The role of Fusarium oxysporum effector protein Avr2 in resistance and pathogenicity

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CHAPTER 8

General discussion

Fusarium oxysporum is ranked fifth in the top 10 most notorious plant pathogens, (Dean *et al.*, 2012). The fungus combines a wide host range with the ability to cause severe yield losses in diverse crops such as tomato, cotton, banana and others (Dean *et al.*, 2012). The interaction between tomato and *F. oxysporum* f.sp. *lycopersici* is an excellent model to study the molecular basis of resistance and susceptibility in plants against vascular pathogens. The *Fol*-tomato interaction complies with the gene-for-gene model as proposed by Flor in the 1940's. At the start of this PhD project, two *Fol* avirulence genes (*AVR1* and *AVR3*) and one resistance (R) gene *I-2* were cloned, but no complete *R-AVR* pair was available (Houterman *et al.*, 2008; Rep *et al.*, 2004; Simons *et al.*, 1998). This thesis describes the cloning of the *AVR2* gene and its functional characterization in resistance and disease. In this chapter, I summarize and discuss my results and suggest directions for future studies.

Recognition of Avr2 by the resistance protein I-2

As described in chapter 2, perception of Avr proteins by R proteins can occur either directly by a physical interaction between the two proteins or, indirectly, via an intermediate host protein. Direct interaction between a NB-LRR protein and an effector protein can result, through antagonistic co-evolution, in diversifying selection and sequence polymorphisms in the effector gene in pathogen populations and the *R* gene in host populations (Dodds and Rathjen, 2010; Ma and Guttman, 2008; Stavrinides *et al.*, 2008). In the effector protein, point mutations can prevent the recognition by its corresponding NB-LRR protein (Bent and Mackey, 2007; Stergiopoulos and de Wit, 2009). As presented in chapter 3, breaking of *I-2*-mediated resistance by *Fol* race 3 is caused by three separate single point mutations in Avr2, resulting in its inability to activate I-2. Apparently, recognition of Avr2 is determined by a part of the protein that includes these three amino acids. Based on these findings, it is possible that Avr2 is directly recognized by I-2, although our results from yeast two-hybrid and co-immunoprecipitation experiments did not show direct association between both proteins (data not shown). Alternatively, the proteins might interact indirectly. Indirect effector recognition has been suggested for a number of cases (Dodds and Rathjen, 2010). So far, three conceptual models have been proposed to describe indirect recognition mechanisms (Dodds and Rathjen, 2010). Among them, the "refined switch model" and "bait-and-switch model" postulate that accessory proteins (co-factors), referred to either as guardees or decoy proteins, function as molecular adaptors used by R proteins to detect the presence or action of an effector (Collier and Moffett, 2009; Lukasik and Takken, 2009). Although a plant accessory protein is required in this model, recognition could still be mediated by direct contact between the effector bound to the co-factor and the NB-LRR protein. This perception concept might apply to the interaction between Avr2 and I-2 as it fits our findings that the polymorphic residues in Avr2 determine its recognition specificity and that there is no direct interaction detectable in yeast or *in planta* between Avr2 and I-2.

In this model (Figure 1), a plant accessory protein is bound to the CC domain of I-2. Subsequently the accessory protein interacts with Avr2 enabling it to interact with the LRR domain of I-2 thereby activating I-2 allowing elicitation of downstream defence signalling. In the future, when a plant target of Avr2 is identified, yeast three-hybrid or *in vivo* co-immunoprecipitations will be required to verify this model.

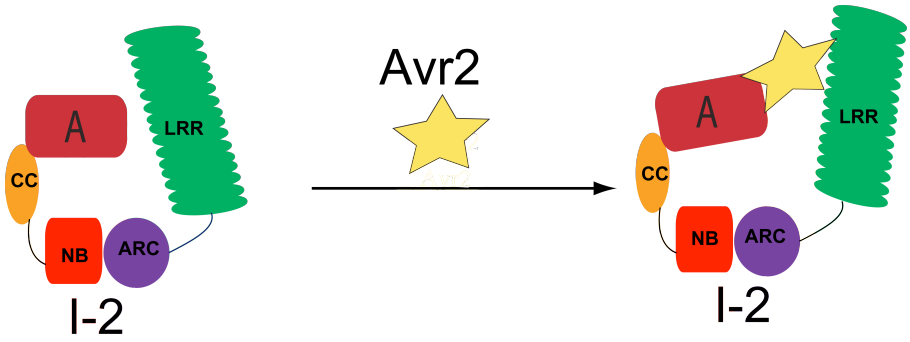

Figure 1. Predicted recognition model of Avr2 by I-2. A plant accessory protein (A) is bound to the CC domain of I-2. Avr2 interacts with the accessory protein enabling Avr2 to interact with the LRR domain of I-2. The domains are depicted as coloured boxes: CC domain (orange), NB (red), ARC (purple) and LRR domain (green).

Avirulence functions of Avr2

Cell death response triggered by Avr2 and I-2 recognition

In general, an avirulence protein functions as an elicitor to trigger its corresponding *R*-gene-mediated immune response that often involves a hypersensitive response (HR) culminating in a local cell death response at the front of infection site to halt spread of the pathogen (Ashida *et al.*, 2011). However, in an incompatible *Fol*-tomato interaction, discernible cell death has not been observed. Instead, tomato defence responses mainly involve the accumulation of phenolics and antimicrobial proteins in the xylem vessels to restrict the immediate sites of infection with substances that harm the fungus, and the deposition of callose in parenchyma cells adjacent to the infected xylem vessels (Beckman, 2000; Beckman *et al.*, 1989). Therefore, when *AVR2* was cloned from *Fol* (Chapter 3), we were very curious to test whether Avr2 can trigger an *I-2*-dependent hypersensitive response with activation of programmed cell death. Strikingly, transiently expressed *AVR2* did trigger *I-2*-mediated cell death in *N. benthamiana* and tomato plant leaves (Chapter 3). In addition, the offspring of two parental tomato plants, one constitutively expressing *AVR2* and the other carrying the native *I-2* gene, showed restricted cell death in its leaves (Chapter 6). This was the first time that one (over) expressed avirulence effector from a xylem-colonizing fungus was reported to trigger a robust *NB-LRR*-dependent cell death response. More recently, Ave1, an effector from the xylem-invading fungus *Verticillium dahliae* was also shown to be able to activate its corresponding resistance gene *Ve1,* not an NB-LRR, resulting in a cell death

response when transiently expressed in *N. tabacum* or tomato plant leaves (de Jonge *et al.*, 2012). These two cases show that avirulence effectors from xylem-colonizing fungi have the potential to trigger *R*-mediated cell death responses like effectors of leaf-invading pathogens. The contradiction between the obvious *I-2*-mediated cell death response triggered by (over) expressed Avr2 and the absence of visible cell death response in *I-2*-mediated resistance suggests that a cell death response might not be coincident with the *I-2*-mediated resistance. So far, there are several other examples where *R*-mediated resistance can occur without a cell death response, such as for potato Rx and barley Rdg2a (Bendahmane *et al.*, 1999; Bulgarelli *et al.*, 2010; Coll *et al.*, 2011). Apparently, the cell death response and resistance are separable (Figure 2), in which cell death may occur simply as a consequence of escalated defense signalling at the plant-pathogen interface (Coll *et al.*, 2011). This cell death might not pose a restriction on pathogen growth, but might serve to induce systemic acquired resistance (Coll *et al.*, 2011). It would be very interesting to determine more firmly whether *I-2*-mediated cell death occurs in the incompatible interaction of *Fo*l-tomato by characterizing expression of marker genes that have been shown to be involved in the cell death response or signalling, such as *Hin1* or *Hsr203J* (Gopalan *et al.*, 1996; Pontier *et al.*, 1994). In addition, physiological and molecular changes accompanied with cell death, such as production of an oxidative burst, reactive oxygen species (ROS), an oxidative cross-linking of various cell wall compounds, and the production of pathogenesis-related (PR) proteins should be investigated (Radwan *et al.*, 2005).

Although intracellularly (over) expressed *AVR2* activates *I-2*-dependent cell death, the hyphae of a race 2 are still able to penetrate the root cortex of the *I-2* tomato plants and subsequently enter xylem vessels, but they are limited in further, extensive colonization (Martijn Rep, personal communication). This delayed defence response indicates that Avr2/I-2 recognition might occur only in cells adjacent to the xylem vessels, possibly because *AVR2* expression in *Fol* is induced only upon entry of xylem tissue. This speculation is in agreement with our finding that expression of *AVR2* is strongly induced in the hyphae growing in the xylem vessel (Chapter 4). Furthermore, previous studies found that *I-2* is specifically expressed in the parenchyma cells in contact with xylem vessel (Mes *et al.*, 2000). These findings rank the xylem contact parenchyma cells as the principal place where recognition of Avr2 by I-2 occurs. It is therefore likely that any cell death response induced by Avr2 and I-2 recognition will preferably occur in these cells. Future studies, such as using microscopy combing with cell death staining are required to show whether cell death response is involved in *I-2*-mediated *Fol* resistance.

I-2-mediated resistance

Besides *AVR2 (SIX3)*, a knockout of *SIX5,* that shares the promoter region with *AVR2*, also resulted in the loss of *I-2*-mediated resistance. Obviously, this discovery could raise the

question whether Six3 or Six5 is in fact Avr2. However, we ascertained that *SIX3* is *AVR2* since only polymorphic Six3 exists in the *I-2*-breaking *Fol* race 3 strains and these Avr2 variants can avoid the recognition by I-2 (Chapter 3). Furthermore, only Six3 can activate I-2 in *N. benthamiana* leaves and in tomato plants. Nevertheless, Six5 and Avr2 are both required for *I-2*-mediated resistance (Chapter 5). This provides a first example of an effector pair that is involved in ETI mediated by a single R protein. According to the gene-for-gene hypothesis (Flor, 1942), specific recognition of a pathogen by a plant is determined by the coincidence of a plant resistance gene and its corresponding pathogen avirulence gene. However, a growing number of studies on interactions between plants and pathogens have reported deviations of this simple gene-for-gene concept. For instance, resistance against a single pathogen or the response to a single *AVR* gene product may require a pair of *R* genes (Eitas and Dangl, 2010). There are several characterized examples of pairs of *R* genes involved in recognizing a single Avr gene product or conferring resistance against a single pathogen. The tobacco *N* and *NRG1* pair, both encoding the NB-LRR proteins, together mediates recognition of the P50 protein of Tobacco Mosaic Virus (Peart *et al.*, 2005). Similarly, the Arabidopsis gene pair *RPM1-TAO1* is required for the resistance against *Pseudomonas syringae* carrying AvrB (Eitas *et al.*, 2008). Furthermore, distinct Avr proteins from different pathogens can activate the same NB-LRR protein (Spoel and Dong, 2012). Our finding that two effectors, derived from one pathogen, are required for a single NB-LRR-mediated resistance is different from other known examples. Our finding not only broadens the range of gene-for-gene exceptions, but also provides a warning that *Fol* could either modify Avr2 or Six5 to avoid *I-2*-mediated resistance.

So far, the mechanisms underlying our observation that the effector pair of Six5 and Avr2 is required for *I-2*-mediated resistance remain unclear. The physical interaction between Avr2 and Six5 in yeast suggests that both could interact *in planta* to form an Avr2-Six5 complex to be recognized by I-2 (Chapter 5 and Figure 2). Unfortunately, we were unable to confirm this interaction by co-immunoprecipication (IP) of Six5 with tagged Avr2 when both are transiently expressed *in planta* (data not shown). A possible explanation of this failure is that the proteins interact in a transient or weak manner or that the protein tag interferes with complex formation. Besides recognition of a Six5/Avr2 complex by I-2, Six5 could be required for an interaction between Avr2 and I-2 during infection, for instance by assisting delivery of Avr2 into plant cells. We do not favour the latter hypothesis since expression of a full length Avr2 that is secreted into the apoplast is able to trigger cell death in tomato and *N. benthamiana*, implying Six5-independent Avr2 uptake (Chapter 3). Future studies to determine their interaction *in planta* are required to identify the role of Six5 in *I-2*-mediated Avr2 recognition. To that aim, secreted Avr2 and Six5 proteins present in the xylem sap isolated from infected tomato plants should be subject to Co-IP. Alternatively, both proteins may be labelled with different fluorescent proteins and fluorescence resonance energy

transfer (FRET) could be used to determine their interaction after secretion in xylem or in plant cells upon *Fol* infection.

It must be noted that Six5 is only required for *I-2*-mediated resistance and not for *I-2*-mediated cell death (Chapter 5 and Figure 2). Possibly, intracellular (over) expression of *AVR2* in the *N. benthamiana* or tomato leaves obviates the requirement of Six5 for *I-2* activation (Chapter 3 and 6). Alternatively, cell death and resistance might be independent process as discussed before (Figure 2), the first being induced by *AVR2* overexpression while the second only occurs upon recognition of an Avr2-Six5 complex.

Localization of Avr2 *in planta*

As presented in Chapters 3 and 4, Avr2 is recognized inside plant cells and its nuclear localization is required to activate *I-2*-dependent cell death (Figure 2). No discernible NLS motifs were found in the Avr2 amino acid sequence by available NLS prediction software (NLStradamus and PredictNLS), so it is possible that Avr2 enters the nucleus by passive diffusion due to the small molecular mass of the Avr2 mature protein (Chapter 3). In any case, we propose that during infection *Fol* Avr2 localizes to the host nucleus to promote virulence. Although, RFP-labelled Avr2 could only be visualized in extracellular spots and there was no intracellular RFP signal in plant cells after secretion by *Fol* (Chapter 4), we cannot exclude that the Avr2-RFP fusion protein is translocated into plant cells. The absence of intracellular RFP signal might be due to either undetectable low levels of Avr2 in the cytosol or cleavage of RFP from the fusion protein in the apoplastic space. Alternatively, the observation time point, four-day-post inoculation, could be too early to visualize intracellular RFP signals. Avr2 was identified as one of the most abundant *Fol* proteins in the xylem sap of infected tomato plants 2-3 weeks after infection, but the gene is hardly expressed during the first days after inoculation (Chapter 3 and 5). If Avr2 can be translocated into plant cells, the contact cells of xylem vessels are therefore expected to perceive and contain a relatively high concentration of Avr2. To test these possibilities different approaches to localize Avr2 upon *Fol* infection should be considered: i) generating *Fol* transformants, in which the wild-type *AVR2* is replaced in locus with an *AVR2:RFP-NLS* construct. The advantage is that the nuclear import signal (NLS) will target the secreted and translocated fusion protein to the plant nucleus resulting in a high local concentration that might be visualised using the confocal microscope, ii) a time course observation of fluorescently labelled Avr2 in xylem vessel contact cells upon *Fol* infection. In case that both strategies fail, the detection of translocated Avr2 in plant cells might be feasible by immunolocalization using the Avr2 specific antibody that was recently generated (Chapter 5). Another option is to use *Magnaporthe oryzae* as a system to investigate the translocation of Avr2. This system has been successfully used to show localization to the plant nucleus of the secreted SP7 effector from the Arbuscular mycorrhizal (AM) fungus *Glomus intraradices*. The mRFP-labeled

effector was secretion by *M. oryzae* in a modified onion epidermis assay (Kloppholz *et al.*, 2011). An advantage of this system is that onion epidermis cells are much thinner than tomato root cells and allow easier visualization and manipulation.

An increasing number of studies indicate that the plant cell nucleus is one of the main targets of pathogen effectors (Rivas, 2012). For instance, subcellular localization analysis of oomycete *Hyaloperonospora arabidopsidis* (Hpa) RxLR effectors during the compatible interaction between Hpa and its host Arabidopsis revealed that twenty-one percent of the Hpa effectors tested, including ATR13Maks9 and ATR13Emoy2, localize in the host nucleus, which suggests that they play roles in the plant cell transcriptional machinery in order to prevent the induced defence responses (Caillaud *et al.*, 2012). Studies on the *P. infestans* CRINKLER (CRN) effectors revealed that this large family of oomycete effector proteins also target the host nucleus (Schornack *et al.*, 2010). Nuclear accumulation of at least one of these (CRN8) is required to induce plant cell death. In our study, nuclear localized Avr2 is also required to induce cell death. However, whether nuclear localization of Avr2 is required to activate *I-2*-mediated resistance remains to be elucidated. Future studies, such as determining whether nuclear or cytoplasmically localized Avr2 is able to complement avirulence of *Fol AVR2* knockout strains on *I-2* tomato plants are required.

Virulence function of Avr2

Mechanisms underlying the virulence function of fungal avirulence effectors have been revealed only in a few cases (Dodds and Rathjen, 2010). Most of these studies involved the intercellular leaf pathogen *Cladosporium fulvum* (Stergiopoulos and de Wit, 2009). Apparently, the discovery of the virulence function of avirulence effector is not as easy as may have been expected. Attempts to identify Avr2 virulence or avirulence targets *in planta* by screening cDNA libraries generated from the tomato plants infected with *Fusarium oxysporum* race 2 strain using yeast two-hybrid, or pull-down with transiently expressed Avr2 in *N. benthamiana* plants did result in some candidates (data not shown). Currently it is unclear whether they are genuine targets. Interestingly, tomato plants constitutively expressing *AVR2* display a hyponastic phenotype, in which the internodes of plants are elongated and the leaves are tilted upwards (Chapter 6), suggesting that Avr2 might interferes with ethylene sensing or signalling. An additional link between Avr2 and ethylene perception comes from quantitative proteomic analysis of xylem sap of tomato plants infected with Fol007 (WT) and Fol∆*AVR2* (Chapter 5 and data not shown). This analysis showed that a PR5a-like protein appears to be down-regulated by Avr2. In Arabidopsis, *PR-5* is a root-specific ethylene-responsive gene activated by the colonization of fluorescent *Pseudomonas* spp. (Leon-Kloosterziel *et al.*, 2005). Reduced accumulation of PR5 is hence in line with a role for Avr2 in blocking ethylene perception/signalling of the plant.

Previously, it was demonstrated that many plant proteins are secreted into the xylem sap in response to *Fol* colonisation, including pathogenesis-related (PR) proteins PR-1, PR-2 (glucanases), PR-3 (chitinases), PR4 and PR-5 (Houterman *et al.*, 2007; Rep *et al.*, 2002). PR-5 belongs to a family of defense-related proteins of which some have antifungal activity (van Loon *et al.*, 2006). For instance, AP24, a PR-5 homolog from tobacco, has been shown to exert an inhibitory activity against *Phytophthora infestans* (Woloshuk *et al.*, 1991). In addition, ATLP3, a PR-5 protein from *Arabidopsis thaliana*, displayed extensive antifungal activity against fungal pathogens such as *Fusarium oxysporum*, *Verticillium albo-atrum* and *Verticillium dahliae* as well as non-pathogenic fungi like *Trichoderma reesei* and *Candida albicans* (Hu and Reddy, 1997). Taken together, we might speculate that the virulence function of Avr2 is to interfere with plant ethylene sensing or signalling, resulting in reduced accumulation of PR5a in the xylem sap of infected tomato plants, which facilitates the colonization of *Fol* in the xylem. In future, this hypothesis could be tested by assessing disease development on ethylene insensitive tomato mutants infected with *Fol* and *Fol AVR2* knockout strains. If both strains are equally capable of causing disease this is a strong indication that Avr2 exerts the virulence function by interfering with ethylene signalling. To test involvement of PR-5a in disease development tomato plants in which *PR5a* is either silenced or overexpressed should be evaluated for their sensitivity to both *Fol* strains.

A working model for the Avr2/Six5 pair in tomato resistance and susceptibility

Based on the results described in this thesis, a tentative model for the involvement of Avr2 and Six5 in tomato resistance and susceptibility is proposed which is shown in Figure 2. When *Fol* grows in the xylem vessel of its host tomato, it secretes Avr2 and Six5. After secretion Avr2 undergoes N-terminal processing. Subsequently, processed Avr2 forms homodimers, and the dimeric Avr2 interacts with Six5, resulting in the formation of a temporary complex. Formation of the complex might be required to resist the proteases in the xylem vessels or to mediate efficient uptake by the host cell. After entry into plant cells, the temporary Avr2-Six5 complex, possibly through interaction with a plant accessory protein, triggers *I-2*-dependent resistance. To trigger cell death, Avr2 has to enter the nucleus to activate I-2. For its virulence function, Avr2 may interfere with ethylene perception or signalling, resulting in reduced accumulation of PR5a in the xylem vessel. The virulence function of Six5 remains unclear.

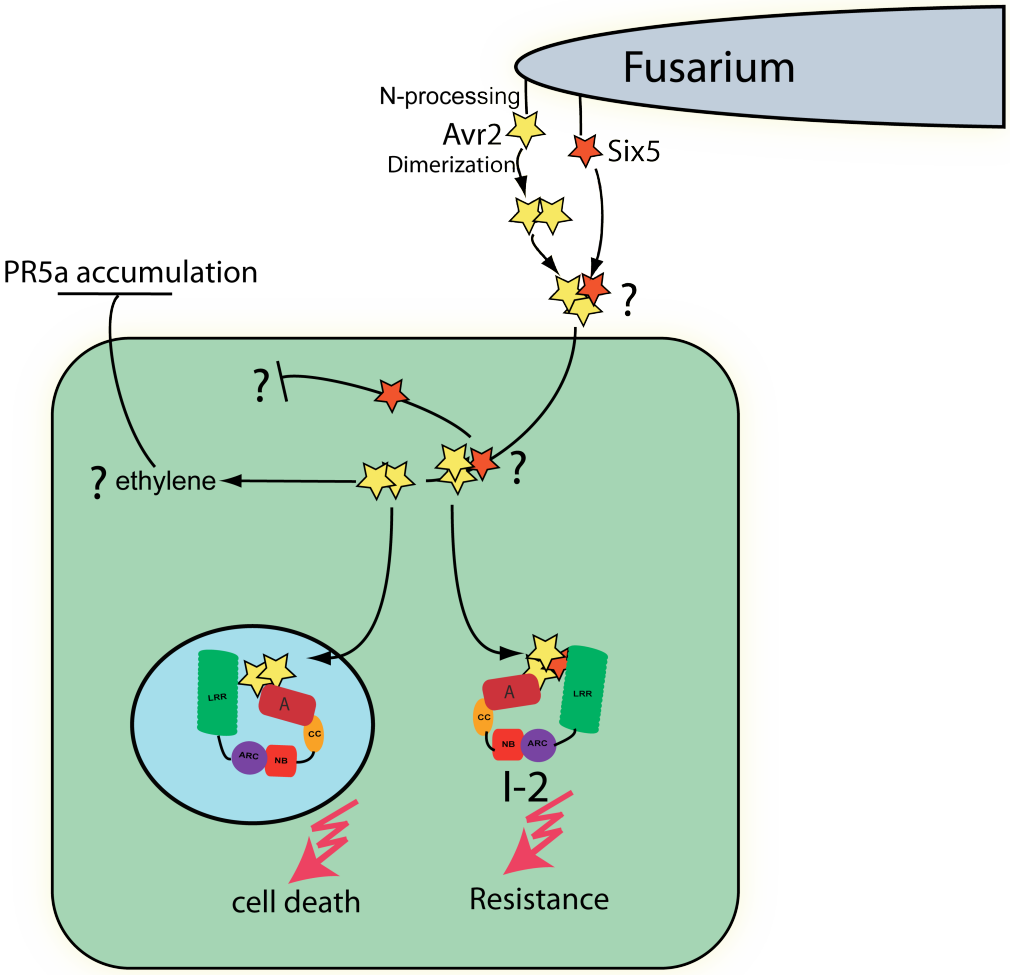

Figure 2. A working model for Avr2/Six5 in tomato *I-2*-mediated resistance and susceptibility. N-terminal processed Avr2 in xylem vessel forms homodimers, and the dimeric Avr2 interacts with Six5, resulting in the formation of a temporary complex. After entry into plant cells, the temporary Avr2-Six5 complex, possibly through interaction with a plant accessory protein (A), triggers *I-2*-dependent resistance. To trigger cell death, Avr2 has to enter the nucleus to activate I-2. For its virulence function, Avr2 may interfere with ethylene perception or signalling, resulting in reduced accumulation of PR5a in the xylem vessel.

Concluding remarks

Despite an apparently simple set of three gene-for-gene interactions dominating the outcome, the interaction between *Fol* and tomato is, perhaps not surprisingly, complex. Results described in this thesis on the functional roles of effector protein Avr2 during *Fol* infection and plant resistance provides some limited insights into the principles of the interaction between *Fol* and tomato. Much more work is required to verify and extend the proposed models. However, the developed concepts and technological approaches in this thesis might enable us to initiate such further investigation with confidence to fill in the gaps and questions left open in these models. Subsequent studies devoted to the determination of Six5

and Avr2 interaction in xylem vessels, investigation of the translocation mechanisms of Avr2 and Six5 into plant cells and identification of Avr2 and Six5 plant target(s) will be essential and challenging, but will undoubtedly result in a better understanding of the functional roles of Avr2 together with Six5 in *I-2*-mediated resistance and in *Fol* infection. Together, these studies will provide insights into how *Fol* manipulates its host and how a resistant host counteracts this attempt. Such new knowledge in plant immunity may provide potential for developing new strategies to control wilt diseases.

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