Brothers in arms

Fusarium oxysporum vs. Fusarium oxysporum

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CHAPTER 1

General Introduction

Fusarium oxysporum

Fusarium oxysporum (Fo) is a haploid and asexual filamentous ascomycete (Michielse and Rep, 2009). This soil borne fungus is an infamous root vascular pathogen causing 'Fusarium wilt disease'. Pathogenicity of Fo is highly host-specific and strains causing disease in a specific host are classified into so-called 'forma specialis' (f.sp.). A f.sp. typically cannot cause disease in other crop species, Fo f.sp. *radicis-cucumerinum* being an exception as it causes disease in melon, watermelon and cucumber (Edel-Hermann and Lecomte, 2019). Nevertheless, the majority Fo strains are non-pathogenic soil borne saprotrophs that are able to endophytically colonize root surfaces and the outer root cortex of different plant species (Bao et al., 2004; Gordon, 2017). The germination of Fo spores, and the subsequent hyphal chemotaxis toward the root is triggered by root exudates (Turrà et al., 2015; Nordzieke et al., 2019). When Fo reaches the root surface the hyphae swell before penetrating the roots. Depending on the f.sp. and its host, root entry can occur through wounds in the epidermis, or at sites of lateral root formations, or at the root tip. Once in the cortex the colonization of the root by Fo endophytes is mostly apoplastic and restricted to the outer cortex. Fo pathogens, however, are able to enter into the vascular stele and to colonize above-ground tissues through the xylem vessels (Gordon, 2017). Typically, the extensive colonization of the vasculature causes the characteristic disease symptoms, such as wilting, stunting and sometimes death of the infected plant. Fo pathogens, like the tomato pathogen Fo f.sp. *lycopersici* (Fol), carry a pathogenicity chromosome that can be horizontally transferred to the endophyte Fo47. Acquiring this pathogenicity chromosome enables the latter to become a tomato pathogen (Ma et al., 2010). Pathogenicity chromosomes harbor host-specific effector genes contributing to fungal virulence. As the encoded proteins are secreted into the vasculature they are referred to as Secreted-in-xylem (Six) proteins (Ma et al., 2010; van Dam et al., 2016). In Fol 14 *Six* genes have been identified, of which *Six1* (*Avr3*), *Six3* (*Avr2*), *Six5* or *Six6* are indispensable for full pathogenicity towards tomato (Rep et al., 2004; Rep et al., 2005; Houterman et al., 2008; Gawehns et al., 2014), classifying them as genuine effector proteins. Contrarily, Fo endophytes carry fewer (or none) *Six* genes.

Plant innate immunity

Plants harbor a sophisticated innate immune system that allow them to counteract invasion attempts of pathogens (Jones and Dangl, 2006). The innate immune system can be broadly divided into two layers; the first relies on the detection of conserved microbe-associated molecular patterns (MAMPs) from pathogens by pattern-recognition receptors (PRR) at the plant cell surface. The two best characterized examples of PRR/MAMP pairs are the receptor-like kinase (RLK) FLAGELLIN-SENSING 2 (FLS2) and the CHITIN ELICITOR RECEPTOR KINASE (CERK1), mediating respectively recognition of bacterial flagellin (or the immunogenic peptide flg22) or fungal chitin (Gomez-Gomez and Boller, 2000; Miya et al., 2007). MAMP recognition by a PRR triggers a broad spectrum, but localized, immune response effective against many pathogens, which is called pattern-triggered immunity (PTI) (Jones and Dangl, 2006). To date, the only receptor proposedly involved in a specific PTI response to *Fusarium* is MALE DISCOVERER1-INTERACTING RLK 2 (MIK2) that recognizes an unknown factor in Fo extracts (Coleman et al., 2019).

During the evolutionary arms race between plants and pathogens, the latter acquired the ability of suppressing and/or evading PTI through specific effectors that either manipulate host targets, or modify or hide MAMPs jeopardizing their recognition by PRRs (Wawra et al., 2016; Di et al., 2017; Gao et al., 2019). An example of a Fol effector protein is Avr2, which enters plant cells compromising PTI signaling (Di et al., 2016; Di et al., 2017). Some plants harbor resistance (*R*) genes encoding R proteins that can detect the action or presence of specific pathogen produced effectors resulting in the activation the second layer of innate immunity, effector-triggered immunity (ETI) (Jones and Dangl, 2006). For example, tomato varieties carrying the *R* genes *I*, *I-2* or *I-3* can trigger ETI upon recognition of the Fol effector proteins Avr1, Avr2 or Avr3, respectively (Simons et al., 1998; Catanzariti et al., 2015; Catanzariti et al., 2017). However, the boundaries between PTI and ETI are blurred as their immune outputs; e.g. reactive oxygen species (ROS) burst, increase in the intracellular Ca^{2+} levels or transcriptional reprogramming affecting, for instance, the levels of the defense phytohormones jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) often overlap (Bozso et al., 2016). Furthermore, some of the tomato R proteins, such as I or I-3, are receptor-like proteins (RLPs) or RLKs located at the cell surface and they act analogous as PRRs detecting MAMPs. RLPs and RLKs often recruit other RLKs upon ligand binding, which results in homomers (CERK1) or heteromers by for instance interaction with BRI1-ASSOCIATED KINASE 1 (BAK1) (Chinchilla et al., 2007). BAK1 recruitment by a PRR or dimerization of a cell surface receptor typically leads to transphosphorylation of the intracellular kinase domains starting a downstream signaling response. This response results in the subsequent phosphorylation of cytoplasmatic kinases and mitogen-activated protein kinases (MAPKs) triggering the production of ROS and a transcriptional reprogramming, resulting in callose depositions and cell wall fortifications (Frey et al., 2014). BAK1 is required for the downstream 'PTI' signaling of FLS2 upon flg22 recognition (Chinchilla et al., 2007), and for 'ETI' signaling of the tomato I protein upon recognition of the effector Avr1 from pathogenic Fol (Catanzariti et al., 2017). This implies that the PTI and ETI signaling originating from the cell surface might employ similar pathways and could rely on shared immune responses.

Plant immune response upon *Fusarium oxysporum* infection

Although Fo endophytes and Fo pathogens trigger local PTI responses, the latter are known to manipulate their hosts through effector secretion, which facilitates infection and concomitant wilt disease (de Sain and Rep, 2015; Di et al., 2017; Gao et al., 2019). A well described effector compromising PTI is Fol Avr2, which suppresses ROS production, MAPK phosphorylation and callose deposition upon flg22 application (Di et al., 2017). The cotton-infecting strain Fo f.sp. *vasinfectum* secretes the effector PDA1 during infection, a chitin deacetylase that converts immunogenic chitin into chitosan thereby thwarting detection by the host cells (Gao et al., 2019). Fo endophytes in contrast, trigger a strong PTI response leading to an increased accumulation of host cytosolic Ca^{2+} concentration, ROS accumulation and host cell death (Olivain et al., 2003; Humbert et al., 2015). Also, unlike Fo pathogens, the endophyte Fo47 triggers host papillae accumulation upon plant cell wall penetration attempts (Benhamou and Garand, 2001).

Notably, most Fo isolates can confer biocontrol to root pathogens (Bao et al., 2004). Biocontrol can be mediated directly through mycoparasitism and competition (Benhamou et al., 2002) or

indirectly by inducing a plant-mediated response called endophyte-mediated resistance (EMR) (See Chapter 2). Co-inoculation of tomato with endophytic Fo47 and a pathogenic Fol results in severe disease reduction (**Figure 1**). The nature of the activated immune response is unclear, but split-root systems imply that Fo endophytes trigger a systemic EMR response that reduces susceptibility to Fo pathogens (Duijff et al., 1998; Kaur and Singh, 2007; Pantelides et al., 2009). This systemic response is effective in controlling Fo pathogens and other root pathogens such as the oomycetes *Pythium ultimum* in cucumber (Benhamou et al., 2002) and *Phytophthora capsici* in pepper (Veloso and Díaz, 2012). The molecular requirements by which Fo endophytes induce EMR in the host (e.g., an intact PTI/ETI or JA-, ET- or SA-signaling) is the main question addressed in this thesis.

The Tomato-Fusarium system

The biocontrol-conferring Fo47 strain has been extensively studied and the fungus has been shown to reduce susceptibility to Fol in tomato when pre- or co-inoculated on roots or added to soils or provided to split-root systems (Fuchs et al., 1997; Duijff et al., 1998; Fuchs et al., 1999; Larkin and Fravel, 1999; Olivain et al., 2006; Nahalkova et al., 2008; Aimé et al., 2013). Different experimental setups by different labs showed that this tri-partite interaction results in a reduction of wilt disease identifying Fo47 as a genuine and robust EMR-inducing Fo strain. Furthermore, tomato is an important crop; production of this Solanaceae in 2017 was 181 millions of tons (http://www.fao.org/faostat/en/) of which pathogens account for a yield loss of around 17 million of tons. At the Molecular Plant Pathology (MPP) group, various tomato varieties carrying dominant *R* genes towards Fol are available. This allowed comparison of resistance levels of tri-partite interactions to those of resistant tomato lines. Moreover, both tomato and Fo are accessible to genetic modification and mutant collections are available. For instance, tomato lines have been generated showing a compromised PTI or an impaired JA, SA and ET biosynthesis and perception. Also, various GFP-labelled Fo strains and different effector knockout of Fol are available. Finally, the group developed an effective setup to isolate xylem sap from (non-) Fo-infected tomato plants and to analyze these samples using mass spectrometry to identify the proteome (Gawehns et al., 2015). In fact, with this system the first Fol effector was detected in tomato xylem sap (Rep et al., 2004). Together these features make the tomato-Fo47-Fol system an excellent choice to study the mechanisms underlying Fo47-triggered EMR, such the role of PTI and ETI and involvement of the xylem sap proteome.

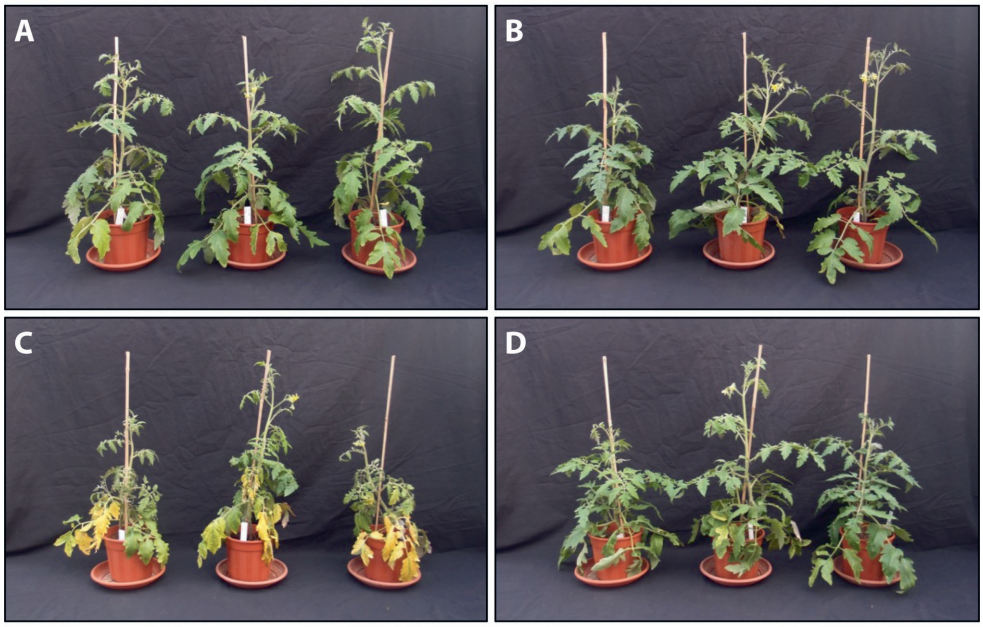

Figure 1. Fo47 reduces tomato susceptibility to pathogenic Fol. Four-weeks-old tomato plants were inoculated with **(A)** water (mock), **(B)** Fo47, **(C)** Fol007 or **(D)** a mixture of Fo47:Fol007.

Thesis outline

Chapter 2 reviews the literature about Fo-triggered EMR. EMR appeared to be an indirect, plant-mediated, resistance response that reduces susceptibility to mostly root pathogens. The role of specific xylem sap-localized proteins, such as PR-5, as well as the potential involvement of localized host cell death in Fo-based EMR are discussed. Furthermore, the differences between Fo endophytes and pathogens are described at the level of host colonization, their effector gene content and the host responses induced upon colonization. **Chapter 3** investigates the role of PTI is controlling the interaction of tomato with endophytic Fo and their concomitantly induced EMR. Thereto, the transgenic MM-Δ*spAvr2-30* tomato line was employed. This line heterologously produces a cytosol-localised Fol Avr2 effector protein that compromises flg22-induced PTI signalling. To test whether this line was also PTI-compromised to fungal MAMPs, leaves of MM-Δ*spAvr2-30* and wild-type MoneyMaker were infiltrated with chitosan. After 24h, a significantly reduced number of callose deposits were found in MM-Δ*spAvr2-30* as compared to wild-type MoneyMaker, showing that this PTI response was also compromised. PTI was found to limit root and stem colonization of both Fo47 and Fo f.sp. *melonis* (Fom) as their colonization was highly increased in MM-Δ*spAvr2-30* plants. Surprisingly, regardless of a compromised PTI the disease symptoms were still reduced in the before-mentioned interactions, showing that EMR is not affected. In **Chapter 4**, it was investigated whether ETI confers biocontrol to pathogenic Fol and, if so, whether disease reduction by ETI was comparable, additive and/or independent of EMR.

Different Fol strains secreting the effector proteins Avr1 and/or Avr2/Six5 were inoculated in tomato cv. Motelle. This variety carries the *R* genes *I* and *I-2* conferring resistance to Fol strains carrying either Avr1 or the Avr2/Six5 effector pair. These Fol strains are avirulent as they trigger ETI via I, I-2, or both R proteins. Bioassays confirmed avirulence of these strains, and co-inoculation with a virulent Fol029 revealed that those Fol strains can reduce susceptibility to a virulent Fol (from now on referred to as 'cross-protection'). The different nature of I (cell surface receptor) and I-2 (intracellular receptor) allowed us to test if they trigger distinct ETI cross-protection responses. Both R genes where found to quantitatively confer similar levels of protection to Fol029 and no additive effect was observed using strains activating both immune receptors, indicating that they mount the same cross-protection response. When cv. Motelle was co-inoculated with Fo47 and Fol029 disease symptoms were reduced to a higher extent than those observed upon co-inoculation of Fol029 with avirulent pathogens. This indicates that EMR is distinct from cross protection and more effective in controlling Fusarium wilt disease. **Chapter 5** focuses on the molecular responses underlying EMR by analyzing the xylem sap proteome dynamics upon Fo47 and/or Fol infection. Xylem sap is an important interface of the tomato-Fo interaction, where Fol secretes its effectors and the plant accumulates antimicrobial proteins. Xylem sap proteome changes upon EMR (i.e., in tomato co-inoculated with Fo47 and Fol) were compared to those in incompatible interactions between Fol and an *I-2*-containing resistant tomato cultivar. The proteome profiles of EMR and incompatible interactions where found to be remarkably similar, suggesting that resistant tomato varieties and Fo47-triggered EMR induce a mechanistically similar response. Upon Fol infection of resistant plants PR-5x was the only differentially accumulated protein out of more than 300 xylem sap proteins (158-fold), while a ß-glucanase (45-fold) and NP24 (33-fold) were also positively accumulated upon EMR. Strikingly, PR-5x and NP24 are highly similar PR-5 isoforms. EMR allowed a more extensive colonization of the tomato vasculature by Fol than I-2 resistant tomato as 13 fungal proteins were detected in the xylem sap of the first. In xylem sap of a susceptible plant many Fol proteins (39) were detected. Notably, no Fo47 proteins were detected in xylem sap either when inoculated alone, or in combination with a pathogen, which indicates that Fo47 does not colonize the vasculature. **Chapter 6** explored the possibility to translate Fo47-based EMR, which was observed under laboratory conditions, to an agricultural setting. A methodology for medium-large scale production of Fo47 inoculum was developed. Different media were analyzed for Fo47 propagation and 1% mung bean medium appeared to be the most prolific and cost-effective. Viability of the mung bean-grown spores was confirmed together with the ability of the endophyte to colonize potato grown under greenhouse conditions. Subsequently, with the collaboration of INOQ GmbH and beneficiaries from the International Training Network BestPass (https://bestpass.ku.dk/), a potato field trial was performed in Clenze (Germany). Fo47 inoculation was shown to be successful, as the fungus could be re-isolated from potato tubers and roots throughout the growing season. Importantly, the tuber yield and starch were not affected by Fo47, showing that the endophyte does not negatively affect plant performance. Increased resistance to other endogenous pathogens could not be assessed as due to the unprecedented dry summer of 2018 most of the typical potato diseases, like late blight caused by *Phytophthora infestans,* were not observed in the field, with the exception of *Rhizoctonia solani.* **Chapter 7** summarizes and discusses the main results described in this thesis and how these contribute to our mechanistic and molecular understanding of this tri-partite system. Also the implications for future research and applicability of the obtained knowledge in improved crop protection strategies is discussed.

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