Brothers in arms

Fusarium oxysporum vs. Fusarium oxysporum

de Lamo Ruiz, F.J.

General discussion

Fusarium oxysporum (Fo) is a soilborne filamentous ascomycete (Michielse and Rep, 2009). The fungus is best known as the causal agent of Fusarium wilt disease but it can also trigger foot- and root-rot. Wilt disease is a consequence of extensive colonization of the xylem vasculature by the fungus leading to typical disease symptoms, such as wilting, stunting and eventual death. Pathogenic Fo strains secrete small proteins during infection, of which some are effectors that facilitate host infection (Edel-Hermann and Lecomte, 2019). Strains pathogenic on one plant species do not usually cause wilt disease in other species, presumably because they lack the cognate host-specific effectors (Validov et al., 2011). The number of putative effector proteins in Fo strains differs; typically, pathogenic strains harbor larger numbers of effector gene candidates than non-pathogenic isolates (van Dam et al., 2016; Constantin, 2020). Most of these effector-encoding genes are localized on a pathogenicity chromosome and together these determine host-specificity (Ma et al., 2010; van Dam et al., 2017). Remarkably, these pathogenicity chromosomes of Fo can be horizontally transferred (Ma et al., 2010). For instance, the pathogenicity chromosomes of the tomato pathogen Fo f.sp. *lycopersici* (Fol) and the cucurbit-infecting pathogen Fo f.sp. *radicis-cucumerinum* can be transferred to the non-pathogenic Fo47 isolate, converting the latter into a vascular-infecting pathogen (Ma et al., 2010; van Dam et al., 2017).

Notwithstanding the large number of pathogenic isolates, pathogenicity is actually the exception as the majority of Fo strains are harmless for plants and the host-specificity makes most strains non-pathogens on most plant species (Gordon, 2017). Notably, these non-pathogenic strains often retain the ability to enter and colonize the roots of a wide variety of host plants and often behave as endophytes when inoculated onto a non-host. Root colonization by endophytic strains is typically confined to the outer cell layers of the roots (epidermis and outer cortex), whereas pathogenic strains usually colonize the xylem vessels of their host resulting in disease symptom development (Gordon, 2017). Many endophytic Fo isolates are reported to confer biocontrol to root-infecting pathogens such as pathogenic Fo (Elmer, 2004; Bolwerk et al., 2005; Olivain et al., 2006; Kaur and Singh, 2007; Nahalkova et al., 2008; Aimé et al., 2013), *Verticillium dahliae* (Veloso and Díaz, 2012), *Pythium ultimum* (Benhamou et al., 2002) or *Phytophthora capsici* (Veloso and Díaz, 2012). Co-inoculation, or prior inoculation, of an endophytic and a pathogenic strain can reduce disease symptom development and in some cases fully prevent disease onset (Alabouvette, 1986; Tamietti et al., 1993). Biocontrol is the result of the combination of two actors : 1) direct antagonism of the endophyte to the root pathogen, reducing its virulence (Benhamou et al., 2002) and 2) indirect protection by triggering a plant response called endophyte-mediated resistance (EMR). The EMR response results in a reduced disease susceptibility of the colonized roots. The ability to induce EMR appears to be a shared property among members of the *Fusarium oxysporum* species complex (Constantin, 2020). The mechanisms underlying EMR are not well understood and form the main focus of this thesis. In this chapter, our data generated concerning the tri-partite Fo47-Fol-tomato system are discussed in the context of the current molecular understanding of EMR in plants. A thorough understanding of the molecular mechanisms underlying EMR is not only of fundamental interest, but also aids in translating the use of biocontrol strains to agriculture to combat pathogens.

Reduced tomato susceptibility to Fol by Fo47: direct antagonism or a plant-mediated response?

As detailed in this thesis, Fo47 reduces susceptibility of tomato to Fol when the roots are co-inoculated with both strains. Fo47-mediated biocontrol can be caused by direct antagonism to Fol and/or to the induction of EMR. The contribution of the latter can be monitored using a split-root assay in which the endophyte and pathogen and physically separated. Split-root inoculation of tomato, chickpea and eggplant have revealed that various Fo endophytes, including Fo47, can trigger a systemic EMR response that reduces susceptibility to pathogenic Fo (Duijff et al., 1998; Kaur and Singh, 2007; Pantelides et al., 2009). The relative contribution of direct antagonism and EMR to biocontrol in our Fo47-Fol-tomato setup is unknown. Some Fo strains are better in competing with pathogens while others are better EMR-inducers (Fravel et al., 2003). The effective spore ratio, endophyte versus pathogen, required to suppress disease can provide clues to the main mechanism involved. When high endophyte doses are required, then protection is more likely to rely on direct competition between the strains e.g. for niches on the roots where the fungus can enter and/or acquire nutrients. When low inoculum doses suffice to mount a robust resistance response, then protection is likely to be mostly plant-mediated (Fravel et al., 2003). In previous studies, the concentration of Fo47 employed to reduce susceptibility to Fol was ten-fold higher than that of Fol (Duijff et al., 1999). However, in our setup a ratio 1:1 confers an equally strong disease reduction as higher Fo47 doses, which suggest that the observed biocontrol involves plant-mediated EMR. Furthermore, despite co-existing on the root surface and in the outer cortex, Fo47 and Fol are physically separated at later stages of infection as only the pathogen colonizes the stele (Nahalkova et al., 2008; Gordon, 2017). Although Fol can still colonize the vasculature and secrete effectors such as Avr2 (chapter 5) disease progression and extensive Fol proliferation is strongly reduced in the presence of Fo47. These observations provide additional support for a plant-mediated component to be involved in the observed biocontrol. Of note, in cucumber osmiophylic depositions in the vasculature and papillae are formed around invading hyphae of *P. ultimum,* but only in the presence of Fo47 (Benhamou et al., 2002), implying a plant resistance response triggered by Fo47. Furthermore, Fo47 and Fol do not appear to compete for infection sites on the root (Olivain et al., 2006). When both are co-inoculated on tomato roots they colonize the same spots on the surfaces and cortex, but even when excess Fo47 spore concentrations are used then Fol is still able to colonize these root niches and infect the host. This observation suggests a marginal role for niche competition between Fo47 and Fol as main cause of biocontrol. Altogether, although antagonism between Fo47 and Fol cannot be excluded, the co-inoculation setup employed in this study is suitable to study EMR.

Is *Fusarium oxysporum*-triggered EMR independent of Pattern-triggered immunity?

Plants can recognize microbe-associated molecular patterns (MAMPs) from invading microbes by pattern recognition receptors (PRRs) located at the plant cell surface (Macho and Zipfel, 2014). MAMP detection leads to induction of a local and broad-spectrum resistance response known as pattern-triggered immunity (PTI) (Jones and Dangl, 2006). Typical PTI responses include

generation of reactive oxygen species (ROS), phosphorylation of mitogen-activated protein kinases (MAPKs) leading to the transcriptional activation of defence-related genes, and accumulation of callose deposits at the cell wall confining invading microbes (Bigeard et al., 2015; Chuberre et al., 2018). Virulence of Fo pathogens is facilitated by effector secretion, some of which can result in evasion of, or compromised PTI responses. For instance, virulence of Fo f.sp. *vasinfectum* toward cotton requires secretion of a chitin deacetylase (PDA1) to convert the highly immunogenic MAMP chitin into the less immunogenic compound chitosan (Gao et al., 2019). This conversion allows the fungus to evade recognition by the plant's chitin PRR receptor to trigger PTI. Full virulence of Fol towards tomato involves secretion of the effector Avr2 (Houterman et al., 2009). Avr2 enters tomato cells and compromises PTI signalling upon exposure to a bacterial MAMP derivative (Di et al., 2016; Di et al., 2017). Contrarily, unlike Fo pathogens endophytes such as Fo47 are reported to trigger PTI responses in plant cell cultures (Olivain et al., 2003; Humbert et al., 2015) and to trigger papillae formation in pea root upon host colonization (Benhamou and Garand, 2001). Whether triggering PTI responses in roots is key for Fo endophytes to trigger EMR was unknown. Therefore, the potential involvement of PTI to mount an EMR response in tomato was studied (chapter 3). To assess this, a transgenic MM-Δ*spAvr2-30* tomato line that produces the fungal effector protein Avr2 was selected. This line exhibits a compromised PTI response upon treatment with the bacterial MAMP-derivative flg22 and is hyper-susceptible to Fol, *Verticillium dahliae* and to the bacterial pathogen *Pseudomonas syringae* (Di et al., 2017). Since this line accumulates a fungal effector conferring hyper-susceptibility to fungal vascular pathogens, it was hypothesized that PTI elicited by fungal elicitors would also be compromised. Indeed, in chapter 3 it is shown that MM-Δ*spAvr2-30* plants exert a compromised PTI towards a fungal MAMP, as callose depositions were reduced as compared to wild-type plants. Subsequently, colonization of this PTI-compromised tomato by Fo endophytes was assessed in roots by qPCR and in stems by incubating stem sections on agar plates and monitoring fungal outgrowth. The tomato-Fo endophyte interaction was found to be restricted by PTI as the MM-Δ*spAvr2-30* tomato line allowed more extensive root- and stem-colonization than wild-type plants. Noteworthy, the biocontrol conferred by tomato Fo endophytes towards Fol was unaltered in MM-Δ*spAvr2-30* plants. This observation suggests that EMR acts independent of PTI signalling. Since a local PTI response had been reported to be indispensable to trigger systemic resistance responses in plants (Houterman et al., 2009), this finding was unexpected.

A limitation of the MM-Δ*spAvr2-30* line is that PTI responses like the ROS burst, MAPK phosphorylation or callose depositions are not fully blocked (Di et al., 2017) and can be restored by high MAMP concentrations (data not shown). These observations imply that PTI is merely reduced and not abolished. Hence, to fully exclude the potential involvement of PTI in EMR a tomato line should be generated that has a completely compromised PTI response. In Arabidopsis, a PTI-compromised line is available; the *bak1-5/bkk1-1/cerk1* (*bbc)* mutant that is blind to bacterial infection (Xin et al., 2016). BAK1 is a leucine-rich repeat (LRR) receptor-like kinase (RLK) located at the plasma membrane that is recruited by numerous PRRs as a co-receptor upon MAMP recognition. BAK1 recruitment allows transphosphorylation of the respective kinase domains of the interacting RLKs resulting in the activation of downstream signaling (Macho and Zipfel, 2014). BKK1 is a homologue of BAK1 showing functional redundancy in *bak1-5* lines (Roux et al., 2011). CERK1 is LysM-RLK PRR that mediates recognition of chitin and bacterial peptidoglycan and subsequently activates PTI (Miya et al., 2007; Willmann et al., 2011). However, it is imaginable that

there are Fo MAMPs that are recognized in a BAK1/BKK1/CERK1-independent manner, resulting in a residual PTI response in a such a mutant. The presence of such potential Fo MAMPs can be investigated upon infection of the Arabidopsis *bbc* mutant and monitoring whether PTI is induced. If Fo MAMPs appear to be absent, then mutation of the *BAK1/BKK1/CERK1* orthologs in tomato could result in a line that is blind to Fo and does not trigger PTI upon inoculation. An alternative approach to generate a PTI mutant is by mutating the PRR receptors themselves. Unfortunately, tomato Fo PRRs have not yet been identified, but the RLK MIK2 in Arabidopsis was shown to be required to activate PTI upon exposure to Fo MAMPs (Coleman et al., 2019). Knocking out homologues of *MIK2* in tomato might be a strategy to generate a mutant that does not trigger PTI towards exposure to Fo. However, as it is unlikely that Fo produces only a single MAMP being recognised by a single tomato PRR receptor, a knockout of all putative Fo-detecting PRRs will be required to generate a fully 'Fo-blind' tomato line using this strategy.

Another possible approach to compromise PTI is to identify and mutate the tomato host target of Avr2. Avr2 compromises PTI responses suggesting that Avr2 interacts with a host protein involved in PTI signaling. Currently this target is unknown but the fact that Avr2 compromises PTI responses upon treatments with both bacterial (flg22) and fungal (chitosan) MAMPs, indicates that the host target of Avr2 is unlikely a PRR, as these MAMPs have different receptors (Gomez-Gomez and Boller, 2000; Hind et al., 2016; Gubaeva et al., 2018). The effector target therefore must be a shared signalling component downstream of PRR receptors and be localised intracellularly, as the effector exerts its virulence function in the cytosol (Di et al., 2016). Since in MM-Δ*spAvr2-30* plants early PTI responses such as ROS accumulation and phosphorylation of MAPKs are compromised upon flg22 treatment (Di et al., 2017) and also a late response (callose depositions) is reduced upon chitosan application (chapter 3), the effector target must act at an early step in signalling where the response pathways have not yet diverged. Potential targets are components of the MAPK pathway, whose phosphorylation in Arabidopsis activates transcription factors regulating defence gene expression and callose deposition (Frey et al., 2014; Xu et al., 2016; Jiang et al., 2019). Other potential targets acting upstream in the signalling pathway are receptor-like cytoplasmic kinases (RLCK) interacting with the plasma membrane-located PRRs complexes. In Arabidopsis, the RLCK Botrytis-induced kinase (BIK1) is required for activating flg22- and chitin-triggered MAPK cascade (Macho and Zipfel, 2014) and for phosphorylation of RbohD that generates reactive oxygen species (ROS) upon MAMP perception (Li et al., 2014). BIK1 is a known target for effectors and the *Pseudomonas syringae* effector AvrPphB has been shown to catalyse its cleavage, resulting in compromised PTI signalling (Block and Alfano, 2011). *TPK1b* is a *BIK1* orthologue of tomato (AbuQamar et al., 2008) that can functionally complement disease resistance of the Arabidopsis *bik1* mutant, indicating that they have a similar function (AbuQamar et al., 2008). TPK1b represents a potential target of Avr2, but other RLCKs currently cannot be excluded as potential targets for this effector. Further research will hopefully unveil the target(s) of Avr2 through e.g. co-immunoprecipitation and/or pulldown assays. A knockout of this host gene might fully compromise PTI signaling, unlike the effect conferred by Avr2 on the encoded protein. All in all, generating a 'PTI-blind' tomato mutant is required to fully exclude involvement of PTI in Fo-induced EMR. Nevertheless, the functional EMR response observed in PTI-knockdown Avr2 tomato lines suggest that these two immune responses act independently from each other.

Has the xylem sap proteome of tomato the potential to contain Fol and prevent disease development?

The vasculature represents an important interface between tomato and Fol. Fol colonizes aboveground tissues through the xylem vessels, which is a prerequisite for the onset and development of wilt disease. The fungus secretes effectors, metabolites and enzymes in the extracellular spaces of the host to facilitate infection, but the plant secretome may counteract infection and restrict fungal growth. The interaction between both secretomes determines whether the fungus or the plant will thrive, or whether both co-exist and the plant can contain the fungus in the absence of disease symptoms (Vincent et al., 2020). Phytoalexins, phenolic compounds and pathogenesis-related (PR) proteins are reported to have antimicrobial activity towards vascular pathogens (Yadeta and Thomma, 2013). Since EMR dampens Fusarium wilt disease progression, it was hypothesized that specific xylem sap-localized tomato proteins and/or metabolites targeting Fol could be involved. To identify potential EMR-induced changes the tomato xylem sap proteome was analyzed in response to the presence of the endophyte, pathogen or both (chapter 5). Like EMR, Resistance (R) proteins can prevent Fusarium wilt disease of isolates harboring a corresponding Avirulence (Avr) factor upon detection of the latter, thereby starting effector-triggered immunity (ETI). To investigate whether *R* gene and EMR induce similar responses also the xylem sap proteome changes of *I-2* resistant lines inoculated with an avirulent Fol isolate were determined (chapter 5). Subsequently, all identified proteomes were compared and over 300 proteins could be identified. Whereas not a single fungal protein was found in xylem sap of resistant plants, 13 were identified in Fo47:Fol co-inoculated plants. This implies a more extensive colonization of the vasculature by Fol in the latter interaction, which correlates with the higher disease index and reduced fresh weight observed upon EMR as compared to resistant plants infected with Fol (chapter 5). Induction of EMR was found to correlate with increased accumulation of a ß-glucanase (45-fold) and NP24 (33-fold), while in Fol-infected resistant tomato plants a significantly higher accumulation of only one protein was observed: PR-5x (158-fold). PR-5x and NP24 are both PR-5 isoforms sharing high sequence similarity, suggesting a similarity in the response towards Fol in both immune responses.

PR-5 proteins can be classified into two groups, osmotins and thaumatin-like proteins (TLPs) (Anil Kumar et al., 2015). TLPs taste sweet unlike osmotins (Richardson et al., 1987; Anil Kumar et al., 2015), which were originally identified in tobacco being induced upon salt stress (Singh et al., 1985). Osmotin overexpression in transgenic tomato, potato or tobacco confers increased tolerance to salinity (Anil Kumar et al., 2015). Many osmotins and TLPs exert antimicrobial activity towards filamentous plant pathogens such as, *Phytophthora infestans* (Woloshuk et al., 1991), *Fo*, *Phytophthora capsici* (Mani et al., 2012), *Fusarium verticilloides*, *Fusarium solani* (Zhang et al., 2018b) and *Alternaria alternata* (Guo et al., 2016). Some PR-5s exert their antimicrobial activity by increasing membrane permeability via formation of pores in the plasma membranes of fungi and oomycetes (Vigers et al., 1992). PR-5 isoforms have also been suggested to exert their antimicrobial effect by binding to cell wall-localized phosphomannoproteins or receptor-like proteins in the plasma membrane. (Woloshuk et al., 1991; Vigers et al., 1992; Abad et al., 1996; de Freitas et al., 2011; Misra et al., 2016). In addition, a barley TLP has been shown to bind 1,3-β-glucans that are present in microbial cell walls (Osmond et al., 2001) and several peach TLPs also bind glucans and exert

β-glucanase activity (Palacin et al., 2010). Notably, the other protein significantly upregulated in xylem sap during EMR is a β-glucanase, whose antimicrobial activity is linked to its ability to bind and hydrolyze β-glucans (Stintzi et al., 1993). Besides a direct antimicrobial effect by degrading the fungal cell wall, β-glucanases can also enhance plant immunity by releasing immunogenic β-1,6-glucans, which are fungal- and oomycete-specific MAMPs that can trigger immune responses in plants (Fesel and Zuccaro, 2016). A tobacco osmotin has been shown to trigger apoptosis in yeast upon binding to a plasma membrane receptor involved in the RAS2/cAMP signaling, providing another means of how these proteins act as antimicrobial agents (Narasimhan et al., 2005). To assess the potential contribution of NP24 and PR-5x in restricting xylem vessel colonization by Fol, two pilot experiments were performed in my project (data not shown). Simultaneous silencing of *NP24* and *PR-5x* in tomato by Tobacco Rattle Virus-induced gene silencing resulted in a slightly increased susceptibility to Fol, together with an unexpected increase in plant weight. Heterologous overexpression of tomato *NP24* and *PR-5x* in *N. benthamiana* leaves through agroinfiltration using the pTRBO vector (Lindbo, 2007) resulted in high accumulation of PR-5x or NP24 in the apoplast. Fol microconidia exposed to isolated apoplastic fluid containing high concentrations of these PR-5 proteins showed growth deformations and formation of vacuolar structures, implying a direct antifungal activity of these proteins, as such responses were not observed using apoplastic fluid isolated from plants overexpressing RFP.

PR-5x (x = xylem) was originally identified in tomato xylem sap as being induced upon Fol infection. Expression of the gene is root-specific and strongly induced upon Fol inoculation of *I*-resistant lines (Rep et al., 2002). *In situ* RNA:RNA hybridization using a *PR-5* probe showed that expression of the gene is restricted to the tomato vasculature within root and crown (Kavroulakis et al., 2006). Interestingly, PR-5x expression is upregulated when tomato is grown in suppressive compost, but not when cultivated in fertilized Sphagnum peat (Kavroulakis et al., 2006). Possibly the presence of beneficial microbes in the compost triggers *PR-5* expression like Fo47 induces expression of the *PR-5*-isoform *NP24*. Analogously, the biocontrol yeast *Cryptococcus laurentii,* which reduces black rot caused by *A. alternata* in tomato fruits, was found to induce *PR-5* expression in tomato. Some plant pathogens, like *V. dahliae*, *Botrytis cinerea* or *Blumeria graminis,* specifically target PR-5s with secreted effectors (Pennington et al., 2016; Gonzalez et al., 2017; Zhang et al., 2019), indicating that these PR-5 proteins are important host factors for the pathogen to manipulate and cause disease. Indeed, Arabidopsis lines overexpressing *PdPR5-1* from plum exhibited increased tolerance towards *Monilinia fructicola* (El-kereamy et al., 2011). CRISPR/Cas9 could be employed to generate *PR-5x* and *NP24* knockout lines in tomato cv. Motelle. With this Fol race 1- and race 2-resistant variety it will be possible to evaluate whether avirulent Fol races (especially race 2 was found to strongly increase PR-5x levels in xylem sap) can break I or I-2 mediated resistance. If resistance is broken, then it would identify these PR-5 isoforms as the main contributor to Fol resistance in ETI. Inoculation with a virulent race 3 on these knockout lines could unveil whether these proteins affect disease susceptibility. Furthermore, these lines will also be instrumental to assess involvement of these proteins in Fo-induced EMR when they are co-inoculated with Fo47 and Fol. PR-5x and NP24 overexpressing lines can provide additional support for a potential involvement of these proteins in controlling Fol. To mimic the vasculature-specific expression of PR-5 (Kavroulakis et al., 2006), and to minimize the risk of unwanted phenotypes due to aberrant accumulation patterns of the protein, it would be preferable to use a xylem-specific promoter, like

the one from the xylem-specific expressed *Xsp10* (Krasikov et al., 2011) to drive expression of PR-5.

Effective containment of Fol by Fo47-based EMR is unlikely to be attributed solely to the accumulation of a single PR-5 isoform within the xylem vessels during later stages of infection. Xylem sap was collected two weeks post inoculation, but it is known that the proteome is affected already one week post infection (Rep et al., 2002). Even earlier changes in the xylem sap composition are likely as the root transcriptome is altered as early as 72 hours Fo post inoculation (Lanubile et al., 2015). Also, the tomato metabolome may play a role in EMR, and xylem sap metabolites could be a subject of study. In fact, the phenolic compounds caffeic and chlorogenic acid are upregulated or primed by Fo47 in pepper and these are toxic towards *V. dahliae* (Veloso et al., 2016). These phenolic acids can also be cross-linked to cellulose in a ROS-catalyzed process resulting in lignification of the cell wall (McLusky et al., 1999; Bubna et al., 2011; O'Brien et al., 2012). Fo47 triggers the formation of callose-containing papillae when penetrating host cells and induces a systemic response resulting in enhanced papilla formation upon subsequent infection with the root pathogen *P. ultimum* (Benhamou et al., 2002). Whether callose depositions and lignification contribute to EMR could be investigated using GFP- or RFP-expressing Fo and histochemical staining of these cell wall components (Ursache et al., 2018), or by the deployment of, e.g. a callose synthase mutants in tomato (Huibers et al., 2013).

The nature of the mobile signal(s) triggering a systemic EMR response in roots is unknown. The defence phytohormones jasmonic acid (JA) and salicylic acid (SA) are transported through the plant vasculature (Tamogami et al., 2012; Maruri-Lopez et al., 2019). However, these phytohormones, and ethylene, appear not to be involved in EMR (Constantin et al., 2019). It is imaginable that the EMR signal moves through the vasculature and the compounds transducing the signal could be identified using metabolomics of the xylem or phloem sap. Identification of the compounds involved could result in triggering EMR to protect plants toward pathogens without the need of an endophyte.

Unlike Fo endophytes, avirulent Fol protects poorly against virulent Fol in tomato: What is going on?

As mentioned above, specific PR-5 isoforms accumulate in tomato xylem sap during EMR (i.e., in tomato co-inoculated with endophytic Fo47 and virulent Fol) and in an incompatible interaction between avirulent Fol and *I-2*-containing tomato. The similarity of these two resistance responses, and the notion that avirulent Fol strains behave as endophytes in resistant tomato varieties (van der Does et al., 2018), incited me to test whether avirulent Fol reduces susceptibility to virulent Fol to a similar extent as Fo47. An avirulent Fol infecting *I-2*-containing resistant tomato plants was expected to strongly reduce susceptibility of the plant to simultaneously inoculated virulent Fol isolate. This hypothesis was based on the finding that PR-5x accumulates to high levels in resistant plants inoculated with an avirulent Fol strain, where ETI is activated. ETI responses are typically enhanced and accelerated PTI responses, resulting sometimes in a hypersensitive (cell death) response (HR) that together contain the pathogen at the infection site (Jones and Dangl,

2006). *I-2*-containing tomato constitutively expresses the *I-2* R gene in the xylem adjacent cells and in cell around sites of lateral root formation (Mes et al., 2000). I-2 is a nucleotide-binding LRR (NB-LRR)-type immune receptor that induces ETI upon recognition of the Fol effector protein Avr2 (van Ooijen et al., 2007; Ma et al., 2013). Notably, not all R proteins are localized intracellularly. For instance, the tomato *I* resistance gene encodes a receptor-like protein (RLP) that is localized at the plasma membrane where it mediates recognition of the Fol effector Avr1 (Catanzariti et al., 2017). We set out to investigate whether ETI triggered at the plasma membrane by I and/or intracellularly by I-2 reduces susceptibility towards a co-inoculated virulent Fol isolate. In addition, we assessed whether these immune responses are additive and whether they reduce susceptibility to a similar extent as Fo47-induced EMR. The tomato variety Motelle, which harbors *I* and *I-2* conferring resistance to Fol race 1 and race 2, respectively (Laterrot, 1993), was selected (Chapter 4). Race 1 Fol secretes the effectors Avr1 and Avr2, whose recognition by tomato I and I-2 triggers ETI, leading to resistance. Race 2 Fol does not have *Avr1* and therefore only triggers I-2 (Houterman et al., 2008). Motelle seedlings were co-inoculated with a pathogenic race 3 isolate (Fol029) and an avirulent Fol strain activating I and/or I-2. Avirulent Fol strains were found to only slightly reduce susceptibility to Fol029 (a phenomenon called 'cross protection') and no additive protection was observed using isolates that carry both effectors. EMR triggered by Fo47 was assessed in parallel and was observed to result in a stronger protection to wilt disease than cross protection. Therefore, either PR-5x does not accumulate when avirulent Fol is co-inoculated with virulent Fol029, or its accumulation in the sap is not sufficient to contain virulent Fol.

While avirulent pathogens confer marginal protection to a virulent Fol isolate, protection conferred by the non-host pathogen Fom (melon pathogen) is as strong as that induced by Fo47 (chapter 3). Likewise, the non-host pathogen Fo f.sp. *cucumerinum* (cucumber pathogen) has been reported to significantly reduce susceptibility to pathogenic Fo f.sp. *niveum* in watermelon (Biles and Martyn, 1989). Also Fol has been shown to reduce disease symptom development caused by the vascular pathogen *V. dahliae* in pepper (Díaz et al., 2005). Why protection conferred by avirulent Fol pathogens is much weaker than that of non-host pathogens towards virulent strains is unknown. Enhanced protection by the former was foreseen as avirulent pathogens trigger ETI and, as the fungus can be detected in the plant and behaves as an endophyte (van der Does et al., 2018), possibly also EMR. Indeed, avirulent Fol was found colonize the hypocotyls of resistant tomato and to reach the cotyledon level, while Fo47 or non-host Fom are poor stem colonizers and were not observed above the crown region (chapter 4). It is tempting to speculate that the lack of particular host-specific effectors in the non-host strain prevents the fungus from hijacking/compromising the host's immune system, thereby allowing the latter to mount an EMR response. In support of this idea, the non-host pathogen Fom has been shown to induce host cell death and immune responses in tomato roots (Alabouvette et al., 2009), while host cell death and other immune signaling responses like Ca^{2+} influx, ROS accumulation, extracellular alkalization or papillae formation upon host cell penetration are barely induced upon Fol inoculation (chapter 2) (Benhamou and Garand, 2001; Olivain et al., 2003; Humbert et al., 2015; Gordon, 2017). Possibly these responses are required to trigger EMR. Host-specific effectors suppressing immune responses have indeed been identified in Fol. One example of a host-specific effector secreted by Fol that reduces host defense response is Fol Avr2. This effector enters the host cells and compromises PTI signaling including ROS burst, MAPK signaling, growth reduction and callose deposition (Di et al., 2016; Di et al., 2017). Interestingly, the effector moves from cell-to-cell

through plasmodesmata in a process facilitated by another Fol effector (Six5) to suppress immune response ahead of the spreading fungus (Cao et al., 2018). Altogether, these data suggest that Fo47 or Fom, both lacking the proper tomato-specific effectors, induce host responses like ROS generation, callose depositions or host cell death limiting endophyte colonization and resulting in an EMR response. Future research should clarify the (in)dispensability of these host responses for EMR. Co-inoculation of a Fo47 strain carrying the Fol pathogenicity chromosome (with the *Six* effector genes) with Fol on susceptible tomato plants did not result in a reduction in disease susceptibility (data not shown), implying that the induction of EMR requires the absence of host-specific effectors in the biocontrol-conferring strain.

Recently, host cell damage has been reported as essential factor to trigger a PTI response in the differentiated zone of Arabidopsis roots (Zhou et al., 2020). Spontaneous cell death, or cell death induced by the formation of lateral roots, nematode feeding, or pathogen presence, triggers PRR expression in adjacent neighbouring cells, enabling them to perceive MAMPs and mount a PTI response. Whether Fo-induced EMR relies on induction of host cell death, and hence enables the root to mount PTI, is currently unknown. Of interest, biocontrol-conferring fungal endophytes like *Harpophora oryzae* or *Serendipita indica* have been shown to induce cell death upon root colonisation (Deshmukh et al., 2006; Su et al., 2013). Cell death triggered by *S. indica* has been proposed to be instrumental for biocontrol, as overexpression of the cell death-suppressing *Bax inhibitor 1* (*BI-1*) gene compromises the biocontrol potential of this endophyte in barley (Zuccaro et al., 2019). Whether a similar mechanism applies to Fo endophytes is unclear, but a mutagenesis screen of tomato Fo endophytes such as Fom24 and Fo47 showed that the mutants that had lost the capacity to induce cell death in the root also lost their ability to trigger EMR, despite retaining the capability of colonizing the host (Alabouvette et al., 2009). Cloning these genes in the mutagenized Fom24 and Fo47 strains (Alabouvette et al., 2009) could provide molecular insight in the underlying processes. Furthermore, tomato lines in which cell death is suppressed by overexpressing the cell death inhibitor gene *BI-1* (*Bax inhibitor 1*) (Scotton et al., 2017) or a caspase inhibitor (Lincoln et al., 2002) could be instrumental to assess involvement of cell death in EMR. Alternatively, tomato lines showing spontaneous cell death in roots could be monitored for an altered EMR response. If EMR is potentiated in these mutants it provides support for a functional relation between these responses. *mlo* mutants resistant to powdery mildew often show spontaneous cell death in their leaf mesophyll (Piffanelli et al., 2002; Acevedo-Garcia et al., 2014). Of note, a *mlo* barley mutant shows a reduced colonization of its roots by the biocontrol-conferring root endophyte *S. indica* (Hilbert et al., 2019). Reduced colonization correlated with a stronger host defence response including enhanced papillae formation. *S. indica* triggers host cell death during later colonization stages of barley, but this late response was impaired in the *mlo* mutant. Whether early host cell death occurs in roots of an *mlo* mutant is unknown. Finally, Arabidopsis PTI-reporter lines where mVenus accumulates in the nucleus upon MAMP recognition (Vermeer et al., 2014; Poncini et al., 2017) could be (co-) inoculated with wild-type Fo47 and/or GFP-labelled Arabidopsis pathogen Fo5176 to assess whether root cells become responsive to the pathogen only in the presence of the endophyte, and whether this response is preceded by cell death.

Does Fo47 have the potential of being used as a biocontrol agent in agriculture?

Fo47 has been reported to confer biocontrol in different setups by various research groups worldwide (see chapter 2). The endophyte was originally isolated from wilt disease-suppressive soils (Alabouvette, 1986), where it was found to increase resilience of crops towards pathogenic Fo isolates. Notwithstanding these beneficial properties, and despite being investigated for over three decades, Fo endophytes are not commercially used as biocontrol agents. Two attempts to market Fo endophyte formulations that I know of are Fusaclean (Nature Plant Protection, France) and Biofox (SIAPA, Italy). To study the potential to apply Fo47 in agriculture, we joined a potato field trial organized by INOQ GmbH in 2018 with the goal of developing a framework for large-scale production of Fo47 propagules, to assess its endophytic ability under field conditions and to monitor its effect on the crop (chapter 6). High amounts of viable Fo47 microconidia could be produced on 1% mung bean media. Fo47 was found to colonize potato plants in the field and the fungus could be re-isolated from roots or tubers of around 70% of the plants. The fungus did not negatively affect yield nor starch content of the plants. Unfortunately, biocontrol by Fo47 to typical potato diseases could not be assessed due to the extremely dry summer, which resulted in the absence of most common potato diseases. Only black scurf caused by *Rhizoctonia solani* could be scored and Fo47 was found to not affect disease incidence. Whether the fungus can provide protection to other pathogenic microbes remains a question for future research.

Previous trials with Fo endophytes revealed that the positive effects observed under controlled laboratory settings do not always translate well to field conditions. For instance, application of Fo47 in tomato fields reduced susceptibility to Fol only transiently and to a different extent within subsequent years (Fuchs et al., 1999). Moreover, application of Fo47 in asparagus fields did not reduce susceptibility to Foa despite doing so under lab conditions (Blok et al., 1997). So while Fo endophytes in disease suppressive soils can protect crops to Fusarium wilt disease (Alabouvette, 1986; Tamietti et al., 1993), these findings cannot be reproduced in agricultural settings upon Fo application, implying the presence of unknown factors that contribute to the effectiveness of biocontrol. To identify these factors, research could focus on the contribution of soil composition and microbiome to the effectiveness of Fo47-mediated biocontrol, or to the formulation, preparation and application of the inoculum. In fact, the soil type influences the biocontrol performance of different Fo endophytes (Larkin and Fravel, 2002) and Fo47-mediated biocontrol is enhanced when applied together with biocontrol-conferring bacteria like actinomycetes (Zhang et al., 2018a) or *Pseudomonas putida* (Duijff et al., 1999). A potential stumbling block to implementing Fo endophytes in the field is the fact that endophytism and pathogenicity are one chromosome away. Horizontal transfer of Fo pathogens to Fo47 turns the latter into a pathogen (Ma et al., 2010; van Dam et al., 2017) and occurrence of this phenomenon in nature could jeopardize efforts to implement biocontrol with Fo. This potential threat should be evaluated prior to implementing a biocontrol strategy with Fo endophytes.

Outlook

Resistance breeding generally focusses on introgressing dominant resistance genes into crops to control plant pathogens. This resistance is sooner or later overcome, and although it can take decades for Fo to do so (probably due to its asexual reproduction), there is a need for alternatives. Future research should focus on identifying plant traits involved in recruitment of beneficial endophytes and in potentiating EMR. Incorporation of these traits in breeding schemes could result in crops with an increase resilience towards root pathogens.

References

Abad, L.R., DUrzo, M.P., Liu, D., Narasimhan, M.L., Reuveni, M., Zhu, J.K., et al. (1996). Antifungal activity of tobacco osmotin has specificity and involves plasma membrane permeabilization. *Plant Sci* 118(1)**,** 11-23. doi: Doi 10.1016/0168-9452(96)04420-2.

AbuQamar, S., Chai, M.F., Luo, H.L., Song, F.M., and Mengiste, T. (2008). Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. *Plant Cell* 20(7)**,** 1964-1983. doi: 10.1105/tpc.108.059477.

Acevedo-Garcia, J., Kusch, S., and Panstruga, R. (2014). Magical mystery tour: MLO proteins in plant immunity and beyond. *New Phytol* 204(2)**,** 273-281. doi: 10.1111/nph.12889.

Aimé, S., Alabouvette, C., Steinberg, C., and Olivain, C. (2013). The endophytic strain *Fusarium oxysporum* Fo47: a good candidate for priming the defense responses in tomato roots. *Mol Plant Microbe Interact* 26(8)**,** 918-926. doi: 10.1094/MPMI-12-12-0290-R.

Alabouvette, C. (1986). Fusarium-Wilt Suppressive Soils from the Châteaurenard Region: Review of a 10-Year Study. *Agronomie* 6(3)**,** 273-284. doi: 10.1051/agro:19860307.

Alabouvette, C., Olivain, C., Migheli, Q., and Steinberg, C. (2009). Microbiological control of soil-borne phytopathogenic fungi with special emphasis on wilt-inducing *Fusarium oxysporum*. *New Phytol* 184(3)**,** 529-544. doi: 10.1111/j.1469-8137.2009.03014.x.

Anil Kumar, S., Hima Kumari, P., Shravan Kumar, G., Mohanalatha, C., and Kavi Kishor, P.B. (2015). Osmotin: a plant sentinel and a possible agonist of mammalian adiponectin. *Front Plant Sci* 6**,** 163. doi: 10.3389/fpls.2015.00163.

Benhamou, N., and Garand, C. (2001). Cytological analysis of defense-related mechanisms induced in pea root tissues in response to colonization by nonpathogenic *Fusarium oxysporum* Fo47. *Phytopathology* 91(8)**,** 730-740. doi: 10.1094/Phyto.2001.91.8.730.

Benhamou, N., Garand, C., and Goulet, A. (2002). Ability of Nonpathogenic *Fusarium oxysporum* Strain Fo47 To Induce Resistance against *Pythium ultimum* Infection in Cucumber. *Appl Environ Microbiol* 68(8)**,** 4044-4060. doi: 10.1128/aem.68.8.4044-4060.2002.

Bigeard, J., Colcombet, J., and Hirt, H. (2015). Signaling Mechanisms in Pattern-Triggered Immunity (PTI). *Mol Plant* 8(4)**,** 521-539. doi: 10.1016/j.molp.2014.12.022.

Biles, C.L., and Martyn, R.D. (1989). Local and Systemic Resistance Induced in Watermelons by Formae Speciales of *Fusarium oxysporum*. *Phytopathology* 79(8)**,** 856-860. doi: 10.1094/Phyto-79-856.

Block, A., and Alfano, J.R. (2011). Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? *Curr Opin Microbiol* 14(1)**,** 39-46. doi: 10.1016/j.mib.2010.12.011.

Blok, W.J., Zwankhuizen, M.J., and Bollen, G.J. (1997). Biological control of *Fusarium oxysporum* f.sp. *asparagi* by applying non-pathogenic isolates of *F. oxysporum*. *Biocontrol Sci Techn* 7(4)**,** 527-541. doi: 10.1080/09583159730596.

Bolwerk, A., Lagopodi, A.L., Lugtenberg, B.J.J., and Bloemberg, G.V. (2005). Visualization of interactions between a pathogenic and a beneficial *Fusarium* strain during biocontrol of tomato foot and root rot. *Mol Plant Microbe In* 18(7)**,** 710-721. doi: 10.1094/Mpmi-18-0710.

Bubna, G.A., Lima, R.B., Zanardo, D.Y.L., dos Santos, W.D., Ferrarese, M.D.L., and Ferrarese, O. (2011). Exogenous caffeic acid inhibits the growth and enhances the lignification of the roots of soybean (*Glycine max*). *J Plant Physiol* 168(14)**,** 1627-1633. doi: 10.1016/j.jplph.2011.03.005.

Cao, L., Blekemolen, M.C., Tintor, N., Cornelissen, B.J.C., and Takken, F.L.W. (2018). The Fusarium oxysporum Avr2-Six5 Effector Pair Alters Plasmodesmatal Exclusion Selectivity to Facilitate Cell-to-Cell Movement of Avr2. *Mol Plant* 11(5)**,** 691-705. doi: 10.1016/j.molp.2018.02.011.

Catanzariti, A.M., Do, H.T.T., Bru, P., de Sain, M., Thatcher, L.F., Rep, M., et al. (2017). The tomato / gene for Fusarium wilt resistance encodes an atypical leucine-rich repeat receptor-like protein whose function is nevertheless dependent on SOBIR1 and SERK3/BAK1. *Plant J* 89(6)**,** 1195-1209. doi: 10.1111/tpj.13458.

Chuberre, C., Plancot, B., Driouich, A., Moore, J.P., Bardor, M., Gugi, B., et al. (2018). Plant Immunity Is Compartmentalized and Specialized in Roots. *Front Plant Sci* 9**,** 1692. doi: 10.3389/fpls.2018.01692.

Coleman, A.D., Raasch, L., Maroschek, J., Ranf, S., and Hückelhoven, R. (2019). The Arabidopsis leucine-rich repeat receptor kinase MIK2 is a crucial component of pattern-triggered immunity responses to *Fusarium* fungi. *bioRxiv***,** 720037. doi: 10.1101/720037.

Constantin, M.E. (2020). *Fusarium oxysporum: beyond good and evil* University of Amsterdam.

Constantin, M.E., de Lamo, F.J., Vlieger, B.V., Rep, M., and Takken, F.L.W. (2019). Endophyte-Mediated Resistance in Tomato to *Fusarium oxysporum* Is Independent of ET, JA, and SA. *Front Plant Sci* 10**,** 979. doi: 10.3389/fpls.2019.00979.

de Freitas, C.D.T., Lopes, J.L.D., Beltramini, L.M., de Oliveira, R.S.B., Oliveira, J.T.A., and Ramos, M.V. (2011). Osmotin from *Calotropis procera* latex: New insights into structure and antifungal properties. *Bba-Biomembranes* 1808(10)**,** 2501-2507. doi: 10.1016/j.bbamem.2011.07.014.

Deshmukh, S., Hueckelhoven, R., Schaefer, P., Imani, J., Sharma, M., Weiss, M., et al. (2006). The root endophytic fungus *Piriformospora indica* requires host cell death for proliferation during mutualistic symbiosis with barley. *PNAS* 103(49), 18450-18457. doi: 10.1073/pnas.0605697103.

Di, X., Cao, L., Hughes, R.K., Tintor, N., Banfield, M.J., and Takken, F.L.W. (2017). Structure-function analysis of the *Fusarium oxysporum* Avr2 effector allows uncoupling of its immune-suppressing activity from recognition. *New Phytol* 216(3), 897-914. doi: 10.1111/nph.14733.

Di, X., Takken, F.L.W., and Tintor, N. (2016). How Phytohormones Shape Interactions between Plants and the Soil-Borne Fungus *Fusarium oxysporum*. *Front Plant Sci* 7, 170. doi: 10.3389/fpls.2016.00170.

Díaz, J., Silvar, C., Varela, M.M., Bernal, A., and Merino, F. (2005). *Fusarium* confers protection against several mycelial pathogens of pepper plants. *Plant Pathol* 54, 773-780. doi: 10.1111/j.1365-3059.2005.01285.x.

Duijff, B.J., Pouhair, D., Olivain, C., Alabouvette, C., and Lemanceau, P. (1998). Implication of systemic induced resistance in the suppression of fusarium wilt of tomato by *Pseudomonas fluorescens* WCS417r and by nonpathogenic *Fusarium oxysporum* Fo47. *Eur J Plant Pathol* 104(9), 903-910. doi: 10.1023/A:1008626212305.

Duijff, B.J., Recorbet, G., Bakker, P.A.H.M., Loper, J.E., and Lemanceau, P. (1999). Microbial antagonism at the root level is involved in the suppression of Fusarium wilt by the combination of nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358. *Phytopathology* 89(11), 1073-1079. doi: 10.1094/Phyto.1999.89.11.1073.

Edel-Hermann, V., and Lecomte, C. (2019). Current Status of *Fusarium oxysporum Formae Speciales* and Races. *Phytopathology* 109(4), 512-530. doi: 10.1094/Phyto-08-18-0320-Rvw.

El-kereamy, A., El-sharkawy, I., Ramamoorthy, R., Taheri, A., Errampalli, D., Kumar, P., et al. (2011). *Prunus domestica* pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. *PLoS One* 6(3), e17973. doi: 10.1371/journal.pone.0017973.

Elmer, W.H. (2004). Combining nonpathogenic strains of *Fusarium oxysporum* with sodium chloride to suppress fusarium crown rot of asparagus in replanted fields. *Plant Pathol* 53(6), 751-758. doi: 10.1111/j.1365-3059.2004.01096.x.

Fesel, P.H., and Zuccaro, A. (2016). beta-glucan: Crucial component of the fungal cell wall and elusive MAMP in plants. *Fungal Genet Biol* 90, 53-60. doi: 10.1016/j.fgb.2015.12.004.

Fravel, D.R., Olivain, C., and Alabouvette, C. (2003). *Fusarium oxysporum* and its biocontrol. *New Phytol* 157, 493-502.

Frey, N.F.D., Garcia, A.V., Bigeard, J., Zaag, R., Bueso, E., Garmier, M., et al. (2014). Functional analysis of *Arabidopsis* immune-related MAPKs uncovers a role for MPK3 as negative regulator of inducible defences. *Genome Biol* 15(6). doi: ARTN R87

10.1186/gb-2014-15-6-r87.

Fuchs, J.G., Moënne-Loccoz, Y., and Défago, G. (1999). Ability of nonpathogenic *Fusarium oxysporum* Fo47 to protect tomato against Fusarium wilt. *Biol Control* 14(2), 105-110. doi: 10.1006/bcon.1998.0664.

Gao, F., Zhang, B.S., Zhao, J.H., Huang, J.F., Jia, P.S., Wang, S., et al. (2019). Deacetylation of chitin oligomers increases virulence in soil-borne fungal pathogens. *Nat Plants* 5(11), 1167-1176. doi: 10.1038/s41477-019-0527-4.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: An LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol Cell* 5(6), 1003-1011. doi: Doi 10.1016/S1097-2765(00)80265-8.

Gonzalez, M., Brito, N., and Gonzalez, C. (2017). The *Botrytis cinerea* elicitor protein BcIEB1 interacts with the tobacco PR5-family protein osmotin and protects the fungus against its antifungal activity. *New Phytol* 215(1), 397-410. doi: 10.1111/nph.14588.

Gordon, T.R. (2017). *Fusarium oxysporum* and the *Fusarium* Wilt Syndrome. *Annu Rev Phytopathol* 55, 23-39. doi: 10.1146/annurev-phyto-080615-095919.

Gubaeva, E., Gubaev, A., Melcher, R.L.J., Cord-Landwehr, S., Singh, R., El Gueddari, N.E., et al. (2018). 'Slipped Sandwich' Model for Chitin and Chitosan Perception in *Arabidopsis*. *Mol Plant Microbe In* 31(11), 1145-1153. doi: 10.1094/Mpmi-04-18-0098-R.

Guo, J., Zhao, X., Wang, H.L., Yu, T., Miao, Y., and Zheng, X.D. (2016). Expression of the LePR5 gene from cherry tomato fruit induced by *Cryptococcus laurentii* and the analysis of LePR5 protein antifungal activity. *Postharvest Biol Tec* 111, 337-344. doi: 10.1016/j.postharvbio.2015.09.002.

Hilbert, M., Novero, M., Rovenich, H., Mari, S., Grimm, C., Bonfante, P., et al. (2019). *MLO* Differentially Regulates Barley Root Colonization by Beneficial Endophytic and Mycorrhizal Fungi. *Front Plant Sci* 10, 1678. doi: 10.3389/fpls.2019.01678.

Hind, S.R., Strickler, S.R., Boyle, P.C., Dunham, D.M., Bao, Z.L., O'Doherty, I.M., et al. (2016). Tomato receptor FLAGELLIN-SENSING 3 binds flgII-28 and activates the plant immune system. *Nat Plants* 2(9). doi: 10.1038/Nplants.2016.128.

Houterman, P.M., Cornelissen, B.J., and Rep, M. (2008). Suppression of plant resistance gene-based immunity by a fungal effector. *PLoS Pathog* 4(5), e1000061. doi: 10.1371/journal.ppat.1000061.

Houterman, P.M., Ma, L., van Ooijen, G., de Vroomen, M.J., Cornelissen, B.J., Takken, F.L., et al. (2009). The effector protein Avr2 of the xylem-colonizing fungus *Fusarium oxysporum* activates the tomato resistance protein I-2 intracellularly. *Plant J* 58(6), 970-978. doi: 10.1111/j.1365-313X.2009.03838.x.

Huibers, R.P., Loonen, A.E., Gao, D., Van den Ackerveken, G., Visser, R.G., and Bai, Y. (2013). Powdery mildew resistance in tomato by impairment of SlPMR4 and SlDMR1. *PLoS One* 8(6), e67467. doi: 10.1371/journal.pone.0067467.

Humbert, C., Aimé, S., Alabouvette, C., Steinberg, C., and Olivain, C. (2015). Remodelling of actin cytoskeleton in tomato cells in response to inoculation with a biocontrol strain of *Fusarium oxysporum* in comparison to a pathogenic strain. *Plant Pathol* 64(6), 1366-1374. doi: 10.1111/ppa.12375.

Jiang, Y., Han, B., Zhang, H., Mariappan, K.G., Bigeard, J., Colcombet, J., et al. (2019). MAP4K4 associates with BIK1 to regulate plant innate immunity. *EMBO Rep* 20(11), e47965. doi: 10.15252/embr.201947965.

Jones, J.D.G., and Dangl, J.L. (2006). The plant immune system. *Nature* 444(7117), 323-329. doi: 10.1038/nature05286.

Kaur, R., and Singh, R.S. (2007). Study of induced systemic resistance in *Cicer arietinum* L. due to nonpathogenic *Fusarium oxysporum* using a modified split root technique. *J Phytopathol* 155(11-12), 694-698. doi: 10.1111/j.1439-0434.2007.01300.x.

Kavroulakis, N., Papadopoulou, K.K., Ntougias, S., Zervakis, G.I., and Ehaliotis, C. (2006). Cytological and other aspects of pathogenesis-related gene expression in tomato plants grown on a suppressive compost. *Ann Bot* 98(3), 555-564. doi: 10.1093/aob/mcl149.

Krasikov, V., Dekker, H.L., Rep, M., and Takken, F.L.W. (2011). The tomato xylem sap protein XSP10 is required for full susceptibility to Fusarium wilt disease. *J Exp Bot* 62(3), 963-973. doi: 10.1093/jxb/erq327.

Lanubile, A., Muppirala, U.K., Severin, A.J., Marocco, A., and Munkvold, G.P. (2015). Transcriptome profiling of soybean (*Glycine max*) roots challenged with pathogenic and non-pathogenic isolates of *Fusarium oxysporum*. *BMC Genomics* 16. doi: 10.1186/s12864-015-2318-2.

Larkin, R.P., and Fravel, D.R. (2002). Effects of Varying Environmental Conditions on Biological Control of Fusarium Wilt of Tomato by Nonpathogenic Fusarium spp. *Phytopathology* 92(11), 1160-1166. doi: 10.1094/PHYTO.2002.92.11.1160.

Laterrot, H.S. (1993). Revised list of near isogenic tomato lines in Moneymaker type with different genes for disease resistances. *Report of the Tomato Genetics Cooperative* 43, 79-80.

Li, L., Li, M., Yu, L.P., Zhou, Z.Y., Liang, X.X., Liu, Z.X., et al. (2014). The FLS2-Associated Kinase BIK1 Directly Phosphorylates the NADPH Oxidase RbohD to Control Plant Immunity. *Cell Host Microbe* 15(3), 329-338. doi: 10.1016/j.chom.2014.02.009.

Lincoln, J.E., Richael, C., Overduin, B., Smith, K., Bostock, R., and Gilchrist, D.G. (2002). Expression of the antiapoptotic baculovirus p35 gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. *PNAS* 99(23), 15217-15221. doi: 10.1073/pnas.232579799.

Lindbo, J.A. (2007). TRBO: a high-efficiency tobacco mosaic virus RNA-based overexpression vector. *Plant Physiol* 145(4), 1232-1240. doi: 10.1104/pp.107.106377.

Ma, L.J., van der Does, H.C., Borkovich, K.A., Coleman, J.J., Daboussi, M.J., Di Pietro, A., et al. (2010). Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. *Nature* 464(7287), 367-373. doi: 10.1038/nature08850.

Ma, L.S., Cornelissen, B.J.C., and Takken, F.L.W. (2013). A nuclear localization for Avr2 from Fusarium oxysporum is required to activate the tomato resistance protein I-2. *Frontiers in Plant Science* 4. doi: 10.3389/fpls.2013.00094.

Macho, A.P., and Zipfel, C. (2014). Plant PRRs and the Activation of Innate Immune Signaling. *Mol Cell* 54(2), 263-272. doi: 10.1016/j.molcel.2014.03.028.

Mani, T., Sivakumar, K.C., and Manjula, S. (2012). Expression and Functional Analysis of Two Osmotin (PR5) Isoforms with Differential Antifungal Activity from *Piper colubrinum*: Prediction of Structure-Function Relationship by Bioinformatics Approach. *Mol Biotechnol* 52(3), 251-261. doi: 10.1007/s12033-011-9489-0.

Maruri-Lopez, I., Aviles-Baltazar, N.Y., Buchala, A., and Serrano, M. (2019). Intra and Extracellular Journey of the Phytohormone Salicylic Acid. *Front Plant Sci* 10, 423. doi: 10.3389/fpls.2019.00423.

McLusky, S.R., Bennett, M.H., Beale, M.H., Lewis, M.J., Gaskin, P., and Mansfield, J.W. (1999). Cell wall alterations and localized accumulation of feruloyl-3 '-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarisation, peroxidase activity and suppression of flavonoid biosynthesis. *Plant J* 17(5), 523-534. doi: 10.1046/j.1365-313X.1999.00403.x.

Mes, J.J., van Doorn, A.A., Wijbrandi, J., Simons, G., Cornelissen, B.J.C., and Haring, M.A. (2000). Expression of the *Fusarium* resistance gene *I-2* colocalizes with the site of fungal containment. *Plant J* 23(2), 183-193. doi: 10.1046/j.1365-313x.2000.00765.x.

Michielse, C.B., and Rep, M. (2009). Pathogen profile update: *Fusarium oxysporum*. *Mol Plant Pathol* 10(3), 311-324. doi: 10.1111/J.1364-3703.2009.00538.X.

Misra, R.C., Sandeep, Kamthan, M., Kumar, S., and Ghosh, S. (2016). A thaumatin-like protein of *Ocimum basilicum* confers tolerance to fungal pathogen and abiotic stress in transgenic Arabidopsis. *Sci Rep* 6, 25340. doi: 10.1038/srep25340.

Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., et al. (2007). CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in Arabidopsis. *PNAS* 104(49), 19613-19618. doi: 10.1073/pnas.0705147104.

Nahalkova, J., Fatehi, J., Olivain, C., and Alabouvette, C. (2008). Tomato root colonization by fluorescent-tagged pathogenic and protective strains of *Fusarium oxysporum* in hydroponic culture differs from root colonization in soil. *FEMS Microbiol Lett* 286(2)**,** 152-157. doi: 10.1111/j.1574-6968.2008.01241.x.

Narasimhan, M.L., Coca, M.A., Jin, J., Yamauchi, T., Ito, Y., Kadowaki, T., et al. (2005). Osmotin is a homolog of mammalian adiponectin and controls apoptosis in yeast through a homolog of mammalian adiponectin receptor. *Mol Cell* 17(2)**,** 171-180. doi: 10.1016/j.molcel.2004.11.050.

O'Brien, J.A., Daudi, A., Butt, V.S., and Bolwell, G.P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta* 236(3)**,** 765-779. doi: 10.1007/s00425-012-1696-9.

Olivain, C., Humbert, C., Nahalkova, J., Fatehi, J., L'Haridon, F., and Alabouvette, C. (2006). Colonization of tomato root by pathogenic and nonpathogenic *Fusarium oxysporum* strains inoculated together and separately into the soil. *Appl Environ Microbiol* 72(2)**,** 1523-1531. doi: 10.1128/AEM.72.2.1523-1531.2006.

Olivain, C., Trouvelot, S., Binet, M.N., Cordier, C., Pugin, A., and Alabouvette, C. (2003). Colonization of flax roots and early physiological responses of flax cells inoculated with pathogenic and nonpathogenic strains of *Fusarium oxysporum*. *Appl Environ Microbiol* 69(9)**,** 5453-5462. doi: 10.1128/Aem.69.9.5453-5462.2003.

Osmond, R.I.W., Hrmova, M., Fontaine, F., Imberty, A., and Fincher, G.B. (2001). Binding interactions between barley thaumatin-like proteins and (1,3)-beta-D-glucans - Kinetics, specificity, structural analysis and biological implications. *Eur J Biochem* 268(15)**,** 4190-4199. doi: DOI 10.1046/j.1432-1327.2001.02331.x.

Palacin, A., Tordesillas, L., Gamboa, P., Sanchez-Monge, R., Cuesta-Herranz, J., Sanz, M.L., et al. (2010). Characterization of peach thaumatin-like proteins and their identification as major peach allergens. *Clin Exp Allergy* 40(9)**,** 1422-1430. doi: 10.1111/j.1365-2222.2010.03578.x.

Pantelides, I.S., Tjamos, S.E., Striglis, I.A., Chatzipavhdis, I., and Paplomatals, E.J. (2009). Mode of action of a non-pathogenic Fusarium oxysporum strain against Verticillium dahliae using Real Time QPCR analysis and biomarker transformation. *Biol Control* 50(1)**,** 30-36. doi: 10.1016/j.biocontrol.2009.01.010.

Pennington, H.G., Gheorghe, D.M., Damerum, A., Pliego, C., Spanu, P.D., Cramer, R., et al. (2016). Interactions between the Powdery Mildew Effector BEC1054 and Barley Proteins Identify Candidate Host Targets. *J Proteome Res* 15(3)**,** 826-839. doi: 10.1021/acs.jproteome.5b00732.

Piffanelli, P., Zhou, F.S., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., et al. (2002). The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. *Plant Physiol* 129(3)**,** 1076-1085. doi: 10.1104/pp.010954.

Poncini, L., Wyrsch, I., Tendon, V.D., Vorley, T., Boller, T., Geldner, N., et al. (2017). In roots of *Arabidopsis thaliana*, the damage-associated molecular pattern AtPep1 is a stronger elicitor of immune signalling than flg22 or the chitin heptamer. *Plos One* 12(10). doi: ARTN e0185808
10.1371/journal.pone.0185808.

Rep, M., Dekker, H.L., Vossen, J.H., de Boer, A.D., Houterman, P.M., Speijer, D., et al. (2002). Mass spectrometric identification of isoforms of PR proteins in xylem sap of fungus-infected tomato. *Plant Physiol* 130(2)**,** 904-917. doi: 10.1104/pp.007427.

Richardson, M., Valdesrodriguez, S., and Blancolabra, A. (1987). A Possible Function for Thaumatin and a Tmv-Induced Protein Suggested by Homology to a Maize Inhibitor. *Nature* 327(6121)**,** 432-434. doi: DOI 10.1038/327432a0.

Roux, M., Schwessinger, B., Albrecht, C., Chinchilla, D., Jones, A., Holton, N., et al. (2011). The Arabidopsis leucine-rich repeat receptor-like kinases BAK1/SERK3 and BKK1/SERK4 are required for innate immunity to hemibiotrophic and biotrophic pathogens. *Plant Cell* 23(6)**,** 2440-2455. doi: 10.1105/tpc.111.084301.

Scotton, D.C., Azevedo, M.D.S., Sestari, I., Da Silva, J.S., Souza, L.A., Peres, L.E.P., et al. (2017). Expression of the *Theobroma cacao Bax-inhibitor-1* gene in tomato reduces infection by the hemibiotrophic pathogen *Moniliophthora perniciosa*. *Mol Plant Pathol* 18(8)**,** 1101-1112. doi: 10.1111/mpp.12463.

Singh, N.K., Handa, A.K., Hasegawa, P.M., and Bressan, R.A. (1985). Proteins Associated with Adaptation of Cultured Tobacco Cells to NaCl. *Plant Physiol* 79(1)**,** 126-137. doi: 10.1104/pp.79.1.126.

Stintzi, A., Heitz, T., Prasad, V., Wiedemannmerdinoglu, S., Kauffmann, S., Geoffroy, P., et al. (1993). Plant Pathogenesis-Related Proteins and Their Role in Defense against Pathogens. *Biochimie* 75(8)**,** 687-706. doi: Doi 10.1016/0300-9084(93)90100-7.

Su, Z.Z., Mao, L.J., Li, N., Feng, X.X., Yuan, Z.L., Wang, L.W., et al. (2013). Evidence for Biotrophic Lifestyle and Biocontrol Potential of Dark Septate Endophyte *Harpophora oryzae* to Rice Blast Disease. *Plos One* 8(4). doi: 10.1371/journal.pone.0061332.

Tamietti, G., Ferraris, L., Matta, A., and Gentile, I.A. (1993). Physiological Responses of Tomato Plants Grown in *Fusarium* Suppressive Soil. *J Phytopathol* 138(1)**,** 66-76. doi: DOI 10.1111/j.1439-0434.1993.tb01361.x.

Tamogami, S., Noge, K., Abe, M., Agrawal, G.K., and Rakwal, R. (2012). Methyl jasmonate is transported to distal leaves via vascular process metabolizing itself into JA-Ile and triggering VOCs emission as defensive metabolites. *Plant Signal Behav* 7(11)**,** 1378-1381. doi: 10.4161/psb.21762.

Ursache, R., Andersen, T.G., Marhavy, P., and Geldner, N. (2018). A protocol for combining fluorescent proteins with histological stains for diverse cell wall components. *Plant J* 93(2)**,** 399-412. doi: 10.1111/tpj.13784.

Validov, S.Z., Kamilova, F.D., and Lugtenberg, B.J. (2011). Monitoring of pathogenic and non-pathogenic *Fusarium oxysporum* strains during tomato plant infection. *Microb Biotechnol* 4(1), 82-88. doi: 10.1111/j.1751-7915.2010.00214.x.

van Dam, P., Fokkens, L., Ayukawa, Y., van der Gragt, M., ter Horst, A., Brankovics, B., et al. (2017). A mobile pathogenicity chromosome in *Fusarium oxysporum* for infection of multiple cucurbit species. *Sci Rep* 7. doi: 10.1038/s41598-017-07995-y.

van Dam, P., Fokkens, L., Schmidt, S.M., Linmans, J.H.J., Kistler, H.C., Ma, L.J., et al. (2016). Effector profiles distinguish *formae speciales* of *Fusarium oxysporum*. *Environ Microbiol* 18(11), 4087-4102. doi: 10.1111/1462-2920.13445.

van der Does, H.C., Constantin, M.E., Houterman, P.M., Takken, F.L.W., Cornelissen, B.J.C., Haring, M.A., et al. (2018). *Fusarium oxysporum* colonizes the stem of resistant tomato plants, the extent varying with the R-gene present. *Eur J Plant Pathol* 154(1), 55-65. doi: 10.1007/s10658-018-1596-3.

van Ooijen, G., van den Burg, H.A., Cornelissen, B.J.C., and Takken, F.L.W. (2007). Structure and function of resistance proteins in solanaceous plants. *Annu Rev Phytopathol* 45, 43-72. doi: 10.1146/annurev.phyto.45.062806.094430.

Veloso, J., Alabouvette, C., Olivain, C., Flors, V., Pastor, V., García, T., et al. (2016). Modes of action of the protective strain Fo47 in controlling verticillium wilt of pepper. *Plant Pathol* 65(6), 997-1007.

Veloso, J., and Díaz, J. (2012). *Fusarium oxysporum* Fo47 confers protection to pepper plants against *Verticillium dahliae* and *Phytophthora capsici*, and induces the expression of defence genes. *Plant Pathol* 61, 281-288. doi: 10.1111/j.1365-3059.2011.02516.x.

Vermeer, J.E.M., von Wangenheim, D., Barberon, M., Lee, Y., Stelzer, E.H.K., Maizel, A., et al. (2014). A Spatial Accommodation by Neighboring Cells Is Required for Organ Initiation in *Arabidopsis*. *Science* 343(6167), 178-183. doi: 10.1126/science.1245871.

Vigers, A.J., Wiedemann, S., Roberts, W.K., Legrand, M., Selitrennikoff, C.P., and Fritig, B. (1992). Thaumatin-Like Pathogenesis-Related Proteins Are Antifungal. *Plant Sci* 83(2), 155-161. doi: 10.1016/0168-9452(92)90074-V.

Vincent, D., Rafiqi, M., and Job, D. (2020). The Multiple Facets of Plant-Fungal Interactions Revealed Through Plant and Fungal Secretomics. *Front Plant Sci* 10. doi: ARTN 1626
10.3389/fpls.2019.01626.

Willmann, R., Lajunen, H.M., Erbs, G., Newman, M.A., Kolb, D., Tsuda, K., et al. (2011). Arabidopsis lysin-motif proteins LYM1 LYM3 CERK1 mediate bacterial peptidoglycan sensing and immunity to bacterial infection. *PNAS* 108(49), 19824-19829. doi: 10.1073/pnas.1112862108.

Woloshuk, C.P., Meulenhoff, J.S., Selabuurlage, M., Vandenelzen, P.J.M., and Cornelissen, B.J.C. (1991). Pathogen-Induced Proteins with Inhibitory Activity toward Phytophthora-Infestans. *Plant Cell* 3(6), 619-628. doi: DOI 10.1105/tpc.3.6.619.

Xin, X.F., Nomura, K., Aung, K., Velasquez, A.C., Yao, J., Boutrot, F., et al. (2016). Bacteria establish an aqueous living space in plants crucial for virulence. *Nature* 539(7630), 524-+. doi: 10.1038/nature20166.

Xu, J., Meng, J., Meng, X.Z., Zhao, Y.T., Liu, J.M., Sun, T.F., et al. (2016). Pathogen-Responsive MPK3 and MPK6 Reprogram the Biosynthesis of Indole Glucosinolates and Their Derivatives in Arabidopsis Immunity. *Plant Cell* 28(5), 1144-1162. doi: 10.1105/tpc.15.00871.

Yadeta, K.A., and Thomma, B.P.H.J. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Front Plant Sci* 4, 97. doi: 10.3389/fpls.2013.00097.

Zhang, J., Chen, J., Jia, R.M., Ma, Q., Zong, Z.F., and Wang, Y. (2018a). Suppression of plant wilt diseases by nonpathogenic *Fusarium oxysporum* Fo47 combined with actinomycete strains. *Biocontrol Sci Techn* 28(6), 562-573. doi: 10.1080/09583157.2018.1468996.

Zhang, M., Xu, J.H., Liu, G., and Yang, X.P. (2018b). Antifungal properties of a thaumatin-like protein from watermelon. *Acta Physiol Plant* 40(11). doi: 10.1007/s11738-018-2759-8.

Zhang, Y., Gao, Y.H., Liang, Y.B., Dong, Y.J., Yang, X.F., and Qiu, D.W. (2019). *Verticillium dahliae* PevD1, an Alt a 1-like protein, targets cotton PR5-like protein and promotes fungal infection. *J Exp Bot* 70(2), 613-626. doi: 10.1093/jxb/ery351.

Zhou, F., Emonet, A., Denervaud Tendon, V., Marhavy, P., Wu, D., Lahaye, T., et al. (2020). Co-incidence of Damage and Microbial Patterns Controls Localized Immune Responses in Roots. *Cell* 180(3), 440-453 e418. doi: 10.1016/j.cell.2020.01.013.

Zuccaro, A., Sarkar, D., Rövenich, H., Nizam, S., and Langen, G. (2019). "Plant immunity and cell death in fungal root symbiosis", *IS-MPMI XVIII Congress*, Lomond Auditorium, Scottish Event Campus (Glasgow)