Function and targets of Fusarium oxysporum effectors
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Chapter 5

General discussion
Six proteins are small proteins that are secreted by the fungus *Fusarium oxysporum* f. sp. *lycopersici* in the xylem sap of infected tomato plants. Their primary structures do not correspond to known functional domains present in the Conserved Domains Database (CDD). In 2004 the first Six protein, Six1, was described (Rep et al., 2004). Exactly ten years later over 14 Six proteins have been identified (Houterman et al., 2007; Schmidt et al., 2013) and we have gathered significant understanding of their biological function and targets (Takken and Rep, 2010). However, also many new questions arose about i.a. their molecular role. This thesis expands our knowledge on Six proteins by functionally characterizing two of them: Six6 and Six8. Furthermore, it describes the identification of the first direct host target of a Six protein, TPL, and the quantitative analysis of the xylem sap proteome and its changes induced by specific Six proteins.

**What defines a Six protein as true effector?**

The definition of “effector” is heavily debated in plant pathology (Tab. 1). Hence it is important to point out which criteria apply before a Six protein is designated an effector. The definitions used in the plant pathology field, cited in Table 1, have in common that effectors are molecules that play a role during disease. This aspect only defines their biological function, which strongly deviates from the biochemical description of an “effector” (Tab. 1). On the mechanistic level, the cited literature is less uniform. A distinction can be made between the virulence functions of an effector, that define the colonization potential of a pathogen, and its role in pathogenicity, which is determined by the contribution of the effector in the development of disease symptoms. The latter definition is for instance used by de Jonge et al. (2011). I propose to combine both activities and define “effectors” for fungal plant-pathogens as follows: “Effectors are pathogen derived molecules that are secreted to manipulate or circumvent plant defenses or otherwise contribute to pathogenicity. Often they are extraordinary examples of biological innovation.” The latter part of the definition excludes all molecules with a known enzymatic mechanism and/or with significant homologies to conserved domains, such as the *Magnaporthe oryzae* derived protein AVR-Pita, which has homologies to metalloproteinases (Orbach et al., 2000). Six proteins *a priori* match this “effector” definition as they have an unknown molecular function and structures are secreted during infection, which makes them excellent effector candidates (Fig. 1). Whether they indeed aid to manipulate or circumvent plant defense or contribute to pathogenicity during infection, has to be identified experimentally as described in detail below.
### Table 1: Representative definitions of the term "effector".

<table>
<thead>
<tr>
<th>Reference</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dictionary: (Biochemistry)</td>
<td>“A small molecule that when bound to an allosteric site of an enzyme causes either a decrease or an increase in the activity of the enzyme.”</td>
</tr>
<tr>
<td>Giraldo and Valent, 2013</td>
<td>“Generally small unique proteins, many of which function to defeat PTI.”</td>
</tr>
<tr>
<td>Rovenich et al., 2014</td>
<td>“Molecules that deregulate immune responses and facilitate colonization.”</td>
</tr>
<tr>
<td>de Jonge et al., 2011</td>
<td>“(Avr) molecules that target host components to establish disease.”</td>
</tr>
<tr>
<td>Win et al., 2012</td>
<td>“Molecules that are secreted by plant-associated organisms that alter plant processes. Many pathogen effectors are extraordinary examples of biological innovation.”</td>
</tr>
<tr>
<td>Hogenhout et al., 2009</td>
<td>“Molecules, that are secreted to different cellular compartments of their host to modulate plant defense circuitry and enable parasitic colonization.”</td>
</tr>
</tbody>
</table>

**How to determine, whether a Six protein is an effector?**

Obviously, it is a challenging task to unravel the biochemical role of a molecule with unknown function. In Figure 1 I have schematically depicted the pipeline that we used to determine whether a Six protein represents a genuine effector. Of single copy SIX genes knockouts can be generated in Fol, and the transformants can be used in bioassays to test for a phenotype different from that of the wild type strain on susceptible plants. The disease index and the weight of the inoculated plants can be determined to assess alteration in pathogenicity of the knockout strains. Complementation of the knockouts with the deleted gene is required to assign the compromised pathogenicity to the deleted effector gene.

A naïve assumption is that disease symptoms (pathogenicity) proportionally increase with fungal biomass (virulence) and *vice versa*. Hence, reduced disease symptoms should correlate with a reduced fungal biomass. However, this correlation is not strict as for instance fungal growth in *coil1* mutant plants was the same as in wild type Col-0, while less disease symptoms were observed in the mutants (Thatcher et al., 2009). Also in *A. thaliana tir3* mutants disease severity and *F. oxysporum* infection are uncoupled (Diener, 2012). So, solely scoring disease symptoms can result in an under- or overestimation of the degree of colonization in the host. A decrease in disease symptoms, but unaltered host colonization, implies a major role of the effector candidate in pathogenicity rather then in facilitation of fungal growth. To identify effectors that effect fungal growth but not symptom development (virulence factors), the amount of fungal biomass could be quantified e.g. by qPCR or by
determining the activity of \( F. \text{oxysporum} \) specific enzymes such as arabinofuranosidase using 5-bromo-4-chloro-3-indoxyl-\( \alpha \)-L-arabinofuranoside (X-Ara) (Diener, 2012).

![Diagram](image)

**Figure 1:** Pipeline for Fol effector identification. A Six protein is a small secreted protein lacking homology to conserved domains in other proteins. For single copy genes a knockout can be generated and pathogenicity of the knockout strain can be analyzed in bioassays. Disease symptoms are scored upon inoculation of susceptible plants and the xylem sap proteome composition is determined. When differences are observed in comparison with the wild type strain, the Six protein might function as effector. A different strategy for analyzing Six protein function is the stable or transient transformation, of a host plant or a close relative with a \( \text{SIX} \) gene. Transgenic plants are phenotyped and tested for altered disease susceptibility. If transgenic lines differ from the wild type in susceptibility, the Six protein is a genuine effector. Often immune responses are accompanied by a hypersensitive response (HR). Hence, Six proteins that alter the HR can also be considered effectors.

A different strategy to assign an activity to a \( \text{SIX} \) gene is to determine the xylem sap proteome composition of inoculated plants (Fig 1). We report a correlation between \( \text{SIX} \) knockout specific changes in the xylem proteome and a reduced pathogenicity in bioassays (chapter 5). Six proteins, in whose absence the xylem sap proteome significantly changes compared to the one obtained after infection with wild type Fol,
can be considered genuine effectors. So far only for one out of six SIX knockouts tested, notably Six2, no clear effect on pathogenicity or xylem sap proteome was found (chapter 5). However, this does not immediately exclude Six2 as an effector, as its function might be masked by redundancy in the fungus caused by another effector molecule exhibiting a similar activity. Alternatively, Six2 is a virulence factor. In addition, agro-infiltration assays (unpublished results) showed that Six2, like Six6 and Avr1, suppresses I-2 mediated cell death in *N. benthamiana*. Modulation (both suppression or enhancement) of programmed cell death, which is induced by an immune response, is a selection criterion to assign effector function, designating Six2 as a genuine effector. Another method to assess a potential effector function is the stable expression of a SIX gene in a host plant, or in a related plant species, and phenotyping the obtained transgenics (Fig. 1). Characterization of the transgenic plants includes screening for phenotypes such as altered susceptibility/resistance to other pathogens, preferably to those having a comparable lifestyle like hemi-biotrophy or xylem-colonization. Six proteins that do not show a phenotype in any of the above-mentioned experiments could still potentially function to evade plant immunity and represent effectors. Hence, Six proteins that obtain a “Yes” in the end of the experimental pipeline (Fig. 1) represent genuine effectors. A “No” is inconclusive with regard to their function, and those Six proteins have to be further investigated to include or exclude them as true effectors.

**Six proteins in resistance and pathogenicity – pieces of a puzzle**

Besides their avirulence function, effector Avr1 suppresses I-2 and I-3 mediated resistance and effectors Avr3 and Avr2 are required for full pathogenicity on susceptible plants (Fig. 2). Furthermore, Avr2 and Six5 both contribute to full virulence and both are required for I-2 mediated resistance (Fig. 2A). Surprisingly, Six5 is not needed for I-2 mediated cell death (Ma, 2012). In this thesis I report that both Avr1 and Six6 can suppress I-2 mediated cell death in *Nicotiana benthamiana* (Fig. 2B), but that Six6 does not suppress resistance in tomato (chapter 3). Notably, Avr1 does not suppress resistance in all Fol strains either (Mes et al., 1999). Some strains, which carry AVR1, do not overcome I-2 resistance. Introduction of AVR1 in strains not carrying the gene is sufficient to restore the ability to suppress I-2 and I-3 mediated resistance (Houterman et al., 2008). The sequence of AVR1 in all race 1 strains is identical like their expression (Biju Chellappan, personal communication). Hence, additional factors might be required to aid suppression of resistance. These factors might also be involved in Six6 functioning. Suppression of I-2 mediated resistance by Avr1 could be achieved by: I) a direct interaction with Avr2, II) perturbation of the (sub)cellular translocation of Avr2, or III) by interference with downstream signaling of I-2. There is no biochemical data supporting a direct interaction between Avr1 and Avr2, disqualifying the first upon.
As Avr2 must localize in the nucleus to mediate I-2-triggered cell death (Ma et al., 2013), Avr1 (and Six6 respectively) might interfere with translocation of Avr2 from the cytoplasm to the nucleus. Confocal microscopy of N. benthamiana leaf epidermis cells agro-infiltrated with e.g. GFP-labeled Avr2 and Avr1 (or respectively Six6) could be used to challenge this hypothesis by monitoring changes in Avr2-localization in the presence of these other effectors. Suppression of cell death due to inhibition of Avr2 uptake by the plant cell is unlikely, as suppression is also observed when both effectors are expressed without their signal peptides assuring a cytosolic localization. Alternatively, Avr1 and Six6 could indirectly interfere with the structure of Avr2 and alter the redox state of the cysteine residue, which was shown to be important for I-2-mediated recognition. Like described below, Six6 might affect the redox state of the cell, which could alter the reduction state of the thiol-groups of Avr2.

Figure 2: An updated model for the molecular functions of Six proteins in tomato. A) Three resistance proteins, namely I (or I-1), I-2 and I-3 were found to mediate recognition of Avr1 (Six4), Avr2 (Six3) and Avr3 (Six1) respectively. Six5 is also required for I-2 mediated resistance, however Avr2 alone is sufficient to induce I-2 mediated cell death. Of the tomato R genes conferring resistance to Fusarium only I-2, encoding a CC-NB-LRR class protein, has been cloned. While Avr2 and Avr3 contribute to virulence, possibly by suppressing pathogen associated molecular pattern (PAMP) triggered immunity, Avr1 suppresses I-2 and I-3 mediated resistance. B) We here show that Avr1 and Six6 can suppress I-2 mediated cell death. Six8 interacts with TOPLESS (TPL), which is recognized by SNC1 in A. thaliana Col-0. Subsequently SNC1 is activated, triggering a stunted phenotype. Furthermore, in SIX6 and AVR2 transgenic A. thaliana plants expression of respectively NIA1, a nitrate-reductase, and PDF1.2 were increased. While Avr2 and Six5 both inhibit accumulation of PR-5a, Avr3 and Six6 increase accumulation of a superoxide dismutase (SOD) and peroxidases respectively.

In chapter 4 we describe the identification of a potential susceptibility target of a Six protein, namely TPL (Fig. 2B). TPL interacts with Six8 in both pull-down assays and Y2H. The protein functions as a co-repressor affecting a broad range of plant developmental and signaling processes. TPL is conserved among land-plants and a key
regulator, making it a potential target for a pathogen to hijack cellular processes like e.g. JA signaling. The interaction of Six8 with TPL could conceivably result in I) stabilization of TPL, II) competition with proteins, that interact with TPL, or III) co-repression of genes by direct binding of the Six8-TPL complex to the DNA. To test hypothesis I) TPL amounts could be quantified in the absence or presence of SIX8. The competition of Six8 with other TPL interactors could conceivably be tested in a Yeast Three-Hybrid assay to assess whether Six8 can disturb the interaction of TPL with negative regulators such as JAZ or AUX/IAAs. For hypothesis III) first the DNA binding capacity of Six8 has to be determined as discussed in chapter 4. Then a Chromatin immunoprecipitation with parallel DNA sequencing (CHIP-seq) could be performed to identify the bound DNA sequences.

These data allow us to refine the current working model (Fig. 2B). However, also new questions arise, such as how Fol effectors are taken up from the xylem sap to the host cells. We showed for Avr1, Six6 and Six8 that, when transiently expressed in N. benthamania cells without their endogenous signal peptide encoding sequence, they affect cell death (chapters 3 and 4). Hence, it is likely that they function inside the cell, which, however, does not exclude a function in the xylem sap as well. Kale et al. (2010) proposed that plant cells take up Avr2 via a PI3P-dependent mechanism in an in vitro assay. However, in vivo studies should be performed to confirm uptake during regular Fol infection.

**Six proteins – involved in modulation of the host’s cellular redox state?**

No effect on immunity or susceptibility of A. thaliana upon Six6 expression was found (chapter 3). However, deletion of SIX6 in Fol caused, besides an increase of plant weight, a significant change in the tomato xylem sap proteome. Most remarkably, accumulation of several peroxidases was decreased upon inoculation of the SIX6 knockout (chapter 5).

To survey expression changes of plant genes by Six6, the transcriptome of SIX6 transgenic A. thaliana plants was analyzed. Microarray experiments were performed as described in chapter 4 and a FDR<0.1 was considered as statistically significant. Four significantly differentially-expressed genes were identified in one of the two independent homozygous SIX6 transgenic lines as compared to the Col-0 wild type (Tab. 2). In the second, independent transgenic line the same expression trend was observed, although expression levels were not significantly different compared to those in the wild type. The significantly differential expression of these four genes in the first line indicates that Six6 might have an activity in A. thaliana, although we did not observe an effect on immunity or susceptibility in this host. One of the genes showing
an increased expression is the nitrate reductase \textit{NIA1}. Together with \textit{NIA2}, \textit{NIA1} is one of the most important enzymes that catalyze the production of nitric oxide (NO). The NO signaling mechanism involves S-nitrosylation, which consists of the covalent addition of an NO moiety to the sulfhydryl group of cysteine residues in target proteins, resulting in the formation of a S-nitrosothiol (SNO) (Lindermayr and Durner, 2009). NO signaling affects the redox state of the plant cell and the activity of numerous proteins is regulated by S-nitrosylation. Many of these proteins are enzymes or transcriptional activators, or co-activators, regulating plant immunity or HR cell death (Bellin et al., 2013). One of them is the nonexpresser of PR genes 1 (NPR1), which acts as a master positive regulator in plant immunity. S-nitrosylation also reduces activity of the NADPH oxidase AtRbohD thereby compromising ROS accumulation and limiting the later stages of HR cell death (Yu et al., 2012). Furthermore, there is evidence that thioredoxins or thioredoxin-reductases can modulate the SNO content during defense signaling (Tada et al., 2008; Spoel and Loake, 2011). Notably, a thioredoxin-peroxidase was identified as a potential Six6 interactor, suggesting a functional link between these proteins (Mara de Sain, unpublished). Besides Six6 also Avr3 might affect the redox state and/or ROS production of the plant cell as we observed a decrease in accumulation of a superoxide dismutase (SOD) (chapter 5), which is a scavenger of ROS, in the tomato xylem sap proteome following infection with a Fol strains lacking \textit{AVR3}.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene ID</th>
<th>Description</th>
<th>U(p) / D(own)</th>
</tr>
</thead>
<tbody>
<tr>
<td>256999.at</td>
<td>AT3G14200</td>
<td>DNAJ heat shock N-terminal domain-containing protein</td>
<td>U</td>
</tr>
<tr>
<td>246490.at</td>
<td>AT5G15950</td>
<td>adenosylmethionine decarboxylase family protein</td>
<td>U</td>
</tr>
<tr>
<td>259681.at</td>
<td>AT1G77760</td>
<td>NIA1 (NITRATE REDUCTASE 1)</td>
<td>U</td>
</tr>
<tr>
<td>265414.at</td>
<td>AT2G16660</td>
<td>nodulin family protein</td>
<td>D</td>
</tr>
</tbody>
</table>

Table 2: Genes with altered expression in \textit{SIX6} transgenic \textit{A. thaliana} plants.

In the xylem sap of tomato plants inoculated with the Fol \textit{AVR2} knockout the accumulation of a Translationally Controlled Tumour Protein (TCTP) was significantly decreased compared to that of plants infected with wild type Fol (chapter 5). TCTP plays a role as negative regulator of the hypersensitive response. Silencing the corresponding gene in \textit{N. benthamiana} increased the \textit{NbrbohB}- and \textit{NbMEK2}-mediated reactive oxygen species production, leading to accelerated HR (Gupta et al., 2013). Also \textit{INF1} induced cell death involves ROS (Asai et al., 2008; Kato et al., 2008) and hence, its enhancement by Six8 might also be mediated by manipulation of ROS production or ROS signaling.

In summary, Six6 might suppress I-2-mediated cell death by affecting the redox state and ROS signaling by targeting enzymes regulating redox homeostasis. Previous studies showed that ROS-induced cell death and defense are separated pathways (Zhu et al., 2013), which might explain why we did not observe altered susceptibility in \textit{SIX6}
transgenic plants. Future studies should address whether the increased expression of NIA1 results in an increase in nitrate reductase activity and increased NO levels in the SIX6 transgenic plants. Furthermore, the role of the thioredoxin-peroxidase in I-2-mediated cell death should be investigated.

**Avr2 and Six8 – interference with hormone signaling?**

Previously it has been suggested that Avr2 might interfere with ethylene signaling or sensing, which could explain the reduced accumulation of PR5a in the tomato xylem sap following infection with a Fol strain lacking Avr2 (chapter 5; Ma, 2012). A transcriptome analysis of AVR2 transgenic A. thaliana, performed as described for the SIX6 transgenic plants (see above), revealed altered expression profiles of 40 genes in one of the two homozygous AVR2 transgenic lines. Ontology analysis showed that these genes were distributed over seven DAVID Ease enrichment clusters. Cluster 2 contained all genes belonging to defense responses (to fungi) and cluster 3 to genes with a function related to light and/or abiotic stimuli or radiation (Tab. 3). Notably, expression of PDF1.2 (cluster 2) was found to be specifically up-regulated in the AVR2 transgenic plants. This gene encodes a JA- and ethylene responsive defense gene and is frequently used as a marker for JA-induced defense. Upon A. thaliana infection by F. oxysporum Fo5176, PDF1.2 expression is increased in both roots and shoots (Berrocal-Lobo and Molina, 2008; Thatcher et al., 2009; Chen et al., 2014). Interestingly, the jin1/myc2 mutant with elevated PDF1.2 expression levels shows increased resistance against F. oxysporum (Anderson et al., 2004). In contrast, the cpr5/hys1 mutant with constitutive PR-1 and PDF1.2 expression and a hyper-senescing phenotype, shows enhanced susceptibility to F. oxysporum (Schenk et al., 2005) as compared to wild type A. thaliana. This is in line with the increased susceptibility to F. oxysporum and Verticillium dahliae that was observed in the AVR2 transgenic A. thaliana (chapter 4; Ma, 2012). A COI1 mutant, lacking a functional JA co-receptor, is more resistant to F. oxysporum (Thatcher et al., 2009) and hence COI1 is required for susceptibility. The involvement of COI1 in expression of foliar symptoms seems to be forma specialis dependent and might be correlated with whether the strain produces jasmonates itself (Cole et al., 2014). Fol does not produce any jasmonates and wilt disease seems to be independent of the tomato COI1 orthologue JAI. In summary, while the role of plant endogenous JA in resistance and susceptibility towards F. oxysporum seems to be important, the mechanism remains elusive yet.

The possible interference of Six8 with JA-signaling via TPL/TPR has been discussed in chapter 4 and a model has been proposed in which Fol hijacks JA-signaling via TPL. As an alternative model auxin signaling could be targeted since the negative regulators of auxin-dependent expression, namely AUX/IAAs, also interact with TPL (Perez and Goossens, 2013).
In summary, Foll effectors potentially modulate hormone signaling of a plant to facilitate susceptibility. In *A. thaliana* this might be mediated by hijacking JA-signaling and thereby accelerating senescence (Thatcher et al., 2009). However, hormone dependent defense signaling might differ between different plant-pathogen systems. Studies such as those determining the transcriptome of *AVR2* transgenic tomato could unravel the role of Avr2 on tomato hormone signaling.

**Table 3:** Genes with altered expression in *AVR2* transgenic plants belonging to DAVID Ease enrichment clusters 2 and 3.

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene ID</th>
<th>Description</th>
<th>U(p) / D(own)</th>
</tr>
</thead>
<tbody>
<tr>
<td>254818_at</td>
<td>AT4G12470</td>
<td>protease inhibitor/seed storage/lipid transfer protein (LTP) family protein</td>
<td>D</td>
</tr>
<tr>
<td>253535_at</td>
<td>AT4G31550</td>
<td>WRKY11 (WRKY DNA-binding protein 11); transcription factor</td>
<td>D</td>
</tr>
<tr>
<td>257365_x_at</td>
<td>AT2G26020</td>
<td>PDF1.2b (plant defensin 1.2b)</td>
<td>U</td>
</tr>
<tr>
<td>249052_at</td>
<td>AT5G44420</td>
<td>PDF1.2 (Low-molecular-weight cysteine-rich 77)</td>
<td>U</td>
</tr>
<tr>
<td>265837_at</td>
<td>AT2G14560</td>
<td>similar to unknown protein</td>
<td>U</td>
</tr>
<tr>
<td>260146_at</td>
<td>AT1G52770</td>
<td>phototropic-responsive NPH3 family protein</td>
<td>U</td>
</tr>
<tr>
<td>255636_at</td>
<td>AT4G00730</td>
<td>similar to unknown protein</td>
<td>D</td>
</tr>
<tr>
<td>246908_at</td>
<td>AT5G25160</td>
<td>Dehydration-responsive protein RD22 (responsive to dessication)</td>
<td>D</td>
</tr>
<tr>
<td>261570_at</td>
<td>AT1G01120</td>
<td>3-ketoacyl-CoA synthase 1</td>
<td>D</td>
</tr>
</tbody>
</table>

**SNC1 – a bona fide resistance gene?**

As proposed in chapter 4 the NB-LRR protein SNC1 might recognize the interaction of Six8 with TPL (Fig. 2B) and subsequently trigger an immune response and a concomitant stunted phenotype in *A. thaliana* (discussed below). It is likely that the interaction between Six8, TPL and SNC1 is localized in the nucleus, as a nuclear localization for an autoactivated *snc1-1* mutant is required for stunting (Cheng et al., 2009). The autoactive *snc1-1* allele mediates resistance to pathogens such as *Pseudomonas syringae* pv. *maculicola* ES4326 and *Hyaloperonospora arabidopsidis* Noco2 (Zhang et al., 2003). Although to date no cognate *AVR* gene recognized by *SNC1* has been identified, *SNC1* has been shown to contribute to effector-triggered immunity (ETI) as *mos7*, which suppresses the constitutive *snc1-1* phenotype affects ETI (Cheng et al., 2009), and *SNC1* contributes to AvrRps4-triggered immunity (Kim et al., 2010).

In this thesis it is shown that *SIX8* and *SNC1* are both required to induce stunting in *A. thaliana* accession Col-0. Furthermore, transcriptome analysis implies that a
constitutive resistance response is responsible for the Six8 phenotype, as marker genes for a SA-dependent defense response like PR-1, PR-2, EDS1 and PAD4 were up-regulated. Assuming that SNC1 is indeed an R gene that recognizes Six8, then TPL might be most likely guarded by SNC1. So far, only for a limited number of R and Avr proteins a direct interaction could be demonstrated, which led to postulation of alternative models explaining effector recognition: I) the guard model, which suggests that susceptibility targets are monitored by R proteins and II) the (guarded) decoy model, which is based on the assumption that Avr proteins interact with host proteins that resemble guardees, but are no true virulence targets themselves. The bait and switch model (Lukasik et al. 2009, Collier and Moffett, 2009) generalizes those two models and postulates - generally speaking - recognition-factors or adaptors that are Avr protein targets and mostly interact with the N-termini of their corresponding R proteins. TPL could be such a recognition factor and as its diverse functions in the cell designate, it is rather a susceptibility target than a decoy (chapter 4). In conclusion, in this thesis we present both a potential AVR and a guardee for SNC1, which would imply that SNC1 is a bona fide R gene.

Applications of TPL and SNC1 in plant breeding?

Due to the availability and introduction of the I genes in tomato resistance against Fol is well established. In banana, however, no resistance genes are available to halt panama disease. This devastating disease is caused by a newly emerged strain of Fusarium oxysporum f. sp. cubense. Hence this pathogen is a serious threat to the production of banana in Southeast Asia, and potentially worldwide. So the demand for resistance in this crop is urgent and high (Consonni et al., 2006; Swarupa et al., 2014).

In chapter 2 we discussed the potential use of effector targets in plants as these genes could encode Susceptibility (S) genes and a mutation in such a gene could render a plant insensitive to disease. One example of a successfully used S gene is MLO, and loss-of-function mutations in this gene confer loss of susceptibility in several crops (Consonni et al., 2006; Bai et al., 2008). Therefore, it will be interesting to test whether TPL is a susceptibility target for F. oxysporum, and whether a loss of function mutation in this gene in tomato could confer resistance to F. oxysporum. As TPL is a member of a gene family the specificity of Six8 to interact with those homologs will have to be determined first. In case Six8 functionally interacts with more than one TPL homolog, it likely requires mutation of several of these genes to affect susceptibility. It is very likely that such a mutant will exert a tpl-1 phenotype hallmarked by the transformation of the embryonic shoot pole into a second root pole at 29°C and/or the failure to form a shoot apical at lower temperatures (Long et al., 2006), possibly limiting the applicability of this strategy.
Alternatively, it will be worthwhile to test whether expression of At\textit{SNC1} in tomato, banana or another host for a \textit{SIX8} carrying \textit{forma specialis} of \textit{F. oxysporum} could confer resistance to this pathogen. A few examples exist of \textit{R} genes that have successfully been transferred between closely related species, like \textit{Bs2} from wild tomato to eggplant or \textit{Mi-1} from pepper to tomato (Goggin et al., 2006; Tai et al., 1999). Also cross-species introduction has successfully been shown, like for the \textit{R} gene pair \textit{RPS4} and \textit{RRS1} from \textit{A. thaliana} to \textit{Brassica rapa} and \textit{B. napus}, tobacco and cucumber (Narusaka et al., 2013). \textit{RPS4} and \textit{RRS1} form a complex, which is required for direct effector recognition and leading to i.a. resistance against \textit{P. syringae} pv. \textit{pisi} and \textit{R. solanacearum} (Williams et al., 2014). Extended resistance to the fungi \textit{Colletotrichum orbiculare} was observed in cucumber. If \textit{SNC1} is functional in other hosts as well it might also mediate resistance to a broader range of pathogens than just \textit{F. oxysporum} in other plant species if these pathogens have effectors manipulating TPL. A potential pleiotropic effect of transgenic \textit{SNC1} expression might be autoactivation due to recognition of the non-native TPL as a modified-self (Spoel and Dong, 2012).

In summary, in this thesis the \textit{R} gene analogue \textit{SNC1}, which might confer resistance against \textit{F. oxysporum}, was identified by characterization of the \textit{SIX8} effector and its host target TPL. In chapter 2 we proposed that identification of effector targets could reveal \textit{S} genes. In addition I now also suggest that key effector targets might be guardees that can guide identification of their guards, which might be deployed as \textit{R} genes.

**Concluding remarks and outlook**

The characterization of Six proteins is an important task as these proteins are critical for the outcome of the battle between fungus and plant. Understanding effector functions provides insights about the molecular mechanism underlying susceptibility and resistance. However, characterization is a laborious task as each Six protein has its unique functions and characteristics regarding e.g. \textit{in planta} expression upon agro-infiltration and stability. With this thesis we contributed some new pieces to the puzzle. Now, the characterization of Six proteins should focus on their targets as they provide information about the molecular function of effectors and the targeted cellular processes.
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