The different levels of gene regulation in plant immunity

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SUMMARY

Altering gene expression is one of the key regulatory mechanisms for plants to respond to pathogens. This alteration can and must be achieved via various means. In this thesis I investigated three different aspects of gene regulation; chromatin remodelling, histone modifications, and post-transcriptional regulation via miRNAs.

In detail, we first analysed the importance of chromatin remodelling during establishment of systemic acquired resistance (SAR). SAR describes the phenomenon that plants can globally prime immune responses after local infection. We could find first hints that nucleosome occupancies at loci involved in the establishment of SAR might be altered after treatment with SAR-inducing signals and that this alteration might be dependent on the defective in mating-type switching/sucrose nonfermenting (SWI/SNF) chromatin remodelling complex. Moreover, we found first hints that nucleosome changes might persist even when expression is at non-detectable level again, possibly representing a piece of the so-called plants' memory function after infection.

Second, we asked whether the bromodomain-containing protein, BRD5, which is able to bind acetylated histones, plays a role during pathogen infection. We were able to show, that *Arabidopsis brd5* mutants are less resistant towards the necrotrophic fungus *Botrytis cinerea*. Even though *in planta* salicylic acid (SA) levels were unaltered, SA signalling, e.g. *PR1* expression levels might be altered in *brd5* mutants. Since SA signalling negatively regulates jasmonic acid (JA) signalling we propose that BRD5 might be important for early response towards necrotrophic pathogens and is possibly part of the crosstalk between SA and JA signalling.

Third, we analysed the role of miR827 during pathogen response. We were able to show that miR827 might be important for the proper response towards *Alternaria brassicicola*, since *miR827* mutants were less susceptible. Additionally, we could proof that miR827 is specifically up-regulated by *A. brassicicola* treatment and that this severe up-regulation is dependent on sufficient phosphate supply. Our results propose that miR827 and its target mRNA *NLA* might be part of the JA-mediated signalling response towards the necrotrophic pathogen *Alternaria brassicicola*.

In summary, we were able to show that many more processes play a role in pathogen response than initially assumed, further refining the classical zig-zag model of plant immunity.

ZUSAMMENFASSUNG

Veränderung von Expressionslevel ist eine der zentralen Mechanismen einer Pflanze, um auf Pathogenbefall zu reagieren. Diese Veränderung kann und muss auf verschiedenste Weise erreicht werden. Im Zuge dieser Arbeit wurden drei unterschiedliche Aspekte dieser Genregulation untersucht; Veränderung auf Chromatin-Ebene, Histonmodifikation und post-transkriptionelle Regulation durch miRNAs.

Im Detail haben wir die Rolle von Chromatin Remodelling während der Etablierung der „systemic acquired resistance“ (SAR) untersucht. SAR beschreibt das Phänomen, dass Pflanzen global auf eine lokale Infektion reagieren können. Wir konnten erste Hinweise darauf finden, dass Nukleosomen an bestimmten, für die SAR-Etablierung entscheidende, Loci durch SAR-induzierende Substanzen langfristig verändert werden könnten. Diese Veränderung könnte durch einen bestimmten Komplex, dem „defective in mating-type switching/sucrose nonfermenting” (SWI/SNF) Chromatin-Remodelling Komplex, reguliert werden. Diese Regulation könnte somit ein Teil des so genannten „Immun-Gedächtnisses“ von Pflanzen sein.

Zusätzlich haben wir untersucht in wie fern das „bromodomain-containing protein“ BRD5 eine Rolle bei der Pathogenantwort spielt. Arabidopsis *brd5* Mutanten waren weniger resistent gegenüber dem Nekrotrophen *Botrytis cinerea*. Obwohl Salicylsäure Messungen keine Unterschiede zwischen WT und *brd5* Mutanten feststellen konnten, waren Komponenten des SA-Signalwegs möglicherweise fehlreguliert (PR1). Da SA und Jasmonsäure Signalwege antagonistisch wirken, wäre es möglich, dass BRD5 wichtig bei der frühen Antwort auf nekrotrophe Pathogene ist und ein Teil des komplexen Hormon-Netzwerkes ist.

Darüber hinaus analysierten wir die Bedeutung der miR827 bei der Antwort auf Pathogene. Wir konnten zeigen, dass *miR827* Mutanten möglicherweise weniger anfällig für einen Befall mit *Alternaria brassicicola* waren. Zusätzlich konnten wir eine Induktion von miR827 durch *A. brassicicola* Behandlung beobachten. Diese Induktion war zudem abhängig vom Phosphat Angebot. Unsere Ergebnisse weisen darauf hin, dass miR827 und die Ziel-mRNA *NLA* eventuell wichtig sind während eines Befalls mit dem Nekrotrophen, *A. brassicicola.*

Zusammenfassend, konnten wir zeigen, dass möglicherweise weitaus mehr Prozesse bei der Immunantwort eine Rolle spielen und dass das klassische Zick-Zack-Modell der Immunantwort somit deutlich komplexer ist als zunächst angenommen.

INTRODUCTION INTO PLANT IMMUNITY

Plants utilize a myriad of different mechanisms to regulate genes in response to biotic stresses. It is easy to imagine why these mechanisms need to be thoroughly fine-tuned. Plants constantly have to cope with different stresses, abiotic as well as biotic, and energy resources need to be used as economically as possible (Bloom et al., 1985; Herms and Mattson, 1992). However biotic stress is the major reason for severe crop losses of up to 50%, in rare cases even 80% (Lobell et al., 2009; Oerke, 2006; Oerke and Dehne, 2004). In order reduce the crop loss by plant diseases in future, it is of great importance to understand the complex regulatory network of plant immunity.

PAMP-triggered immunity and effector-triggered immunity

The basic concept of the plant immune system is illustrated by the so called "zig-zag model" (see Figure 1), describing the first line of defence, PAMP-triggered immunity (PTI), and the second line of defence, effector-triggered immunity (ETI) (Jones and Dangl, 2006). During PTI, transmembrane pattern recognition receptor (PRRs) recognize microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs, (Zipfel and Felix, 2005). The recognition of PAMPs, such as flagellin or chitin, leads to downstream signalling and the appropriate response depending on the attacker. Amongst other things, this includes stomatal closure, a reactive oxygen species burst, increased callose-deposition and the activation of mitogen-activated protein (MAP) kinase cascades (Bigeard et al., 2015; Nicaise et al., 2009).

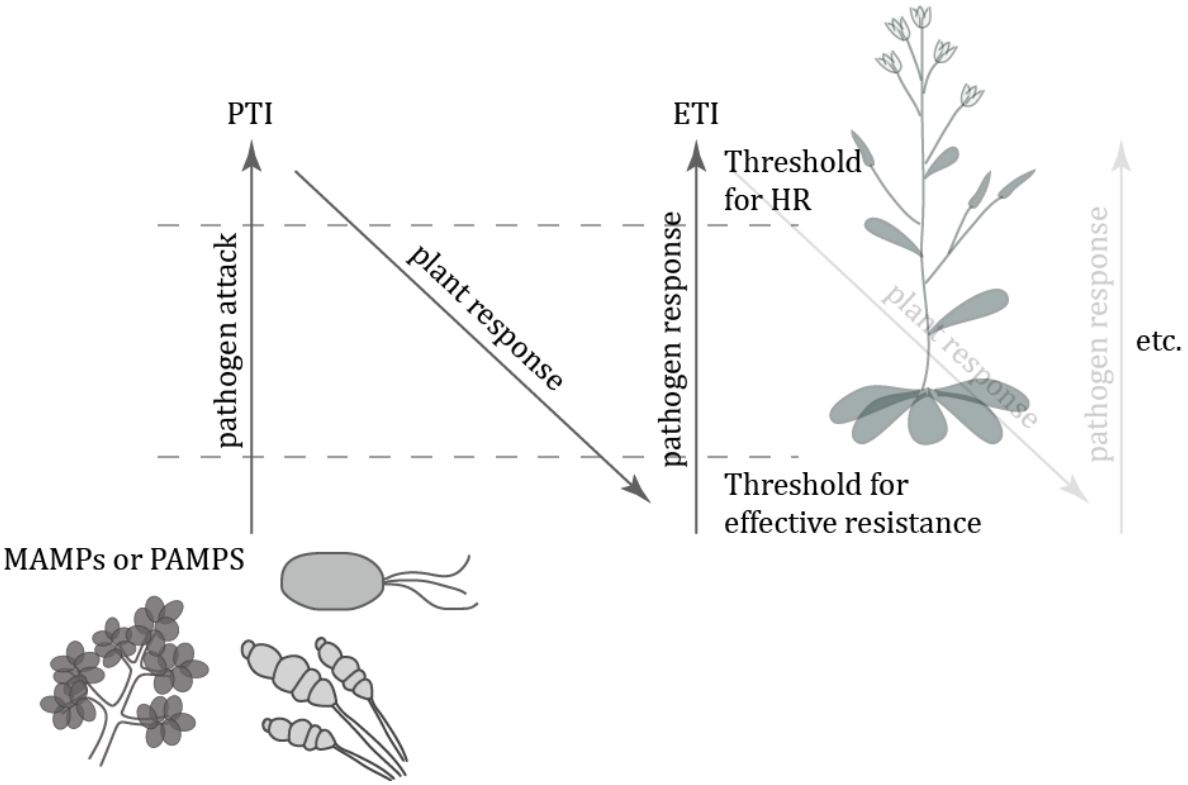


Figure 1: Schematic view of the plant's immune response. Depicted is the so-called zig-zag model, describing initial infection by pathogens and the plants first line of defense, PAMP-triggered immunity (PTI) and subsequent pathogen response and the plants second line of defense effector-triggered immunity (ETI; Jones and Dangl, 2006).

During ETI intracellular receptors are able to recognize pathogen effector proteins from pathogens that were able to breach the first line of defence (Cui et al., 2015). This recognition is facilitated by nucleotide-binding/leucine-rich repeat (NB-LRR) proteins and is often accompanied by hypersensitive responses (HR) and in the end leads to programmed cell death (PCD). This defence mechanism is only effective against pathogens that depend on a living host or plant tissue, such as biotrophic or hemi-biotrophic pathogens (Jones and Dangl, 2006). Necrotrophic pathogens are unaffected by ETI, since their life style requires them to kill plant tissues (Glazebrook, 2005).

During PTI and ETI a complex network of phytohormones is activated, regulating proper pathogen response (Bari and Jones, 2009; Howe and Jander, 2008; Katagiri and Tsuda, 2010). Even though many plant hormones seem to contribute to this network, salicylic acid (SA) and jasmonic acid (JA) represent the main hormonal regulators of plant defence (Browse, 2009; Vlot et al., 2009).

Interplay between salicylic acid and jasmonic acid

In general infection of plants with biotrophic pathogens leads to SA induced signalling and infection with necrotrophic pathogens or herbivores leads to JA induced signalling (Glazebrook, 2005). However, both signalling pathways are affected by other hormones as well as by each other (Caarls et al., 2015; Pieterse et al., 2012).

SA is a phenolic compound that in Arabidopsis is synthesized in chloroplasts via two different pathways, the PAL pathway, involving the PHENYLALANINE AMMONIA LYASE (PAL) enzyme, and the IC pathway, involving ISOCHORISMATE SYNTHASE (ICS)(Dempsey et al., 2011). Upon attack by biotrophic pathogens SA biosynthesis is triggered leading to strong accumulation of SA in the cell (Zheng et al., 2015). The mechanism of subsequent perception of SA is heavily discussed. It is assumed that it mainly involves the proteins NON EXPRESSOR OF *PR* GENES 1 (NPR1), NPR3 and NPR4 (Fu et al., 2012; Kuai et al., 2015; Wu et al., 2012; Yan and Dong, 2014; Zhang et al., 2006). It is proposed that accumulation of SA can directly or indirectly be sensed by NPR1, which acts as a transcriptional co-activator of downstream defence-related genes (Dong, 2004; Moore et al., 2011). By contrast, NPR3 and 4 seem to be negative regulators of downstream signalling (Fu et al., 2012; Moreau et al., 2012; Shi et al., 2013). Interestingly, SA-dependent processes that are not immune-related are still working in *npr1* mutants and additionally there are some immune responses that are SA-dependent but not NPR1-dependent (An and Mou, 2011; Lu, 2009; Moreau et al., 2012). Therefore, it is proposed that additional SA-binding proteins (SABPs) are important for SA perception and that the detailed SA signalling pathway depends on developmental stage, pathogen infection and resulting spatial and temporal concentration gradients of SA (Klessig et al., 2016; Kumar, 2014; Manohar et al., 2015). Downstream of SA perception many so-called defence-related (PR) genes are being regulated (van Loon et al., 2006; Van Loon and Van Strien, 1999). PR proteins is a term for small, 5-75kDa, proteins with antimicrobial activity (Sels et al., 2008). One of the main PR genes that is induced by SA is *PATHOGENESIS-RELATED 1* (*PR1*) (Breen et al., 2017; Sels et al., 2008; van Loon et al., 2006; Van Loon and Van Strien, 1999). Even though the actual function of PR1 is still unknown, it is regarded as the most stable marker for SA signalling and biotrophic pathogen responses (Breen et al., 2017).

JA is a lipid-derived compound, that is initially synthesised in the chloroplasts from α-linolenic acid released from membranes in a complex enzymatic pathway (Browse,

2009; Gfeller et al., 2010; Wasternack, 2007). After synthesis JA can be metabolised to the biologically active form, methyl jasmonate (MeJA) by JA CARBOXYL METHYLTRANSFERASE (JMT), or for example be conjugated to isoleucine by JASMONATE RESISTANT 1 (JAR1; (Seo et al., 2001; Staswick and Tiryaki, 2004). The latter leads to the biologically most active enantiomer jasmonyl-isoleucine (JA-Ile)(Fonseca et al., 2009). In non-induced cells, JA signalling is repressed by transcriptional repressor JASMONATE ZIM-domain (JAZ) proteins. Upon necrotrophic pathogen or herbivorous insect attack JA-Ile is synthesised, which can be bound by the F-box protein CORONATINE-INSENSITIVE 1 (COI1) and together with the rest of the Skp1-Cul1-F-box protein (SCF) ubiquitin E3 ligase complex enable targeting of JAZ proteins for proteasomal degradation (Sheard et al., 2010). This leads to the release of transcriptional activators, which in turn can activate transcription of downstream target genes. Depending on the attacker, necrotrophic pathogen or herbivorous insects, two distinct pathways are induced in *A. thaliana*. For necrotrophic pathogens this includes the activation of the so-called ERF-branch which is controlled by members of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family and leads to activation of target genes such as PLANT DEFENSIN 1.2 (PDF1.2, (Lorenzo et al., 2003; McGrath et al., 2005; Pré et al., 2008). For herbivorous insects however the transcriptional activators MYC2, 3 and 4 and downstream target genes such as VEGETATIVE STORAGE PROTEIN 2 (VSP2) are induced, representing the so-called MYC-branch of JA signalling (Dombrecht et al., 2007; Fernandez-Calvo et al., 2011; Lorenzo et al., 2004). What further distinguishes both pathways is the involvement of different additional hormones, for the ERF-branch ethylene is essential, for the MYC-branch ABA seems to play an important role, adding another layer of complexity (Pieterse et al., 2012).

Gene regulation in plant immunity

Many of the above described processes depend on massive expression changes (Caarls et al., 2015; Coolen et al., 2016). The amount of functional protein was once thought to be solely influenced along the lines of the central dogma of gene expression (Stent, 1968). The central dogma implies that DNA is transcribed into RNA and thereafter RNA is translated into protein. For many years it has been known now that apart from the central dogma many processes influence the actual amount of functional protein (Schneider-Poetsch and Yoshida, 2018). Figure 2 shows an overview of some of these

processes. It needs to be said that this overview is far from complete, since there are many exceptional examples of gene regulation.

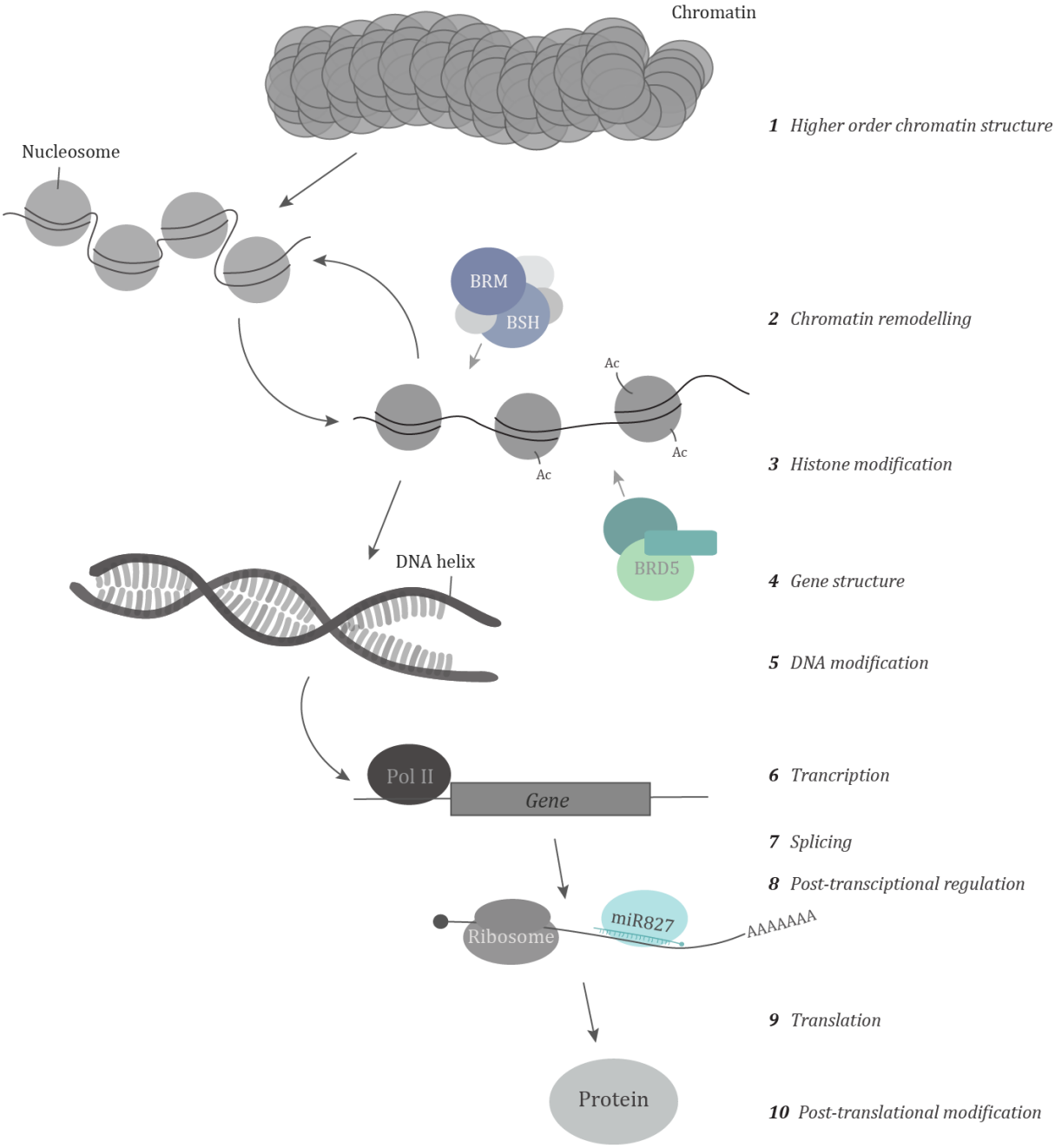


Figure 2: Schematic view of the current state of knowledge on gene regulation. Chromatin forms higher order structures and consist of DNA wrapped around histone octamers. DNA itself is a helix and contains information for genes that are subsequently transcribed and translated into protein.

Objectives of this work

Apart from the basic intensively studied immune system in plants often depicted in the well-known zig-zag model, there are many pathways and mechanisms that were not initially identified as part of the immune system. Recent data clearly show that much more processes are involved in plant immunity and so the relatively simple zig-zag model is becoming more and more complex as crosstalk pathways are added constantly. The goal of this work was to analyse if three different heavily discussed levels of gene regulation play a role in plant immunity.

First, in the past few years it has become clear that plants can not only respond to local infections but that they developed a mechanism, namely SAR, that enables the whole plant to turn local infection into global resistance towards a pathogen. Here, we wanted to investigate the influence of several SAR-inducing chemicals on the chromatin state and to find out whether this is dependent on the action of the SWI/SNF chromatin remodelling complex and whether this influences the plants ability to establish SAR.

Secondly, the influence of histone acetylation on plant immunity is becoming more and more of interest. The acetylation state of histones is influenced by 'writing' as well as 'erasing' enzymes but also by accessory proteins that can recognize and bind acetylated histones. We analysed the role of such an accessory protein, BROMODOMAIN CONTAINING PROTEIN 5 (BRD5), in response to biotrophic as well as necrotrophic pathogens.

Thirdly, microRNAs (Pré et al.) and the posttranscriptional regulation of their target transcripts play major roles in plant development. Lately however there have been reports stating that they also modulate immune responses. Here, we analysed the role of one miRNA, miR827, in response to the necrotrophic fungus *Alternaria brassicicola*.

MATERIAL AND METHODS

Plant material and growth conditions

For all pathogen assays plants were grown under short day conditions (8 h light, 22 °C) and high humidity in GS90 soil for 6 weeks. Except for *brd5-1* and *brd5-3* lines, all mutant plants used for this thesis were described before. *brm-3* and *syd-11* lines were kindly provided by Prof. Dr. Doris Wagner, *miR827* (SALK_020837) were kindly provided by Prof. Dr. Steven J. Rothstein (Kant et al., 2011). All mutant plants are in the Colombia-0 (Col-0) background. For sterile conditions, if not mentioned otherwise, plants were grown on ½ MS media in continuous light conditions (22 °C).

For expression analysis and MNase assays if not mentioned otherwise plants were grown on ½ MS media for 7 days before treatment. Treatment with SA, MeJA, Isotianil or flg22 was performed by spraying in indicated concentrations.

For MeJA plate assays plants were sown on ½ MS media with 1% sucrose and grown vertically under short day conditions (22 °C). After 8 days plants were transferred to ½ MS+1% sucrose plates containing mock (EtOH), 25 µM MeJA or 100 µM MeJA and again grown vertically for another 4 days under short day conditions (22 °C).

For SA plate assays plants were germinated on ½ MS media and grown vertically under short day conditions (22 °C). After 8 days plants were transferred to ½ MS plates containing mock (EtOH), 25 µM SA or 100 µM SA and again grown vertically for another 4 days under short day conditions (22 °C).

Bioassays

For all disease symptom-scoring experiments, 6-week plants were infected with respective pathogens.

Alternaria brassicicola assays where conducted according to (Kemmerling et al., 2007) (strain: MUCL 20297). Two *Arabidopsis thaliana* leaves were inoculated with two drops of 5 µl of *A. brassicicola* spore solution (1 x 10^6 spores/ml). Disease symptoms were scored 7, 10 and 12 days after infection.

Botrytis cinerea assays were done according to (Van Wees et al., 2013) (strain: B05.10). Five leaves of *A. thaliana* were inoculated with 5 µl *B. cinerea* spore solution (5 x 10^5 conidia/ml). Disease symptoms were scored 3, 5 and 7 days after infection. Quantification of *Botrytis cinerea* biomass was performed as previously described (Gachon and Saindrenan, 2004).

Pseudomonas syringae pv. tomato (Pto) were done according to (Van Wees et al., 2013)(strains: DC3000 hrcC$^-$). Two leaves of *A. thaliana* were pressure infiltrated with bacterial solution (1x10^9 cells/ml).

DNA constructs

For cloning cDNA was amplified using Phusion proof-reading polymerase (Thermo Fisher Scientific). Subsequently purified PCR products were cloned into the ENTRY vector pCR8-GW-TOPO (Life Technologies) according to the user guide. cDNAs were transferred from ENTRY vectors into the pGADT7 and pGBKT7 destination vectors via recombination catalysed by LR Clonase II (Life-Technologies) (Horak et al. 2008).

MNase assay

Nuclei extraction

All experiments were performed at 4 °C to prevent protein degradation. Nuclei were extracted according to (Kaster and Laubinger, 2016). 2 g of ground *A. thaliana* material were resuspended in 50 ml Honda buffer (0.44 M sucrose, 1.25 % Ficoll, 2.5 % Dextran T40, 20 mM HEPES-KOH, 10 mM Mg-CL_2, 0.5 % Triton, 5 mM dithiothreitol, 1 mM Pefa Bloc, 1 % protease inhibitor (Roche; Basel, Switzerland)). To isolate nuclei the extract was filtered through two layers of Miracloth (Merck Millipore; Billerica, Massachusetts) and filtrates were centrifuged at 2300 g for 15 min at 4 °C. Isolated nuclei were resuspended in 1 ml Honda buffer, and washed until nuclei were clean (2300 g for 15 min at 4 °C). Extracted nuclei were washed once with 1 ml MNase reaction buffer (20 mM Tris/HCl pH 8.0, 5 mM NaCl, 2.3 mM $CaCl_2$; 2300 g for 15 min at 4 °C). Finally, pellets were resuspended in 660 µl MNase reaction buffer.

MNase digestion

For MNase digestion DNA content of samples was determined and according amounts of MNase (Takara; Paris, France) were added (for 70-80 ng DNA final concentration of 0.01-0.02 units/µl MNase). Samples were incubated at 37 °C for 8 min. Reaction was stopped by addition of stop buffer (10 mM EDTA, 10 mM EGTA) and 1% SDS. After digestion samples were incubated with 40 ng of Proteinase K (Roche; Basel, Switzerland) at 60 °C for 1 h. Finally, samples were incubated with RNase A (10 µg/µl) and incubated at 37 °C for 1 h and at 4 °C over night.

DNA extraction

DNA was purified by phenol:chloroform:isoamyl alcohol. Samples were mixed with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1 ratio) and centrifuged at maximum speed for 5 min. The aqueous phase was mixed with 1 volume of phenol:chloroform:isoamyl alcohol and samples were centrifuged at maximum speed for 5 min. These steps were repeated until the interphase was clear. The aqueous phase was washed twice with 1 volume of chloroform and samples were centrifuged at maximum speed for 5 min. DNA was precipitated using 1/10 volume of 3 M NaAc and 2-3 volumes of 100 % ethanol and incubated at 4 °C for 2 h. After incubation samples were centrifuged at maximum speed for 30 min. Pellets were washed once with 70 % ethanol, air-dried and resuspended in 50 µl ddH2O.

Total RNA extraction

Total RNA was extracted using TRIZOL (Life technologies) reagent. Ground plant material was resuspended in 1 ml TRIZOL and centrifuged. 1/5 Vol of chloroform was added to the supernatant and mixed by vigorous vortexing. Organic and aqueous phases were separated by centrifugation at 16.000 g for 5 min at 4 °C. The aqueous phase was washed with 1 Vol of chloroform until the interphase was clear (16.000 g for 5 min at 4 °C). For precipitation of RNA the aqueous phase was supplemented with 1 Vol of isopropanol and incubated for 1 h at -80 °C. Precipitates were collected by centrifugation at 16.000 g for 30 min at 4 °C. RNA pellets were washed with 80 % (v/v) ethanol and resuspended in 20 – 50 µl nuclease-free water. Integrity and quality of RNA was analysed on a 1 % (w/v) agarose gel.

cDNA synthesis

Up to 1 µg of total RNA was used for DNase treatment and reverse transcribed with oligo-dT and for microRNA analysis a miRNA specific stem-loop oligonucleotide using the First Aid cDNA synthesis Kit (Thermo Fisher) according to (Varkonyi-Gasic et al., 2007).

quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed using the Bio-Rad CFX384 system and SYBR green 2xPCR Master-Mix (Thermo Fisher Scientific). For all experiments two technical and two biological replicates were performed. Amplification efficiency was calculated using a standard curve of amplification and PP2A was used for normalization. Relative expression levels were calculated using the ΔΔct-method and all oligonucleotides are listed in Table 1.

Table 1: List of oligonucleotides.

Chapter	*Name*	*Sequence 5' to 3'*
all	Actin2 qPCR F	CTTGCACCAAGCAGCATGAA
	Actin2 qPCR F	CCGATCCAGACACTGTACTTCCTT
	Tubulin qPCR F	GAGCCTTACAACGCTACTCTGTCTGTC
	Tubulin qPCR R	ACACCAGACATAGTAGCAGAAATCAAG
	PR1 qPCR F	TTGTGGTCACTACACTCAAG
	PR1 qPCR R	CACATAATTCCCACGAGGAT
Chapter I	PR5 qPCR F	AATATTGCTGCCGTGGAG
	PR5 qPCR R	TTCGTCGTCATAAGCGTAG
	AZI1 qPCR F	AAGGTTTGGTTGACGTCGAC
	AZI1 qPCR R	TTGAGAAGAACGCTGAGAG
	WRKY6 qPCR F	ACAGCAAATGACGAACTTACC
	WRKY6 qPCR R	TGTGAGTGCCGTTATTGTATC
	WRKY53 qPCR F	AGAGTCAAACCAGCCATTAC
	WRKY53 qPCR R	TCGGAGAACTCTCCACGTGG
	LOX2 F	AGCATCTTGGGCCAATGAGCCTG
	LOX2 R	TGTTCACGTTTCTCTCATCTATCAC
	VSP2 qPCR F	ATGCCAAAGGACTTGCCCTA
	VSP2 qPCR R	CGGGTCGGTCTTCTCTGTTC
	NPR3 qPCR F	CAACACAACATGAAACTGCAC
	NPR3 qPCR R	GAACGTTTTGGGTTAATCAGC
	SNC1 qPCR F	CAACCAACAGACTACGAGAA
	SNC1 qPCR R	TAGGCCATAGGGTAAGTTGA
	TGA4 qPCR F	CGGCTAAAGGAAATGTTTGA
	TGA4 qPCR R	CCTTCCCCCATTCTATTATTAC

Chapter	*Name*	*Sequence 5' to 3'*
Chapter I	PR1 MNase -3N F	CAACTATATACAATGTTTCTTA
	PR1 MNase -3N R	GTCAATCTTGTCCTTACACACATTTTTCCTA
	PR1 MNase -1N F	TACAAAAACGTGAGATCTATAG
	PR1 MNase -1N R	GGTTATTGTTGTGTTATGATTTTG
	PR1 MNase +1N F	AAAGCTCAAGATAGCCCACA
	PR1 MNase +1N R	TAGGCTGCAACCCTCTCG
	PR1 MNase +2N F	TAGCGGTGACTTGTCTGG
	PR1 MNase +2N R	TGACCACAAACTCCATTGC
	NPR3 MNase -3N F	TCATCAATAGTGACGGCTATTTCTT
	NPR3 MNase -3N R	AACTTGTAATGATATGCCAATTAATGC
	NPR3 MNase -1N F	CACGGAGAACCTTTGGCATC
	NPR3 MNase -1N R	AAAGAAAGACTCAAATGAAAGTTGACA
	NPR3 MNase +1N F	CTTGCACCTGTTGTGTCCTG
	NPR3 MNase +1N R	GGAAGAGAATTTAGAGGAAAAGTCAAG
Chapter II	BRD5 qPCR R1	TCACTATTCTCACACTCAACAG
	BRD5 qPCR F2	GGAAGTGTAGATCTAGCTGAT
	BRD5 qPCR R2	TGTCTCATTGGGTTCAGGTG
	BRD5 qPCR F3	GAAAGCACCTGAACCCAATGA
	BRD5 qPCR R3	TTTCTCAACAGGCACAACAG
	BRD5 qPCR F4	GTTCAGGATGTGGTAGTAGG
	BRD5 qPCR R4	CTTCCAGTTCTTGCTTATTG
	BRD5 qPCR F5	CTGAGGATGTTTACAAGGATG
	BRD5 qPCR R5	TTTCTTCACTCGCTTCATCAG
	BRD5 qPCR F6	CGATATGTGTTTTGAAGCGTC
	BRD5 qPCR R6	ATGAATTATCCACAGACGGAC
	BRD5 qPCR F7	GTCGAAGTGGAGAGTGAAAAT
	BRD5 qPCR R7	TTCTCTGCAGCACTATTCACA
	SALK_062612	TATTGCCCTTGAGGGATAACC
	SALK_062612	AGTTTCAGCGACATACCCATG
	SALK_033645, SALK_033638	TGCATGTAGCTCCGTTTTAGG
	SALK_033645, SALK_033638	GACGTTTTCCTCTTCCTCCAC
	BRD5 qPCR F	AACCCGCAGCTTCTAAGTCTC
	BRD5 qPCR R	GACGTCTAAACAGTGAGTGAG
	a-Shaggy kinase qPCR F	CTTATCGGATTTCTCTATGTTTGGC
	a-Shaggy kinase qPCR R	GAGCTCCTGTTTATTTAACTTGTACATACC
	Cutinase A qPCR F	AGCCTTATGTCCCTTCCCTTG
	Cutinase A qPCR R	GAAGAGAAATGGAAAATGGTGAG
Chapter III	NLA qPCR F	ACAATTGTTCTCGTGAATGCCC
	NLA qPCR R	GAGCATGCTCGTTAAACCATCC
	mir827 stemloop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTTTG
	mir827 qPCR F	CCGTCGTTAGATGACCATCAA
	mir827 pri-mi	CACATGTTGATCATCCTTGTG
	mir827 pri-mi	CGAGATTCCAAGAAGCGATG
	399a pri-mi F	AGGGTAAGATCTCTATTGGCAGGAAAC
	399a pri-mi R	GCAGAAGAATTACAGGGCAAATCTCC
	PXMT1 qPCR F	GATTGGAGGAGACGGTCCTGAGA
	PXMT1 qPCR R	GGCTGAGATCGCCTTGGTCAT
	miR163 stemloop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCGAA
	miR163 qPCR F	CCGACGTTGAAGAGGACTTGGAAC
	RT-universal-Reverse-primer	GTGCAGGGTCCGAGGT
	FRK1 qPCR F	ATGAGTCAGGTCGTTATGGA
	FRK1 qPCR R	TCTCGGTGTCTAGATTCACT
	WRKY53 qPCR F	AGAGTCAAACCAGCCATTAC
	WRKY53 qPCR R	TCGGAGAACTCTCCACGTGG

mRNA library preparation

For mRNA library preparation polyadenylated transcripts were isolated from total RNA using the NE Next ® Poly(A) mRNA Magnetic Isolation Module (NEB). The polyadenylated fraction was used for the ScriptSeq ™ v2 RNA-Seq Library Preparation Kit (Epicentre). For each sample libraries were prepared in triplicates, all libraries were pooled and sequenced on Illumina HiSeq 3000.

Microscopy

For confocal microscopy plants were grown vertically under sterile conditions (continuous light at 22 °C) and pictures were taken with a Leica SP8 confocal microscope.

Salicylic acid measurements

For salicylic acid measurements 200 mg of plant material was snap frozen in liquid nitrogen. Material was ground using a Retsch Mixermill (MM200). Free analytes were extracted with 1.5 ml ethyl acetate, containing 0.1 % (v/v) formic acid and the internal standards 3-hydroxybenzoeic acid. Samples were sonicated in an ultrasonic bath and incubated at 28°C for 60 min. Samples were centrifuged at 18 500 g and 1.2 ml of the supernatant was transferred into a new tube. The ethyl acetate was removed in an Eppendorf vacuum concentrator. Derivatization was performed with 70 µl of a 1 : 1 mixture of TMSDM (2.0 M in diethyl ether) and methanol for 20 min at 25°C. Determination of the analytes was performed using Triple Quadrupol GC-MS/MS (Shimadzu TQ8040) in splitless MRM mode. Separation of the compounds was achieved using a glass capillary column from Restek (SH-Rxi-17SIL-MS) with a diameter of 0.25 mm, film thickness of 0.25 µm and a length of 30 m.

CHAPTER I: SYSTEMIC ACQUIRED RESISTANCE AND CHROMATIN REMODELLING

Author contributions

Margaux Kaster and Sascha Laubinger designed the research; Margaux Kaster performed research; gDNA isolations were partially performed by Corinna Speth and Philipp Reichert; Salicylic acid measurements were performed by Dr. Joachim Kilian.

Introduction

Systemic acquired resistance

Apart from basal resistance against a single infection by pathogens, plants can acquire resistance during or after infections to be able to respond quicker or better to a spreading disease or a second infection. This concept is very similar to the human immune response (Gourbal et al., 2018). It is proposed that once a plant is infected locally, a mobile signal is released through-out the plant that will prepare the whole organism for secondary infections by inducing systemic expression of antimicrobial *PR* genes (Durrant and Dong, 2004). This process is generally described as systemic acquired resistance (SAR) if it is referring to spreading of the same infection or priming if it is referring to subsequent infections (Dempsey and Klessig, 2012; Durrant and Dong, 2004; Fu and Dong, 2013; Gao et al., 2015; Kachroo and Robin, 2013; Shah and Zeier, 2013). Many putative SAR signals have been proposed such as methyl salicylate (MeSA) (Park et al., 2007; Shulaev et al., 1997), azelaic acid (AzA) (Cecchini et al., 2015b; Jung et al., 2009; Yu et al., 2013), dehydroabietinal (DA) (Chaturvedi et al., 2012), glycerol-3-phosphate dependent factor (G3P) (Chanda et al., 2011; Nandi et al., 2004) and pipecolic acid (Pip) (Bernsdorff et al., 2016; Hartmann et al., 2017; Návarová et al., 2012). The fact that so many different mobile signals are proposed suggests that

there is not one universal SAR signal but that more likely dependent on circumstances such as pathogen or treatment the plant produces a different subset of mobile SAR signals, which then leads to resistance of the whole plant (Liu et al., 2011).

Apart from questions concerning the mobile signal there is still the open question of how the "prepared-for-attack" state of the whole organism is achieved. Some reports claim that the primed state can last for months or even generations (Luna et al., 2012). A constantly active immune system however would mean unnecessary energy loss for a plant. Therefore, there must be a mechanism by which the plant can prime defence-related genes, enabling them to be transcribed faster or to a larger extent. It is generally accepted that this process involves the induction of salicylic acid (SA; (Gao et al., 2015; Metraux et al., 1990; Park et al., 2007; Vernooij et al., 1994) since SA biosynthesis as well as SA signalling mutants are defective in the establishment of SAR (Adam et al., 2018; Gao et al., 2015). Additionally, it is proposed that strong induction of *PR1* in systemic tissues is important for the establishment of SAR (Bernsdorff et al., 2016). But how is the strong induction of *PR1* expression enabled? How is it memorised?

Chromatin remodelling in SAR

For some time, it has been discussed if the chromatin state might play a role during SAR and priming (Brzezinka et al., 2016; Espinas et al., 2016; Jaskiewicz et al., 2011; Luna et al., 2012; Mozgova et al., 2015). This hypothesis is founded on the assumption that open chromatin is more easily accessible and thereby genes in open chromatin regions more easily transcribed. The chromatin state can basically be influenced on four different levels; DNA methylation, histone modification, noncoding RNA-mediated chromatin remodelling and ATP-dependent chromatin remodelling (Chen et al., 2017).

Chromatin remodelling is one of the central mechanisms that alter nucleosome occupancy and can be facilitated by large multi-protein complexes. These complexes are called chromatin remodelling complexes (CRCs) and are helicase-like enzymes that can slide histone octamers along the DNA, disassemble whole nucleosomes or exchange histone variants in an ATP-dependent manner (Clapier and Cairns, 2009; Clapier et al., 2017; Kwon and Wagner, 2007). In Arabidopsis roughly 40 different CRCs are identified, which are divided into different subfamilies depending on their SNF2 ATPase

subunit (Flaus et al., 2006; Knizewski et al., 2008). Even though most of the CRCs play important roles during plant development, several also have shown to be important for plant immune responses (Ramirez-Prado et al., 2018). One of these CRCs is the SWI/SNF chromatin remodelling complex. The SWI/SNF complex was initially identified in two independent screens for yeast mutants either defective in mating-type switching (SWI) or sucrose nonfermenting (SNF) and consists of one ATPase of the Snf2 subfamily (SPLAYED or BRAHMA), two SWI3 proteins (SWI3A-D) and one SNF5-like protein (BSH) (Jerzmanowski, 2007; Winston and Carlson, 1992).

The Arabidopsis ATPases of the SWI/SNF complex, SPLAYED and BRAHMA, have been implicated in the regulation of defence related pathways (Bezhani et al., 2007; Han et al., 2012; Johnson et al., 2015; Peirats-Llobet et al., 2016; Walley et al., 2008a). Mutants of the BRAHMA (BRM) ATPase showed a miss-regulation of *PR* genes indicating a role during plant immunity (Bezhani et al., 2007). The other ATPase, SPLAYED (SYD), has been shown to be required for specific regulation of JA- and ET-related genes and *syd-5* mutants are more susceptible to *Botrytis cinerea* infection (Walley et al., 2008b). Additionally, it has been reported that SYD is important for repression of *SUPPRESSOR OF npr1-1, CONSTITUTIVE1* (*SNC1*). SNC1 in turn is an intracellular NOD-like receptor (NLR) protein, which is activated during ETI and is known to be one of the factors important for SAR (Li et al., 2010a; Zhu et al., 2010). These data already indicate that the SWI/SNF chromatin remodelling complex might play an important role in the establishment of SAR, however the detailed mechanism remains to be eluded.

Here, we investigate the role of chromatin remodelling in SAR and in detail whether SA treatment changes the chromatin state of SAR-related genes and whether these changes are dependent on the SWI/SNF chromatin remodelling complex.

Results

SA-induced SAR on an epigenetic level

In general, our idea was, that induction and establishment of SAR might lead to changes on the chromatin level enabling certain genes, such as *PR* genes, to be more easily transcribed. Appliance of SA is known to lead to severe short-term up-regulation of *PR1* transcription in *A. thaliana* (De Vos et al., 2005; van Loon et al., 2006). To determine when *PR1* expression levels return to non-treated levels under our conditions, we analysed *PR1* transcripts over a period of six days in mock and SA-treated wild-type *Arabidopsis thaliana* plants (see Figure 3).

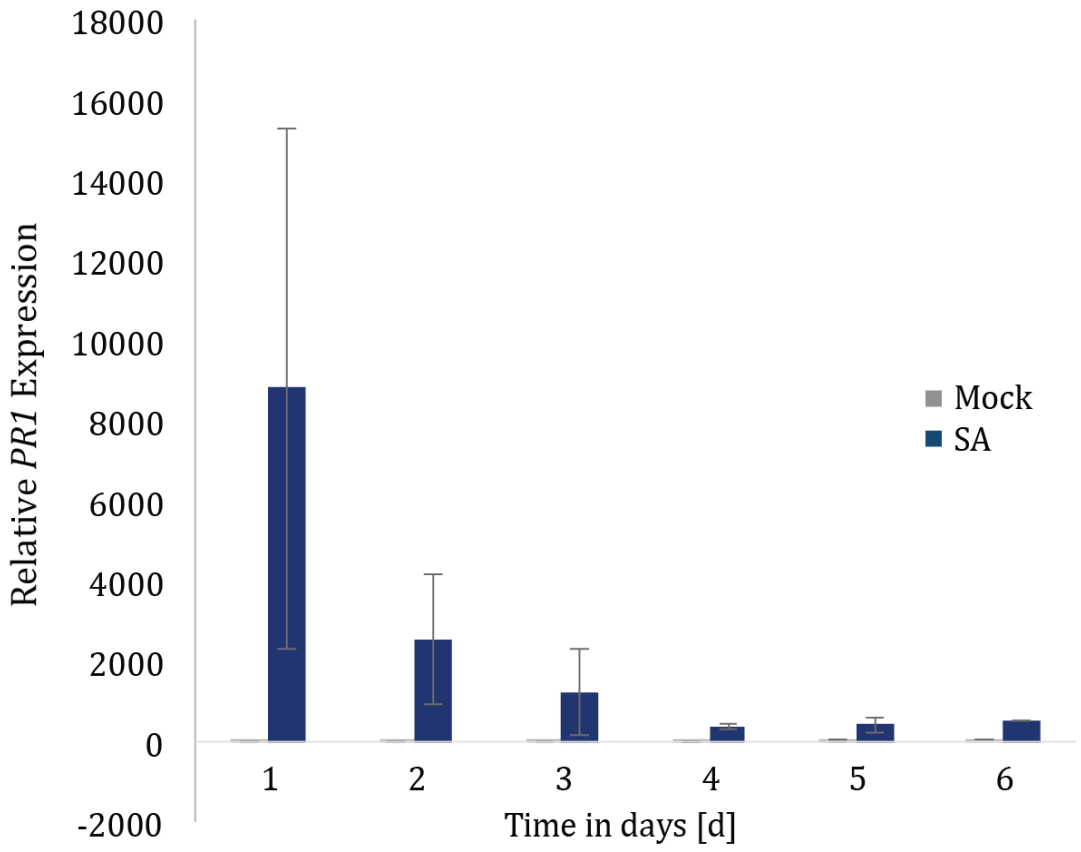


Figure 3: Long-term analysis of *PR1* expression levels shows down-regulation to base-level after 5 days. Relative expression levels of *PR1* of sterile grown WT Col-0 plants treated with 1 mM SA are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*.

After 5 days expression levels are comparable to mock-treated plants. Statistical significance was tested via student's t-test however variation of expression levels between experiments is always very high for *PR1* (f-test for unequal variances for all time points highly significant; *p*-values < 0.002). Therefore, significance of three biological replicates could not be shown. In summary all experiments independently showed the same trend as visualized in Figure 3.

We also checked long-term expression profiles of other factors known to be involved in SAR after SA treatment (Cecchini et al., 2015b; Jung et al., 2009). *PR5* expression (see Figure 4) behaved very similar to *PR1* with significant induction 1 and 2 days (student's t-test; *p*-values 0.0021 and 0.0161 respectively) after treatment that over time goes down to background levels. In general, we observed that *PR5* expression in average is much lower compared to *PR1* expression.

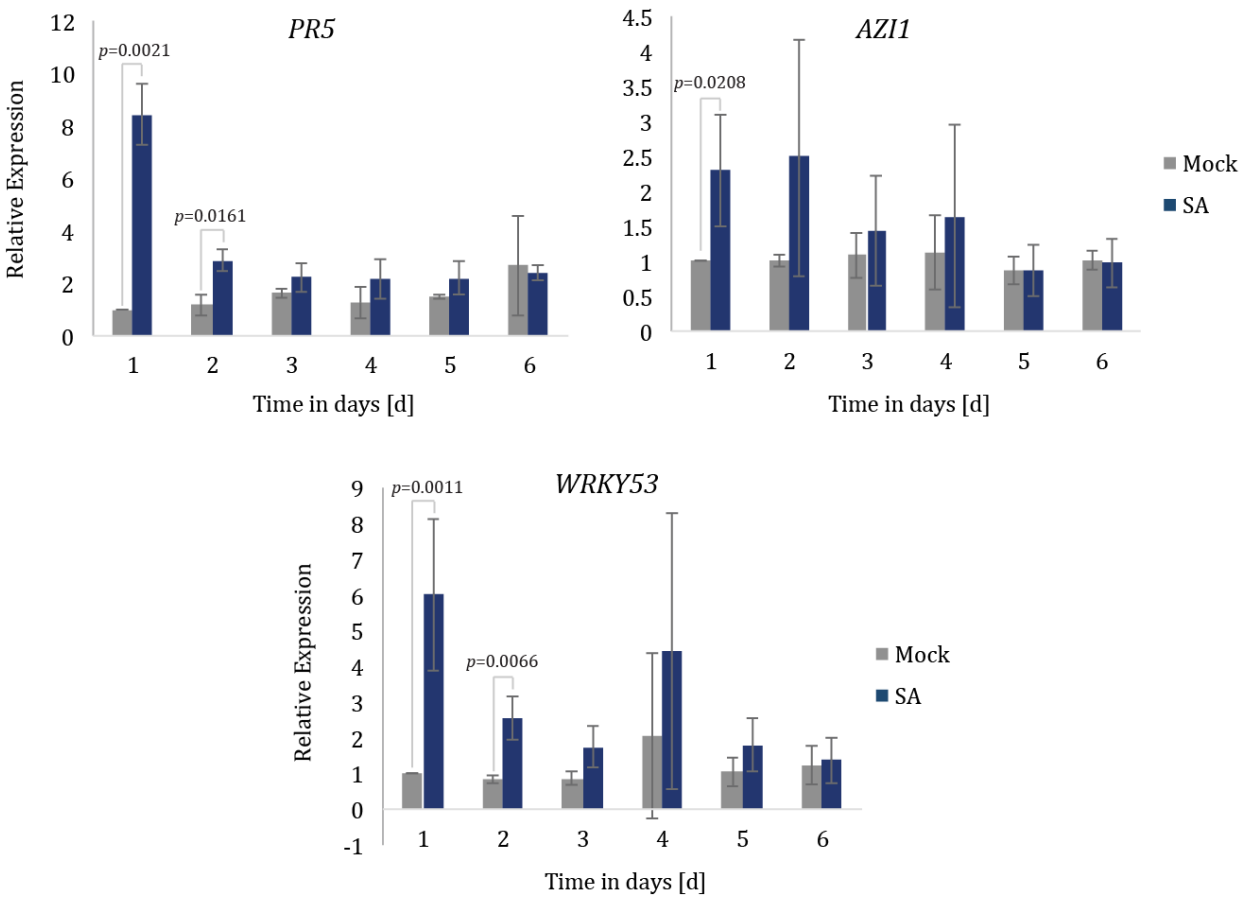


Figure 4: Long-term analysis of *PR5*, *AZI1* and *WRKY53* expression levels Relative expression levels of sterile grown WT Col-0 plants treated with 1 mM SA are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Results of three biological replicates are shown, statistical significance was tested via student's t-test, p-values are shown.

Apart from *PR1* and *PR5* we also analysed *AZELAIC ACID INDUCED 1* (*AZI1*). AZI1, together with DEFECTIVE IN INDUCED RESISTANCE 1 (DIR1) is proposed to be involved in the translocation of a lipid-derived mobile signal (azelaic acid) crucial for induction of SAR (Cecchini et al., 2015a; Yu et al., 2013). *AZI1* expression was significantly induced by SA 1 day after treatment (*p*-value 0.0208) but variation was very high. We also looked at *WRKY53* expression levels after SA treatment. *WRKY53* is induced by BTH (Wong et al 2016) and plays a putative role in SAR. In our hands *WRKY53* expression was also significantly induced by SA treatment, showing strongest induction 1 day after treatment that goes down over 5-6 days and comes down to background levels again. Two other factors, known to play roles in plant immunity *LOX2* and *WRKY6* did not show any significant changes upon SA treatment (see Supplementary Figure S 1).

In addition, we also analysed long-term expression profiles of all factors during treatment with another substance, called Isotianil, that might lead to induction of SAR (Ogawa et al., 2011). This, however, did not lead to any significant induction of transcripts (see Supplementary Figure S 2). Therefore, we focused on SA application in the following experiments.

In theory, even though expression of *PR1* is at background level again, it might be that the chromatin is somehow altered, 'primed', thereby enabling faster and or higher expression during second treatment or attack and that this is a part of SAR establishment. We were interested to analyse the chromatin state of SAR-induced genes before and after SA treatment. To capture biotic-stress-induced nucleosome changes, we used micrococcal nuclease (MNase) from the microorganism *Staphylococcus aureus.* The MNase enzyme is able to cleave free DNA (Alexander et al., 1961; Noll, 1974). Therefore, DNA wrapped around histones/nucleosomes is protected from cleavage and can be used to precisely map nucleosomes on a base pair level (Zentner and Henikoff, 2012; Zhang and Pugh, 2011). A short overview of the procedure is shown in Figure 5.

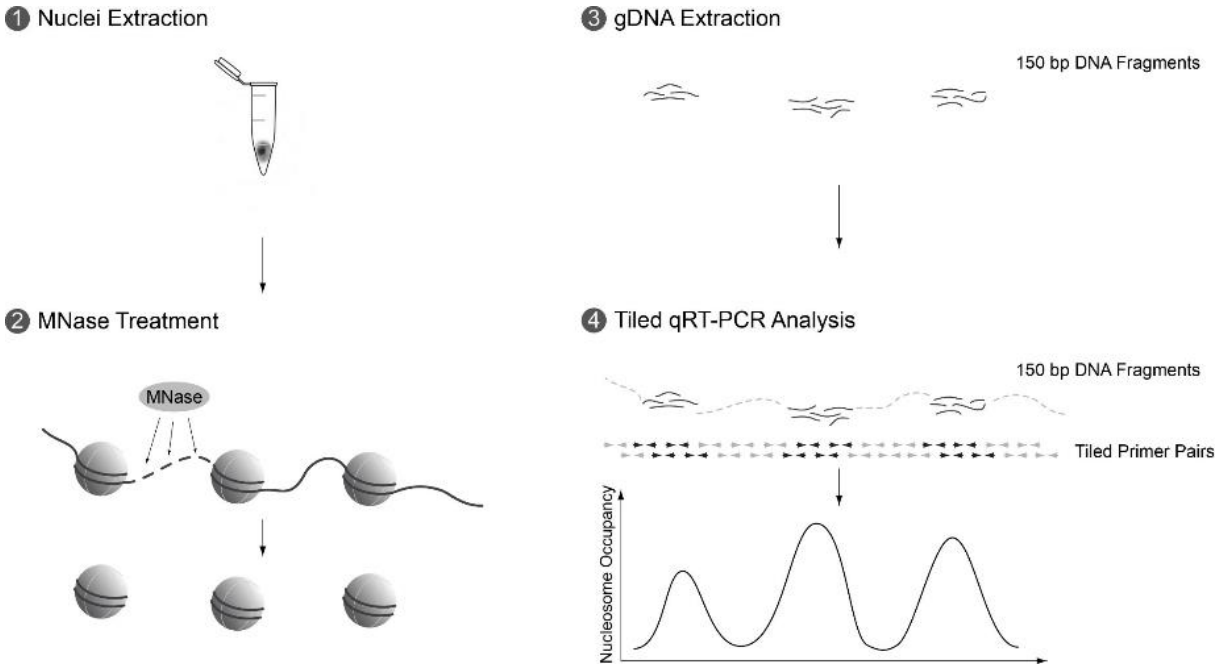


Figure 5: Schematic overview of MNase assay. Protein-free DNA is cleaved by MNase enzyme. Afterwards mono-nucleosomal DNA can be purified and analysed by tiled qPCR reactions.

First, we analysed the *PR1* locus 1 day after SA appliance to see if changes can be observed while expression of *PR1* is strongest (see Figure 6). Indeed, nucleosome occupancies of the first nucleosome upstream (-1 N) of the transcription start site (TSS) and the first and second nucleosomes downstream (+1 N and +2 N) of the TSS are significantly lower during SA treatment, possibly enabling rapid and extensive transcription. It needs to be mentioned that three biological replicates is a small sample size and more replicates need to be produced, to be able to make final statements. We also checked the effect of other substances that might lead to induction of SAR. Even though treatment with a 22-amino acid long peptide of the flagellin N-terminus (flg22) also lead to a reduction in nucleosome occupancy at the *PR1* locus, SA treatment seemed to be the most effective and reproducible treatment (see Supplementary Figure S 3; Garcia et al., 2014).

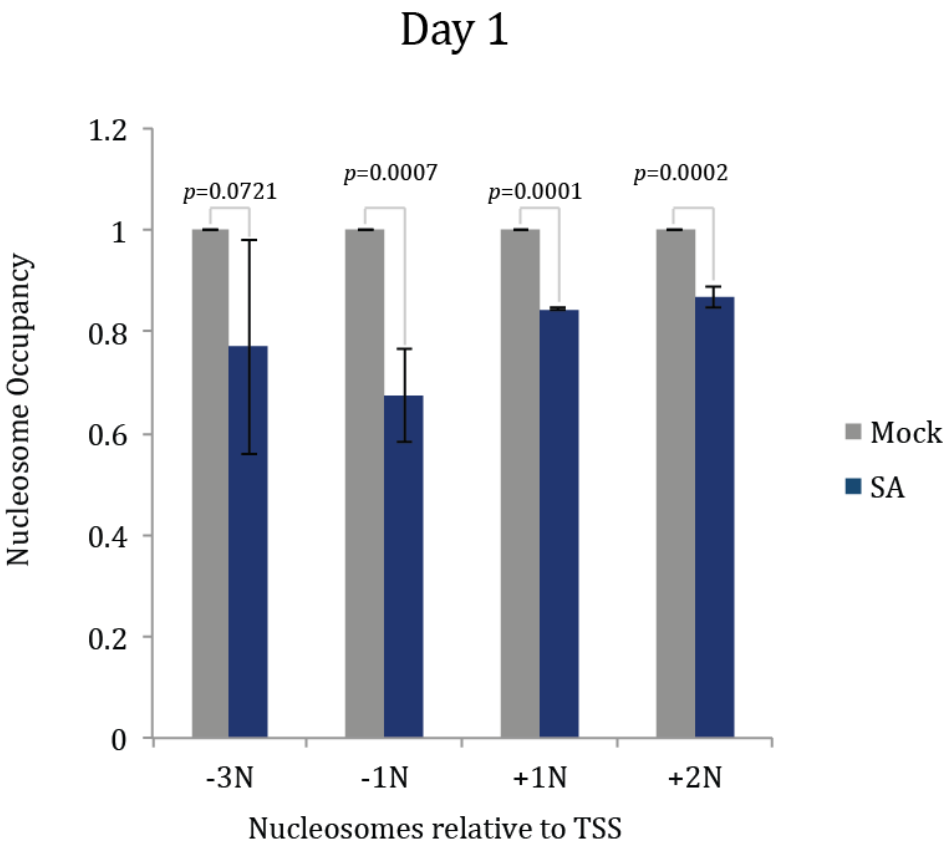


Figure 6: Nucleosome occupancy at the *PR1* locus is lower during SA treatment. Sterile grown Arabidopsis WT Col-0 plants were treated with mock solution or 500 µM SA and samples were harvested 1 day after treatment. Nucleosome occupancy was analysed using MNase treatment and subsequent tiled qPCR analysis. Relative nucleosome occupancies of at least three biological replicates are shown and were normalized to the *gypsy-like transposon* (AT3G32010). Statistical significance was tested via student's t-test and resulting *p*-values are indicated.

Having established, that *PR1* expression goes back to mock levels after 5 days of treatment we now asked whether these possible chromatin changes we observed after 1 day of treatment are still evident after 5 days of treatment or if nucleosome occupancy is comparable to mock treated plants. Therefore, we analysed nucleosome occupancy at the *PR1* locus 5 days after SA treatment (see Figure 7).

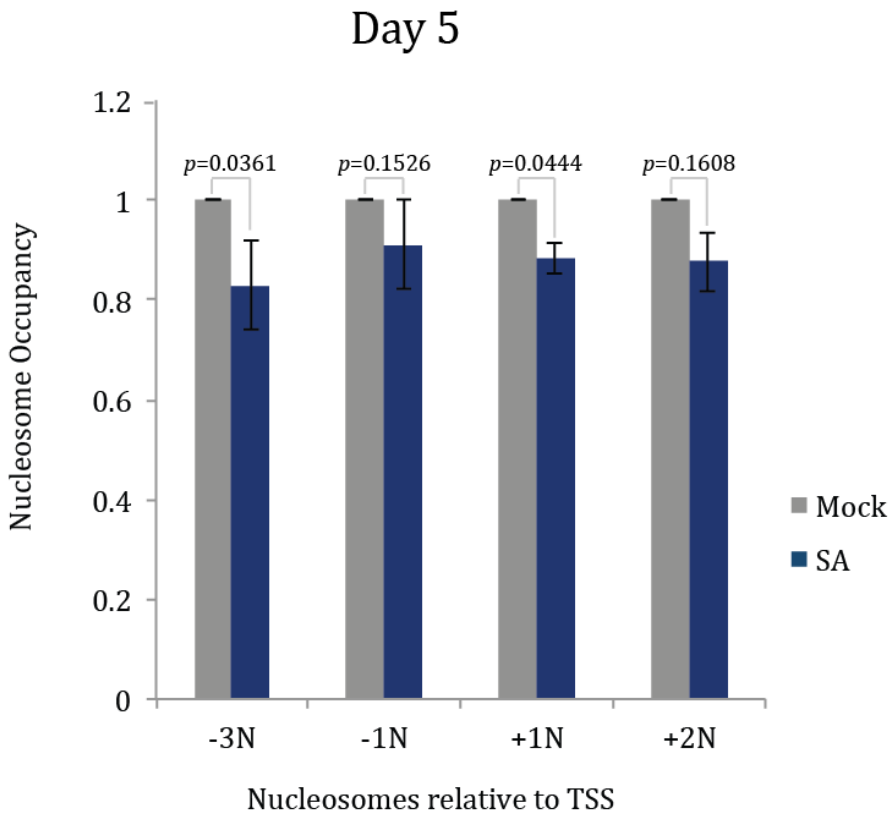


Figure 7: Nucleosome occupancy at the *PR1* locus 5 days after SA treatment. Sterile grown Arabidopsis WT Col-0 plants were treated with mock solution or 500 µM SA and samples were harvested 5 days after treatment. Nucleosome occupancy was analysed using MNase treatment and subsequent tiled qPCR analysis. Relative nucleosome occupancies of three biological replicates are shown and were normalized to the *gypsy-like transposon* (AT3G32010).

We were able to find first hints that nucleosome occupancy of the -3 N and +1 N nucleosomes at the *PR1* locus stays slightly lower even though expression levels are on mock level again. Even though these changes were statistically significant (student's t-test; *p*-values see Figure 7), these data are also from only three replicates, therefore experiments will have to be repeated to obtain sufficient amount of data to be able to make final statements. These results suggest that chromatin remodelling might play a role in establishing SAR and might lead to priming of the *PR1* locus, leaving it ready for transcription during second stress conditions.

The role of SWI/SNF mediated chromatin remodelling during SA signalling and SA-induced SAR

Since our results might indicate that after SA treatment the *PR1* locus is altered on an epigenetic level, we asked whether specific CRCs might play a role in the regulation of nucleosome occupancy at the *PR1* locus after SA treatment. Since several components of the SWI/SNF complex have been implicated to be important for plant immunity we analysed mutants of this complex in more detail.

Behaviour of SAR-induced genes in *swi/snf* mutants after SA treatment

First, we analysed *PR1* expression profiles during SA treatment after 1 and 5 days in Col–0 and three mutants of the SWI/SNF complex, *bsh–2, brm–3* and *syd–11* plants (see Figure 8). The first thing worth mentioning is that variation between experiments on day 1 after treatment is extremely high for all samples, therefore it was not possible to see statistical significance (f-test for unequal variances; $p < 0{,}03$). All lines showed the typical up-regulation of *PR1* after 1 day of SA treatment, however differences between lines are hard to define due to the high variation. After 5 days of SA treatment *bsh-2* mutants still show significantly elevated *PR1* expression levels in comparison to its own mock sample and also in comparison to WT mock samples. Additionally, in *bsh-2* mutants *PR1* levels under mock conditions seem to be lower compared to WT, which is noticeable in all *bsh-2* mock samples (1 and 5 days). *Brm-3* mutant lines also show this lower *PR1* expression levels compared to wild type 5 days after treatment. Since after 1 day of mock treatment, *brm-3* mutants show higher *PR1* expression levels, these results should be doubted. All in all, our results indicate that in *bsh-2* mutants transcription of *PR1* might be altered.

Apart from *PR1* expression, we also analysed expression levels of other factors that might play a role during SAR and several direct targets of BRM (Archacki et al., 2017; Walley et al., 2008a). One of these factors is the proposed negative regulator of SA signalling, NON EXPRESSOR OF *PR* GENE 3 (NPR3). *NPR3* transcript levels were significantly up-regulated by SA treatment after 24 h and returned to mock levels after 5 days of SA treatment in WT plants. In all mutant lines *NPR3* was significantly induced and to a very similar extent compared to WT plants after 1 day of treatment.

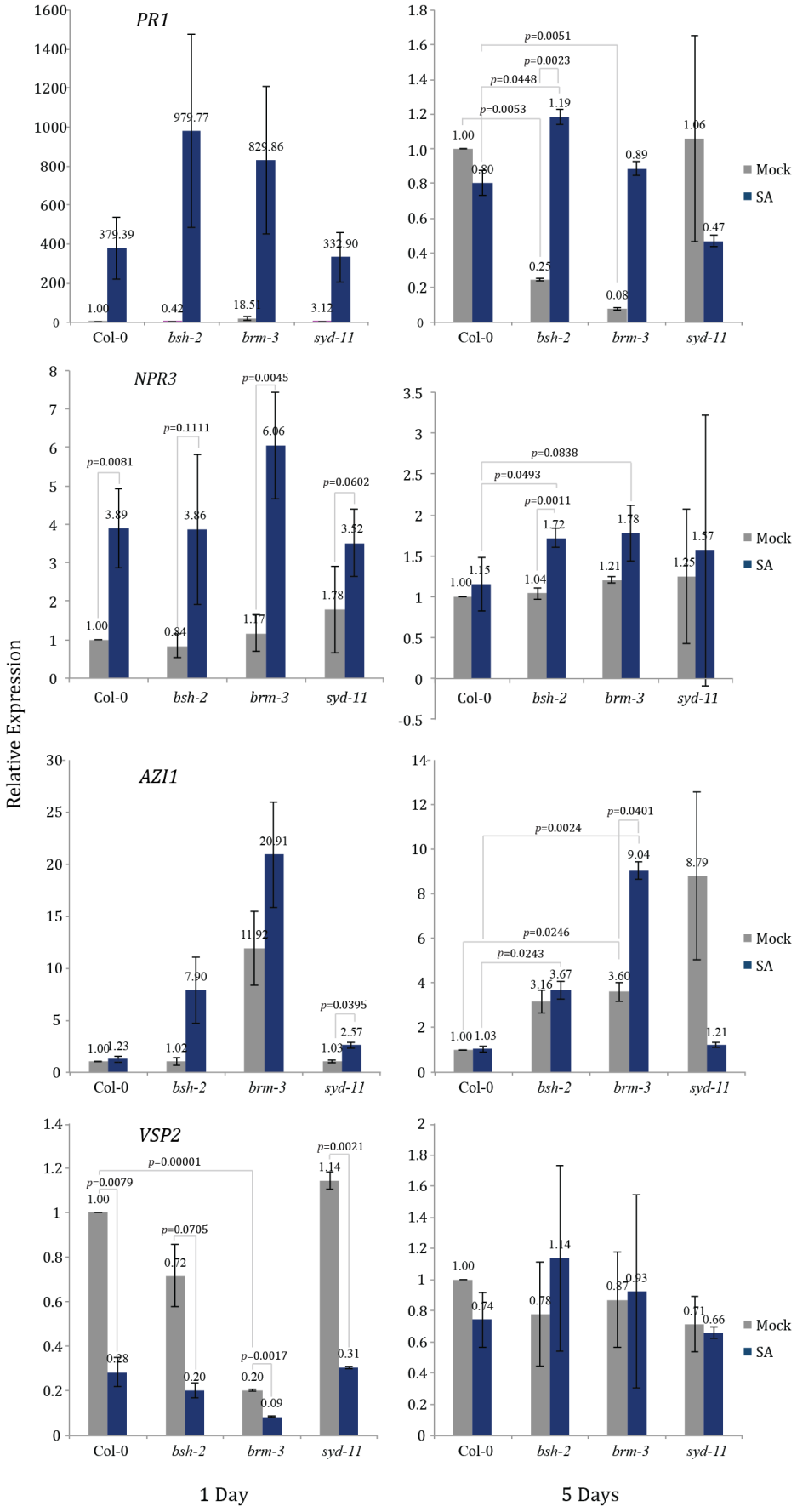


Figure 8: *swi/snf* mutants show altered expression profiles under mock and SA conditions. Relative expression level of *PR1, NPR3, AZI1* and *VSP2* of sterile grown WT plants treated with mock solution or 1 mM SA are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of at least two biological replicates, statistical significance was determined via student's t-test, *p*-values are indicated.

In *bsh-2* mutant plants *NPR3* expression remained at a higher level 5 days after SA appliance compared to WT plants. In *brm-3* mutants a similar trend was observed.

We also analysed expression levels of *AZI1* in all mutant lines and could observe stronger induction in *bsh-2* and *brm-3* lines after 1 and 5 days after SA treatment. For the first time point results were not significant and therefore have to be repeated. However, 5 days after SA treatment *AZI1* expression stays at a higher level in *brm-3* plants compared to its own mock conditions as well as WT SA treatment conditions. In addition, *AZI1* levels under mock conditions seem to be slightly higher compared to WT mock samples. In *bsh-2* mutant plants a similar trend is visible, but only differences between *bsh-2* and WT SA treated plants were statistically significant.

In addition we looked at expression levels of *VSP2*, since it was previously reported that *VSP2* expression is miss-regulated in *syd* mutants (Walley et al., 2008a). As expected, *VSP2* levels are significantly reduced after SA treatment, since *VSP2* is a typical JA signalling marker and SA and JA signalling pathways act antagonistically. All plant lines showed the typical down regulation after 1 day of treatment, apart from *bsh-2* plants. These plants did show a similar trend, with slightly lowered *VSP2* levels. In *brm-3* mutants *VSP2* expression seemed overall significantly reduced under mock conditions. This effect was not evident after 5 days of treatment, due to the strong variation in these samples; still, the results of the first time point might still be considered.

Since the *PR1* expression profiles might be somehow altered in *bsh-2* and *brm-3* mutants, we now analysed whether the nucleosome occupancy at the *PR1* locus is altered under mock conditions as well as after SA treatment in these mutants (see Figure 9). Unfortunately, we could not observe any significant changes for any of the mutant lines after 1 day of treatment. After 5 days of treatment we could observe slightly lower nucleosome occupancies for *brm-3* and *syd-11* plants under mock conditions and for *brm-3* plants also under SA conditions compared to WT plants. This raises the question why we were not able to see these changes 1 day after treatment. In general experiments have to be repeated to make any statements. If these results can be repeated it would indicate a negative role for the SWI/SNF chromatin remodelling complex in priming the *PR1* locus.

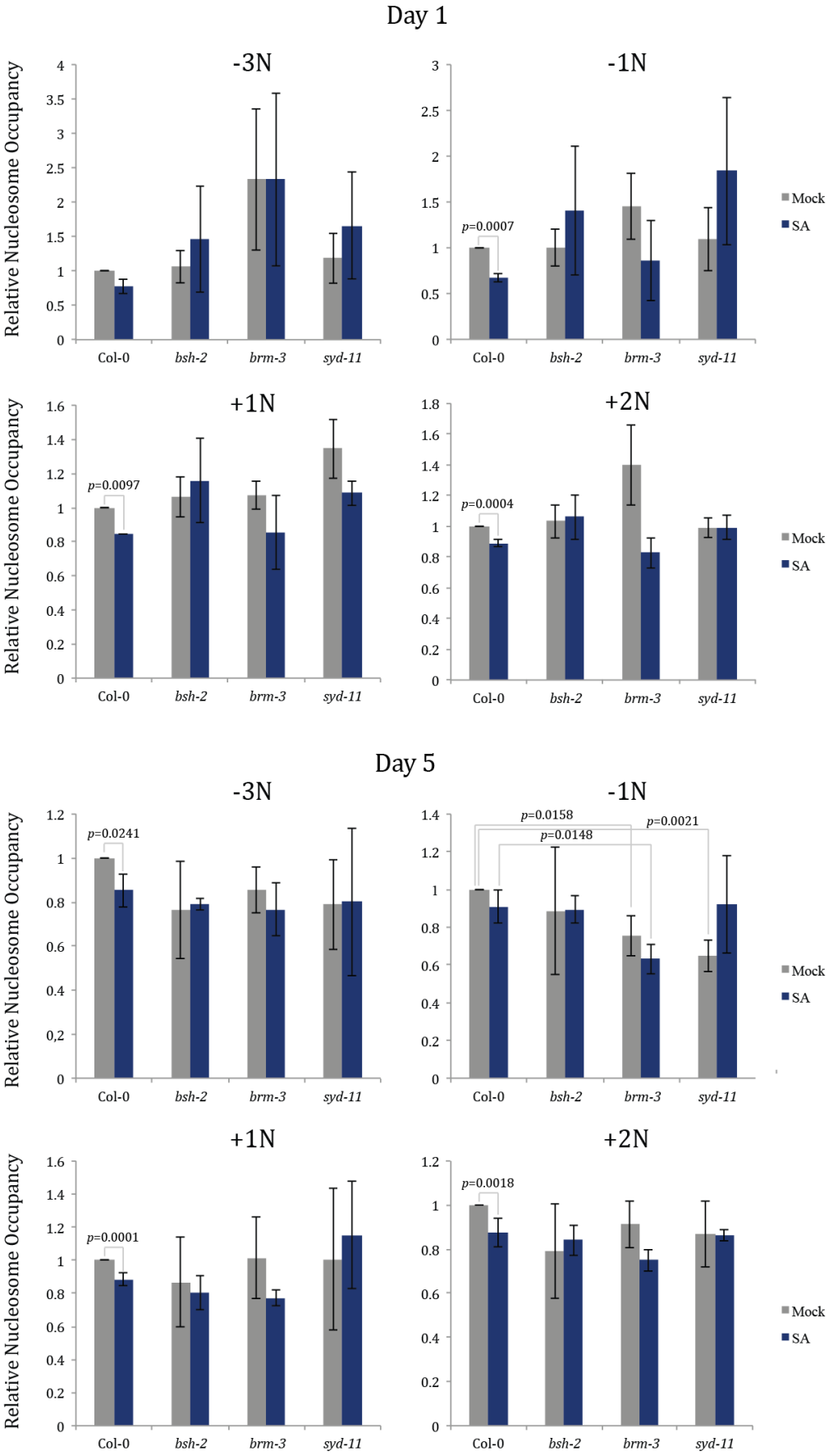


Figure 9: Nucleosome occupancy at the *PR1* locus is changed in *swi/snf* mutants during SA treatment. Sterile grown Arabidopsis WT, *bsh-2, brm-3* and *syd-11* plants were treated with mock solution or 500 µM SA and samples were harvested 1 day and 5 days after treatment. Nucleosome occupancy was analysed using MNase treatment and subsequent tiled qPCR analysis. Relative nucleosome occupancies of three biological replicates are shown and were normalized to gypsy-like trans-poson locus (AT3G32010).

Li et al (2016) identified *NPR3* as a direct target of BRM using ChIP-seq experiments. Since *NPR3* expression levels were slightly higher in *brm-3* mutants we also analysed the nucleosome occupancy at the *NPR3* locus (see Figure 10). Nucleosome occupancy was slightly higher in WT plants after 1 day and 5 days of SA treatment on all nucleosomes, but none of the observed changes were statistically significant and therefore have to be repeated. In *brm-3* mutants, nucleosome occupancy at the –2 N and +1 N nucleosome were higher under mock conditions.

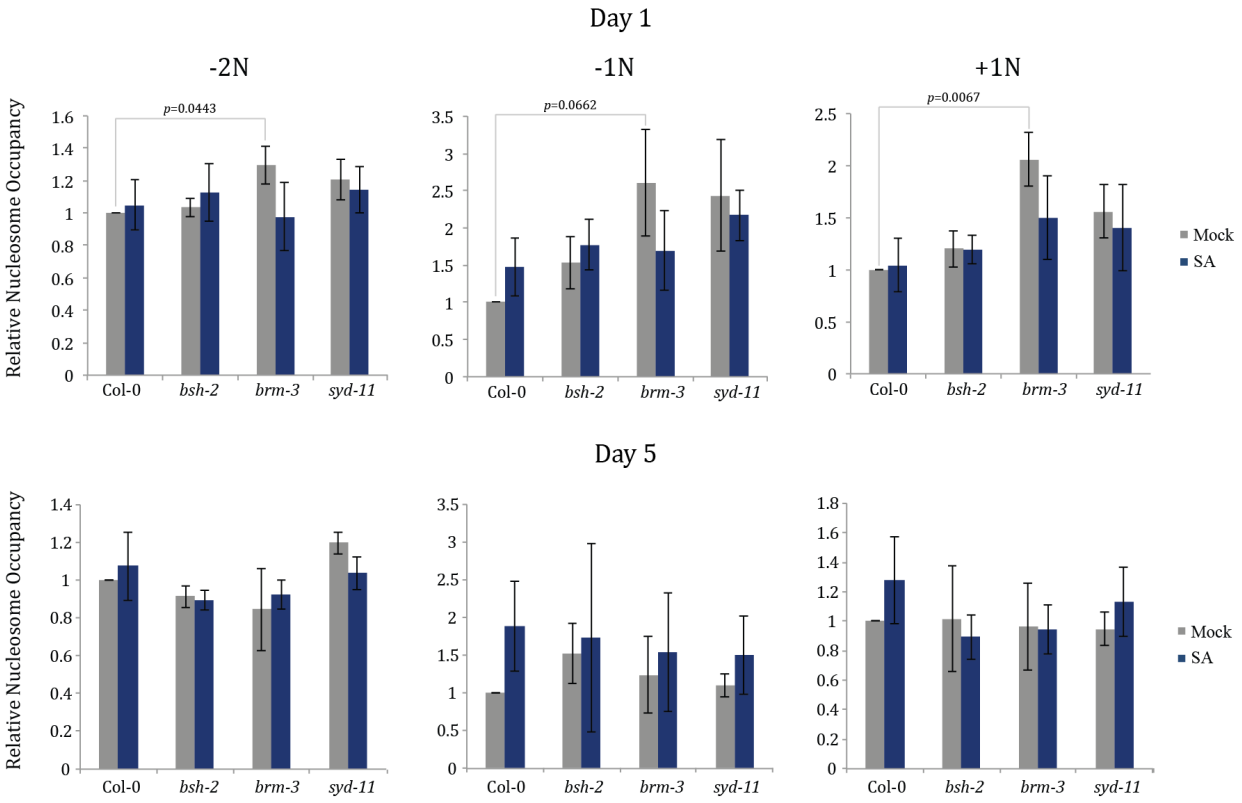


Figure 10: Nucleosome occupancy at the *NPR3* locus is changed in *swi/snf* mutants during SA treatment. Sterile grown Arabidopsis WT, *bsh-2, brm-3* and *syd-11* plants were treated with mock solution or 500 µM SA and samples were harvested 1 day and 5 days after treatment. Nucleosome occupancy was analysed using MNase treatment and subsequent tiled qPCR analysis. Relative nucleosome occupancies of three biological replicates are shown and were normalized to gypsy-like transposon locus (AT3G32010).

Overall the obtained expression as well as nucleosome occupancy data give first indications that chromatin remodeling might play a role in establishment of SAR at the *PR1* locus and that the SWI/SNF chromatin remodeling complex might be involved in this process.

swi/snf mutants have altered SA levels but show no altered response to *Pseudomonas syringae* pv. *Tomato*

To be further investigate whether the SWI/SNF chromatin remodelling complex plays a role during establishment of SAR we analysed *in planta* SA levels in WT, *bsh-2*, *brm-3* and *syd-11* plants (see Figure 11). In *bsh-2* and *brm-3* mutants SA levels were significantly reduced. Indicating that the SWI/SNF complex might not only play a role during signalling but also during biosynthesis of SA. This is counterintuitive to the finding that *PR1* expression is increased under mock conditions.

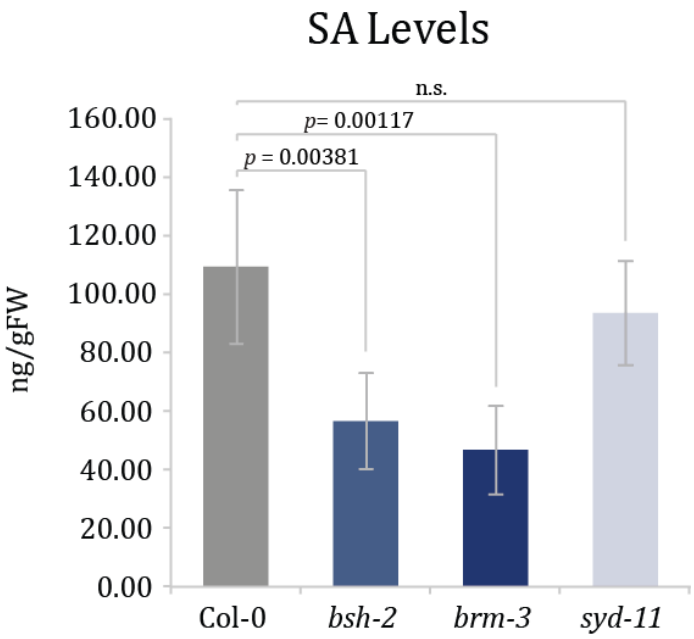


Figure 11: *swi/snf* mutants show reduced internal levels of SA. SA levels [ng/gFW] in sterile grown WT, *bsh-2, brm-3* and *syd-11* plants are shown. Error bars indicate standard deviation of at least 5 biological replicates. Statistical significance of this experiment was evaluated via student's t-test, resulting p-values are shown.

Altered *in planta* SA levels as well as altered *PR1* expression profiles often coincide with weaker or stronger resistance towards biotrophic pathogens (Glazebrook, 2005). Therefore, we analysed the phenotypes of WT Col-0, *bsh-2*, *brm-3* and *syd-11* plants after *Pseudomonas syringae* pv. *Tomato* treatment. To test whether SAR also influences this phenotype we included a pre-treatment of all plant lines with either mock or SA solution in the experiment. First, we analysed resistance against the avirulent *Pseudomonas syringae* pv. *Tomato* strain hrcC- (see Figure 12). This strain is non-pathogenic, since it carries a mutation in the type 3 secretion system and can therefore

not induce effector-triggered immunity (Roine et al., 1997; Yuan and He, 1996). We could not observe differences for any of the mutant plant lines.

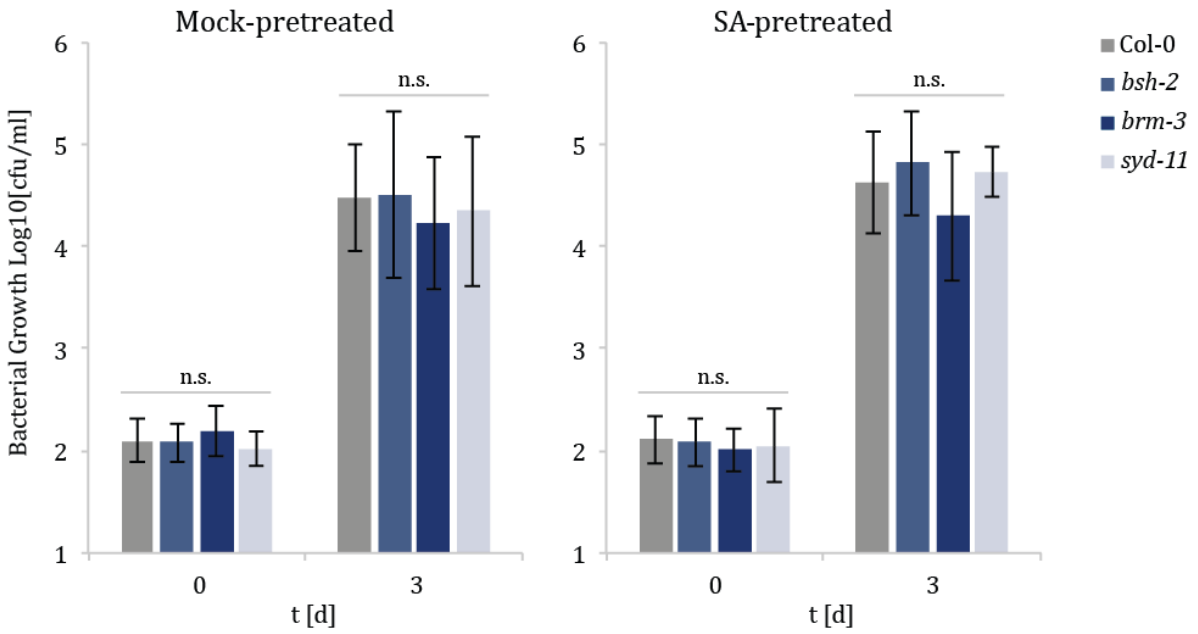


Figure 12: *swi/snf* mutant plants show no altered phenotype against *Pseudomonas syringae* pv. *tomato* hrcC-. Arabidopsis WT, *bsh-2, brm-3* and *syd-11* mutant plants were pre-treated with mock solution or 500µM SA solution and after 5 days inoculated with hrcC-. Bacterial growth was observed after indicated time points on plates in serial dilutions and counted after 2 days. Error bars indicate standard deviation of five technical replicates. Statistical significance of this experiment was evaluated via student's t-test, none of the results showed significant differences.

Next, we analysed resistance against *Pseudomonas syringae* pv. *Tomato* strain DC3000. This strain is a virulent *Pseudomonas syringae* strain that can induce ETI during infection (Buell et al., 2003; Whalen et al., 1991). In our first experiment, results were very promising, since *bsh-2* and *brm-3* mutant plants showed statistically significant stronger susceptibility towards DC3000 (see Figure 13 (a)). SA pre-treatment could eradicate this effect further indicating a negative role for the SWI/SNF chromatin remodelling complex in SAR establishment. When we repeated this experiment, we were not able to reproduce the previously obtained data (see Figure 13 (b)). Even though the *bsh-2* mutant showed a similar trend. In summary our results were not conclusive. There might be involvement of chromatin remodelling and the SWI/SNF complex in SAR establishment but to find final proof all experiments will have to be repeated and more research will be needed.

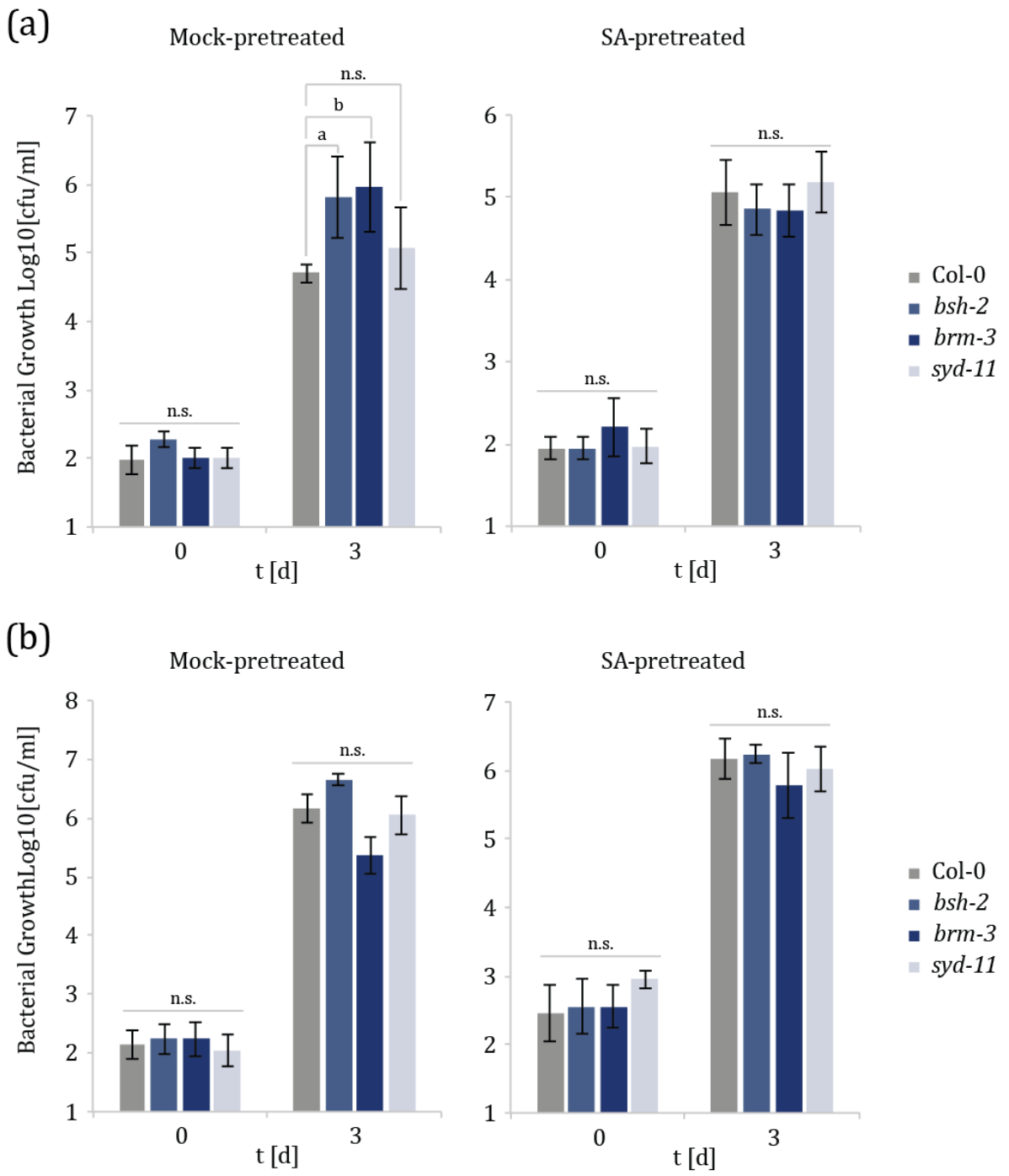


Figure 13: *swi/snf* mutant plants show no altered phenotype against *Pseudomonas syringae* pv. *tomato* DC3000. Arabidopsis WT, *bsh-2, brm-3* and *syd-11* mutant plants were pre-treated witch mock solution or 500 µM SA solution and after 5 days inoculated with DC3000. Bacterial growth was observed after indicated time points on plates in serial dilutions and counted after 2 days. Error bars indicate standard deviation of five technical replicates. Results of biological replicate 1 (a) and replicate 2 (b) are shown. Statistical significance of both experiments was evaluated via student's t-test (a, p-value = 0.008; b, p-value = 0.007)

Discussion

SAR is of great interest to agriculture, since it might be a mechanism to increase resistance towards a variety of pathogens without the use of herbicides or genetically modified organisms. Although the concept is generally accepted and proven, it is not at all clear which molecular mechanisms are involved in the induction as well as the establishment of SAR (Adam et al., 2018; Espinas et al., 2016; Kumar, 2014; Shine et al., 2018). To attack the question how SAR is established and what the cause for stronger and faster transcription after induction of SAR could be, we analysed the chromatin state of various PR genes, known to be involved in SAR, after SA treatment and investigated the involvement of a specific chromatin remodelling complex in this process, namely the SWI/SNF complex.

First, we could demonstrate that treatment with the plant hormone SA might lead to a short-term as well as long-term loss of the third nucleosome upstream and the first nucleosome downstream of the transcription start site (TSS) in the *PATHOGENESIS RELATED 1* (*PR1*) gene. This lowered occupancy might still be evident even after *PR1* expression levels are at mock-level again.

Secondly, we observed that the *PR1* locus and *PR1* expression levels are altered in some *swi/snf* mutants. In *bsh-2* and *brm-3* mutants *PR1* expression was lower under mock conditions. In *brm-3* mutants nucleosome occupancy after 5 days of treatment was lower under mock conditions. Considering the expression data, which showed lower expression levels under mock conditions, this might be contradicting, but since *PR1* expression is still responsive to SA treatment it might also explain why this is the case. It does not explain why we do not see lower nucleosome occupancies in 1-day mock treated samples. The variation in these samples was much higher though, and further repetitions will be necessary. In *bsh-2* mutants *PR1* expression in SA treated plants after 5 days was higher even though nucleosome occupancies were not significantly altered. Also in this case more repetitions might eliminate the strong variance and therefore give more reliable results. Considering the most reliable data, our results hint in the direction that a SWI/SNF complex containing the BSH and BRM components might be regulating the *PR1* locus.

Thirdly, we were able to demonstrate that *in planta* levels of SA were reduced in *bsh-2* and *brm-3* mutants. Due to the fact, that SA biosynthesis is regulated by positive as well as negative feedback loops, this result is neither expected nor unexpected (Shah, 2003). It still hints in the direction that the SWI/SNF complex most likely has several target genes in the SA-dependent defence pathway.

Assuming that altered *PR1* expression profiles and SA hormone levels could lead to altered resistance towards biotrophic pathogens we analysed phenotypes of *swi/snf* mutants after *Pseudomonas syringae* infection. In one experiment we observed that *bsh-2* as well as *brm-3* mutants were less resistant towards the virulent *P. syringae* strain DC3000. Interestingly in both mutants the observed phenotype could be abolished by pre-treatment with SA. Since we were not able to reproduce these results it is difficult to interpret them.

Should these results be valid it also needs to be considered that if SA treatment activates similar pathways as *Pseudomonas syringae* infections, our expression data after 24 h of SA treatment (only single treatment) might be conflicting with the observed phenotypes. At a first glance one might expect that higher *PR1* transcription levels lead to higher resistance towards biotrophic pathogens. However overexpression of PR1 proteins in tobacco doesn't necessarily lead to enhanced resistance towards pathogens (Cutt et al., 1989; Linthorst et al., 1989). Unfortunately, available data on *PR1* overexpression in Arabidopsis are very scarce. So far pathogen assays with transgenic Arabidopsis lines have only been made with lines overexpressing *PR* proteins from other organisms and not with the endogenous Arabidopsis *PR1* (Broekaert et al., 2000; Hong and Hwang, 2005; Pecenkova et al., 2017). Therefore, our obtained *PR1* expression data might not be seen as contradicting and might indicate that a too strong induction of *PR1* during initial responses towards a pathogen is obstructive. To substantiate this hypothesis, it will be interesting to analyse expression patterns in mock/*Pseudomonas* and SA/*Pseudomonas* double treated plants.

For some *syd* mutant alleles altered disease phenotypes have already been reported (Johnson et al., 2015; Walley et al., 2008b). Johnson et al. (2015) could show that in the mutant alleles *syd-10* and *syd-4* expression levels of *SNC1* are significantly higher. SNC1 is one of the intracellular NOD-like receptor (NLR) proteins that are activated upon recognition of R-proteins during ETI and therefore is important for defence responses

(Johnson et al., 2015). The fact that the altered phenotype towards *P. syringae* is not evident in all mutant alleles and the fact that the *syd-11* mutant is a very weak allele that shows no developmental defects might explain why we did not observe any altered *PR1, VSP2* or *SNC1* expression, SA hormone levels or resistance towards *P. syringae* in these mutants (Johnson et al., 2015; Walley et al., 2008b).

Apart from *PR1* we also analysed other factors known to be involved in SA signalling. Among them we analysed *NPR3*. NPR3 is assumed to be a negative regulator during SA perception (Kuai et al., 2015) and was identified as a direct target of the BRM ATPase of the SWI/SNF complex (Li et al., 2016). In general, *NPR3* expression was up-regulated by SA treatment in all plant lines. In *bsh-2* and *brm-3* mutant plants expression remained at a higher level 5 days after SA application. Surprisingly, our MNase experiments investigating the *NPR3* locus showed conflicting data. Nucleosome occupancy was slightly higher in *brm-3* mutants after 1 day of SA treatment, but we could not observe these changes after 5 days of treatment. Since it is generally assumed that higher nucleosome occupancies coincide with lower expression levels this would be unexpected. In general, we conclude that *NPR3* is no direct target of the SWI/SNF complex and that observed slight changes in expression and nucleosome occupancy are an indirect effect.

We also analysed *AZI1* expression levels in all mutants after SA treatment. AZI1 is shown to be involved in the transport of two of the putative mobile signals that induce SAR and therefore AZI1 is proposed to be an important factor for SAR induction (Cecchini et al., 2015a; Jung et al., 2009; Yu et al., 2013). Interestingly we could show that expression of *AZI1* was not altered by SA treatment in WT plants at any time. In *bsh-2* and *brm-3* mutants, however, expression was up-regulated under mock and SA conditions 5 days after treatment. After 1 day of treatment we observed similar results, but these were not statistically significant. These data suggest that apart from *PR1*, *AZI1* might be another target that might be directly or indirectly regulated by the BSH- and BRM-containing SWI/SNF chromatin remodelling complex during SA signalling, preventing a premature or unwanted establishment of SAR.

Additionally, we could observe altered *VSP2* levels. In WT plants *VSP2* expression was down-regulated upon SA treatment. Since *VSP2* is part of the JA-dependent signalling pathway and SA has been shown to negatively influence JA signalling this is as expected

(Caarls et al., 2015). Interestingly all mutants showed the characteristic down-regulation after SA treatment. In *brm–3* mutants *VSP2* levels were lower after 1 day of mock treatment. Again, we were not able to observe these results after 5 days of mock treatment, shedding some doubt on our results. But, this again hints in the direction that BSH and BRM might somehow regulate the SA signalling pathway thereby effecting JA signalling components. Together with the lowered SA hormone levels these results also indicates that there might be a factor upstream of the analysed pathway components, which is a target of the SWI/SNF complex. Such a factor could be *ENHANCED DISEASE SUSCEPTIBILITY 1* (*EDS1*) or its interacting partner, *PHYTOALEXIN DEFICIENT 4* (*PAD4*; (Wiermer et al., 2005). Both factors are known to influence the cross-talk between SA and JA signalling and are influenced by MITOGEN-ACTIVATED KINASE 4 (MPK4), which is known to be important for induction of SAR (Petersen et al., 2000). Interestingly both factors were identified as a direct target of BRM in ChIP-seq experiments (Li et al., 2016). These data show that further research is necessary to investigate the role of the SWI/SNF complex in the establishment of SAR.

Altogether we propose a putative model in which a SWI/SNF complex containing BSH and BRM might play a role in the regulation of a specific subset of SA- induced SAR genes This regulation might influence and prevent untimely and unwanted establishment of SAR (see Figure 14). However, this model is highly speculative and further experiments will have to be performed to find final proof.

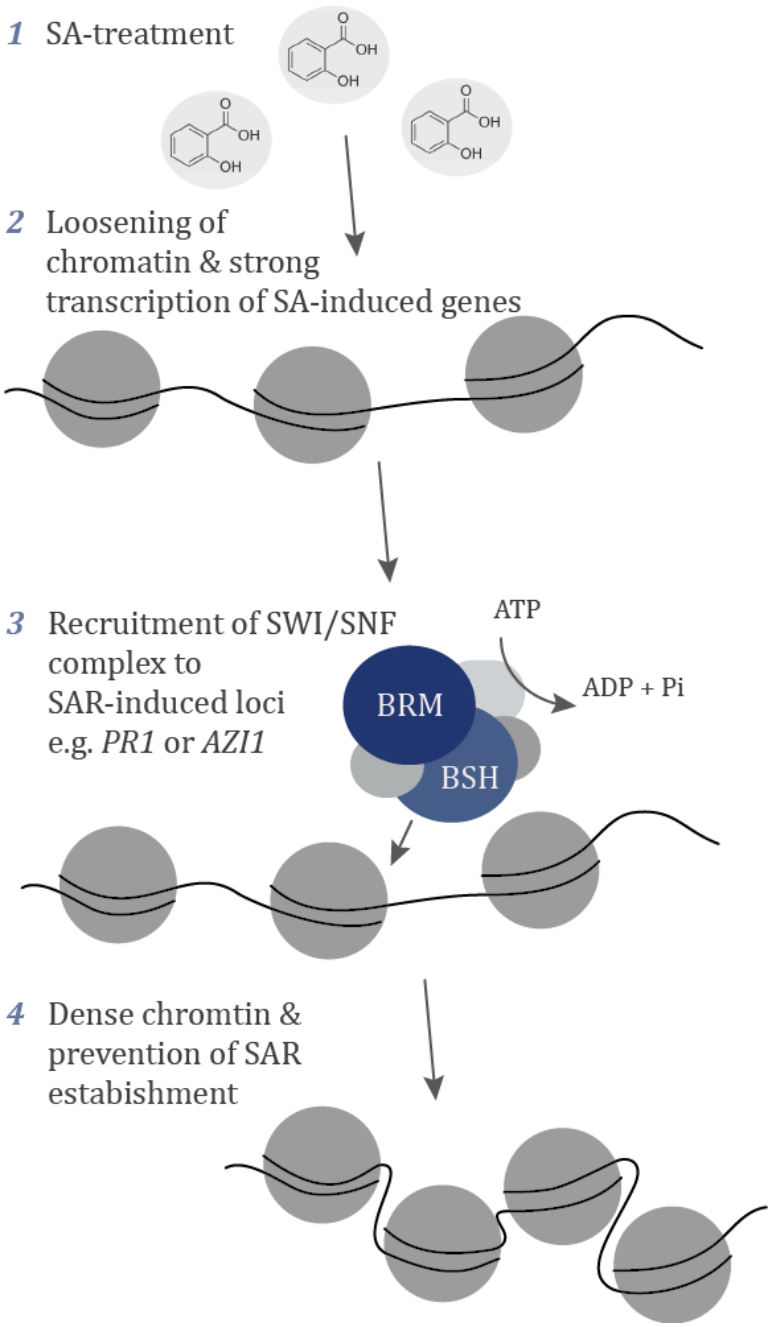
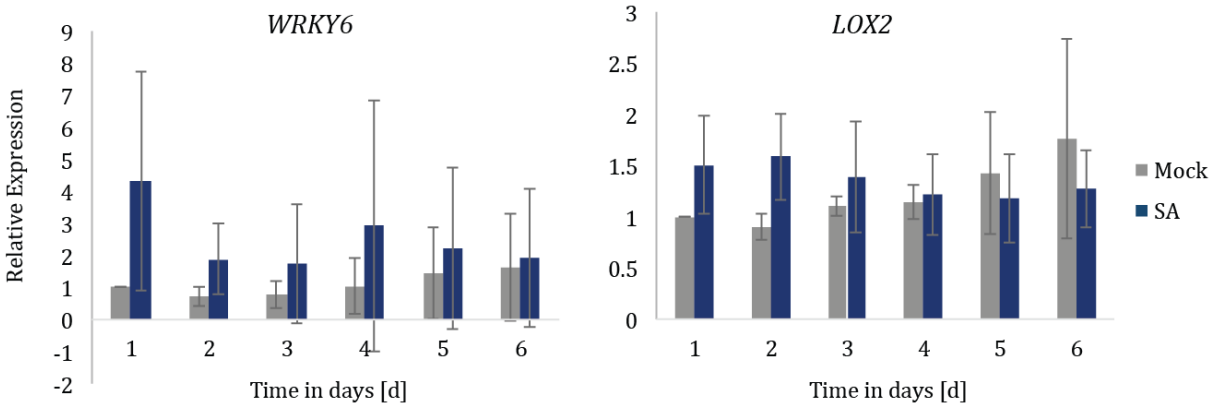
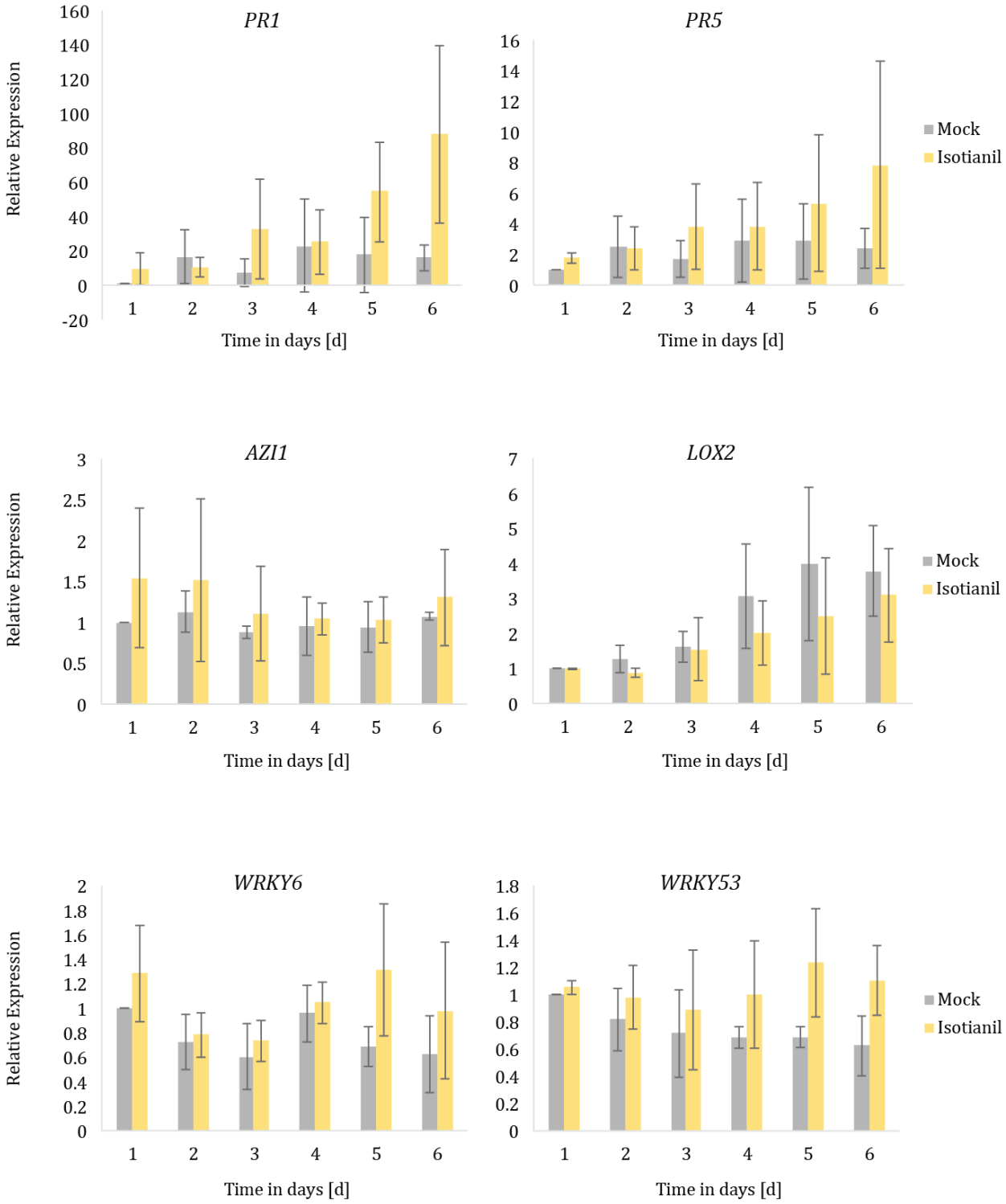


Figure 14: Mode of action of SWI/SNF complex during SA treatment. ***1*** Strong production of SA (during SA treatment or biotrophic pathogen infection). ***2*** Loosening of chromatin SA-induced genes and consequently strong expression (e.g. *PR1* and *PR5*). ***3*** Recruitment of the SWI/SNF complex, containing BRM and BSH, to loci important for SAR establishment (e.g. *PR1* and *AZI1*). ***4*** Stronger packaging of chromatin of SAR-induced genes and thereby negative regulation of SAR.

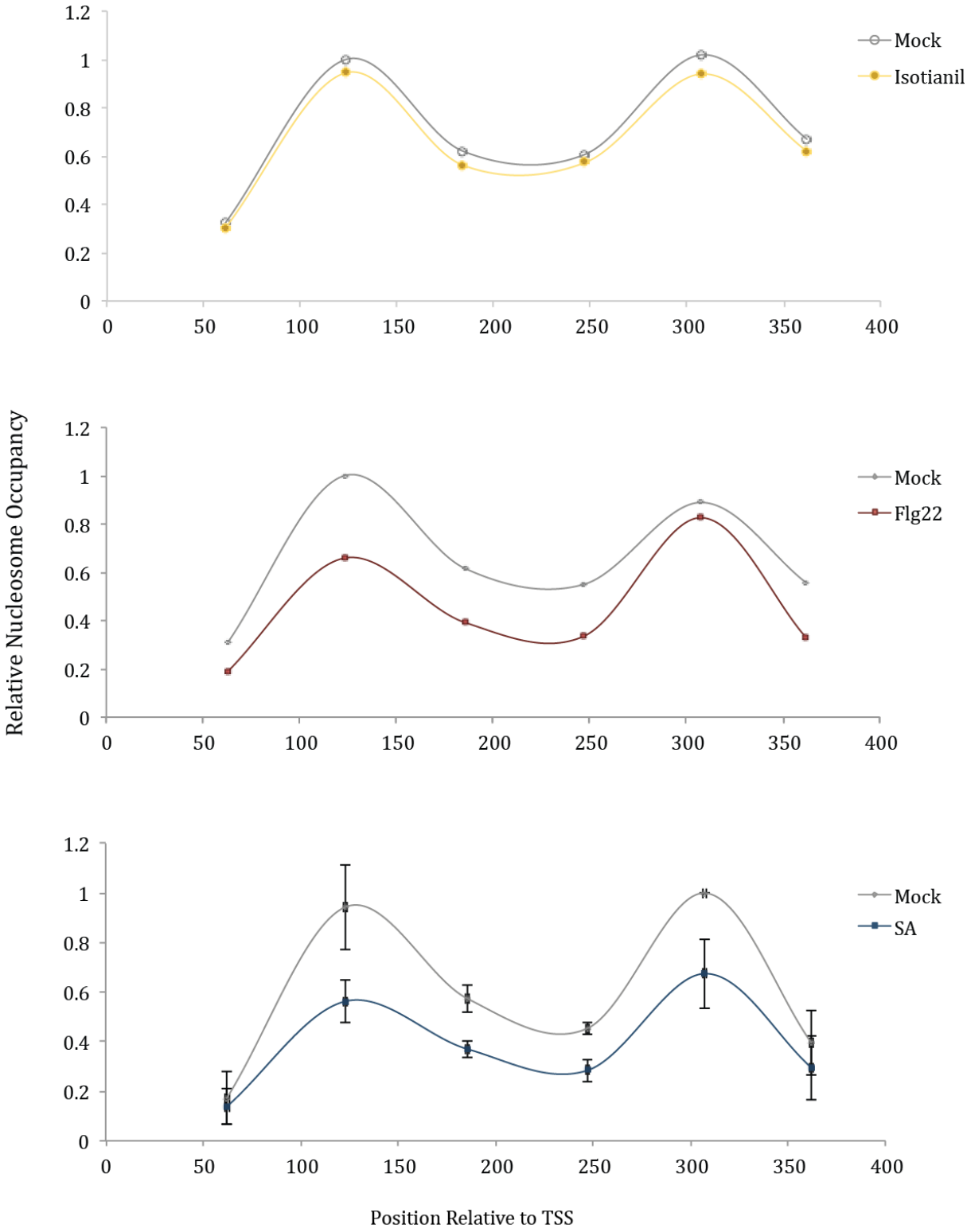
Supporting information



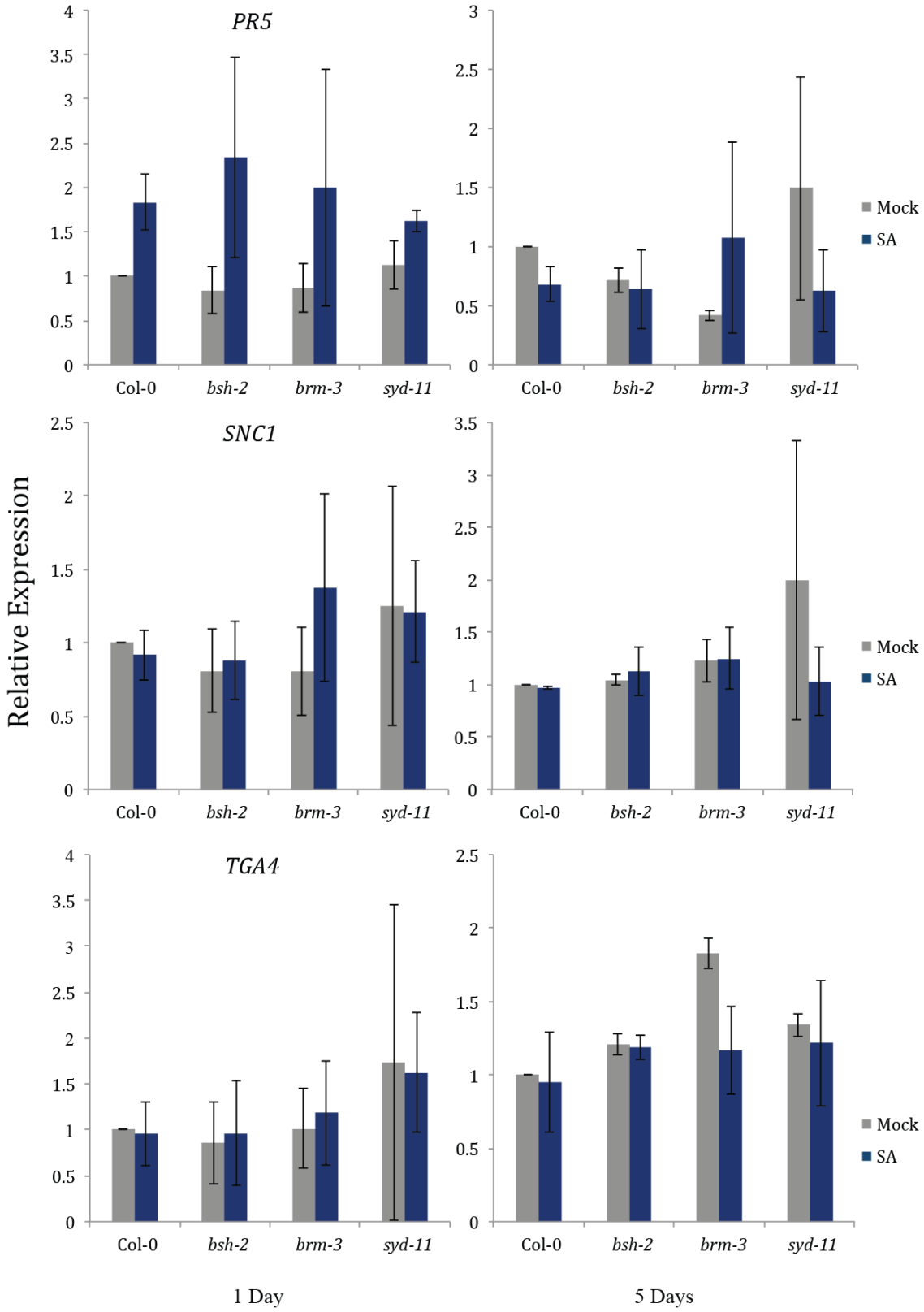
Supplementary Figure S 1: Long-term expression profiles of defence related genes during SA treatment. Relative expression level of *WRKY6* and *LOX2* of sterile grown WT plants treated with 1 mM SA are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates; student's t-test was performed and showed no significant changes.



Supplementary Figure S 2: Long-term expression profiles of defence related genes during Isotianil treatment. Relative expression level of *PR1, PR5, AZI1, LOX2, WRKY6* and *WRKY53* of sterile grown WT plants treated with 100 µM Isotianil are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates; student's t-test was performed and showed no significant changes.



Supplementary Figure S 3: Nucleosome occupancy at the *PR1* locus is altered during isotianil and flagellin treatment. Sterile grown Arabidopsis WT plants were treated with mock solution, 10 µM flagellin (Flg22) or 100 µM isotianil and samples were harvested 1 day after treatment. Nucleosome occupancy was analysed using MNase treatment and subsequent tiled qPCR analysis. Relative nucleosome occupancies of three biological replicates are shown and were normalized to the *gypsy-like transposon* (AT3G32010).



Supplementary Figure S 4: *swi/snf* mutants show altered expression profiles under mock and SA conditions. Relative expression level of *PR5, SNC1* and *TGA4* of sterile grown WT plants treated with mock solution or 1 mM SA are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates; student's t-test was performed and showed no significant changes.

CHAPTER II: HISTONE ACETYLATION AND THE PLANT IMMUNE SYSTEM

Author contributions

Margaux Kaster and Sascha Laubinger designed the research; Margaux Kaster performed research; Stephanie Rausch transformed *brd5* mutant lines with 35S:BRD5 constructs; Salicylic acid measurements were performed by Dr. Joachim Kilian; Emese X. Szabó did the analysis of *brd5-3* transcriptome data shown.

Introduction

DNA is organized into higher order structures to ensure a differential spatial and temporal accessibility of genes. This organisation is basically achieved by wrapping the DNA around histone octamers, consisting of the highly conserved histone proteins H3, H4, H2A, H2B and H1 (Kornberg and Lorch, 1999; Luger and Richmond, 1998).The resulting chromatin structure has been shown to have severe impact on gene regulation and is highly dynamic (Ramirez-Prado et al., 2018). In addition to this basic structure it has been shown that histone tails can extensively be post-translationally modified resulting in a complex so-called histone code (Jenuwein and Allis, 2001). The histone code consists of eight different types of modifications that can be found on over 60 different histone tail residues (Kouzarides, 2007). The most studied modifications are acetylation methylation, phosphorylation, ubiquitination and sumoylation (Kouzarides, 2007). Most of these modifications cannot only be combined with each other but can also appear in different grades (e.g. di- or tri-methylation (Brodersen et al., 2006)) resulting in a complex combinatory system. Considering that Arabidopsis promoter regions contain in average 18 nucleosomes (Yamamoto et al., 2011) that can be modified in at least 8 different ways at at least 60 different residues (Kouzarides, 2007) each gene ends up with a regulatory module comparable to a lock with at least 18000 combinations. This does not even yet include the differential influence of regulatory modules such as higher order chromatin structures, DNA binding motifs, transcription

factors, the transcription machinery and posttranscriptional and posttranslational modifications.

Acetylation of histone tails has been intensively studied and identified as one of the key regulatory mechanisms to increase or repress transcription of genes (Grunstein, 1997; Shahbazian and Grunstein, 2007). In general it is assumed that acetylation is more associated with active genes while the lack of acetylation is rather associated with inactive genes (Carrozza et al., 2003; Hebbes et al., 1988; Marmorstein and Roth, 2001). This is explained by the fact that acetylation negatively perturbs the interaction between nucleosomes, which leads to looser, more accessible chromatin (Berger, 2007; Shahbazian and Grunstein, 2007). Histone acetylation states are mainly regulated by histone acetyltransferases (HATs), that acetylate lysine residues on histone tails, and histone deacetylases (HDACs) that deacetylate such acetylated residues (Lusser et al., 2001; Pandey et al., 2002). HATs and HDACs are therefore also compared to writers and readers of histone acetylation, respectively. Recently it has been shown that in general H3K9 and 14 acetylation is responsive to nitric oxide, which is induced upon pathogen attack and that in particular HDA19 seems to play an important role in the negative regulation of JA-mediated pathogen responses (Choi et al., 2012; Mengel et al., 2016). Another HDAC, HD2B, has been reported to positively regulate innate immunity. Latrasse et al. (2017) could show that a mitogen-activated protein (MAP) kinase, MPK3, directly interacts and most likely phosphorylates HD2B, which leads to binding of the MPK3-HD2B regulatory module to specific target defence genes and their deacetylation. They also show that upon flagellin treatment major changes H3K9 acetylation take place (Latrasse et al., 2017). These data indicate that histone acetylation might be another key regulatory mechanism of plant defence.

Bromodomain-containing proteins

Apart from HATs and HDACs some accessory proteins influencing the acetylation state of histones were identified. So called bromodomain-containing proteins can recognize and bind acetylated residues via bromodomains, are highly conserved and can be found in many species (Haynes et al., 1992; Hudson et al., 2000; Jeanmougin et al., 1997; Owen et al., 2000). Bromodomains were first analysed in connection with the Drosophila SWI/SNF chromatin remodeller and consist of ~110 amino acids that can form four α-

helices (Dhalluin et al., 1999; Haynes et al., 1992; Zeng and Zhou, 2002). In humans bromodomain-containing proteins often play central roles in a variety of cancers, obesity and inflammation (Belkina and Denis, 2012; Fujisawa and Filippakopoulos, 2017).

The Arabidopsis genome encodes 29 bromodomain-containing proteins. (Pandey et al., 2002; Zhao et al., 2018). Analysis of several proteins from that family uncovered important functions in seed germination, the determination of leaf shape and mitotic cell division (Chua et al., 2005; Della Rovere et al., 2010; Duque and Chua, 2003). Among these 29 proteins, a hypothetical protein (At1g58025) was identified, which due to its similarity to other bromodomain-containing proteins will be referred to as AtBRD5 or BRD5 in short (Pandey et al., 2002). BRD5 contains one bromodomain and is ubiquitously expressed under non-stress conditions (Klepikova et al., 2016). Additionally transcript levels of BRD5 have been reported to be severely up-regulated in plants overexpressing MAP kinase 4 substrate 1 (MKS1 (Andreasson et al., 2005)). MKS1 is a substrate of MPK4 and is known to be a negative regulator of SA signalling and a positive regulator of JA signalling (Kiba et al. 2007; (Petersen et al., 2000). Arabidopsis knockout mutants of MAP kinase 4 (MPK4) show elevated levels of SA and stronger resistance against the virulent *Pseudomonas syringae* pv. *tomato* strain DC3000 (Petersen et al., 2000). Arabidopsis mutants overexpressing the substrate, MKS1, show elevated levels of SA and *PR1* and are also more resistant against *Pseudomonas syringae* pv. *tomato* DC3000 (Andreasson et al., 2005). Since *BRD5* levels are also severely changed in plants overexpressing *MKS1* we asked the question whether BRD5 plays a role in pathogen defence (Andreasson et al., 2005).

Results

To investigate the role of BRD5 in pathogen response we isolated two independent T-DNA lines (*brd5-1* and *brd5-3*), that evidently show lower *BRD5* transcript levels (see Figure 15 (b)). Since in both *mpk4* knockout mutants and MKS1 overexpressing plants, SA levels were increased we checked whether SA levels were also changed in *brd5-1* and *brd5-3* knockout plants (see Figure 15 (c)). In both lines SA levels were not significantly changed compared to WT Col-0 levels. Next, we wanted to see if *BRD5* transcript levels

are influenced by SA or the active JA derivate MeJA (see Figure 15 (d)). However, treatments did not alter *BRD5* transcript levels.

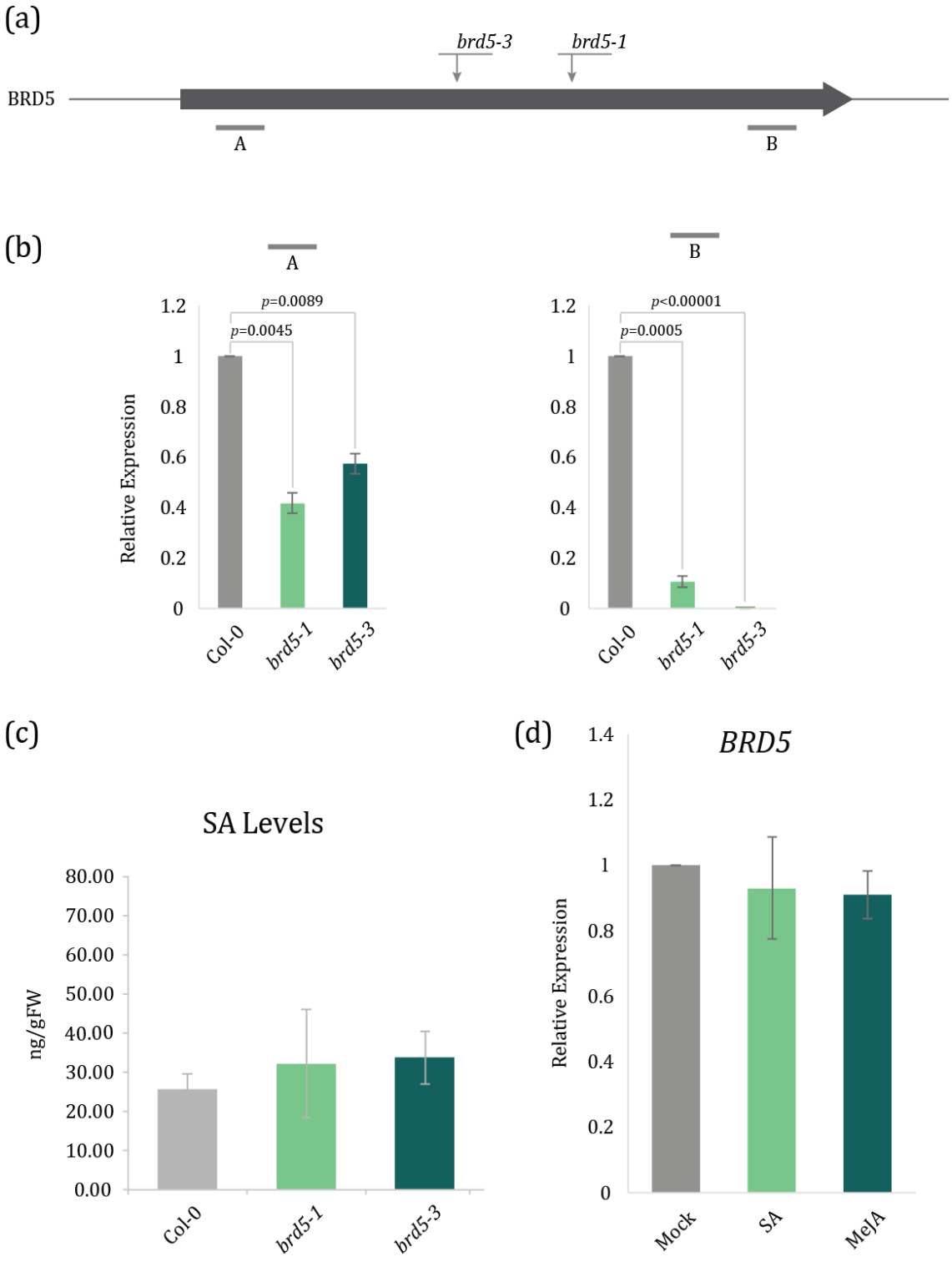


Figure 15: *brd5-1* and *brd5-3* mutants show reduced levels of *BRD5*. (a) Gene model of BRD5 and location of T-DNA insertion in *brd5-1* and *brd5-3* mutant lines. Lines indicate binding site of oligonucleotides used for expression level analysis of *BRD5* transcripts. (b) Relative expression level of *BRD5* of sterile grown WT Col-0, *brd5-1* and *brd5-3* plants are shown. Lines indicate binding site of oligonucleotides used for expression level analysis of *BRD5* transcripts. Relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates. (c) SA levels [ng/gFW] in sterile grown WT, *brd5-1* and *brd5-3* plants are shown. Error bars indicate standard deviation of at least 5 biological replicates. (d) Relative expression levels of *BRD5* of sterile grown

WT plants treated with mock solution, 500 µM SA or 25 µM MeJA are shown. Relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates, statistical significance was determined via student's t-test, *p*-values are indicated.

Whole transcriptome analysis of *brd5-3* mutants

To get a more detailed idea what might be the direct target of BRD5 we analysed the transcriptome of *brd5-3* mutants (see Table 2). Overall the changes that we observed were not as severe as expected. Among the list of down-regulated genes we found *PR1*.

Table 2: Genes down-regulated in *brd5-3* mutants (adjusted p-value <0,05).

Gene	log2 Fold Change	p-value	Gene Name
AT1G47600	-2.94242	4.9957E-204	BETA GLUCOSIDASE 34. BGLU34
AT2G14610	-2.19588	1.75543E-73	PATHOGENESIS-RELATED GENE 1. PR 1
AT5G20230	-2.13738	7.07227E-90	ATBCB. BCB. BLUE COPPER BINDING PROTEIN
AT1G14880	-1.60405	4.31463E-45	ATPCR1. PCR1. PLANT CADMIUM RESISTANCE 1
AT4G31940	-1.45598	3.85651E-53	"CYTOCHROME P450. FAMILY 82. SUBFAMILY C. POLYPEPTIDE 4". CYP82C4
AT5G20240	-1.45009	6.86413E-31	PI. PISTILLATA
AT3G28510	-1.32613	3.97821E-53	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT2G41640	-1.20966	1.29196E-55	Glycosyltransferase family 61 protein
AT1G49860	-1.18645	1.51748E-39	ATGSTF14. GLUTATHIONE S-TRANSFERASE (CLASS PHI) 14. GSTF14
AT2G14560	-1.15449	2.18989E-56	LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA. LURP1
AT1G08630	-1.15176	2.50095E-33	THA1. THREONINE ALDOLASE 1
ATCG00350	-1.10595	2.94967E-25	PSAA
ATCG00340	-1.09484	1.28922E-29	
AT5G54610	-1.09065	9.14222E-26	ANKYRIN (ANK)
AT1G13609	-1.07509	4.44647E-22	
ATCG00120	-1.04926	1.16332E-25	ATP SYNTHASE SUBUNIT ALPHA (ATPA)
AT4G22470	-1.02715	1.09507E-16	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT3G12900	-1.02224	3.40774E-15	

To verify the results from the transcriptome analysis we analysed *PR1* expression levels in *brd5-1* and *brd5-3* mutants under mock and SA treatment conditions via qPCR (see Figure 16(a)). In both lines *PR1* expression was lower compared to WT Arabidopsis plants, but expression could still be induced by SA treatment. Even though the level of induction was slightly lower compared to WT plants, this difference was not statistically significant. Therefore, it needs to be assumed that *PR1* expression might be impaired under mock conditions but not during SA induced processes.

Additionally, we generated complemented EYFP lines of *brd5-1* and *brd5-3,* analysed the localisation of BRD5-EYFP fusion proteins using confocal microscopy (see Figure 16 (b)). Indeed BRD5-EYFP localises to the nucleus and could not be detected anywhere else. It also seems to be omitted in the nucleolus. Since BRD5 is a putative chromatin binding protein this localisation is exactly as expected.

We further analysed *BRD5* expression levels in all complemented lines. In all complemented we observed *BRD5* expression levels could be restored to WT levels or higher. Additionally, we observed that *BRD5* overexpression does not change the fact that *BRD5* levels do not respond to SA treatment.

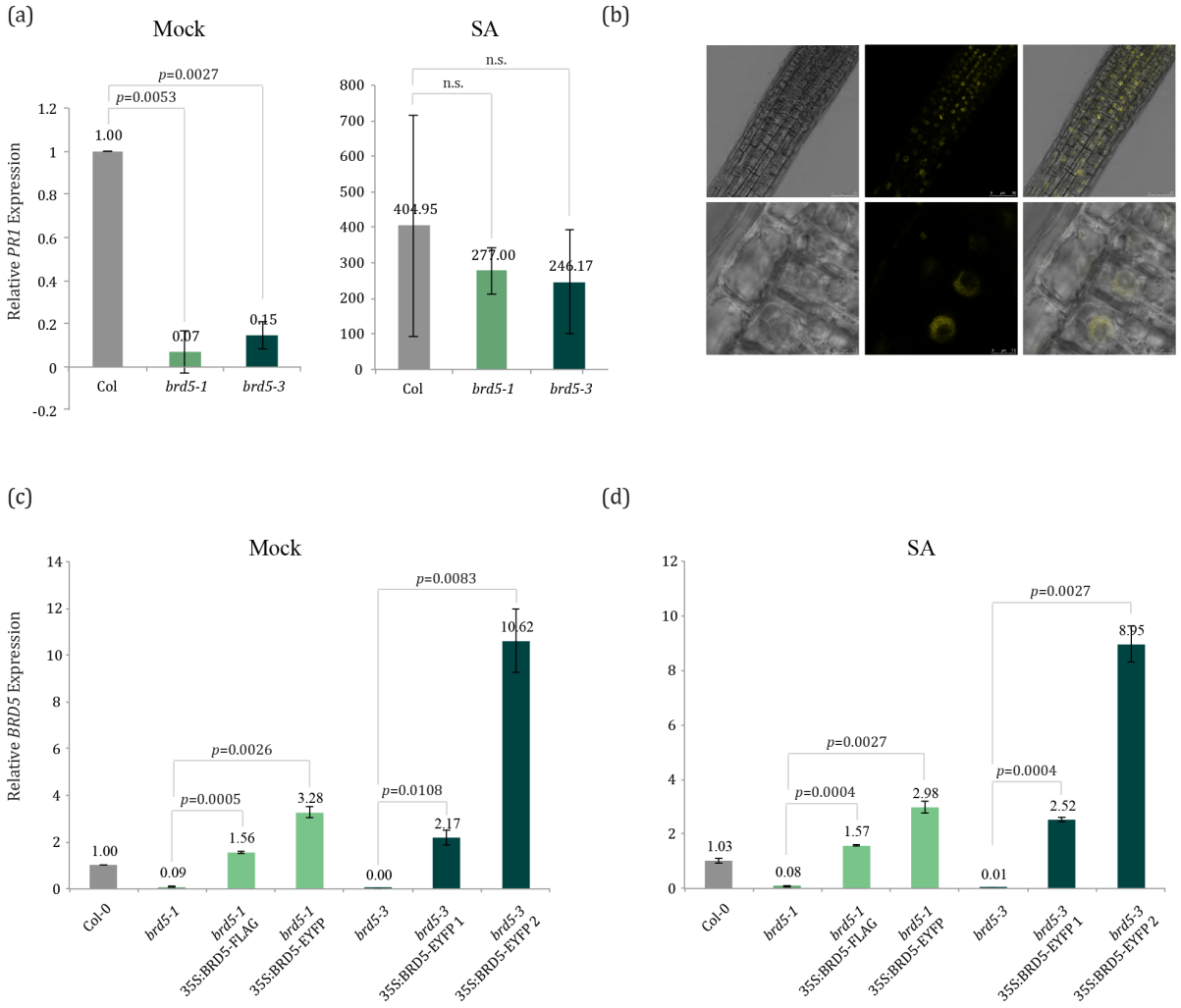


Figure 16: *brd5-1* and *brd5-3* mutant plants show altered *PR1* expression profiles. (a) Relative expression levels of *PR1* of sterile grown WT, *brd5-1* and *brd5-3* plants are shown. Relative expression values were normalized to *PP2A;* error bars indicate standard deviation of two biological replicates and statistical significance was evaluated via student's t-test. (b) BRD5-EYFP localises to the nucleus. Analysis of the subcellular localisation of BRD5–EYFP proteins. Roots of 35S:BRD5-EYFP transgenic lines were analysed by fluorescence microscopy. (b) Relative expression level of *BRD5* of sterile grown WT, *brd5-1*, *brd5-1*-BRD5-FLAG, *brd5-1*-BRD5-EYFP, *brd5-3,* and *brd5-3*-BRD5-EYFP 1 and 2 plants are shown. Relative expression values were normalized to *PP2A;* error bars indicate standard deviation of two biological replicates and statistical significance was evaluated via student's t-test. (a), (c) and (d) sterile grown WT, *brd5-1*, *brd5-1*-BRD5-EYFP, *brd5-3,* and *brd5-3*-BRD5-EYFP plants treated with mock solution or 500 µM SA.

brd5-1 and *brd5-3* show no altered response to *Pseudomonas syringae* pv. *tomato*

Interestingly *BRD5* is miss regulated in *MKS1* overexpression mutants and these mutants are more resistant against *Pseudomonas syringae* pv. *tomato* DC3000. Additionally, *PR1* levels might be altered in *brd5* mutants. Therefore we wanted to check whether mutants of *BRD5* are more or less susceptible against DC3000 (Andreasson et al., 2005). However, infection in both mutants was comparable to WT plants (see Figure 17).

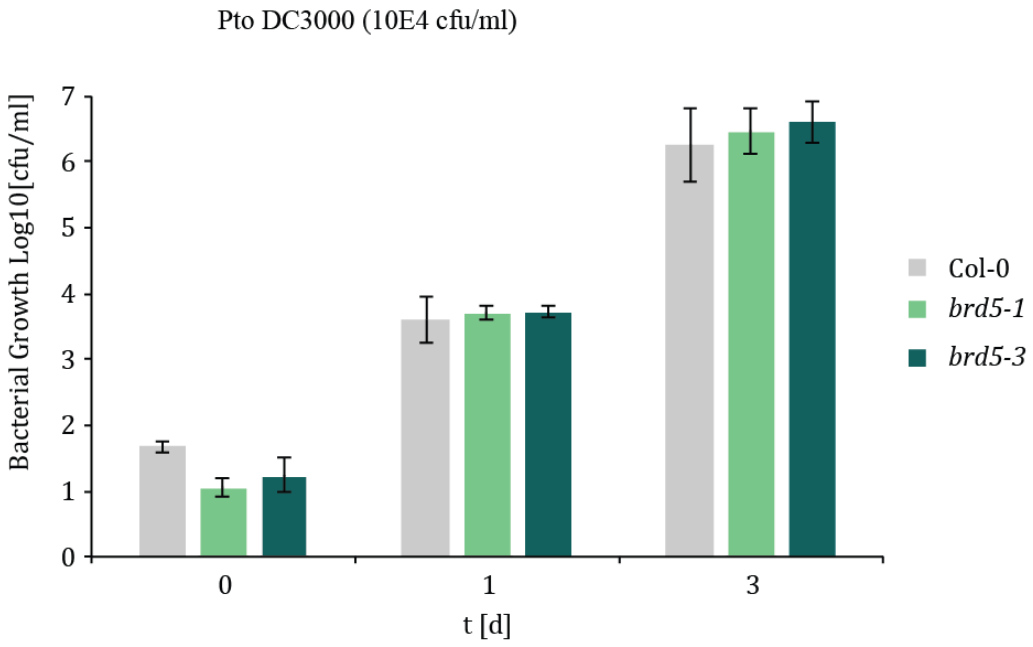


Figure 17: *brd5*-1 and *brd5-3* mutant plants show no altered phenotype under DC3000 treatment compared to WT plants. Arabidopsis WT, *brd5-1* and *brd5-3* mutant plants were inoculated with DC3000. Bacterial growth was observed on plates after indicated time points in serial dilutions and counted after 2 days. Error bars indicate standard deviation of five technical replicates, statistical significance was evaluated via student's t-test.

The role of BRD5 during *Botrytis cinerea* infection

It is known that miss-regulation of SA signalling can negatively affect resistance against necrotrophic pathogens and vice versa (Pieterse et al., 2012; Spoel et al., 2003). Since this well-studied cross-talk between SA and JA pathways has been described we were wondering if BRD5 might play a role in the response to necrotrophic pathogens instead of biotrophic pathogens. Therefore, we treated WT, *brd5-1* and *brd5-3* plants with *Botrytis cinerea* and performed four independent experiments (see Figure 18).

The *brd5-1* mutant line mostly showed the same trend of slightly stronger susceptibility towards *B. cinerea* infection in all experiments, but these results were only statistically

significant in replicate 2 and 4. The *brd5-3* only showed significantly stronger susceptibility towards *B. cinerea* infection in replicate 4. The differences observed in replicate 4 were statistically significant using the disease index and student's t-test and when classifications were compared using the chi-square test (see Figure 18 (b)). Exemplary pictures of all plant lines are shown in Figure 18 (c). To analyse these observations in more detail we analysed the accumulation of *B. cinerea Cutinase A* transcript levels in all lines after infection (see Figure 18(d)). The analysis of *Cutinase A* transcript levels enables a quantitative measurement of *B. cinerea* infection *in planta*. The *brd5-1* mutant showed significantly stronger accumulation of *Cutinase A* transcript levels than WT plants (see Figure 18(d)). Since the results of our first experiments were not convincing further replicates will be needed to make final statements. Additionally we could not observe altered susceptibility towards another necrotrophic pathogen, *Alternaria brassicicola* (see Supplementary Figure S 5).

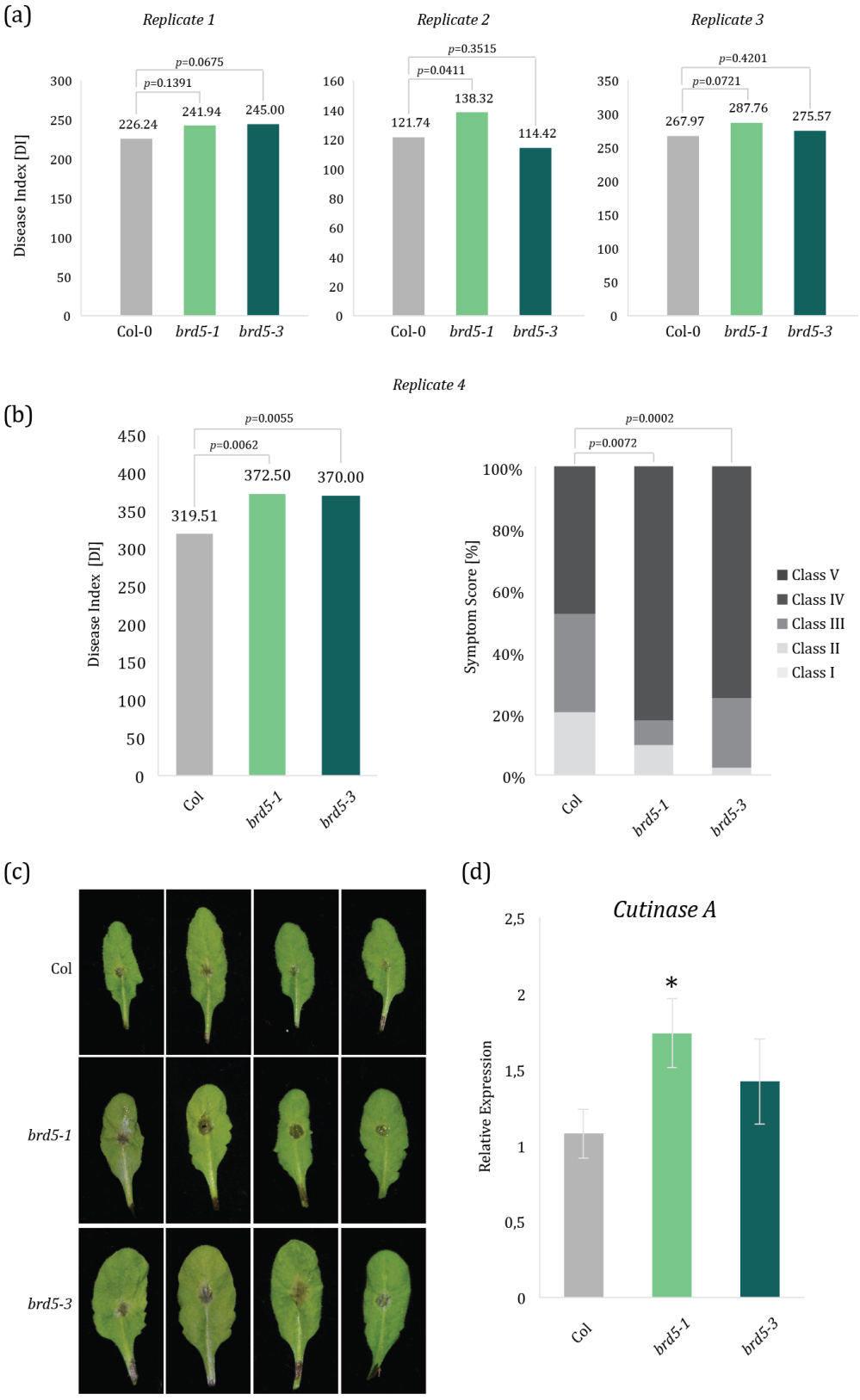


Figure 18: *brd5-1* and *brd5-3* mutant plants might show slightly higher susceptibility towards *Botrytis cinerea.* (a) Symptom score of Arabidopsis WT, *brd5-1* and *brd5-3* mutant plants 9 days after inoculation with spores of *B. cinerea* (replicates 1-3) and replicate 4 (b, left panel) Statistical significance was determined via student's t test, *p*-values are indicated. (b, right panel) Classification of *B. cinerea* infected WT, *brd5-1* and *brd5-3* mutant plants 9 days after infection. Statistical significance was determined via chi-square test, *p*-values are indicated. (c) Representative *B. cinerea* infected leaves of WT, *brd5-1* and *brd5-3* mutant plants shown 9 days after infection. (d) Relative expression level of *B. cinerea Cutinase A* in WT, *brd5-1* and *brd5-3* mutant plants harvested 9 days after infection. Relative expression values were normalized to Arabidopsis *α-shaggy kinase* (At5g26751). Error bars indicate standard deviation of three biological replicates.

Discussion

Plant microbe interactions are a heavily discussed topic and it has been shown that many processes influence the plant immune system. Mengel et al. (2016) and Latrasse et al. (2017) could show that histone deacetylases and H3K9 acetylation play major roles during pathogen response. MPK4 has been shown to be crucial for JA-dependent defence. *mpk4* mutants show increased resistance against the hemi-biotrophic pathogen, *Pseudomonas syringae* pv. *tomato* (DC3000) and decreased resistance against the necrotrophic pathogen *A. brassicicola* (Brodersen et al., 2006; Petersen et al., 2000). BRD5, a bromodomain-containing protein that putatively binds acetylated histone residues, is strongly up-regulated in *mpk4* mutants. Therefore, we wanted to investigate whether BRD5 also plays a role in pathogen response.

In summary we were able to identify two T-DNA insertion lines with severely diminished *BRD5* transcript levels. Both lines showed lower *PR1* transcript levels under mock conditions. Even though pathogen response to biotrophic pathogens is mainly mediated via SA-signalling, *brd5* mutants did not show altered resistance against *Pseudomonas syringae* pv. *tomato* (DC3000). Additionally, in our experiments we could not observe any altered resistance towards *A. brassicicola*. However, we found first hints that *brd5-1* lines might be less resistant against the necrotrophic pathogen *B. cinerea*. Since transcriptome data from Alternaria and Botrytis infected Arabidopsis plants show that expression changes are very different depending on the invading pathogen it is not surprising that *brd5* mutants might show altered resistance towards Botrytis but not Alternaria (Sham et al., 2014; van Wees et al., 2003).

Interestingly we did not observe an overlap between the published *mpk4* and *brd5-3* transcriptome data, indicating that BRD5 acts on a different set of target genes. Interestingly we found overlap of differentially expressed genes in *brd5-3* and the transcriptome data published by Latrasse et al. (2017) (see Table 3). In this publication the direct interaction of another MAP kinase MPK3 and the histone deacetylase HD2B is shown. This interaction between MPK3 and HD2B is important for the binding to target genes in an flagellin-dependent manner and for subsequent pathogen response.

Table 3: Comparison of *brd5-3* differentially expressed genes and transcriptome data from Latrasse et al. (2017).

brd5-3	log2 Fold Change	HD2B target genes in mock condition	HD2B target genes in flg22 condition	Flg22 up-regulated genes in Wt	Up-regulated genes in *mpk3* mutant	Down-regulated genes in *mpk3* mutant	Up-regulated genes in *hd2b* mutant	Down-regulated genes in *hd2b* mutant	H3K9 hyper-acetylated genes after Flg22 treatment
AT2G14610	-2.19587584		+	+					
AT5G20230	-2.13738212	+		+					
AT1G14880	-1.60405491			+	+		+		
AT4G31940	-1.45597678	+		+					
AT3G28510	-1.32612688			+	+		+		
AT2G41640	-1.20966110		+	+					+
AT2G14560	-1.15448595			+					
AT1G08630	-1.15175832	+	+						
ATCG00350	-1.10594952	+				+		+	
ATCG00340	-1.09483528					+		+	
AT5G54610	-1.09064775			+	+		+		
ATCG00120	-1.04926166					+			
AT4G22470	-1.02714643		+	+					
AT3G12900	-1.02223732	+							
AT2G38940	0.61250867	+	+						
AT1G14960	0.617675727	+	+						
AT5G59320	0.622402504		+						
AT5G06760	0.631751074	+	+		+				
AT1G52690	0.791436318		+						

Particularly interesting is the overlap of genes that are down-regulated in *brd5-3* and up-regulated during flagellin treatment. Additionally, many of the top-genes up-regulated in *brd5-3* mutants are specific targets of HD2B after flagellin treatment. Even though flagellin is mostly found in bacterial membranes it is not unlikely that MKP3 and HD2B are not only responsive to flagellin but also to other MAMPs. This could indicate that BRD5 is an important accessory protein for the regulation of a subset of HD2B target genes during the specific response to Botrytis. This is in accordance with the fact that *mpk3* mutants also show increased susceptibility towards Botrytis (Ren et al., 2008) and the fact that PR1 is known to negatively regulate JA-mediated signalling (Caarls et al., 2015). Additionally MPK4 has been shown to be an important regulator of SA-JA crosstalk, since it negatively regulates SA signalling and positively regulates JA signalling (Petersen et al., 2000). It is possible that differential activation and downstream regulation of MPK3 and MPK4 is one of the first levels of SA-JA crosstalk.

To investigate this further it will be crucial to analyse BRD5 interaction partners, direct BRD5 target genes and H3K9 acetylation patterns in *brd5* mutants.

Additionally, it has been shown that *A. brassicicola* can produce depudecin, a toxin that specifically inhibits HDACs and thereby negatively influences the plant immune system (Wight et al., 2009). Since other pathogens have been shown to produce mycotoxins that

inhibit HDACs (Brosch et al., 1995; Ransom and Walton, 1997) a similar mechanism could be used by *Botrytis cinerea* to change histone acetylation patterns during infection.

Apart from its role in immune response it should be discussed whether BRD5 also plays a role in root growth or development in Arabidopsis. We measured the relative shortening of roots from plants which were grown on ½ MS media containing SA, MeJa or mock (see Supplementary Figure S 6). On MeJA containing plates *brd5* mutants did not show increased or decreased shortening of roots compared to WT plants. On SA-containing plates however *brd5-1* as well as *brd5-3* mutants showed decreased relative shortening. This effect could be rescued by overexpression of BRD5 in WT plants. Therefore, both mutant lines showed a hypersensitive response towards SA, which is in line with our observed Botrytis phenotypes. Interestingly in our MeJA experiments *brd5-1* and *brd5-3* mutants showed significantly longer roots on mock plates; this difference was not evident on mock plates during our SA plate experiments. The sole difference between both experiments was the addition of 1 % sucrose on MeJA mock plates. It is known that sugars, such as sucrose as well as most MAP kinase cascades can influence various developmental processes in *A. thaliana* (Gibson, 2005; Xu and Zhang, 2015). Therefore, it is possible that the MKP3-HD2B-BRD5 regulatory module under non-treatment conditions is also responsible for the regulation of root growth or development. This assumption is supported by the fact that *PR1* expression is altered in *brd5* mutants under mock conditions but not during SA treatment and the knowledge that many PR proteins, including PR1, have been reported to have functions in processes other than pathogen response (van Loon et al., 2006).

Altogether we hypothesise a putative model in which *BRD5* plays a dual role. On the one hand *BRD5* influences root growth under non-treatment conditions. On the other hand, *Botrytis cinerea* infection leads to MAMP-triggered immunity via activation of the MPK3-MAP kinase cascade and to binding of a MPK3-HD2B-BRD5 regulatory module to a specific subset of target genes. This places BRD5 downstream of MPK3 in the MAP kinase cascade (see Figure 19).

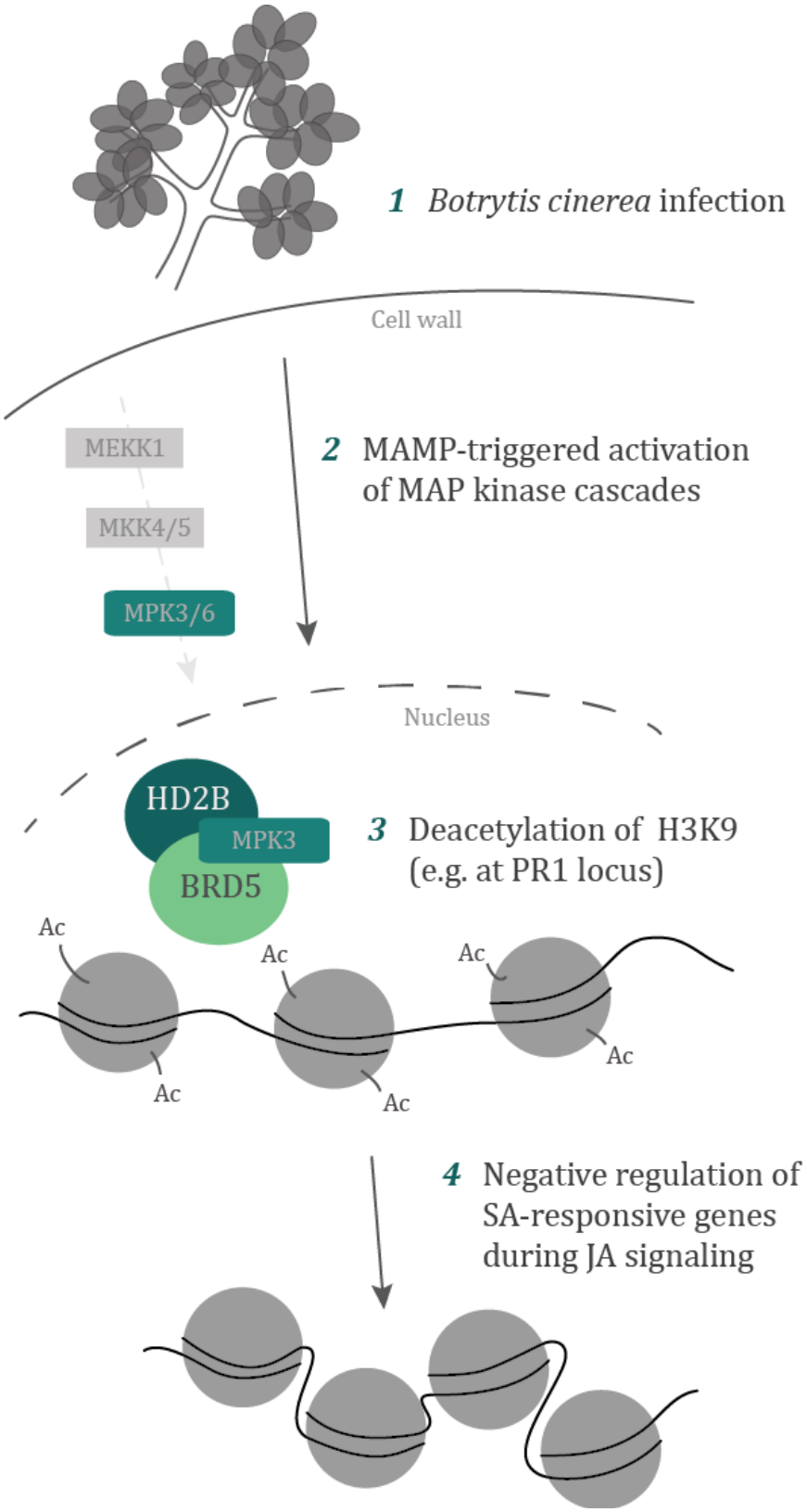
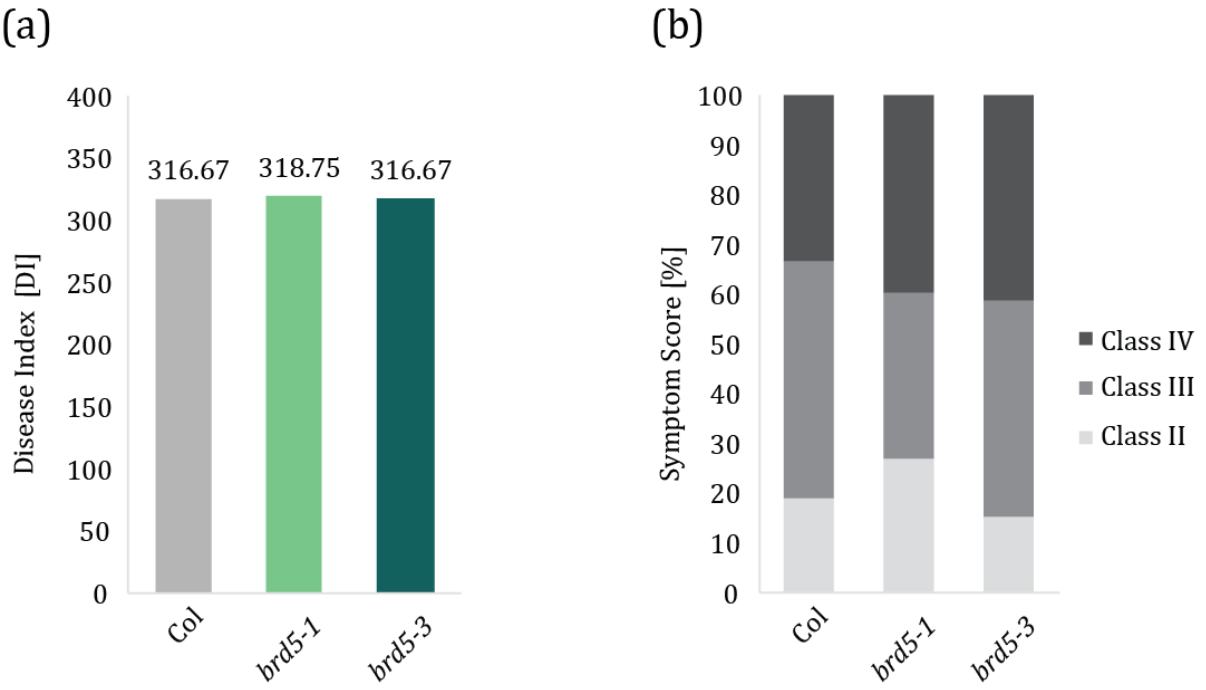


Figure 19: Mode of action of BRD5 during *Botrytis cinerea* infection. ***1*** During *Botrytis cinerea* infection LRR-receptor kinases recognize MAMPs at the cell wall. ***2*** Inside the cell MAP kinase cascades are activated. ***3*** A putative MPK3-HD2B-BRD5 regulatory module deacetylates H3K9 residues at specific target genes. ***4*** SA-induced genes are repressed during JA signaling processes.

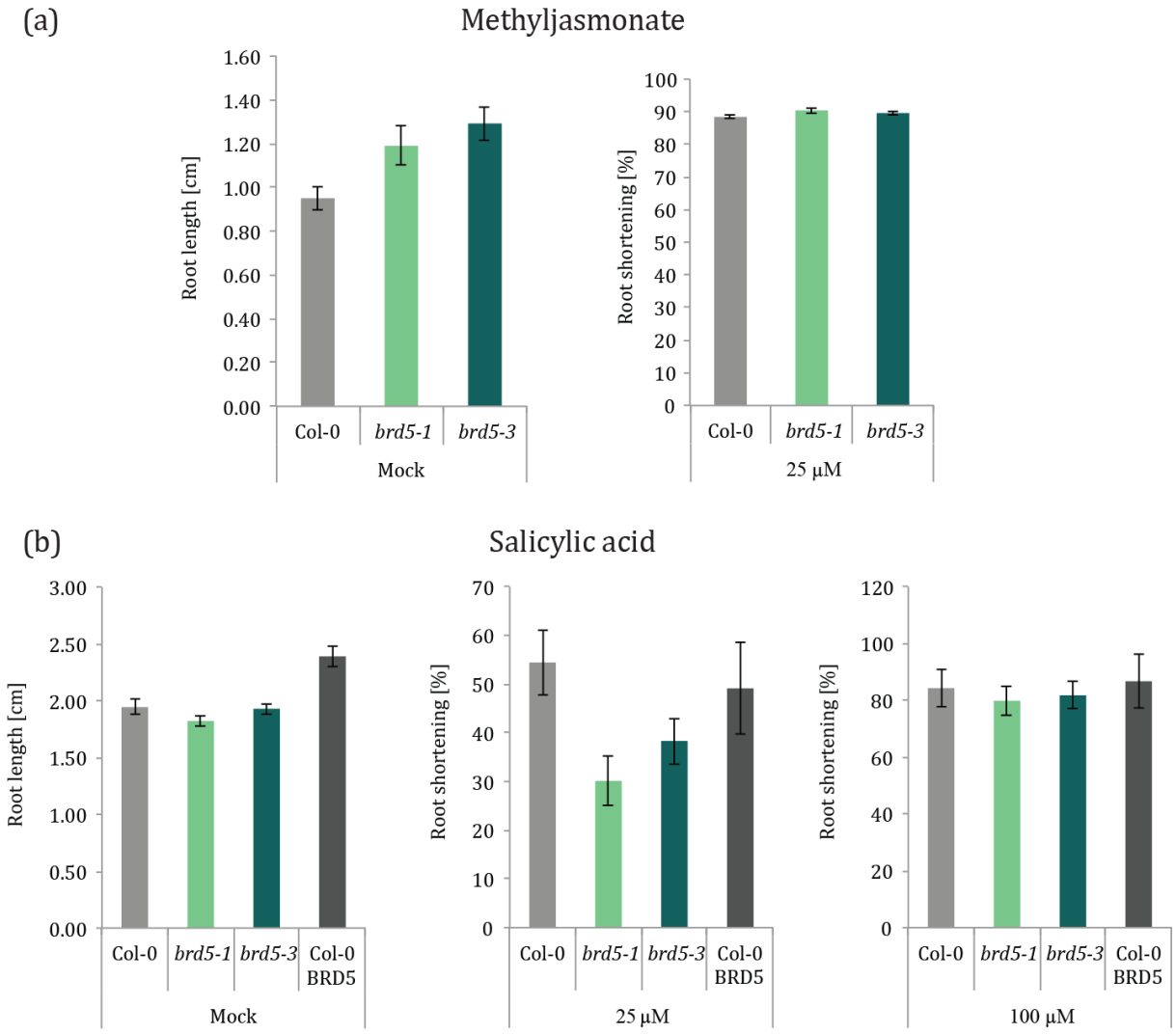
Supporting information



Supplementary Figure S 5: *brd5-1* and *brd5-3* mutant plants show no altered susceptibility towards *Alternaria brassicicola.* (a) Symptom score of Arabidopsis WT, *brd5-1* and *brd5-3* mutant plants 9 days after inoculation with spores of *A. brassicicola.* (b) Classification of *A. brassicicola* infected WT, *brd5-1* and *brd5-3* mutant plants 9 days after infection.

Supplementary Table T 1: Genes up- or down-regulated in *brd5-3* mutants (adjusted p-value <0,05)

Gene	log2 Fold Change	p-value	Gene Name
AT1G47600	-2.94242	4.9957E-204	BETA GLUCOSIDASE 34. BGLU34
AT2G14610	-2.19588	1.75543E-73	PATHOGENESIS-RELATED GENE 1. PR 1
AT5G20230	-2.13738	7.07227E-90	ATBCB. BCB. BLUE COPPER BINDING PROTEIN
AT1G14880	-1.60405	4.31463E-45	ATPCR1. PCR1. PLANT CADMIUM RESISTANCE 1
AT4G31940	-1.45598	3.85651E-53	"CYTOCHROME P450. FAMILY 82. SUBFAMILY C. POLYPEPTIDE 4". CYP82C4
AT5G20240	-1.45009	6.86413E-31	PI. PISTILLATA
AT3G28510	-1.32613	3.97821E-53	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT2G41640	-1.20966	1.29196E-55	Glycosyltransferase family 61 protein
AT1G49860	-1.18645	1.51748E-39	ATGSTF14. GLUTATHIONE S-TRANSFERASE (CLASS PHI) 14. GSTF14
AT2G14560	-1.15449	2.18989E-56	LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA. LURP1
AT1G08630	-1.15176	2.50095E-33	THA1. THREONINE ALDOLASE 1
ATCG00350	-1.10595	2.94967E-25	PSAA
ATCG00340	-1.09484	1.28922E-29	
AT5G54610	-1.09065	9.14222E-26	ANKYRIN (ANK)
AT1G13609	-1.07509	4.44647E-22	
ATCG00120	-1.04926	1.16332E-25	ATP SYNTHASE SUBUNIT ALPHA (ATPA)
AT4G22470	-1.02715	1.09507E-16	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT3G12900	-1.02224	3.40774E-15	
AT5G59220	0.60254	5.86069E-09	HIGHLY ABA-INDUCED PP2C GENE 1 (HAI1)
AT2G38940	0.61251	6.03381E-11	PHOSPHATE TRANSPORTER 1;4 (PHT1;4)
AT1G14960	0.61768	4.10731E-07	Polyketide cyclase/dehydrase and lipid transport superfamily protein
AT1G55152	0.61901	1.34454E-09	unknown protein
AT5G59320	0.62240	5.61523E-09	LIPID TRANSFER PROTEIN 3 (LTP3)
AT5G20150	0.62526	1.07688E-11	SPX DOMAIN GENE 1 (SPX1)
AT5G06760	0.63175	2.26687E-07	LATE EMBRYOGENESIS ABUNDANT 4-5 (LEA4-5)
AT2G28990	0.63668	2.78542E-07	Leucine-rich repeat protein kinase family protein
AT3G56275	0.64794	3.1747E-08	pseudogene of unknown protein
AT5G26270	0.65195	1.36532E-14	unknown protein
AT3G17520	0.65304	2.49068E-06	Late embryogenesis abundant protein (LEA) family protein
AT3G02480	0.66096	3.02131E-06	Late embryogenesis abundant protein (LEA) family protein
AT4G12545	0.66549	2.49232E-10	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT1G56150	0.67501	7.9824E-07	SAUR-like auxin-responsive protein family
AT1G69490	0.72712	1.76132E-10	NAC-LIKE. ACTIVATED BY AP3/PI (NAP)
AT5G35940	0.74712	5.82476E-08	Mannose-binding lectin superfamily protein
AT4G12550	0.75698	3.8082E-16	AUXIN-INDUCED IN ROOT CULTURES 1 (AIR1)
AT5G03545	0.79020	3.13546E-17	AT4 INDUCED BY PI STARVATION 2 (ATIPS2)
AT1G52690	0.79144	2.68309E-13	LATE EMBRYOGENESIS ABUNDANT 7 (LEA7)
AT2G05540	0.80780	9.39931E-28	Glycine-rich protein family
AT5G23990	0.80892	1.47834E-16	FERRIC REDUCTION OXIDASE 5 (FRO5)
AT5G42600	0.86726	5.15157E-13	MARNERAL SYNTHASE (MRN1)



Supplementary Figure S 6: Root lengths of *brd5-1* and *brd5-3* mutant lines during SA and MeJA treatments. (a) For MeJA plate assays WT, *bard5*-1 and *brd5*-3 mutant plants were grown vertically on 1/2 MS media with 1% sucrose under short day conditions (22 °C). After 8 days plants were transferred to 1/2 MS+1% sucrose plates containing mock (EtOH), 25 µM MeJA or 100 µM MeJA and grown vertically for another 4 days under short day conditions (22 °C). (b) For SA plate assays WT, *brd5-1, brd5-3* and WT 35S:BRD5 plants were grown on 1/2 MS media and grown vertically under short day conditions (22 °C). After 8 days plants were transferred to 1/2 MS plates containing mock (EtOH), 25 µM SA or 100 µM SA and again grown vertically for another 4 days under short day conditions (22 °C).

CHAPTER III: THE ROLE OF POSTTRANSCRIPTIONAL REGULATION IN RESPONSE TO NECROTROPHIC PATHOGENS

Author contributions

Margaux Kaster and Sascha Laubinger designed the research; Margaux Kaster performed research; Stephanie Rausch and Emese X. Szabó provided results of small RNA library.

Introduction

MicroRNAs (miRNAs) are a class of small RNAs (sRNAs) that play major roles during plant development, such as leaf differentiation and phase change (Sun, 2012). More recently miRNAs have also been implicated to play important roles during plant-microbe interactions (Fei et al., 2016; Weiberg and Jin, 2015; Weiberg et al., 2014). sRNAs in general are short regulatory noncoding RNAs that are able to induce silencing of specific target mRNAs at a transcriptional and posttranscriptional level (Baulcombe, 2004). miRNAs in particular are encoded by *MIR* genes and are transcribed by Polymerase II (Pol II). The primary transcript (pri-miRNA) forms a unique hairpin structure and is processed by DICER-LIKE 1, an RNaseIII family enzyme, into miRNA/miRNA* duplexes. The miRNA* strand is removed during loading of the mature miRNA into the ARGONAUTE 1 protein forming an active RNA-induced silencing complex (RISC; (Rogers and Chen, 2013; Yu et al., 2017); see Figure 20).

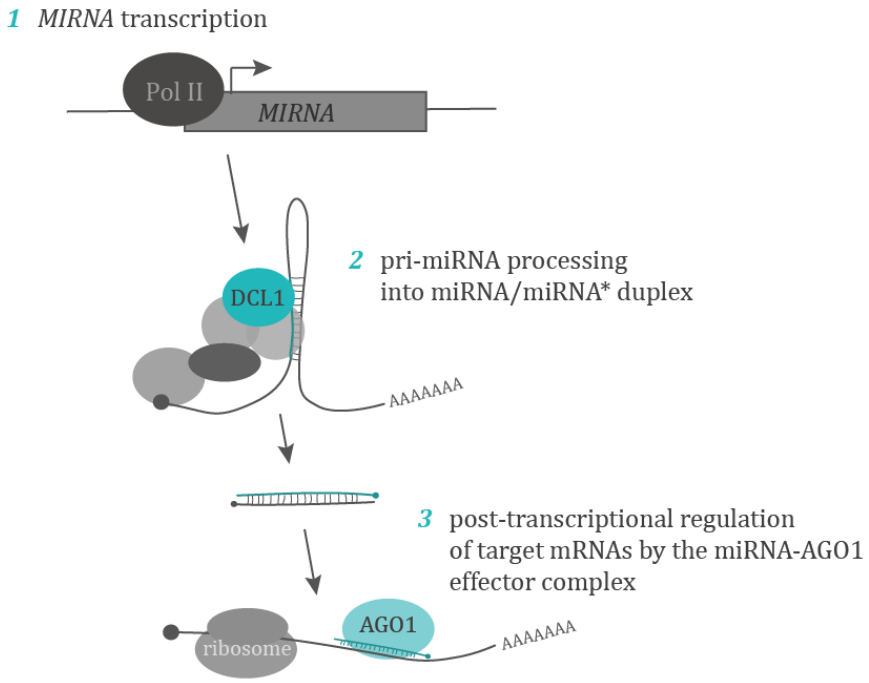


Figure 20: microRNA biogenesis overview. 1 *MIRNA* genes are transcribed by Pol II. **2** pri-miRNA transcripts form hairpin structures and are processed by DCL1 into miRNA/miRNA* duplexes. **3** miRNA targets are regulated by AGO1-miRNA effector complex.

miRNAs and plant immunity

It is known that miRNA biogenesis mutants in general, such as *hyl1* and *hen1*, show altered resistance phenotypes towards various pathogens (Balmer et al., 2017; Balmer and Mauch-Mani, 2013). Interestingly, specific miRNAs have been reported to regulate pathogen response on the level of PTI as well as ETI (Li et al., 2010b; Navarro et al., 2006; Zhai et al., 2011; Zhang et al., 2011; Zhu et al., 2013). This involves responses to various pathogens such as bacteria, fungi, viruses and nematodes.

One nice example is the induction of the miR393 duplex. After flagellin treatment miR393 is induced and specifically loaded into AGO1 effector proteins (Navarro et al., 2006). miR393 then targets F-box auxin receptors *TIR1*, *AFB2* and *AFB3*, which leads to enhanced resistance towards *Pseudomonas syringae* (Navarro et al., 2006). It is known that auxin signalling represses SA levels and that SA signalling leads to a general down-regulation of auxin-related genes thereby influencing resistance towards biotrophic pathogens (Robert-Seilaniantz et al., 2011; Wang et al., 2007). Interestingly, it has also been reported that the star strand of miR393 is also involved in regulating disease resistance towards *Pseudomonas syringae* (Zhang et al., 2011). Before it was assumed that miRNA* strands were merely a by-product necessary for biogenesis,

without any regulatory function. The miR393*, however, is loaded into AGO2 effector proteins and targets the golgi-localised SNARE, MEMBRIN 12 (MEMB12), thereby negatively regulating the exocytosis of *PR1* (Zhang et al., 2011). Sequencing of AGO1 bound small RNAs after flagellin treatment revealed that miR393-mediated regulation is not a unique case, involvement of small RNAs in plant defence responses is a more general mechanism (Li et al., 2010b).

This regulation of plant immunity can not only be induced by the host plant. Several pathogen-derived small RNAs have been reported to target plant immunity components and modulate plant pathogen responses (Navarro et al., 2008; Wang et al., 2017; Weiberg et al., 2013). Weiberg et al. (2013) were able to show that *B. cinerea* small RNAs can bind to Arabidopsis AGO1 proteins and specifically target parts of the MAP kinase signalling pathway (*MPK1* and *MPK2*) that is very important during PTI.

Recent studies postulate that host plants might have evolved a mechanism to generate small RNAs from pathogen derived RNA transcripts. Since (Wang et al., 2016) were able to show that small RNAs that target Botrytis *DCL1* are taken up by the pathogen and can inhibit pathogen growth. This very recent development will need further investigation.

Overall these examples nicely demonstrate the importance of small RNAs during pathogen response and show that it is an intriguing research field. Therefore, we asked whether there are more miRNAs in *Arabidopsis thaliana* that have a role in response to *Alternaria brassicicola.* In our previous studies we could identify miRNAs that are specifically regulated, and are specifically loaded into AGO1 proteins during infection of *Arabidopsis thaliana* by *A. brassicicola* (Rausch, 2017 unpublished; see Table 4). Among these miRNAs, miR827 was found to be one of the most prominent candidates.

Table 4: Differential expression (top panel) and specific loading into AGO1 (lower panel) of miRNAs during *Alternaria brassicicola* treatment (adjusted p-value <0,1; Rausch, 2017 unpublished).

miRNA	log2 fold change	p value
mir169	-2,668	5,67E-08
mir827	2,507	1,49E-07
mir6173	-2,836	1,37E-04
mir163	3,570	2,09E-04
mir866	3,858	7,12E-04
mir8175	-3,781	6,95E-03
mir840	2,064	9,88E-03
mir846	2,177	1,08E-02
mir172	1,042	1,21E-02
mir399	1,253	1,26E-02

miRNA	log2 fold change	p value
mir163	1,202	1,54E-05
mir5651	1,547	3,51E-05
mir399	1,302	3,80E-04
mir827	1,091	9,05E-04
mir846	0,975	1,95E-03
mir164	-1,260	5,06E-03

The Arabidopsis miR827 is encoded by a single gene and can perfectly bind its target mRNA *NITROGEN ADAPTATION LIMITATION (Qiu et al.),* an ubiquitin E3 ligase, between the nucleotides 257 and 278. Initially miR827 was identified to play a major role during phosphate homeostasis (Kant et al., 2011; Lin et al., 2013; Liu et al., 2014). Under normal nutrient conditions, in particular sufficient phosphate supply, miR827 is not induced, NLA is not negatively regulated and can thereby degrade the phosphate plasma membrane transporter PHOSPHATE TRANSPORTER 1 (PHT1). Under limited phosphate conditions the miR827 is severely induced. This increase negatively regulates NLA transcript levels, which in then leads to sustaining of PHT1 and finally increased phosphate uptake (Liu et al., 2014). More recently NLA has also been implicated to play a role during plant defences (Hewezi et al., 2016; Yaeno and Iba, 2008). Hewezi et al. (2016) were able to show that depletion of miR827 as well as overexpression of NLA leads to an over-accumulation of SA signalling markers (*PR1, PR5*) as well as JA signalling markers (*PR4, PDF1.2*) with *PR1* showing the strongest up-regulation.

These data suggest that in addition to its role during phosphate homeostasis miR827 and its target NLA might play a role during *A. brassicicola* infection.

Results

miR827 and its role in response to *Alternaria brassicicola*

To test whether miR827 plays a role during *A. brassicicola* infection, we first analysed mature miR827 levels after *A. brassicicola* treatment to verify our small RNA library data (see Figure 21). miR827 was clearly induced 48 h after inoculation with *A. brassicicola*. Even though the miR827 target, *NLA*, showed down-regulation during *A. brassicicola* treatment, this down-regulation was also evident in mock treated plants.

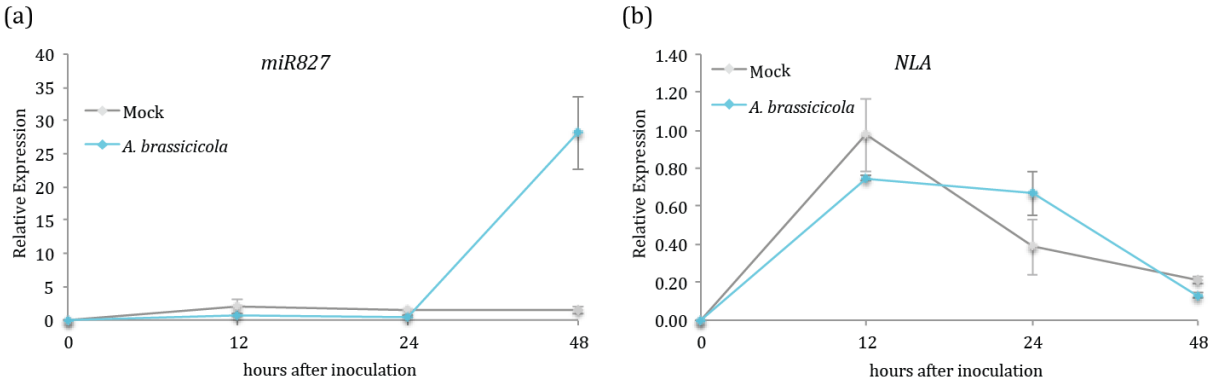


Figure 21: miR827 is severely up-regulation by *A. brassicicola* infection. Relative expression level of mature *miR827* of hydroponically grown WT plants after inoculation with mock solution or spores of *A. brassicicola* are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates.

Is the miR827 up-regulation upon pathogen attack a general mechanism?

To analyse whether the regulation of miR827 is specific to *A. brassicicola* or a general response to any fungi, we analysed miR827 induction after chitin treatment. Chitin is a component of fungal cells as well as insect exoskeletons and therefore is often used as elicitor to induce PTI that is not species specific (Wan et al., 2008).

A. thaliana WT plants were treated with C7, a chitin oligomer. Afterwards *MIR827* transcripts and mature miR827 were analysed (see Figure 22). Even though pri-miR827 is significantly up-regulated after 2 and 12 h, it is significantly down-regulated after 24h.

In concert with this finding the mature miR827 is also significantly down-regulated after 24h.

Since miR163 and miR399 were also candidates in our small RNA library, we also analysed transcript levels of *MIR163*, mature miR163, *MIR399B,C* and mature miR399a after C7 treatment. Even though previously obtained data in our lab (Rausch, 2017 unpublished) showed that miR163 is also up-regulated after *A. brassicicola* treatment, we observed significant down-regulation of *MIR163* after 48 h of C7 treatment. The mature miR163 showed the same trend, but none of the differences that we observed was significant. On the contrary neither *MIR399B,C* transcript levels nor mature miR399a levels were significantly altered by C7 treatment. In addition, transcript levels of *FRK1* and *WRKY53* were used as a control for C7 treatment and both transcripts showed the characteristic up-regulation.

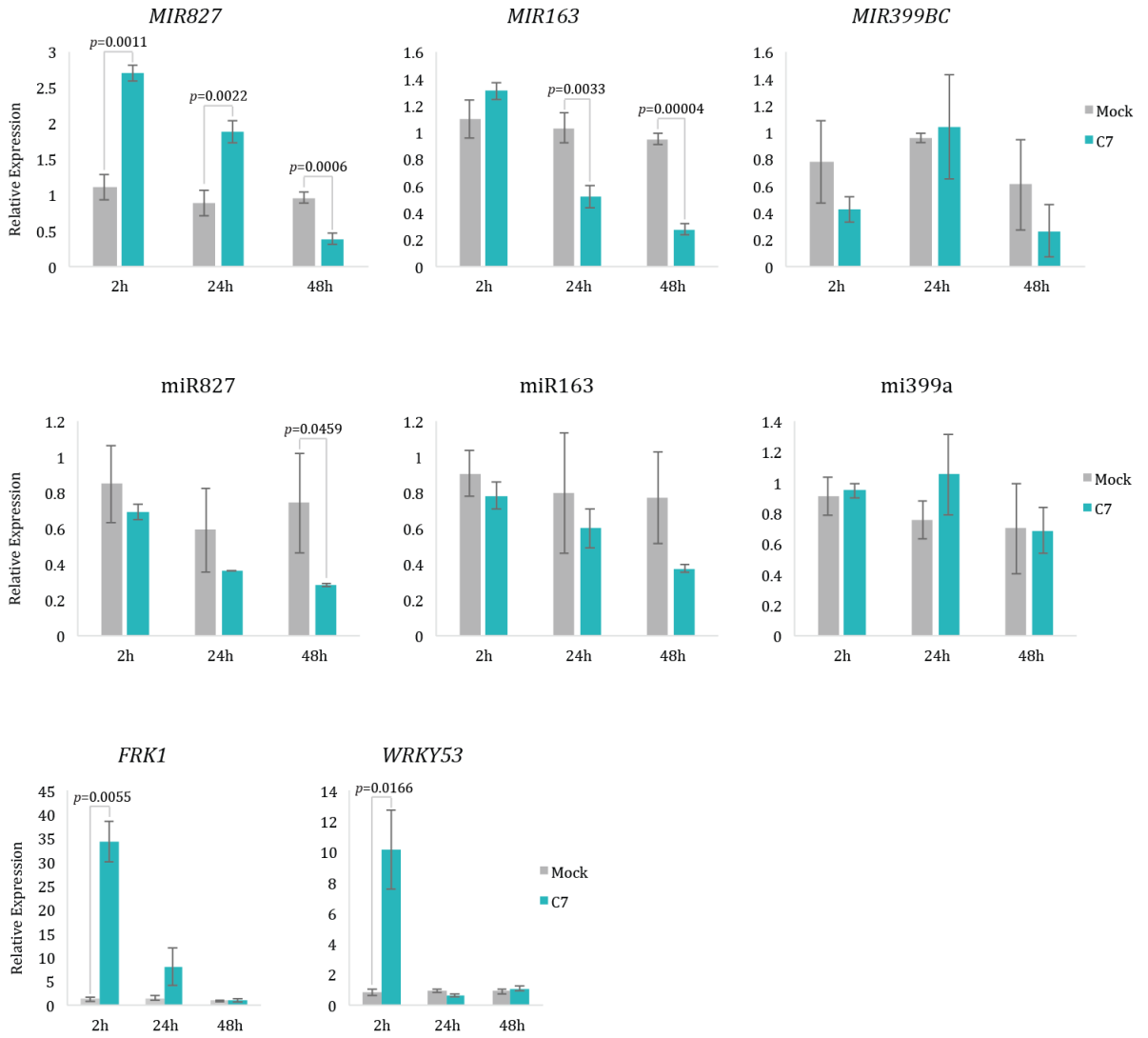


Figure 22: Chitin treatment can also induce mirR827 transcription. Relative expression levels of mature miR827 of sterile grown WT plants after treatment with mock solution or chitin (C7) are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates, statistical significance was tested via student's t-test, significant *p*-values are indicated.

Is the miR827 up-regulation a conserved mechanism in plants?

MiRNAs and their function in general are very conserved among plant species (Cui et al., 2017). Therefore, we asked the question if miRNAs are also up-regulated in *Nicotiana benthamiana* after pathogen treatment. Unfortunately, we were not able to establish *Alternaria brassicicola* treatment on *N. benthamiana* leaves. Even though previously obtained data indicated that *Botrytis cinerea* treatment at least in Arabidopsis does not lead to elevated miR827 levels (Stephanie Rausch, unpublished data) we tested *Botrytis cinerea* on *N. benthamiana* leaves to investigate whether this might be different in other organisms. We observed successful infection of *N. benthamiana* by *B. cinerea* (data not

shown). However, in our miRNA expression analysis standard deviation was too strong to make final statements (see Figure 23), therefore further research will be necessary to finally answer this question.

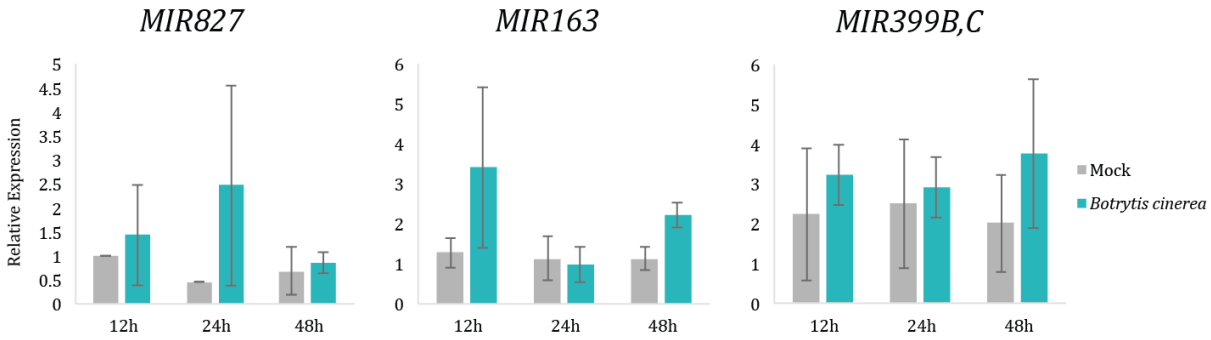


Figure 23: pri-*miR827* can also be induced by *Botrytis cinerea* treatment in *Nicotiana benthamiana*. Relative expression levels of pri-*miR827*, pri-*miR163,* and pri–*miR399b,c* of sterile grown WT plants that were inoculated with spore of *B. cinerea* are shown. Samples were harvested at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates, statistical significance was tested via student's t-test.

Investigation of the crosstalk between phosphate homeostasis and *A. brassicicola* response

miR827 and its target have been shown to play an important role during phosphate homeostasis. Therefore we analysed the disease phenotype of WT and *miR827* mutant plants during *A. brassicicola* infection and whether this phenotype can be influenced by different phosphate (Pi) conditions (Liu et al., 2014). We infected Arabidopsis WT and *miR827* mutant plants with *A. brassicicola* under low and high Pi conditions (see Figure 24). In two independent experiments we could show that the *miR827* mutant is more susceptible to *A. brassicicola* infection than the WT (chi-squared test, p-value= 0.049871206 and 0.029137937 respectively, see Figure 24. Even though the *miR827* mutants still showed stronger susceptibility to *A. brassicicola* infection under low Pi conditions, the phenotype was less severe or comparable to high Pi conditions.

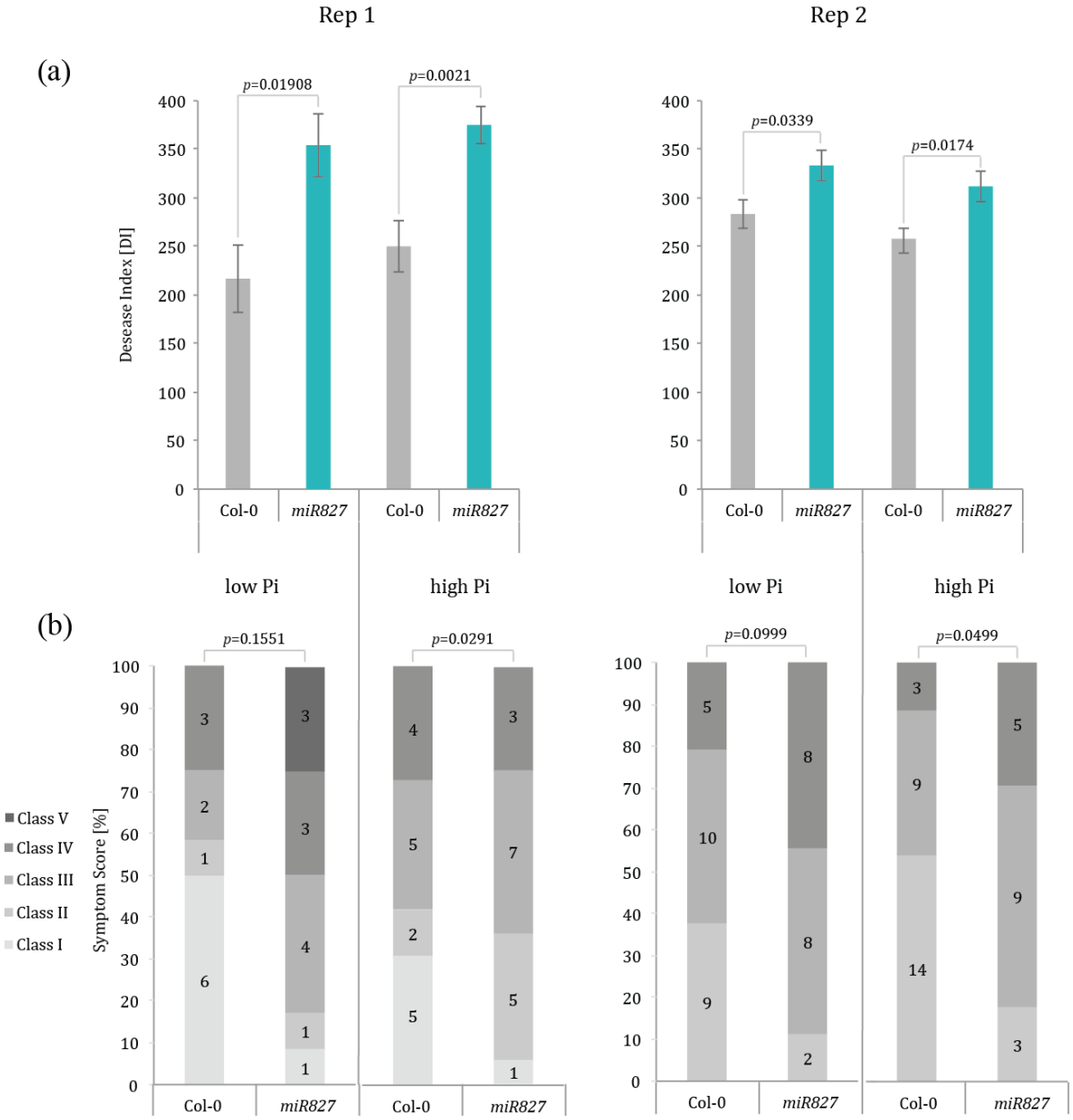


Figure 24: miR827 mutants during double treatments. (a) Disease index of Arabidopsis WT and *miR827* mutant plants 9 days after inoculation with spores of *A. brassicicola.* Two independent biological replicates are shown, statistical significance was evaluated via student's t-test, *p*-values are indicated. (b) Classification of *A. brassicicola* infected WT and *miR827* mutant plants 9 days after infection. Two independent biological replicates are shown, statistical significance was evaluated via chi square test, *p*-values are indicated.

To understand the role of miR827 during the crosstalk between Pi homeostasis and *A. brassicicola* infection in more detail we analysed miR827 levels during double treatments (see Figure 25). As known from published data we saw a general up-regulation of mature miR827 during phosphate starvation 48 h after the second treatment (equals 4 days of phosphate starvation in total). This increase is even more severe in *A. brassicicola* treated WT Col-0 plants. Initially it seems that miR827 expression is stronger induced under low phosphate conditions (WT *A. brassicicola*

treated low compared to high phosphate, 12 h and 24 h). After 48 h, miR827 induction is much stronger under high phosphate conditions. No notable change in mir827 expression was observed in the *miR827* mutant plants.

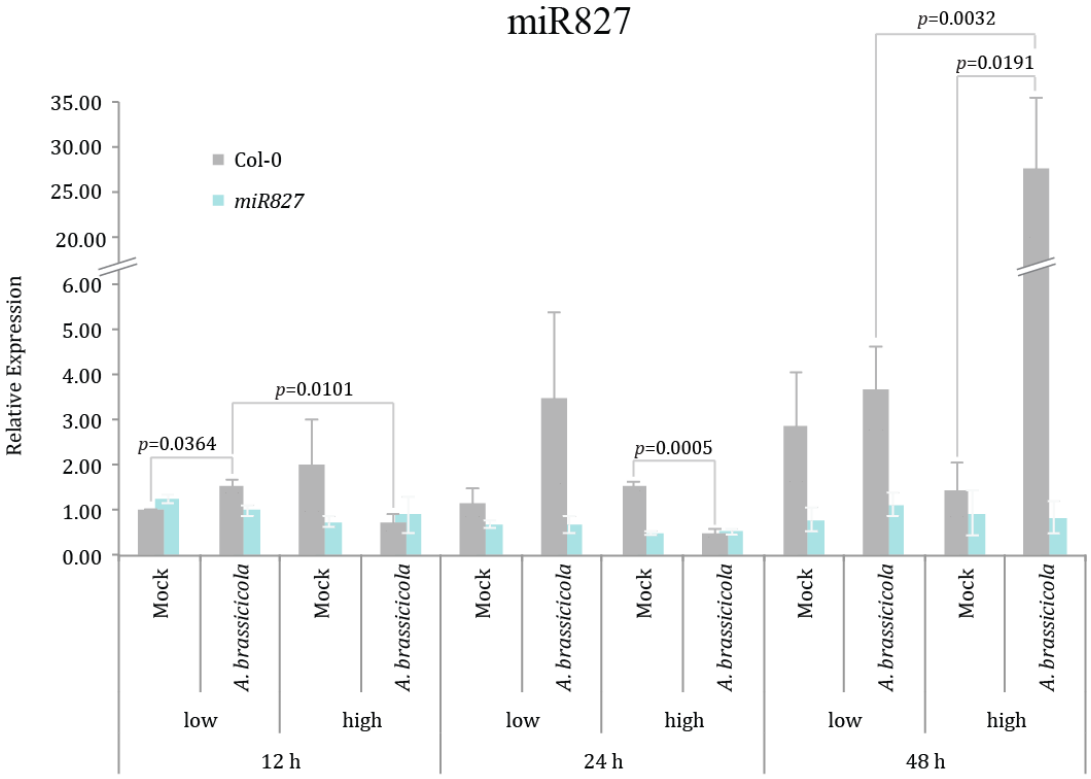


Figure 25: miR827 expression during double treatments. Relative expression level of mature miR827 of WT and *miR827* plants grown hydroponically under low Pi (10 µM Pi) or high Pi (1 mM Pi) conditions for 48 h before *A. brassicicola treatment* are shown. Samples were harvested after inoculation with mock solution or spores of *A. brassicicola* at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates, statistical significance was tested via student's t-test, significant *p*-values are indicated.

Since miR827 up-regulation should lead to down-regulation of its target mRNA *NLA* we wanted to analyse *NLA* transcript levels during double treatment in WT and *miR827* mutants (see Figure 26). In general, we could observe down-regulation of *NLA* transcript levels, which is most severe in WT under high Pi conditions during *A. brassicicola* treatment after 48h. This observation is in accordance with the miR827 expression analysis. However, a general down-regulation of *NLA* transcript levels can be observed over time, including the samples taken from *miR827* mutant plants. This questions if these results are dependable. There seems to be a general down-regulation of *NLA* transcripts independent of miR827 regulation. This down-regulation might be caused by growth or light conditions, affecting all plants in a similar manner.

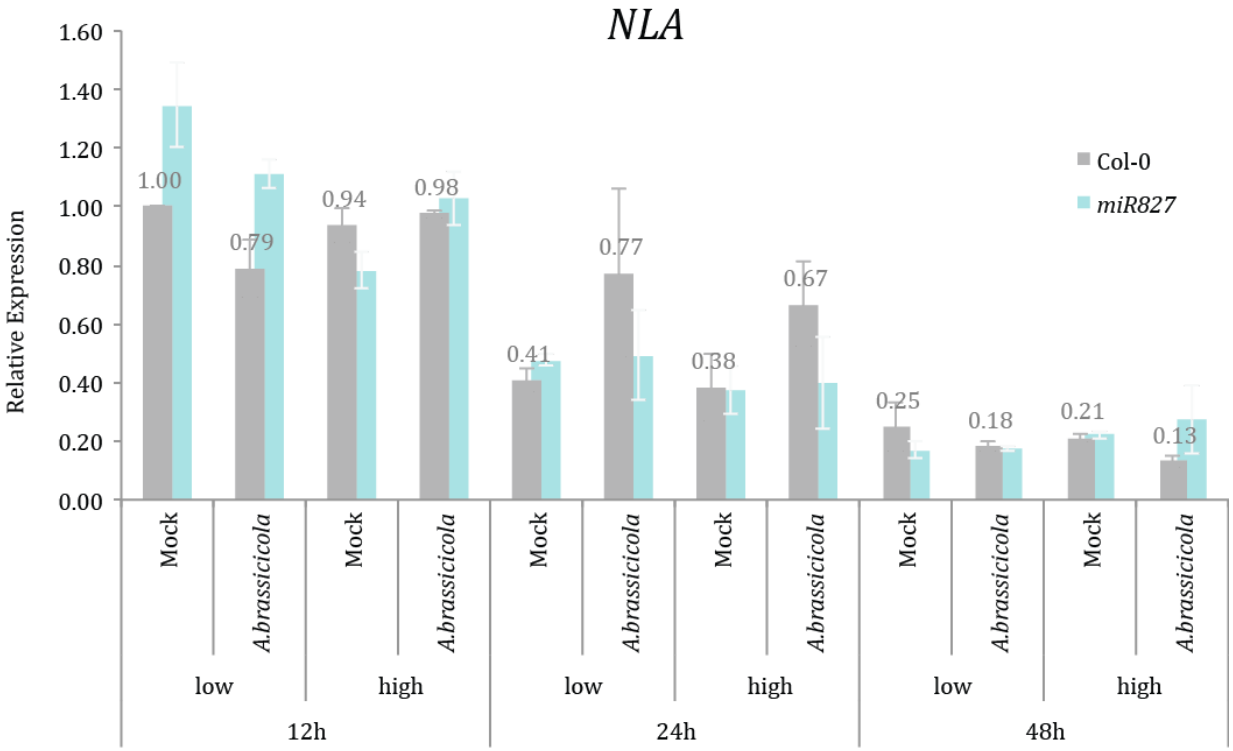


Figure 26: *NLA* expression during double treatment. Relative expression level of *NLA* of WT and *miR827* plants grown hydroponically under low Pi (10 µM Pi) or high Pi (1 mM Pi) conditions for 48 h before *A. brassicicola treatment* are shown. Samples were harvested after inoculation with mock solution or spores of *A. brassicicola* at indicated time points and relative expression values were normalized to *PP2A*. Error bars indicate standard deviation of three biological replicates.

Discussion

Small RNAs and their role in pathogen response have been heavily discussed and have been established as new contributors to plant immunity (Fei et al., 2016; Islam et al., 2018; Weiberg and Jin, 2015). Recent data show that miRNAs are involved in the defence against many fungal pathogens such as *Plectosphaerella cucumerina, Botrytis cinerea*, *Fusarium oxysporum* and *Colletotrichum higginsianum.* (Baldrich et al., 2014; Salvador-Guirao et al., 2017; Soto-Suarez et al., 2017). However, these are few examples and the role of miRNAs during defence against the fungal pathogens is in dire need of further investigation.

Therefore, we analysed the role of Arabidopsis miR827 in response to *A. brassicicola* infection. We were able to show that miR827 is up-regulated during *A. brassicicola* infection and that *miR827* mutants might show increased susceptibility towards this fungal pathogen. We could further demonstrate that the induction of miR827 is most likely not mediated via chitin-dependent signalling and according to Li et al. (2010b) miR827 is also not up- or down-regulated by flagellin treatment, indicating that this might be a mechanism specifically regulated during infection by *A. brassicicola.* This is in accordance with our findings that miR827 is also not up- or down-regulated by infection with *B. cinerea* in Arabidopsis (Rausch, 2017 unpublished data).

Furthermore, these findings raise the question whether miR827 expression is influenced during insect infestation. In general JA-mediated signalling that is induced by necrotrophic fungi is also induced by insect infestation. After initial induction of JA hormone production, the downstream signalling cascades that are induced differ between necrotrophic fungi and insect infestation. While necrotrophic fungi mainly activate a signalling pathway including ERF as a main node, insect infestation mainly activates a signalling pathway involving MYC. Both pathways have antagonistic effects on each other. To find out whether miR827 can also be induced by insect infestation, we produced very preliminary data. We analysed pri-miRNA transcript levels of *MIR827*, *MIR163* and *MIR399B,C* in *A. thaliana* WT Col-0 plants infected with two different caterpillar species, *Pieris rapae* and *Mamestra brassicae* (our unpublished data). *Pieris rapae* is a generalist, infecting a range of different plant species, while *Mamestra brassicae* is a specialist mainly infecting Brassicaceae. In both treatments pri-miR827 seems down-regulated. The fact that miR827 expression shows a similar pattern in two independent herbivore treatments is interesting, even though, it must be clearly stated

that this experiment is very preliminary since it has only been done once and therefore have to be repeated.

Previously published data show that plants overexpressing miR827 have significantly lower levels of *PR4, PR5* and *PDF1.2* (Hewezi et al., 2016). In theory these findings should indicate that infection with a necrotrophic pathogen in *miR827* mutants leads to lower resistance. Thereby contradicting our observed *A. brassicicola* phenotype. Interestingly in the same publication they show that overexpression of *NLA* as well as overexpression of a miR827 resistant *NLA* transcript have different, partially opposite, effects on expression profiles of various PR genes, resulting in different expression patterns. This indicates that the miR827-NLA regulatory module might be much more complex than the so far established gene regulation models of plant immunity.

The only rather consistent finding is that *PR1* seems to be induced when *NLA* expression levels are higher (Hewezi et al., 2016). It is known that higher levels of *PR1* in general antagonise JA-mediated signalling and therefore this might explain the reduced resistance towards *A. brassicicola*. Surprisingly, our obtained *NLA* expression data cannot support this model. Our finding that *NLA* expression was overall down-regulated in a miR827-independent manner and, more importantly, was not up-regulated in *miR827* mutants indicates that NLA might be regulated by additional factors that are influenced by e.g. growth or light conditions. It should also be considered that *NLA* expression is spatially regulated (Hewezi et al., 2016). In non-infected plants under Pi-sufficient conditions *NLA* expression could only be detected in cotyledons, root caps, vascular root tissues and in lateral root, not in leaves. This suggests that our obtained *NLA* expression data might be misleading in general since we analysed whole seedlings. To investigate our hypothesis, it will be interesting to analyse *NLA* transcripts in root tissues only. Some of our results could be also explained by miR827 having several targets, which is actually common among miRNAs. One possible alternative target of miR827 is the SPX domain-containing protein VACUOLAR PHOSPHATE TRANSPORTER 1 (Hsieh et al., 2009). Under normal phosphate conditions VPT1 is important for the transport and thereby storage of P_i in the vacuole. If phosphate levels reach a toxic concentration, VPT1 is important for the detoxification process (Liu et al., 2015). Previously we could show that during Alternaria treatment *VPT1* transcripts are induced (Rausch, 2017 unpublished data). This indicates that VPT1 might be important during pathogen response. Though it still needs to be proven, if VPT1 is in fact a miR827

target. So far there is no indication in the literature that VPT1 is involved in pathogen response. Therefore, much more research will be needed to investigate this hypothesis and it will be interesting to analyse *nla* as well as *vpt1* mutant plants during Alternaria infection.

Apart from the classical SA- JA-signalling pathways in plant immunity other independent pathways that influence resistance towards pathogens have been reported. (Balmer et al., 2017) analysed the metabolome of wild-type plants and the miRNA biogenesis mutants, *hen1-1* and *hyl1-2* after *Colletotrichum higginsianum* infection. *Colletotrichum higginsianum* is also a fungus that initially shows a biotrophic lifestyle and then switches to necrotrophy (O'Connell et al., 2012). Results showed that in wild-type plants the putative antifungal phytoalexin, camalexin, was strongly induced after *C. higginsianum* infection and that this infection was slightly less severe in *hyl1-2* mutants and much less severe in *hen1-1* mutants (Balmer et al., 2017). Interestingly, the *pad3-1* mutant, deficient in camalexin biosynthesis, is specifically more susceptible towards *A. brassicicola*, not towards the bacterium *Pseudomonas syringae*, the biotrophic fungi *Peronospora parasitica* and *Erysiphe orontii* and also not towards the necrotrophic fungus *Botrytis cinerea* (Thomma et al., 1999). This very much resembles the phenotype that we observe for the *miR827* mutant. Additionally, it has been shown that overexpression of miR393 leads to increased resistance towards biotrophic pathogens and that miR393 negatively regulates camalexin biosynthesis (Robert‐Seilaniantz et al., 2011). Our results indicate that apart from its putative role in mediating the cross-talk between SA and JA signalling, miR827 might also be a part of the camalexin regulatory network.

We also investigated whether *A. brassicicola* infection is influenced by Pi homeostasis in WT Col-0 and *mir827* mutant plants. We had hypothesised that infection during low Pi conditions might be less severe, since miR827 is already induced under these conditions, leaving the plant in a 'primed state'. In general, we could show that miR827 is induced by low Pi conditions as was published (Hsieh et al., 2009) and that induction was strongest 48 h after *A. brassicicola* infection under high Pi conditions. Indicating that plants need sufficient Pi supply to be able to respond properly to pathogen attack. Unfortunately, the phenotypic results were not consistent. The *miR827* mutant was reproducibly more resistant under high Pi conditions as compared to low Pi conditions, WT plants on the other hand showed sometimes lower and sometimes stronger

resistance towards *A. brassicicola* under low and high Pi conditions. In general, both results would not have been surprising because the host plant as well as the pathogen, *A. brassicicola,* need sufficient Pi supply for survival. Unfortunately, we cannot make a final conclusion concerning the link between Pi homeostasis and miR827 mediated response towards *A. brassicicola*.

In our small RNA library, we also found miR399 to be up-regulated during *A. brassicicola* infection. miR399 is also known to be up-regulated during Pi deficiency (Bari et al., 2006; Fujii et al., 2005; Liu et al., 2014) and has also been linked to pathogen response in citrus plants (Zhao et al., 2013). However, miR399 did not seem responsive to any treatment in our obtained expression data. This questions its role in response to *A. brassicicola* in Arabidopsis and further research will be needed.

Taken together we propose a model (see Figure 27) in which infection by *Alternaria brassicicola* leads to up-regulation of miR827 and down-regulation of its target *NLA* only in specific tissues, e.g. in roots. This in turn prevents up-regulation of *PR1* and thereby negatively regulates SA-mediated signalling during JA-mediated signalling. In *miR827* mutants, *PR1* expression is higher, thereby negatively regulating JA-mediated defence response towards *A. brassicicola*. Therefore, miR827 and its target NLA might be another module fine-tuning the antagonistic cross-talk between SA- and JA-mediated signalling. Additionally, miR827 induction might induce camalexin biosynthesis, which synergistically to JA-mediated responses enhances resistance towards *A. brassicicola* infection.

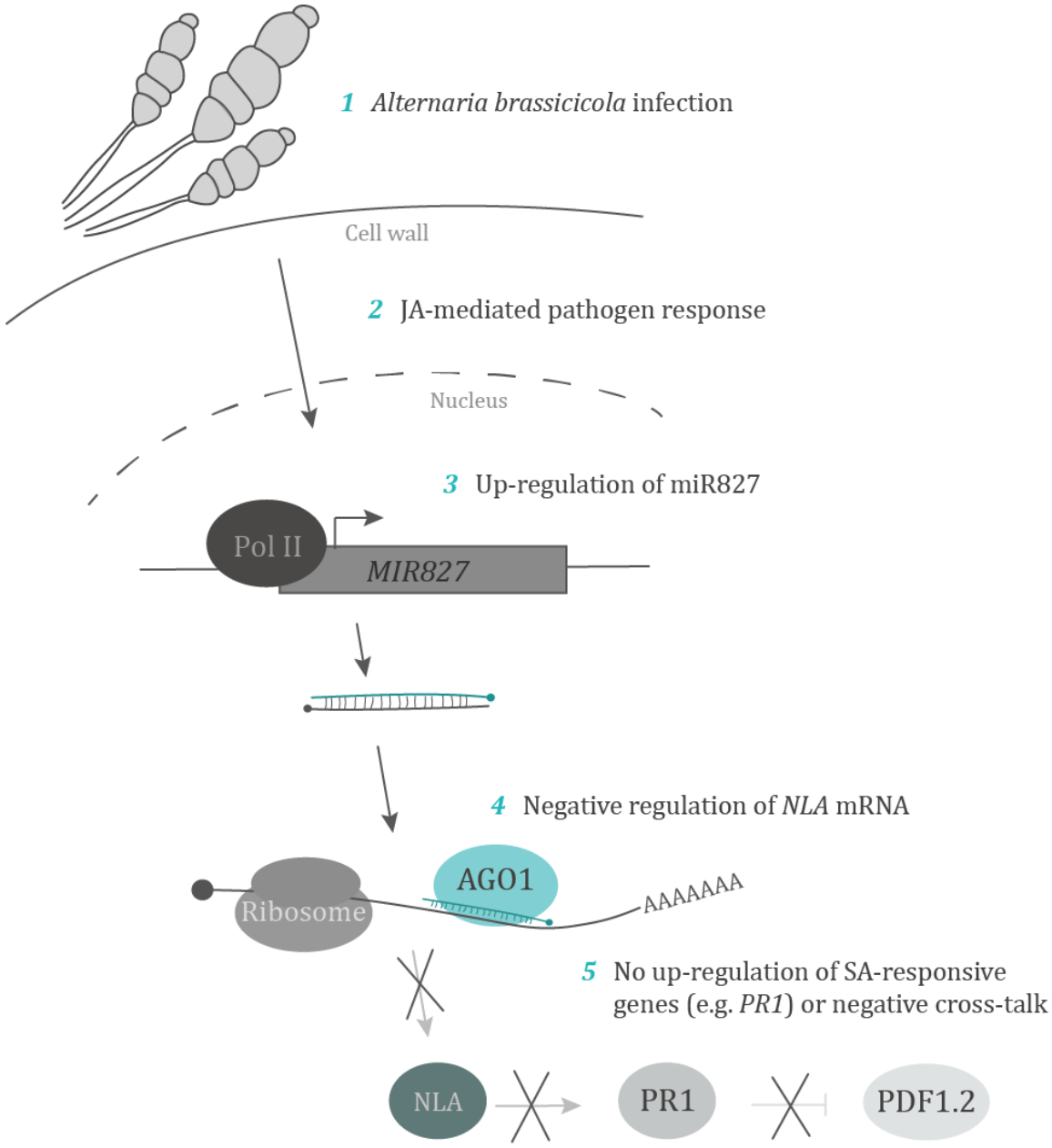


Figure 27: Mode of action of miR827 during *Alternaria brassicicola* infection. ***1*** Infection of *Arabidopsis thaliana* by *Alternaria brassicicola* spores. ***2*** Induction JA-mediated pathogens responses. ***3*** Strong up-regulation of miR827 and ***4*** post-transcriptional down-regulation of *NLA* mRNA transcripts. ***5*** Prevention of negative cross talk.

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APPENDIX

Curriculum vitae

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Kaster, M. and S. Laubinger (2016). "Determining Nucleosome Position at Individual Loci After Biotic Stress Using MNase-qPCR." Methods Mol Biol 1398: 357-372.