Molecular cues of Cuscuta and their effects in host plants

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1 Introduction

1.1 Plant pathogens

Green autotrophic plants obtain their carbohydrates through photosynthesis and are therefore primary producers of energy-dense macromolecules. Autotrophic plants form therefore a rich nutrient source for heterotrophic organisms to feed on. As sessile organisms plants need to fend off these attackers, including among others insects, nematodes, fungi, bacteria and oomycetes, but also parasitic plants. Plants have developed multiple mechanisms to recognize and repel different pests and pathogens. Immune receptors play an important part in those interactions for the survival of the potential host. They usually recognize microbe-associated molecular patterns (MAMPs) which are highly conserved microbial structures like bacterial flagellin or fungal chitin or pathogen-associated molecular patterns (PAMPs). Furthermore, phytohormones can take on an important role during those stress responses, including the stress hormones jasmonate, salicylic acid and ethylene.

1.1.1 Parasitic Plants

An especially interesting host parasite interaction is the one between plant and parasitic plant. Because of the high similarity of both systems it is harder to identify a reliable molecular pattern for the host's immune system in comparison to other pests like fungi or insects. The parasitic lifestyle developed at least 12 times independently during the evolution of flowering plants. This heterotrophic lifestyle benefits from water and nutrients provided by the host. Approximately 1% of dicotyledonous angiosperms, which comprise of approximately 4500 species belonging to 28 families, live a parasitic life (Westwood *et al.*, 2010; Nickrent *et al.*, 2020). Parasitic plants can be classified by the site of infection at the host plant and by degree of host dependency. Hemiparasites usually obtain water and nutrients from their host but retained photosynthetic abilities to produce carbohydrates. By contrast, holoparasites lost their photosynthetic capabilities completely. A differentiation can also be made between facultative and obligate parasites, respectively. Facultative parasites can complete their lifecycle without a host whereas obligate parasites cannot. Furthermore, parasitic plants can be classified as stem and root parasites. Agronomical important members of root parasites are the Orobanchaceae with

Orobanche spp. as obligate root holoparasite and *Striga* spp. as hemiparasite. *Triphysaria* spp. and *Phtheirospermum* spp., which also belong to the Orobanchaceae, are facultative root hemiparasites. Two examples for stem parasites are mistletoes (*Viscum* spp.) as hemiparasites and dodder (*Cuscuta* spp.) as holoparasite (Heide-Jorgensen, 2013).

All of those parasitic plants display a great danger for the worldwide food production. Especially, *Striga* is one of the main biotic constraints on the African continent (Spallek *et al.*, 2013). There it is responsible for 68-79% of maize losses (Kim *et al.*, 2002). Another member of the *Orobanchaceae* called *Phelipanche ramosa* can infect crops like carrot, potato, tomato and tobacco (Buschmann *et al.*, 2005). The shoot parasite *Cuscuta* spp. is distributed worldwide representing another big threat to crop plants (Parker & Riches, 1993) with the highest species diversity located in America (Navas, 1979). In Europe, *C. europaea* is the most dominant species of the five native members of the genus and exposes the greatest threat (Mabberley, 1997). On a global scale *C. campestris* is the most economical devastating species member affecting 25 crop species in 55 countries (Lanini, 2005). Figure 1 depicts examples of such infected crops.

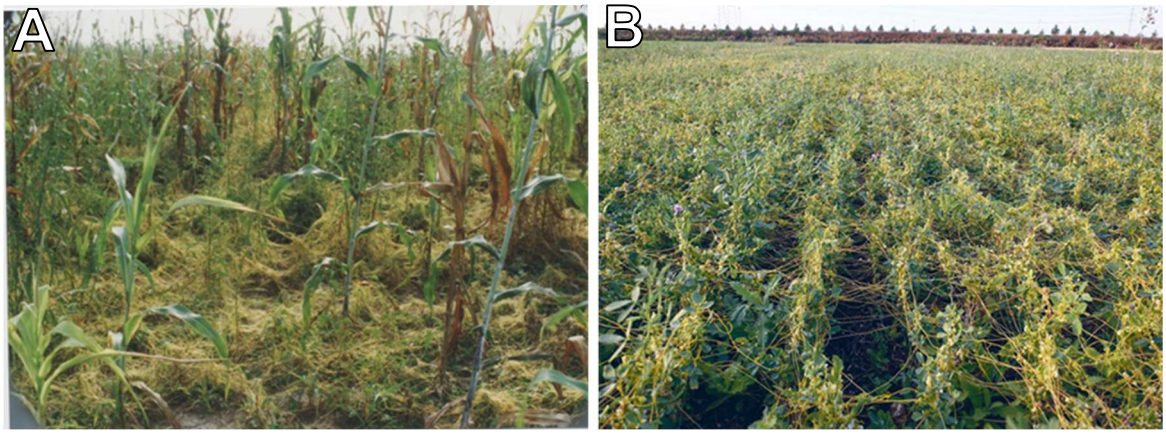


Figure 1: Crop infection through *Cuscuta campestris*. A, Infection of a sorghum maize field (© Nuhu Gworgwor). B, Infection of a clover field („Die Kleeseide ist wieder auf dem Vormarsch“, 21.08.2015, bauernzeitung.at).

Parasitic plants developed several strategies to maximize their chances of successful host infection. These include the formation of small seeds which can easily be spread by wind and the endurance of a long dormancy period until a host is recognized. The most known signals for germination for a lot of parasitic plants e.g. *Striga* spp. are the host-derived strigolactones. Those are naturally occurring carotenoid derivatives and are a class of plant hormones with diverse functions (Faizan *et al.*, 2020). For the

host plant strigolactones are important for plant architecture regulation and interaction with symbiotic arbuscular mycorrhizal fungi (Akiyama *et al.*, 2005; Gomez-Roldan *et al.*, 2008).

1.1.2 Cuscuta reflexa

The genus *Cuscuta* belongs to the plant family of Convolvulaceae and is one of the most ecologically and economically important kind of parasitic plants (Mishra, 2009). *Cuscuta spp.* is also known as dodder, devil's hair, witch's hair, love vine and amarbel. These plants usually have a yellow to orange colouring and lack roots or expanded leaves. As holoparasites they require other plants to survive and reproduce (Runyon *et al.*, 2010). The genus of *Cuscuta* comprises of around 200 species. They feed on a large number of dicotyledones, among them important crop plants like tomato, tobacco and forage legumes (Kaiser *et al.*, 2015). Therefore, dodder is a nonspecific parasite only avoiding grasses and monocotyledonous weeds (Lanini & Kogan, 2005).

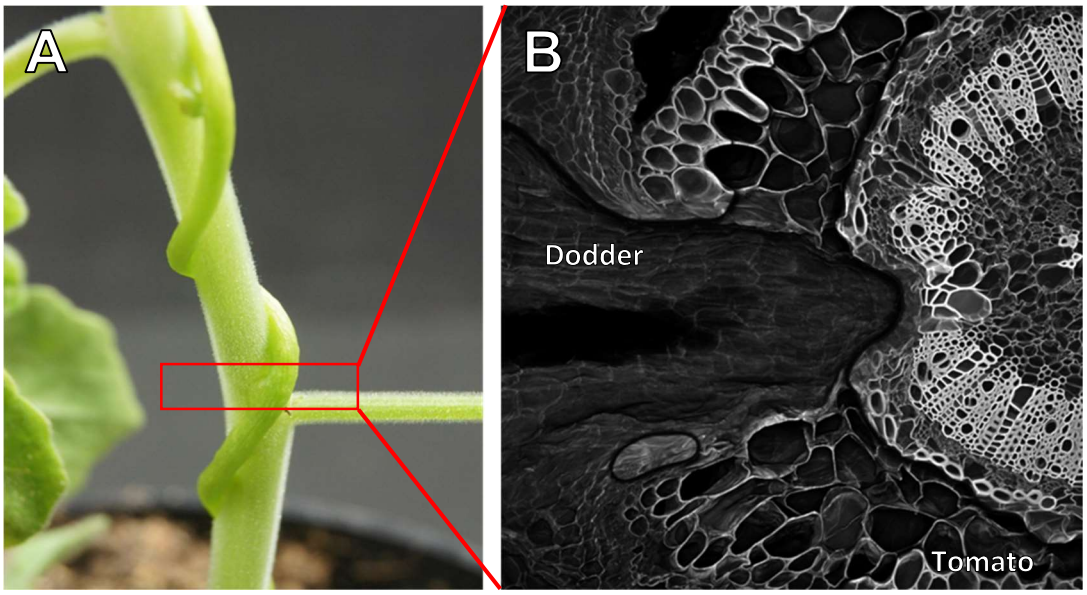


Figure 2: *Cuscuta reflexa* and the susceptible *Solanum pennellii*. *C. reflexa* winding counter clockwise around its host stem (A) documented macroscopically. The parasite penetrating the tissue to access the hosts vasculature is shown microscopically as stem crosssection of propidiumiodid stained tissue (B).

Cuscuta lacks obvious chlorophyll in most cases but conserved the necessary genes for photosynthesis, most likely to synthesize lipids for seed production (McNeal *et al.*, 2007). A *C. reflexa* plant parasitizing a susceptible *S. pennellii* host is displayed in Figure 2A. *Cuscuta* spp.'s success of host infection is greatly dependent on massive

seed production and a dormant period which can be as long as 10 or 20 years (Lanini & Kogan, 2005). The seed‘s dormancy is broken through microbial activity and weathering and happens at the temperature optimum (30°C for *Cuscuta campestris*) in a light-independent manner (Benvenuti *et al.*, 2005). *Cuscuta* flowering period begins in late spring and can continue until autumn e.g. *C. pentagona* needs 60 days for seed production (Lanini & Kogan, 2005).

The germinating *Cuscuta* seedlings rely on the energy stored in the seeds to grow towards the host possibly influenced through volatiles from their potential victim (Runyon et al., 2006). The location of the host is supported by the recognition of chemoattractants as described in Runyon *et al.*, 2006 were unknown plant volatiles seem to be responsible for directed growth. The growing seedling rotates counter-clockwise until host contact is achieved. Additionally, far red light conditions and tactile stimuli seem to be important for production of parasitic invasion organs. The application of both treatments can be utilized to induce this organ formation without a host’s presence (Tada *et al.*, 1996).

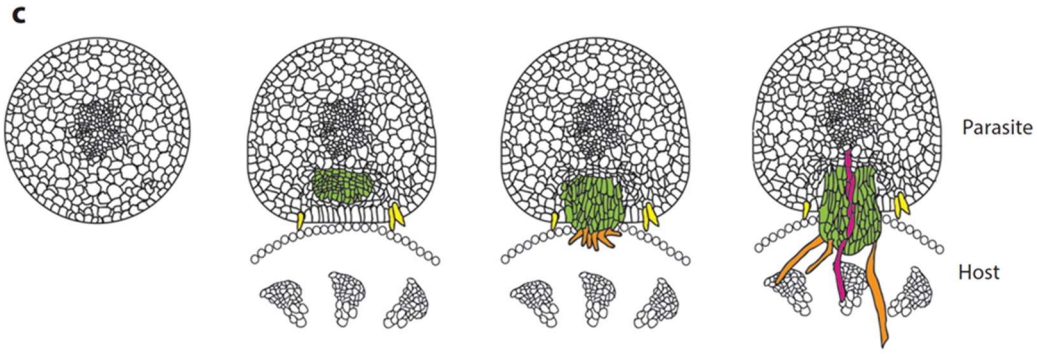


Figure 3: Scheme of haustorial development stages of *Cuscuta* spp. (modified after Yoshida *et al.*, 2016). Upon host contact a disc-like meristem (green) is produced by the parasite in the prehaustorium. The epidermal cells divide and produce trichome-like elongated cells (yellow). The meristem cells penetrate the host‘s tissue and produce elongated searching hyphae (orange), which grow towards the vascular tissue. As soon as the hyphae make contact with the vasculature a permanent connection is formed (pink).

To obtain nutrients and water from their hosts, parasitic plants form multicellular organs called haustoria emanating either from their shoots or roots (Yoshida *et al.*, 2016). The term ‘haustorium’ developed from the Latin ‘*haustor*’ or ‘*haurire*’ meaning ‘water drawer’, a term also used for fungal organs important for symbiosis. Even though fungi and parasitic plant haustoria share the same name they can be separated by two main characteristics. First, fungal haustoria develop from unicellular hyphae and second, fungal haustoria grow through host cells and surround

themselves with a host-derived extrahaustorial membrane whereas parasitic plant haustoria penetrate between cells (Yoshida *et al.*, 2016). Haustoria can be divided into two classes according to their formation site in either lateral or terminal haustoria. The terminal haustoria develop at root tips, transform those into a new organ and terminate the meristematic activity at the same time (Olivier *et al.*, 1991). Lateral haustoria are initiated at the side of a root or shoot without disturbing the meristematic activity (Tomilov *et al.*, 2005).

Haustoria from *Cuscuta* belong to the group of lateral haustoria and penetrate the host shoots. For this process epidermis cells enlarge and secrete a mixture of compounds mainly consisting of de-esterified pectins which stick to the host (Vaughn, 2002). Additionally, an arabinogalactan protein is produced which further enhances the adherence of the parasite (Albert *et al.*, 2006). The connection continues through elongation of haustorial cells. These cells are called searching hyphae and penetrate the host tissue utilizing mechanical pressure and enzymes (Nagar *et al.*, 1983) The searching hyphae extend to phloem and xylem cells and form connections through interspecific plasmodesmata (Vaughn, 2003). The hyphae then build a connection to the vasculature and withdraw water, sugars, amino acids and other nutrients (Birschwilks *et al.*, 2007). The haustorium formation is illustrated in Figure 3 and a microscopic view of a *Cuscuta* haustorium is shown in Figure 2B.

Cuscuta spp. were classified as phloem feeders and take up almost all their carbon from the phloem (Hibberd *et al.,* 2001). Upon developmental stress the transfer of xylem has also been observed (Christensen *et al.*, 2003). Besides the uptake of nutrients, viruses and RNA can be exchanged between species (Bennet, 1944; Westwood *et al.*, 2009).

Ten days after infection a connection between host plants and *Cuscuta* has been developed (Birschwilks *et al.*, 2007). After the first successful connection the parasite can grow up to 7 cm per day and extends and connects to neighbouring plants. Eventually, this leads to a dense blanket-like covering of the host plants (see Figure 4A).

A minor number of plants exhibit resistance to *C. reflexa* infection, including *S. lycopersicum* (see Figure 5). Resistance against *Cuscuta* species can be achieved either through anatomical barriers or not yet fully elucidated signalling pathways like in monocotyledons (Dawson et al., 1994) but also through a specific defence response like hypersensitive-like responses (HLRs; see Figure 5), phytoalexin

production and pathogenesis-related (PR) gene expression (Bringmann *et al.*, 1999; Borsics & Lados, 2002) dependent on the infected plant.

Figure 4: *Coleus blumeii* infected with *C. reflexa*. In the left picture the blanket like covering of the host *C. blumeii* by the pathogen *C. reflexa* is visible. The right picture shows the infection site in detail with swelling of the *Cuscuta* stem at the site of haustoria formation.

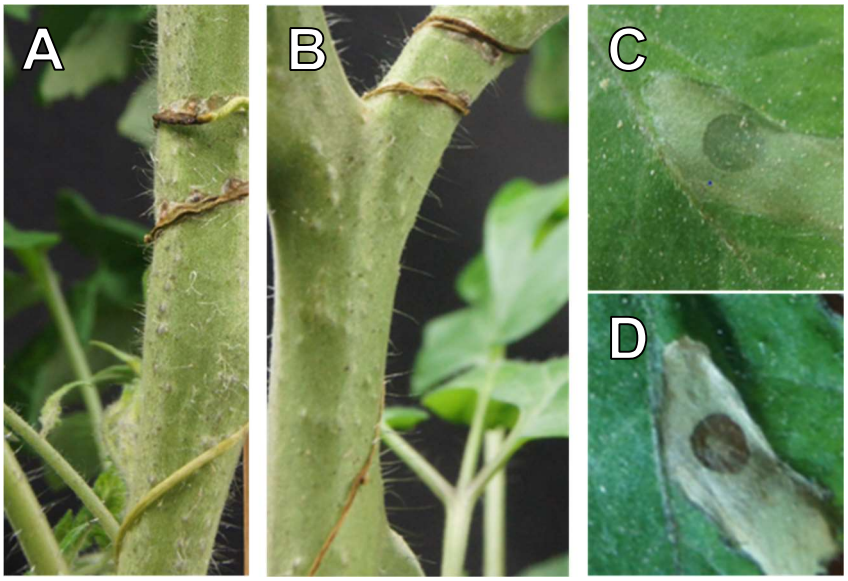


Figure 5: HLR of resistant *S. lycopersicum* against *C. reflexa*. Infection of a resistant plant leads to a HLR defence response and is followed by the starvation of the parasite (3 weeks after infection). A, B, The infiltration of crude extract from the parasite leads to similar symptoms of an early (3 days past infection (dpi; C) and late HLR (8 dpi; D).

HLR responses are best studied in resistant tomato cultivars and are accompanied by accumulation of phenolics and peroxidases at the attachment site which create a mechanical barrier (Ihl *et al.*, 1988) but similar reactions can also be observed in other plants like *Gossypium hirusutum* and *Hibiscus rosa-sinensis* which are able to fend the parasite off at their vasculature through a development of such a wounding

tissue (Capderon *et al.*, 1985). During the *C. reflexa* attachment process the cultivated tomato *S. lycopersicum* reacts therefore with cell elongation and division in hypoderm and cortex thus strengthening the cell wall through resembling wound suberin which prevents penetration and is visible as necrotic tissue spots (Kaiser *et al.*, 2015). The *Cuscuta* starves during a time period of 3 to 4 weeks. This reaction resembles an active resistance described for pathogens which is defined through localized cell death preventing pathogen spread (Ramachandran *et al.*, 2017) and is therefore a hypersensitive-like response (HLR). HLR differs from the well-described HR through a more complex pattern of events e.g. cells dividing first, followed by lignification and formation of several cell layers deep, hardened layer of woody tissue (Kaiser *et al.*, 2015). The effectiveness of this defence seems to be also dependent on age and trichomes of the tomato, at least for the defence against *C. pentagona* (Runyon *et al.*, 2010).

1.2 Signalling during host-pathogen interactions

1.2.1 Plant immune system

The challenges of plants through pests and pathogens are omnipresent. To fend off *Cuscuta* and other pests, plants developed a sophisticated immune system. The immune response can be expressed either constitutively or following a pathogen challenge.

Plants block the majority of invading pathogens through a non-host resistance. Physical barriers like waxy cuticles, rigid cell walls and antimicrobial secondary metabolites are essential for this first resistance trait. Usually if pathogens overcome these barriers the next steps in plant defence can be activated (Boller & Felix, 2009).

Because of the lack of mobile defender cells like in animals, plants relay on each cell exerting innate immunity with systemic signals being produced by infected cells. Also, the ability to remember previous infections is essential (Reimer-Michalski & Conrath, 2016).

This immune system consists of multiple layers, the MAMP-triggered (MTI) or innate immunity and the effector-triggered (ETI) or adaptive immunity. The first layer of the immune system often recognizes signals from parasites of diverse nature which usually are microbe- or pathogen- or damage-associated patterns (MAMPs, PAMPs, DAMPs, respectively) (Albert *et al.*, 2020). Those signals are usually recognized by

plant cell surface-anchored pattern recognition receptors (PRRs). PRRs often belong to the class of leucine-rich repeat (LRR)-receptor kinases (RKs) or –receptor proteins (RPs) depending on presence or absence of intracellular kinase domains (Albert *et al.*, 2019). The LRR part as ectodomain often has a high variance between the different PRRs to allow the detection of a wide variety of PAMPs/DAMPs (Saijo *et al.*, 2018). Furthermore, LRR-RLKs usually detect proteinacous ligands and induce downstream developmental and immunogenic processes, sometimes in a signalling complex with somatic embryogenesis receptors (SERKs; Chinchilla *et al.*, 2009). In contrast, LRR-RLPs usually show only genus-specific distribution unlike the often widespread LRR-RLKs in different plant species (Albert *et al*., 2010). RLPs often interact with LRR-RKs like Suppressor of BIR1-1/EVERSHED (SOBIR1/EVR; Liebrand *et al.* 2014) to provide further intracellular signalling (Gust & Felix, 2014).

Well studied MAMPs that induce plant immune responses are the flagellin peptide flg22, the elongation factor-Tu epitope elf18 and fungal chitin as well as bacterial peptidoglycan recognized by their corresponding PRRs FLAGELLIN-INSENSITIVE 2 (FLS2), elongation factor-Tu (EFR) and chitin elicitor receptor kinase 1 (CERK1), respectively (Bauer *et al.*, 2001; Zipfel *et al.*, 2006; Miya *et al.,* 2007; Willmann *et al.*; 2011).

Phtheirospermum japonicum, a member of the *Orobanchacea*, produces 2,6-dimethoxy-1,4-benzoquinone (DMBQ), a PAMP which can be recognized by the potential host plants CANNOT RESPOND TO DMBQ 1 (CARD1) receptor (Laohavisit *et al.*, 2020; summarized in Körner *et al.*, 2020). Quinones, a class of organic compounds derived from aromates (Goor *et al.*, 2019), are already well-known in the animal and bacteria kingdoms as inducers of the expression of cytoprotective genes (Hillion & Antelmann, 2015, Yamamoto *et al.,* 2018) and also in the plant kingdom they show an enhancement of immune responses. But often those PAMPs are also relevant for the pest. DMBQ for example seems to be important for the haustorium formation in *P. japonicum* (Laohavisit *et al.*, 2020).

The second layer of the immune system utilizes microbial effectors for recognition by resistance (R) proteins to initiate effector-triggered immunity (ETI). To counter MTI, pathogens produce effector proteins which shall repress immune responses. Those effectors can be recognized by the host by intracellular immune receptors called nucleotide binding (NB) leucine rich-repeat (LRR) receptor (NLR) proteins (Dangl & Jones, 2001). Those NLR proteins belong to a class of signal transduction adenosine

triphosphatases with numerous domains (STANDs). The conserved domain structure consists of three parts, a central NB domain flanked by a c-terminal LRR domain and a non-conserved n-terminal domain. Based on their n-terminal domain, NLRs can be separated into coiled coil (CC)-NLRs and Toll-interleukin-1 receptor (TIR)-NLRs also called CNLs and TNLs, respectively (Nishad *et al.*, 2020; see Figure 6). They act as switches depending on binding of ADP for autoinhibition countered through ATP binding for activation (Hu *et al.*, 2013) upon effector recognition at the c-terminal LRR domain (Krasileva *et al.*, 2010; Ravensdale *et al.*, 2012).

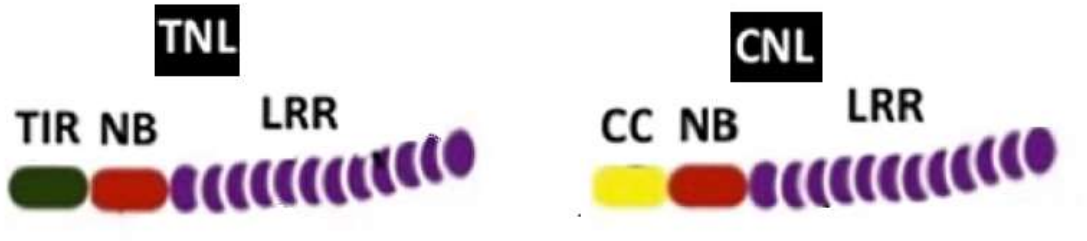


Figure 6: Structure scheme of NLR proteins (modified from Nishad *et al.*, 2020). Displayed are two categories of NLRs (TNLs and CNLs) both containing a c-terminal leucine rich-repeat (LRR) domain and a central nucleotide binding (NB) domain. The categories can be separated by the c-terminal domain which is either a coiled coil (CC) domain or a Toll-interleukin-1 receptor (TIR) domain.

Pathogen effectors can either be recognized directly or indirectly through either effector modified host target proteins called guardee or modified plant decoy proteins which resemble host target proteins (Chung *et al.*, 2014; Li *et al.*, 2014; Ntoukakis *et al.*, 2014). A recent example from the *Arabidopsis* coiled-coil-NLR protein HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) gives insight into the mechanism of plant NLR resistance. ZAR1 and the resistance-related kinase 1 (RKS1) form an oligomer where an uridylated PBS1-like protein 2 (PBL2) can bind. PBL2 gets uridylated through AvrAC, an effector of *Xanthmonas campestris pv. campestris.* This leads to ADP/ATP-dependent conformational change in ZAR1 which results in the creation of a wheel-like pentameric structure called resistosome, that affects plasma membrane integrity and ionic homeostasis (Wang *et al.*, 2019a, b).

The activation of either of the immunity layers triggers a cascade of complex signalling events like calcium influx, reactive oxygen species accumulation (ROS), mitogen-activated protein kinase (MAPK) phosphorylation cascades, cell wall alterations and defence gene expression, finally leading to the suppression of the pathogen (Windram & Denby, 2015; de Lorenzo *et al.*, 2018). The described complex immune responses are illustrated in Figure 7.

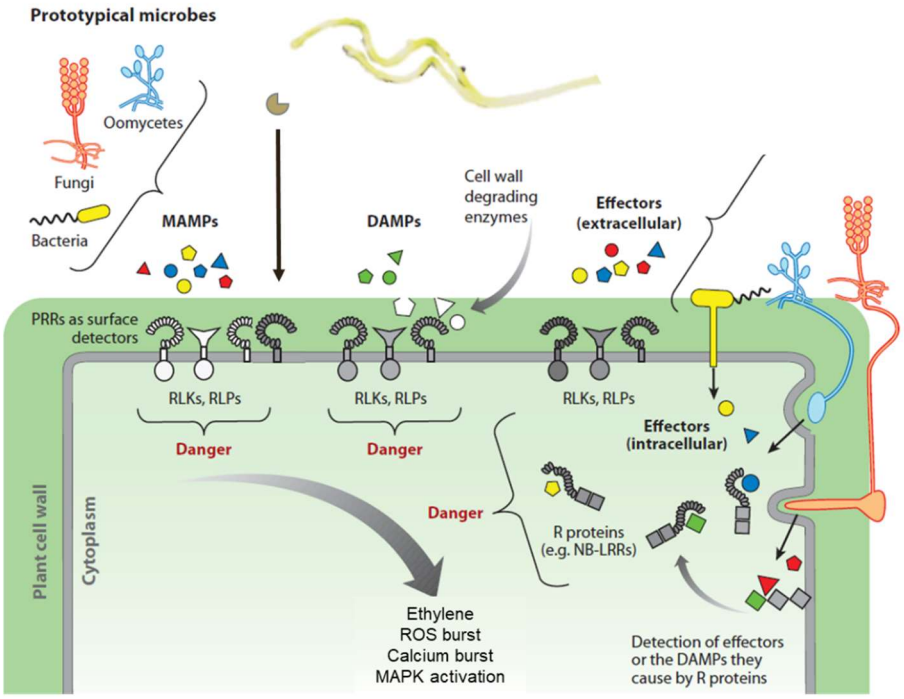


Figure 7: Plant immunity (modified after Boller & Felix, 2009). Microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) are perceived through pattern recognition receptors (PRRs). During coevolution pathogens develop effectors to suppress PRR effects which in turn lead to the development of new plant PRRs and resistance (R) proteins. If pathogen recognition by the plant is successful a complex signalling cascade is activated including ethylene production, reactive oxygen species (ROS) burst, calcium burst and MAPK activation. RLK = receptor-like kinase; RLP = receptor-like protein; NB-LRR = nucleotide binding-site-leucine-rich repeat.

1.2.2 LRR-RLPs and Cuscuta

Since plants possess the formerly described immune responses, they usually have the ability to resist most potential pathogens. Therefore, plant diseases are not common in the natural ecosystems since a breach of the immune system is rare (Ponce de Léon & Montesano, 2013). But once these first defence lines are overcome the pathogen can use the plants metabolites and reproduces, which often also leads to obvious disease symptoms (Jones & Takemoto, 2004). The pathogens developed effectors to suppress MTI (Pel & Pieterse, 2013) and even manipulate host proteins. Such manipulations can be found across the kingdoms e.g. effectors from phytoplasma bacteria can bind to Teosinte branched 1/Cincinnata/proliferating cell factor (TCP)/TFs which in turn inhibits synthesis of the phytohormone jasmonate (JA; Sugio *et al*., 2011) and oomycetes can secrete proteins supressing defence like *Phytophthora infestans* secreting AVR3a which stabilizes ubiquitin ligases and prevents HLR host cell death (Stassen & Van den Ackerveken, 2011).

An example for a parasite able to dodge the immune system of a great variety of plants is the parasitic plant *Cuscuta reflexa.*

Since most pathogens are microbes or arthropods with evolutionary distance to plants the latter can use PRRs to detect molecular patterns of the invaders (Hegenauer *et al.*, 2020). Parasitic plants like *Cuscuta* spp. usually go undetected by their host because of the limitation of the innate immune system to recognize parasitic plants.

Nevertheless, there are a few known examples of parasitic plant-plant incompatibility. A legume especially important in West and Central sub-Saharan Africa is cowpea (*Vigna unguiculata (L.) Walp.*), which exhibits resistance against the hemiparasitic angiosperm *Striga gesnerioides* through a CC-NBS-LRR encoded by the *RSG-301* gene (Li & Timko, 2009). Another important crop is the sunflower (*Helianthus spp.*) which is able to fend off the obligate plant parasite *Orobanche cumana* with the help of a LRR-RLK encoded by the *HaOr7* gene (Duriez *et al.*, 2019).

The cultivated tomato (*S. lycopersicum*) is one of the few plants that are able to detect the parasite *C. reflexa*. Upon infection the tomatoes innate immune response is activated resulting in ethylene production, ROS burst and HLR (Hegenauer *et al.*, 2016). An important signalling component to induce this resistance is the LRR-RLP *Cuscuta* receptor 1 (CuRe1) which detects the *C. reflexa* derived cell wall protein CrGRP (Hegenauer *et al.*, 2020). The close *Solanaceaen* relatives *N. tabacum, N. benthamiana* and *S. pennellii* do not exhibit these defence responses.

But also the resistance of *S. lycopersicum* against *C. reflexa* seems to be rather specific. Several other *Cuscuta* species like *C. campestris* and *C. pentagona* native to North America are able to infect tomato and induce yield losses in the US and India up to 50-75% (Lanini & Kogan, 2005; Mishra, 2009). Since *C. reflexa* is indigenous to Asia and tomato is native to South America it is possible that the lack of co-evolution leads to missing adaption of the parasite to its potential host and therefore a resistance in the latter one.

Additional to the PRRs and R proteins, phytohormones are often important during plant immune reactions in such plant-pathogen interactions either in a role of influencing the host to favour the parasite or to be influenced by the parasite to avoid immune or other unfavourable reactions. In resistant plants, e.g. during interaction of *C. pentagona* on tomato, typical defence related phytohormones like salicylic acid

and jasmonic acid can be induced maybe even connected to a HLR (Runyon *et al.*, 2010).

1.3 Phytohormones

The plant immune system activates a complex signalling network that is regulated by plant hormones in response to pathogen attacks (Wasternack *et al.*, 2007). Originally recognized as regulators of growth and development, phytohormones have been shown to act downstream of PTI and ETI activation or in other early recognition events of pathogens (Bari & Jones, 2009; Howe & Jander, 2008; Katagiri & Tsuda, 2010; Pieterse *et al.,* 2009). The major hormones involved in this defence are salicylic acid (SA) and jasmonic acid (JA) and its derivatives (Browse, 2009, Vlot *et al*., 2009). But also other hormones like ethylene (ET; Van Loon *et al*., 2006), abscisic acid (ABA; Ton *et al.,* 2009), gibberellins (GA; Navarro *et al.,* 2008), auxins (AUX; Kazan & Manners, 2009), cytokinins (CK; Walters & McRoberts, 2006) and brassinosteroids (Nakashita *et al.,* 2003) play a role as modulators in the plant immune signalling network. During parasitic interactions with a host plant, those hormones mediate a wide variety of plant responses, often in exchange for growth and developmental drawbacks (Walters & Heil, 2007). Additionally, through cross talk between hormones a complex network of further regulations is possible allowing a precise response to pathogen attack and a maximum of cost-efficiency (Jaillais & Chory, 2010; Mundy *et al*., 2006). An understanding of the function of phytohormones during plant pathogen interaction is essential to get deeper knowledge of the whole plant immune system.

1.3.1 General answer to parasitism

Phytohormones are key players in the defence of plants against a wide variety of parasites like oomycetes, fungi, herbivores, parasitic plants and others. The most important hormones in this process are SA and JA.

JA and its derivatives are lipid-derived compounds synthesized through the oxylipin pathway (Gfeller *et al*., 2010) from α-linolenic acid from membrane lipids (Wasternack, 2007). The JA pathway is known to be important for herbivore-induced responses. A scheme summarizing the JA pathway is shown and described in Figure 8.

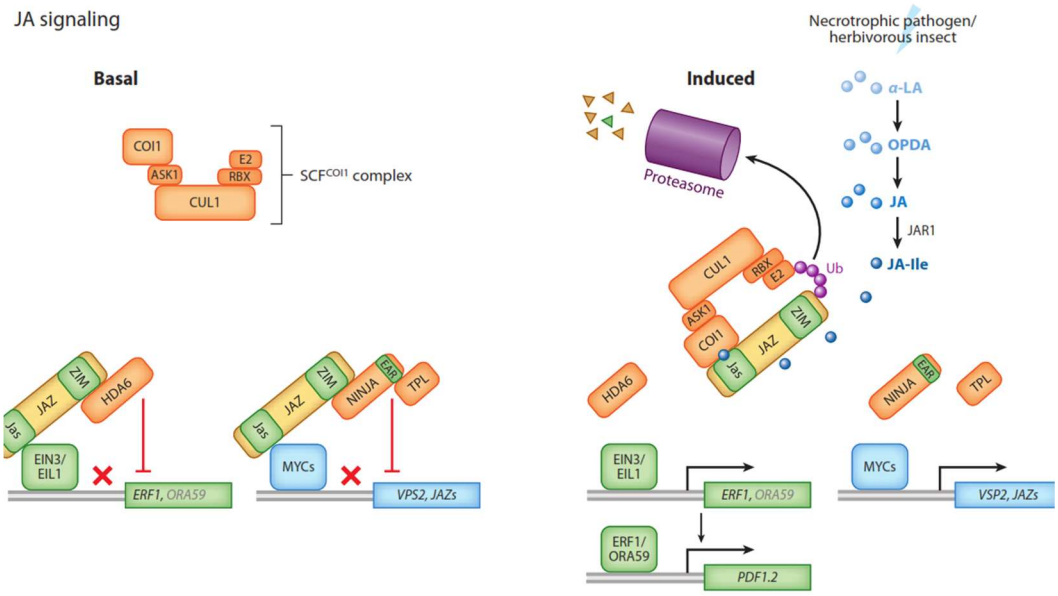


Figure 8: Jasmonate pathway (modified from Pieterse *et al.*, 2012). In JA absence the transcriptional repressor Jasmonate Zim (JAZ) binds to positive transcriptional regulators of basic helix-loop-helix leucine zipper proteins like MYC2, 3 and 4. The ZIM domain of JAZ proteins works here as binding point for the Novel Interactor Of JAZ (NINJA) which in turn can bind corepressors like Topless (TPL) through its ERF-Associated Amphiphilic Repression (EAR) motif eventually preventing JA pathway activation. The F-Box protein Coronatine Insensitive 1 (COI1) together with the JAZ domain transcriptional repressor protein can recognize the presence of the highly active JA conjugate jasmonoyl-isoleucine (JA-Ile). In JA-activated cells COI1 as part of an E3 ubiquitin ligase (SCF^{COI1}) can ubiquitylate JAZ which subsequently leads to degradation removing the transcriptional repressing functions of JAZ. In *Arabidopsis* two major activation classes exist, the MYC branch activating amongst others the marker gene *Vegetative Storage Protein*2 (*VSP*2) and ERF branch regulated by Apetala2/Ethylene Response Factor (AP2/ERF) family transcription factors activating i.a. *Plant Defensin1.2* (*PDF1.2*). ERF factors are e.g. ERF1 or Octadecanoid-Responsive Arabidopsis59 (ORA59). The ERF branch additionally needs ethylene for activation and the corresponding transcription factors Ethylene Insensitive3 (EIN3) and EIN3-Like1 (EIL1) can interact with JAZ and recruit the corepressor Histone Deacetylase6 (HDA6).

The produced metabolites can affect herbivorous insects through e.g. synthesis of proteineous defence compounds like proteinase inhibitors (PINs) which than impair the nutrient consumption through the pests. Formation of toxic compounds like nicotine and the emission of insect predator attracting volatiles are also JA dependent (Wasternack *et al.*, 2007). Studies using the parasitic plant *Orobanche ramose* showed an upregulated gene expression of several JA dependent genes in *A. thaliana* (Dos Santos *et al.*, 2003). However, resistance against *Orobanche minor* was not affected through treatment with JA analogues (Kusumoto *et al.*, 2007).

In general, two branches of JA-dependent gene activation exist, the Myc and the ERF branch, whereas the ERF branch is often associated with enhanced resistance to necrotrophic pathogens (Berrocal-Lobo *et al.*, 2002, Lorenzo *et al.*, 2003) and the

Myc branch with wounding response and defence against insect herbivores (Kazan & Manners, 2012; Lorenzo *et al.*, 2004). Once JA responses are triggered, similar responses can also be seen in distant undamaged plant parts providing resistance.

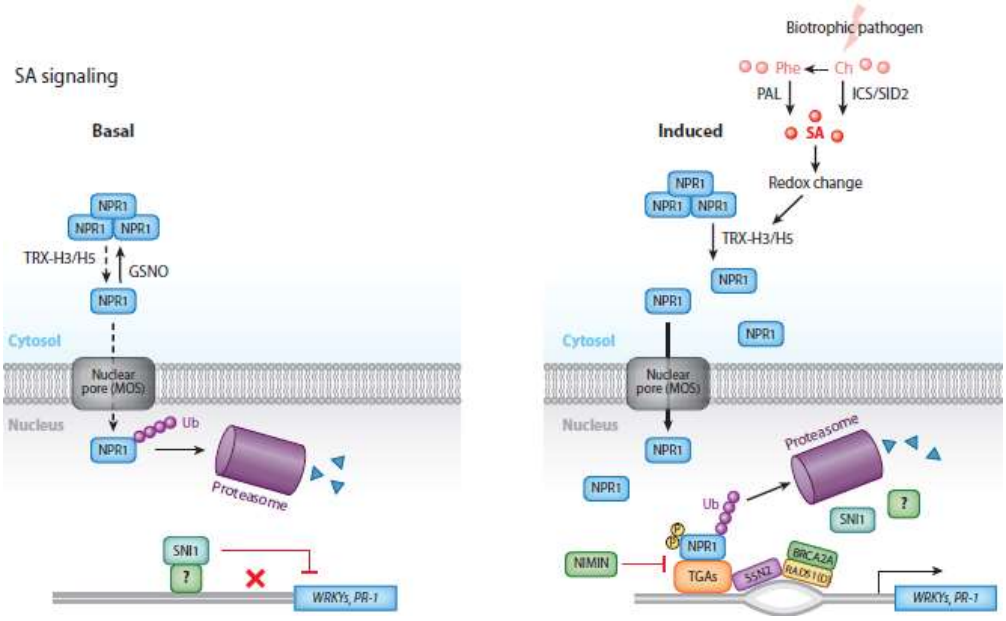


Figure 9: Salicylic acid pathway (modified from Pieterse *et al.*, 2012). NPR1 exists as oligomers connected through disulfide bonds in the cytoplasma during resting state. The small number of monomers that is translocated to the nucleus is continuously ubiquitylated and degraded by the proteasome preventing signalling activation. Upon SA induction the cellular redox state is changed leading to monomerization of NPR1 through the thioredoxins TRX-H3 and TRX-H5. Monomeric NPR1 continues to translocate through nuclear pores like Modifier Of *snc1* (MOS) 3, 6 and 7. Inside the nucleus NPR1 interacts with basic leucine zipper (bZIP) transcription factors TGA binding SA responsive genes activating them. During this process NPR1 is ubiquitylated by E3 ubiquitin ligases followed by degradation further facilitating the induction process possibly through reinitiation of the induction loop. NPR1 is further controlled through NPR1-Interacting protein NIM1-Interacting1 (NIMIN1) and Suppressor Of *npr1* Inducible1 (SNI1). SNI1 can be removed from the promoter region through DNA damage repair proteins like Suppressor Of *sni1* 2 (SSN2) and Ras Associated With Diabetes51D (RAD51D). Additionally, a complex of RAD51 and Breast Cancer2A (BRC2A) can be recruited to the *PR-1* promoter positively regulation immune gene expression.

SA is a phenolic substance that can be synthesized from chorismate either via the Phenylalanine Ammonia Lyase (PAL) pathway or the Isochorismate Synthase (ICS/SID2) pathway (Garcion & Métraux, 2006). A scheme summarizing and describing the SA pathway is shown in Figure 9. The SA biosynthesis can be triggered during microbe infection through changes in calcium levels followed by processing through the lipase-like protein Enhanced Disease Susceptibility1 (EDS1) and Phytoalexin Deficient4 (PAD4) during PTI (Du *et al.*, 2009). The same happens during TIR-NBS-LRR triggered ETI (Wiermer *et al.*, 2005) but during CC-NBS-LRR triggered ETI SA production is induced through the function of Non-Race-Specific Disease Resistance1 (NDR1).

SA downstream signalling is mainly controlled through Non-Expressor of *PR* Genes1 (NPR1) working as transcriptional coactivator of a multitude of defence genes (Dong, 2004; Moore *et al.*, 2011).

SA activates and regulates a hypersensitive response (HR) and induces the production of antimicrobial phytoalexins, pathogenesis-related (PR) proteins, like *PR-1* and transcription factors, like WRKY, which in return lead to a systemic acquired resistance (SAR; Durrant & Dong, 2004; Rushton *et al.*, 2010; Wang *et al*., 2006). Introduction of benzothiadiazole-7-carbothioic acid, a functional analogue of SA, was able to induce resistance in several hosts e. g. against parasitic plants of *Orobanchen* spp. (Sauerborn *et al.*, 2002).

Activation of the SA pathway often leads to further hormone-dependent gene activation in distant plant parts to provide protection for undamaged tissue (Vlot *et al.*, 2009).

Furthermore, other plant hormones have shown effects in plant immunity often in cross-talk with JA or SA responses.

In interaction between *C. reflexa* and a resistant host the production of ET could already be observed as essential response in the immune defence (see 1.2.2) but also in other plant immunity signalling pathways it already showed diverse functions. The gaseous hormone ET is an important modulator of defence signals on its own and is interacting extensively with SA and JA signalling (Glazebrook *et al.*, 2003; Sato *et al.,* 2010). The effects can be diverse. ET can positively regulate plant immunity as shown in *Arabisopsis* in potentiation of SA-responsive *PR-1* expression (De Vos *et al.,* 2006; Lawton *et al.,* 1994) or in tobacco as important activator of SAR (Verberne *et al.*, 2003). On the contrary, ET can also negatively regulate plant immunity as detected for the transcription factors EIN3 and EIL1 repressing the PAMP response in *Arabidopsis* (Chen *et al.,* 2009). The authors state that EIN3 and possibly EIL1 regulate the SA synthesis gene SALICYLIC ACID INDUCTION DEFICIENT2 (SID2) in a negative manner possibly in a mechanism of downregulation of immunity response during times without pathogen attacks to preserve energy for other biological processes.

Besides important functions in development and adaptation to abiotic stress like drought or salinity, abscisic acid (ABA) also takes part as modulator in the plant immune network (Asselbergh *et al.,* 2008; Cao *et al.*, 2011; Ton *et al.*, 2009). ABA balances the abiotic-biotic triggered stress responses through affection of SA

biosynthesis at several levels (Yasuda *et al.,* 2008). It can also act synergistically with JA further enhancing the MYC branch dependent responses and therefore shifting immunity with a priority to herbivore instead of necrotroph defence (Anderson *et al.*, 2004; Dombrecht *et al.,* 2007; Fernandez-Calvo *et al.,* 2011).

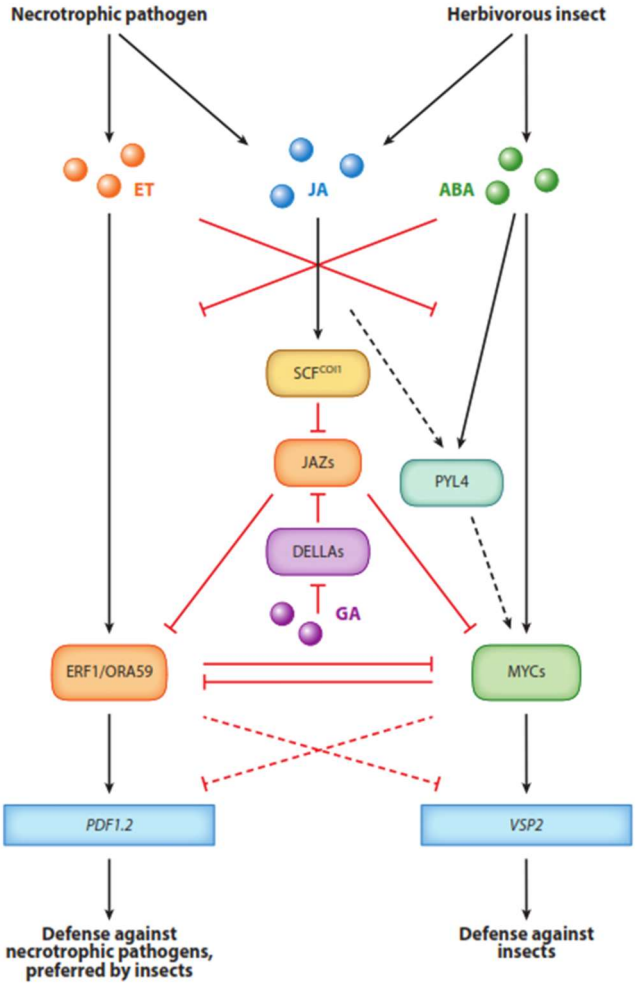


Figure 10: Modulation of JA pathway by different phytohormones (Pieterse *et al.*, 2012). The crosstalk of ET, ABA and GA is displayed in this figure. JA- and ET-dependent signalling pathways can be induced by necrotrophic pathogens and JA- and ABA-dependent pathways can be induced by herbivorous insects. ET and ABA act mutually antagonistic in their regulation of JA. Solid lines indicate established interactions, dashed lines represent hypothesized interactions, arrows indicate positive effects and red inhibition lines represent negative effects.

Auxin has a ubiquitous role in plant development (Benjamins & Scheres, 2008) and a great number of microbes are able to either manipulate the hormone signalling or produce auxin themselves (Kazan & Manners, 2009; Robert-Seilaniantz *et al.*, 2011a).

Especially the repression potential of auxin for SA levels and signalling encouraged some biotrophic pathogens to evolve mechanisms to exploit this effect (Chen *et al.*, 2007). The bacterial PAMP flagellin for example targets auxin receptors resulting in a

signal suppression and therefore also a prevention of SA antagonism resulting in an enhanced resistance against *P. syringae* and *H. arabidopsis* indicating a role in the intrinsic SA-dependent response against biotrophs (Navarro *et al.*, 2006; Robert-Seilaniantz *et al*., 2011b).

Gibberellins (GAs) are important hormones for plant growth control by repressing DELLA proteins (Sun 2011). The letters DELLA indicate here the amino acid motif characteristic for this class of proteins. DELLA proteins have been shown to modulate JA and SA signalling and therefore promoting susceptibility to necrothrophs and resistance to biotrophs (Navarro *et al*., 2008). In detail DELLA proteins interact with JAZ and therefore allow MYC2 activated JA-gene expression (Hou *et al*., 2010). If GAs degrade DELLA proteins the JA-response gets supressed shifting the balance to SA and therefore enhancing biotroph resistance.

Cytokinins (CKs) also play important roles in plant growth. CKs can also act synergistically with the SA pathway e.g. through binding of the CK-activated transcription factor ARR2 to the SA transcription factor TGA3 inducing *PR-1* gene expression and therefore resistance against *P. syringae* (Choi *et al*., 2010). The different possible modulations of phytohormone responses through crosstalk between the individual hormones are schematically shown in Figure 10 for the JA pathway.

1.3.2 Plant hormones during infection with parasitic plants

As described before plant defence against pathogen attacks involves a complex signalling network. This network is regulated by phytohormones, especially by JA and SA.

Defence against parasitic plants like the genus *Cuscuta* is less well studied. It has been shown that application of benzothiadiazole-7-carbothioic acid, a SA analogue, enhanced resistance in some susceptible hosts including tobacco against *Orobanche* spp. (Sauerborn *et al*., 2002; Gonsior *et al*., 2004; Perez-de-Luque *et al.*, 2004; Kusumoto *et al*., 2007).

On the contrary, infection of tobacco with *Orobanche aegyptiaca* did not induce expression of *PR-1a* a marker gene for the SA pathway and SAR (Griffitts *et al.*, 2004). *A. thaliana* gene expression changes in response to *Orobanche ramosa* led to changes of JA regulated genes but no SA-dependent genes (Dos Santos *et al*.,

2003a,b) but treatment of the susceptible host with JA did not affect resistance to *Orobanche minor* (Kusumoto *et al*., 2007).

Table 1: Roles of hormones in the control of nutrient homeostasis (modified from Rubio *et al.*, 2009).

Hormone	Role in nutrient signaling or plant adaptive responses
CK	Long-distance (systemic) signal in nitrate resupply
	Repressor of genes responsive to various nutrient starvation stresses
	Potential link between meristematic activity and activation of nutrient starvation responses
ABA	Lateral root inhibition by nitrate resupply
	Positive modulatory effect on the inducibility of PSR genes
	Potential role as nutrient starvation stress-protecting hormone
ET	Root hair proliferation and elongation in response to low-iron supply
	Positive modulator of the expression of low-iron-responsive genes
	Lateral root growth inhibition in potassium starvation stress
Auxin	Bidirectional antagonistic effect with sulfur deprivation signaling
	Upregulation of the accumulation of potassium transporters
JA	Positive modulator of the expression of sulfur metabolism genes
	Possible role in nutrient recycling under potassium starvation stress
GA	Repressor of several PSR (changes in root architecture, root hair production, anthocyanin accumulation)

Even the age of the host plant seems to influence the parasitism process. As shown by Runyon *et al*., 2010, *C. pentagona* induced typical resistance HLR on 20-day-old potential hosts but not on 10-day-old ones. In accordance with this finding, *C. pentagona* grew better on JA-insensitive and SA-deficient tomato hosts.

Phytohormones also take over different functions during the infection process. The twining of the stem parasites *Cuscuta* and *Cassytha* is strongly dependent on hormones, especially on brassinolides and cytokinins which act downstream of light induced recognition pathways (Furuhashi *et al.,* 2021).

By contrast, haustoria differentiation proved to be at least not exclusively dependent on phytohormones. The application of phytohormones inducing differentiation into

xylem vessel cells did not induce *C. campestris* haustoria differentiation (Kaga *et al.*, 2020).

During parasitization *Cuscuta* may utilize phytohormones of the host and repurpose them for its own growth or nutrient acquisition possibly even actively degrading important defence hormones (Furuhashi *et al.*, 2014). Some possible functions are summarized in Table 1.

1.3.3 The role of ethylene in stress response

As mentioned in 1.2.2 the LRR-RLP CuRe1 is an important PRR for the recognition of the parasitic plant *C. reflexa* through the resistant *S. lycopersicum*. One of the CuRe1 prominent features is the induction of ethylene, a plant stress hormone. This is triggered through the recognition of a *C. reflexa* specific GRP (Hegenauer *et al.*, 2020, summarized in Slaby *et al.*, 2021). Ethylene production as response to stress is caused by the fast activation of the ACC synthase and is already known for several decades to play a role in MTI (Spanu *et al.*, 1994).

As described earlier pathogen resistances are often linked to PRRs. The identification of the relevant receptors is possible through the help of immune assays e.g. ethylene production. During immune reactions the plant stress hormone ethylene is often produced as response. Crude extracts from the parasitic plant *C. reflexa* tissue and a library of 49 introgression lines (ILs) were used to identify the corresponding receptor for *S. lycopersicum* resistance trait (Hegenauer *et al*., 2016). As mentioned in 1.2.2 the cultivated tomato *S. lycopersicum* exhibits a resistance against the parasitic plant *C. reflexa*. The introgression lines are crossings between the green-fruited *S. pennellii* and the cultivated *S. lycopersicum* (cv. M82), in which each line has a single homozygous restriction fragment length polymorphism-defined *S. pennellii* chromosome segment in the *S. lycopersicum* background (Eshed & Zamir, 1995). Those lines were analysed for their ethylene production capacity as response to *C. reflexa* plant extracts (see Figure 11).

Expression analysis of the PRR candidates in the mapped chromosome region of IL8-1 led to the identification of the leucine-rich repeat receptor-like protein CuRe1 (Solyc08g016270) as *C. reflexa* recognizing immune receptor (Hegenauer *et al.*, 2016; see Figure 11).

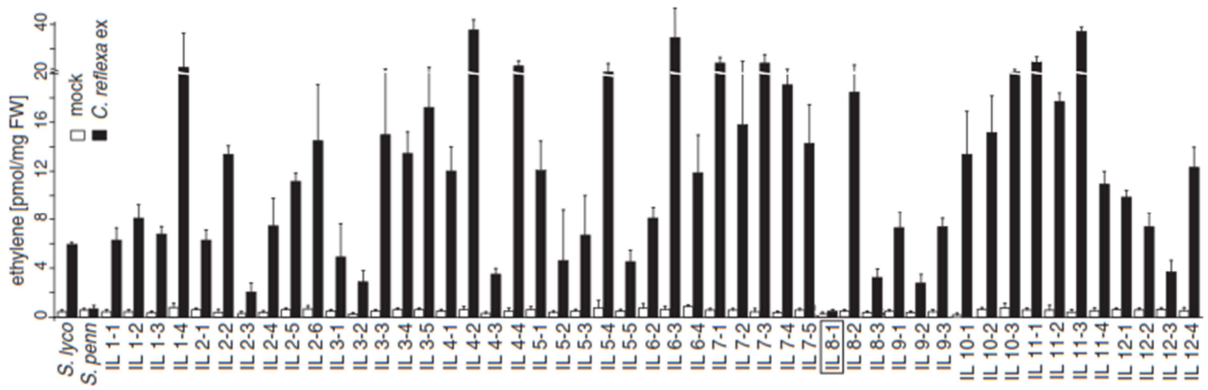


Figure 11: Ethylene production of ILs as response to *C. reflexa* plant extract (modified from Hegenauer *et al.*, 2016). Two tomato cultivars (*S. lycopersicum* and *S. pennellii*) and their crossings, the IL library, were screened for ethylene production in response to *C. reflexa* extract (black) and BSA buffer as mock control (white). The plants belonging to IL8-1 showed comparably low levels of ethylene response to the susceptible *S. pennellii.*

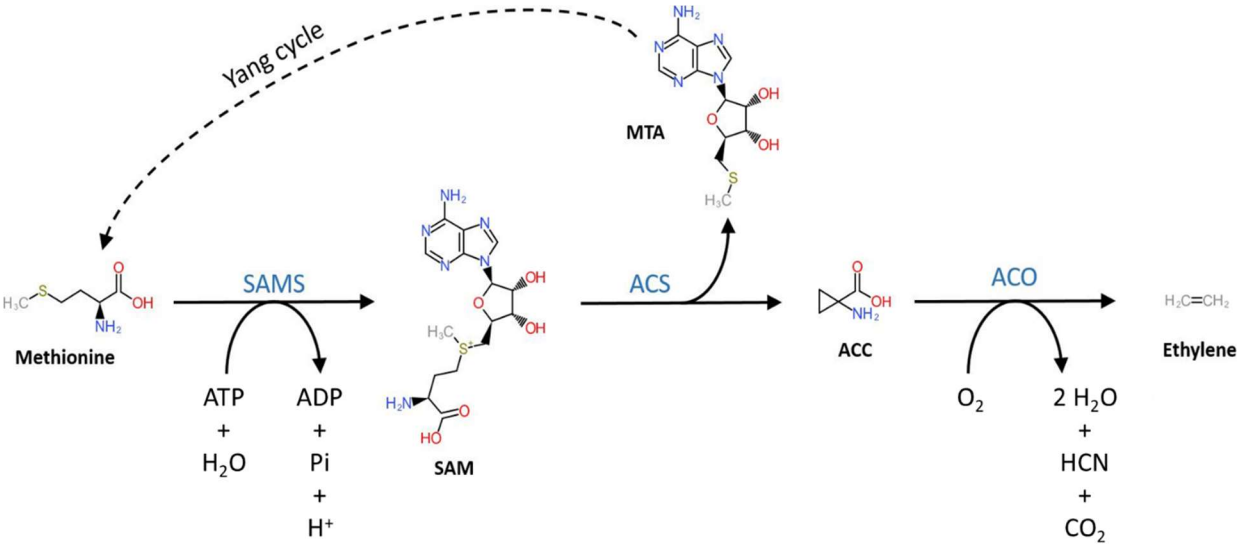


Figure 12: Ethylene biosynthesis (modified from Houben & Van de Poel, 2019). The SAM synthetase (SAMS) converts methionine to SAM (S-adenosyl methionine) with the help of ATP. Afterwards, SAM is transformed into methylthioadenosine (MTA) and 1-aminocyclopropane-1-carboxylic acid (ACC) through the ACC synthase. The MTA can be recycled via the Yang cycle. Finally, the ACC is converted to ethylene by the ACC- oxidase (ACO) in an oxygen-dependent manner.

The analysed stress hormone for this identification, ET, is synthesized by a three-step procedure, which is shown in Figure 12. First, methionine is converted into *S*-adenosyl methionine (SAM). Afterwards, the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) converts SAM to ACC, the ET precursor. Finally, the 1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO) produces the ET (Houben & Van de Poel, 2019).

Methyladenosine can be recycled for rapid ethylene synthesis (Sauter *et al.*, 2013). During ethylene synthesis, the ACS is the rate limiting enzyme. Activation of the ACS works through initiation of transcription factors induced by e.g. drought (Dubois, 2017) or shade (Nomoto, 2012). Post-translational control through phosphorylation followed by ubiquitin-mediated degradation (Thomann, 2009; Yoon, 2015) is also possible. Activation on a post-translational level usually is achieved through MAPK-phosphorylation (Xu & Zhang, 2014). Additionally, ACC levels are regulated through construction and degradation of conjugates such as malonyl- or jasmonyl-ACC (Van de Poel, 2014). ACC can be transported through the plant xylem, e.g. in *A. thaliana* with the help of the amino acid transporter LHT1 (LYSINE HISTIDINE TRANSPORTER1; Shin *et al.*, 2015).

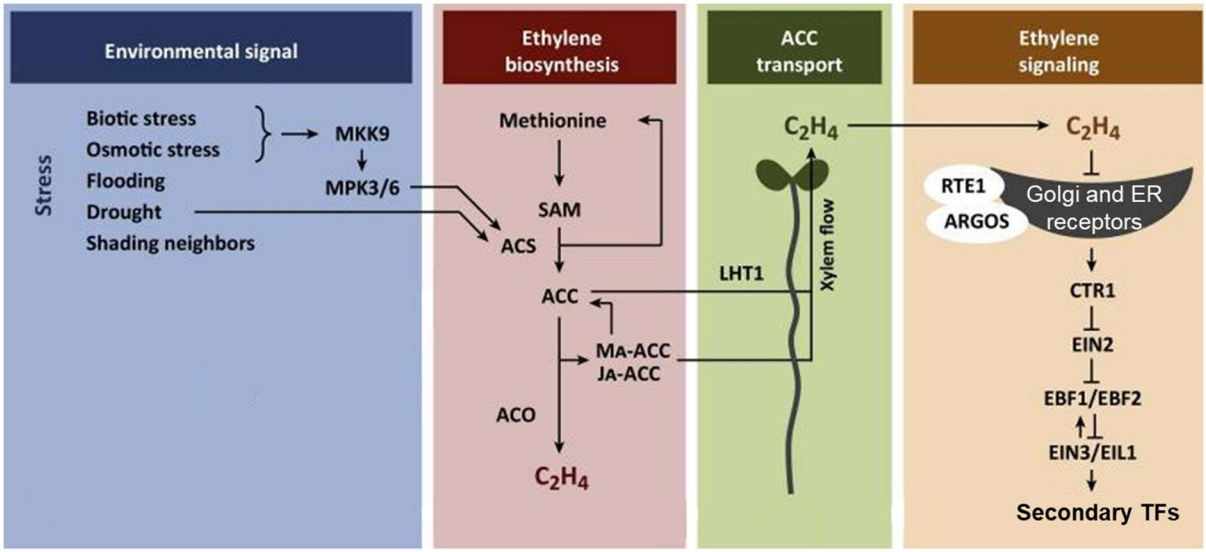


Figure 13: Ethylene signalling (modified from Dubois, 2018). Several plant stress initiators induce the production of ethylene (blue box). Ethylene is synthesized from methionine in three steps over SAM and ACC with the help of the enzymes ACS and ACO. Methyladenosine can be recycled for rapid ethylene production and ACC conjugates help to regulate ACC levels (red box). Ethylene precursors and conjugates can be transported through the plant xylem using e.g. amino acid transporters like LHT1. At its destination ethylene inactivates several Golgi and ER receptors blocking CTR1 mediated degradation of EIN2 which in turn inactivates the F-Box protein production of EFB1 and EFB2. This finally stabilizes the transcription factors EIN3 and EIL1 leading to the activation of numerous downstream TFs.

The effect of ethylene is mediated through a complex network of proteins summarized in Figure 13. At its destination, ethylene is detected by receptors in endoplasmic reticulum (ER) and Golgi membranes and leads to their inactivation through complex formation with RTE1 (REVERSION TO ETHYLENE SENSITIVITY) and ARGOS (AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE) proteins which are positive regulators of ethylene receptors and thus negative regulators for

ethylene sensitivity (Rai *et al.*, 2015; Resnick, 2006). The inactivated receptor no longer stabilizes CTR1 (constitutive triple response1, Shakeel *et al.*, 2015) which then can no longer repress EIN2 (ETHYLENE INSENSITIVE 2). EIN2 is dephosphorylated, cleaved and the C-terminal fragment is released (Li *et al.*, 2015). This fragment binds to the 3' UTR of *EBF1* and *EBF2* (EIN3 BINDING F-BOX1 and 2) transcripts repressing their translation. Therefore, the two F-box proteins are no longer present to target the transcription factors (TFs) EIN3 and EIN3-LIKE (EIL1) for degradation which in turn leads to the activation of secondary TFs producing numerous responses (Potuschak, 2003).

1.3.4 Resistance-Loci in S. lycopersicum

As described above, the LRR-RLP CuRe1 is essential for the resistance of *S. lycopersicum* against *C. reflexa* (Hegenauer *et al.*, 2016). Nevertheless, there are striking differences between the CuRe1-mediated resistance transferred into susceptible plants and the full resistant phenotype of *S. lycopersicum* (Kaiser, 2019). Therefore, the tomato introgression lines were screened again but this time for susceptibility phenotypes. Most introgression lines showed the typical HLR symptoms except for the line 12-2. This was evidence for the necessity of another component in the immunity answer of *S. lycopersicum* against *C. reflexa*. Especially the line 8-1, which was lacking the CuRe1 receptor and still showing a resistance phenotype, underlines the importance of a second immunity component. The IL screening also revealed that this second component should be located on chromosome 12, more precisely in the IL 12-2 since no other ILs showed a corresponding phenotype (see Figure 14). The introgressions are categorized in BINs. 'Bins' are unique overlapping regions of recombination events between *S. lycopersicum* and *S. pennellii* (Chitwood *et al.*, 2013).

Another set of precisely sequenced ILs genotyped by Chitwood *et al.*, 2013 allowed a further fine mapping of the potential resistance locus. The introgression line 12-3-1 was used to further reduce the gene candidate range to a few hundred genes (see Figure 15).

These genes are located in the bin d-12E (see Figure 16; Chitwood *et al.,* 2013).

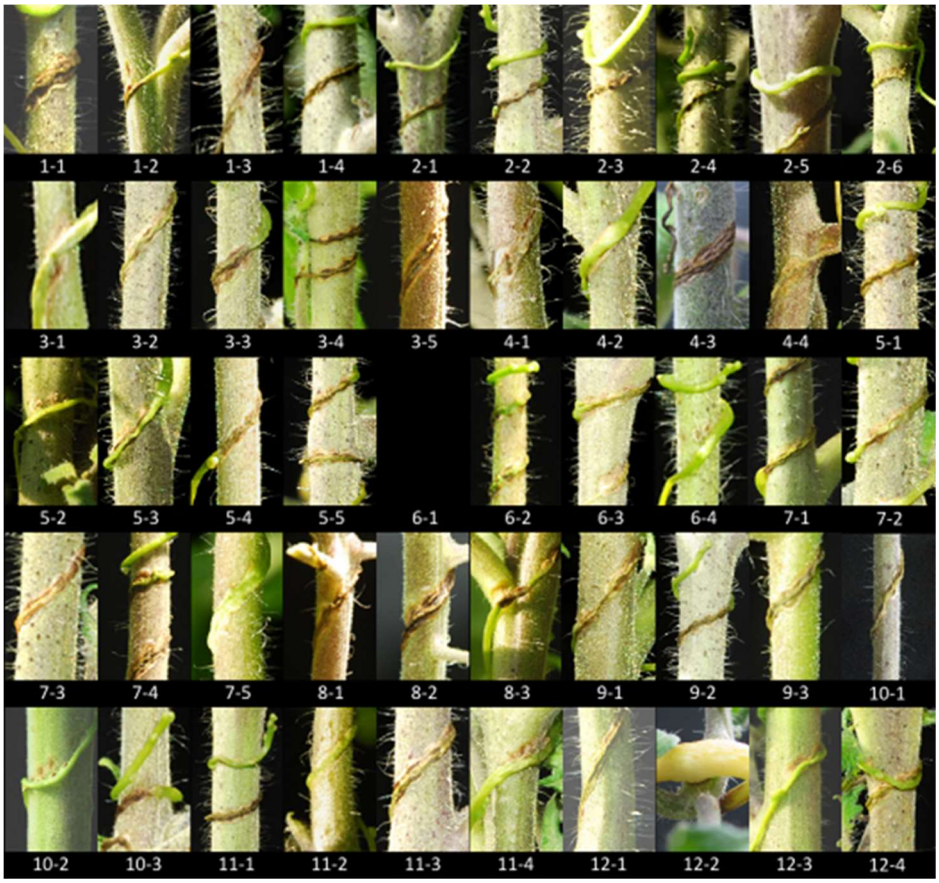


Figure 14: Infection assay of tomato introgression lines with *C. reflexa* (Kaiser, 2019). Shown are *S. lycopersicum* x *S. pennellii* introgression lines 5 weeks after infection with *C. reflexa*. Most plants show HLR symptoms and starving parasites. The line 12-2 does not show any resistance symptoms (Kaiser, 2010).

Figure 15: Infection of IL 12-3-1 with *C. reflexa* (Kaiser, 2019). The *Cuscuta* induces a less prominent defense response.

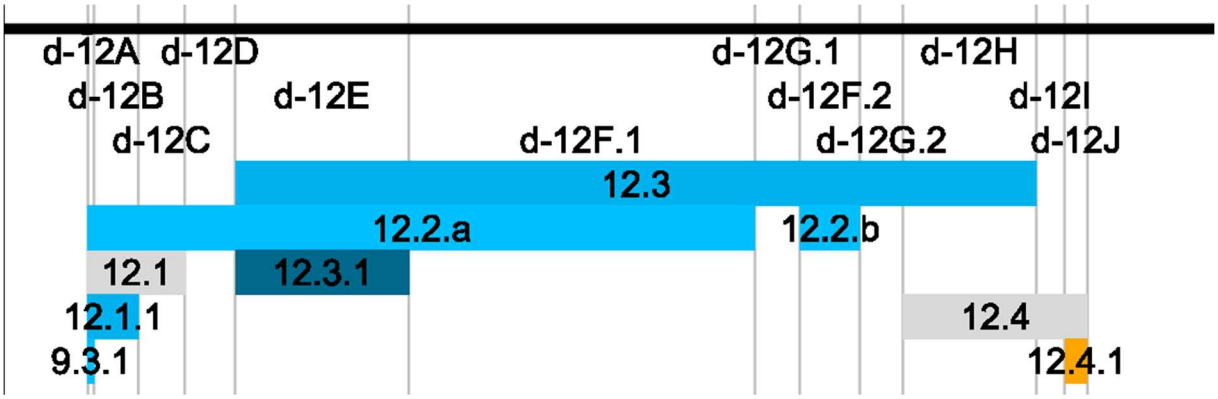


Figure 16: Scheme of overlapping parts of different ILs on tomato chromosome 12 (modified from *Chitwood et al.*, 2013). The different coloured bars indicate recombination events where chromosome fragments of *S. lycopersicum* were exchanged with fragments of *S. pennellii* in the ILs with the respective numbers. The letter-number combinations between the vertical lines indicate bins, genetic distances defined by overlap of recombination events.

However, which of the remaining candidates are the resistance responsible genes? The *Cuscuta* growth assay involving the ILs 12-2 and 12-3-1 both supported the thesis of an existence of other resistance related genes for *C. reflexa* interaction with *S. lycopersicum* (Kaiser, 2019). The corresponding chromosomal region bin d-12E is located on chromosome 12. It is around 5 Mbps in size and consists of approximately 430 genes. Some of them have already been tested in a stable transformation assay in *N. benthamiana* with potential low impact on *C. reflexa* viability (Welz, 2017). In contrast to the IL 8-1, which allowed the identification of CuRe1, the lines 12-2 and 12-3-1 were still able to produce ethylene and ROS as an answer to treatment with *Cuscuta* extract (Kaiser, 2019). The screening for infection impairment or visual HLR seems to be the most feasible way. HLR-responses like those induced by *Cuscuta* are often controlled by R-genes. Therefore, it is possible that the second resistance gene is involved in an ETI response and potentially is an NBS-LRR protein. This can be explained by coevolutionary pressure. Upon development of a pathogen detection system in a potential host an immune response against a specific invader is established. To further be able to feed on the potential host, the pathogen is forced to develop avoidance strategies, often in form of effector proteins which interfere with PTI. The potential host again, needs to develop new recognition tools against the invader like NB-LRR proteins which can trigger ETI. This arms race is a continuous process e.g. between NB-LRRs encoded by the flax *L* locus and the AvrL proteins (Jones & Dangl, 2006).

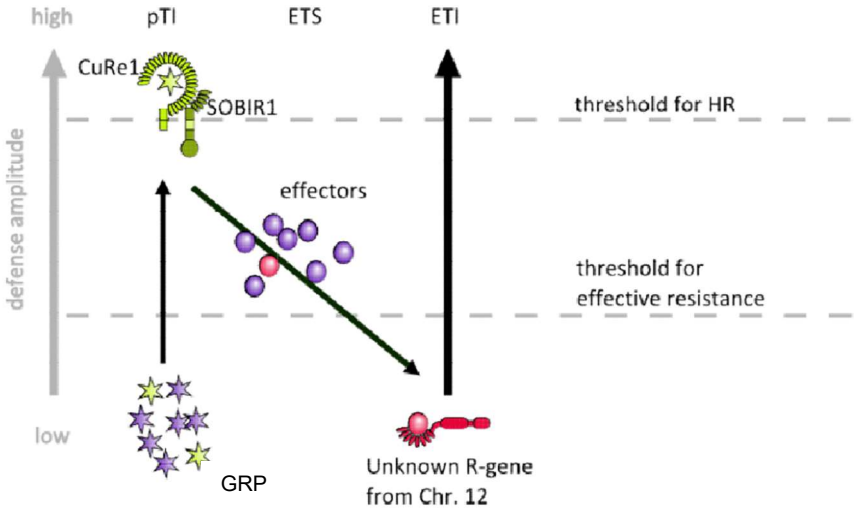


Figure 17: Model for *Cuscuta* resistance (Kaiser, 2019). The *Cuscuta* derived PAMP (GRP) is recognized by the LRR-RLP CuRe1 which induces upon complex formation with SOBIR a PTI response. Potential effectors from *Cuscuta* can lead to an ETS which are therefore perceived by unknown R-genes located on chromosome 12. Those genes than induce ETI.

A putative model for *Cuscuta* resistance integrated in this so called zig-zag model of coevolution of the plant immune system is shown in Figure 17. The zig-zag model was introduced in 2006 by Jones and Dangl and illustrates the co-evolution of the plant's innate immune system. MAMPs, which can be recognized by plant PRRs like the CuRe1, can be masked by effectors from the pathogens which are currently unknown for *C. reflexa*. These effectors can in return potentially be detected by unknown R-proteins which can also initiate an immune response (see 1.2.1).

1.4 Aims of the thesis

In this work the resistance mechanism of the cultivated tomato *S. lycopersicum* against the parasitic plant *C. reflexa* should be further elucidated. The resistant tomato relies on the LRR-RLP CuRe1 to recognize a *C. reflexa* derived PAMP, the CrGRP, with its minimal peptide epitope Crip21. Since the full resistance phenotype is not exclusively dependent on CuRe1, as seen in growth assays with introgression lines lacking the LRR-RLP but still showing resistance (see Figure 14) or by transformation of susceptible plants with *CuRe1* not showing a full resistance but only reduced parasite growth (Hegenauer *et al.*, 2016), further candidate genes for immune signalling should be identified. Mapping of a second resistance trait to chromosome 12 of tomato gave further hints for the above-mentioned hypothesis. The bin d-12e, identified as genetic region for at least one other resistance-related

gene (see 1.3.4), should be further reduced with regard to potential genes of interest by genetic methods e.g. CRISPR-Cas9. Finally, the resistance related genes should be identified by single knockouts in resistant tomatoes.

Additionally, the influence of *C. reflexa* on different susceptible host's metabolisms should be analysed. To achieve this, levels of different phytohormones should be measured in dependence of *Cuscuta* infection. Taking into account the different stages of infection (e.g. haustoria formation) for the first few days, a time series for hormone content during *C. reflexa* connection to a host should be analysed.

2 Material and Methods

2.1 Materials

2.1.1 Media and Antibiotics

The composition of used media is listed in the following table.

Table 2: Composition of bacterial and plant growth media

Medium	Components
LB	10 g/l Bacto-Tryptone, 5 g/l Bacto-Yeast Extract, 10 g/l NaCl (5 g/l for low salts medium), for plates add 15 g/l Bacto Agar Difco direct into the bottle, Autoclave
YEB	5 g/l Beef-Extract, 1 g/l Yeast-Extract, 5 g/l Peptone, 5 g/l Sucrose, 0.49 g/l MgSO4 • 7H2O, for plates add 15 g/l Bacto Agar Difco direct into the bottle, Autoclave
Bacteria growth medium	10 g/l Yeast-Extract, 10 g/l Bacto-Peptone, 5 g/l NaCl, Autoclave, 0.2 mM Acetosyringone (from 400 mM stock-solution in DMSO), Antibiotics
Germination Medium	dissolve in 900 ml ddH2O: 4.3 g/l Murashige & Skoog Salt, 30 g/l sucrose, 100 mg/l myo-Inositol, 1 ml/l NPT Vitamins stock-solution, adjust pH to 5.8, with about 8-10 droplets of a KOH stock 1 M and fill up to 1 l, add agar direct into the bottle, autoclave
Liquid Germination Medium	dissolve in 450 ml ddH2O: 4.8 g/l Murashige & Skoog Salt, 33.3 g/l sucrose, 111.1 mg/l myo-Inositol, 1.11 ml/l NPT Vitamins stock-solution, adjust pH to 5.8, autoclave
Conditioning medium	the same as Germination Medium, cool to 60°C, add hormones: 0.1 mg/l BAP and 1 mg/l NAA
Selection medium	the same as Germination Medium, cool to 60°C, add 1 mg/l trans-Zeatin, add antibiotic against Agrobacterium: 250 mg/l Ticarcillin-clavulanate, add antibiotics to select: Kanamycin mg/l 35, 50 or 100, or Basta 2 mg/l, or Hygromycin 6 mg/l
Rooting medium	the same as Germination Medium, cool to 60°C, add 0.1 mg/l auxin (IAA), add antibiotics to select: 20 mg/l Kanamycin, or 2 mg/l Basta or

	6 mg/l Hygromycin, add antibiotic against Agrobacterium: 500 mg/l Vancomycin
Medium A	20 g/l Bacto Tryptone; 5 g/l Yeast Extract; 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$; 10 mM $MgSO_4$
TB-Medium	10 mM PIPES pH 6.8; 55 mM $MgCl_2$; 15 mM $CaCl_2$; 250 mM KCl

All antibiotics (Table 2.2) were added with following concentrations when the media were cooled down to a temperature of ~60°C.

Table 3: Antibiotics

Antibiotic	Stock	Final concentration	Solvent
Carbenicillin	50 mg/ml	50 µg/ml	H_2O
Gentamycin	40 mg/ml	40 µg/ml	H_2O
Kanamycin	50 mg/ml	50 µg/ml	H_2O
Rifampicin	50 mg/ml	100 µg/ml	DMSO
Spectinomycin	100 mg/ml	100 µg/ml	H_2O
Streptomycin	100 mg/mL	100 µg/mL	H_2O
Amphotericin B	100x	1x	respective medium

2.1.2 Bacterial strains

The *Escherichia coli* strains were used for cloning and amplification of DNA vectors. *Agrobacterium tumefaciens* strains were used for expression plants.

Table 4: Bacterial strains

Strain	Genotype
A. tumefaciens strain GV3101	T-DNA- vir+ rifr, pMP90 genr
E. coli strain ccdB survival	F-mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araΔ139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG fhuA::IS2
E. coli strain DH10B	str. K-12 F– Δ(ara-leu)7697[Δ(rapA'-cra')] Δ(lac)X74[Δ('yahH-mhpE)] duplication(514341-627601)[nmpC-gltI] galK16 galE15 e14–(icdWT mcrA) φ80dlacZΔM15 recA1 relA1 endA1 Tn10.10 nupG rpsL150(StrR) rph+ spoT1 Δ(mrr-hsdRMS-mcrBC) λ– Missense(dnaA glmS glyQ lpxK mreC murA) Nonsense(chiA gatZ fhuA? yigA ygcG) Frameshift(flhC mglA fruB)

E. coli strain DH5α	(F-(Φ80lacZΔM15) Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1))
E. coli strain Top10	F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(StrR) endA1 λ-
E. coli strain XL1-Blue	endA1 gyrA96(nalR) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB+ lacIq Δ(lacZ)M15] hsdR17(rK- mK+)

2.1.1 Plant genotypes

Table 5: Plant genotypes

Genotype	Mutation
Col-0	Wild type
Solanum lycopersicum M82	Wild type
Solanum pennellii	Wild type
Cuscuta reflexa	Wild type
S. lycopersicum mutant list is located in the digital supplemental data.	

2.1.2 Plasmids

Table 6: Plasmid List

Plasmid	Features	Reference
Level 0 vectors		
pAGM11311	Ubi10 Promoter	Grützner *et al*., 2020
pICH49477	dsRed	Engler *et al.*, 2014
pICH41432	Ocs Terminator	Engler *et al.*, 2014
pICH51288	2x35S Promoter	Engler *et al.*, 2014
pICSL11021	Cas9	Belhaj *et al*., 2013
pICH49344	Nos Terminator	Engler *et al.*, 2014
pAGM38869	U6 promoter	Grützner *et al*., 2020
pAGM9037	template for target site PCR	Grützner *et al*., 2020
pICH51277	35S promoter	Engler *et al.*, 2014
pICH41531	GFP	Engler *et al.*, 2014
pICH72400	G7 terminator	Engler *et al.*, 2014
pAGM1311	level -1 vector	Weber *et al.*, 2011

pICH41308	level 0 vector	Weber *et al.*, 2011
pICH51266	level 0 35S promoter	Engler *et al.*, 2014
pICH42301	Basta Lvl0 (with nos-P & nos-T)	Leibniz-Institut für Pflanzenbiochemie (IPB), 2017 (WO2017133738A1)
Level 1 vectors		
pICH47732	position 1 level 1	Weber *et al.*, 2011
pICH47742	position 2 level 1	Weber *et al.*, 2011
pICH47751	position 3 level 1	Weber *et al.*, 2011
pICH47761	position 4 level 1	Weber *et al.*, 2011
pICH47772	position 5 level 1	Weber *et al.*, 2011
pICH47781	position 6 level 1	Weber *et al.*, 2011
pICH47791	position 7 level 1	Weber *et al.*, 2011
pICH41744	end linker position 2	Weber *et al.*, 2011
pICH41766	end linker position 3	Weber *et al.*, 2011
pICH41780	end linker position 4	Weber *et al.*, 2011
pICH41800	end linker position 5	Weber *et al.*, 2011
pICH41822	end linker position 6	Weber *et al.*, 2011
pICH50866	end linker position 7	Weber *et al.*, 2011
BCJJ344	UBI10_Cas9-IV_E9	Castel *et al.*, 2019
pICH47732_Basta	Lvl1 Basta	This work
Level 2 vectors		
pAGM4673	level 2 vector	
pAGM4673_GB (Cas neu)	Lvl2 CRISPR/Cas9	This work
pAGM4673_B1 (Cas neu)	Lvl2 CRISPR/Cas9	This work
pAGM4673_B2 (Cas alt)	Lvl2 CRISPR/Cas9	This work
pAGM4673_VB(Cas alt)	Lvl2 CRISPR/Cas9	This work
pAGM4673_B3 (Cas neu)	Lvl2 CRISPR/Cas9	This work
pAGM4673_B3 (Cas alt)	Lvl2 CRISPR/Cas9	This work
pAGM4673_GB (Cas alt)	Lvl2 CRISPR/Cas9	This work
pAGN4673_B2 (Cas neu)	Lvl2 CRISPR/Cas9	This work
pAGM4673_B1 (Cas alt)	Lvl2 CRISPR/Cas9	This work

pAGM4673_VB (Cas neu)	Lvl2 CRISPR/Cas9	This work
pAGM4673_B4	Lvl2 CRISPR/Cas9	This work
Gateway vectors		
pCR8	Entry vector blunt	Thermo Fisher Scientific
pENTR/D-TOPO	Entry vector with 5' overhang	Thermo Fisher Scientific
pENTR-BsaI	Entry vector with Golden Gate overhangs	Parniske Lab
pCR8_RNAi1	Entry vector for RNAi	This work
pCR8_RNAi3	Entry vector for RNAi	This work
pCR8_RNAi1_2	Entry vector for RNAi	This work
pCR8_RNAi3_2	Entry vector for RNAi	This work
pB7GWIWG2(II)	RNAi Silencing vector	This work
pB7GWIWG2(II)_RNAi1	RNAi LRR-RLPs	This work
pB7GWIWG2(II)_RNAi1_2	RNAi LRR-RLPs	This work
pB7GWIWG2(II)_RNAi3	RNAi disease resistance	This work

2.1.3 Primers

Primers were designed using OligoCalc-Software or Tm Calculator and ordered by Sigma. The product was diluted to a final concentration of 100 µM.

Table 7: List of oligonucleotides

Name	Sequence 5'→ 3'	Characteristics
RNAi target site		
RNAi_1_fw	TCCTTGATGTGGGGAACAATGCTATA	Targets: Solyc12g009690, …9720, …9730, …9740, …9745, …9770
RNAi_1_rev	ATCTAATTTGATCATTGCCTTGAAG	Targets: Solyc12g009690, …9720, …9730, …9740, …9745, …9770
RNAi_2_fw	GATATGTGGACAGGAGAATAT	Target: Solyc12g009870
RNAi_2_rev	CACTGAGTTCCTTTATCCTC	Target: Solyc12g009870
RNAi_3_fw	CCGACTGCAAAAGCATC	Target: Solyc12g010660

	TGAAAGGC	
RNAi_3_rev	CGTCAAAATTCATGAGG CGCATGTG	Target: Solyc12g010660
RNAi_1_2_fw	GGTCCACTACCTTCATC CAT	Targets: Solyc12g009690, …9720, …9730, …9740, …9745, …9770
RNAi_1_2_rev	AATGGTACTCAATGAAG TGCT	Targets: Solyc12g009690, …9720, …9730, …9740, …9745, …9770
RNAi_2_2_fw	ATGGGAAGTGTTTGTTC ATCAAAG	Target: Solyc12g009870
RNAi_2_2_rev	CTTACGTATTCTATGAAC GCATAATTCC	Target: Solyc12g009870
RNAi_3_2_fw	GGAGTATTACCTCTAGC AATTTCTG	Target: Solyc12g010660
RNAi_3_2_rev	CGTTCATCCTCTGCGAA AATTG	Target: Solyc12g010660
CRISPR/Cas9 target site		
Del_GB_1_Anfan g	TTGGTCTCAATTGCGGG GCCCCGTATAGAACACG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_GB_1_Ende	TTGGTCTCAATTGTGTT GAGTTATGGGCCGTAG GTTTTAGAGCTAGAAAT AGCAAG	fw Targetsite PCR CRISPR-Cas9
Del_GB_1_2_Anf ang	TTGGTCTCAATTGAATAA CATCACCCTTGACGCGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_GB_1_2_End e	TTGGTCTCAATTGTGCG GAGAATGTAAGTTCGGG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_B1_1_Anfang	TTGGTCTCAATTGCGGG	fw Targetsite PCR CRISPR-Cas9

	GCCCCGTATAGAACACG TTTTAGAGCTAGAAATA GCAAG	
Del_B1_1_Ende	TTGGTCTCAATTGTGGA ACCATAGGCGGATCCGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_B1_2_Anfang	TTGGTCTCAATTGAATAA CATCACCCTTGACGCGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_B1_2_Ende	TTGGTCTCAATTGAGTC GTTCCTTCTTGGGCGGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_B2_1_Anfang	TTGGTCTCAATTGTGGA ACCATAGGCGGATCCGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_B2_1_Ende	TTGGTCTCAATTGTGTT GAGTTATGGGCCGTAG GTTTTAGAGCTAGAAAT AGCAAG	fw Targetsite PCR CRISPR-Cas9
Del_B2_2_Anfang	TTGGTCTCAATTGAGTC GTTCCTTCTTGGGCGGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_B2_2_Ende	TTGGTCTCAATTGTGCG GAGAATGTAAGTTCGGG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_B3_1_Anfang	TTGGTCTCAATTGCTTTA GATCTCTCCTGGAATGT	fw Targetsite PCR CRISPR-Cas9

	TTTAGAGCTAGAAATAG CAAG	
Del_B3_1_Ende	TTGGTCTCAATTGCTCT CTTGGAATCGGCTGACG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_B3_2_Anfang	TTGGTCTCAATTGTTATC TTATAATCATTTCACGTT TTAGAGCTAGAAATAGC AAG	fw Targetsite PCR CRISPR-Cas9
Del_B3_2_Ende	TTGGTCTCAATTGCTTG GATTTACTAGCGGCGCG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_VB_1_Anfang	TTGGTCTCAATTGTCCA TGGACCTATAAGTGCTG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_VB_1_Ende	TTGGTCTCAATTGCGGG GCCCCGTATAGAACACG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_VB_2_Ende	TTGGTCTCAATTGAATAA CATCACCCTTGACGCGT TTTAGAGCTAGAAATAG CAAG	fw Targetsite PCR CRISPR-Cas9
Del_B4_1_Anfang	TTGGTCTCAATTGTCCA TGGACCTATAAGTGCTG TTTTAGAGCTAGAAATA GCAAG	fw Targetsite PCR CRISPR-Cas9
Del_B4_1_Ende	TTGGTCTCAATTGTCCC TATCTGGCCCACTGCCG TTTTAGAGCTAGAAATA	fw Targetsite PCR CRISPR-Cas9

	GCAAG	
Del_B4_2_Ende	TTGGTCTCAATTGCTTTCTGCTACTTATATGCGGTTTTAGAGCTAGAAATAGCAAG	fw Targetsite PCR CRISPR-Cas9
Del_B4_2_Anfang_neu	TTGGTCTCAATTGCAATCCCACACAGCATAGGCGTTTTAGAGCTAGAAATAGCAAG	fw Targetsite PCR CRISPR-Cas9
Citarev	ATGTACGGCCAGCAACGTCG	rev Targetsite PCR CRISPR-Cas9
Expression with cDNA		
Solyc12g009680.2.1_FW	GAACCTTTTCTTAGTCCAAATGG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009680.2.1_REV	GACTATGACCAACTAGATCAAAC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009560.2.1_FW	GATATTAGACTTGCTGCAATTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009560.2.1_REV	CAGTCCTTAATTGTAAGAGACTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009565.1.1_CDS_FW	ATGTTAACTATGAATCCAGTTGG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009565.1.1_CDS_REV	ATGTTCTGCACTGGGGC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009565.1.1_FW	CACCATTTTGCCAATTTTTGTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009565.1.1_REV	CGTTTGACCTTCAAAATGGG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009580.2.1_CDS_FW	GTTCCATGCAGCAACTTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009580.2.1_CDS_REV	GATGAAGATGATTTTGCCAAG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009580.2	GACTTTTTCCTAGCTCAT	RT-PCR *S. lyc.* Chr. 12 candidate

.1_FW	GG	genes
Solyc12g009580.2 .1_REV	CTTTCTACATCTGCCTC G	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009600.2 .1_FW	CCACTAGAAAACAAATA ATTTTGTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009600.2 .1_REV	CTATCCTTTTTAACTTTT TCCATC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009660.3 .1_FW	GACCATAGTTTAAATCC GAAAAATC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009660.3 .1_REV	CATTATTTTGCAATTTCA AGTAC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009670.1 .1_FW	GTGTGCTTATTATTCCC ATAG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009670.1 .1_REV	GCATGATGAGTGGTATG G	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009590.2 .1_FW	GAAACCCCAAGAAGATC G	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009590.2 .1_REV	CATTATCAGCCATCTGA ACG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009630.3 .1_FW	GAATGAAGAAGAAGTTG CTAA	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009630.3 .1_REV	GGAACAAGAGTTCCACA AG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009620.2 .1_FW	GTGTGGCTACAACTAAT GG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009620.2 .1_REV	CTTCTGGAAACGGCAGG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009650.2 .1_FW	GTAAACCTTCTCACAAG CC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009650.2 .1_REV	GTTTTACAATTGGTGGA ACTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009570.3 .1_FW	ATGGCAGGATATCTTCC AT	RT-PCR *S. lyc.* Chr. 12 candidate genes

Solyc12g009570.3.1_REV	GCCCCAATTAATCTAATAAAAAAC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009640.1.1_FW	GATAATGAGATGACACATGAGC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009640.1.1_REV	CTATTGACTTCTTCTTCCTACG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009510.1.1_FW	GTCTGTTGTGTCTTTCTAGG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009510.1.1_REV	CTATGCTGTGTGGGATTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009610.2.1_FW	GTTTGAATTTGAACAAACGATG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009610.2.1_REV	CATATGATCATCATCATCTTGTTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009690.1.1_FW	GAATGGTAATCAATTCGAAGG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009690.1.1_REV	CACCAACCTCAATGAATCC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009530.1.1_FW	CATAATTCATTAACAGGCCAC	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009530.1.1_REV	GTTACTGAAGTTGTTATGTGAT	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009520.2.1_FW	CATTTATTCACTTTTCTGTTGTG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009520.2.1_REV	GGAAAGGTATGAGATTTCTGT	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009550.2.1_FW	CAATTATCTGGTTCCATTCCT	RT-PCR *S. lyc.* Chr. 12 candidate genes
Solyc12g009550.2.1_REV	GTATGACAAAACCAGTTCAG	RT-PCR *S. lyc.* Chr. 12 candidate genes
Cloning of single GOIs		
9510_fw	TTGGTCTCAACATATGGAATGTCTCTTTTTCTTTT	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)

	ATTC	
9510_rev	TTGGTCTCAACAATCAA GTCCGTCTTCTTTGAG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9520_fw	TTGGTCTCAACATATGT GGCCTCTCCTTTTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9520_rev	TTGGTCTCAACAATTAA GTCCGTCGTCTCAGA	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9530_fw	TTGGTCTCAACATATGA ACCCAACACTAAGAGA	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9530_rev	TTGGTCTCAACAACTAC GGATTCCCACGTAA	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9550_fw	TTGGTCTCAACATATGG AGCTACTTTCCCTT	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9550_rev	TTGGTCTCAACAAATTC AGTGAGTTGTATGACAA	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9560_fw	TTGGTCTCAACATATGC CTACTCTTGTTAATTACA	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9560_rev	TTGGTCTCAACAATTAG GAGAGGATGTCACAC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9565_fw	TTGGTCTCAACATATGTT AACTATGAATCCAGTTG G	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9565_rev	TTGGTCTCAACAACTAA TGTTCTAATGGACGTCC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9570_fw	TTGGTCTCAACATATGA AGAAAGTGAAGAGAAAG C	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9570_rev	TTGGTCTCAACAATCAG CGAGTCCTTGTCC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9580_fw	TTGGTCTCAACATATGT CATCAAGCTACATTGAT	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9580_rev	TTGGTCTCAACAATTAG AAGGTGAAGGTCTTTAT	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)

	TT	
9590_fw	TTGGTCTCAACATATGGCGAAATCAGTGGAGAAAAATG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9590_rev	TTGGTCTCAACAATCAAGGCTTGGCCTCGTTG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9600_fw	TTGGTCTCAACATATGGCAACTGTGTGTCTCTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9600_rev	TTGGTCTCAACAATCAAAGCCCTTTTAGCTCTTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9610_fw	TTGGTCTCAACATATGCATGAAATTGACGTTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9610_rev	TTGGTCTCAACAATTATAACATATGATCATCATCATCTT	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9620_fw	TTGGTCTCAACATATGTCTGCTATATTGTGCG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9620_rev	TTGGTCTCAACAACTAAAAGACATCGGGGTTG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9630_fw	TTGGTCTCAACATATGTTGACACTATTAACCATTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9630_rev	TTGGTCTCAACAATTAAGAAAATGGAACAAGAGTT	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9640_fw	TTGGTCTCAACATATGGCAACCCCAGAAACCG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9640_rev	TTGGTCTCAACAACTAAATGGGGACTTCCATTTTTATTGG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9650_fw	TTGGTCTCAACATATGGAGTTCTCTAAGATAACTTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9650_rev	TTGGTCTCAACAATCAA	*S. lyc.* GOIs, gene name

	ATTTCAGATTGGAAACAAG	Solyc12g00... (primer name)
9660_fw	TTGGTCTCAACATATGTTAAAGAGATTCAAAGTGTG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9660_rev	TTGGTCTCAACAATCAATTAGCAAGTCTAAAATTAAGTC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9690_fw	TTGGTCTCAACATATGAAGAATAAGAAGAATATTGAAAG	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
9690_rev	TTGGTCTCAACAACTATCTATTATGACATCCGAATC	*S. lyc.* GOIs, gene name Solyc12g00... (primer name)
Sequencing		
M13 fw	GTAAAACGACGGCCAG	Sequencing pENTR, pCR8
M13 rev	CAGGAAACAGCTATGAC	Sequencing pENTR, pCR8
pB7GWIWG2_fw_seq1	GCGGACTCTAGCATGGCCG	Sequencing pB7GWIWG2 first insert location
pB7GWIWG2_rev_seq1	CGTGTTTGCAGGTCAGCTTG	Sequencing pB7GWIWG2 first insert location
pB7GWIWG2_fw_seq2	CATTTCCATGAGGTTGCTTCTG	Sequencing pB7GWIWG2 second insert location
pB7GWIWG2_rev_seq2	GGAGAGGACTGCAGGACG	Sequencing pB7GWIWG2 second insert location
L1f	GTGGTGTAAACAAATTGACGC	Golden Gate Lvl2 sequencing
RBf1	GGATAAACCTTTTCACGCCC	Golden Gate Lvl2 sequencing
Cas9 for	CCACCAAGACTTGACTTTGC	Cas9 sequencing
Cas9 rev	TCCTTTCGATGAAGGACTGG	Cas9 sequencing

GB_Seq_fw	TAAATGTATCTAAGAGA AATTTAA	Genotyping, sequencing GB
GB_Seq_rev	CCACCTCTTCTATGCTTT TTCCCT	Genotyping, sequencing GB
Solyc12g009690_ Seq_fw	CTTCTTTCTAATGGGCC TTATG	Genotyping, sequencing GB, B1
Solyc12g009690_ Seq_rev	GACTAGGAAAGTTGATA TGTGTTC	Genotyping, sequencing GB, B1
Solyc12g010660_ Seq_fw	GTAAAACATTGGATGAG TGG	Genotyping, sequencing GB, B2
Solyc12g010660_ Seq_rev	TTATTCATTCAAAAACCC CT	Genotyping, sequencing GB, B2
Solyc12g009870_ Seq_FW1	TTAAAGTTGGGAGATCG AGTTG	Genotyping, sequencing B1, B2
Solyc12g009870_ Seq_REV1	GCGGTAACATTAAACTA TACTAAACG	Genotyping, sequencing B1, B2
Solyc12g009870_ Seq_FW2	GTT TCT CGA ATT TGA CTT ATT TGA G	Genotyping, sequencing B1, B2
Solyc12g009870_ Seq_REV2	CTGCATGTCTGAATCCA TTATG	Genotyping, sequencing B1, B2
Solyc12g009720_ Seq_FW	GATAAACTAATTACGGA GTTAGAT	Genotyping, sequencing B3
Solyc12g009720_ Seq_rev	GAACCACTGAGTTGATT ATTGC	Genotyping, sequencing B3
Solyc12g009720_ Seq_FW2	CGC TTT GGC TAC TCA ATT TA	Genotyping, sequencing B3
Solyc12g009720_ Seq_REV2	ACTGATTCCCACGTAAA TCC	Genotyping, sequencing B3
Solyc12g009770_ Seq_FW	CTG TTC CAC ATT GCT TGG G	Genotyping, sequencing B3
Solyc12g009770_ Seq_REV	GTCAATTCCTGTGGAAT CTTTC	Genotyping, sequencing B3
Solyc12g009510_	CCA ATA GCA GCC TCT	Genotyping, sequencing VB, B4

Seq_FW	TCG	
Solyc12g009510_ Seq_REV	CCAACAGAGTGTTGCTC G	Genotyping, sequencing VB, B4
Solyc12g009510_ Seq_FW2	CAT TGA CGA CCA TTG TCT TAA AT	Genotyping, sequencing VB, B4
Solyc12g009510_ SEQ_REV2	TGATTCTTTCTAGATCCA TATCG	Genotyping, sequencing VB, B4
Solyc12g009550_ Seq_FW	GAG CTA CTT TCC CTT GTT CT	Genotyping, sequencing B4
Solyc12g009550_ Seq_REV	GCCTGTGTTTGAAATAT CCAAC	Genotyping, sequencing B4
Solyc12g009550_ Seq_FW2	GGT GAA ATT CCT GAC GTT TTC T	Genotyping, sequencing B4
Solyc12g009550_ Seq_REV2	GATTGCGGGTCTAACAG TAC	Genotyping, sequencing B4
GB_Del_heterose q_FW	CTATGTTTCCCGGACTG TAACAATGGG	Heterozygoty sequencing GB
Reverse transcription		
Oligo dT	[Phos]T15	Reverse transcription
qRT-PCR		
RNAi1_qRT-PCR_fw	AGCACTTCATTGAGTAC CATT	qRT-PCR RNAi1; in combination with RNAi_1_rev

2.1.4 Chemicals

Chemicals, used in this work, were purchased from different companies: Sigma-Aldrich (Taufkirchen, Germany), Carl-Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Duchefa (Haarlem, The Netherlands) or Applichem (Darmstadt, Germany). Enzymes used for nucleic acid studies (PCR, cloning etc.) were obtained either from Thermo Scientific (Karlsruhe, Germany), NEB Biolabs (Frankfurt am Main, Germany) or Promega (Walldorf, Germany).

2.2 Methods

2.2.1 DNA-Analysis

2.2.1.1 Production of chemocompetent *Escherichia coli* cells

E. coli pre-culture was grown at 37 °C in 3 ml LB medium. The solution was added to 250 ml Medium A and incubated at 37 °C until an OD_{600} of 0.6 was reached. The cell suspension was divided into 50 ml tubes and incubated on ice for 10 min. Afterwards it was centrifuged at 200 x *g* for 10 min followed by another incubation on ice for 10 min. The centrifugation was repeated once and the cell pellet was resuspended in 20 ml TB medium. While cooled on ice 1.4 ml DMSO was slowly added to the suspension. After a final incubation of 10 min on ice the cells were collected in 200 µM aliquots and stored at -80 °C.

2.2.1.2 Transformation of *Escherichia coli*

The used bacterial stocks (XL-1 Blue, DH5α, DH10B, Top10) were thawed on ice. Chemical competent *E. coli* were incubated with 1 µl purified plasmid DNA on ice for 20 min. The cells were heatshocked for 60 s at 42 °C. Afterwards, the cells were cooled on ice, 500 µl LB medium were added and the reaction mixtures was incubated at 37 °C for 1 h while shaking. Ultimately, the cell suspension was plated on solid LB medium containing the appropriate antibiotics and incubated at 37 °C for 24 h. The *E. coli* strain DH10B was exclusively used for transformation of Golden Gate compatible vectors.

2.2.1.3 Production of *Agrobacterium tumefaciens*

Bacteria of the strain *A. tumefaciens* GV3101 were spread onto LB plates containing the appropriate antibiotics. They were incubated for 2 days at 28 °C. A bacterial colony was chosen and incubated in 10 ml LB medium containing the appropriate antibiotics at 28 °C and 200 rpm overnight. This pre culture was inoculated into 400 ml LB with appropriate antibiotics until an OD_{600} of 0.6 was reached. Afterwards, the bacterial culture was split in 50 ml reaction tubes, centrifuged for 15 min at 4°C with 4500 rpm and the supernatant was discarded. The cell pellet was washed with one volume ice cold 10% glycerol, centrifuged at 4500 rpm and 4°C for 15 min and the supernatant was discarded. This step was repeated only changing the glycerol

amount to 0.5 volumes. During the last glycerol washing step the bacterial pellets were resuspended in the 0.02 volume 10% glycerol and combined into one fraction. The cells were again centrifuged for 15 min at 4 °C and 4500 rpm. Finally, they were resuspended in 3 ml 10% ice col glycerol, 50 µl aliquots were created and stored at -80 °C.

2.2.1.4 Transformation of *Agrobacterium tumefaciens*

The electrocompetent *A. tumefaciens* GV3101 were thawed on ice and 1 µl purified plasmid DNA was added. The cells were mixed gently and transferred into a pre-cooled cuvette. An electric pulse was applied by an electroporator (Gene Pulser II, Bio-Rad Laboratories GmbH, Feldkirchen, Germany). Afterwards, 500 µl of LB medium were added and the cells were transferred to a fresh tube. The cells were incubated on a shaker at 28 °C for 120 min. The bacterial suspension was plated on LB medium containing the appropriate antibiotics and incubated at 28 °C for 2 days.

2.2.1.5 Plasmid extraction from bacteria

Plasmid DNA was extracted from bacteria liquid culture with the GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, Waltham, Massachusetts) according to the manufacturer's protocol.

2.2.1.6 Restriction enzyme digestion of DNA

From *E. coli* extracted plasmid DNA (see chapter 2.2.1.5) was digested using different digestion enzymes. The digestion products were analysed on an agarose gel (see section 2.2.1.12) for expected fragment pattern to confirm correct insertion of GOIs.

2.2.1.7 Genomic DNA extraction from plants

The genomic DNA (gDNA) isolation was performed according to Edwards's protocol (Edwards *et al.*, 1991). Frozen leaf material was shred and taken up in 200 µl Edwards buffer (200 mM Tris/HCl, pH7.5; 250 mM NaCl; 25 mM EDTA, pH 8; 0.5% SDS (w/v)). The mixture was centrifuged at 14000 rpm for 5 min and the supernatant was transferred into a fresh reaction tube. Isopropanol was added in equal volume. The resulting solution was mixed thoroughly and incubated for 45 min at room temperature. Subsequently it was centrifuged at 14000 rpm and 4 °C for 10 min. The

supernatant was discarded and the pellet was washed with 70% ethanol. A drying step was performed at 37 °C and the final DNA pellet was resuspended in 50 µl buffer (10 mM Tris/HCl, pH 8.0). The gDNA was stored at -20 °C.

2.2.1.8 RNA extraction from plants

RNA isolation was performed using the RNeasy® Plant Mini Kit (50) from Qiagen (Hilden, Germany) according to the manufacturer's guidelines except for DNase treatment. For samples used for qRT-PCR an on-column digest with DNase (RNase-Free DNase Set, Qiagen, Hilden, Germany) was performed to exclude DNA contamination. RNA quantity was determined using a NanoDrop (Nanophotometer P330, Implen, Munich, Germany) and quality control was performed by visualization of abundance of 60S and 40S ribosomal RNA on a agarose gel (see 2.2.1.12). The RNA was stored at -80 °C.

2.2.1.9 cDNA synthesis

For cDNA synthesis 1 µg RNA in 10 µl RNase-free water was incubated at 70 °C for 10 min. The solution was cooled on ice and 10 µl of RT-Mix (4 µl RT-Buffer; 2 µl 30 µM Oligo-dT; 2 µl 2.5 mM dNTPs; 1 µl RevertAid Reverse Transcriptase (200 U/µl, Thermo Fisher Scientific, add 20 µl H_2O) were added. The mixture was incubated at 42 °C for 90 min and finally heated to 70 °C for 10 min. The cDNA was stored at -20 °C.

2.2.1.10 Polymerase-Chain Reaction (PCR)

PCR (Saiki *et al.,* 1985) was performed using different polymerases depending on the purpose. For cloning purposes, the Phusion Polymerase (ThermoFisher Scientific, Waltham, Massachusetts) was used because of the higher accuracy due to the proof reading ability. Genotyping of bacterial DNA and plant gDNA was accomplished using the DreamTaq Polymerase (ThermoFisher Scientific, Waltham, Massachusetts). Genotyping of fresh plant material was achieved through the Phire polymerase (ThermoFisher Scientific, Waltham, Massachusetts). PCRs were executed according to the affiliated instruction manuals. The annealing temperatures and elongation times were adjusted depending on DNA template and primer combination.

2.2.1.11 Colony-PCR

Potential positive bacterial colonies, which were expected to contain a DNA vector with a gene of interest (GOI) insert, were picked from plate. Bacterial material was added directly to the prepared PCR solution with DreamTaq DNA Polymerases (ThermoFisher Scientific, Waltham, Massachusetts) according to manufacturer's protocol. PCR products were separated according to their length and checked for presence of the correct fragment size by agarose gel electrophoresis (see 2.2.1.12).

2.2.1.12 DNA-agarose gel electrophoresis

Agarose gels were casted using 1 to 1.5 % of Agarose (Agarose NEEO Ultra-Qualität, Roth, Am Seerain 2, 35232 Dautphetal-Buchenau) in 1x TAE (1 mM EDTA, 40 mM Tris, 20 mM acetic acid). Extracted DNA or RNA was mixed with TriTrack DNA Loading Dye (ThermoFisher Scientific, Waltham, Massachusetts) according to manufacturer's guidelines. Thermo Scientific™ GeneRuler 1 kb DNA Ladder (ThermoFisher Scientific, Waltham, Massachusetts) was used as size standard for nucleic acid fragmentation. The agarose gel was stained with GelRed™ Nucleic Acid Gel Stains (VWR International GmbH, Darmstadt, Germany).

2.2.1.13 Gel-Purification of DNA-fragments

After PCR amplification of desired DNA fragments unwanted byproducts were removed using the GeneJet Gel Extraction Kit (ThermoFisher Scientific, Waltham, Massachusetts). The lane with the expected amplification product was cut form an agarose gel and purified according to the manufacturer's instructions.

2.2.1.14 Entry Cloning

For cloning purposes using the gateway system purified DNA fragments were ligated either blunt (pCR™8/GW/TOPO™ TA Cloning Kit, Thermo Fisher Scientific), with 5' overhang (pENTR™/D-TOPO™ Cloning Kit, Thermo Fisher Scientific) or with BsaI overhangs (pENTR-BsaI, pENTR-BsaI was a gift from Martin Parniske (Addgene plasmid #54340; http://n2t.net/addgene:54340; RRID: Addgene_54340). The ligation into the commercially available vectors was performed according to the manufacturer's protocol. To obtain the pENTR-BsaI vector, a Golden Gate reaction was performed as described in section 2.2.1.15.

2.2.1.15 L/R-reaction – Gateway

Transfer of DNA amplicons from entry to destination vector was performed using Gateway™ LR Clonase™ II Enzyme mix (Thermo Fisher Scientific). The reaction was performed according to manufacturer's guidelines.

2.2.1.16 Golden Gate Cloning

The Golden Gate cloning method was introduced by Engler and Marillonnet (2008) and makes use of type IIs endonucleases which cut DNA distal to their recognition site. By exploiting this unique way of site recognition and different sites for cutting, specific overhangs are created which allow unidirectional ligation of various fragments in one reaction. If the module to be inserted already contains type IIs restriction sites of either BpiI or BsaI, those need to be removed by targeted nucleotide exchange without interrupting the reading frame to avoid any kind of frame shift mutation (Engler et al., 2008).

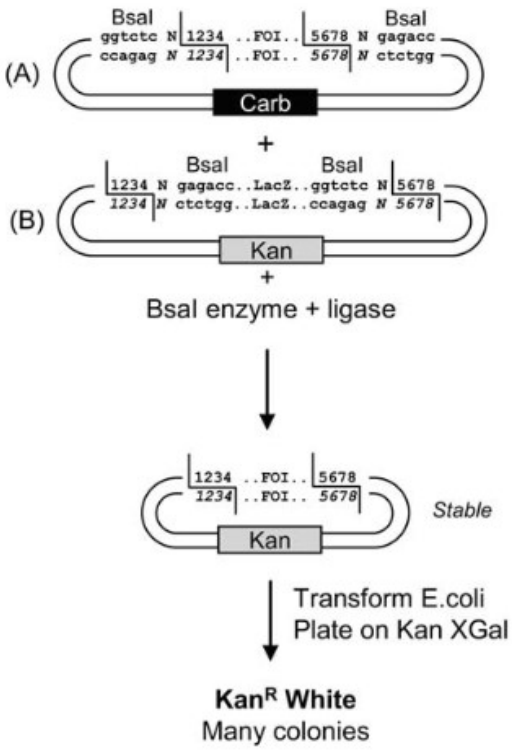


Figure 18: Golden Gate Cloning (modified after Engler et al., 2008). Entry clone (A) and expression vector (B) are mixed in one tube together with BsaI and ligase. Only the desired product is stable. Numbers 1 to 8 enote any nucleotide of choice, and numbers in italics denote the complementary nucleotides. FOI means DNA fragment of interest.

2.2.1.17 DNA sequencing

Cloned DNA fragments integrated into either Gateway or Golden Gate vectors as well as amplicons derived from gDNA or cDNA as PCR fragments were analysed via sequencing prior and after transfer into DNA vectors. The sequencing was performed through GATC Biotech (Konstanz, Germany) and evaluated via ApE - A plasmid Editor (by M. Wayne Davis).

2.2.1.18 RNA interference (RNAi)

RNAi is a cellular mechanism which is able to target and silence specific genes on the RNA level (reviewed in Wilson & Doudna, 2013). Small double stranded RNAs are recognized and processed by several enzymes included in the RNAi pathway. The processed RNA fragments are afterwards used for downregulation of genes with complementary structures (see Figure 19).

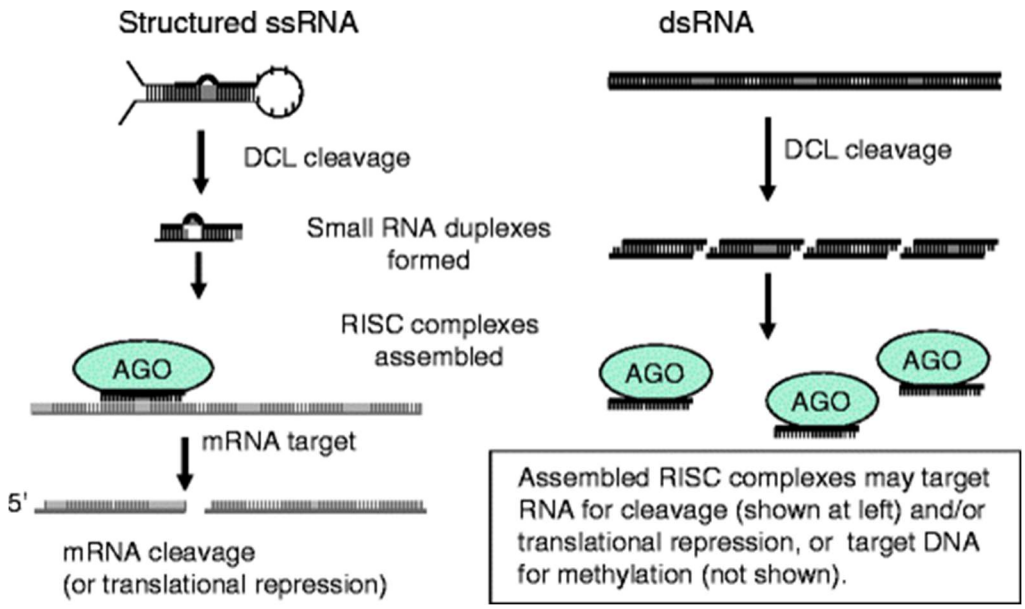


Figure 19: Simplified overview of RNAi system in plants RNAi is ultimately triggered by RNA molecules with duplexed structure. The source of structured RNAs can come from a variety of sources including endogenous plant transcripts, plant viruses, or ssRNAs converted to dsRNAs by the action of RNA-dependent RNA polymerases. Both dsRNAs and structured ssRNAs can be processed by DCL enzymes into short (~21–24 nt) duplexes. Short ssRNAs associate with AGO effector proteins in an RNA-induced silencing complex (RISC). Small RNA guides RISC to specific RNA or DNA sequence by complementary basepairing. RISCs can downregulate the expression of target RNA by degradation and/or translational repression. DNA targets can be downregulated by DNA methylation/heterochromatin formation (Lindbo, 2012).

In this work artificial double stranded RNA was created with the help of the Gateway vector pB7GWIWG2(II) (Karimi *et al*., 2002). RNAi templates homologous to several

GOIs mainly belonging to the class of LRR-RLPs were created to silence several genes at once. Therefore, the primer pairs RNAi_1_fw and RNAi_1_rev, RNAi_2_fw and RNAi_2_rev, RNAi_3_fw and RNAi_3_rev, RNAi_1_2_fw and RNAi_1_2_rev, RNAi_2_2_fw and RNAi_2_2_rev and also RNAi_3_2_fw and RNAi_3_2_rev were used to amplify RNAi targets from *S. lycopersicum* M82 cDNA (see Table 7). Each amplicon was cloned into the vector pB7GWIWG2(II) and stably transformed into *S. lycopersicum* M82. Expression of target genes was analysed via qRT-PCR.

2.2.1.19 CRISPR-Cas9 (reviewed in Adli, 2018)

Deletion of chromosomal parts of *S. lycopersicum* from several 10 kb up to several 100 kb was performed using the CRISPR/Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated proteins) system. It belongs to the typ II CRISPR/Cas systems and targets a DNA sequence with the help of guide RNAs (gRNAs). The gRNA consists of a crRNA and tracerRNA. The crRNA is complementary to a target sequence of a GOI. To create those target sites for the chromosomal regions (named GB, B1, B2, B3, B4, VB see Figure 20), which were analysed in this work, the CRISPR-P 2.0 tool (Lei *et al*., 2014) was used. The CRISPR-P tool analyses the sequence of a gene of interest in the background of the used model organism (*Solanum lycopersicum* (SL3.0)) for suitable target sites. For each region of interest four target sequences were selected. The 20 nucleotide long sequences (23 nucleotides with PAM sequence included) were selected in a way that as few as possible off-targets were present in the genome. From those target sites primers were derived (see Table 7, CRISPR/Cas9 target site) which together with the primer critarev (see Table 7) and the template vector pAGM9037 (see Table 6) create the gRNA during PCR. This gRNA was afterwards cloned into a level 1 vector (see Table 6) under the control of an U6 promotor derived from the small ribonucleoprotein from *A. thaliana* (Belhaj *et al*., 2013). Finally, the gRNAs were cloned together with either the sequence of Cas9 from the pICSL11021 plasmid or another Cas9 from the BCJJ344C plasmid into a binary level 2 Golden Gate vector. These vectors could then be integrated via *A. tumefaciens*-mediated transformation in a plant genome. A Basta resistance gene encoded on the same plasmid as the gRNA and the Cas9 was used for selection of transgenic plants. The utilized plant specific promoters are responsible for the constitutive expression of Cas9 and gRNA in the host organism. The Cas9 enzyme can than create a double strand break

through the gRNA at the target site (Belhaj et al., 2013) which possibly leads to a mutation through incorrect non-homologous end joining (NHEJ) or homology directed repair (HDR; Haber, 2000). Figure 20 displays a scheme of the chromosomal regions (GB, B1, B2, B3, B4, VB) that should be deleted through CRISPR/Cas9.

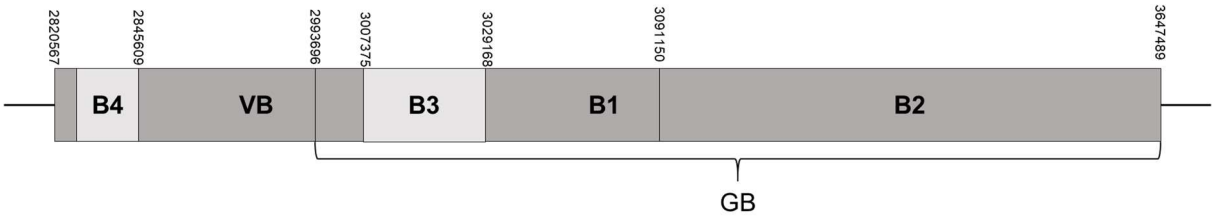
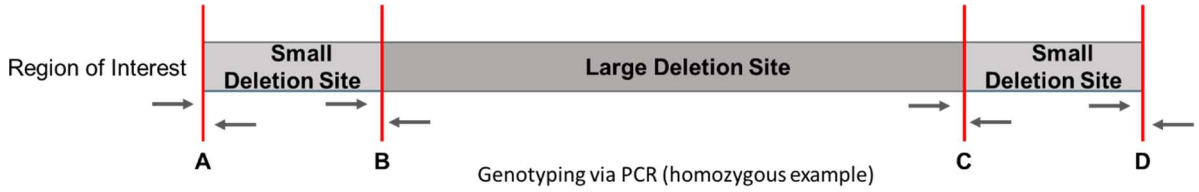


Figure 20: Region of interest on S. *lycopersicum* chromosome 12. Chromosomal parts for deletion were named B1, B2, B3, B4, GB and VB. The approximate positions of introduced double strand breaks through CRISPR/Cas9 are shown through basepair numbers from chromosome 12.

2.2.1.20 Genotyping of CRISPR/Cas9 mediated mutations

Gene-specific primers were designed which enclose the target sites of the potential CRISPR/Cas9 deletions. Those primers (see Table 7 Sequencing) were designed for all chromosomal regions (GB, B1, B2, B3, B4, VB) that should be deleted and were also used to confirm if a large deletion has taken place through simultaneous double strand breaks at two distinct locations (see Figure 21). Genomic DNA was extracted from the mutant plants (as described in section 2.2.1.7) and analysed via PCR (see section 2.2.1.10). If the amplification of DNA fragments was successful, those fragments were further analysed through Sanger sequencing (Eurofins Genomics).



Primer Pair	Small Deletions	Large Deletion	Single Basepair Deletion
A	-	+	+
B	-	-	+
C	-	-	+
D	-	+	+
A/D	-	+	-

Figure 21: Genotyping of CRISPR/Cas9 mutants. Red lines resemble target sides of the guide RNA for CRISPR/Cas9 deletion. Each of them is enclosed by a primer pair (A, B, C, D). Several mutations are possible either small deletions or large deletions or single base pair mutations at the target sites. PCRs were performed and checked for amplicon presence (+) or absence (-). The resulting pattern of the different mutation possibilities for homozygous candidates is shown in the table.

2.2.1.21 Quantitative Real-Time-PCR (qRT-PCR)

Analysis of gene expression on transcript level was performed by qRT-PCR. A fluorescent dye binds to the double stranded DNA and with an increase in amplicon number during PCR, fluorescence intensity increases. The released fluorescence is directly proportional to the amount of amplified nucleic acid. If the sample has a high number of transcribed gene copies, fluorescence will appear earlier during the PCR. The cycle at which the fluorescence can be detected first is termed as Quantitation cycle (Cq) or Threshold cycle (Ct) and is used for the calculation of transcript levels. Transcript levels of GOIs are calculated relative to reference genes (also called housekeeping genes).

Maxima™ SYBR™ Green/ROX 2x qPCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts) was used for qRT-PCR in combination with gene specific primers according to manufacturers protocol. The binding specificity of the primers was checked by BLAST search on NCBI, efficiency was tested and exclusivity of binding was analysed via melting curve. For this work a qRT-PCR instrument (Rotor-Gene Q) from Qiagen was used. Data evaluation was done with Rotor Gene Q Series Software.

The mean value of technical triplicates per sample was calculated and used for analysing the relative expression of a gene in the sample. The negative potency of the ΔCt-value was finally calculated for more accuracy for gene expression.

2.2.2 Plant methods

2.2.2.1 Plant growth conditions

Nicotiana benthamiana (N. benthamiana) was used for infiltration assays for hypersensitive response and overexpression of candidate genes. The plants were grown under long day conditions for four weeks (14 h light at 23 °C, 10 h dark at 20 °C, 60% humidity).

Solanum lycopersicum (S. lycopersicum) was grown in the same conditions as *N. benthamiana* for 4 to 104 weeks.

Arabidopsis thaliana (A. thaliana) was used for confocal laser scanning microscopy. The plants were grown under long day conditions for 2 to 4 weeks (16 h light at 18 °C and 8 h dark at 15 °C, 55-60% humidity).

2.2.2.2 Transient transformation of *N. benthamiana* leaves

Agrobacterium tumefaciens carrying the desired DNA vector were cultured in liquid medium containing the appropriate antibiotics for 2 days at 28 °C. The OD_{600} was measured and the desired concentration (OD_{600} 0.1 or 0,5) for the infiltration was calculated. The needed volume of bacterial suspension was centrifuged at 8000 rpm for 3 min. The cells were resuspended in 5 ml 10mM $MgCl_2$ with 150 µM acetosyringone and incubated for 3 h at room temperature. The bacterial suspension was infiltrated into the abaxial side of a *N. benthamiana* leaves. The leaves were subject to different analysis.

2.2.2.3 Stable transformation of *S. lycopersicum*

Until plants were transferred to the greenhouse every step was performed in a sterile environment with sterile tools. Per transformation approximately 1000 *S. lycopersicum* seeds were sterilized through 3 min shaking in 70% ethanol followed by another shaking incubation in 1.5% hypochlorite containing 0.001% Triton W-100. The seeds were washed 3 times in distilled water, dried and stored for 2 days at 4 °C.

The seeds were sown on Germination medium (see table Table 2) and kept for 10 days in the dark at 22 °C. Afterwards, the cotyledons of the germinated seedlings were cut at top, at the bottom and in the middle of the leaf. During this process leaves were kept moist with Liquid Germination Medium (see table Table 2). Cut leaves were placed on petri dishes containing Conditioning medium (see Table 2) and incubated for 2 days in the dark at 22 °C.

A. tumefaciens containing the desired transformation vectors were grown on LB agar plates containing appropriate antibiotics for 2 days at 28 °C. Liquid LB medium containing antibiotics was inoculated with a single bacterial colony and incubated at 28 °C for 24 h while shaking. The cell suspension was added to 100 ml of Bacteria growth medium (see Table 2) and incubated overnight at 28°C while shaking.

For stable transformation the bacteria needed to have an OD_{590} of 1 in 10 mM $MgSO_4$ with 0.2 mM acetosyringone. The suspension was dropped on the prepared cotyledons and incubated for 2 days in the dark at 22 °C.

Afterwards, the cotyledons were placed on petri dishes containing Selection medium (see Table 2) with the appropriate plant selection substance followed by incubation

for 3 days under long day conditions at 23 °C and 50% humidity. The leaves were transferred to fresh Selection medium every seven days.
After approximately two months shoots, which regenerated from callus tissue, were cut and placed on Rooting medium (see Table 2). Plants were then transferred into pots and grown in the green house. Callus tissue was kept for more shoots to form.

2.2.2.4 Hypersensitive like response (HLR) Test

HLR was determined on living plants either through direct effector infiltration into leaves or infection of shoots through the parasite *C. reflexa*.
For direct effector infiltration 20 µl of CrCrip21 (Hegenauer *et al.*, 2020) from *C. reflexa* with a concentration of 100 nM or crude *C. reflexa* extract was infiltrated into the abaxial side of a *N. benthamiana* (wildtype or CuRe1-transformed) or *S. lycopersicum* (wildtype or CRISPR/Cas9-mutated) leaf. As positive control and HLR trigger Xylanase with the same concentration was used. HLR was evaluated 4 to 7 days after infiltration.
For HLR test through infection approximately 10 cm long *C. reflexa* shoots were connected to the shoot of the host plant. The parasite was left to grow for 4 weeks and regularly checked for HLR.
The results were documented by photography using a Sony α500 3.5-5.6/18-55 SAM digital camera.

2.2.2.5 Ethylene assay

Plant leaves were collected and cut into small squares (leaflets). After overnight incubation in water at room temperature, three leaflets were put into 6 ml-test tubes together with 500 µl water. Elicitors of different concentration and identity were added to the liquid phase inside the test tube. Afterwards the tube was sealed with a rubber stopper and incubated at room temperature for 3 h while shaking. The gaseous phase was collected with a 1 ml syringe and directly injected into the flame ionization detector (FID) of a gas chromatograph for analysis. (Felix *et al.*, 1991; GC-2014, Shimadzu, Duisburg, Germany). The samples were analysed for the presence of ethylene. The results were normalized against an untreated sample and the effectiveness of the used elicitor was evaluated.

2.2.2.6 Growth assay

Growth assay with *C. reflexa* on the host *N. benthamiana* was performed as described in Hegenauer *et al.*, 2017. Therefore, cut *Cuscuta* shoots of approximately 15cm length were grown on a wooden stick for one day. Afterwards, the coiled plants were transferred to potential hosts. The shoots kept growing for at least 21 days.
For growth assays on tomato, data evaluation was performed through HLR documentation rather than biomass production.
Phytohormone level determination was performed with *A. thaliana* plants. Because of the short lifetime of *Arabidopsis* shoots the growth assay was only performed for a maximum of 14 d. For each day of this time period shoot material at the infection site was harvested and further analysed.

2.2.2.7 Confocal laser scanning microscopy

Live cell imaging of *A. thaliana* shoot cuts was performed with Leica TCS SP8 (Leica, Wetzlar, 35578 Germany). GFP, YFP and mKate fluorescence was excited with 488 nm, 514 nm and 594 nm lasers, respectively. The detection of GFP was performed with a hybrid photodetector (HyD) with single molecule detection (SMD) in a range of 496 nm -522 nm. YFP was detected using a HyD in a range between 521 nm and 576 nm. mKate was detected with a photomultiplier tube (PMT) in a range between 601 nm and 651 nm. Pinhole, detector gain and digital gain settings were adjusted to provide an optimal balance between fluorescence intensity and background signal. The resulting images were further processed with Fiji (Schindelin *et al.*, 2012).

2.2.3 Bioinformatical analysis

2.2.3.1 Fluorescence analysis through Fiji and Python

Fluorescence images taken with confocal laser scanning microscopy (see 2.2.2.7) were processed using Fiji software (version 1.52i). Using the LIF projector plugin maximum intensity projections for every picture were created. Ratiometric quantification was performed using the Ratiometry Plugin. The constitutive mKate2 fluorescence was used for the normalization of the phytohormone-dependent Venus fluorescence. Plugins were provided by Mohamed El-Sayed (member of Prof. Volker Lipka laboratory). The intensity values and the number of fluorescent nucleii were collected in an excel file (Excel, Microsoft, Redmond, Washington, USA).

A custom Python program (Van Rossum & Drake, 2009) was used to get mean intensity and number of nuclei automatically. The code of the program is stored in the digital supplemental data. Final results were summarized in Excel bar diagrams.

2.2.3.2 Whole Genome Sequencing (WGS)

Two stably transformed *S. lycopersicum* M82 plants of separate plant lines were selected and gDNA was extracted. The DNA was then sent to Quantitative Biology Center (QBiC, University of Tübingen, Tübingen, Germany) for whole genome sequencing.

2.2.3.3 Gas Chromatographie Mass Spectrometry

A. thaliana shoots were harvested during *C. reflexa* growth assays (see 2.2.2.6). Samples were transferred to the GC-MS facility and further processed by Joachim Killian after the following protocol:
Around 150 mg of Arabidopsis material were snap frozen in liquid N_2 and stored at -80 °C. The material was transferred to 2 ml Screwcap tubes from Sarstedt. The exact weight was noted and one steel ball (5 mm) was added. Samples were ground with a Retsch Mixermill two times 0.5 min at 30 Hz, before and in-between milling steps they were cooled with liquid N_2. Two times 750 µl of Extraction solution (ethyl acetate with 0.1% formic acid containing the internal standard (3HOBA 60 ng, DHJA 80 ng and 5IFA 50 ng per ml)) were added and the samples incubated shaking horizontally. Sonification was performed 10 min in an Ultra sonic bath. The incubation was done shaking for 1 h at 28 °C at 1400 rpm in Eppendorf Shaker (Type Thermomixer C). Afterwards the samples were spun down 10 min at 13000 rpm in a Hettich table top Micro 220C centrifuge equipped with a swing out rotor. Two times 600 µl (1200 µl in total) of the extracts supernatant were transferred to a fresh 1.5 ml test-tube. For hydrolysates continue with the pellet. To remove the solvent to dryness the Eppendorf Vacuum concentrator (Mode HV) at 30 mbar at the "Vaculan" was used for about 1 h. Derivatisation was performed through adding 70 µl of 1:1 fresh mix of methanol and TMSDM (Trimethylsilyldiazomethan) to the dry samples. The samples were again incubated for 35 min shaking at 24 °C and 1200 rpm in an Eppendorf Shaker. The solution was transferred into vials and submitted to GCMS (Shimadzu TQ8040) in splitless MRM Mode.

For the rest of the sample a hydrolysis was performed removing all of the remaining ethyl acetate phase without touching the "water phase". Let the rest evaporate (approx. 45 min). The steel balls were removed and 300 µl of 3M HCl were added. Incubation was done shaking for 1h at 50°C at 1400rpm in Eppendorf Shaker (Type Thermomixer C). Afterwards 300 ul 3 M NH3 were added followed by vortexing. The extraction solution was added (1000 µl). Incubation was continued shaking for 1 h at 28 °C at 1400 rpm in Eppendorf Shaker (Type Thermomixer C). Samples were again spun down for 10 min at 13000 rpm in a Hettich table top Micro 220C centrifuge equipped with a swing out rotor. 700 µl supernatant were transferred to a fresh 1.5 test-tube. The solvent was removed with an Eppendorf Vacuum concentrator (Mode HV) at 30 mbar at the "Vaculan" for about 40 min. Derivatisation was performed with 70 µl of 1:1 fresh mix of methanol and TMSDM (Trimethylsilyldiazomethan Aldrich) added to the dry samples. The derivatisation was followed by an incubation for 35 min shaking at 24 °C at 1200 rpm in an Eppendorf Shaker. Samples were transferred to vials and submitted to GC-MS (Shimadzu TQ8040) in splitless MRM Mode.

2.2.3.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS measurements were performed by the research group jasmonate function & mycorrhiza from Prof. Bettina Hause in the department of cell and metabolic biology in the Leibniz Institute of Plant Biochemistry (Halle (Saale)). The used method is described in Balcke et al., 2012.

3 Results

3.1 Resistance against *C. reflexa*

The obligate stem holoparasite *C. reflexa* has a broad host spectrum. Nevertheless, there are a few plants that are able to recognize the pest. One of those plants is the cultivated tomato *S. lycopersicum*. Upon recognition of the parasite different immune responses are triggered inside the tomato like ethylene production and hypersensitive response at the infection site, making the tomato resistant to *C. reflexa.* In this work further resistance relevant genes besides the already described LRR-RLP encoding gene *CuRe1* should be identified.

3.1.1 Relation of ethylene production and hypersensitive response

The responsible receptor for ethylene response upon *C. reflexa* infection of the resistant *S. lycopersicum* could be mapped to chromosome 8, more precisely to the LRR-RLP encoding gene *CuRe1* (Hegenauer *et al.*, 2016).

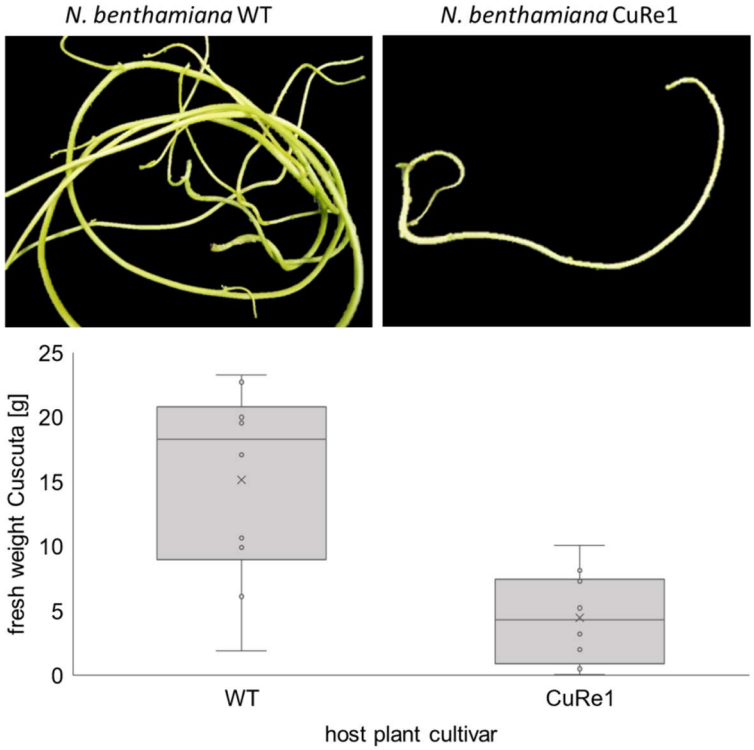


Figure 22: Biomass reduction in stably transformed CuRe1 *N. benthamiana*. *C. reflexa* was grown on *N. benthamiana*, either wildtype or CuRe1 expressing, for four weeks. Biomass increase was measured (n=10). T-Test: p=0,00023.

The ability of the CuRe1 to influence ethylene dependent resistance is described in chapter 1.3.3.

Overexpression of the receptor in the susceptible host *N. benthamiana* confirmed a reduction in biomass production of the parasite during infection (Hegenauer *et al.*, 2016). These results could be reproduced in this work (see Figure 22).

A second resistance trait, which should be analysed, is the hypersensitive like response (HLR). This trait should be analysed using a library of introgression lines. Introgression lines are crossings between the resistant *S. lycopersicum* and the susceptible *S. pennellii* which were selected for different homozygous recombinations per separate line, spanning the whole genome (Eshed & Zamir, 1995, Chitwood, *et al.,* 2013).

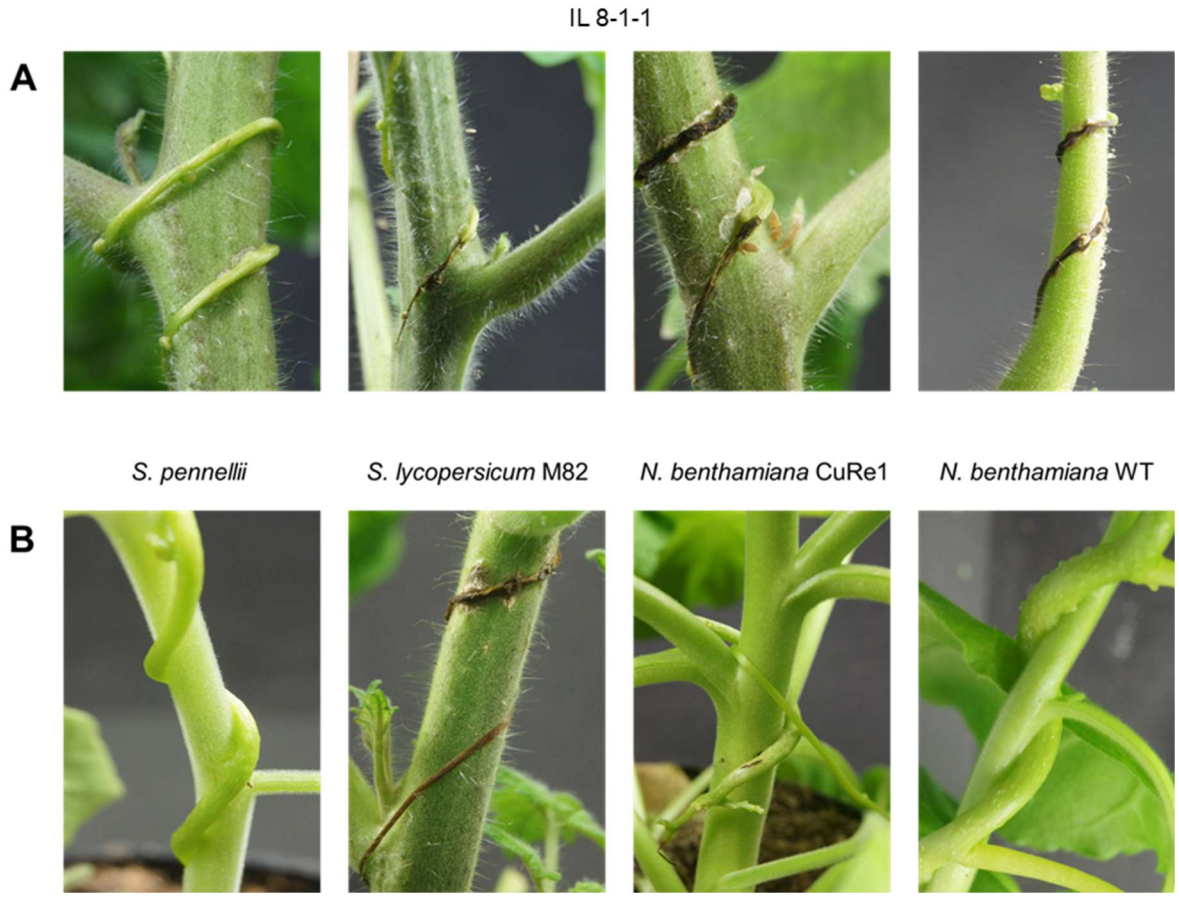


Figure 23: HLR phenotypes in CuRe1 relation. Figure A displays different *S. lycopersicum* plants belonging to the introgression line 8-1-1. They show differences in susceptibility to *C. reflexa* and HLR symptoms. Figure B displays a comparison of the HLR phenotype of the resistant *S. lycopersicum*, the susceptible *S. pennellii* and *N. benthamiana* as well as the CuRe1 expressing *N. benthamiana*. *C. reflexa* was grown on each plant for 4 weeks.

Regarding this trait, the introgression line 8-1-1, which lacks *CuRe1*, shows an inconsistent phenotype. Some of the plants allow little growth of *C. reflexa*

resembling the before mentioned *N. benthamiana* overexpression phenotype, some do not show *Cuscuta* growth at all and all of them show HLR symptoms, characterized through necrotic cell lesions, although in differing strength (see Figure 23A). Especially the pictures shown in Figure 23A display the different phenotypes of surviving *Cuscuta* (first picture) and dying *Cuscuta* (second, third and fourth picture) with most prominent HLR lesions in the third picture at the region of connection between *C. reflexa* and *S. lycopersicum* IL 8-1-1 and the close bordering regions. This underlines the inconsistent phenotype of the IL8-1-1 lacking *CuRe1* but still displaying resistance. Furthermore, the stably *CuRe1*-transformed *N. benthamiana* plants did not show an HLR phenotype upon *C. reflexa* infection and resemble, leaving the biomass reduction aside, more the susceptible phenotype of wildtype *N. benthamiana* or *S. pennellii* (see Figure 23B).
Concluding from the aforementioned seemingly independent HLR relation to CuRe1 the idea of a second resistance locus in *S. lycopersicum* arose.

3.1.2 Screening of introgression lines

For further mapping of HLR resistance related responses in the *S. lycopersicum* genome the introgression lines (ILs) were screened for hypersensitive response upon *C. reflexa* infection similar to Kaiser, 2019. The library screen of all introgression lines allowed the localization of a putative HLR responsible locus on chromosome 12 (see Figure 24). A less prominent HLR and susceptibility against *C. reflexa* was visible for plants belonging to the introgression lines 12-2 and 12-3A. These lines contain *S. pennellii* introgressions in *S. lycopersicum* background on chromosome 12 and therefore indicate the existence of a resistance trait in this region.
The original IL 12-3 (Eshed & Zamir, 1995) did show a varying susceptibility against *C. reflexa.* In conclusion, seeds of resistant and susceptible plants were collected and designated 12-3B and 12-3A respectively (Kaiser, unpublished). The screen from Kaiser, 2019 also found IL 12-3-1 susceptible. A repetition of this could not be achieved, all of the tested plants (n=19) were resistant to *C. reflexa* infection.

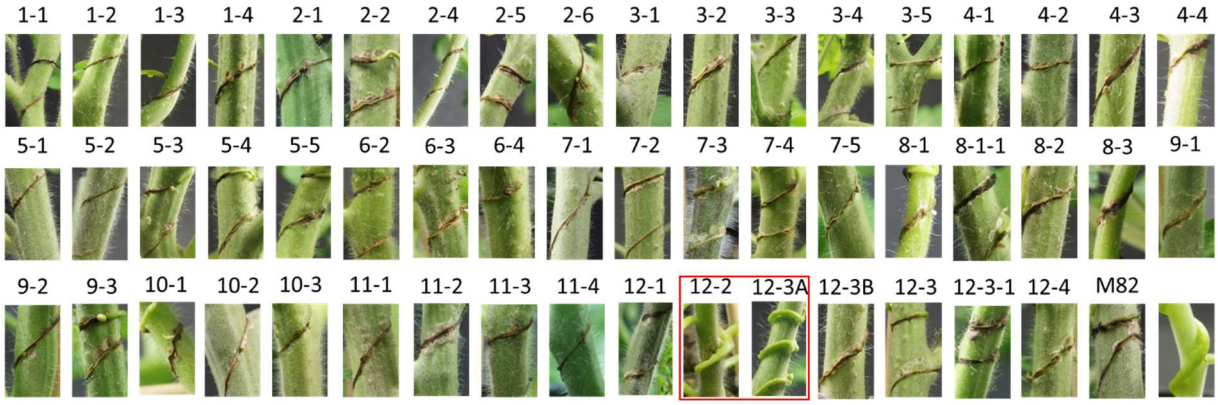


Figure 24: Hypersensitive response of introgression lines against *C. reflexa*. *C. reflexa* was grown for four weeks on different introgression lines (1-1 until 12-4) and the wildtype parent species *S. lycopersicum* M82 and *S. pennellii*. The tomato lines 12-2 and 12-2A marked in red were susceptible.

3.1.3 Genotyping of unconfirmed lines

Addressing the discrepancy of the original Kaiser, 2019 screen of the introgression lines and the screen performed in this work the plant lines of question should be genotyped. Therefore, a selection of genes which is located inside the expected introgression spanning across the recombination border (Eshed & Zamir, 1995; Chitwood *et al.,* 2013) of the line 12-3-1 was chosen for genotyping. Table 8 shows the results of genotyping of 19 genes (Fernandez-Pozo *et al.*, 2015, ITAG4.0) compared with the expected results for this genotyping attempt. The tested genes *Solyc12g009660*, *Solyc12g009670*, *Solyc12g009680* and *Solyc12g009690* are located inside the recombination site of the ILs 12-3 and 12-3-1 (Chitwood *et al.*, 2013). The other genes shown in Table 8 are located directly at the border of outside of the expected recombination site. For all of those genes a 500 bp fragment was attempted to be amplified.

Several genes that were expected to be present on the tested tomato wildtype and introgression lines were not amplifiable and vice versa. Especially the ability to amplify gene fragments of the *S. lycopersicum* genes in *S. pennellii* gDNA indicates a high similarity of both genomes in the ROI. Even though the primers were tested *in silico* for their uniqueness in the *S. lycopersicum* and against the *S. pennellii* genome with the help of the Solgenomics database (Fernandez-Pozo *et al.*, 2015) this unexpected amplification was possible. Thus, the genotyping seemed not to be suitable to tackle the discrepancy of the different HLR screens of the introgression lines and possibly a more in depth view e.g. via sequencing would be necessary. Since the *S. lycopersicum* genes showed between 84 % and 99 % identity with

corresponding regions and genes in the *S. pennellii* genome (ncbi blastn; Altschul *et al.*, 1990) no great differences through sequencing can be expected. Additionally, there is a possibility of slightly differing introgression sizes since, at least the original ILs (Eshed & Zamir, 1995), were identified through DNA markers and not through in-depth sequencing. Those differing introgression sizes could include or exclude genes which were originally expected to be inside or outside of the recombination side.

Table 8: Genotyping of different tomato cultivars. Shown is the presence (Yes, green) or absence (No, red) of 500 bp gene fragments after genotyping of tomato gDNA in comparison to the expectation according to sequence data (Fernandez-Pozo *et al.*, 2015; Chitwood *et al.*, 2013).

	***S. lycopersicum* M82**		***S. pennellii***		**IL 12-3**		**IL 12-3-1**	
gene name	expected	genotyped	expected	genotyped	expected	genotyped	expected	genotyped
Solyc12g009510	Yes	Yes	No	Yes	No	Yes	Yes	No
Solyc12g009520	Yes	Yes	No	Yes	No	Yes	Yes	Yes
Solyc12g009530	Yes	Yes	No	Yes	No	Yes	Yes	Yes
Solyc12g009550	Yes	Yes	No	No	No	Yes	Yes	No
Solyc12g009560	Yes	Yes	No	Yes	No	Yes	Yes	No
Solyc12g009565	Yes	No	No	Yes	No	No	Yes	Yes
Solyc12g009570	Yes	Yes	No	Yes	No	No	Yes	No
Solyc12g009580	Yes	No	No	Yes	No	No	Yes	No
Solyc12g009590	Yes	Yes	No	No	No	Yes	No	No
Solyc12g009600	Yes	Yes	No	No	No	No	No	No
Solyc12g009610	Yes	Yes	No	Yes	No	No	No	Yes
Solyc12g009620	Yes	Yes	No	No	No	Yes	No	Yes
Solyc12g009630	Yes	No	No	No	No	No	No	No
Solyc12g009640	Yes	Yes	No	Yes	No	No	Yes	No
Solyc12g009650	Yes	Yes	No	Yes	No	Yes	No	No
Solyc12g009660	Yes	Yes	No	Yes	No	Yes	No	Yes
Solyc12g009670	Yes	No	No	Yes	No	No	No	No
Solyc12g009680	Yes	Yes	No	No	No	No	No	No
Solyc12g009690	Yes	Yes	No	Yes	No	Yes	No	No

3.1.4 Search for chromosome 12 candidates

In order to reduce the number of potential resistance related candidate genes the chromosomal ROI should be limited. Taken into consideration the susceptibility of the IL12-2, IL12-3A and IL12-3-1 from Kaiser, 2019, a first reduction of candidate genes could be achieved (see Figure 25). The IL12-2 includes introgressions beginning at BIN d-12A up to and including d-12F.2 (Ofner *et al*. 2016). Concerning gene ranges this means genes between Solyc12g005000 and Solyc12g082750 were exchanged

for *S. pennellii* genes on the resembling region on their chromosome. Actually, IL12-2 contains two introgressions named 12-2A and 12-2B with a smaller gap in between (Chitwood *et al.*, 2013). IL 12-3 contains gene exchanges for BIN d-12E, d-12F.1, d-12G.1, d-12F.2, d-12G.2 and 12-12H and all genes between Solyc12g009660 and Solyc12g099090. Finally, IL 12-3-1 possesses a chromosomal exchange for BIN d-12E meaning from Solyc12g009660 to Solyc12g019020. The overlapping region of all those ILs should contain the second resistance trait but because of the aforementioned differences in the original Kaiser, 2019 screen and the screen performed in this work, the chosen ROI was enlarged. Afterwards it was analysed for promising candidates genes. Due to the high abundance of LRR-RLP encoding genes in the region and their well-known function in pathogen defence related responses, the ROI was focused to a part containing most of PRR encoding genes.

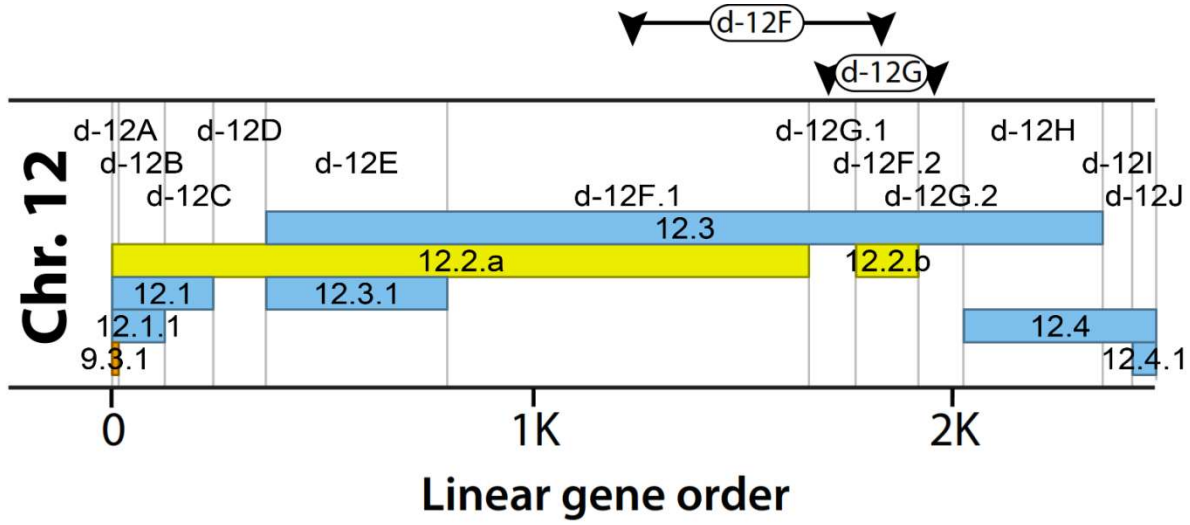


Figure 25: Map of *S. pennellii* introgression lines, chromosomes 12 (modified from Chitwood *et al.*, 2013). A map showing the architecture of ILs based on precisely defined introgression boundaries determined from the sequenced tomato genome and next-generation sequencing data. IL size is proportional to the number of annotated genes harboured in each introgression. Bins, or intervals defined by unique combinations of IL overlap, are indicated with a "d-" prefix. Note that ILs can be non-contiguous (indicated yellow) as well as bins (indicated above graphs with arrowheads and lines). Recombination can also happen on different chromosomes like 9.3.1 were the main introgression is on chromosome 9 and a smaller one on chromosome 12 (indicated in orange).

This region will be further referred to as GB and starts with Solyc12g009690, a putative receptor-like protein 12, and ends with Solyc12g010660, a putative NBS-LRR protein also known for immune related responses. Additionally, another chromosomal region bordering this region should be analysed. This region also contains several putative LRR-RLP encoding genes and includes the genes from Solyc12g009510 to Solyc12g009690. It is further referred to as VB and was chosen

taking possible inaccuracies during IL sequencing into consideration. The importance of those regions of interest for HLR response of tomato during *Cuscuta* infection was further analysed.

3.1.5 Silencing of LRR-RLP and other possible disease resistance genes by RNAi approach

The importance of LRR-RLP encoding genes in the ROI GB for resistance should be elucidated. An appealing technique for this purpose was RNA interference (RNAi). It uses artificially created DNA fragments which are cloned inside an appropriate DNA vector and are then transcribed inside the target organism to create double stranded RNA (dsRNA). These RNA fragments are used by the cells innate immune system to destroy transcribed mRNA of genes with sequences homologous to the dsRNA. It can be used to reduce the expression of GOIs. Therefore, RNAi silencing constructs were created that target homologous regions of all LRR-RLP encoding genes inside the GB ROI and enable a silencing of several of those genes at once. An *in silico* alignment of the GOIs Solyc12g009690, Solyc12g009720, Solyc12g009730, Solyc12g009740, Solyc12g009745, Solyc12g009770, Solyc12g009870 was performed. An alignment of homologous fragments of those genes can be seen in Figure 26.

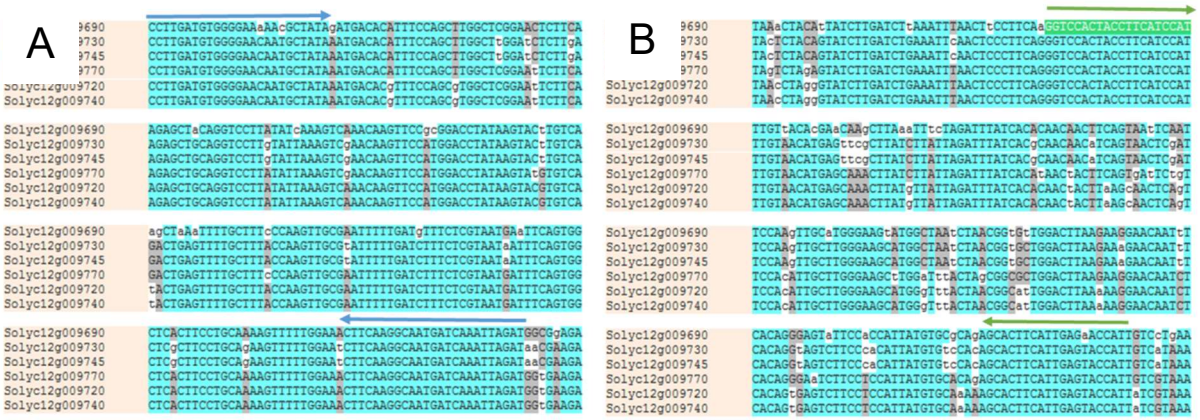


Figure 26: Alignment of LRR-RLP encoding genes inside the ROI GB (created with CLUSTAL W; Thompson *et al.* 1994). Letters shown in blue indicate homology between the different genes, letters highlighted in grey and white indicate similarity between only a few of the aligned genes. The blue arrows in the left figure (A) indicate the start and end regions for RNAi target site amplification. The green arrows in the right figure (B) indicate the primer pair used for qRT-PCR. Figure 26 A and B display distinct sequence sites of the GOIs.

For the shown genes in Figure 26A one common RNAi silencing construct could be generated but the LRR-RLP gene Solyc12g009870 and the NBS-LRR gene

Solyc12g010660 were too dissimilar to be silenced by the same RNAi construct. Accordingly, separate constructs for these two genes were created. The final silencing constructs were stably transformed into *S. lycopersicum* M82 and checked for expression. The generated plants were then checked for *C. reflexa* susceptibility. A total number of 57 separate RNAi plant lines were created. The silencing success was evaluated using qRT-PCR. For this purpose primers were designed which bind also in the homologous region of the LRR-RLP encoding genes but at a different position (see Figure 26B). The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method as described in Livak & Schmittgen, 2001. The expression reduction in the stably transformed tomato lines was compared to the wild type expression in *S. lycopersicum* M82. Selected representative results are shown in Figure 27. The representatives were tested for expression of all genes and with expression primers shown in Figure 26B.

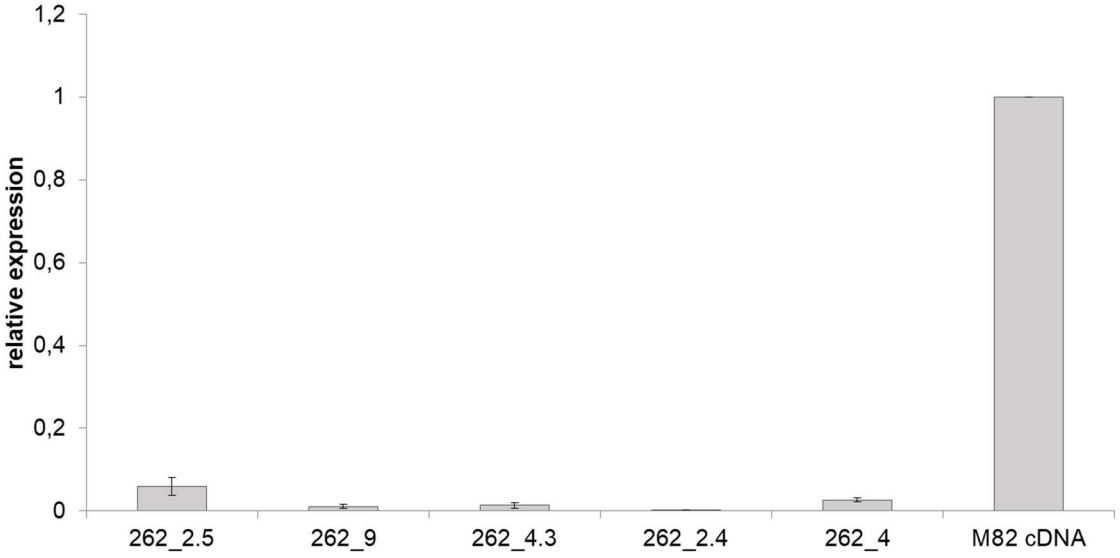


Figure 27: qRT-PCR of *S. lycopersicum* stably transformed with RNAi silencing constructs. Shown is the relative expression of several mutant plants carrying the same silencing construct (plant line named 262). Plants are not numbered continuously (detailed list of mutant plants in digital supplemental data). The wild type expression of M82 cDNA is also shown. Every bar indicates the expression of a single independent mutant plant line with 3 technical replicates in relation to wild type expression (M82 cDNA). Data represent mean values.

As shown exemplarily in Figure 27 the general expression of the RNAi target genes could be reduced in comparison to wild type. The targeted genes in this approach are *Solyc12g009690*, *Solyc12g009720*, *Solyc12g009730*, *Solyc12g009740*, *Solyc12g009745* and *Solyc12g009770* (see Figure 26) in all of the tested mutant plant lines 262_2.5, 262_9, 262_4.3, 262_2.4 and 262_4 (see Figure 27).

Nevertheless, the growth assay using *C. reflexa* to infect these mutant plants showed no changed phenotype concerning HLR and resistance to resistant wild type (see Figure 28).

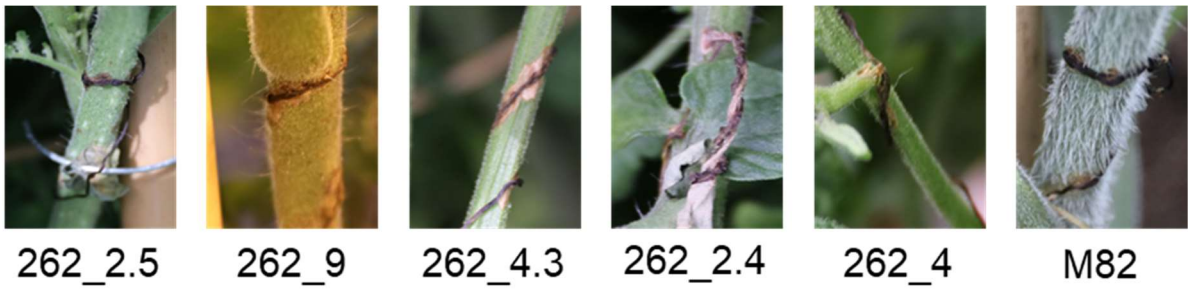


Figure 28: Growth assay of *C. reflexa* on RNAi tomato mutants. Shown are infection sites on *S. lycopersicum* M82 wild type and stably transformed RNAi silenced plants. The dying *Cuscuta* was documented 4 weeks after infection.

Since, even though the silencing of the GOIs was a success, no change in infection phenotype could be observed the tested LRR-RLPs seem to be not essential for immunity. Some obvious conclusions are that either minimal expression of receptors is still sufficient for an immune response or the LRR-RLPs and the NBS-LRR are not responsible for the HLR/resistance. To address this issue another mutation approach was used.

3.1.6 CRISPR-Cas9 Deletions

The CRISPR-Cas9 system is a tool for genetic editing using the Cas9 enzyme. This enzyme is guided by a single guide RNA (sgRNA) containing a specific target site to its designated location of action and induces a double strand break at said position. Through mistakes of the cells innate repair machinery small mutations at the target site can be induced. In this approach the Cas9 enzyme was combined through Golden Gate cloning with four different sgRNAs. The target sites were located at the borders of chromosomal loci flanking regions as small as 35 kb and as big as 668 kb. With this approach six smaller regional deletions inside the ROI (see 2.2.1.19) should be introduced named B1, B2, B3, B4, GB and VB (see Figure 29).

The deletion constructs containing the Cas9 and sgRNA expressing modules were stably transformed into *S. lycopersicum* M82 and genotyped for success and character of the deletion (see Figure 21 in section 2.2.1.19). Single site mutation, small deletions between the two flanking target site and large deletions between two of the target sites at opposite borders of the ROI were possible. Additionally, different

combinations of those mutations were also possible as well as homo- and heterozygosity and no mutations at all.

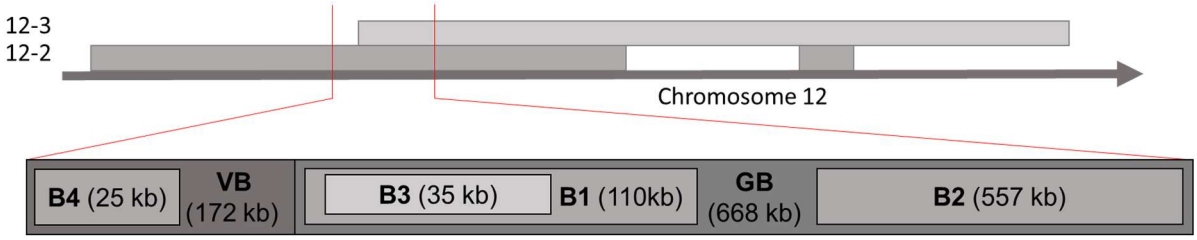


Figure 29: CRISPR-Cas9 deletion scheme in IL context. Displayed are the chromosomal regions B1, B2, B3, B4, GB and VB which should be deleted through a CRISPR-Cas9 approach. The introgressions of the ILs 12-3 and 12-2 are also schematically shown on chromosome 12. The red lines indicate the complete target region for the CRISPR-Cas9 mutagenesis in relation to those introgression lines. The numbers indicate the expected deletion size in kb.

In total a number of 278 stably transformed tomato lines carrying CRISPR-Cas9 deletion constructs for the 6 chromosomal regions (B1, B2, B3, GB, VB) were created and further analysed (see digital supplemental data).

3.1.7 Analysis of mutant lines containing CRISPR-Cas9 deletion constructs

The generated stably transformed tomato plants, which were potential subject to CRISPR-Cas9 mutation, were tested for a change in susceptibility against *C. reflexa* in a growth assay. Therefore, the tomato plants main shoot was infected with *C. reflexa* and growth of the parasite was documented over a course of four weeks. In resistant plants the parasite tries to invade the host plant through haustoria which is countered by a HLR of the resistant target plant. The HLR symptoms get stronger shutting of the parasitic organs and the lack of nutrients finally leads to the starvation of the parasite. During this time nearly no growth of the parasite is visible. The whole process usually takes four weeks on a resistant tomato. Mutants belonging to potential deletion plant lines for B1, B2, B3, B4, GB showed the same pattern as a resistant tomato plant, a representative example is shown in Figure 30. The exact phenotype of the individual mutant plants is summarized in the mutant list (digital supplemental data). Not all plants were genotyped and tested for ethylene production but all of them were phenotyped.

Figure 30: Growth assay of *C. reflexa* on stably transformed *S. lycopersicum* M82. Shown is an example of a stably transformed tomato line which showed a large deletion in the GB region through genotyping. At week 1 first advances of *C. reflexa* trying to infect the plant are visible. Afterwards in week 2 first HLR symptoms are visible which get stronger at week 3 and are usually followed by death of the parasite at week 4.

Additionally, ethylene production of all plants was measured with crude *C. reflexa* extract as elicitor. This should give a hint at potential CuRe1 involvement as it is known that wild type tomato produces ethylene as response to *C. reflexa* infection and an unchanged ethylene metabolism would indicate a CuRe1 independent resistance. Unfortunately, it was not possible to get a reliable and stable response from the first generation of generated plants, possibly through transformation effects but most of them showed ethylene production to some extent (data for measured plants in digital supplemental data).

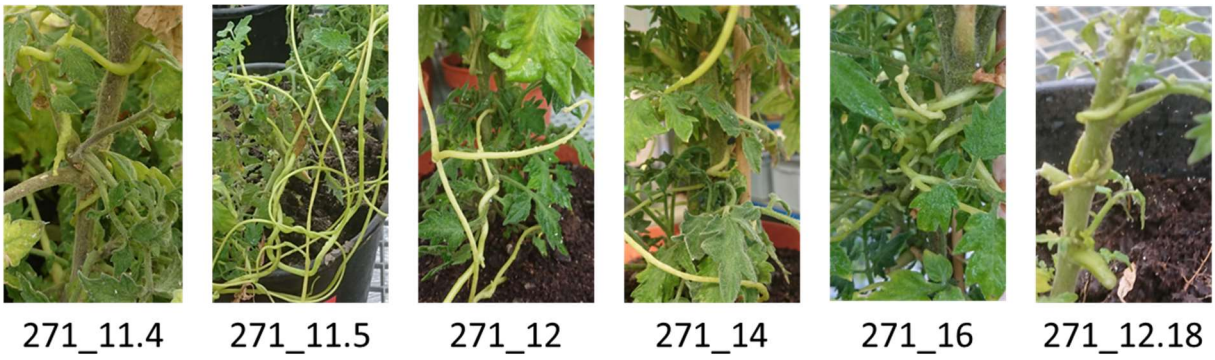


Figure 31: Susceptible tomato mutants. Plants of six different plant lines are shown 4 weeks after *C. reflexa* infection. All of them show *C. reflexa* biomass increase and less or no HLR symptoms. The plant 271_12.18 is a special case and shows no significant *Cuscuta* biomass increase but also no starvation or HLR symptoms. All of those plant lines lack the VB region (see Figure 29).

During the growth assays several plants belonging to the deletion line for VB (see Figure 29, also named 271) showed *C. reflexa* susceptibility and weakened HLR symptoms. Out of 65 plant lines created through stable transformation with CRISPR-

Cas9 deletion constructs for VB six lines showed susceptibility to *C. reflexa* with slightly varying degree (see Figure 31). To further narrow down the number of candidates the six susceptible plants were genotyped for presence and size of deletion as described in section 2.2.1.20. All of those susceptible transformants showed the presence of a large deletion of approximately 172 kb but probably in a heterozygous manner (see Figure 32). The resulting shortened chromosomal sequence of chromosome 12 at the deletion site was amplified, cloned and sequenced. This showed that the deletion site resembled the expected shortened chromosome version based on the possible double strand breaks through the Cas9 enzyme.

Primer Pair	Amplification	Conclusion
A+B	Succesful	Small or no mutation at the target site
C+D	Succesful	Small or no mutation at the target site
E+F	Succesful	Small or no mutation at the target site
A+F	Succesful	Big deletion (heterozygous)

Figure 32: Scheme for genotyping of VB deletion mutants. The upper bar represents the whole VB region on *S. lycopersicum* M82 chromosome 12 and is bordered by the two genes Solyc12g009510 and Solyc12g009690. The used target sites for CRISPR-Cas9 deletion of the whole region are displayed with grey arrows. The primer pairs flanking those regions and used for genotyping (A-F) are indicated by red arrows. The table summarizes the genotyping result (Amplification) and their interpretation (Conclusion) of the six susceptible *S. lycopersicum* plant lines.

The genotyping results for the three plants used for follow up experiments utilizing the sequence amplification method displayed in Figure 32 are summarized in Table 9. The results indicate also several homozygous deletions but since such deletions are unlikely for such big chromosomal regions smaller mutations at the target sites resulting in smaller deletions are more probable. To further validate that the shown effect is exclusively correlated to the deletion and not to off-targets of the CRISPR-Cas9 system a whole genome sequencing was performed (see section 2.2.3.2).

Table 9: Gentoyping of CRISPR/Cas9 mutant plants.

Mutant Plant	Primer Pair	Amplification	Conclusion
271_12	A+B	No	Heterozygous 172 kb deletion
	C+D	No	
	E+F	Yes	
	A+F	Yes	
271_14	A+B	No	Homozygous 172 kb deletion
	C+D	No	
	E+F	No	
	A+F	Yes	
271_16	A+B	No	Homozygous 172 kb deletion
	C+D	No	
	E+F	No	
	A+F	Yes	

This should also give a conclusion about the homo- or heterozygosity of the mutant plants. Unfortunately, no new insights could be obtained through the sequencing except for a possible confirmation of heterozygosity since no feature counts of candidate genes could be obtained except for Solyc12g009520, Solyc12g009540, Solyc12g009670 and Solyc12g009690. Those genes possibly were homozygously deleted but the accuracy of the sequencing approach was too low to give certainty on this matter (data not shown).

3.1.8 Functional Analysis of the New Candidate Genes found by CRISPR-Cas9 deletion

The deletion of the VB region, which led to susceptibility of *S. lycopersicum,* revealed 19 potential resistance relevant genes. These genes are located inside the 172 kb deletion in *S. lycopersicum* (see 3.1.7) and are listed in Table 10.

The candidate genes (see Table 10) were extracted from Genome Browser provided by the Sol Genomics Network (Fernandez-Pozo *et al.*, 2015, database: ITAG 4.0). To reveal their relevance in resistance phenotypes several analyses were performed. First of all, the increase of *Cuscuta* biomass and the weakened HLR already described in chapter 3.1.7 seem to indicate an importance of one or several of those genes in immunity. Therefore, amplification of those genes was performed and the

resulting amplicons of those genes were cloned into expression vectors and transiently expressed in *N. benthamiana*.

Table 10: New Candidate genes located in the region deleted by CRISPR-Cas9 approach

Gene name	Annotation (ITAG4.0; Fernandez-Pozo *et al.*, 2015)
Solyc12g009510.1.1	receptor-like protein 12
Solyc12g009520.2.1	receptor-like protein 12
Solyc12g009530.1.1	receptor-like protein 12
Solyc12g009550.2.1	Leucine-rich repeat receptor-like protein kinase PXL1
Solyc12g009560.2.1	EIN3-binding F-box protein 1
Solyc12g009565.1.1	Unknown protein
Solyc12g009570.3.1	calcineurin B-like interacting protein kinase
Solyc12g009580.2.1	CRABS CLAW 5b
Solyc12g009590.2.1	Methyl-CpG-binding domain-containing protein 11
Solyc12g009600.2.1	Thylakoid lumenal 16.5 kDa protein
Solyc12g009610.2.1	RabGAP/TBC domain-containing protein
Solyc12g009620.2.1	Ubiquitin system component Cue protein
Solyc12g009630.3.1	Calmodulin-like protein 5
Solyc12g009640.1.1	zinc finger BED domain-containing protein DAYSLEEPER-like
Solyc12g009650.2.1	Sl proline-rich protein
Solyc12g009660.3.1	Exostosin-like protein
Solyc12g009670.1.1	Exostosin-like
Solyc12g009680.2.1	Heptahelical transmembrane protein 1
Solyc12g009690.1.1	receptor-like protein 12

It was not possible to get expression vectors for all of the candidate genes. Afterwards, the immune response of those plants was tested in different immunologic assays.

First, the ability to induce an HLR was analysed in *N. benthamiana* CuRe1 plants. This is a tobacco line which is stably transformed with CuRe1 - a *C. reflexa* recognizing receptor - and is therefore suitable to analyse possible downstream functions of the tomato candidate genes. A transient transformation of *N. benthamiana* CuRe1 with different tomato candidate genes did not show an HLR

production in response to plant treatment with crude *C. reflexa* extract. Figure 33 displays representative results of lacking HLR production for several GOIs also true for all other tested candidate genes. These results indicate that either transient transformation of *N. benthamiana* is not suitable for production of HLR phenotype since a different plant system could lack essential genes of the resistance pathway or the genes are involved in resistance but not in HLR production.

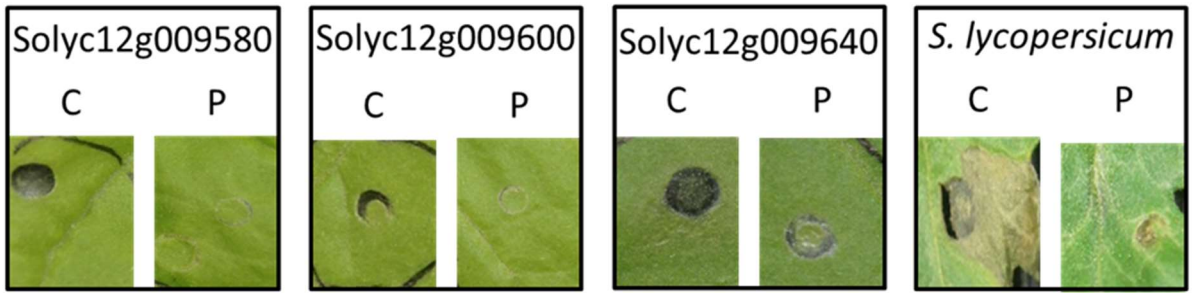


Figure 33: HLR test of candidate genes in *N. benthamiana* CuRe1 and in the resistant *S. lycopersicum*. Tobacco leaves were infiltrated with *A. tumefaciens* containing an expression vector for different *S. lycopersicum* genes (first three figures). Two days after infiltration infiltrated leaves were treated with 20 µl crude extract from *C. reflexa* (C) MES buffer (P). Additionally, *S. lycopersicum* (figure four) is shown as positive control for HLR development. Leaves displayed 4 days after extract treatment.

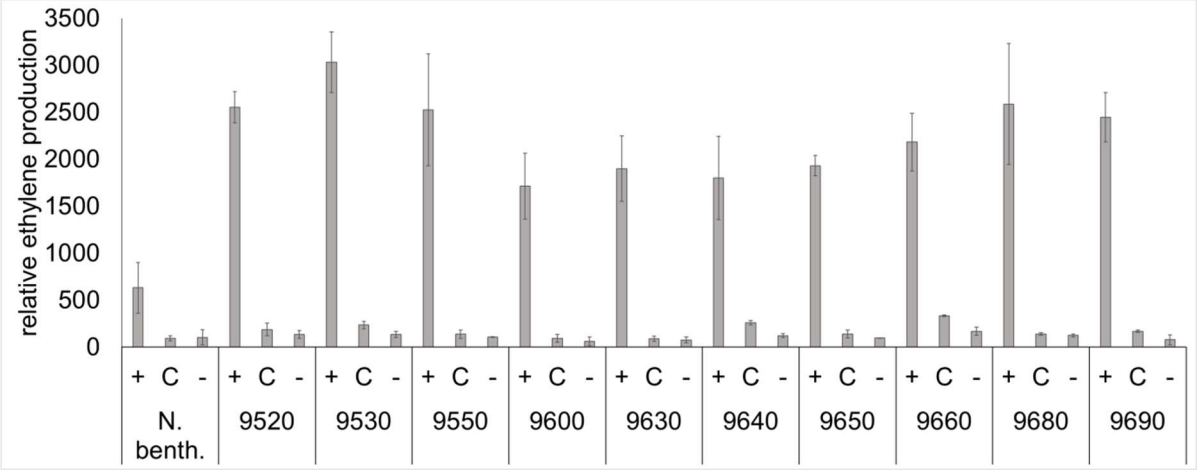


Figure 34: Relative ethylene production of transiently transformed *N. benthamiana*. Shown is the ethylene production of *N. benthamiana* wild type or transient transformants infiltrated with *A. tumefaciens* carrying expression vectors for different tomato genes. Gene names are displayed as abbreviations. Full names start with Solyc12g00 followed by the numbers in the diagram. The plants were treated with 100 nM flg22 as positive control (+), buffer as negative control (-) or crude *C. reflexa* extract (C). n = 3; Data represent mean values +/- standard deviation.

Another assay to test the capability of plants to react to different pathogens is the observation of the ethylene production. For this purpose, transiently transformed *N. benthamiana* containing different candidate genes from tomato (see Table 10) were

treated with flg22 (positive control), *C. reflexa* crude extract or buffer (as negative control) and the amount of ethylene produced was measured (see section 2.2.2.5). The results for the currently available candidate genes are displayed in Figure 34. The results are true for all tested GOIs.

Since *N. benthamiana* CuRe1 plants are able to produce ethylene in response to *C. reflexa* extract on their own, wildtype *N. benthamiana* was used for this assay. The treatment of the transiently transformed plants with *C. reflexa* extract did not reveal the production ethylene as specific response compared to wild type *N. benthamiana*.

Together, the results indicate that the transient transformation of susceptible host plants with single immune candidate genes from *S. lycopersicum* is not sufficient to render the plants resistant to *C. reflexa*, possibly through limitations of the method, downstream dependencies of CuRe1 with the candidate genes or quantitative trait properties of the combined candidate genes.

3.1.9 Genotyping Analysis

Similar to the initial genotyping of the *S. lycopersicum* wildtype and introgression lines as well as the *S. pennellii* wildtype (see 3.1.3) a genotyping of the mutant lines was performed. Since *S. pennellii* possesses DNA sequences that share a high similarity with *S. lycopersicum* for the GOIs, a new genotyping of deletion mutant lines could reveal genetic changes in a more obvious manner. Deletion of whole regions or single genes should be unaffected by similarities of *S. lycopersicum* and *S. pennellii* genomes and therefore be visible in genotyping PCRs. The results of such amplifications are shown in Table 11. The green fields indicate a successful amplification of fragments of the respective genes for the different plant lines. The wild type lines *S. lycopersicum* and *S. pennellii* showed nearly the same amplification results and so did the CRISPR-Cas9 created deletion mutant lines 271_12, 271_14 and 271_16. The remaining confirmed CRISPR-Cas9 deletion lines could not be further analysed since they did not survive. These results confirm the limitations of the genotyping method via PCR for the mutant lines as described in 3.1.3 and could again be to some extent a result of a high similarity of the genetic loci in both tomato cultivars. Nevertheless, the primer pairs for genotyping were tested for homology inside the *S. pennellii* genome and most of them showed high mismatch potential or wrong orientation on the chromosomal region. However, also off target amplifications are possible on distant regions in the genome. More likely in this case is the

heterozygosity of the CRISPR-Cas9 mutated plants. Since during a deletion the highly homologous regions should be deleted and therefore not be amplifiable in any way.

Table 11: Genotyping of deletion lines in comparison with tomato wildtypes. Shown is the presence (Yes, green) or absence (No, red) of 500 bp gene fragments after genotyping of tomato gDNA in comparison to the expectation according to sequence data (Solgenomics database, Fernandez-Pozo *et al.*, 2015).

Gene name	M82	S. penn.	271_12	271_14	271_16
Solyc12g009510.1.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009520.2.1	Yes	No	Yes	Yes	Yes
Solyc12g009530.1.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009550.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009560.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009565.1.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009570.3.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009580.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009590.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009600.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009610.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009620.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009630.3.1	No	No	No	No	No
Solyc12g009640.1.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009650.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009660.3.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009670.1.1	No	Yes	Yes	Yes	Yes
Solyc12g009680.2.1	Yes	Yes	Yes	Yes	Yes
Solyc12g009690.1.1	Yes	Yes	Yes	Yes	Yes

3.1.10 Single Knock Out

Since transient expression of the candidate genes in *N. benthamiana* seemed not to be a suitable method to obtain immune responses, *S. lycopersicum* plants containing single knock out mutations of all candidate genes should be created. Therefore, 3 to 4 target sites for CRISPR-Cas9 mediated mutation were chosen for each of the

candidate genes (see Table 10). The newest version of the *S. lycopersicum* genome annotations (ITAG4.1; Fernandez-Pozo *et al.*, 2015) does no longer contain the genes Solyc12g009550 and Solyc12g009600. Therefore, those genes were also no target for CRISPR-Cas9 mediated single knock out. Similar to the results presented in section 3.1.6, vectors containing the appropriate expression cassettes for the mutation were created and stably transformed into *S. lycopersicum* M82 (see section 2.2.1.19).

Table 12: Single knock out CRISPR-Cas9 mutation of candidate genes. The table displays the number of stable *S. lycopersicum* transformants carrying the expected CRISPR-Cas9 mutation for the different GOIs.

Gene name	**Annotation**	**No. of CRISPR/ Cas9 mutants**
Solyc12g009510	receptor-like protein 12	8
Solyc12g009520	receptor-like protein 12	6
Solyc12g009530	receptor-like protein 12	14
Solyc12g009560	EIN3-binding F-box protein 1	7
Solyc12g009565	Unknown protein	9
Solyc12g009570	calcineurin B-like interacting protein kinase	5
Solyc12g009580	CRABS CLAW 5b	3
Solyc12g009590	Methyl-CpG-binding domain-containing protein 11	8
Solyc12g009610	RabGAP/TBC domain-containing protein	5
Solyc12g009620	Ubiquitin system component Cue protein	12
Solyc12g009630	Calmodulin-like protein 5	13
Solyc12g009640	zinc finger BED domain-containing protein DAYSLEEPER-like	12
Solyc12g009650	Sl proline-rich protein	8
Solyc12g009660	Exostosin-like protein	2
Solyc12g009670	Exostosin-like	16
Solyc12g009680	Heptahelical transmembrane protein 1	13
Solyc12g009690	receptor-like protein 12	6

The mutations were then confirmed by sequencing of the according genes. Successfully mutated plants were subjected to a *C. reflexa* growth assay. The current results are summarized in Table 12. The numbers in column 3 indicate the number of generated plant calli containing CRISPR-Cas9 vectors. They are independent plant lines containing mutations confirmed through sequencing of PCR amplicons of the

genetic region predicted to contain CRISPR-Cas9 induced double strand breaks. The genes *Solyc12g009535, Solyc12g009550* and *Solyc12g009600,* missing in the latest *S. lycopersicum* genome annotation, were not subject to CRISPR-Cas9 mediated mutation. As shown in Table 12 several mutant plants for every GOI could be generated, further analysis is underway. Furthermore, genes that were not amplifiable (see 3.1.8) were also target for CRISPR-Cas9 mutation, taking challenges during the cloning process into account.

3.1.11 Transient overexpression of Cuscuta resistance candidate genes in N. benthamiana

A quantitative trait effect of the candidate genes cannot be excluded based on the results described in the previous chapters. Sometimes several genes display cumulative effects against a specific trait. This can be a result of inheritance of slightly differing cultivars and their allelic variations. Those effects are called quantitative trait loci (QTLs) and can enhance or repress signalling pathways (Kumar *et al.*, 2017). Even a QTL effect between one or several chromosome 12 candidate genes and *CuRe1* is possible.

Therefore, a complementation of the susceptible introgression line 12-2 should be achieved through an overexpression of multiple candidate genes. First, the candidate genes where cloned and afterwards several of them were combined in one expression vector with the help of the Golden Gate system (see 2.2.1.16). The in section 3.1.10 excluded genes (*Solyc12g009535, Solyc12g009550, Solyc12g009600*) were used as cloning targets if the corresponding region was successfully amplified. The currently available vectors are shown in Figure 35.

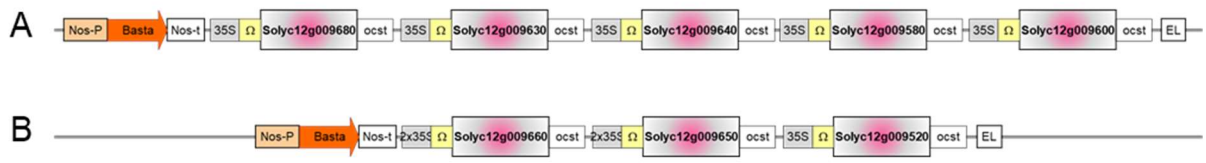


Figure 35: Scheme of multigene vectors for overexpression in IL12-2. Shown are two vectors with a varying number of expression cassettes. The orange box displays the plant selection marker and the grey/pink boxes display the different candidate genes. The utilized promoters (35S with omega(Ω) sequence, see also Gallie *et al.*, 1987) and terminators (ocst; octopine synthase gene; see also Shao *et al.*, 2009; nos-t; nopaline synthase terminator; see also Pierboni *et al.*, 2015) are indicated. EL=endlinker (fusing varying numbers of expression cassettes in one vector backbone)

Before stable transformation into the IL 12-2 the constructs were tested transiently in the susceptible host *N. benthamiana*. For this purpose, *N. benthamiana* wild type and

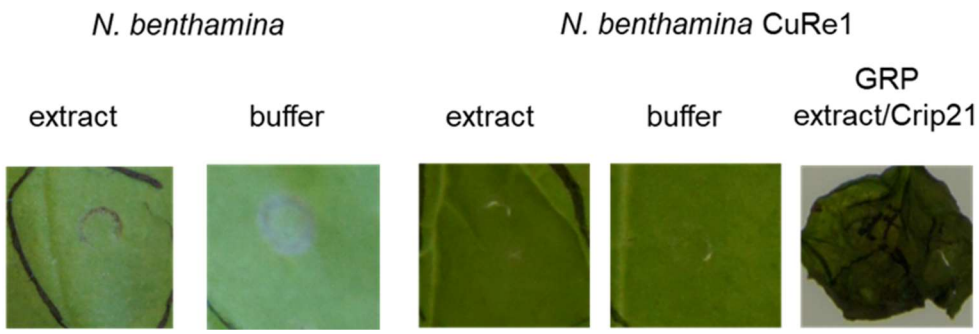


Figure 36: Transient expression of a vector containing Solyc12g009680 + Solyc12g009630 + Solyc12g009640 + Solyc12g009580 + Solyc12g009600. The multigene construct was transiently transformed in either *N. benthamiana* wild type or CuRe1. Two days after transient transformation the plants were infiltrated either with *C. reflexa* crude extract, 25 mM MES buffer (pH 5,5) or 20 µl 100nm Crip21. One leaf was co-transformed with a vector expressing *C. reflexa* GRP. n = 3

N. benthamiana CuRe1 were transformed with the DNA vector constructs containing GOIs exemplarily displayed according to Figure 35A as described in section 2.2.1.16 and the transiently transformed leaves were then infiltrated with either *C. reflexa* crude extract or a buffer control (see Hegenauer *et al.*, 2016).

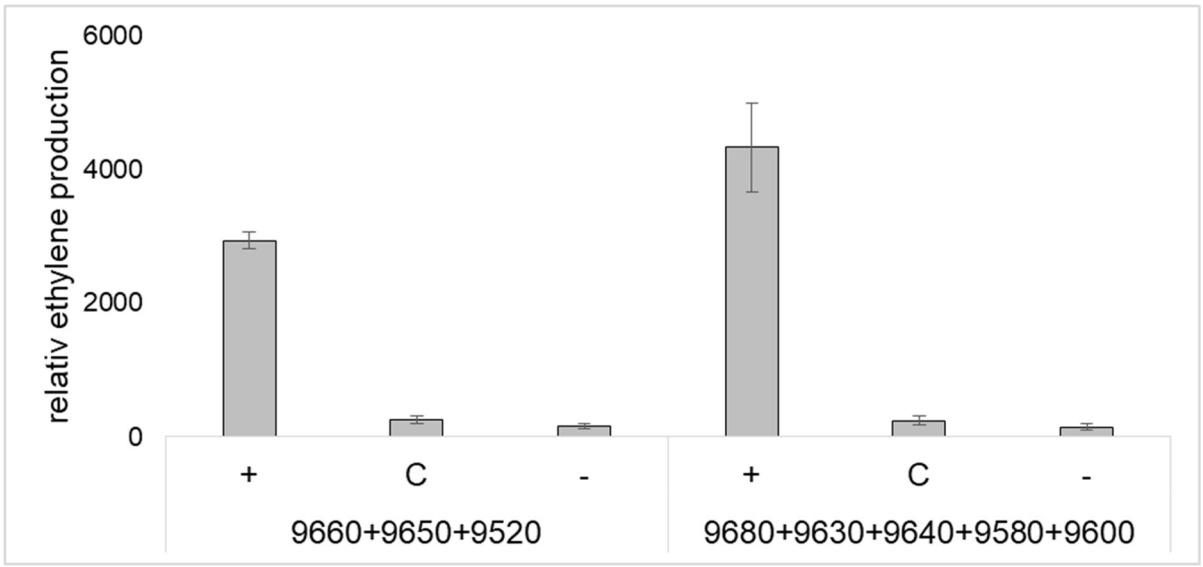


Figure 37: Ethylene measurement of transiently transformed *N. benthamiana* with a multigene construct. The ethylene production of *N. benthamiana* transformed with *Solyc12g009660* + *Solyc12g009650* + *Solyc12g009520* or *Solyc12g009680* + *Solyc12g009630* + *Solyc12g009640* + *Solyc12g009580* + *Solyc12g009600* is shown after treatment with 100 nM flg22 (+), crude *C. reflexa* extract (C) or 25 mM MES buffer pH 5,5 (-). n = 3; Data represent mean values +/- standard deviation.

The coexpession of the CuRe1 ligand GRP together with the construct for the candidate genes in the *N. benthamiana* CuRe1 lines was used as positive control for

the HLR. The results are presented in Figure 36. Neither the transformed wild type plants nor the CuRe1 plants showed any HLR. In comparison the overexpression of the CuRe1 ligand GRP in the according plant could induce a strong HLR.
Since no HLR could be observed, additionally the ethylene production in transiently transformed *N. benthamiana* wild type background was analysed according to section 2.2.2.5. The result is presented in Figure 37.
A transient transformation of a multigene vector (Figure 35) into *N. benthamiana* WT followed by a treatment with *C. reflexa* extract was also not sufficient to induce an immune response in form of ethylene production.
A stable transformation of those gene constructs into the susceptible IL12-2 followed by a growth assay seems to be necessary to obtain insight into the immunity function of the candidate genes.

3.2 *Cuscuta's* influence on host metabolism during infection

The parasitic infestation of a plant often requires some modification on the hosts' metabolism. Alteration of developmental phytohormones in plants like auxin or primary defence relevant hormones like JA to promote susceptibility could already be observed for infections with *Pseudomonas syringae* (Chen *et al.*, 2007; Shang *et al.*, 2006), *Fusarium oxysporum* (Anderson *et al.*, 2004) or *Meloidogyne javanica* (Lambert *et al.*, 1999). These few examples belonging to the distinct domains namely bacteria, fungi and nematodes suggest an importance for parasitism in general. To study those interactions a susceptible host is needed which can display natural growth conditions of plant and parasite. Therefore, effects on phytohormone balance during infection of a host (*A. thaliana* and *N.* tabacum) with the plant parasite *C. reflexa* were analysed by microscopic approach, GC-MS, LC-MS and hormone-deficient knockout mutants.

3.2.1 *A. thaliana* COLORFUL lines

Changes in phytohormone abundance should be observed using *A. thaliana* plants stably transformed with the COLORFUL-Circuit (Ghareeb *et al.*, 2016) provided by Volker Lipka. They exhibit three fluorescence proteins, two of them constitutively expressed as cell organelle markers (nucleus and plasma membrane, respectively) and one responsive to a specific phytohormone (see Figure 38).

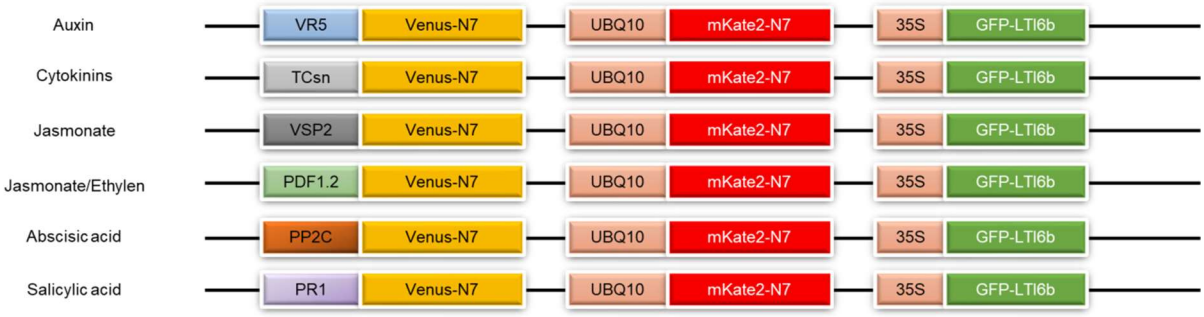


Figure 38: COLORFUL-Circuit. Shown is the scheme for 6 independent DNA vectors (gifted from Volker Lipka) expressing a yellow Venus fluorescence in response to the respective phytohormone. The green GFP fluorescence is constitutively expressed and located at the plasma membrane and the constitutively expressed red mKate2 fluorescence is located in the nucleus. (TCS = Two Component signaling Sensor, VSP2 = VEGETATIVE STORAGE PROTEIN 2; PDF1.2 = PLANT DEFENSIN 1.2; PR1 = PATHOGENESIS-RELATED GENE 1; PP2C = Protein phosphatases type 2C; LTI6B = Low temperature-induced protein 6B; N7 = nuclear localisation signal)

A. thaliana COLORFUL plants were infected with *C. reflexa* for 6 to 10d. During the infection period plants were harvested for each time point (day) and fluorescence was evaluated through confocal laser scanning microscopy (see section 2.2.2.7). For this purpose, cuttings of *Arabidopsis* shoots at the infection site were prepared using a razor blade and immediately analysed by confocal laser scanning microscopy. A representative image of an *A. thaliana* COLORFUL plant infected with *C. reflexa* is depicted in Figure 39 as example.

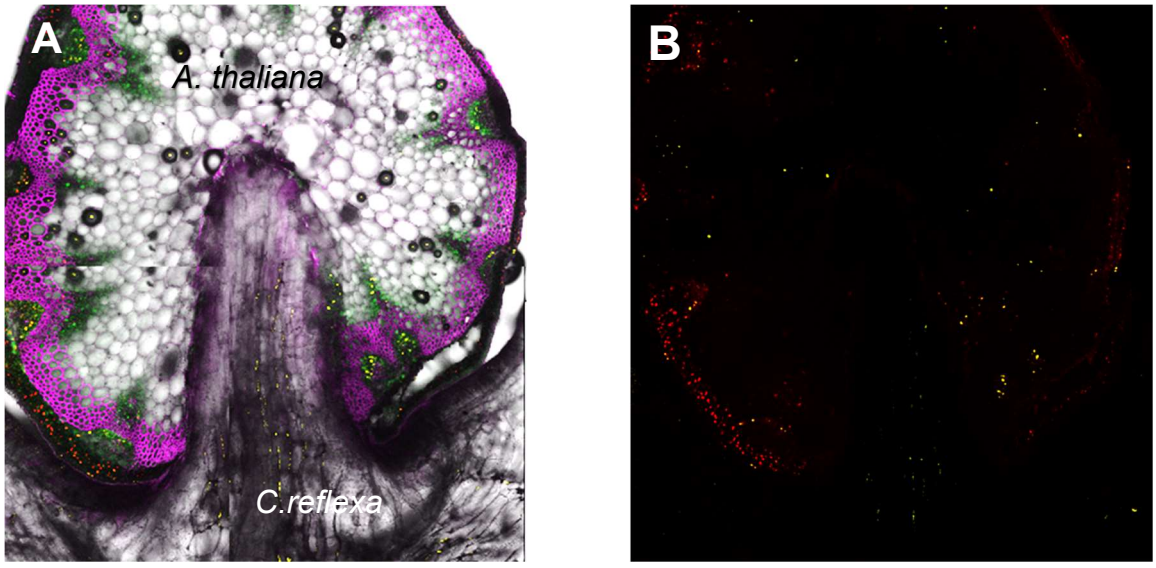


Figure 39: Example fluorescence image of *A. thaliana* razor blade cutting infected with *C. reflexa* 8 days past infection. Figure A displays an overlay of the infection site as bright field with several fluorescence channels. Violet colour displays cell wall autofluorescence, green shows the membrane bound GFP fluorescence, red shows nuclear localized constitutive expressed mKate2 fluorescence and yellow displays nuclear localized Venus fluorescence. Figure B shows the same picture as overlay of mKate2 and Venus fluorescence.

Venus fluorescence is shown in yellow, GFP-plasma membrane fluorescence in green and mKate2 fluorescence in red. Different from the application in Ghareeb *et al.*, 2020 cross sections from plants were analysed. In such sections the GFP fluorescence is less defined then in epidermis pictures (data not shown).

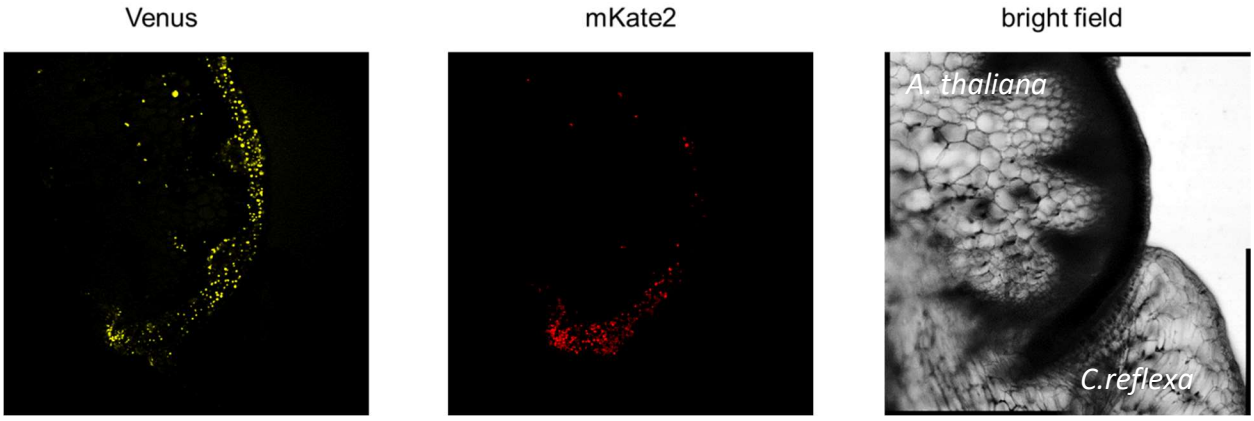


Figure 40: Normalization example. Phytohormone dependent Venus fluorescence, constitutively produced mKate2 fluorescence and a bright field picture of a shoot cutting from *A. thaliana* at the *C. reflexa* infection site.

The number of Venus fluorescent nuclei, which is only produced as response to the respective phytohormone, and the corresponding fluorescence intensity were normalized against the constitutively expressed nuclear marker fused to mKate2 and bioinformatically analysed with Fiji and Python (see 2.2.3.1). Figure 40 shows the Venus and mKate2 fluorescent nucleii in single pictures in comparison to bright field. GFP is not shown in this figure since the fluorescence was overall weak and not very evenly distributed.

An evaluation of the normalized phytohormone induced Venus fluorescence intensity at the different time points compared to the wild type was performed (see Figure 41).

It was not possible to obtain any consistent data from the SA and jasmonate/ethylene (JAEt) *A. thaliana* COLORFUL line. However, also the other COLORFUL lines showed a huge variability of Venus fluorescence during measurements. Four of them are shown in Figure 41. Furthermore, the intensity differs greatly between the different plant lines and individual experiments as indicated by the vertical axis in Figure 41.

No significant increase or decrease of any tested phytohormone (auxin, cytokinins, jasmonate, ethylene, absisic acid, salicylic acid) could be observed during several repetitions of the measurements (T-Test: $p>0,05$). Possibly the phytohormone

content is too low to determine it with this fluorescence based method or the infection site is too large to differentiate between local concentrational changes.

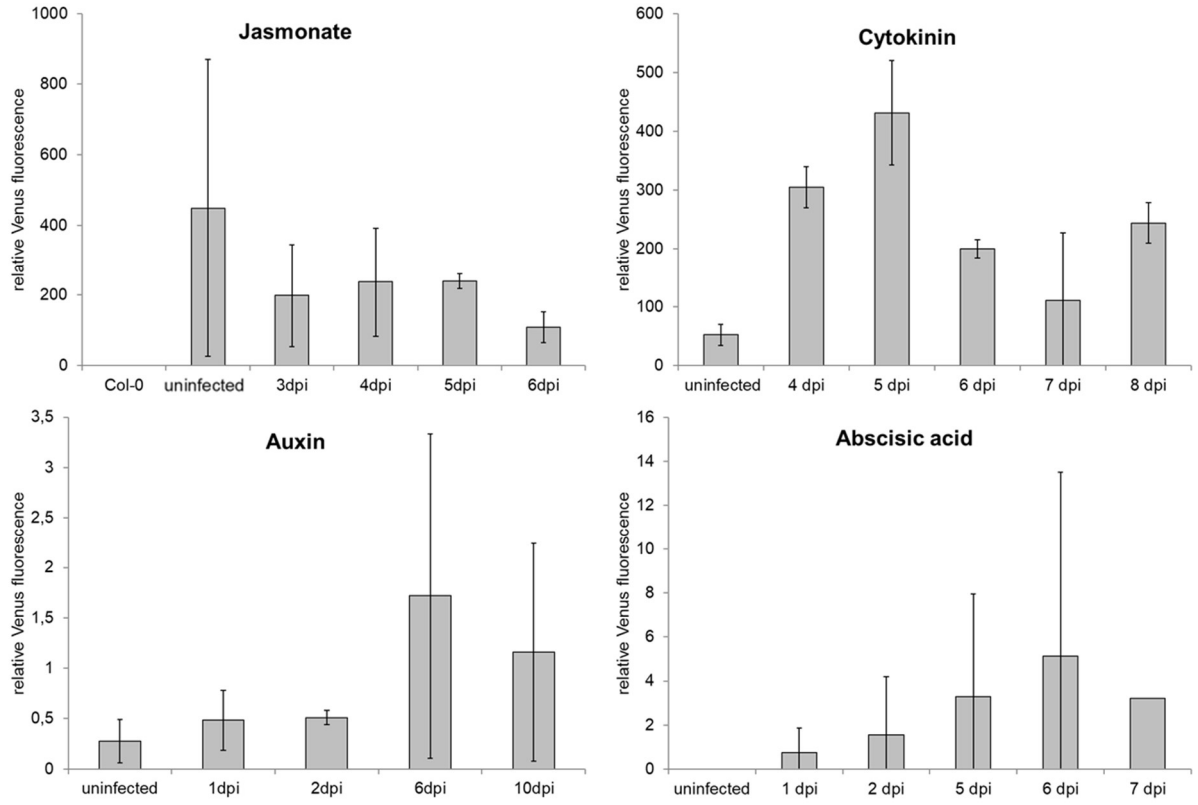


Figure 41: Relative YFP fluorescence of different *A. thaliana* COLORFUL lines during *C. reflexa* infection. The *A. thaliana* COLORFUL lines responsive to jasmonate, cytokinin, auxin and abscisic acid are displayed. *C. reflexa* infection occurred in the course of several days. The time points during infection at which shoots were harvested and checked for phytohormone dependent YFP fluorescence are indicated in the horizontal axis. n = 3; dpi = days past infection; T-Test: p>0,05; Data represent mean values +/- standard deviation.

3.2.2 Gas chromatography-Mass spectrometry (GC-MS)

In parallel to the fluorescence based phytohormone level determination an approach using GC-MS measurements were performed. *A. thaliana* plants were again infected with *C. reflexa* over a time course from 8 to 10d. Afterwards the phytohormone content for salicylic acid, jasmonate, auxin, absisic acid and camalexin was determined. The measurement of the free analytes (see 2.2.3.3) is shown in Figure 42.

As shown in Figure 42 the phytohormone content range and also the variability per biological replicate differ. JA and SA show a tendency to increase their abundance around day 5.

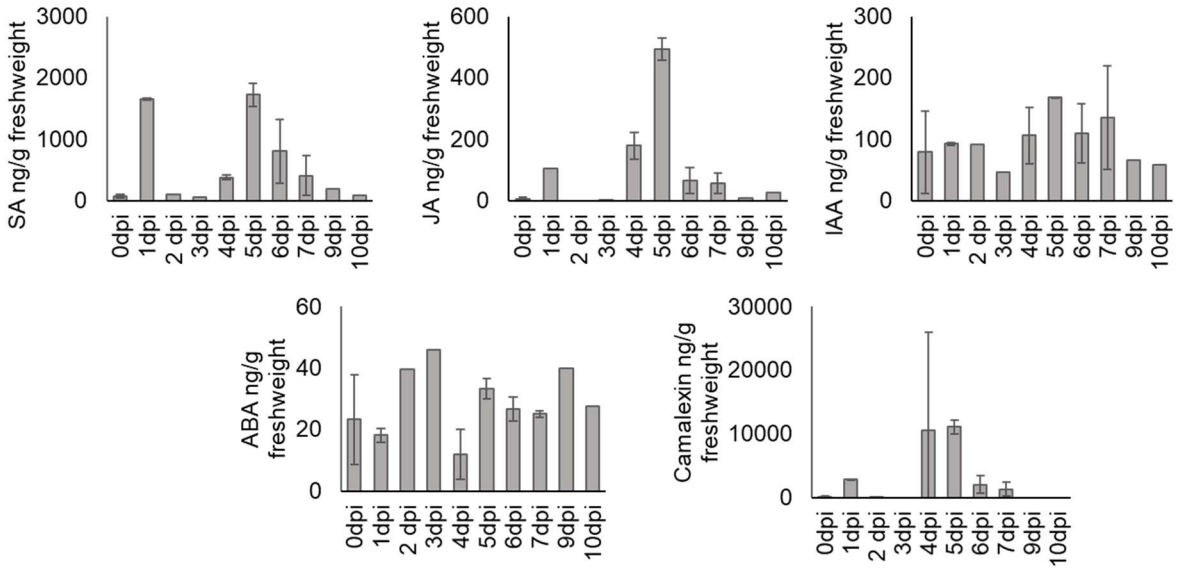


Figure 42: GC-MS measurement of *A. thaliana* phytohormone content during *C. reflexa* infection. *A. thaliana* was infected with *C. reflexa* over a timeperiod of 10 days. At each timepoint (indicated by the horizontal axis) the content of the phytohormones SA, JA, IAA, ABA and camalexin in ng per g freshweight were determined. Data represent mean values +/- standard deviation.

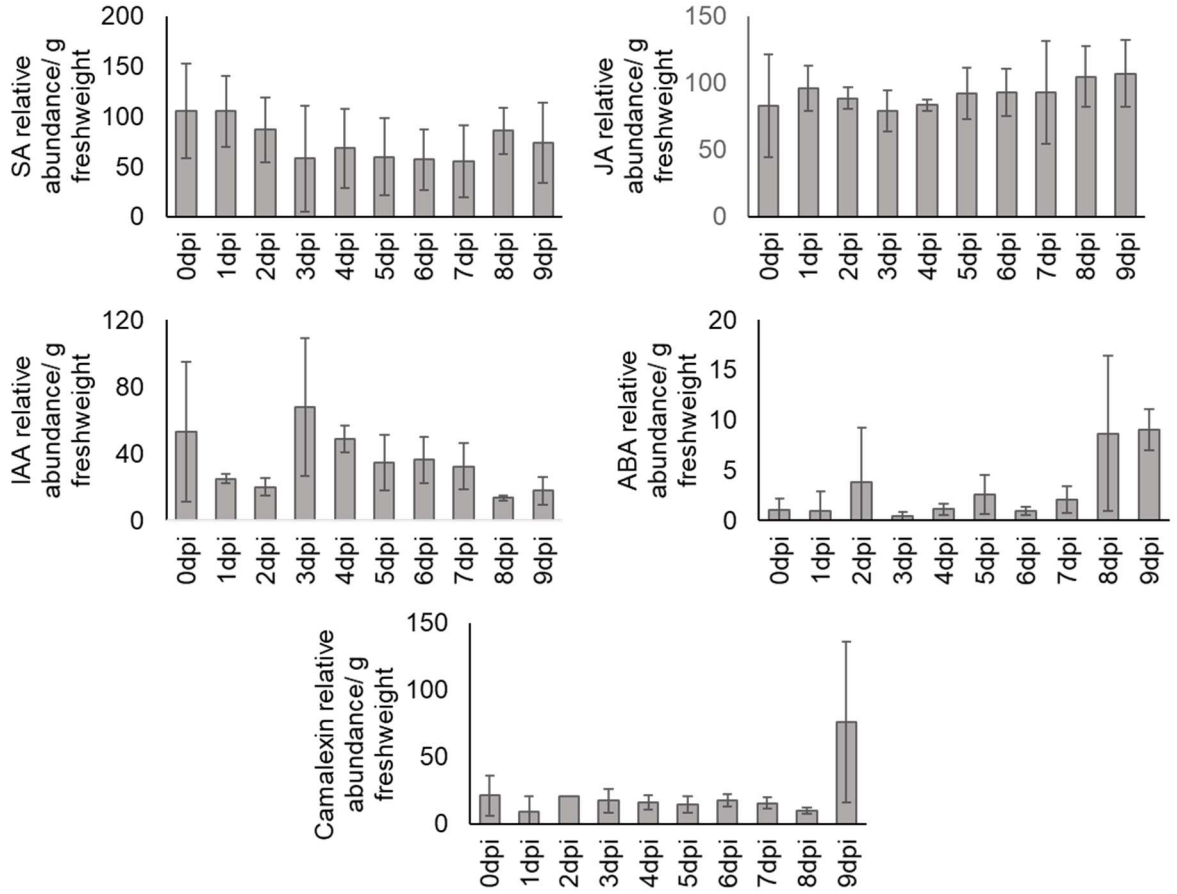


Figure 43: Second GC-MS measurement of *A. thaliana* phytohormone content during *C. reflexa* infection. *A. thaliana* was infected with *C. reflexa* over a timeperiod of 9 days. At each timepoint (indicated by the horizontal axis) the relative abundance of the phytohormones SA, JA, IAA, ABA and camalexin were measured. n=2; Data represent mean values +/- standard deviation.

For confirmation of this observation and to get further insights into the changes of other phytohormone levels the GC-MS measurement was repeated with new plants from another growth assay. The results of this second GC-MS measurement are displayed in Figure 43.
The general low concentration of phytohormones in a plant impedes the measurement. Therefore, only the relative abundance of the phytohormones could be determined in the second measurement unlike the first one where the amount of phytohormone in ng per g freshweight was used. Nevertheless, the tendencies assumed from the first GC-MS measurement could not be replicated. Possibly the change is too subtle to detect it through the used GC-MS method. It is also possible that local phytohormone changes only occur in a very limited region in the plant which is not possible to separate since GC-MS needs a minimal amount of tissue to work.

3.2.3 Cuscuta growth on hormone deficient *Arabidopsis* lines

To observe if alterations in the hormone system negatively influence the host plant during *C. reflexa* infection, hormone/immune deficient *A. thaliana* plants were used for a growth assay. The used plants were *A. thaliana npr 1-1*, *ndr 1-1* and *pad 4*. The *npr1* gene (ARABIDOPSIS NONEXPRESSER OF PR GENES 1) is a key regulator of the SA-mediated systemic acquired resistance pathway but also interplay with other phytohormones like ABA could already be observed (Khan *et al.*, 2022). The *ndr1* (NON RACE-SPECIFIC DISEASE RESISTANCE 1) gene is required for non-race specific resistance to bacterial and fungal pathogens in *Gylcine max* an impairment of parasitism related to this gene was observable (McNeece *et al.*, 2017). The *pad4* gene (ARABIDOPSIS PHYTOALEXIN DEFICIENT 4) encodes a lipase-like gene that is important for SA signalling and function in R gene-mediated and basal plant disease resistance it is also involved in the regulation of plant – pathogen interactions (Ślesak *et al.,* 2015).
Cuscuta was grown for two weeks on the different *Arabidopsis* mutants and afterwards the fresh weight of the host plants was determined and compared to the fresh weight of uninfected plants. The results are presented in Figure 44. A significant change in *C. reflexa* fresh weight was not expected, since the parasite usually needs more than two weeks to start a rapid growth phase, and was therefore not analysed.

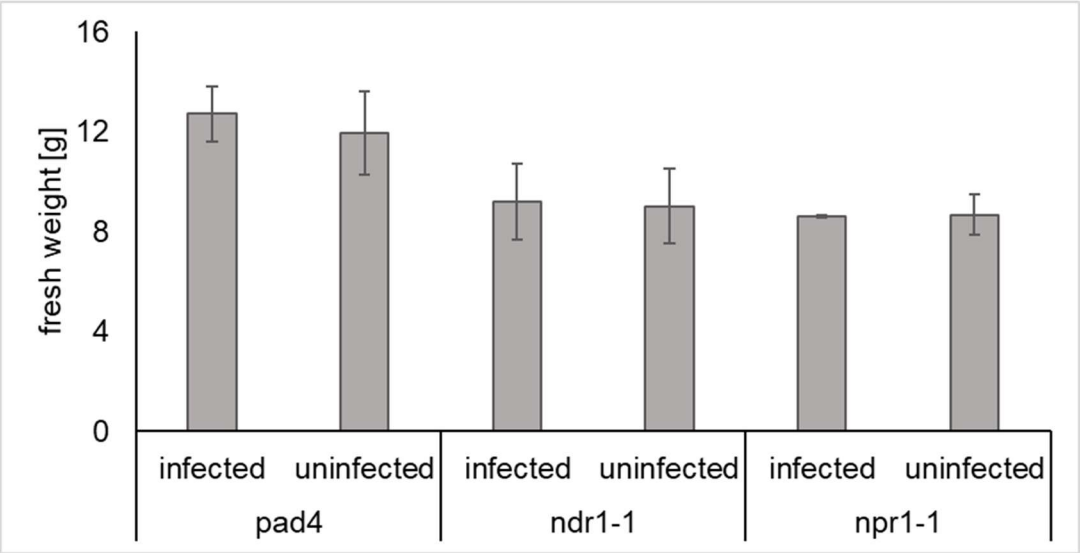


Figure 44: Growth assay of *A. thaliana* infected with *C. reflexa*. *C. reflexa* was grown on the susceptible host *A. thaliana* for two weeks. Afterwards the fresh biomass of the host was determined and compared to an uninfected plant. (ndr1-1 infected: n=10; ndr1-1: n=10; npr1-1 infected: n=2; npr1-1: n=2; pad4 infected: n=5; pad4: n=6) Data represent mean values +/- standard deviation.

During the infection process there was no delay or acceleration of the *C. reflexa* attachment on the host visible. The end of the growth assay revealed no significant differences between the *A. thaliana* wild type and mutant plants concerning the infection process with *Cuscuta*.

Similar to the previously described phytohormone analysis no significant changes could be detected (T-Test: p>0,05).

3.2.4 Liquid Chromatography-Mass Spectrometry (LC-MS)

For analysis of phytohormone contents besides GC-MS another independent method was chosen. Here a similar experiment as described in 3.2.2 was performed. *N. tabacum* was infected for 9 days with *C. reflexa* shootsand phytohormone contents were determined. The new host was used because of its thicker stem resulting in more biomass at the infection site usable for analysis. Additionally, the utilized tobacco plants expressed GFP under the control of the AtSUC2 promoter, which regulates the expression of companion cell-specific *AtSUC2* sucrose-H^+ symporter gene in wildtype *Arabidopsis* plants. Therefore, the tobacco plants produced fluorescence protein freely moving through phloem from source to sink (Imlau *et al.*, 1999). Upon connection with *C. reflexa* the GFP can move from host to parasite exclusively through connected phloem cells. This allows for a more precise evaluation of infection stages since *Cuscuta* haustorium development varies slightly

between individual organisms. Figure 45 displays an example of *N. tabacum* expressing phloem mobile GFP while infected with *C. reflexa* whereas Figure 45A shows an incomplete and Figure 45B shows a complete phloem connection.

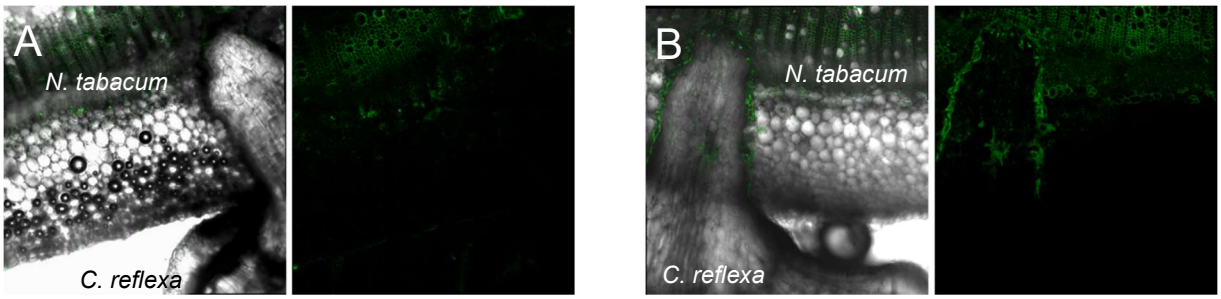


Figure 45: Infection of *N. tabacum* expressing phloem transported GFP with *C. reflexa*. Figure A displays the infection site 5 dpi without GFP transport between the host and the parasite (left picture: bright field and GFP combined; right picture: GFP fluorescence). Figure B shows the infection 8 dpi with GFP transported from the host to the parasite proving a successful infection (left picture: bright field and GFP combined; right picture: GFP fluorescence).

For each time point (infection day) up to 5 plants were analysed for ABA, JA and OPDA content. They were chosen dependent on their visual infection state also taken the GFP transfer during infection into account. The results of these measurements are summarized in Figure 46.

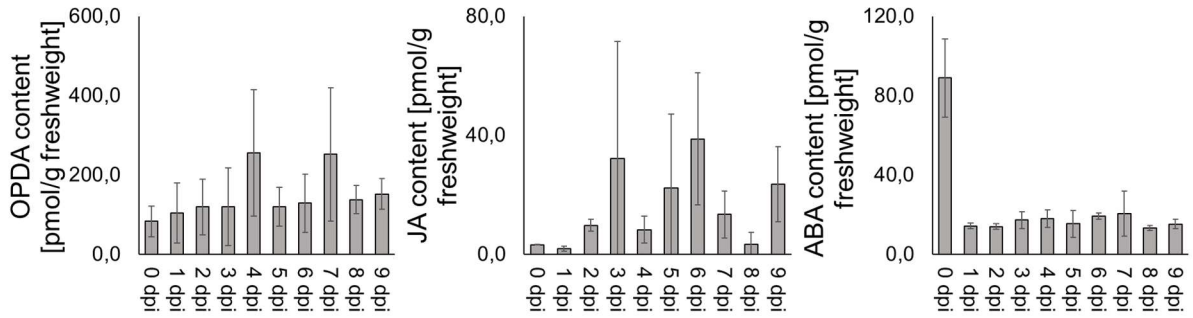


Figure 46: Phytohormone content in *N. tabacum* stems infected by *C. reflexa*. LC-MS measurement of samples collected from day 0 to day 9 post infection (dpi; x-axis). Contents of OPDA, JA and ABA in the host were measured as pmol per g freshweight (y-axis). Data represent mean values +/- standard deviation. n = 3 - 5

The phytohormone content of the analysed plants again displayed inconsistence with previous measurements with GC-MS (see 3.2.2) or with the fluorescence-based (see 3.2.1) microscope analyses, probably because of the low phytohormone content. This seemed to be the case at least for some samples, since the phytohormone levels have been below the technical detection limit. Additionally, it is also possible that *C. reflexa* infection does not trigger the expression of the tested phytohormones or maybe it even suppresses unfavourable ones. Most likely JA and SA need to be

regulated since these hormones are the key players in a lot of plant defence processes (see 1.3.1).

4 Discussion

4.1 Resistance of Solanum lycopersicum against Cuscuta reflexa

Parasitic plants of the genus *Cuscuta* belong to the most relevant crop pests. Especially, *C. campestris* occurs to be the most devastating species member which affects 25 crop species in 55 countries (Lanini, 2005). The control of *Cuscuta* proved to be very difficult because of their persistent seedbank formation. Those seeds are characterized by a long dormant period, hard seed coats and their potential to infect a wide variety of species. Additionally, the intimacy of connection between host and parasite hampers the application of control methods which do not affect the host itself (Cudney *et al.*, 1992; Parker & Riches *et al.*, 1993; Fernández-Aparicio *et al.*, 2020). Therefore, controls for most *Cuscuta*-crop interactions are limited or non-existent. In most cases either phloem-mobile herbicides are applied to crops which carry a resistance against them (Guza, 2000; Nadler-Hassar *et al.*, 2003; Nadler-Hassar *et al.*, 2009) or parasite-resistant crops (Goldwasser *et al.*, 2012; Córdoba *et al.*, 2021).

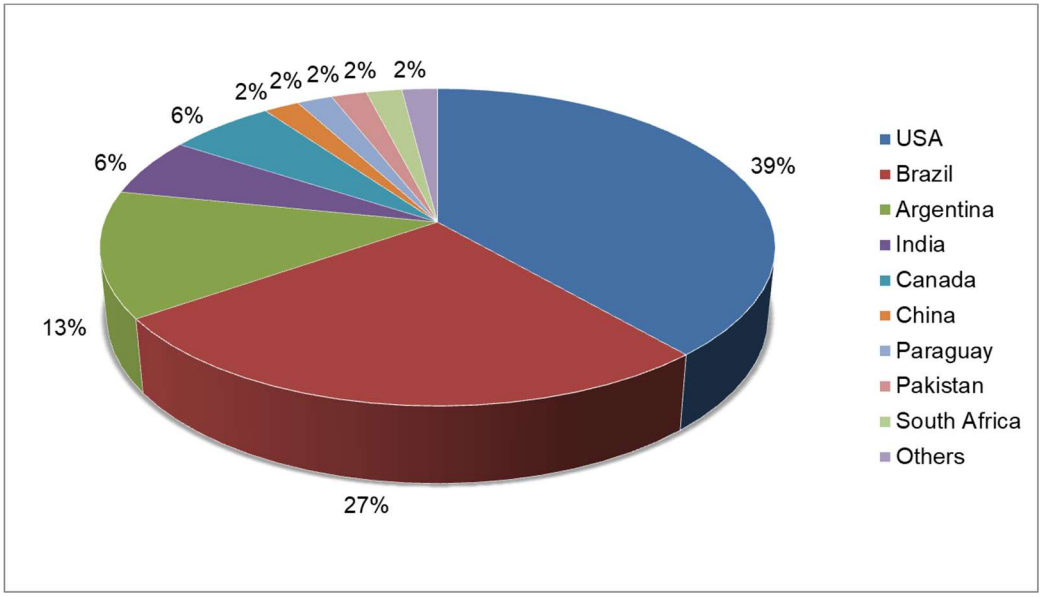


Figure 47: Worldwide GMO distribution per country (Source: ISAAA 2017).

Even though usage of genetically modified organisms is not forbidden in the European Union (RL 2001/18/EG; Vives-Vallés & Collonnier, 2020) more and more countries stop the distribution of genetically modified organisms (GMOs) in their territory (e.g. Germany, Gesetzentwurf November 2016 zur Änderung des Gentechnikgesetzes). In fact, cultivation of GMO plants is mostly limited on 9

countries shown in Figure 47 (International Service for the Acquisition of Agri-biotech Applications (ISAAA), BRIEF 53, 2017).
Those limitations impede the sustainability of food for the growing world population. Therefore, a better understanding of the plant immune system with respect to resistance against pathogens is important to allow breeding and crossing of resistant cultivars and faster identification of resistance traits in naturally occurring plants. Especially the field of parasitic plants needs a better understanding how friends and foes are distinguished on a cellular level.
In this work, the incompatible interaction between the parasite *C. reflexa* and the resistant *S. lycopersicum* was analysed.

4.1.1 The *Solanum lycopersicum* resistance traits

One important resistance mediating factor of the cultivated tomato against *C. reflexa* was already identified. It is the leucine-rich repeat receptor-like protein CuRe1 (Solyc08g016270) which identifies the CrGRP from *C. reflexa* through its minimal motif Crip21 (Hegenauer *et al.*, 2016; Hegenauer *et al.*, 2020). Besides this resistance factor, there exists a second resistance trait which has been identified (described in 1.3.4). The accurate composition of the corresponding chromosomal region as well as the identity of resistance related genes mapped to this region have been major goals of this work.
Pathogen resistance mediated through a single gene is often the main focus in discovery of host immune components but introduction of such a resistance bears a stronger risk of defence breakdown in a higher frequency (Martins *et al.*, 2020). Therefore, methods to discover and to analyse multi-layered resistance with possibly very distinctively located genes is of great importance. Multi-layered resistance is already well known for several crop plants. For example, *Medicago truncatula* A17 displays a resistance against fungus *Erysiphe pisi* through three different genes at distinct positions namely *Epp1* on chromosome 4, *Epa1 and Epa2* on chromosome 5 (Ameline-Torregrosa *et al.*, 2007). Also resistance of *Lathyrus cicera* against *Erysiphe pisi* and *E. trifolii* was mapped to several QTLs (Santos *et al.*, 2020).
Multi-layered responses to plant parasites could already be observed in *S. lycopersicum* M82 against *C. campestris* where *SlPR1* and *SlNLR* CRISPR knockout resulted in more susceptible plants (Jhu *et al.*, 2022). As described in the introduction, *PR1* is a gene tightly connected to the SA response usually associated

with SAR related defence. *NLRs* often play roles in ETI (see 1.2.1). So both genes may act together at different points in the plants defence pathway to confer resistance. Similar mechanisms could act in concert with the CuRe1-dependent resistance between *S. lycopersicum* and *C. reflexa*.

For the *S. lycopersicum* M82 resistance against *C. reflexa* the recognition of the parasite by CuRe1 is accompanied by a HLR response (Hegenauer *et al*., 2016). This HLR acts not completely dependent of CuRe1 since the ILs lacking the receptor are still able to produce this defence response against the parasite. Recent studies of the related parasite *C. campestris* on specific resistant Heinz tomato cultivars revealed several key genes important for lignin-based HR resistance (Jhu *et al.*, 2021). The genes *LIF1, SlMYB55* and *CuRLR1* seem to act together to create resistance dependent on a 30 kDa – 100 kDa heat-sensitive protein with CuRLR1 probably acting as subcellular NBS-LRR inducing ETI. These observations further enhance the idea of a multi-layered resistance against *Cuscuta* in tomato.

In this work, the existence of another resistance trait in *S. lycopersicum* against *C. reflexa* could be confirmed. A lack of this genomic region on chromosome 12 in the corresponding IL allowed *C. reflexa* survival on *S. lycopersicum* M82. This region is distinct from the *CuRe1* locus and also from the mentioned lignin related locus in Jhu *et al.*, 2021. Furthermore, the generation of CRISPR-Cas9 mutants reduced the number of candidate genes to a selection of 19 genes between Solyc12g009510 and Solyc12g009690 (see Table 10). Some of those genes (Solyc12g009510, Solyc12g009520) were already tested in a stably transformed *N. benthamiana* background to reduce penetration ability of *C. reflexa* (Welz, 2017). In the same study Solyc12g013680, another LRR-RLP located on chromosome 12, was observed to have the same growth reducing effect even though it is not located in the region of interest identified through IL screening. Transient approaches with other candidate genes in that study could also not give further insight into immune answers in response to *C. reflexa*. Therefore, a possible redundancy of the LRR-RLPs, a quantitative trait effect or transformation effects in the stable approach cannot be excluded. Even the existence of multiple resistance traits inside the chromosome 12 region is possible.

In this work, the problem should be addressed through stable mutations in the actual resistance background *S. lycopersicum* which should result in a similar phenotype to the susceptible introgression lines e.g. IL12-2. A susceptible phenotype could be

achieved (see 3.1.7) resembling the expected one. Both phenotypes still show lesions around the haustoria penetration site but usually later and weaker than the ones visible at stems of resistant plants. Therefore, the deleted genes could be in a minor way also directly or indirectly be involved in HLR pathways. A difference between the phenotype of susceptible ILs and susceptible CRISPR-Cas9 mutants is an irregularly occurring stronger branching of the attached *C. reflexa*. This phenotype resembles the one described in Christensen *et al.,* 2003 where *Euphorbia pulcherrima* is introduced as partially incompatible host to *C. reflexa*. The parasite shows a similar coral-like structure (see Figure 31) but there were also several differences. First of all, *Cuscuta* did not need a primary susceptible host to infect the CRISPR-Cas9 mutated plants. Second, the parasite did not show any signs of preliminary death during growth, parasites were grown on the tomato until the host's death. Finally, *Cuscuta* growth seemed not impaired to the extent described by Christensen *et al.*, 2003. There are also still HR-like lesions visible resembling those from the initially screened susceptible introgression lines.

The deletion of the mutant plant lines could be confirmed, by phenotyping and genotyping including sequencing. The chromosomal region lacking the 172 kb region could be amplified, cloned into a DNA vector and sequenced for an expected fusion of two distant chromosomal parts. Furthermore, the amplification of smaller fragments of genes inside the 172 kb region implies the existence of the genes on another allele and therefore heterozygosity for some plant lines.

Including the results of this work, some preliminary assumptions about the resistance mediating gene inside the CRISPR-Cas9 mutated part on chromosome 12 can be made.

The CRISPR-Cas9 deletion of a 172 kb chromosome fragment in this work revealed 19 candidate genes with a potential function in immunity. Taken together with the overlapping regions of the susceptible ILs 12-2, 12-3 and the presumably susceptible line 12-3-1 (Kaiser, 2019) combined with the information gained by the newly created CRISPR-Cas9 deletion lines, the candidate genes for the second resistance trait can be narrowed down to Solyc12g009660, Solyc12g009670, Solyc12g009680 and Solyc12g009690 leaving the other 14 genes outside of this ROI. Those genes are annotated to encode two Exostosin-like proteins, an Adiponectin receptor protein 2 and a LRR receptor-like serine/threonine-protein kinase. Additionally, the absence of the genes Solyc12g009520, Solyc12g009540, Solyc12g009670 and

Solyc12g009690 from the whole genome sequencing indicates further evidence that a deletion of either one of the latter ones or both leads to susceptibility. Both Solyc12g009660 and Solyc12g009670 encode Exostosin-like proteins. Size analysis of proteins belonging to this protein class revealed usually bigger protein sequences between 300 and 900 amino acids (Yamada, 2020; Madson *et al.*, 2003). Combined with the inability to amplify the gene product of Solyc12g009670 indicate a possible combination of both genes into a single gene leaving Solyc12g009690 as most promising candidate gene for resistance. Since this gene encodes a putative RLP, it is possibly encoding a new resistance receptor recognizing *C. reflexa* infection in *S. lycopersicum.*

Unfortunately, there are some results that oppose that theory. First of all, the susceptibility of IL 12-3-1 described in Kaiser, 2019 could not be confirmed in this work. Without this additional restriction the overlapping chromosomal regions of the IL 12-2, 12-3 and the CRISPR-Cas9 mutant lines become less well defined and none of the identified 19 candidate genes can be excluded as resistance factor since the essential overlap of exchanged chromosomal regions between *S. lycopersicum* and *S. pennellii* is missing. The different behaviour of IL 12-3-1 could be due to unfavourable light or temperature conditions for *C. reflexa*, non-uniform genotype of the tomato seeds and age or fitness of the plants. Also different applied time frames for *Cuscuta* growth assays may explain the different results since the phenotype shown in Kaiser, 2019 displays beginning death symptoms of the parasite which may be completed after a longer infection period. *C. reflexa* shows great variations during infection process dependent on light conditions. Strong light in long day conditions usually enhances infection capabilities of the parasite (personal observation through changing greenhouse conditions) possibly through better detection of R/FR light ratio for haustoria formation (Tada *et al.*, 1996) and also temperatures between 25 and 30 °C seem favourable (personal observation through changing greenhouse conditions). The genomic regions on chromosome 12 of *S. lycopersicum* and *S. pennellii* share a high degree of homology which prevents accurate genotyping for the genes of interest (see e.g. 3.1.9) and therefore also prevents in depth fast genotyping of plants. *C. reflexa* showed the ability to grow on partially incompatible hosts especially when already infecting a compatible host (Christensen *et al.*, 2003). Additionally, tomato plants of different developmental stages have proven to express different strengths of immune responses (Runyon *et al.*, 2010). These variations in plant

fitness may indicate that in different experimental setups outcomes may also display variability.

Moreover, the data quality of the whole genome sequencing of the CRISPR-Cas9 mutant lines which lack the 172kb GOI on chromosome 12 was not very good. A self-assembly from the raw data was not possible and the lack of bioinformatical support renders the results highly questionable. Only the feature count gave a small insight into the actual changes in the plant genome but unfortunately the method was not described in detail by the company. Possibly the heterozygous nature of the CRISPR-Cas9 mutant plants additionally challenged the method.

Finally, the high redundancy in the region of interest between genes of *S. pennellii* and *S. lycopersicum* as well as between the genes of *S. lycopersicum* itself made a sequence analysis for presence or absence of specific genes challenging. Additionally, the usage of a CRISPR-Cas9 system with several target sites at once grants much higher mutation efficiency but also leaves the possibility of different mutations on both alleles. Nevertheless, the high accuracy of CRISPR-Cas9 mutation was already shown in numerous publications and a prove of successful mutations through DNA sequencing was also possible.

4.1.2 Immunity functions of the resistance gene candidates

Besides the mapped LRR-RLPs none of the identified candidate genes were extensively studied for their general role in immune response pathway but for most of them some relation to plant defence could be shown.

As discussed in 1.2.1 and the following chapters, plants recognize MAMPs and DAMPs through PRRs in the first layer of immunity. LRR-RLPs belong to the PRRs and stand out due to a lack of an intracellular kinase domain. They rely on adapter kinases and additional proteins and enzymes to forward the immune signalling (Albert *et al.*, 2020). Several of the through CRISPR-Cas9 identified candidate genes were annotated as receptor-like proteins which belong to the LRR-RLP family and are therefore interesting candidates for potential immune signalling.

The genes Solyc12g009510, Solyc12g009520, Solyc12g009530, Solyc12g009690 and the formerly annotated Solyc12g009550 (genome version SL4.0; annotation ITAG4.1; Fernandez-Pozo *et al.*, 2015) are all predicted to encode receptor-like protein 12. In *A. thaliana* some RLPs perceive CLV3 and CLV3-like peptides that act as extracellular signals regulating meristems maintenance (Wang *et al.*, 2010). The

relation of the putatively encoded proteins is displayed in a phylogenetic tree (see Figure 48) in comparison with the known resistance receptor CuRe1 (Solyc08g016270). It displays a high genetic distance between Solc12g009690 and the other putative proteins possibly enforcing the in 4.1.1 mentioned indications for its putative crucial role. The protein sequences were analysed using MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (Kumar *et al.*, 2018) and a maximum likelihood tree was constructed. Since the predicted sequences of the candidate genes and proteins are not yet fully characterized there is also a chance for sequence inaccuracies.

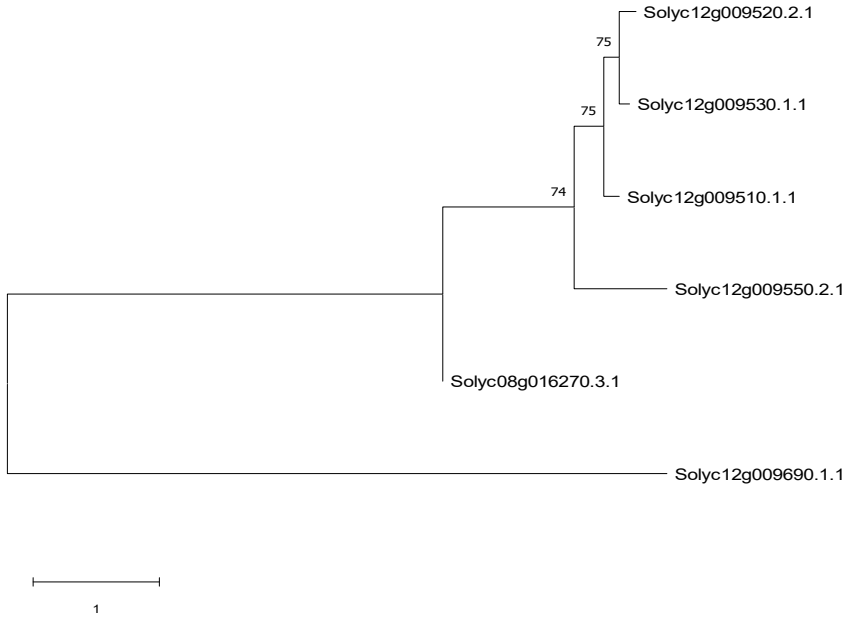


Figure 48: Phylogenetic tree for candidate RLPs and CuRe1 (Solyc12g08g016270). A Maximum Likelihood method and Le_Gascuel_2008 model were used to predict the evolutionary history (Le & Gascuel, 2008). The tree with the highest log likelihood (-1171.32) is shown. The number besides the branches displays the percentage of trees where the associated taxa were clustered together. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Besides the PRRs immune relations a myriad of other proteins where already observed to be involved in pathogen defence sometimes acting downstream of those receptors. Some examples in relation to the revealed candidate genes are discussed in the following text.

Solyc12g009560 putatively encodes an EIN3-binding F-box protein 1 (EBF1). As mentioned in 1.3.1 EIN3 proteins can regulate the ERF branch of JA signalling which is often associated with resistance against necrotrophic pathogens. As part of a E3

ubiquitin ligase complex EBF1 may be responsible for proteasomal degradation and further regulation of the ethylene cascade.

Since Solyc12g009565 is only annotated as unknown protein, a further look into the sequence was necessary. The predicted translated protein is very small (44 amino acids). Furthermore, it has no predicted domain structure and no sequence homologues at DNA or protein level. Therefore, it is probably a wrongly annotated open reading frame (ORF) or part of a bigger unannotated coding region. Possibly a Rapid amplification of cDNA ends (RACE) PCR or a comparable technique could give further insights into the correct reading frame. Through a RACE-PCR unknown 5' or 3' sequence of an ORF starting from a known DNA sequence inside the gene can be elucidated on cDNA level (Frohmann, 1994).

Solyc12g009570 is a calcineurin B-like interacting protein kinase also called non-specific serine/threonine protein kinase (cipk). Such proteins are often involved in abiotic stress signalling e.g. SlCBL10 ensures plant growth under salt stress (Egea *et al.*, 2018). Furthermore, biotic stress signalling for example in plant immunity could be observed e.g. AtCIPK6 negatively regulating ROS in ETI and PTI (Sardar *et al.*, 2017) or *OsCIPK14* and *15* which are induced through MAMPs (Kurusu *et al.*, 2010).

Solyc12g009580 domain architecture is similar to a CRABS CLAW 5b protein. These proteins contain a specific domain called YABBY and act as putative transcription factors. The DNA binding often occurs at target genes involved in carpel developmental processes (Gross *et al.*, 2018). The gene itself seems unlikely to be involved in important immune processes during *C. reflexa* infection but as for all transcription factors a binding and activation or inactivation of a specific gene cannot be excluded.

Solyc12g009590 encodes a putative Methyl-CpG-binding domain-containing protein 11. These proteins are used in the mechanism of DNA methylation (Bartee *et al*., 2001; Lindroth *et al*., 2001; Matzke *et al*., 2004) and are important epigenetic markers for genome stability and regulation of expression in plants and animals (Law & Jacobsen, 2010; He *et al*., 2011). But they are usually not directly linked to plant immunity. Nevertheless, it is possible that they may be involved in regulation of downstream genes by promoter activation or inactivation like AtMBD7 for the Pro35S from the *cauliflower mosaic virus* (Wang *et al.*, 2015).

Solyc12g009600 was only annotated in the previous annotation of the tomato genome (ITAG4.0). It was annotated as Thylakoid lumenal 16.5 kDa protein. Even so

this protein class is usually involved in photosystem II repair cycle, connections to immune genes are not unknown (Järvi *et al.*, 2016). But since the gene is no longer present in the new *S. lycopersicum* genome release, it is most likely not relevant for the researched immune response in this work.

Solyc12g009610 encodes a RabGAP/TBC domain-containing protein. Rabs, together with their regulator proteins guanosine triphosphatases (GTPases), are the best known regulators for endomembrane vesicle formation. They are related to intracellular vesicle trafficking, budding, targeting, docking and fusion (Cherfils & Zeghouf, 2013; Johansen *et al*., 2009; Mizuno-Yamasaki *et al*., 2012). A possible immune relevance could be shown for the movement of *Bamboo mosaic virus* which seems to be positively regulated by Rab-GTPases in *N. benthamiana* (Huang *et al.*, 2013). Also the infection of *A. thaliana* through the fungus *Verticillium longisporum* is influenced through a RabGAP protein (RabGAP22) activating multiple components in the plant immune system (Roos *et al.*, 2014).

Solyc12g009620 is annotated as Ubiquitin system component Cue protein because of the presence of ubiquitin binding domain (UBA, InterPro: Blum *et al.*, 2021) which is usually a ~45 amino acids long with a conserved structure and hydrophobic patch (Mueller & Feigon, 2002). The rest of the protein sequence gives no further function clues and *in silico* modelling also results in uncharacterized protein structures (SWISS-MODEL: Waterhouse *et al.*, 2018). UBA domain containing proteins can be involved in several cellular processes like in the ubiquitin/proteasome pathway, DNA excision-repair, and cell signaling via protein kinases (Mueller & Feigon, 2002). It could already be observed that ubiquitin associated proteins can have effects in plant immunity like the putative E3 ubiquitin ligase UBR7 from tobacco which negatively regulates the resistance against *Tobacco mosaic virus* (Zhang *et al.*, 2019). Therefore, the tomato UBA-containing protein could also show effects on pathogen immunity.

Solyc12g009630 a Calmodulin-like protein 5 shows homology to some related proteins e.g. from *Oryza sativa subsp. japonica* (OsCML5) or *A. thaliana* (AtCML5, ncbi: blastn; Altschul *et al.*, 1990). Both genes have exhibited relations to stress responses where OsCML5 is linked to osmotic and salt stress (Chinpongpanich *et al.*, 2012) and AtCML8 an EF-hand family Ca^{2+}-binding protein acting as positive regulator for plant immunity against *Pseudomonas syringae* (Zhu *et al.*, 2016). Usually CMLs are considered early or transiently responsive to biotic and abiotic

stresses (McCormack *et al*., 2005, Hruz *et al*., 2008) with specific subcellular location (Dong *et al*., 2002, Chigri *et al*., 2012) but several observations about immunity connections could already be made (Heo *et al*., 1999). Consequently, the tomato variant of this protein could possibly also induce a defence response in answer of parasite attack either in response to stresses or through interactions with the SA signaling pathway similar to the AtCML8.

Solyc12g009640 encoded proteins show homology to the class of zinc finger BED domain-containing protein DAYSLEEPER-like. These proteins structurally resemble hAT transposases and can bind DNA e.g. the AtDAYSLEEPER binds the promoter of the DNA-damage response gene *Ku70* (Bundock & Hooykaas, 2005). They are predominantly expressed in the nucleus and related to meristems and flower and fruit development (Knip *et al.*, 2013). No obvious connection between plant immunity and DAYSLEEPER proteins was observed but the BED domain is also present in some NLRs and was shown to confer resistance to bacterial blight in rice (Yoshimura *et al.*, 1998; Das *et al.*, 2014).

Solyc12g009650 is annotated as Sl proline-rich protein but Sundaresan *et al.*, 2018 stated it is also a *HyPRP1* gene which negatively regulates salt and oxidative stress. Like other genes, it may not be included in the primary defence response but act downstream in the signalling cascade.

Solyc12g009660 and Solyc12g009670 are annotated as exostosin-like proteins. This protein class was first annotated in animals as glycosyltransferases through their linkage to hereditary multiple exostosis (e.g. Cook *et al.*, 1993; Le Merrer *et al.*, 1994; Wu *et al.*, 1994; Yamada, 2020). Also in plants this protein family shows activity as glycosyltransferases even though the corresponding polysaccharide structures are completely different e.g. AtMUR3 (Madson *et al.*, 2003). The closest homologues to the tomato exostosin-like protein in *Arabidopsis* At5g20260 and At3g42180 share the predicted glycosytransferase activity and further are suggested to be Golgi located (Jensen, 2008). Even a regulatory defense function for *Atmur3* mutants could be observed (Tedman-Jones, 2008). Therefore, the tomato variants could possibly also contribute to plant-plant defense maybe even in concert with the SlCuRe1-CrGRP pathway (Hegenauer *et al.*, 2020) since exostosin-like glycosyltransferases modify cell wall components as well as LRR receptors and the cell wall is also the location of GRP.

Solyc12g009680 is a heptahelical transmembrane protein (HHP). HHP function can be quite diverse and differentially expressed in various organs. In *Arabidopsis* those genes show significant similarities to human AdipoRs (human adiponectin receptors) and mPRs (membrane progestin receptors) and can be induced by light, sucrose, plant hormones, temperature or salt stress (Hsieh & Goodman, 2005). Besides their diverse induction pattern these proteins can also regulate several functions like plant hormone signalling through ABA (Pandey & Assmann, 2004) by AtGCR1 a putative GPCR (G protein-coupled receptor 1). In barley even immune response coupled to heptahelical transmembrane proteins could be observed where the MLO negatively regulates defence against powdery mildew and cell death (Büschges *et al.*, 1997) supported through interaction with calmodulin (Kim *et al.*, 2002).

The described diverse functions of the candidate proteins and their relatives in different plant species revealed that nearly all candidates can play a potential role in plant immunity. Therefore, an in depth analysis of all GOIs is essential.

Furthermore, it is possible that the initial 172kb deletion resulted in a generation of dominant-negative mutation effects. A classical dominant-negative effect is described as a mutant polypeptide that disrupts the activity of a wild-type gene if overexpressed (Herskowitz, 1987). Initially described as intralocus interactions dominance and dominant-negative effects have been found out to also be able to act in an interlocus manner (Omholt *et al.*, 2000; Veitia, 2002). In immune responses a dominant-negative effect was already visible e.g. in *Arabidopsis* where a mutated PUB13 only consisting of ARMADILLO (ARM) domain led to the inhibition of the ubiquitination of FLS2 (Zhou *et al.*, 2015).

Also in ETI dominant-negative effects could be observed. P-loop mutations in specific *Arabidopsis* NLRs can lead to impairment of autoimmunity (Lolle *et al.*, 2017).

Besides exostosin-like proteins from the animal system (McCormick *et al.*, 2000) none of the candidate genes identified in this work are famous to be an obvious class of proteins building dimers or oligomers but some of them contain DNA binding domains like Solyc12g009590 or Solyc12g009640 which could possibly be influenced through dominant-negative effects. For transcription factors, dominant-negative effects could already be observed in yeast (Dutoit & Jacobs, 2010) and plants (Velten *et al.*, 2010).

Interestingly a study from Kim *et al.* from 2018 analysed two QTL connected to disease resistance from *S. lycopersicum* Hawaii7996 against bacterial wilt caused by

Ralstonia pseudosolabacearum. One of those loci was located on chromosome 12 partially overlapping with the region of interest analysed in this work. Kim *et al.* identified functional SNPs inside the gene *Solyc12g009690* making resistant and susceptible cultivars distinguishable. Observations like this further enhance the idea that the experimental evidence from this work rendering *Solyc12g009690* a putative important resistance gene for plant-plant parasitism.

4.1.3 Homology of *S. lycopersicum* candidate genes to *S. pennellii*

To finally pin down the immunity relevant candidate gene/genes, the generation of single knock out mutants for all genes is important. Stably transformed *S. lycopersicum* CRISPR-Cas9 mutant lines for the candidate genes are already in production as described in 3.1.10. Those lines need to be further tested for their immune relevance especially in their effect on *C. reflexa* infection.

Homology analysis of candidates on gene level revealed a high similarity between *S. lycopersicum* and *S. pennellii* genome sequences (see Table 14) and annotation (see

Table 13).

Therefore, even small base pair exchanges, insertion or deletions may be of high relevance for identifying a susceptible phenotype. Nevertheless, genes that show less similarity between both tomato species seem more likely to be relevant for *Cuscuta* resistance.

Also on protein sequence level most genes showed high homology with regard to sequence (see Table 16) and annotation (see Table 15). Therefore, it is possible that only minor sequence dissimilarities are responsible for the different resistance phenotypes of *S. pennellii* and *S. lycopersicum* e.g. SNPs introducing frame shift mutations resulting. A relatable case was observed with CuRe1 where highly homologues proteins even from the same tomato species were not able to induce the same effect (Fürst *et al.*, 2016).

Finally, a complementation of the lacking candidate genes in the original IL12-2 should be performed where ideally the candidates identified through the single knock out *S. lycopersicum* plants should be used. In this case, the resistant phenotype as seen in the growth assay (see Figure 24) should be reconstitutable.

Table 13: Blast of *S. lycopersicum* genomic sequence (ITAG4.1; SL4.0) of candidate genes against *S. pennellii* (NCBI Solanum pennellii Annotation Release 100).

Gene Name	Solgenomics Description (ITAG4.1; SL4.0)	Accession *S. penn.*	NCBI Blast Description (PREDICTED: *Solanum pennellii*)
Solyc12g009510	receptor-like protein 12	XM_027913548.1	receptor-like protein 32
Solyc12g009520	receptor-like protein 12	XM_015203827.2	receptor-like protein 7
Solyc12g009530	receptor-like protein 12	XM_027913560.1	receptor-like protein 6
Solyc12g009550	Leucine-rich repeat receptor-like protein kinase PXL1	XM_015205175.2	receptor-like protein 7
Solyc12g009560	EIN3-binding F-box protein 1	XM_015205650.2	EIN3-binding F-box protein 1-like
Solyc12g009565	Unknown protein	HG975451.1	chromosome ch12, complete genome
Solyc12g009570	calcineurin B-like interacting protein kinase	XM_015205966.2	CBL-interacting serine/threonine-protein kinase 24
Solyc12g009580	CRABS CLAW 5b	HG975451.1	chromosome ch12, complete genome
Solyc12g009590	Methyl-CpG-binding domain-containing protein 11	XM_015206160.2	methyl-CpG-binding domain-containing protein 11-like
Solyc12g009600	Thylakoid lumenal 16.5 kDa protein	XM_015206215.2	thylakoid lumenal 16.5 kDa protein, chloroplastic
Solyc12g009610	RabGAP/TBC domain-containing protein	XM_015206203.2	TBC1 domain family member 17-like
Solyc12g009620	Ubiquitin system component Cue protein	XM_015203875.2	uncharacterized LOC107005314
Solyc12g009630	Calmodulin-like protein 5	XM_015204332.2	calmodulin-like protein 3
Solyc12g009640	zinc finger BED domain-containing protein DAYSLEEPER-like	XM_015205661.2	zinc finger BED domain-containing protein DAYSLEEPER-like
Solyc12g009650	Sl proline-rich protein	XM_015205850.2	36.4 kDa proline-rich protein
Solyc12g009660	Exostosin-like protein	XM_027913258.1	probable glycosyltransferase At5g20260
Solyc12g009670	Exostosin-like	XM_027913258.1	probable glycosyltransferase At5g20260
Solyc12g009680	Heptahelical transmembrane protein 1	XM_015203870.2	heptahelical transmembrane protein 1-like
Solyc12g009690	receptor-like protein 12	XM_027913548.1	receptor-like protein 32

Table 14: Gene sequence homology of *S. lycopersicum* (ITAG4.1; SL4.0) and *S. pennellii* (NCBI *Solanum pennellii* Annotation Release 100) based on NCBI Nucleotide Blast.

S. lyco. Gene Name	Length	*S. penn.* Accession	Acc. Len	Max Score	Total Score	Query Cover	E value	Per. Ident
Solyc12g009510	5572	XM_027913548.1	3868	4063	8304	75%	0.0	97.76%
Solyc12g009520	2892	XM_015203827.2	3299	4959	4959	100%	0.0	97.62%
Solyc12g009530	1467	XM_027913560.1	3447	2172	2172	99%	0.0	93.46%
Solyc12g009550	684	XM_015205175.2	2826	652	652	99%	0.0	84.01%
Solyc12g009560	3952	XM_015205650.2	2878	4662	5110	72%	0.0	98.89%
Solyc12g009565	1291	HG975451.1	83305730	1605	3650	100%	0.0	88.94%
Solyc12g009570	6632	XM_015205966.2	1856	446	2772	23%	3,00E-122	97.33%
Solyc12g009580	6247	HG975451.1	83305730	8091	83698	99%	0.0	93.76%
Solyc12g009590	7908	XM_015206160.2	1757	2501	2736	19%	0.0	98.45%
Solyc12g009600	2195	XM_015206215.2	895	564	1516	38%	3,00E-158	97.58%
Solyc12g009610	11397	XM_015206203.2	2147	689	3708	18%	0.0	97.52%
Solyc12g009620	6909	XM_015203875.2	1415	680	2225	18%	0.0	97.72%
Solyc12g009630	717	XM_015204332.2	1212	1269	1269	100%	0.0	98.61%
Solyc12g009640	2808	XM_015205661.2	3510	5125	6995	100%	0.0	99.61%
Solyc12g009650	1534	XM_015205850.2	1036	1306	2575	61%	0.0	96.46%
Solyc12g009660	1306	XM_027913258.1	1445	1210	1828	78%	0.0	99.11%
Solyc12g009670	964	XM_027913258.1	1445	377	643	39%	2,00E-102	96.89%
Solyc12g009680	2768	XM_015203870.2	1429	1195	1924	39%	0.0	99.10%
Solyc12g009690	1570	XM_027913548.1	3868	4063	8304	75%	0.0	97.76%

Table 15: Blast of *S. lycopersicum* protein sequence (ITAG4.1; SL4.0) of candidate proteins against *S. pennellii* (NCBI Solanum pennellii Annotation Release 100).

Gene Name	*S. lycopersicum* Description	*S. penn.* Accession	*S. pennellii* Description
Solyc12g009510	receptor-like protein 12	XP_027769349.1	LOW QUALITY PROTEIN: receptor-like protein 32
Solyc12g009520	receptor-like protein 12	XP_015059313.1	receptor-like protein 7
Solyc12g009530	receptor-like protein 12	XP_027769361.1	receptor-like protein 6
Solyc12g009550	Leucine-rich repeat receptor-like protein kinase PXL1	XP_027769361.1	receptor-like protein 6
Solyc12g009560	EIN3-binding F-box protein 1	XP_015061136.1	EIN3-binding F-box protein 1-like
Solyc12g009565	Unknown protein	-	-
Solyc12g009570	calcineurin B-like interacting protein kinase	XP_015061451.1	CBL-interacting serine/threonine-protein kinase 24
Solyc12g009580	CRABS CLAW 5b	XP_015061453.1	axial regulator YABBY 5-like
Solyc12g009590	Methyl-CpG-binding domain-containing protein 11	XP_015061646.1	methyl-CpG-binding domain-containing protein 11-like
Solyc12g009600	Thylakoid lumenal 16.5 kDa protein	XP_015061701.1	thylakoid lumenal 16.5 kDa protein, chloroplastic
Solyc12g009610	RabGAP/TBC domain-containing protein	XP_015061689.1	TBC1 domain family member 17-like
Solyc12g009620	Ubiquitin system component Cue protein	XP_015059361.1	uncharacterized protein LOC107005314
Solyc12g009630	Calmodulin-like protein 5	XP_015059818.1	calmodulin-like protein 3 [Solanum pennellii]
Solyc12g009640	zinc finger BED domain-containing protein DAYSLEEPER-like	XP_015061145.1	zinc finger BED domain-containing protein DAYSLEEPER-like
Solyc12g009650	SI proline-rich protein	XP_015061336.1	36.4 kDa proline-rich protein
Solyc12g009660	Exostosin-like protein	XP_027769059.1	probable glycosyltransferase At5g20260
Solyc12g009670	Exostosin-like	XP_027769059.1	probable glycosyltransferase At5g20260
Solyc12g009680	Heptahelical transmembrane protein 1	XP_015059356.1	heptahelical transmembrane protein 1-like
Solyc12g009690	receptor-like protein 12	XP_027769352.1	receptor-like protein 19

Table 16: Protein sequence homology of *S. lycopersicum* (ITAG4.1; SL4.0) and *S. pennellii* (NCBI *Solanum pennellii* Annotation Release 100) based on NCBI Protein Blast.

S. lyco. Gene Name	Length	*S. penn.* Accession	Acc. Len	Max Score	Total Score	Query Cover	E value	Per. Ident
Solyc12g009510	1671	XP_027769349.1	1279	1613	2848	99%	0.0	75.52%
Solyc12g009520	964	XP_015059313.1	963	1612	1612	98%	0.0	95.27%
Solyc12g009530	489	XP_027769361.1	1009	779	897	98%	0.0	90.21%
Solyc12g009550	209	XP_027769361.1	1009	295	452	100%	6,00E-94	76.59%
Solyc12g009560	637	XP_015061136.1	637	1186	1186	100%	0.0	98.27%
Solyc12g009565	45	-	-	-	-	-	-	-
Solyc12g009570	244	XP_015061451.1	446	501	501	99%	8,00E-180	99.18%
Solyc12g009580	192	XP_015061453.1	191	396	396	99%	3,00E-143	97.91%
Solyc12g009590	435	XP_015061646.1	433	638	638	99%	0.0	97.47%
Solyc12g009600	211	XP_015061701.1	210	347	347	99%	3,00E-123	99.05%
Solyc12g009610	657	XP_015061689.1	656	1283	1283	99%	0.0	98.63%
Solyc12g009620	282	XP_015059361.1	281	565	565	99%	0.0	98.22%
Solyc12g009630	157	XP_015059818.1	238	133	214	84%	2,00E-39	98.75%
Solyc12g009640	963	XP_015061145.1	935	1783	2270	98%	0.0	99.89%
Solyc12g009650	266	XP_015061336.1	262	160	195	41%	6,00E-48	100.00%
Solyc12g009660	339	XP_027769059.1	467	690	690	99%	0.0	97.93%
Solyc12g009670	144	XP_027769059.1	467	216	216	86%	2,00E-69	96.00%
Solyc12g009680	329	XP_015059356.1	328	650	650	95%	0.0	99.04%
Solyc12g009690	386	XP_027769352.1	276	541	541	71%	0.0	96.01%

4.2 Phytohormones during *C. reflexa* infection of a susceptible host

Parasites need to influence the potential host in subtle but efficient manner to gain access to the foreign beneficial nutrients. Especially the plant – parasitic plant interaction is interesting because of the close relation of both partners. Upon infection parasitic plants form haustoria through which they can acquire nutrients as well as small compounds like plant hormones and RNAs (Yoshida *et al.*, 2016; Spallek *et al.*, 2017; Shahid *et al.*, 2018). The induction of haustoria formation in *Orobanchaceae* for example depends on host-derived signal molecules. Several secondary compounds which are relevant for the infection process could already be identified.

They are usually summarized as haustoria-inducing factors (HIFs) which can include diverse substances like quinones or phenolics, such as 2,6-dimethoxy-p-benzoquinone (DMBQ) or syringic acid (Yoshida *et al.*, 2016). The influence on HIFs stretches to the phytohormone synthesis specifically on the infection site like auxin production to support the cell division and expansion for haustorial structures (Ishida *et al.*, 2016) or ethylene upregulation potentially related to haustoria initiation (Tomilov *et al.*, 2005). Therefore, analysis of fluctuation in phytohormone metabolism during *Cuscuta* infections is of great interest for understanding the mechanism behind the infection process.

Detailed information about changes in gene expression patterns or metabolism are often examined at time points 48h or 72h after infection. In this study we aimed to get a better insight into the detailed effects of phytohormone effects for early and later infection stages from 1 up to 10 days post infection.

Phytohormone effects are observable in numerous parasitizing species across different kingdoms. Fungi, for example the biotroph *Ustilago maydis,* convert chorismate into the inconvertable prephenate through Cmu1 depleting the chorismate pool available for isochorismate production resulting in less SA biosynthesis (Djamei *et al.*, 2011) and therefore most likely reduced immunity. Some bacterium pathovars of *P. syringae* produce coronatine a molecular mimic of JA-Ile (Mitchell, 1982) which binds the same receptor complex leading to gene repression (Thines *et al.*, 2007). Even an enhancement of hormone production inside the host is possible where for example the bacterial *Rhodococcus fascians* produces three different CKs to force continued proliferation of host tissue (Pertry *et al.*, 2009).

In a resistant host plant, *C. pentagona* attachment induces JA and SA production, which is no surprise since both hormones have a strong connection to plant defence (Runyon *et al.*, 2010). Also for *C. reflexa* the same is true with the resistant host *S. lycopersicum* cv. Moneymaker (Albert, 2005). The hormone effects of *Cuscuta* infection on a susceptible host remain elusive. Which hormones might be influenced during the infection process in susceptible plants?

Studies exist in which the involvement of phytohormones like auxins and cytokinins in xylem vessel formation has been analysed (Ohashi-Ito *et al.*, 2002; Fukada, 2004). These hormones might also be important for xylem bridge formation in the developing haustoria. Additionally, findings revealed effective injection of cytokinin

from the hemiparasitic plant *Phtheirospermum japonicum* into the host *A. thaliana* (Spallek *et al.*, 2017).

Moreover, the relevance of JA and SA as potential defense hormones should be observed in a susceptible host possibly gaining insight into different avoiding or suppression mechanisms during recognition and repellation of plant parasites.

The COLORFUL plants are an easy way to determine hormone concentration even on a single cell level (Ghareeb *et al.*, 2016; Ghareeb *et al.*, 2020). Those plants are *A. thaliana* plants stably transformed with genes expressing fluorescence proteins. In this work plants were used containing constitutively expressed membrane located GFP and nuclear localized mKate2. In addition, those plants contained an inducible nuclear localized Venus reporter-gene that could be expressed phytohormone-dependent. Specifically, those hormones were auxin, cytokinins, jasmonate, jasmonate/ethylene, abscisic acid and salicylic acid. With the help of these plants stem cross-sections at *C. reflexa* infection sites for time periods up to 10 days could be analysed. In this work complete cross-sections of *Arabidopsis* were analysed on cellular level and the resulting nuclear fluorescence was combined and normalized against the constitutive expressed mKate2.

The results of these measurements showed no reproducible trend in phytohormone production in response to *Cuscuta* infection. They were repeated at least three times for the phytohormones for which COLORFUL marker lines were available (auxin, cytokinins, jasmonate, jasmonate/ethylene, abscisic acid, salicylic acid). This seems to be partly explainable due to a high variability across the measured samples. Possibly, this is because of slightly differing development stages of the tested plants since the biological replicates may grow faster or slower. Additionally, *C. reflexa* may show differing infection behaviour even though the starting weight, developmental stage and growth conditions are the same. Also slightly differing sizes of the cross-sections is possible since the cross-sections had to be prepared on fresh material because of the weak fluorescence retaining properties of fluorescent proteins in fixated, embedded or mounted tissue. Therefore, in some pictures a higher number of fluorescent nuclei is possibly visible, shining through from tissue layers above or below the focus plane.

To tackle the discrepancies of phytohormone production in the different measurements a second method was performed in parallel. The hormone content was determined through GC-MS from shoots infected with *C. reflexa*. The minimal

amount necessary for GC-MS measurements of 150 mg required the use of whole nitrogen-frozen shoots. Again, a high discrepancy between the different measurements and biological replicates in phytohormone content made the identification of *Cuscuta* induced changes in hormone metabolism challenging during the three independent repetitions.

Phytohormones can act in a systemic manner. This could for example be observed for JA and some derivatives (Heyer, 2018). Therefore, the higher amount of plant material needed for GC-MS, usually including a complete infected shoot, could be advantageous for identifying such effects. Unfortunately, also no consistent systemic effects of all tested phytohormones could be observed.

Taking into account the challenges of the two described methods, a final technique, the LC-MS measurement, was performed. The lower minimal amount of plant material needed in this method for analysis allowed the use of a more defined region directly infected by *C. reflexa* haustoria. Additionally, the used susceptible host plant was *N. tabacum* constitutively expressing GFP (Haupt *et al.*, 2001) allowed a better classification of infection stadiums. The possibility to picture the transport of free GFP to *C. reflexa* after completed haustorium formation allows for far better determination of the appropriate time point and a differentiation of faster and slower developing *Cuscutas*. Thus, a better relativization of the analysed plant-parasite interactions is possible but still no significant differences were observed. Neither could changed JA levels, visible in resistant plants tomato plants (Runyon *et al.*, 2010), be observed in susceptible hosts nor could any abnormalities in abscisic acid content be observed in such plants. There exists evidence that *C. reflexa* is able to produce ABA (Qin *et al.*, 2008) which seems, however, host independent. Some evidence for hormone signals travelling from *Cuscuta* to host exist (Zhuang *et al.*, 2018) alongside numerous other molecules (Haupt *et al*., 2001; Birschwilks *et al*., 2006; Furuhashi *et al*., 2011; Kim *et al*., 2014; Smith *et al*., 2016).

There can be various reasons for the inconsistency of the changes in phytohormone content. First of all, phytohormone fluctuation tracked through marker genes like *PR1* for SA signalling (Jiang & Guo, 2010; McCourt, 1999) or hormone deficient mutants (Gazzarrini & McCourt, 2003) may differ from measurements of direct phytohormone content since genetic effects may not take non-linear cross-talk between hormones into account. Therefore, some effects may only be observable if the according technique is used. Furthermore, small deviations in extraction or measurement

protocols for GC-MS may lead to varying outcomes since phytohormones are present only in low concentrations inside the plant cell and maybe more precise methods are necessary for example as described in Cao *et al.*, 2020 where UPLC-MS/MS from low plant material amount allows measurement from several phytohormones and RNA. Also the high variability observable in every performed measurement could hinder the identification of a clear trend in metabolism. Possibly through the use of an even higher number of biological replicates per experiment this could be reduced.

Moreover, it is also possible that changes in hormone abundance at the measured time points are too subtle or even non-existent for most hormones and therefore also not detectable. Since susceptible hosts were used for hormone measurement, major changes in phytohormones involved in defence are not to be expected. Furthermore, it is possible that *Cuscuta* influences the host metabolism on another level than through the tested hormones e.g. RNAs or secondary metabolites.

Finally, the use of some hormone synthesis/defence deficient mutants for NPR1, NDR1 and PAD4 revealed that the lack of those is not essential for a successful infection through *C. reflexa* at least in the tested susceptible plants. The biomass increase of all tested plants was unaltered. This fits to the before discussed results of a mostly unaltered hormone metabolism during infection of a susceptible host.

Taken together the results suggest that the phytohormones tested in this work seem not essential for the infection process of *C. reflexa* on the analysed susceptible hosts *A. thaliana* and *N. tabacum*. It is also possible that the hormonal changes are too subtle to be detectable. Regulation of the infection process might still occur on a different metabolic level or be dependent on other hormones which were not subject for testing in this work.

Interestingly, there was no change in JA or SA content, which may hint at a possible repression of those important defence/stress response hormones during *C. reflexa* infection on a susceptible host.

5 Outlook

5.1.1 Resistance mechanism of *S. lycopersicum* against *C. reflexa*

The existence of a second resistance locus could be confirmed in this work and the identity of the defence relevant GOIs could be limited to 19 candidates.
The reduction of candidate genes through the CRISPR-Cas9 based method described in this work leaves a reasonable low number of GOIs for further genetic analysis. But which gene/genes is/are the responsible defence components?
A knock-out of those genes should reveal the exact identity of the relevant GOIs. Those mutants are in production but the results are not yet available at this time.
In addition to mutant production a RNA sequencing (RNAseq) based experiment could give further insight into regulation and therefore defence relevance of those genes. With RNAseq the differential gene expression of all genes could be observed (reviewed in Stark *et al.*, 2019). A comparison of gene expression profiles between an infected and an uninfected *S. lycopersicum* with regard to the candidate genes identified in this work could give a hint about resistance genes. Especially in the case of multiple involved GOIs such an approach could reveal a quantitative trait effect.
Finally, a complementation of the susceptible *S. lycopersicum* lL12-2 would prove the relevance of the soon to be identified gene/genes of interest for resistance.

5.1.2 *C. reflexa* infection and the resulting changes in phytohormone metabolism

The results of this work indicate an unchanged or only slightly changed phytohormone metabolism for auxin, cytokinins, jasmonate, jasmonate/ethylene, abscisic acid and salicylic acid at the stem infection sites.
It is possible that only few cells respond to the penetrating *C. reflexa* haustoria or other hormones show a stronger involvement during the infection process. Therefore, it would be advisable to check them like e.g. brassionolide or peptide hormones (overview in Hirakawa *et al.*, 2017). Furthermore, a first analysis of hormone metabolism changes on genetic level with the help of marker genes (e.g. with qRT-PCR) could give first insights into variations which are maybe too subtle to detect with methods described in this work.

Especially a lacking response in JA and SA production in susceptible *A. thaliana* and *N. tabacum* plants shown in this work, may indicate an active repression of those signals which may otherwise hinder the invasion of plant cells through haustoria formation.

6 Summary

Parasitic flowering plants live as heterotrophs and obtain inorganic and organic nutrients from their hosts. *C. reflexa*, belonging to the genus *Cuscuta* comprising of approximately 200 species, is a holoparasite with the appearance of a thread-like vine that coils around its hosts shoot. During the infection process, *C. reflexa* forms a specialized haustorium for penetrating the hosts shoot and connecting to the hosts xylem and phloem. While nearly all plants are susceptible hosts for *Cuscuta spp.*, tomato as one of few exceptions shows an active resistance against *C. reflexa*.

The aims of this work were the identification of new resistance related genes in the resistant host *S. lycopersicum* and the identification of changes in phytohormone metabolism during the infection of *C. reflexa* on a susceptible host.

A library of introgression lines between resistant *S. lycopersicum* and susceptible *S. pennellii* carrying different chromosomal recombinations, was screened for susceptibility and a hypersensitive response occurring at the attempted haustorium penetration sites. A region of about 172 kbps on chromosome 12 revealed a function in defence against *C. reflexa*. *S. lycopersicum* plants lacking this region showed susceptibility against the plant parasite. By excising smaller chromosomal parts from the region of interest with a CRISPR-Cas9 technique the number of potential candidate genes could be further narrowed down. The susceptibility mapped to chromosome 12 does not influence immune responses like ROS and ethylene production which are linked to the first identified resistance trait CuRe1.

Single knock outs of the identified candidate genes will reveal the exact gene/genes responsible for the second resistance trait in *S. lycopersicum* against *C. reflexa*. A complementation with respected gene(s) will prove this.

During the interaction of *C. reflexa* and a susceptible host, numerous signals may be exchanged to ensure a successful invasion through the parasite. Most likely, in this process there is a directed influence on phytohormone metabolism. This is to be expected with a high probability since these signalling molecules are vital for almost all processes inside the plant.

To identify those changes several experimental approaches were tested, e.g. GC-MS, LC-MS, fluorescence plant marker lines (COLORFUL lines) and hormone-deficient mutants. These techniques were all used to monitor phytohormone changes during an infection of susceptible *A. thaliana* or *N. tabacum* by *C. reflexa* over a time

of up to 10 days. Changes have been analysed for the phytohormones auxin, cytokinins, jasmonate, jasmonate/ethylene, abscisic acid and salicylic acid. None of those hormones showed consistent reproducible trends for up- or downregulation of metabolism. This may be due to several reasons, like the low total abundance of phytohormones in general and therefore the challenges of measuring their total content or due to an influence of the parasite on a different regulatory level (even different hormonal level). Even an active suppression of unfavourable hormones through the parasite during the infection is possible.

A repetition of the experiments with a much higher number of plants could give better insights into slight changes of phytohormone levels. Also promising could be the analysis of other hormones not included in our studies, like e.g. brassinolide or peptide hormones or even a comparison on genetic level with established marker genes.

7 Zusammenfassung

Blütenbildende parasitäre Pflanzen sind heterotrophe Organismen und beziehen organische und anorganische Nährstoffe von ihren Wirten. *C. reflexa* gehört zur Gattung der *Cuscuta*, die aus etwa 200 Arten bestehen. Dabei handelt es sich um einen Holoparasiten, der wie eine fadenartige Ranke aussieht, welche sich um die Sprossachse des Wirtes windet. Während der Infektion bildet *C. reflexa* ein spezialisiertes Pflanzenorgan, das Haustorium, aus, welches den Wirtsspross durchdringt und sich mit dessen Xylem und Phloem verbindet. *Cuscuta spp.* hat ein breites Wirtsspektrum. Eine der wenigen resistenten Ausnahmen ist die Kulturtomate (*S. lycopersicum*). Diese kann eine effektive Immunantwort gegen *C. reflexa* etablieren.

Das Ziel dieser Arbeit war die Identifikation von neuen Resistenzgenen in der resistenten Wirtspflanze *S. lycopersicum* und außerdem die Identifikation von Änderungen des Pflanzenhormonstoffwechsels während der Infektion von *C. reflexa* bei einem suszeptiblen Wirt.

Introgressionslinien sind Kreuzungen zwischen der *C. reflexa*-resistenten *S. lycopersicum* und der suszeptiblen *S. pennellii*. Die verschiedenen Kreuzungslinien zeigen unterschiedliche chromosomale Rekombinationen. Eine Auswahl dieser ILs wurde analysiert in Hinsicht auf Suszeptibilität und auf Ausbildung einer hypersensitiven Reaktion im Bereich der versuchten *C. reflexa* Penetration. Ein Bereich mit einer Größe von etwa 172 kb auf Chromosom 12 zeigte dabei einen Einfluss auf die Immunantwort der Tomate gegen *C. reflexa*. *S. lycopersicum* Pflanzen ohne diese Region zeigten Suszeptibilität gegen den Pflanzenparasiten. Diese 172 kb wurden durch Nutzung einer CRISPR-Cas9-basierten Technik erzeugt, indem kleinere chromosomale Regionen aus dem Genom ausgeschnitten wurden. Dadurch konnte die Zahl potentieller Kandidatengene weiter eingegrenzt werden. Die Suszeptibilität, welche mit den Genen auf Chromosom 12 in Zusammenhang steht, scheint dabei Immunantworten wie die ROS und Ethylenproduktion nicht zu beeinflussen. Diese sind allerdings abhängig von dem ersten identifizierten Resistenzgen (*CuRe1*).

Die Deletionen einzelner Kandidatengene sollte die Identität des/der relevanten Gens/Gene für den zweiten chromosomalen Resistenzbereich von *S. lycopersicum*

gegen *C. reflexa* offenlegen. Eine Komplementation durch dieses/diese Gen/Gene sollte dies zusätzlich beweisen.

Während der Interaktion von *C. reflexa* und einem suszeptiblen Wirt werden eine Vielzahl von Signalen zwischen beiden ausgetauscht, um eine erfolgreiche Infektion durch den Parasiten zu gewährleisten. Bei diesem Prozess wird höchstwahrscheinlich auch der Hormonmetabolismus der Pflanze beeinflusst, da diese wichtigen Signalmoleküle von vitaler Bedeutung in fast allen Prozessen innerhalb der Pflanze sind.

Um diese Unterschiede zu untersuchen wurden mehrere verschiedene experimentelle Techniken angewendet, wie z.B. GC-MS, LC-MS, Pflanzenlinien mit Fluoreszenzmarkern (COLORFUL Linien) und hormondefekte Mutanten. Diese Methoden wurden genutzt, um die Stoffwechseländerungen bei einer Infektion durch *C. reflexa* in suszeptiblen *A. thaliana* und *N. tabacum* über einen Zeitraum von bis zu 10 Tagen für die Hormone Auxin, Cytokinine, Jasmonate, Ethylen, Abscisinsäure und Salicylsäure zu untersuchen. Für keines dieser Hormone konnte eine konsistente Hoch- oder Runterregulation des Stoffwechsels beobachtet werden. Dafür kann es mehrere Gründe geben, wie z.B. der geringe Gehalt an Hormonen innerhalb der Pflanze im Allgemeinen und die daraus folgenden Schwierigkeiten bei deren Messung oder durch Beeinflussung des Parasiten auf anderen regulatorischen Ebenen (sogar andere hormonelle Bereiche). Auch eine aktive Suppression von unerwünschten Hormonen durch den Parasiten während der Infektion ist möglich.

Eine Wiederholung der Experimente mit einer deutlich höheren Menge an Pflanzen könnte bessere Einblicke über geringe Änderungen im Phytohormonmetabolismus geben. Ein ebenfalls vielversprechender Ansatz könnte die Analyse von anderen Hormonen, wie beispielsweise Peptidhormonen, sein oder sogar der Vergleich der Hormonfluktuation auf genetischer Ebene mit etablierten Markergenen.

8 List of Figures

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12 Supplement

Supplemental data are stored digitally on the server of the Molecular Plant Physiology (MPP), Friedrich-Alexander-University, 91058 Erlangen, Germany (Chair: Prof. Markus Albert).

Supplemental Data 1: Python code for analysis of mean intensity and number of fluorescent nuclei.

Supplemental Data 2: Transiently transformed *N. benthamiana* treated with *C. reflexa* crude extract.

Supplemental Data 3: CRISPR-Cas9 mutants of *S. lycopersicum* (B1, B2, B3, B4, VB, GB)