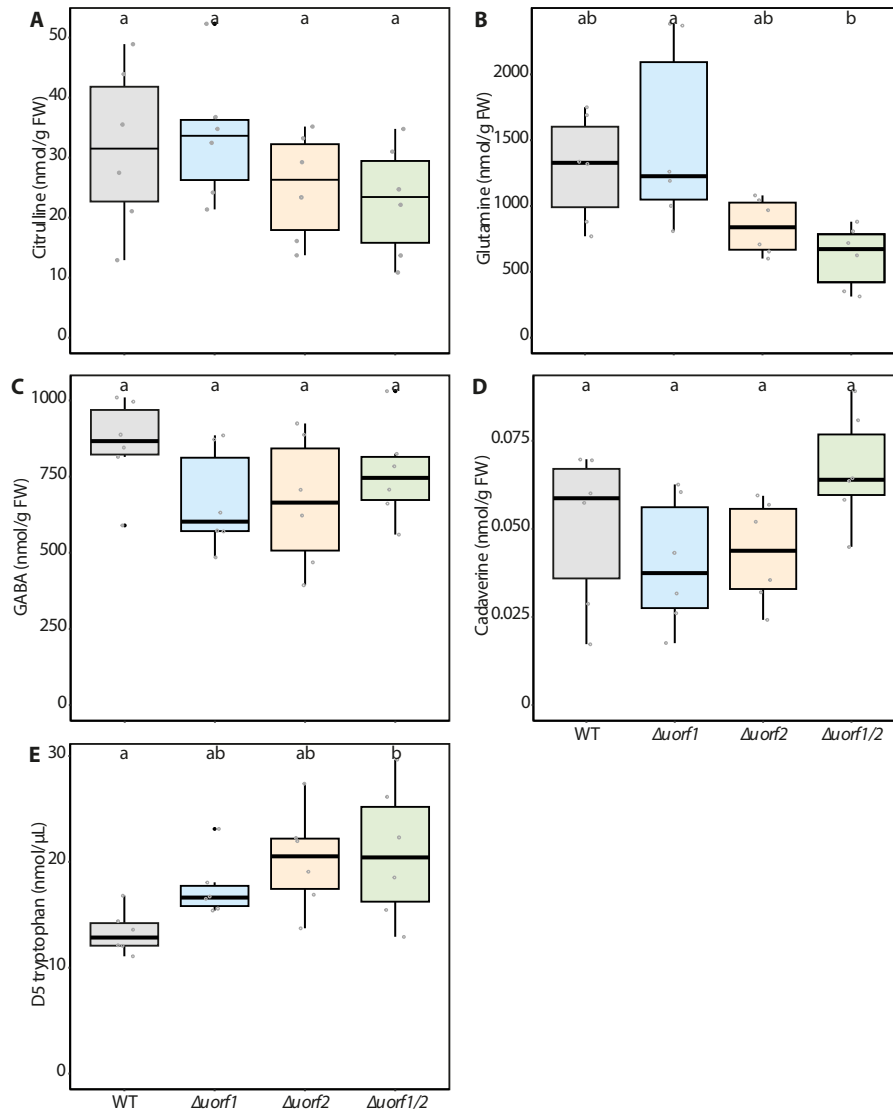
Supplementary Figure 18: Second ADC activity assay of all tomato ADC-box mutants

Samples from cold-treated detached leaves of single and double tomato *ADC-box* mutants were prepared for ADC activity assays and LCMS analysis of their polyamine profile. ADC activity (heavy agmatine) (A), agmatine (B), putrescine (C), and arginine (D) levels are shown in nmol/g FW, normalised to D5 tryptophan. WT and the control line (S11C_-6) are shown in grey, *SLADC1-box* mutants (S11M_-4 and S11M_-108) in blue, *SLADC2-box* mutants (S12M_+1 and S12M_-13) in yellow, and double *SLADC-box* mutants (*brg11-ebe* and *adc1/2-box*) in green. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.



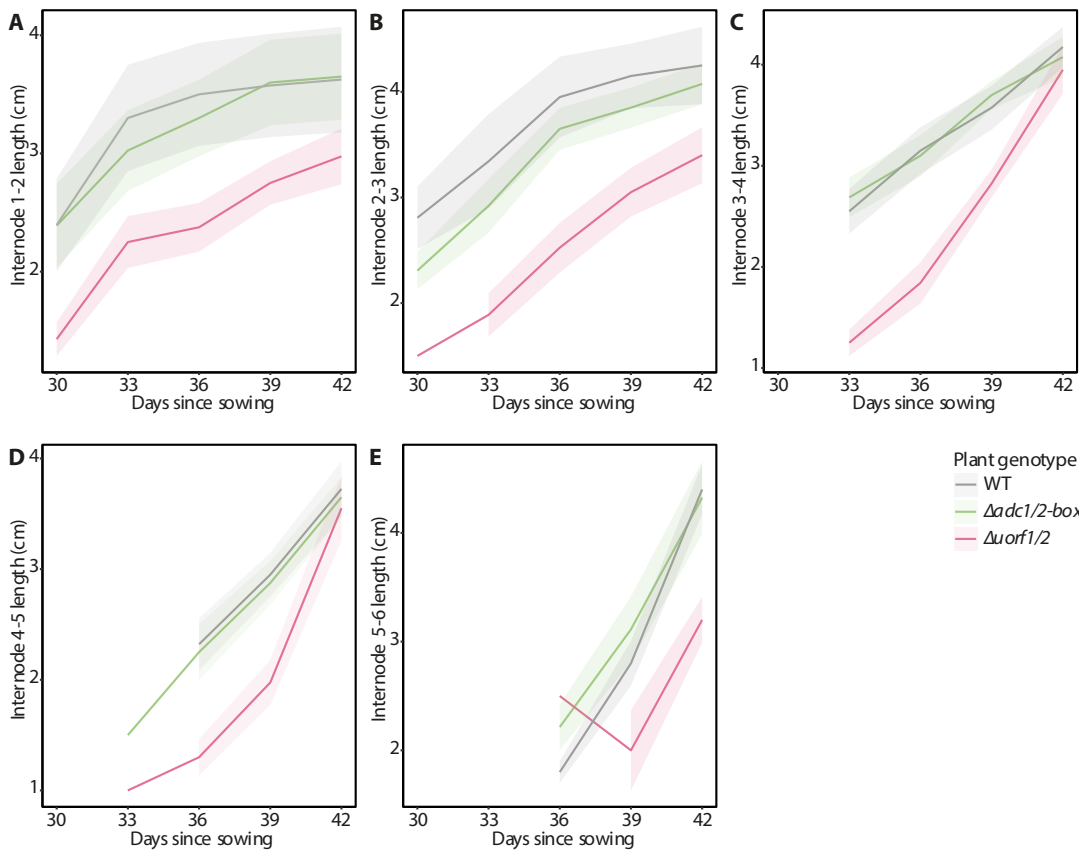
Supplementary Figure 19: Third ADC activity assay with single and double *adc-box* tomato mutants.

Samples from cold-treated detached leaves of single and double tomato *ADC-box* mutants were prepared for ADC activity assays and LCMS analysis of their polyamine profile. WT and the control line (SI1C_-6) are shown in grey, *SIADC1-box* mutants (SI1M_-4 and SI1M_-108) in blue, *SIADC2-box* mutants (SI2M_+1 and SI2M_-13) in yellow, and double *SIADC-box* mutants (*brg11-ebe* and *adc1/2-box*) in green. ADC activity (heavy agmatine) (A), agmatine (B), putrescine (C), arginine (D), spermidine (E), spermine (F), ornithine (G), acetylputrescine (H), citrulline (I), and GABA (J) levels are shown in nmol/g FW, normalised to D5 tryptophan; D5 tryptophan levels (K) shown as nmol/g FW. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.

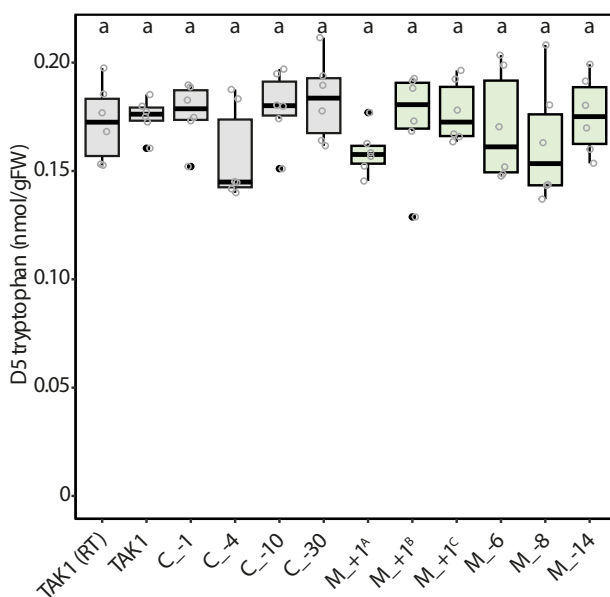


Supplementary Figure 20: *SLADC* $\Delta uorf$ mutants have lower ADC activity and some metabolite levels compared to WT.

Samples from cold-treated detached leaves of WT (grey), $\Delta uorf1$ (blue), $\Delta uorf2$ (yellow) and the double tomato *uorf* mutant ($\Delta uorf1/2$, green) were prepared for ADC activity assays and LCMS analysis of their polyamine profile. Citrulline (**A**), glutamine (**B**), GABA (**C**), and cadaverine (**D**) levels are shown in nmol/g FW, normalised to D5 tryptophan (**E**; presented as nmol/μL of buffer). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.



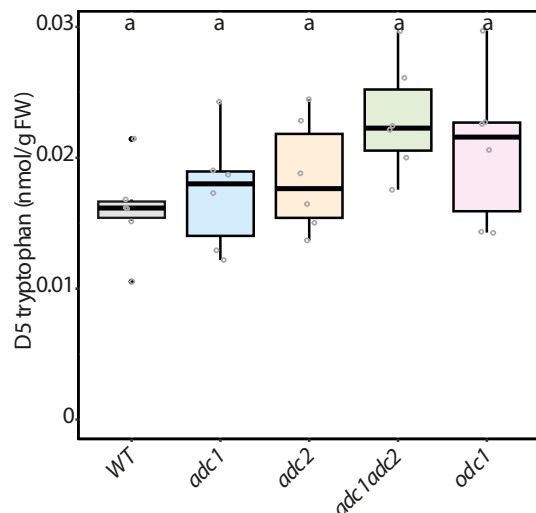
Supplementary Figure 21: Internode lengths of $\Delta uorf1/2$ are smaller than WT and $\Delta adcl/2\text{-box}$ at 30 to 42 days after sowing. After 30, 33, 36, 39, and 42 days after sowing seeds, the internode lengths between leaves 1-2 (A), 2-3 (B), 3-4 (C), 4-5 (D), and 5-6 (E) of WT (grey), $\Delta uorf1/2$ (pink), and $\Delta adcl/2\text{-box}$ (green) tomato plants were measured ($n = 20$). Shaded area shadowing each line represents a 95% confidence interval, where any point that the shaded areas of two lines are not covered by one another, they are statistically significantly different to the $\alpha = 0.05$ level; when the shaded area is lacking, less than 3 plants had those leaves at that measurement day.



Supplementary Figure 22: D5 tryptophan levels are unaffected by *MpADC-box* genotype.

M. polymorpha TAK1 (grey), control mutants (grey), and *Mpadc-box* mutants (green) were cold-treated for 16 hours, before samples were taken and prepared for ADC activity assays and LCMS analysis of the polyamine profile ($n = 6$). As a negative treatment control, an additional plate of TAK1 plants was kept at room temperature in the dark (RT; grey; $n = 6$). D5 tryptophan is used as an internal control in the ADC activity assay buffer and its levels are presented here in nmol/g FW. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

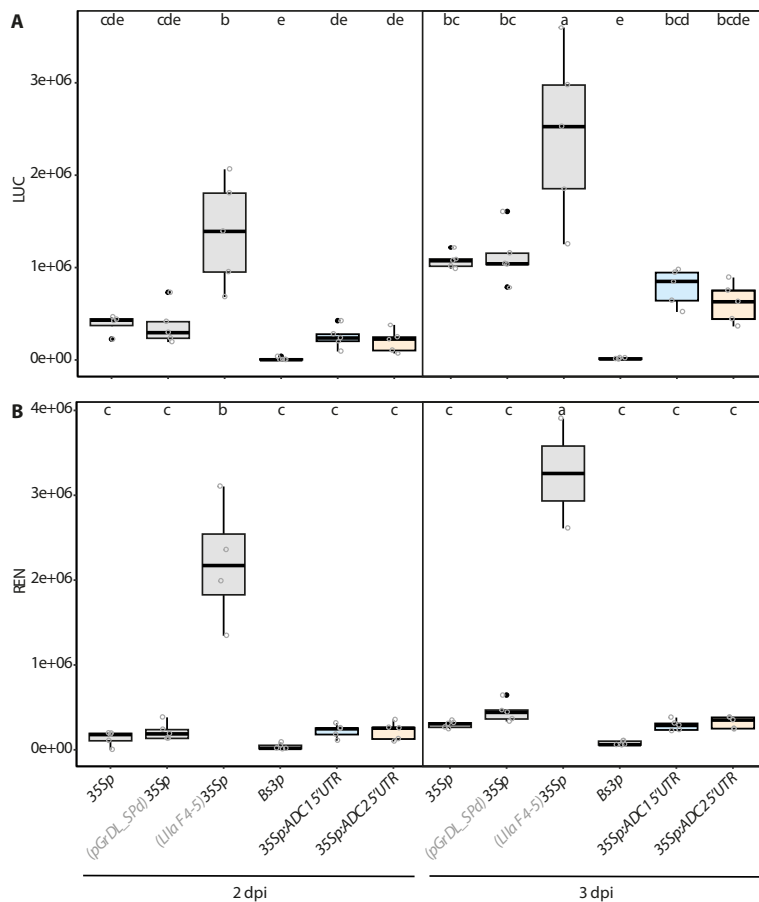
5.6 Analysis of tomato *adc* and *odc* *cds* mutants *ADC-box* mechanism



Supplementary Figure 23: D5 tryptophan levels are consistent across cold-treated *adc1*, *adc2*, *adc1adc2*, and *odc1* mutant tomato genotypes.

Samples from cold-treated detached tomato leaves of WT (grey), single *adc1* (blue), *adc2* (yellow), and double *adc* (green) mutants, and the *odc* mutant (pink) were prepared for ADC activity assays and LCMS analysis of their polyamine profile ($n = 6$). D5 tryptophan is used as an internal control in the ADC activity assay buffer and its levels are presented here in nmol/g FW. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

5.7 *ADC-box* mechanism



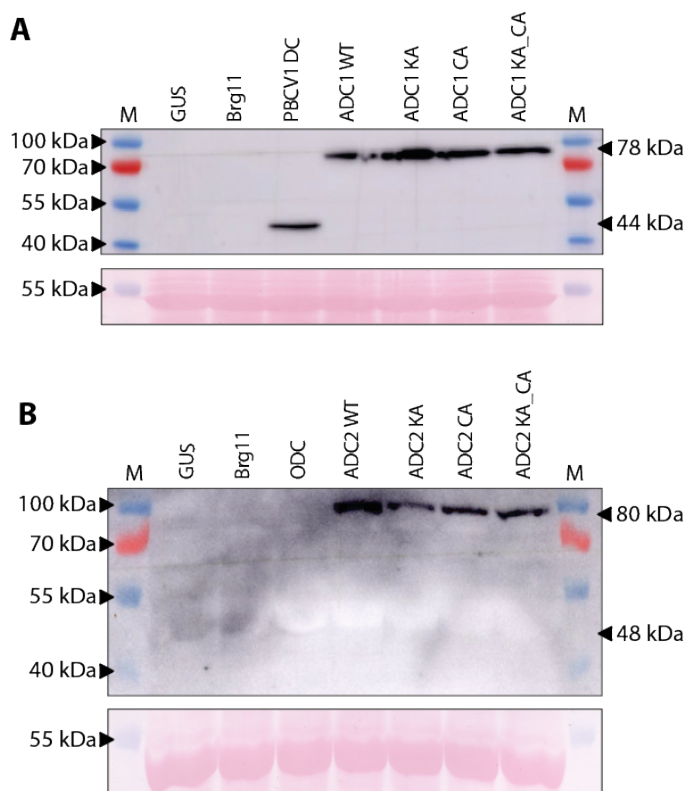
Supplementary Figure 24: Individual luciferase and renilla luminescence of golden gate compatible *pGrDL_SPb* (GG) reporter plasmids, *pGrDL_SPb*, and *35Sp:LUC* in *LIIα F 4-5*.

Golden-gate compatible (*pGrDL_SPb* (GG)) luciferase reporter constructs with a *35Sp* promoter (*35Sp*) as a positive control, *Bs3p* promoter (*Bs3p*) as a negative control, and *35Sp*-driven tomato *ADC* 5'UTRs (*SIADC1/2* 5'UTR) were generated and agroinfiltrated into WT *N. benthamiana* leaves ($n = 5$). The original *pGrDL_SPb* plasmid (Moyle *et al.*, 2017) with a *35Sp:LUC* reporter, as well as the previously generated *35Sp:LUC* construct with an *LIIα F 4-5* backbone were also agroinfiltrated in the same manner. After 2 dpi (left) and 3 dpi (right), samples were taken for luminescence measurement. Luciferase luminescence was normalised to renilla luminescence and LUC/REN is reported here as arbitrary units (AU). An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.

pGrDL_SPb	TAAACGACGGCCAGTGAGCGCGGTAATACGACTCACTATAGGCGAATTGGGTACCGG	3000
pGrDL_SPb_GG	TAAACGACGGCCAGTGAGCGCGGTAATACGACTCACTATAGGCGAATTGGGTACCGG	3000
pGrDL_SPb	GCCACCCCTACTCCAAAAATGTCAAAGATACAGTCTCAGAAGACCAAGGGCTA---	3054
pGrDL_SPb_GG	GCCACCCCTACTCCAAAAATGTCAAAGATACAGTCTCAGAAGACCAAGGGCTA GCG	3060
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	GAGATTAGCCTTTTCAATTTCAGAAAGATGCTAACCCACAGATGGTTAGAGGGCTTAC	3120
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	GCAGCAGACTCATCAAGACGATCTACCCGAGCAATAATCTCCAGGAATCAAATACCTT	3180
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	CCCAAGAAGGTTAAAGATGCAGTCAAAAGATTCAAGACTAAGTGCATCAAGAACACAGAG	3240
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	AAAGATATATTTCTCAAGATCAGAAGTACTATTCCAGTATGGACGATTCAAGGCTTGCTT	3300
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	CACAAACCAAGGCAAGTAATAGAGATTGGAGTCTCTAAAGAGGTAGTTCCTCACTGAATCA	3360
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	AAGGCCATGGAGTCAAAGATTCAAATAGAGACCTAACAGAACTGCGCGTAAAGACTGGC	3420
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	GAACAGTTCATACAGAGTCTCTTACGACTCAATGACAAGAAGAAATCTTCGTCAACATG	3480
pGrDL_SPb	-----	3054
pGrDL_SPb_GG	GTGGAGCACGACACACTGTCTACTCCAAAAATATCAAGATACAGTCTCAGAAGAGCAA	3540
pGrDL_SPb	-----TTGAGACTTTTCAACAAAGGGTAATTCGGGAAACCTCTCGGATTCATTGC	3110
pGrDL_SPb_GG	AGGGCAATTGAGACTTTTCAACAAAGGGTAATATCCGGAACCTCTCGGATTCATTGC	3600
pGrDL_SPb	-----	3170
pGrDL_SPb_GG	CCAGCTATCTGTCACTTCAATCGAAAGGACAGTAGAAAAGGAAGGTGGCTCTCAAAATGC	3660
pGrDL_SPb	-----	3230
pGrDL_SPb_GG	CATCATTCGATAAAGGAAAGGCTATCATTCAAGATGCCTCTGCGACAGTGGTCCCAA	3720
pGrDL_SPb	-----	3290
pGrDL_SPb_GG	GATGGACCCCAACCCAGGAGCATCGTGGAAAAAGAACGTTCCAAACACGCTTCA	3780
pGrDL_SPb	-----	3350
pGrDL_SPb_GG	AAGCAAGTGGATTGATGTGACATCTCCACTGACGTAAGGGATGACGCAATCCCACTAT	3840
pGrDL_SPb	-----	3403
pGrDL_SPb_GG	CCTTCGCAAGACCTTCTCTATATAAGGAAGTTCAATTCATTGGAGAGGAC-----	3900
pGrDL_SPb	CTCGAG ATGGAAGACGCCAAAAACATAAAGAAAGGCCGCGCCATTCTATCCGCTGGAA	3463
pGrDL_SPb_GG	CTCGAG ATGGAAGACGCCAAAAACATAAAGAAAGGCCGCGCCATTCTATCCGCTGGAA	3960
pGrDL_SPb	-----	3523
pGrDL_SPb_GG	GATGGAACCGCTGGAGAGCAACTGCATAAGGCTATGAAGAGATACGCCCTGGTTCTCGGA	4020
pGrDL_SPb	-----	3583
pGrDL_SPb_GG	ACAATTGCTTTTACAGATGCACATATCGAGGTGGACATCACTTACGCTGAGTACTTCGAA	4080

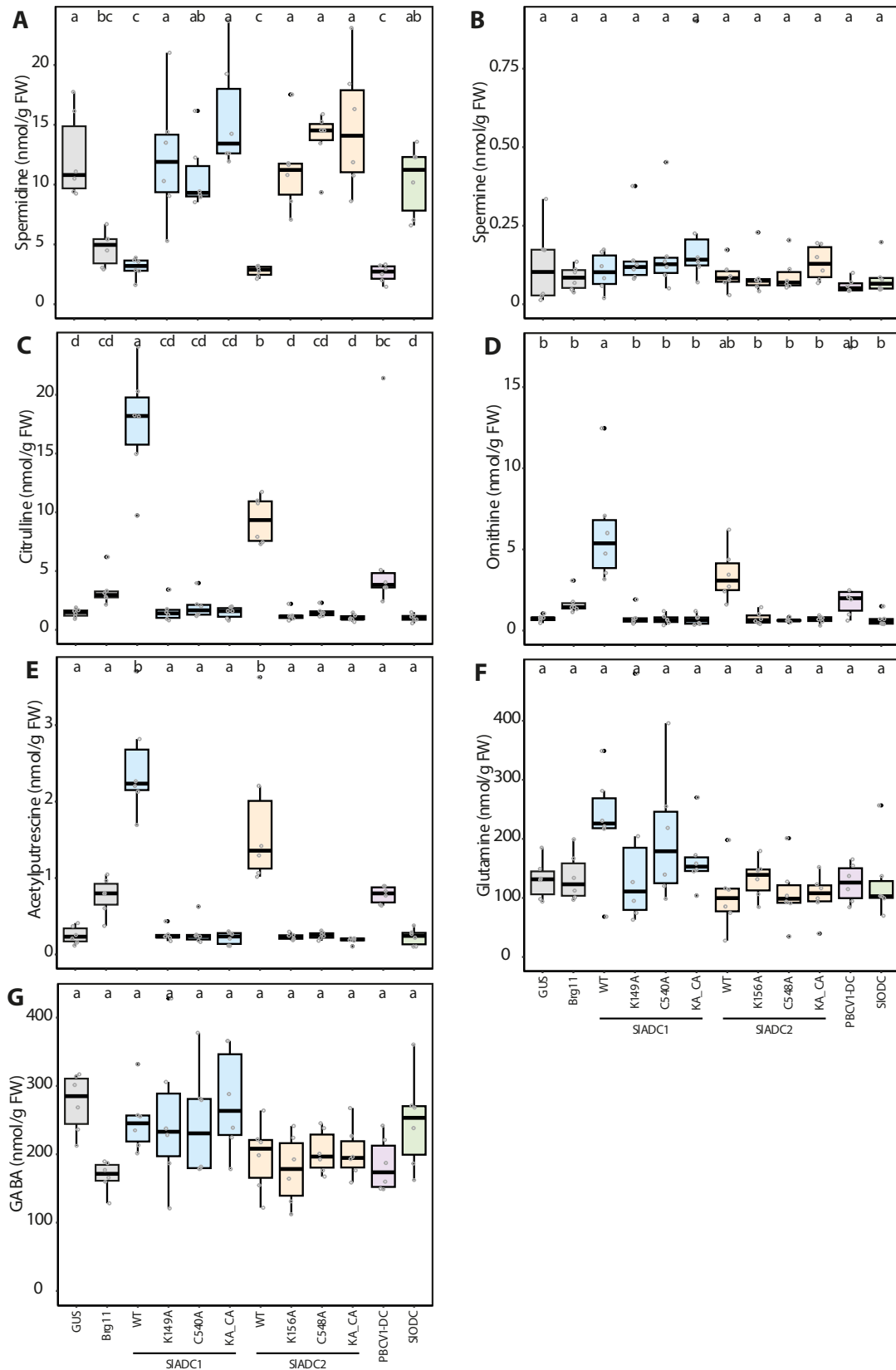
Supplementary Figure 25: Alignment of *pGrDL_SPb* and *pGrDL_SPb (GG)* plasmids, showing differences in *35S* promoters and sequences 5' to the luciferase start codon.

Alignment of full *pGrDL_SPb* and *35Sp:LUC pGrDL_SPb (GG)* plasmid sequences generated via Clustal. A section of the alignment shown here to highlight the differences in the *35Sp* sequences (bold text, grey), the *BsaI* overhangs used for cut ligations (white bold text, black background), and luciferase gene (bold text, green) with its start codon (underlined). Matching sequences are indicated by an asterisk below the alignment.



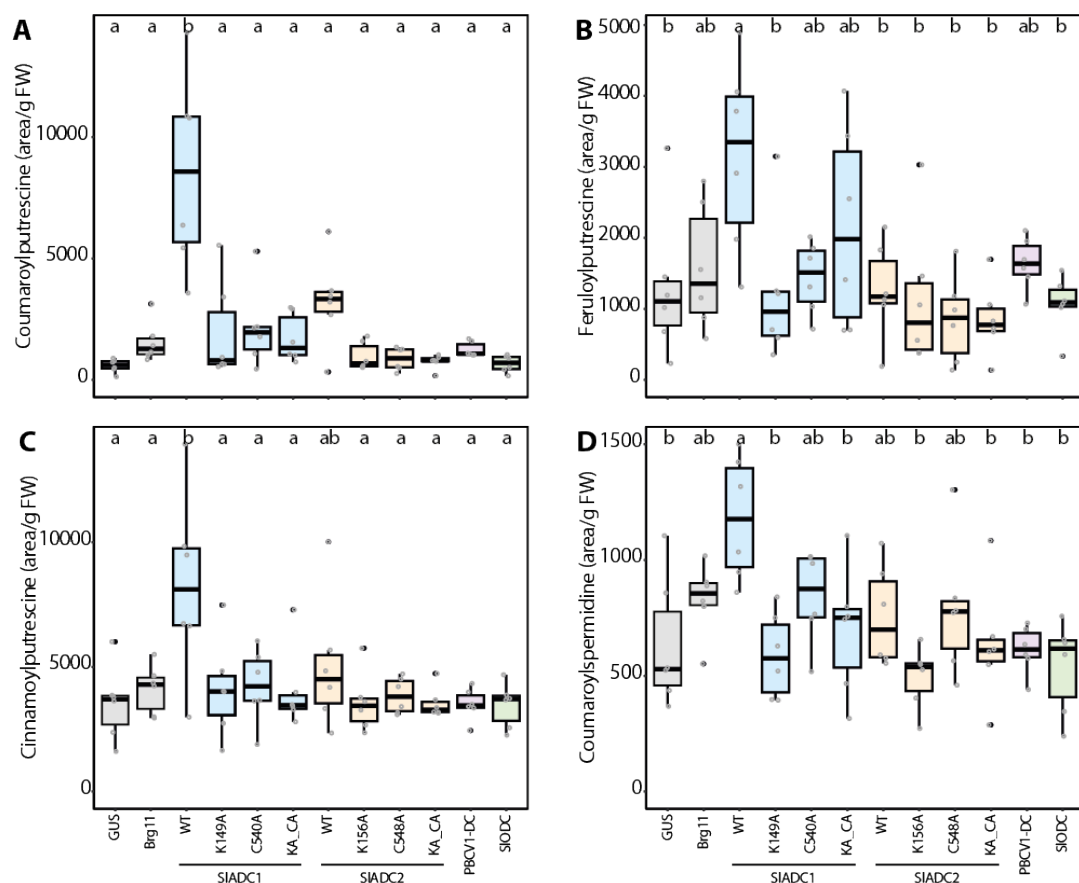
Supplementary Figure 27: Verification of transient expression of SIADC1/2, SIODC, and PBCV-1 DC proteins in WT *N. benthamiana* for LCMS analysis.

Epitope-tagged, WT and catalytic SIADC1/2 mutants, SIODC, and PBCV-1 DC, as well as control GUS (negative) and Brg11 (positive) constructs for LCMS analysis, were transiently expressed in WT *N. benthamiana* leaves for 2-3 days; samples were taken for western blot and LCMS analysis. HA-tagged SIADC1 proteins, HA-tagged PBCV-1 DC, GUS, and Brg11 (**A**), and MYC-tagged SIADC2 proteins, SIODC-MYC, GUS, and Brg11 (**B**) were run on a 10% SDS-PAGE gel. Blots were incubated with anti-HA-HRP (**A**) or with anti-MYC primary and anti-mouse secondary (**B**) antibodies. Molecular weight marker (M) band sizes (left) and expected weights of expressed tagged proteins (right) are shown; SIADC1 78 kDa, PBCV-1 DC 44 kDa, SIADC2 80 kDa, and SIODC 48 kDa. Ponceau stained blot shown below each western blot.



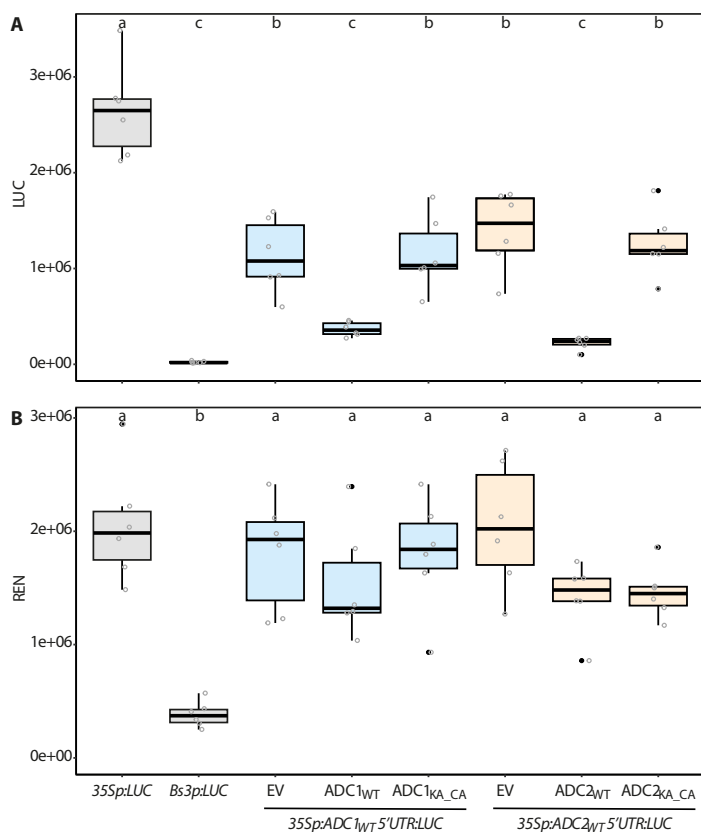
Supplementary Figure 28: Remaining PA levels from transiently expressed WT and catalytically dead ADCs, ODC, and PBCV1-DC proteins.

After 2 dpi, samples of GUS (grey), Brg11 (grey), SIADC1 WT and mutant variants (blue), SIADC2 WT and mutant variants (yellow), PBCV1-DC (purple), and SIODC (green) constitutively expressed in *N. benthamiana* leaves were harvested for LCMS analysis (n=6). Spermidine (A), spermine (B), citrulline (C), ornithine (D), acetylputrescine (E), glutamine (F), and GABA (G) were measured. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.



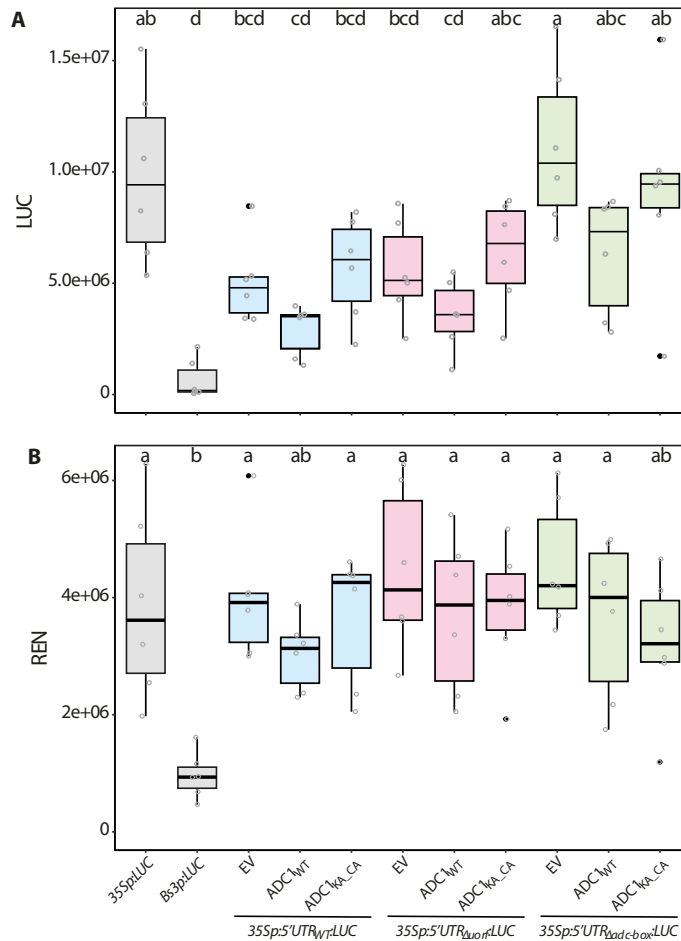
Supplementary Figure 29: Phenolamide levels from transiently expressed WT and catalytically dead ADCs, ODC, and PBCV1-DC proteins.

After 2 dpi, samples of GUS (grey), Brg11 (grey), SIADC1 WT and mutant variants (blue), SIADC2 WT and mutant variants (yellow), PBCV1-DC (purple), and SIODC (green) constitutively expressed in *N. benthamiana* leaves were harvested for LCMS analysis ($n = 6$). Coumaroylputrescine (**A**), feruloylputrescine (**B**), cinnamoylputrescine (**C**), and coumaroylspermidine (**D**) were measured. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.



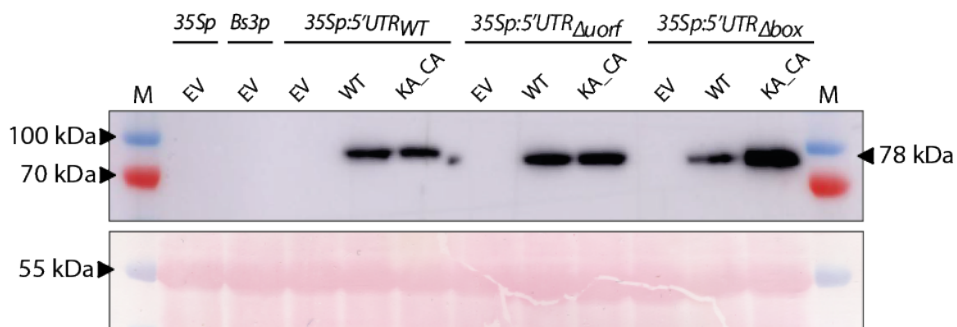
Supplementary Figure 30: Individual luciferase and renilla luminescence of 35Sp:SIADC1/2_{WT} 5'UTR:LUC reporters co-expressed with EV, SIADC1_{WT} or SIADC1_{KA_CA} proteins.

35Sp:SIADC1/2_{WT} 5'UTR:LUC reporters co-expressed with EV, SIADC1/2_{WT}, or SIADC1/2_{KA_CA} proteins in *N. benthamiana* leaves (n = 6). 35Sp:LUC and Bs3p:LUC reporters were used as positive and negative controls for luciferase expression. After 2 dpi, samples were taken for luminescence measurement. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.



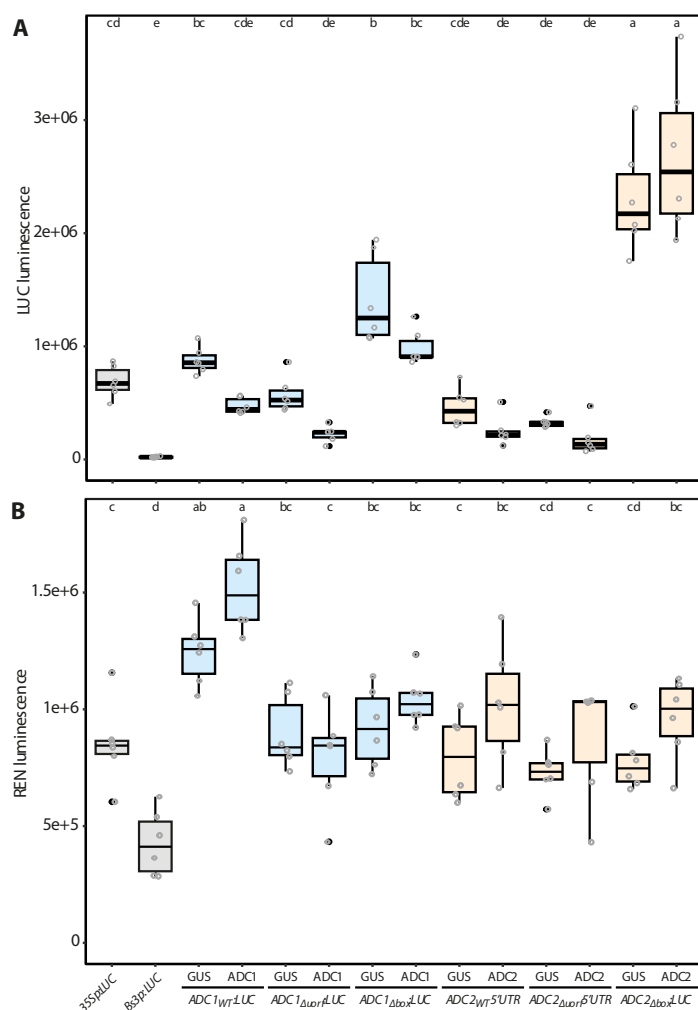
Supplementary Figure 31: Individual luciferase and renilla luminescence of *35Sp:SIADC1_{WT/Δuorf/Δadc-box} 5'UTR:LUC* reporters co-expressed with EV, SIADC1_{WT} or SIADC1_{KA_CA} proteins.

35Sp-driven *SIADC1 5'UTR:LUC* reporters (*WT*, *Δuorf*, and *Δadc-box* variants) co-expressed with EV, SIADC1_{WT}, or catalytically dead SIADC1 (SIADC1_{KA_CA}) proteins in *N. benthamiana* leaves (n = 6). *35Sp:LUC* and *Bs3p:LUC* reporters were used as positive and negative controls for luciferase expression. After 2 dpi, samples were taken for luminescence measurement. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different to $\alpha = 0.05$.



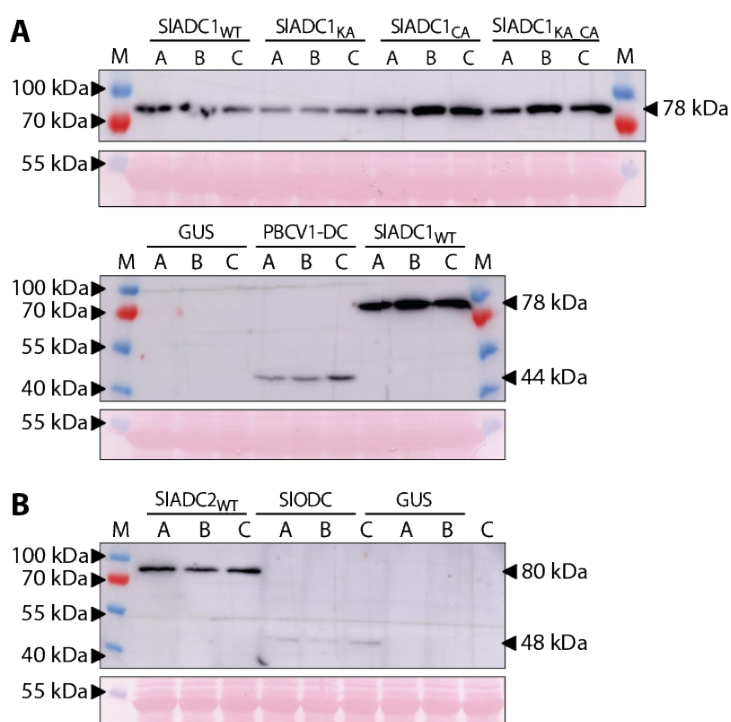
Supplementary Figure 32: Western blot of SIADC1_{WT} and SIADC1_{KA_CA} proteins co-expressed with WT and mutant SIADC1 luciferase reporter constructs.

35Sp:LUC, *Bs3p:LUC*, and *35Sp:SIADC1_{WT/Δuorf/Δadc-box} 5'UTR:LUC* reporters co-expressed with EV, SIADC1_{WT}, or catalytically dead SIADC1 (SIADC1_{KA_CA}) proteins in *N. benthamiana* leaves (n = 6). After 2 dpi, one leaf disk was taken from each infiltration replicate and combined into one pooled sample for a western blot. Blot was incubated with an anti-HA-HRP antibody to detect HA-tagged SIADC proteins. Molecular weight marker (M) band sizes (left) and expected size of tagged SIADC1 proteins (right).



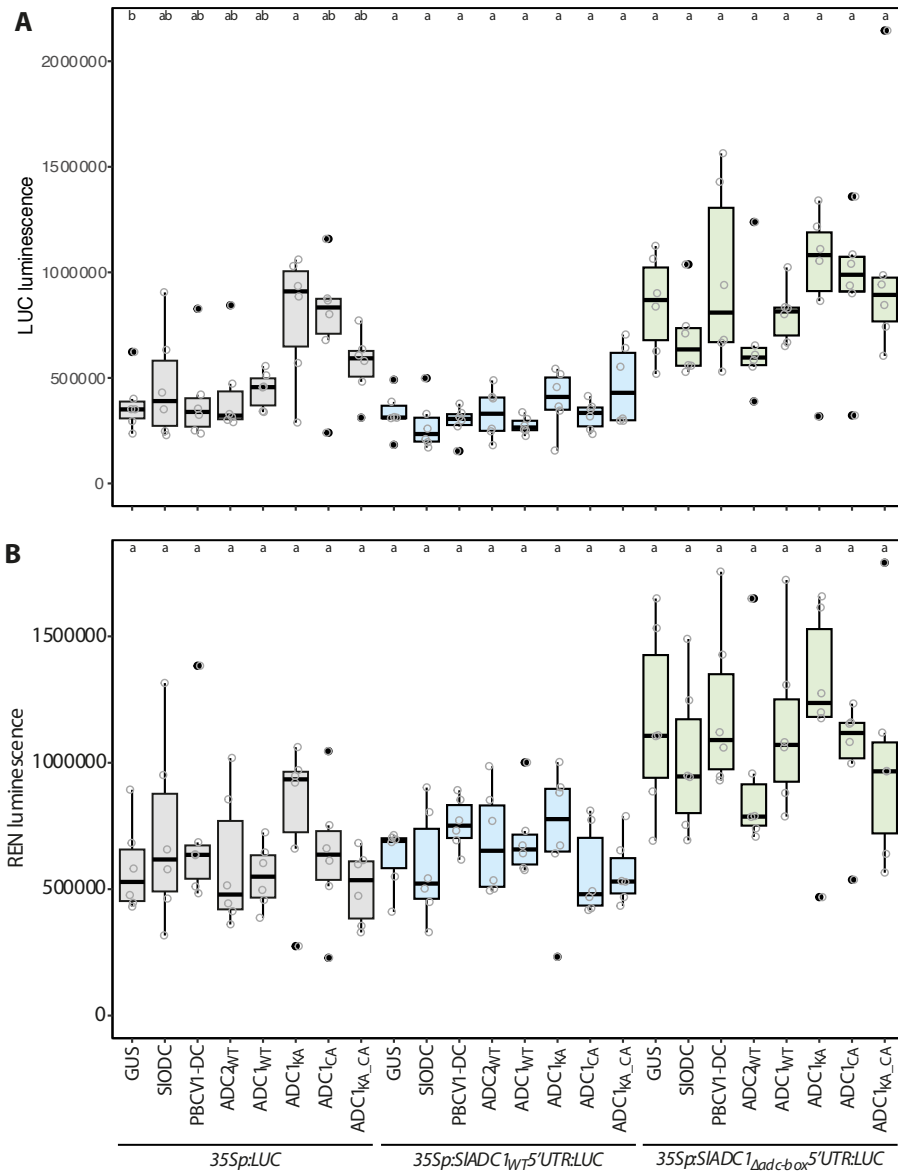
Supplementary Figure 33: Individual luciferase and renilla luminescence of *35Sp:SIADC1/2 5'UTR:LUC* reporters co-expressed with GUS or SIADC1/2_{WT} proteins.

35Sp:SIADC1/2 5'UTR:LUC (WT, $\Delta uorf$ or $\Delta adc-box$) reporters were co-expressed with GUS or SIADC1/2_{WT} proteins in *N. benthamiana* leaves (n = 6). After 2 dpi, samples were taken for luminescence measurement. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.



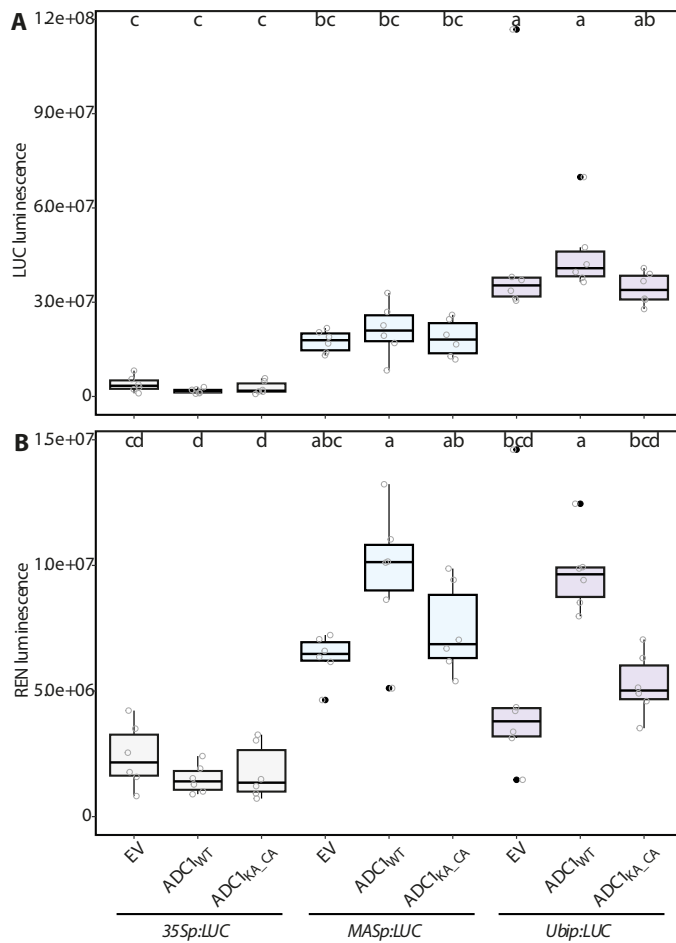
Supplementary Figure 34: Polyamine related proteins are evenly expressed when co-expressed with *35Sp:LUC*, *35Sp:SIADC1_{WT} 5'UTR:LUC*, and *35Sp: SIADC1_{Δadc-box} 5'UTR:LUC* reporters

35Sp:LUC (A), *35Sp:SIADC1_{WT} 5'UTR:LUC* (B), and *35Sp:SIADC1_{Δadc-box} 5'UTR:LUC* (C) reporters co-expressed with GUS, SIODC, PBCV1-DC, SIADC2_{WT}, SIADC1_{WT}, SIADC1_{KA}, SIADC1_{CA}, or SIADC1_{KA CA} proteins in *N. benthamiana* leaves (n = 6). After 2 dpi, one leaf disk was taken from each infiltration replicate and combined into one pooled sample for a western blot. Blot was incubated with an anti-HA-HRP antibody to detect HA-tagged proteins (A) or anti-MYC primary and anti-mouse-HRP secondary antibodies to detect MYC-tagged proteins (B). Molecular weight marker (M) band sizes (left) and expected size of tagged proteins (right).



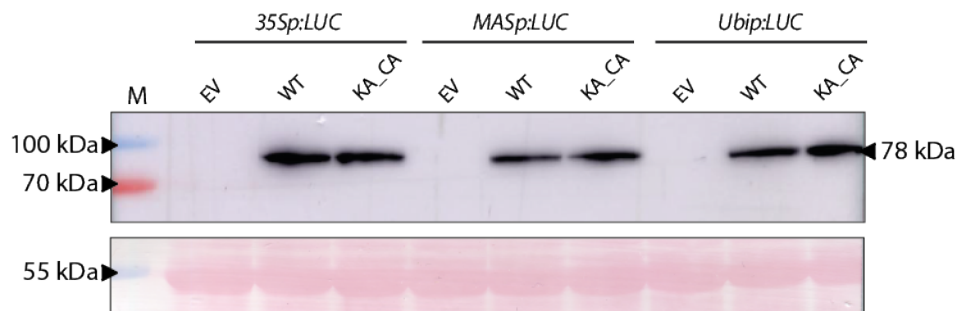
Supplementary Figure 35: Individual luciferase and renilla luminescence of *35Sp:LUC*, *35Sp:SIADC1_{WT} 5'UTR:LUC*, and *35Sp: SIADC1_{Δadc-box} 5'UTR:LUC* reporters co-expressed with polyamine-related proteins.

35Sp:LUC, *35Sp:SIADC1_{WT} 5'UTR:LUC*, and *35Sp:SIADC1_{Δadc-box} 5'UTR:LUC* reporters co-expressed with GUS, SIODC, PBCV1-DC, SIADC2_{WT}, SIADC1_{WT}, SIADC1_{KA}, SIADC1_{CA}, or SIADC1_{KA_CA} proteins in *N. benthamiana* leaves (n = 6). After 2 dpi, samples were taken for luminescence measurement. An ANOVA, followed by a Tukey HSD test, was conducted between samples from the same LUC reporter and not between all other samples; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.



Supplementary Figure 36: Individual luciferase and renilla luminescence of 35Sp:LUC, MASp:LUC, and Ubip:LUC reporters co-expressed with EV, SIADC1_{WT} or SIADC1_{KA_CA} proteins.

35Sp:LUC, MASp:LUC, and Ubip:LUC reporters co-expressed with EV, SIADC1_{WT}, or catalytically dead SIADC1 (SIADC1_{KA_CA}) proteins in *N. benthamiana* leaves (n = 6). After 2 dpi, samples were taken for luminescence measurement. An ANOVA, followed by a Tukey HSD test, was conducted to determine statistical significance; letters displayed above each box indicate significance, where groups with different letters are significantly different at the $\alpha = 0.05$ level.



Supplementary Figure 37: SIADC1_{WT} and SIADC1_{KA_CA} proteins are evenly expressed, despite differences in reporter expression from 35Sp:LUC, MASp:LUC, and Ubip:LUC.

35Sp:LUC, MASp:LUC, and Ubip:LUC reporters co-expressed with EV, SIADC1_{WT}, or catalytically dead SIADC1 (SIADC1_{KA_CA}) proteins in *N. benthamiana* leaves (n = 6). After 2 dpi, one leaf disk was taken from each infiltration replicate and combined into one pooled sample for a western blot. Blot was incubated with an anti-HA-HRP antibody to detect HA-tagged SIADC proteins. Molecular weight marker (M) band sizes (left) and expected size of tagged SIADC1 proteins (right).

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SlADC1      TCATTT CGCAG TTGTT TACAG TTTCCTAAACGGAAC TACCATC TCTTT TTCCGGTT
SlADC2      TCAC TTCA TAA--GC TGTTAGT TTCAC-----GT TC TCT TC GCAAT TTC CATTACAGCC
          ***  ***   *   * *   *****   *       *   *****   *   *   * *   *

SlADC1      ACCGTT TAGGT TTCCGAC CAA TT TTC TTCTT CTCCGGT GAACT ATTCCGGT TAAAAGTTG
SlADC2      GC-----TAGGTTT CCGTCCA--CT TC TTC TTCTCCA-----TTAA TATCTG
          *       *****   * *   *****   *****   *****   * *   *

SlADC1      T ATGGTGGGGTGT TTGTTGA ATCTAGGT TTGTG TTTCTGTTGGTT-TTGA GTTTAAT
SlADC2      T ATGGTGT TCT TCTTTTGA ATTC TAGAT TTTTCTT TGAAT TC TTGTTAATCTTAA T
          *****   *   * *   *****   * *   * *   *   *   *   *   *   *

SlADC1      TCTTGT TT TGATT TAGAAAAT TT TGC TTCTT CTGAA GTCGTGTGGGGATA TTCCGGT C
SlADC2      TTTCTT GTTTT AAATC AAGAAAT TAGCT TCT TC TGAAG TCAGG GTGGGGAT TT TTGATC
          *   *   *   *   *   *   *   * *   *****   *****   * *   *

SlADC1      GTGTAA GAGTGTGTTAGAAGGTGATTATCTT TTGATTAAGT TC TTT TT TGCTCTCTTT G
SlADC2      GTGTCAAAGTG TGTAGAGGGTGATTATCTT TTGAT TC ACTTG TTT TT TGCTCTCTTT G
          *****   *   *****   *****   *   *   *****   *****

SlADC1      AGGGGGTAGCCGGGGCTCTGGCCTCGGCGGGT TCTAA-AGCCCC AGCTATTACAACAT T
SlADC2      AGGGGGTAGCCGGGGCTCGGCCTCGGCGGGT TT TAA TAGCCCC AACTATTACAACAT T
          *****   *****   *****   * *   *****   *****

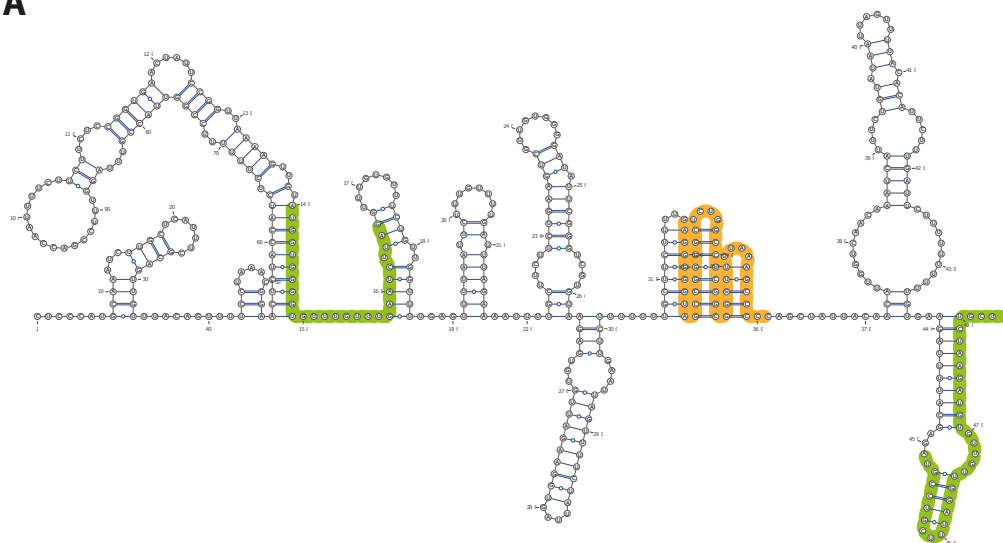
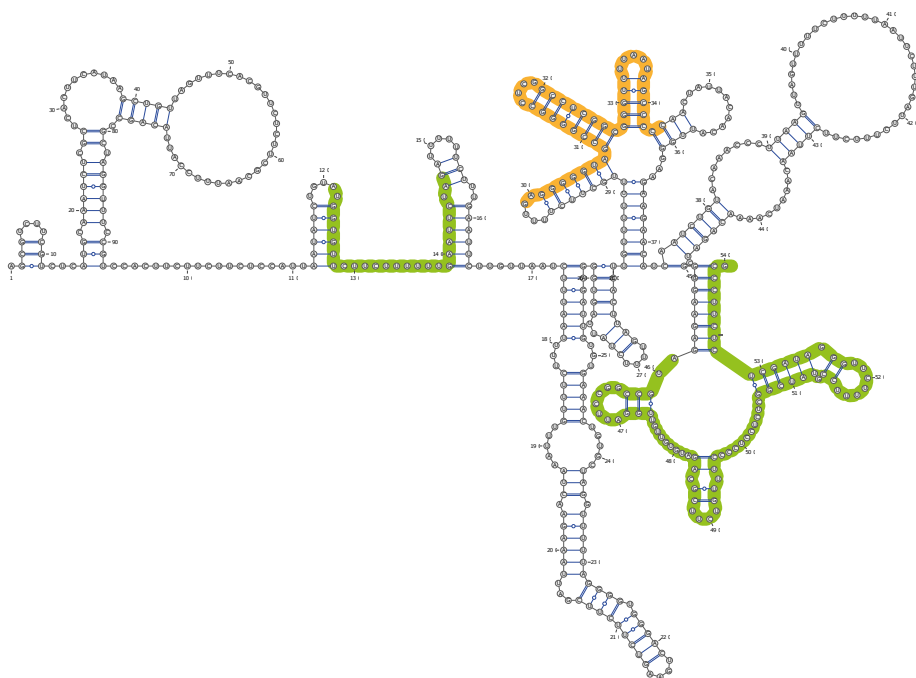
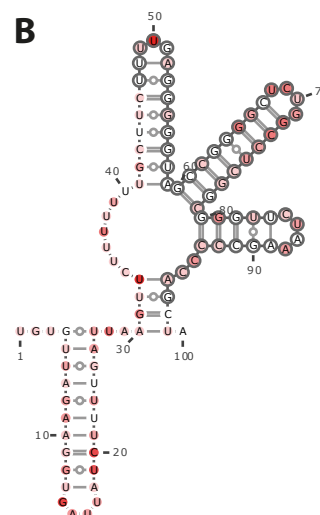
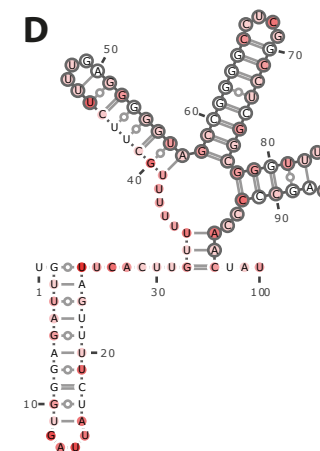
SlADC1      GGTCAA CAAATCA TT--TCTGTATAA-----TTAGTTT TACAC---ATTC TTTGA
SlADC2      GGG AAGAA GAACA TCAATCTGTACAAAC CCTTAAGT TAGTT TT TCT TT TTAAT TCTTTGA
          **   *   *   *   *   *   *****   *   *****   *   *****

SlADC1      TTC TTT TTT TTGTGAAGAT TTT-----ACGAG ATG
SlADC2      TTC TTT CT TCT TAACAAA--TCAAAAC AGATCGGGGAAGAG ATG
          *****   * *   *   *   *   *   *   *****

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Supplementary Figure 38: Nucleotide alignment of *SlADC1/2* 5'UTRs.

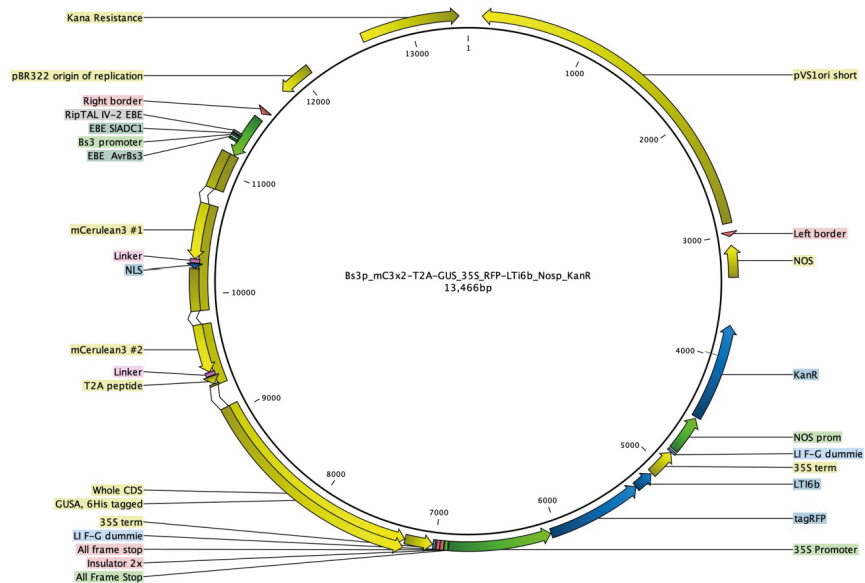
Alignment of *SlADC1/2* 5'UTR from the transcriptional start sites determined by Wu *et al.*, 2019 until the *SlADC* start codon generated via Clustal. Coding sequences (bold, green background) indicate uORF and *SlADC1/2* ATG; start and stop codons are underlined. The *SlADC1/2-box* sequences are bolded and highlighted with an orange background. Matching nucleotides are indicated by an asterisk below the alignment; overall sequence identify of 65.3%.

A**C****B****D**

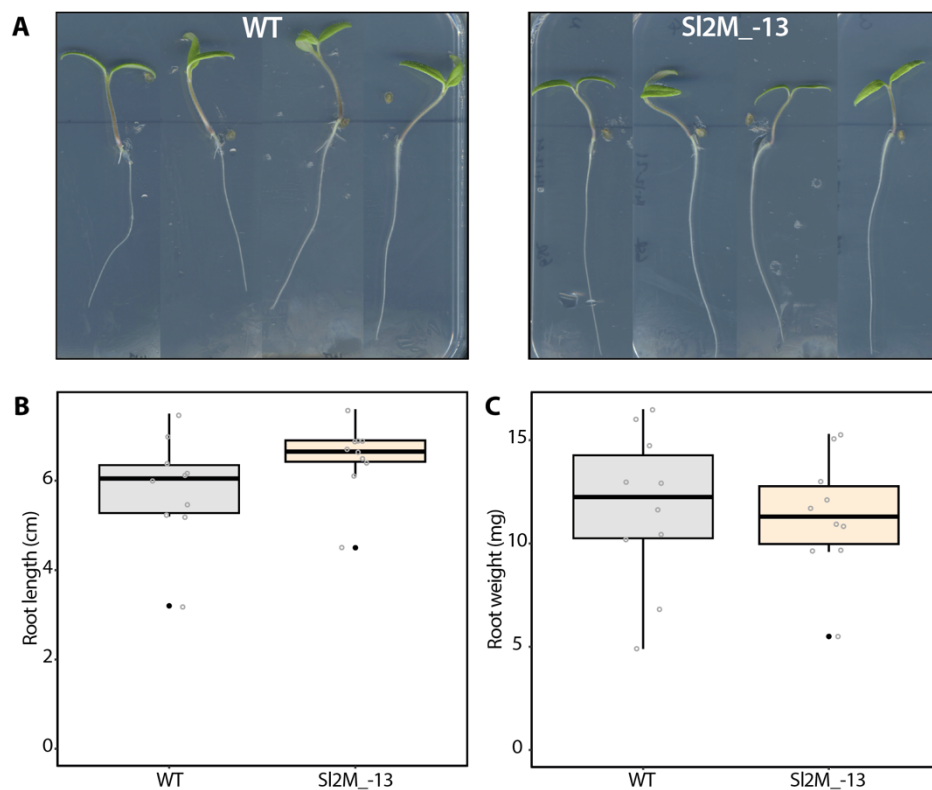
Supplementary Figure 39: Original images of SHAPE generated RNA secondary structures of *SIADC1* and *SIADC2* 5'UTRs

SHAPE RNA secondary structures of *SIADC1* (**A, B**) and *SIADC2* (**C, D**) 5'UTRs were generated from RNA harvested from WT tomato flowers (**A, C**) and mature green tomato fruits (**B, D**). Larger RNA secondary structure images from tomato fruits were provided showing the *ADC-box* (orange), uORF (green), and start of the *ADC* coding sequence (green). Images of RNA structures from tomato fruits were focused on the *ADC-box* sequences (bold ringed nucleotides, **B, D**) and some surrounding sequence; each nucleotide has been shaded darker red to indicate more SHAPE reactivity or conformational flexibility of that nucleotide. Images generated from Yiliang Ding's laboratory (John Innes Centre, UK).

5.8 Developing tools to study RipTALs

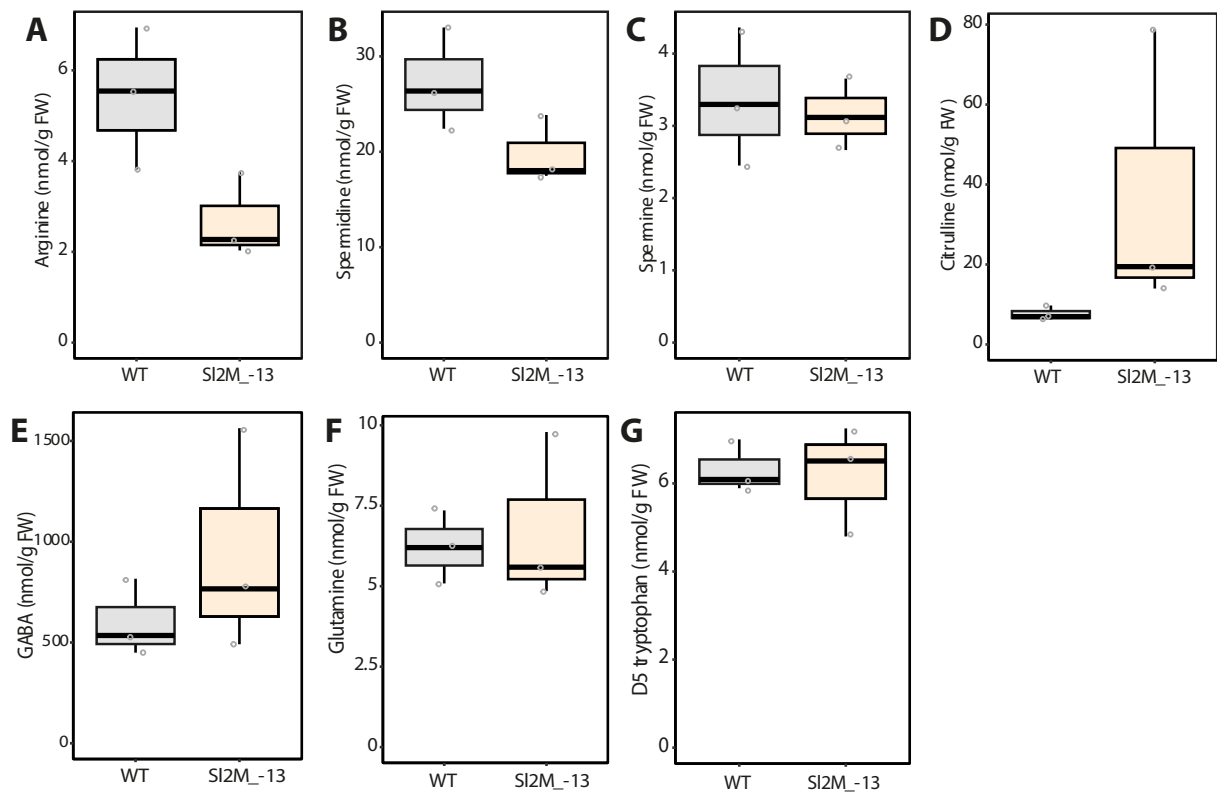


Supplementary Figure 40: Brg11 mCerulean3/GUS reporter plasmid used for tomato and *A. thaliana* stable transformation. Promoters (green), coding sequences (yellow or blue), terminators (yellow), and additional features are annotated around the plasmid. Bacterial resistance gene (“Kana Resistance”) and *in planta* resistance genes (KanR) are also shown.



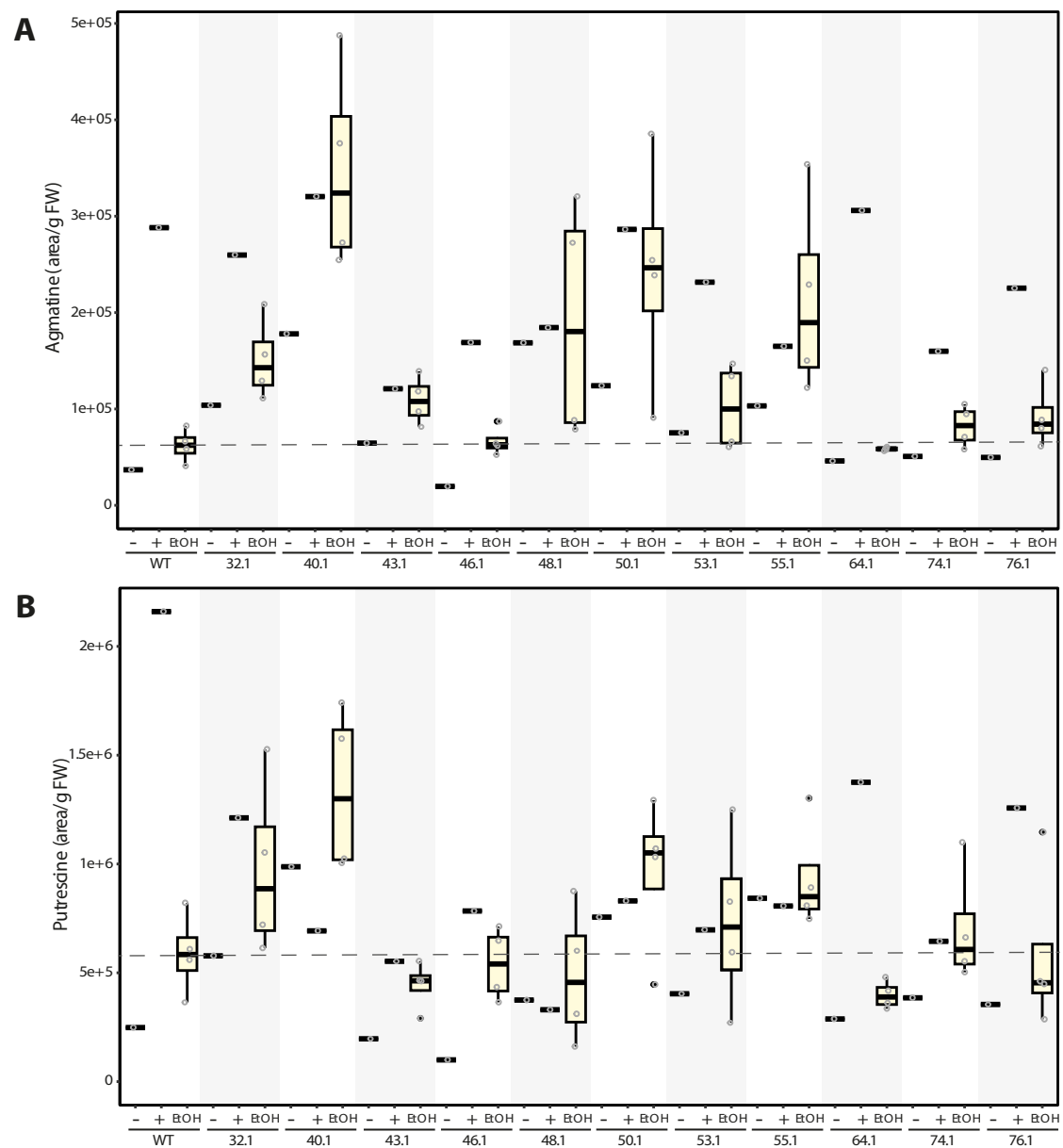
Supplementary Figure 41: No observable differences between WT and SI2M_-13 root phenotypes.

WT and *adc2-box* mutant (SI2M_-13) tomato seeds were sterilised, spread on damp filter paper, and kept in the fridge for 2 days before being taken to the climate chamber to germinate. After 3 days, germinated seeds were transferred to agar plates ($n = 10$) and allowed to grow vertically for another week. Agar plates were scanned (**A**), and the root lengths (**B**) and fresh weights (**C**) were measured.



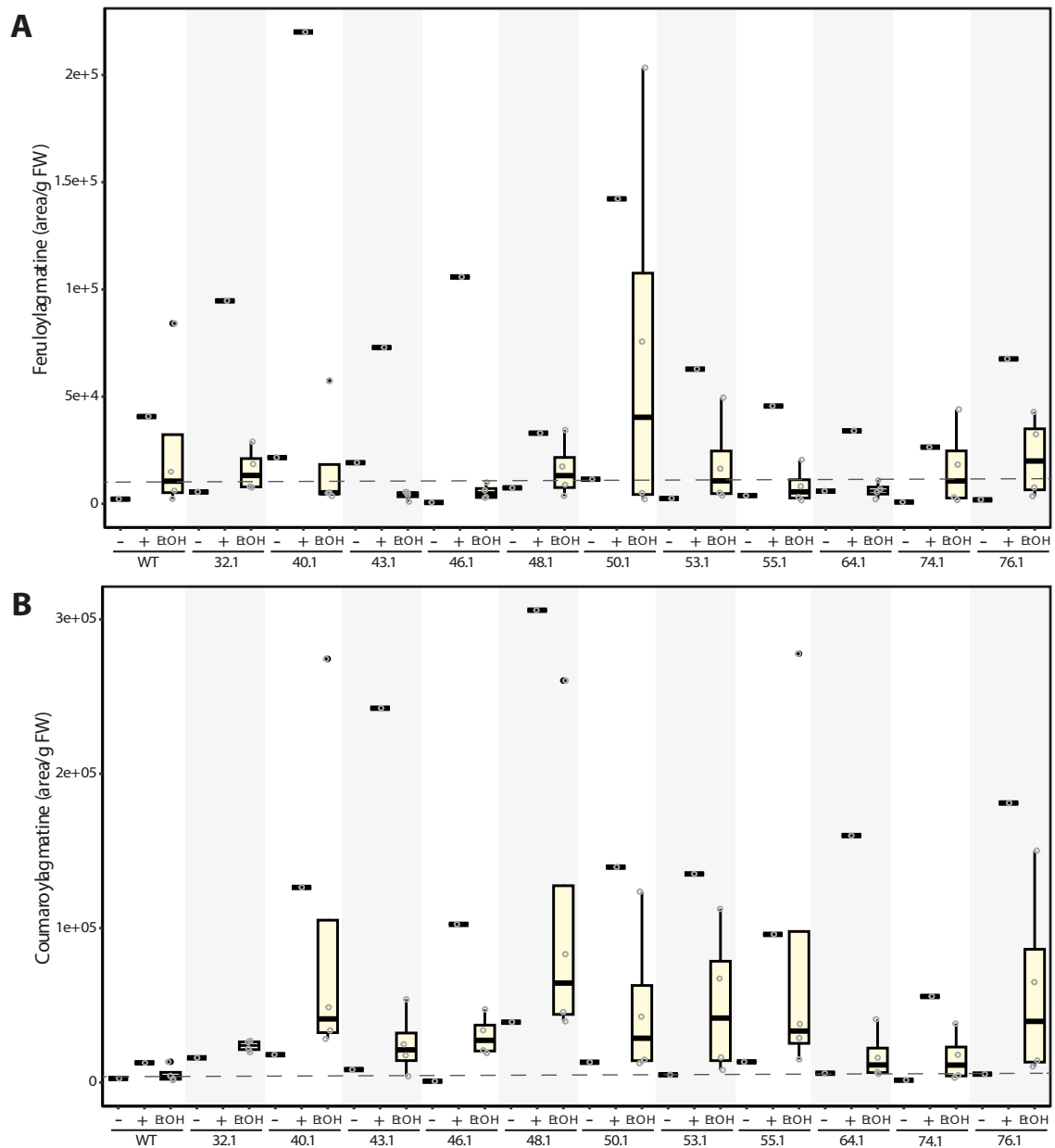
Supplementary Figure 42: Not all PA-related metabolite levels differ between SI2M_-13 mutant and WT tomato roots.

WT (grey) and *adc2-box* mutant (SI2M_-13; yellow) tomato seeds were sterilised, spread on damp filter paper, and kept in the fridge for 2 days before being taken to the climate chamber to germinate. After 3 days, germinated seeds were transferred to agar plates and allowed to grow vertically for another week before being cold treated for 24 hours. The roots of 3-4 seedlings were combined to form one replicate sample ($n = 3$), which were then processed for LCMS measurement of arginine (A), spermidine (B), spermine (C), citrulline (D), GABA (E), and glutamine (F) in nmol/g fresh weight (FW), normalised to the internal standard D5 tryptophan (G).



Supplementary Figure 43: Agmatine and putrescine levels of ethanol-inducible Brg11-GFP tomato lines.

Agmatine (**A**) and putrescine (**B**) levels of positive (+) and negative (-) controls, and ethanol-treated samples (EtOH) from WT and 11 selected ethanol-inducible Brg11-GFP lines were measured via LCMS. The dotted horizontal line represents the median agmatine or putrescine level of the ethanol-treated WT.



Supplementary Figure 44: Feruloylagmatine and coumaroylagmatine levels of ethanol-inducible Brg11-GFP tomato lines. Feruloylagmatine (**A**) and coumaroylagmatine (**B**) levels of positive (+) and negative (-) controls, and ethanol-treated samples (EtOH) from WT and 11 selected ethanol-inducible Brg11-GFP lines were measured via LCMS. The dotted horizontal line represents the median agmatine or putrescine level of the ethanol-treated WT.

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