Verticillium wilt resistance in Arabidopsis and tomato: Identification and functional characterization

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This research was conducted under the auspices of
the Graduate School of Experimental Plant Sciences

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Thesis

submitted in fulfillment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. dr. M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 15 October 2012
at 4 p.m. in the Aula.

Koste A. Yadeta
Verticillium wilt resistance in Arabidopsis
and tomato: Identification and functional
characterization, 148 pages.

PhD thesis, Wageningen University, Wageningen, NL (2012)
With references, with summaries in Dutch and English

ISBN: 978-94-6173-384-9

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Chapter 1

Xylem defence against vascular pathogens

Abstract

Plants are constantly engaged in battles against a wide range of potential pathogens including viruses, bacteria, fungi, oomycetes, protozoa, and nematodes. Vascular wilt pathogens, which comprise bacteria, fungi and oomycetes, are among the most destructive plant pathogens that affect annual crops as well as woody perennials, thus not only impacting world food and feed production but also natural ecosystems. They colonize the xylem vessels of their host plants, which can lead to obstruction of upward water and mineral transport. Cultural, chemical and biological control options against this group of plant pathogens are mostly inefficient, and the most effective management strategy against vascular wilt pathogens thus far is the use of genetic resistance. In general, plants sense xylem-invading vascular wilt pathogens by using extracellular or cell surface receptors or intracellular or cytoplasmic receptors. Extra-or intracellular receptor-mediated recognition of vascular wilt pathogens activates innate immunity which triggers physical and chemical defence responses in the xylem vessels and surrounding parenchyma cells. While physical defence responses halt the pathogen from further spreading in the xylem vessels, chemical defence responses can eliminate the pathogen or inhibit its growth, thereby leading to resistance.

Introduction

Plants are constantly engaged in battles against a wide range of potential pathogens including viruses, bacteria, fungi, oomycetes, protozoa, and nematodes. While some plant pathogens evolved to infect the aerial parts of plants such as stems, leaves and reproductive organs and fruits, others target below-ground organs such as roots and tubers. Since leaves are the plant's main "sugar factories", it is not surprising that most pathogens specifically target these organs. These so-called foliar pathogens often colonize mesophyll tissues while absorbing sugars. Attracted by sugar gradients, they subsequently grow towards the phloem that transports photosynthesis products towards the sinks. A specific group of pathogens targets the plant's vascular system that contains xylem vessels and phloem elements. Xylem vessels transport water, minerals and other nutrients absorbed by the roots to the photosynthetic organs. Paradoxically, most vascular pathogens colonize the relatively nutrient-poor xylem tissues while only few pathogen species occur in the carbohydrate-rich phloem. This can most likely be explained by the accessibility of these tissue types, as the phloem is characterized by living cells with a high osmotic pressure which makes penetration difficult, while the xylem is characterized as "dead" tissue with relatively low

osmotic pressure. While phloem pathogens only comprise rickettsias, spiroplasmas and phytoplasmas that are introduced by vectors, such as phloem feeding insects, or by cultural practices like grafting, xylem-invading vascular pathogens comprise bacterial, fungal, and oomycete microorganisms (Agrios, 2005). The xylem-invading pathogens cause vascular wilt diseases.

Vascular wilt pathogens are among the most destructive plant pathogens that can cause complete production losses. Vascular wilt diseases occur worldwide and affect annual crops as well as woody perennials, thus not only impacting world food and feed production but also natural ecosystems. Most of the symptoms caused by vascular wilt pathogens develop in acropetal direction: from bottom to top. Epinasty is the primary disease symptom, followed by flaccidity, chlorosis, vascular browning and necrosis of the terminal leaflets (Agrios, 2005). Depending on the pathogen species and the host, plants may become stunted, wilt partially or completely, and ultimately die. Plant death may occur within days to weeks or, in case of perennials, may take months to years (Purcell and Hopkins, 1996; Fradin and Thomma, 2006; Niño-Liu et al., 2006; Juzwik et al., 2008; Klosterman et al., 2009; Michielse and Rep, 2009; Genin, 2010; Janse and Obradovic, 2010; Harwood et al., 2011; Klosterman et al., 2011). Environmental conditions, virulence of the pathogen, and age and the nutritional status of the host plants all determine the speed and severity at which symptoms develop (Tjamos and Beckman, 1989; Hayward, 1991; Roncero et al., 2003; Niño-Liu et al., 2006; Chatterjee et al., 2008a). A large range of symptoms is caused by vascular wilt pathogens, and even the same pathogen may cause diverse symptoms on different host plants. In all cases where it is observed, wilting symptoms represent a transitory phase of the disease, and some vascular pathogens typically cause other symptoms. For instance, *Xylella fastidiosa* hardly causes wilting, but mainly causes scorching of leaf margins and shriveling of grape berries (Purcell and Hopkins, 1996; Chatterjee et al., 2008a; Chatterjee et al., 2008b).

Vascular wilt pathogens overwinter in the soil, in plant debris, in watercourses, or in insect vectors (Fradin and Thomma, 2006; Niño-Liu et al., 2006; Juzwik et al., 2008; Klosterman et al., 2009; Michielse and Rep, 2009; Genin, 2010; Janse and Obradovic, 2010; Klosterman et al., 2011; Nadarasah and Stavrinides, 2011). While most vascular wilt pathogens enter their hosts through the roots by penetration via wounds or cracks that appear at the sites of lateral root formation (Vicente et al., 2001; Di Pietro et al., 2003; Fradin and Thomma, 2006; Klosterman et al., 2009; Michielse and Rep, 2009; Genin, 2010), some of them enter via natural openings on leaves such as stomata and hydathodes (Niño-Liu et al., 2006), while some others are directly delivered to the xylem by insect vectors which feed on xylem sap or reach it while chewing young tissues like the bark beetles transmitting conidia of *Ophiostoma*

ulmi, the causal agent of Dutch elm disease (Purcell and Hopkins, 1996; Chatterjee et al., 2008a; Moser et al., 2010; Nadarasah and Stavrinides, 2011). Regardless of the mechanisms used by different vascular wilt pathogens to enter their hosts, they ultimately all reach the xylem vessels and colonize them. Once inside the xylem, vascular wilt pathogens will produce mycelia and quickly produce (micro) conidia for transport (fungi) or multiply by fission (bacteria) rapidly and systemically spread via the xylem vessels to the upper part of the plant, while provoking the characteristic wilting symptoms (Tjamos and Beckman, 1989; Purcell and Hopkins, 1996; Agrios, 2005; Niño-Liu et al., 2006; Klosterman et al., 2009; Genin, 2010).

Controlling vascular wilt pathogens is very difficult, as they are able to survive over long periods of time outside their host plants. Moreover, some of them can infect a broad range of host plants and no efficient treatments exist to cure infected plants. These typical characteristics of vascular wilt pathogens make the use of cultural and chemical disease management ineffective. Soil solarization and fumigation strategies are among the options which can be employed to control vascular wilt diseases. However, limitations in large-scale applicability and the impact of chemical fumigants on public health and the environment are some of the drawbacks of these control practices. Biological agents and organic soil amendments are also used to control vascular wilt diseases (Tsuda et al., 2001; Spadaro and Gullino, 2005; Suárez-Estrella et al., 2007; Ji et al., 2008; Markakis et al., 2008). For instance, injection of the Dutch trig, which contains conidia of the *V. albo-atrum* isolate WCS850 in sterile water, into elm trees has been used to prevent *Ophiostoma ulmi* infection (Scheffer et al., 2008). However, since biological agents are affected by biotic and abiotic factors, performance of biocontrol microorganisms in the field is often inconsistent (Tsuda et al., 2001). The most effective management strategy thus far is the use of genetic resistance in crop plants. Moreover, genetic resistance is also user-friendly, relatively cheap and has no negative impact on public health and the environment.

Traditionally, most research efforts in molecular phytopathology are targeted against foliar pathogens, while vascular pathogens have been understudied. This may be due the fact that vascular wilt pathogens live deep in the heart of their host plants, making studies to investigate their biology relatively difficult. However, their high economic impact, combined with the absence of curative treatments, justifies that they receive more attention. The recent availability of a number of genome sequences of vascular pathogens has inspired novel research efforts to unravel the molecular basis of vascular wilt diseases. To design novel strategies to combat vascular wilt diseases, understanding the (molecular) biology of vascular pathogens and the molecular mechanisms underlying plant defence against these pathogens are

crucial. In this chapter we will summarize and discuss the current knowledge about interactions of vascular wilt pathogens with their host plants, with emphasis on the plant defence responses against this specific group of pathogens.

1. Vascular wilt pathogens

1.1. Fungal vascular wilt diseases

There are four fungal genera containing vascular wilt pathogens: *Ceratocystis* (causing vascular wilts of oak, cocoa, and eucalyptus), *Ophiostoma* (pathogen of elm tree), *Verticillium* (broad host range pathogen) and *Fusarium* (broad host range pathogen) (Tjamos and Beckman, 1989; Agrios, 2005; Juzwik et al., 2008; Schumann and D'Arcy, 2010; Harwood et al., 2011; López-Escudero and Mercado-Blanco, 2011). In contrast to the other three genera, the vast majority of *Fusarium* vascular wilt pathogens all belong to a single species, *F. oxysporum*, which contains morphologically indistinguishable pathogenic as well as non-pathogenic strains (Lievens et al., 2008), of which the pathogenic *F. oxysporum* strains can cause vascular wilt or root rot in over 100 different host species (Di Pietro et al., 2003; Roncero et al., 2003; Michielse and Rep, 2009). Despite the broad host range of these species, individual strains usually infect only a single or a few hosts, and therefore pathogenic strains have been assigned to *formae speciales*. Based on host specificity, currently over 120 *formae speciales* have been described (Michielse and Rep, 2009). Interestingly, Ma and colleagues experimentally demonstrated that the transfer of two lineage specific (LS) chromosomes between strains of *F. oxysporum* converts a non-pathogenic strain into a pathogen, suggesting that host specificity is determined by mobile pathogenicity chromosomes (Ma et al., 2010).

Fungal vascular wilt pathogens overwinter in soil or on dead host tissues in the form of persistent resting structures. These include microsclerotia, chlamydospores, thick-walled mycelium and spore-bearing coremia which all can survive for an extended period of time without losing viability. Compounds released from host plants, often referred to as exudates, are the trigger for germination of the dormant fungal resting structures and subsequent host infection. Except *Ophiostoma* species and *Ceratocystis fagacearum*, which are transmitted by elm bark beetles and the family of *Nitidulidae* beetles, respectively (Hayslett et al., 2008; Juzwik et al., 2008; Harwood et al., 2011), all fungal vascular wilt pathogens penetrate their host plants through the roots. Following penetration, the fungi colonize the cortical cells and hyphae migrate intercellularly towards the vascular parenchyma cells and finally invade the xylem vessels (Di Pietro et al., 2003; Klosterman et al., 2009; Schumann

and D'Arcy, 2010; Nadarasah and Stavrinides, 2011). Once in the xylem, conidia are produced which are disseminated acropetally with xylem sap movement. In living tissues, the fungal vascular wilt pathogens are restricted to the xylem vessels, but once tissues become necrotized they colonize other tissues and produce resting structures, which eventually are released into the soil (Di Pietro et al., 2003; Agrios, 2005; Fradin and Thomma, 2006).

1.2. Bacterial vascular wilt diseases

There are seven bacterial genera that contain vascular wilt pathogens: *Clavibacter* (causing ring rot of potato and bacterial canker and wilt of tomato), *Curtobacterium* (causing bacterial wilt of beans), *Erwinia* (bacterial wilt of cucurbits), *Pantoea* (stewart's wilt of corn), *Ralstonia* (southern bacterial wilt of Solanaceous crops and Moko disease of banana), *Xanthomonas* (black rot of crucifers, bacterial blight of rice), and *Xylella* (Pierce's disease of grape, citrus variegation chlorosis) (Tjamos and Beckman, 1989; Agrios, 2005; Chatterjee et al., 2008a; Schumann and D'Arcy, 2010; Nadarasah and Stavrinides, 2011; Roper, 2011). Unlike the fungal vascular wilt pathogens, bacterial wilt pathogens do not produce special resting structures. Bacterial vascular wilt pathogens overwinter in plant debris in soil, in seeds, in vegetative propagules, or in their insect vectors as dormant cells (Agrios, 2005). Bacterial wilt pathogens enter host tissues only passively, via wounds, cracks or natural openings such as stomata and hydathodes, while some of them, such as *Xylella fastidiosa* (sharpshooter leafhoppers and spittlebugs), *Pantoea stewartii* (corn flea beetles) and *Erwinia tracheiphila* (cucumber beetles) are directly delivered into the xylem by insect vectors (Schumann and D'Arcy, 2010; Nadarasah and Stavrinides, 2011; Roper, 2011). After entrance of their host plants, they rapidly multiply and invade the root cortex and vascular parenchyma cells intercellularly. From there they spread to the xylem vessels which they use as avenues for passive spread to aerial plant parts. During host colonization, bacterial wilt pathogens degrade xylem cell wall components, parenchyma cells and pit membranes, resulting in early rupture of host cells forming cavities filled with slimy masses of bacteria and cellular debris (Agrios, 2005; Schumann and D'Arcy, 2010). Occasionally, bacteria can be seen oozing out from the cracks on the stems and hydathodes of leaves of infected plants (Agrios, 2005).

1.3. Oomycete vascular wilt diseases

Only one Oomycete genus, *Pythium*, contains vascular wilt pathogens. *Pythium* mainly infects seeds or seedlings in the soil causing pre-emergence or post-emergence seedling damping-off disease (Martin and Loper, 1999). It also infects roots, the hypocotyl of seedlings, lower stems, and overall young and juvenile plant tissues (Oliver et al., 2009). The genus *Pythium* comprises many complex species, most of which are plant pathogens while others are saprophytes, or animal parasites (Martin and Loper, 1999). *Pythium* species survive in soil for long periods of time in absence of a suitable host or in organic substrates as dormant oospores (Martin and Loper, 1999). Oospores are thick-walled sexual spores which are capable of withstanding harsh environmental conditions like desiccation (Martin and Loper, 1999). Oospores germinate upon stimulation by exudates released from seeds, roots or plant debris and initiate infection. They often produce a sporangium full of zoospores that are released and encyst after contact with the host (indirect penetration); they can also produce a germinating hyphae to directly penetrate the root epidermis and migrate through the cortex, endodermis and parenchyma cells, and eventually invade the vascular stele causing the typical damping-off symptoms (Rey et al., 1998).

1.4. Genome sequences of vascular wilt pathogens

To design efficient control strategies against devastating vascular wilt diseases, understanding the genetic basis of pathogenicity, biology, as well as the evolution of the pathogens is crucial. Whole genome sequencing of these pathogens can provide much useful information on the biology of the pathogens. Currently, whole genome sequences of various vascular wilt pathogens are available. These include *V. dahliae* and *V. albo-atrum* (Klosterman et al., 2011), *F. oxysporum* (Ma et al., 2010), *R. solanacearum* (Salanoubat et al., 2002), *X. oryzae* pv. *oryzae* (Lee et al., 2005), *X. campestris* pv. *campestris* (Qian et al., 2005), *Xylella fastidiosa* (Simpson et al., 2000), *Clavibacter michiganensis* ssp. *michiganensis* (Gatermann et al., 2008), and various strains of *Erwinia amylovora* (Sebaihia et al., 2010; Smits et al., 2010; Powney et al., 2011). The availability of whole genome sequences facilitates the identification of genes that may have roles in pathogenicity, as well as in nutrition and adaption of the pathogens to the host.

2. Xylem structure

The xylem consists of distinct cells with special wall structures that allow efficient transport of water and solutes from the roots to the upper parts of the plant. The xylem functions not only for long distance transport, but also provides physical strength to the plant. Xylem development occurs in two phases, during which the primary and secondary xylem is produced (Fukuda, 1997; Ye, 2002; Fukuda, 2004; Zhang et al., 2011). Primary development involves the formation of primary xylem from procambium cells, which are derived from the apical meristem. Procambium cells give rise to xylem precursor cells that eventually differentiate into treachery elements, xylem parenchyma cells or fiber cells, which are collectively called the xylem (Ye, 2002; Fukuda, 2004). Treachery elements, which consist of tracheid and vessel elements, are the main conductive tissues. While the xylem parenchyma cells are metabolically active and adapted for storage and transport, the xylem fiber cells together with treachery elements provide physical support (Nieminen et al., 2004). Following xylem differentiation, the treachery elements undergo cell elongation before the initiation of secondary xylem wall development (Ye, 2002; Fukuda, 2004; Nieminen et al., 2004; Zhang et al., 2011). The secondary xylem walls, which are derived from vascular cambium, are deposited onto the primary xylem walls (Fukuda, 1997; De Boer and Volkov, 2003). Secondary xylem is made of cellulose microfibrils, crystalline aggregates of linear polymers of D-glucopyranosyl residues linked in β-(1-4) conformation (Brett, 2000; Emons and Mulder, 2000). The secondary xylem walls are further impregnated with different polysaccharides, such as lignin, hemicellulose, pectin and structural proteins, that add strength and rigidity to the wall (Ye, 2002; Fukuda, 2004; Yokoyama and Nishitani, 2006). Subsequently, the secondary xylem walls are lignified, cross-linked, and eventually waterproofed by polymerization to the aromatic compound monolignol (Fukuda, 1996; De Boer and Volkov, 2003). The patterned secondary xylem walls provide physical strength to the treachery elements to withstand the high negative pressure generated during transpiration and also from the compressive pressure from surrounding cells (Ye, 2002; Nieminen et al., 2004; Choat and Pittermann, 2009; Zhang et al., 2011).

The final step of xylem development is the induction of programmed cell death (PCD) that destroys the cellular contents of treachery elements, leaving behind hollow tube-like vessels through which water and nutrients flow (Fukuda, 1997; Zhang et al., 2011). The PCD is developmentally regulated and is strongly associated with secondary xylem wall formation (Fukuda, 2004). The vessel tubes are dedicated to the unrestricted water and solute movement throughout the plant and individual vessels are interconnected through small openings called pits (De

Boer and Volkov, 2003; Choat and Pittermann, 2009). Pits between vessels typically have overarching secondary walls that form a bowl-shaped chamber, referred to as a border pit (De Boer and Volkov, 2003; Jansen et al., 2004). Border pit exists in pairs and contain a pit membrane at the center, which is formed from primary walls and the intervening middle lamella (De Boer and Volkov, 2003). The pit membrane is made of cellulose microfibrils embedded in polysaccharide matrix of hemicellulose and pectin (Tyree and Zimmermann, 2002; Pérez-Donoso et al., 2010). This fine mesh-like and tightly interlocked polysaccharide structure has minute openings through which water and solutes can move with a minimal resistance between vessels or to neighboring parenchyma cells (Choat and Pittermann, 2009). In angiosperm trees, the pit pore diameter varies between 5 and 20 nm (Choat et al., 2003; Choat et al., 2004), thus acting as a safety mechanism to limiting the spread of embolism within xylem vessels (Tyree and Zimmermann, 2002; De Boer and Volkov, 2003; Choat et al., 2008; Pérez-Donoso et al., 2010).

Because xylem vessels are the home to vascular wilt pathogens to proliferate and spread, their structure and composition determines the success or failure of the pathogens. All vascular wilt pathogens have to breach the highly structured and rigid secondary xylem walls to get access to the xylem vessels. Obviously, the aperture size of pit membranes also determines the fate of vascular pathogens after they have accessed the xylem. *X. fastidiosa*, a rod-shaped bacterium, has a cell size of 0.25 to 0.5 µm in diameter (Mollenhauer and Hopkins, 1974), while the conidia of *Verticillium* species have a diameter of about 2.2 µm (Qin et al., 2008), indicating that vascular wilt pathogens are too large to pass pit membrane pores (Choat et al., 2003; Choat et al., 2004). In general, the structure and composition of the xylem have a significant impact on vascular wilt pathogens.

3. The xylem as an ecological niche for vascular wilt pathogens

As xylem is a conduit for water and inorganic solutes, it is considered as a nutritionally poor environment. This could be one of the reasons why only a limited number of plant pathogens are able to thrive in this environment. Vascular wilt pathogens reside in the xylem for the major part of their lifecycle, implying that they need to obtain all factors that are necessary for their growth, reproduction and survival from the xylem tissue. Possibly, vascular wilt pathogens evolved to occupy xylem vessels as a niche to avoid competition with other microbes, including endophytes (McCully, 2001).

3.1. Nutrient composition of xylem sap

Several studies have shown that the xylem sap contains various organic and inorganic compounds. While nitrate, sulfate, and phosphate are among the most abundant inorganic anions in the xylem sap, calcium, potassium, magnesium, and manganese are the most predominate inorganic cations present in the xylem sap of oilseed rape (Nakamura et al., 2008). Xylem sap also contains various carbohydrates, such as glucose, fructose, saccharose, maltose, raffinose, trehalose and ribose (Alvarez et al., 2008; Nakamura et al., 2008; Fernandez-Garcia et al., 2011; Krishnan et al., 2011). Of these, glucose, fructose and saccharose are predominant and are utilized as a carbon source for growth. Xylem sap furthermore contains various proteins, amino acids, and organic acids, which can also act as a source of organic and inorganic nutrients (Alvarez et al., 2008; Nakamura et al., 2008; Fernandez-Garcia et al., 2011; Krishnan et al., 2011). For instance, the sulphur-containing amino acids methionine and cysteine can be used as a source of inorganic sulphur (Divon and Fluhr, 2007; Krishnan et al., 2011). Nevertheless, the quantities of the organic and inorganic compounds in the xylem sap are extremely low and also fluctuate with day time, growth condition and plant species (Siebrecht et al., 2003). Moreover, several studies have shown that the concentrations of various compounds present in the xylem sap change in response to biotic and abiotic stress (Rep et al., 2002; Zhang et al., 2002; Houterman et al., 2007; Alvarez et al., 2008; Basha et al., 2010; Fernandez-Garcia et al., 2011). Therefore, vascular wilt pathogens must have evolved an efficient and sophisticated nutrient acquisition system to cope with the limited availability of nutrients in the xylem tissue.

3.2. Nutrient acquisition by vascular wilt pathogens

Vascular wilt pathogens satisfy their nutrient requirements by either efficiently acquiring the scarce nutrients available in xylem sap, enzymatic digestion of host cell walls, or by manipulating xylem tissue or invading neighboring cells leading to nutrient leakage (Divon et al., 2005; Möbius and Hertweck, 2009; Klosterman et al., 2011). Nitrogen is one of the limiting nutrients in the xylem sap for vascular wilt pathogens (Divon et al., 2005). For instance, ammonia, glutamine, and glutamate are the preferred primary nitrogen sources for fungal pathogens (Marzluf, 1997; Divon et al., 2006) and in the xylem sap, these nitrogen sources are absent or present in extremely low concentrations. In the absence of the primary nitrogen sources, vascular wilt pathogens can utilize secondary nitrogen sources such as nitrate, nitrite, purines, amides, amino acids, and proteins (Marzluf, 1997; Divon et al., 2005; Divon

et al., 2006). Divon and colleagues have cloned the global nitrogen regulator (*FNR1*), a GATA transcription factor (GATA TF) family protein from *Fusarium oxysporum* f.sp. *lycopersici* (Divon et al., 2006). GATA TF family proteins are known to regulate utilization of secondary nitrogen sources which otherwise are under nitrogen catabolite repression in various microorganisms (Marzluf, 1997; Divon and Fluhr, 2007; Bolton and Thomma, 2008; Donofrio et al., 2009). *FNR1* mutants failed to utilize secondary nitrogen sources such as amino acids, hypoxanthine, and uric acid, while the mutants grow normally on primary nitrogen sources such as ammonia and glutamine (Divon et al., 2006). In addition, disruption of *FNR1* abolishes expression of secondary nitrogen acquisition genes *Gap1*, *Mtd1*, and *uricase* (Divon et al., 2006), showing that *FNR1* of *Fusarium oxysporum* f.sp. *lycopersici* regulates the utilization of secondary nitrogen sources during xylem colonization.

Another possibility for vascular wilt pathogens to obtain nitrogen and carbon during host invasion is by enzymatic digestion of xylem walls or pit membranes. Various studies have shown that vascular wilt pathogens produce an arsenal of enzymes that are capable of degrading xylem walls or pit membranes, so-called cell wall-degrading enzymes (CWDEs) (Di Pietro et al., 2003; Jha et al., 2005; Sun et al., 2005; Fradin and Thomma, 2006; Michielse and Rep, 2009; Klosterman et al., 2011). Some of the CWDEs secreted by vascular wilt pathogens are polygalacturonases, pectin methyl esterases, exo-cellobiohydrolases, cellulases/endoglucanases, xylanases, α-amylases, cellobiosidases, and pectatelyases (Jha et al., 2005; Chatterjee et al., 2008a; Michielse and Rep, 2009; Klosterman et al., 2011). These enzymes are known to cleave different components from the xylem walls and/or pit membranes, releasing carbon and nitrogen sources that can be utilized by vascular wilt pathogens.

Vascular wilt pathogens such as *Verticillium*, *Fusarium*, and *Ophiostoma* are reported to produce high- and low-molecular weight phytotoxins during host colonization (Temple and Horgen, 2000; Wang et al., 2004; Palmer et al., 2005; Stipanovic et al., 2011). In interactions of vascular wilt pathogens with their hosts, phytotoxins are often associated with wilt symptom development (Wang et al., 2004; Palmer et al., 2005). In addition, phytotoxins also disturb plant cell membrane integrity (Möbius and Hertweck, 2009), resulting in leakage of nutrients from cells surrounding the xylem vessels that can be utilized by vascular wilt pathogens.

Although most vascular wilt pathogens are limited to xylem vessels, some fungal and bacterial vascular wilt pathogens are capable of degrading the xylem vessel walls and colonize the adjacent parenchyma cells (Agrios, 2005). Thus, these pathogens can also obtain nutrients form parenchyma cells or attract nutrients from the adjacent cells to satisfy their nutrient requirements. Altogether, vascular wilt pathogens employ different nutrient acquisition strategies, such as efficient

regulation of nutrient acquisition genes, production of cell wall degrading enzymes and phytotoxins that can cause the release of nutrients from the host cell wall constituents and attracting nutrients from the neighboring tissues.

4. Plant defence against vascular wilt pathogens

Plants deploy two types of defences against invading pathogens: pre-existing and inducible plant defence responses. The pre-existing defences are constitutive and provide physical and chemical barriers against attempted host penetration. Once successful pathogens breach pre-existing defences, they encounter inducible defence responses that are generally divided into MTI (MAMP-triggered immunity) and ETI (effector-triggered immunity) (Jones and Dangl, 2006; Dodds and Rathjen, 2010) While MTI is activated upon recognition of conserved microbe-associated molecular patterns (MAMPs), ETI is activated upon recognition of secreted effector proteins. Nevertheless, ample examples suggest that the delineation between MAMPs and effectors, as well as between MTI and ETI, is blurred and they rather represent a continuum (Thomma et al., 2011).

4.1. Perception of vascular wilt pathogens

In general, plants sense invading pathogens by using two types of receptors: extra- and intracellular receptors. While extracellular receptors recognize pathogen molecules on the cell-surface, intracellular receptors recognize pathogen molecules that are delivered inside host cells. This extra-and intracellular receptor-mediated recognition of pathogen molecules (MAMPs, effectors) lead to the activation of plant innate immunity that wards off invading pathogens. Consequently, failure of a host plant to perceive invading pathogens leads to susceptibility and successful infections by pathogens occurs.

4.1.1. Extracellular plant receptors

Upon MAMP recognition, extracellular plant receptors activate MTI. Arabidopsis FLS2, EFR, CERK1, and the rice CEBiP are well-characterized extracellular plant receptors that recognize MAMPs. FLS2 and EFR encode receptor-like kinases (RLKs) that recognize the bacterial MAMPs flg22 and EF-Tu, respectively (Gómez-Gómez et al., 2001; Kunze et al., 2004; Zipfel et al., 2006). CERK1 and CEBiP encode plasma membrane proteins containing three and two LysM domains, respectively (Kaku et al., 2006; Miya et al., 2007; Kishimoto et al., 2010). Both CERK1 and

CEBiP recognize chitin, the main constituent of the fungal cell wall (Miya et al., 2007; Kishimoto et al., 2010). Plant extracellular receptors recognize not only MAMPs but also effectors and trigger ETI. The tomato Cf resistance proteins (Cf for *Cladosporium fulvum*) which include Cf-2, Cf-4, Cf-4E and Cf-9, are examples of this group of plant receptors. Cf-2, Cf-4, Cf-4E, and Cf-9 are receptor-like proteins (RLP) which recognize effector protein Avr2, Avr4, Avr4E, and Avr9, receptively, of *Cladosporium fulvum*, a fungus that causes tomato leaf mold (Thomma et al., 2005; De Wit et al., 2009). Cf-mediated recognition of *C. fulvum* Avrs leads to a hypersensitive response (HR), a type of programmed cell death at and immediately surrounding pathogen infection sites (Agrios, 2005), that stops the fungus from further invasion.

Extracellular plant receptors play also a role in plant defence against vascular wilt pathogens. Rice *Xa21*, that confers resistance against *Xanthomonas oryzae* p.v. *oryzae* (*Xoo*) (Song et al., 1995), is an example of extracellular plant receptor that is involved in xylem defence. *Xa21* recognizes *Ax21* (activator of Ax21), a typeI-secreted sulfated protein (Song et al., 1995; Lee et al., 2009). Similar to *FLS2* and *EFR*, *Xa21* encodes a receptor-like kinase (Song et al., 1995; Park et al., 2010). Xa21 physically interacts with XB24 (Xa21 binding protein 24) (Chen et al., 2010b). *XB24* encodes a protein that contains a C-terminal ATP synthase (ATPase) motif (Chen et al., 2010b). XB24 promotes autophosphorylation of Ser/Thr residues on Xa21 through its ATPase activity, keeping Xa21 in an inactive state (Chen et al., 2010b; Chen and Ronald, 2011). Upon Xa21-mediated Ax21 recognition, the Xa21 kinase becomes activated, triggering rice defence responses (Chen et al., 2010b; Park et al., 2010).

The tomato *Ve1* is another example of extracellular plant receptor that plays a role in xylem defence. *Ve1*, which encodes an extracellular LRR receptor-like protein (RLP) (Kawchuk et al., 2001; Wang et al., 2008), provides resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum* in tomato (Fradin et al., 2009; Fradin et al., 2011). Interestingly, Fradin and colleagues have recently shown that interfamily transfer of *Ve1* gene to Arabidopsis confers resistance against race 1 isolates of *V. dahliae* and *V. albo-atrum* (Fradin et al., 2011).

4.1.2. Intracellular plant receptors

Plants have also evolved intracellular receptors that enable them to recognize pathogen effectors in the cytoplasm. To date, several intracellular plant receptors have been identified and some of them are functionally characterized. Most of the cloned intracellular plant receptors contain a central nucleotide binding site

(NBS) and C-terminal LRR domain. A genome-wide search for NBS-LRR genes in Arabidopsis, rice, and popular (*Populus trichocarpa*) identified 149, 480, and 400 genes, respectively (Meyers et al., 2003; Zhou et al., 2004; Kohler et al., 2008). NBS-LRR receptor proteins are further subdivided into CC-NBS-LRR (contains coiled-coil domain) and TIR-NBS-LRR (contains Toll-interleukin-1 receptor (TIR) domain) receptors based on their N-terminal domain. The majority of the currently characterized intracellular plant receptors are involved in defence against foliar pathogens (Martin et al., 2003; Liu and Coaker, 2008; Chen and Ronald, 2011). Intracellular plant receptors have also shown to mediate plant defence against xylem-invading pathogens. The tomato *I-2* gene is one of the intracellular plant receptors that contribute to xylem-based resistance. I-2 confers resistance to race 2 isolates of *F*. *oxysporum* f.sp. *lycopersici* (Huang and Lindhout, 1997; Takken and Rep, 2010). It encodes a cytoplasmic CC-NBS-LRR receptor protein that recognizes the effector protein Avr2, which was initially identified from the xylem sap of tomato infected by *F*. *oxysporum* f.sp. *lycopersici* and is taken up by tomato cells (Houterman et al., 2007; Houterman et al., 2009; Takken and Rep, 2010).

The Arabidopsis RRS1-R resistance protein is an intracellular plant receptor that confers resistance against *R. solanacearum*. RRS1-R encodes a TIR-NBS-LRR R-protein and contains a C-terminal nuclear localization signal (NLS) and a WRKY domain (Deslandes et al., 2002). It recognizes the *R. solanacearum* type three-secreted effector protein PopP2 (Deslandes et al., 2003). RRS1-R physically interacts with the effector PopP2 and this binding determines the nuclear localization of the RRS1-R (Deslandes et al., 2003). RRS1-R requires RD19, a cysteine protease that also binds to PopP2 (Deslandes et al., 2003; Bernoux et al., 2008). RD19 is localized in the vacuole in absence of PopP2 and re-localizes to the nucleus in the presence of PopP2 (Deslandes et al., 2003; Bernoux et al., 2008). However, no direct interaction between RRS1-R and RD19 has been reported so far. Thus, the current notion is that RRS1-R potentially recognizes the RD19-PopP2 complex in the nucleus and activates the Arabidopsis ETI against *R. solanacearum*.

4.2. Plant defence responses in the xylem vessel

Recognition of vascular wilt pathogens mediated by either extracellular or intracellular receptors leads to the activation of defence responses in the xylem vessels. These comprise physical defence responses which physically halt or contain the pathogen from further spread in the xylem vessels, and chemical defence responses that kill the pathogen or inhibit its growth.

4.2.1. Physical defence responses

A common defence mechanism in xylem vessels against vascular wilt pathogens is the formation of tyloses (Beckman, 1964; Talboys, 1972; Rahman et al., 1999; Fradin and Thomma, 2006). Tyloses are outgrowths of vessel-associated parenchyma cells which protrude into the xylem vessel through pits and block the spread of pathogens (Beckman, 1964; Talboys, 1972; Grimault et al., 1994; Agrios, 2005).They are formed during both compatible and incompatible interactions between the host and vascular wilt pathogens, although the time and extent of tylose formation significantly differs. Tyloses form much faster and more extensively in resistant plants when compared to susceptible plants (Grimault et al., 1994; Fradin and Thomma, 2006).

Often, the generation of tyloses is associated with the production of gels and gums around the differentiated tylose (Clérivet et al., 2000). Using immuno-gold labeling, Clerivent and colleagues observed strong accumulation of pectin-rich materials around the parenchyma cells, pit membrane, and the newly emerging tylose in the xylem vessels of *Platanus acerifolia* cultivar infected by *Ceratocystis fimbriata* f. sp *platani* (Clérivet et al., 2000). Plants potentially accumulate these pectin-rich gels and gums around tyloses to completely seal off a xylem vessel to prevent the vascular wilt pathogen to spread to adjacent healthy xylem vessels (Rahman et al., 1999). However, complete sealing of xylem vessels can be disadvantageous for the plant as well. If tylose formation affects too many vessels and no new vessels are formed, tylose formation can result in drought stress (Fradin and Thomma, 2006).

Another physical defence response observed during xylem colonization is vascular coating. A quick vascular wall coating around the initially infected and the adjacent xylem vessels, infusing the pit membrane and primary walls was observed in resistant chili pepper inoculated with *R. solanacearum*, whereas the xylem wall coating was not observed in susceptible chili pepper (Rahman et al., 1999). Similar coating of xylem parenchyma cells induced by *V. albo-atrum* was reported in tomato (Street et al., 1986) and alfalfa (Newcombe and Robb, 1988), indicating that infusion of pit membranes, primary walls and parenchyma cells with coating materials could prevent lateral and vertical spreading of vascular wilt pathogens in the xylem vessels. Furthermore, callose deposition and swelling of the primary walls of the xylem vessels was reported during the interaction of *R. solanacearum* with chili pepper (Rahman et al., 1999). Previously, a similar deposition of callose in resistant and susceptible tomato infected with *F. oxysporum* f. sp. *lycoperscici* was reported (Beckman et al., 1982). However, the resistant cultivar maintains a stronger level of callose deposition during the course of the infection than the susceptible (Beckman et al., 1982). This high level deposition of callose in the resistant cultivar around the

initially infected cells could inhibit the pathogens from further spreading.

Xylem colonization by *Xanthomonas campestris* pv. *campestris* has also been reported to activate vascular immunity that triggers an HR, referred to as vascular HR (VHR) (Xu et al., 2008). Vascular immunity was proposed based on the fact that $AvrAC_{Xcc8004}$, a type three effector protein of *Xanthomonas campestris* pv. *campestris* that confers avirulence in Arabidopsis ecotype Col-0, provides resistance when exclusively targeted to the vascular system (Xu et al., 2008). Infiltration of $AvrAC_{Xcc8004}$ into leaf mesophyll tissue of Col-0 did not trigger resistance against *Xanthomonas*, implying that $AvrAC_{Xcc8004-}$ mediated activation of Arabidopsis defence response (vascular immunity) occurs in xylem vessel. Castañeda et al. (2005) also previously reported that the *X. campestris* pv. *campestris* effector protein AvrXccFM elicits VHR on Florida mustard seedlings. It is, however, important to note that unlike the HR occurring in leaf mesophyll cells, VHR is very difficult to score (Castañeda et al., 2005; Xu et al., 2008).

4.2.2.Chemical defence responses

Xylem infection causes drastic metabolic changes in xylem parenchyma cells, which are located adjacent to the infected vessels. These metabolic changes lead to the accumulation of different proteins and secondary metabolites in the xylem sap. Some of the proteins and secondary metabolites that accumulate in the xylem sap during xylem colonization include PR-1, PR-2, PR-3, PR-4, PR-5, peroxidases, proteases, xyloglucan-endotransglycosylase (XET), and xyloglucan-specific endoglucanase inhibitor protein (XEGIP), phenols, phytoalexins, and lignin-like compounds (Cooper et al., 1996; Hilaire et al., 2001; Rep et al., 2002; Williams et al., 2002; Rep et al., 2003; Houterman et al., 2007; Basha et al., 2010; Gayoso et al., 2010). These compounds are known to contribute directly or indirectly to plant defence. The PR-1, PR-2, PR-3, and PR-5 were also among the proteins abundantly accumulated in xylem sap during compatible interaction between *Fusarium* and tomato (Rep et al., 2002; Houterman et al., 2007). For instance, PR-2 (β-1, 3-glucanase) and PR-3 (chitinase) hydrolyze the fungal cell wall component β-1,3-glucan and chitin, respectively (Leubner-Metzger and Meins, 1999; van Loon et al., 2006). In addition, antimicrobial activity of PR-5 proteins has also been demonstrated towards multiple pathogens (van Loon et al., 2006), implying that the presence of these proteins in xylem sap could inhibit or slow down the growth of the fungal vascular wilt pathogens in the xylem vessels, but likely it is the speed of accumulation that differs between resistant and susceptible cultivars .

Peroxidases are among the abundantly accumulated enzymes in xylem sap during host colonization of vascular wilt pathogens. The cationic peroxidase, PO-

C1, accumulates in the cytoplasm, the primary and secondary walls of the xylem parenchyma, and lumen cells during incompatible interactions between *X. oryzae* pv. *oryzae* and rice (Hilaire et al., 2001). Peroxidases are heme-containing enzymes that catalyze the oxidation of different substrates using hydrogen peroxides as an electron acceptor (Gayoso et al., 2010). Peroxidases are known to be involved in the production of reactive oxygen species through their enzymatic activity and reactive oxygen species are toxic compounds that can eliminate vascular wilt pathogens. Furthermore, peroxidases are implicated in the polymerization of cell wall compounds, lignin and suberin biosynthesis, and regulation of hydrogen peroxide levels, which all can contribute to defence (Hilaire et al., 2001; Passardi et al., 2005).

Phenolic compounds, which have toxic effects on microbes, are also involved in xylem defence. Plants accumulate different phenolic compounds in the xylem in response to infection. Olive trees accumulate phenols such as rutin, leuropein, luteolin-7-glucoside, and tyrosol at the site of *V. dahliae* infection that were shown to have a toxic effect on *V. dahliae* (Báidez et al., 2007). Interestingly, exogenously treating Dutch elm trees with phenolic compounds induces accumulation of suberin-like compounds in the xylem tissue and thereby increases resistance to *Ophiostoma novo-ulmi* (Martín et al., 2008). This indicates that, in addition to direct toxicity, phenolic compounds could also activate other defence responses against vascular wilt pathogens.

Plants employ not only complex organic phytoalexins as defence mechanism against vascular wilt pathogens, but also employ inorganic compounds such as elemental sulphur and sulphur-containing inorganic compounds (Williams et al., 2002; Cooper and Williams, 2004). During an incompatible interaction between *V. dahliae* and tomato elemental sulphur mainly accumulates in xylem parenchyma cells, xylem vessel walls and around the vascular occluding gels (Williams et al., 2002). Similar accumulation of elemental sulphur has been observed in an incompatible interaction between *V. dahliae* and cacao (*Theobromacacao*) or cotton (Cooper et al., 1996; Cooper and Williams, 2004). The accumulation of inorganic sulphur specifically in xylem vessel walls and around the vascular occluding gels might suggest its role in eliminating vascular wilt pathogens that are arrested by physical defence responses.

Overall, chemical defence responses play major roles in xylem defence. Some chemical compounds accumulated in xylem sap after infection modulate the morphology of xylem tissue and by doing so inhibit vertical and lateral colonization of the pathogens, whereas other compounds accumulate during xylem infection have antimicrobial activity and can eliminate vascular wilt pathogens contained by the physical defence responses.

5. Conclusion

Vascular wilt pathogens have adapted to thrive in the xylem, which is known as a nutrient-poor ecological niche, causing vascular wilt diseases on hundreds of plant species. Plants sense vascular wilt pathogens on their cell-surface by extracellular receptors and inside host cells by intracellular receptors. Recognition of vascular wilt pathogens by both extra-and intracellular plant receptors triggers plant innate immunity that, in turn, results in the activation of physical and chemical plant defence responses. Both those defence responses occur in the xylem vessels in a coordinated manner, where physical defence responses mainly prevent the pathogens from spreading in the xylem vessels and chemical defence responses kill the pathogen or inhibit its growth.

Currently, little is known about the molecular basis underlying the interaction between vascular wilt pathogens and their hosts. This could be due to the fact that interaction between vascular wilt pathogens and their hosts takes place in xylem vessels, which are located deep in the interior of the plant, making investigation more difficult when compared to the interaction between foliar pathogens and their hosts which takes place in leaves. Genetic resistance is the best strategy for controlling vascular wilt pathogens. To develop genetic resistance, however, a deeper understanding of the host defence mechanisms as well as the biology, evolution and pathogenicity of vascular wilt pathogens is required.

6. Objective and scope of the thesis

The objective of this PhD thesis research was to identify resistance factors against Verticillium wilt disease. Verticillium wilt disease is caused by three major *Verticillium* species: *V. dahliae*, *V. albo-atrum* and *V. longisporum*, which collectively infect more than 200 different plant species, including food crops and forest trees, threatening not only food and feed production but also natural ecosystems. In this thesis, we have used Arabidopsis and tomato as model hosts to search for resistance factors, and to study the molecular mechanisms underlying interactions between *Verticillium* and its hosts.

In **chapter 2,** the identification of four activation-tagged Arabidopsis mutants that displayed enhanced resistance to Verticillium wilt disease are described. The mutants were further characterized for their resistance to the necrotrophic fungal foliar pathogens *B. cinerea*, *P. cucumerina*, *A. brassicicola*, the bacterial foliar pathogen *P. syringae* pv. *tomato*, and the vascular wilt pathogens *R. solanacearum* and *F. oxysporum* f.sp. *raphani*. The gene responsible for the enhanced Verticillium

wilt resistance of one of the activation-tagged Arabidopsis mutants was cloned. This gene, *AHL19*, encodes AT-hook DNA binding protein that belongs to a large protein family. Functional analysis confirmed that over-expression of *AHL19* confers enhanced Verticillium wilt resistance.

In **Chapter 3,** the identification of the activation tag insertion site in a second mutant that displayed enhanced Verticillium wilt resistance is described. Over-expression of the gene *EVR1*, for *Enhanced Verticillium Resistance 1*, provides resistance to the fungal vascular wilt pathogens *V. dahliae* and *F. oxysporum* f.sp. *raphani* and also to the bacterial vascular wilt pathogen *R. solanacearum*. Furthermore, *AtEVR1* (*for Arabidopsis thaliana EVR1*) over-expression leads to enhanced resistance to drought stress. Over-expression of an *AtEVR1* homologue from *B. oleracea*, *BoEVR1*, in Arabidopsis also confers resistance to Verticillium wilt disease.

Currently, no resistance in tomato against race 2 isolates of *V. dahliae* and *V. albo-atrum* has been described. **Chapter 4** describes the identification of six wild tomato accessions that showed enhanced resistance to race 2 isolate of *V. dahliae*. The signalling that is responsible for race 2 resistance in the wild tomato accessions was compared with *Ve1*-mediated race 1 resistance signalling by virus-induced gene silencing of candidate genes. This study showed that the signalling cascade that is employed to activate race 1 resistance significantly differs from the cascade that activates race 2 resistance.

In **Chapter 5** all results obtained in this thesis are discussed and placed in a broader perspective including a discussion of recent data reported in literature on related pathosystems.

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Chapter 2

The *Arabidopsis thaliana* DNA binding protein AHL19 mediates Verticillium wilt resistance

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Molecular Plant-Microbe Interactions 24:1582-1591

Abstract

Verticillium spp. are destructive soil-borne fungal pathogens that cause vascular wilt diseases in a wide range of plant species. Verticillium wilts are particularly notorious, and genetic resistance in crop plants is the most favourable means of disease control. In a gain-of-function screen using an activation-tagged Arabidopsis mutant collection we identified four mutants, A1 to A4, which displayed enhanced resistance towards the vascular wilt species *V. dahliae*, *V. albo-atrum* and *V. longisporum*, but not to *F. oxysporum* f. sp. *raphani*. Further testing revealed that mutant A2 displayed enhanced *Ralstonia solanacearum* resistance, while mutants A1 and A3 were more susceptible towards *Pseudomonas syringae* pv. *tomato*. Identification of the activation tag insertion site in the A1 mutant revealed an insertion in close proximity to the gene encoding the AT-hook DNA binding protein AHL19, which was constitutively expressed in the mutant. *AHL19* knock-out alleles were found to display enhanced Verticillium susceptibility, while over-expression of *AHL19* resulted in enhanced Verticillium resistance, showing that AHL19 acts as a positive regulator of plant defence.

Introduction

Verticillium spp. belong to the most destructive soil-borne fungal pathogens and cause vascular wilt disease in a wide range of plant species including annual crops, vegetables, fruits, fiber crops and perennial woody plants (Fradin and Thomma, 2006; Klosterman et al., 2009; Klosterman et al., 2011). Verticillium wilt diseases cause huge economic losses in most, if not all, agro-ecological regions worldwide. While *V. dahliae* and *V. albo-atrum* are closely related, truly plant pathogenic and cover a wide host range of over 200 dicotyledonous plant species, *V. longisporum* causes disease on Brassicaceous plants only (Fradin and Thomma, 2006; Klosterman et al., 2011). Verticillium wilts are particularly notorious since control of these diseases is extremely difficult for a number of reasons: (1) the resting structures can survive in soil in absence of host plants for up to 15 years (Wilhelm, 1955), (2) soil fumigation is expensive and has harmful environmental effects (Rowe et al., 1987), (3) the fungi have broad host ranges which makes crop rotation ineffective, and (4) the pathogens cannot be reached by many fungicides once they have entered the vascular system of host plants, and only few fungicides exist to cure infected plants. Therefore, genetic resistance in crop plants is the most favourable means to control Verticillium wilt diseases (Fradin and Thomma, 2006).

Genetic resistance against Verticillium wilt diseases has been reported for several economically important crop species (Fradin and Thomma, 2006; Klosterman et al., 2009). However, so far the only locus that has been cloned and functionally characterized is the tomato *Ve* locus that contains the *Ve1* gene that provides tomato resistance against isolates of the species *V. dahliae* and *V. albo-atrum* that belong to race 1 (Kawchuk et al. 2001; Fradin et al. 2009; Fradin et al. 2011). However, *Verticillium* race 2 strains are able to overcome *Ve*-mediated resistance and have become a problem for commercial tomato production (Schaible et al., 1951), and presently no resistance against race 2 *Verticillium* isolates has been described. Single dominant Verticillium resistance genes have been identified in other plant species as well, including potato, cotton, sunflower and Arabidopsis, while polygenic Verticillium resistance has been reported for strawberry, potato, alfalfa, cotton, oilseed rape and tomato (Jiang *et al*., 2009, Simko et al. 2004, Fradin and Thomma, 2006, Rygulla et al. 2008). Over the recent years, Arabidopsis has increasingly been used as a model host for studying wilt diseases caused by *V. dahliae* (Veronese et al., 2003; Tjamos et al., 2005; Johansson et al., 2006; Fradin and Thomma, 2006; Ellendorff et al., 2009; Pantelides et al., 2010; Fradin et al., 2011).

In addition to screening germplasm of a plant species or its wild relatives for resistance (Schaible et al., 1951; Veronese et al., 2003), mutagenesis followed by screening for enhanced resistance with the pathogen of interest is a means to identify novel resistance traits. Several technologies have been developed, including EMS- and radiation-induced mutation, transposon and activation tagging, each with its own advantages. Activation tagging involves the random integration of promoter or enhancer sequences in a plant genome using either a T-DNA or a transposon, generally leading to enhanced expression of genes near the integration site and generating gain-of-function mutants (Weigel et al., 2000; Ayliffe and Pryor, 2007). Transposon-based activation tagging has been successfully used in various plant species to identify novel genes involved in various physiological processes (Ayliffe and Pryor, 2007), including pathogen defence (Xia et al., 2004; Grant et al., 2003; Aboul-Soud et al., 2009). To identify genes involved in Verticillium wilt resistance, we screened an Arabidopsis transposon-based activation-tagged mutant collection (Marsch-Martinez et al., 2002). Four mutants were identified that displayed enhanced resistance to *V. dahliae, V. albo-atrum* and *V. longisporum*. Subsequent analysis of one of the mutants has demonstrated that the specific activation of the gene encoding the AT-hook DNA binding protein AHL19 causes the enhanced resistance phenotype.

Results

Screening of an Arabidopsis activation-tagged mutant collection for enhanced Verticillium wilt resistance

In our research, *Arabidopsis thaliana* is used as host to investigate the biology of Verticillium wilt diseases (Ellendorff et al., 2009; Fradin et al., 2011). To identify Arabidopsis genes that can contribute to resistance against Verticillium wilt, a collection of 2000 stable transposon activation tag insertion lines in the Arabidopsis ecotype Ws (Marsch-Martinez et al., 2002) was screened for plants displaying enhanced resistance upon inoculation with *V. dahliae*. This resulted in the identification of four mutants, A1 to A4, which displayed fewer symptoms of Verticillium disease (chlorosis, stunting and wilting) when compared to other mutants in the collection and the corresponding wild type Ws (Figure 1A; Figure S1). At 14 days post inoculation (dpi), leaves of wild type plants showed clear wilting and chlorosis symptoms, while these symptoms were absent from leaves of the four mutants. By 21 dpi, wild type plants were severely stunted while the oldest rosette leaves displayed chlorosis and severe necrosis. Whereas A3 and A4 mutant plants showed only slight symptoms of disease, A1 and A2 mutant plants remained symptomless. With real-time PCR on genomic DNA as target for quantification (Ellendorff et al., 2009), significantly less *Verticillium* DNA (using the internal transcribed spacer region of the ribosomal DNA as a marker) normalized to the amount of host DNA (using the RuBisCo gene as a marker) was detected in the four mutants when compared to wild type plants at both time points (Figure 1B), confirming that the four mutants display enhanced *V. dahliae* resistance.

Similar as to *V. dahliae*, all mutants showed enhanced resistance to *V. albo-atrum* and to *V. longisporum* when compared to wild type plants, and real-time PCR quantification of fungal biomass using genomic DNA as target for quantification confirmed reduced colonization of the mutants when compared to wild type plants for both fungal species (Figure 1; Figure S1; Figure S2).

In addition to the absence of disease symptoms, the four mutants showed clear differences in rosette leaf morphology when compared to wild type plants. Whereas mutant A1 displayed enlarged leaves, delayed flowering and delayed senescence, mutant A2 displayed compact, rounded rosette leaves with short petioles, mutant A3 had curly leaves with lobbed leaf edges, and mutant A4 had small, thick rosette leaves when compared with wild type plants (Figure 1A).

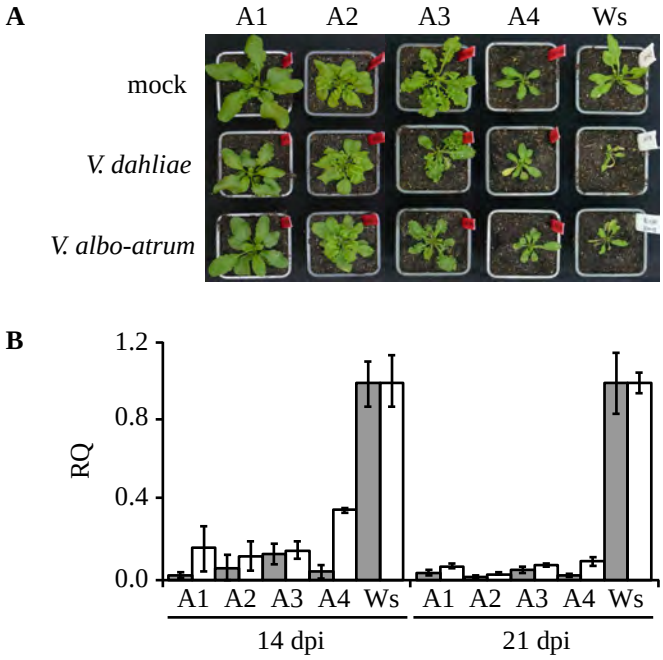

Figure 1. Activation-tagged Arabidopsis mutants are more resistant to *Verticillium* spp. (A) Typical *Verticillium* symptoms (stunting, wilting and chlorosis) on wild type (Ws) and activation-tagged mutants (A1-A4) at 21 days post inoculation (dpi). The upper, middle and lower rows show mock-, *V. dahliae*- and *V. albo-atrum*-inoculated plants, respectively. A representative of three independent experiments is shown. (B) Relative quantification (RQ) by real-time PCR on genomic DNA of *Verticillium* colonization by comparing levels of the *V. dahliae* (grey bars) and *V. albo-atrum* (white bars) internal transcribed spacer region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RuBisCo* gene (for equilibration) at 14 and 21 dpi. Bars represent averages with standard deviation of three independent biological replicates.

Response of Verticillium resistant mutants to other vascular wilt pathogens

We subsequently studied whether the enhanced resistance of the mutants is specific to *Verticillium* spp. or extends to other vascular pathogens as well. However, none of the mutants showed enhanced resistance to *Fusarium oxysporum* f.sp. *raphani* (Diener and Ausubel, 2005, Ellendorff et al., 2009) when compared to wild type plants (Figure 2A; Figure S3). However, while mutant A2 showed clearly enhanced resistance towards the bacterial vascular wilt pathogen *R. solanacearum* (Genin and Boucher, 2002), none of the other mutants showed differential susceptibility when compared with wild type plants (Figure 2A, B). Thus, the enhanced Verticillium wilt resistance of the mutants does not generally extend to other vascular pathogens, except for mutant A2 that is also resistant to *R. solanacearum*.

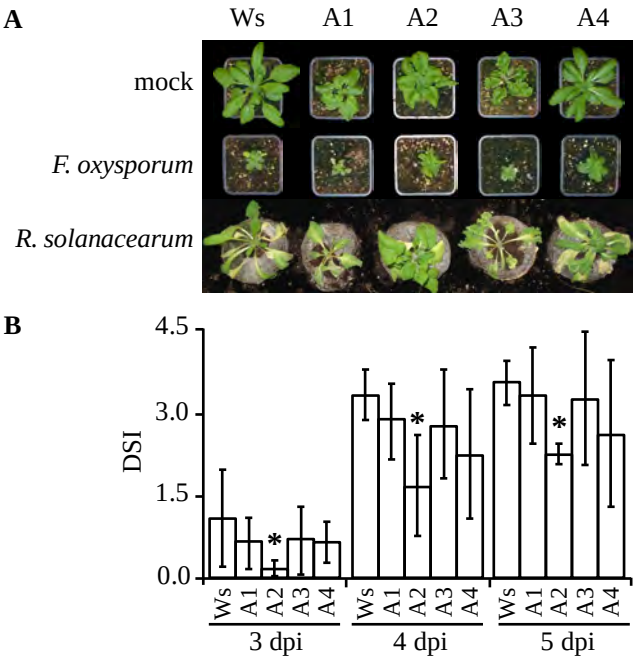

Figure 2. Response of Verticillium wilt resistant mutants to other vascular pathogens. (A) Typical mock-inoculated wild type plants (Ws) and activation-tagged mutants (A1-A4) and symptoms caused by *Fusarium oxysporum* at 12 days post inoculation (dpi) and *Ralstonia solanacearum* at 5 dpi. A representative of three independent experiments is shown. (B) Disease severity index (DSI) scores upon inoculation of at least 21 plants with *R. solanacearum* on a scale of 0 (no infection) to 4 (all rosette leaves diseased) at 3, 4 and 5 dpi. Bars represent averages with standard deviation of three independent biological replicates and the asterisks indicate significant differences ($p<0.05$).

Response of Verticillium resistant mutants to foliar pathogens

In addition to vascular pathogens, three necrotrophic foliar fungal pathogens were tested: *Botrytis cinerea*, *Plectosphaerella cucumerina* and *Alternaria brassicicola*. For *P. cucumerina*, leaves of the four mutants and wild type plants showed clear necrosis at the site of inoculation at 3 dpi, which resulted in lesion expansion and tissue collapse by 5 dpi. No significant differences were observed between the wild type and the mutants (Figure 3). Similar results were obtained for *B. cinerea* and *A. brassicicola* (Figure 3), and all results were confirmed by measurements of lesion diameters (Figure S4). These results showed that the enhanced Verticillium resistance of the mutants cannot be explained by generally enhanced resistance against fungal pathogens.

Subsequently, the response of the mutants to the bacterial pathogen *Pseudomonas syringae* p.v. *tomato* strain DC3000 (*Pst* DC3000) was studied. While the mutants A1, A2 and A4 showed similar disease symptoms when compared to wild type plants, mutant A3 showed clearly enhanced symptoms of the disease (Figure 3). At 5 dpi, severe necrosis of leaves of the A3 mutant plants was observed, while wild type, A1, A2 and A4 plants only showed mild chlorosis and slight necrosis (Figure

3). *In planta Pst* DC3000 proliferation was assessed using real-time PCR (Brouwer et al., 2003) at 3 and 5 dpi. While A1 and A3 showed significantly enhanced levels of bacterial colonization at both time points, mutant A4 showed similar level of colonization when compared to wild type plants. However, mutant A2 showed significant level of *Pst* DC3000 colonization at 3 dpi and wild type colonization at 5 dpi (Figure S5).

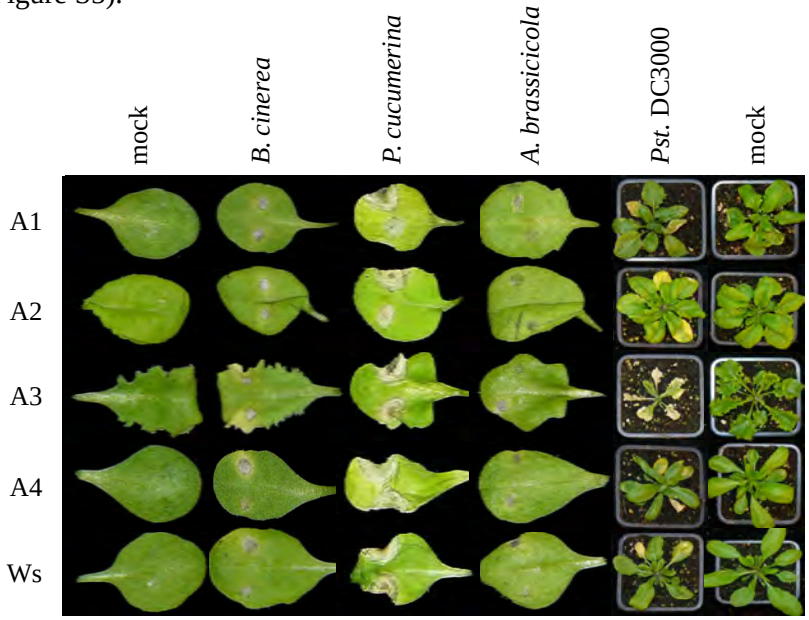

Figure 3. Response of Verticillium wilt resistant mutants to foliar pathogens. Typical disease symptoms caused by the necrotrophic fungal pathogens *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Alternaria brassicicola* and *Pseudomonas syringae* p.v. *tomato* strain DC3000 on the activation-tagged mutants A1 to A4 and the wild type Ws at 5 days post inoculation. A representative of three independent experiments is shown.

Assessment of root development and architecture

Verticillium spp. invade their hosts via the root, penetrate the root cortex and colonize the xylem tissue (Fradin and Thomma, 2006). Since the four Verticillium resistant mutants displayed clear morphological alterations of above-ground organs, possibly also root morphology could be affected, explaining the altered Verticillium susceptibility. To investigate this possibility, root growth and architecture of soil-grown seedlings was assessed. Up to two weeks post germination, the time point when *Verticillium* inoculation is performed, no differences in root development and architecture were observed for any of the mutants when compared to wild type

plants (Figure 4A). Measurements of root length did not show significant differences between wild type roots and those of the mutants (Figure 4B).

Since none of the mutants displayed obvious macroscopic differences in root growth and architecture that can explain the enhanced Verticillium resistance of the mutants, the xylem structure in wild type and mutant plants was analyzed microscopically. To this end, the wild type and the mutants were grown *in vitro*. Similar as for soil-grown plants, no significant differences in root development, architecture or length were monitored up to two weeks post germination (Figure S6). No obvious differences in structure and cellular orientation of xylem elements were observed between the roots of mutants and wild type plants upon microscopic analysis of the toluidine blue stained root cross section (Figure 4C, upper panel). Furthermore, bright field analysis of the (longitudinal) axis of living roots indicated no differences in the development and structure of the epidermis, cortex, endodermis, and vasculature cells between the wild type and activation tagged mutant plants (Figure 4C, middle panel). Measuring the diameter of the epidermis, cortex and endodermis also showed no significance differences between the activation tag mutants and the wild type (Figure S6C). In addition, the root tips, where penetration by *Verticillium* usually occurs, were analysed using confocal laser scanning microscopy after fixing and staining with propidium iodide (for detail, see materials and methods) (Truernit et al., 2008). No obvious differences between the activation tagged mutants and the wild type were observed (Figure 4C, bottom panel). Altogether, these data suggest that the enhanced Verticillium resistance of the mutants cannot be explained by macroscopic differences in root development or architecture.

Determination of the activation tag insertion site in mutant A1

Due to the enhanced resistance phenotype which exclusively concerned *Verticillium* spp. mutant A1 was selected for further molecular characterization of the enhanced resistance phenotype. The activation tag insertion site was cloned using thermal asymmetric interlaced PCR (TAIL-PCR; Liu and Whittier, 1995) and the flanking sequence was determined. BLAST analysis of the flanking sequence using the Arabidopsis genome sequence revealed that the activation tag was inserted in chromosome 3, 81 bp upstream of the predicted translational start codon of the *At3g04570* gene which encodes the AT-hook DNA binding protein AHL19 (Fujimoto et al. 2004; Figure S7). The position of the insertion site was confirmed by PCR amplification and subsequent sequencing of the chromosomal region flanking the activation tag insertion site in the A1 mutant. Several of the genes 10 kb downstream and 20 kb upstream of the activation tag insertion site (Table 1) encode proteins

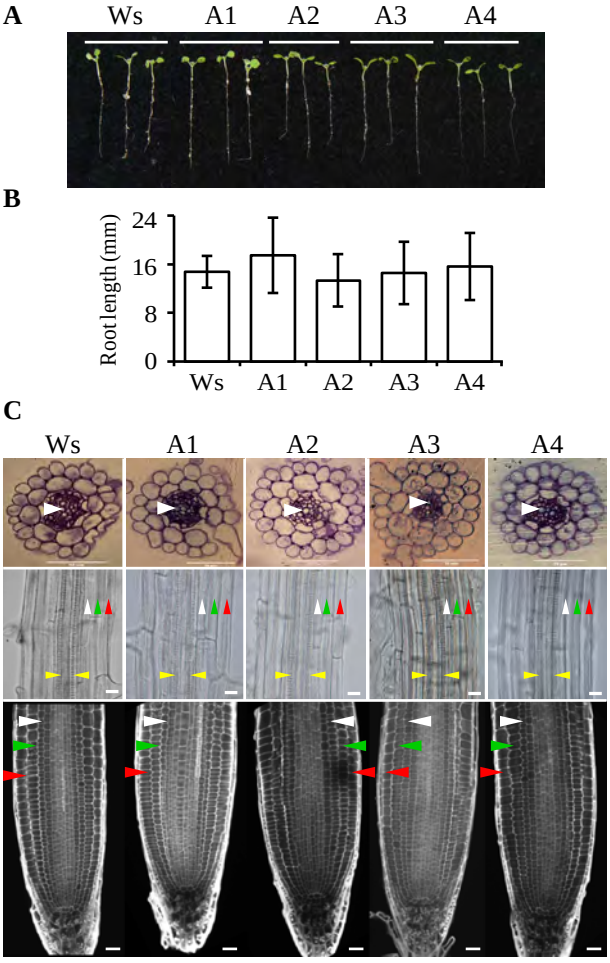

Figure 4. Root development and architecture of Verticillium wilt resistant mutants. (A) Typical appearance of 12-day-old roots of soil-grown activation-tagged mutants (A1-A4) and wild type (Ws) plants. (B) Primary root length of 12-day-old soil-grown on wild type (Ws) and activation-tagged mutants (A1-A4). (C) Microscopic analysis of the 12-day-old roots of *in vitro* grown wild type (Ws) and the activation-tagged mutant (A1-A4) plants showing the epidermal (red arrow), cortical (green arrow), endodermal (white arrow) and the vascular bundle cells (yellow arrow). The upper panel shows image of xylem elements (white arrow) of the root cross section after staining with toluidine blue, the middle panel showed bright field image of the epidermal, cortical, endodermal and the vascular bundle cells of life roots, and the bottom panel shows confocal laser scanning microscopy image of the root cap of the activation tagged mutants and the wild type plants. The scale bar indicates 50 µm in upper and middle panels and 20 µm in bottom panel.

that have previously been implicated in pathogen defence, such as defensin-like proteins (*At3g04540*, *At3g04545*, Thomma et al., 2002) and the ethylene receptor EIN4 (*At3g04580*, Johansson et al., 2006). Furthermore, several genes encoding transcription factors surround the activation tag insertion site (*At3g04590*, *At3g04610*, *At3g04620*).

Subsequently, the expression of genes flanking the insertion site was compared between the A1 mutant and wild type plants in absence of pathogen inoculation. RT-PCR showed a strong induction of gene *At3g04570* (*AHL19*) in mutant A1 when compared with wild type plants (Figure S8). Furthermore, the *At3g04530* gene appeared to be slightly induced while the *At3g04580*, *At3g04605* and *At3g04630* genes were repressed in the mutant when compared to wild type. The transcript levels of all other genes were similar in mutant A1 and wild type (Figure S8). These results

suggest that activation of gene *AHL19* may be causal to the enhanced Verticillium resistance phenotype, as expression of this gene is most significantly induced in the A1 mutant.

Homozygous knock-out alleles of all genes flanking the activation tag insertion site in mutant A1 were tested for their level of Verticillium susceptibility (Table 1). As the vast majority of publicly available knock-out alleles is in the Col-0 ecotype while the A1 mutant is in Ws, the susceptibility of plants of the Col-0 and Ws ecotypes were compared, revealing that plants of the Col-0 ecotype are less susceptible to *V. dahliae* infection than Ws plants. The analysis of the knock-out alleles in Col-0 showed that only the insertion in *AHL19* (*ahl19-1*) displayed significantly enhanced *V. dahliae* susceptibility while all other lines displayed wild type susceptibility levels (Table 1; Figure S9).

To confirm that the enhanced Verticillium resistance in mutant A1 is caused by over-expression of *AHL19*, transgenic lines that constitutively express *AHL19* were generated in both Ws and Col-0. To this end, an over-expression construct

Table 1. Analysis of the genomic region surrounding the activation tag insertion site in mutant A1.

Gene[1]	**Annotation**	**Expression**[2]	**Knock-out allele**	***V. dahliae* phenotype**[3]
At3g04520	Threonine aldolase	Similar	SALK_069973	Similar
At3g04530	Phosphoenolpyruvate carboxylase kinase 2	Induced in A1	SALK_102132	Similar
At3g04540	Defensin-like protein	Similar	None available	Not tested
At3g04545	Defensin-like protein	Similar	FLAG_378H04	Similar
At3g04550	Unknown protein	Similar	SALK_151062C	Similar
At3g04560	Unknown protein	Similar	SALK_110145C	Similar
At3g04570	**DNA-binding protein AHL19**	**Induced in A1**	**SALK_070123C (*ahl19-1*)**	**Enhanced susceptibility**
At3g04580	Ethylene receptor subfamily	Repressed in A1	FLAG_322D05	Similar
At3g04590	DNA-binding family protein	Similar	SALK_081411	Similar
At3g04600	tRNA synthetase class I (W and Y) family protein	Similar	FLAG_048G11	Similar
At3g04605	Transposon element	Repressed in A1	FLAG_404H06	Similar
At3g04610	RNA binding, nucleic acid binding	Similar	SALK_001523	Similar
At3g04620	Nucleic acid binding	Similar	SALK_124178C	Similar
At3g04630	KLEEK domain family protein	Repressed in A1	SALK_020938	Similar

[1] The gene harboring the activation tag insertion site (At3g04570) is indicated in bold.
[2] Gene expression in mutant A1 relative to the expression in wild type.
[3] Phenotype of knock-out alleles upon *V. dahliae* inoculation when compared to wild type plants.

was generated that contained the *AHL19* coding sequence behind the constitutive CaMV 35S promoter and transformed into Arabidopsis. Interestingly, *AHL19* over-expressing plants showed similar morphological phenotypes as A1 mutant plants; larger leaf size, delayed flowering and delayed senescence. Subsequently, the *AHL19* over-expressing lines were challenged with *V. dahliae* together with the corresponding wild type and an *AHL19* knock-out allele in Ws (*ahl19-2*). This analysis showed clear Verticillium wilt symptoms on wild type and *ahl19-2* plants from 14 dpi onwards that resulted in severe wilting, stunting and chlorosis by 21 dpi (Figure 5A; B). In contrast, the over-expression lines remained symptomless. Real-time PCR analysis showed that, while the wild type and knock-out allele accumulated similar levels of *V. dahliae* biomass by 21 dpi, significantly less *Verticillium* biomass was detected in the over-expression lines (Figure 5C; D). Similar results were observed when Col-0, *ahl19-1* and three independent *AHL19* over-expressing lines were challenged with *V. dahliae* (Figure S10). Altogether, these data confirm that *AHL19* over-expression mediates Verticillium resistance in Arabidopsis.

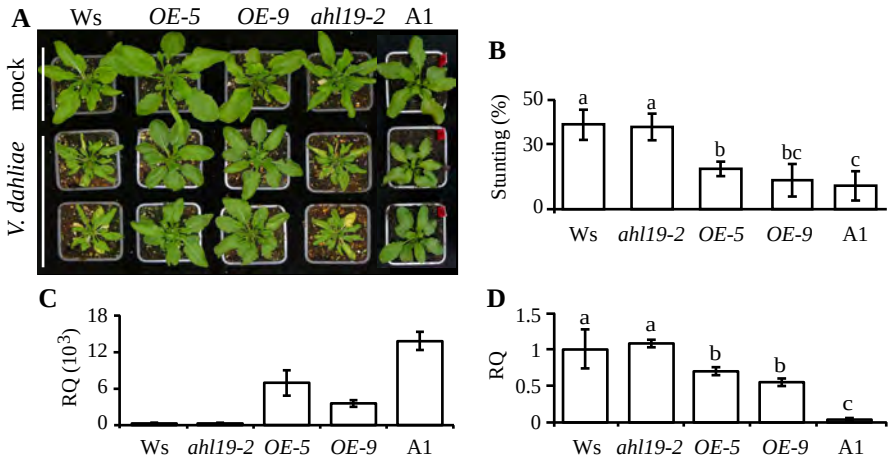

Figure 5. Over-expression of *AHL19* enhances *V. dahliae* resistance. (A) Typical symptoms upon *V. dahliae* inoculation of wild type (Ws) plants, plants of the A1 mutant, two independent *AHL19* over-expression lines (*OE-5* and *OE-9*) and of the *AHL19* knockout line (*ahl19-2*), all in Ws background, when compared with mock-inoculated plants at 22 days post inoculation (dpi). A representative of three independent experiments is shown. (B) *Verticillium*-induced stunting of wild type (Ws) plants, plants of the A1 mutant, two independent *AHL19* over-expression lines (*OE-5* and *OE-9*) and of the *AHL19* knockout line (*ahl19-2*) at 22 days post inoculation (dpi). Rosette diameters of inoculated plants were compared with those of mock-inoculated plants. The bars represent averages of three independent experiments with standard deviation and letter codes indicate significant differences (Dunnett t-test at *P=0.05*). (C) Relative quantification (RQ) of *AHL19* transcription in 4-week-old wild type (Ws) plants, plants of the A1 mutant, two independent *AHL19* over-expression lines (*OE-5* and *OE-9*) and of the *AHL19* knockout line (*ahl19-2*). Bars represent averages with standard deviation of three

biological replicates. (D) Relative quantification (RQ) by real-time PCR on genomic DNA of *V. dahliae* colonization in wild type (Ws) plants, plants of the A1 mutant, two independent *AHL19* over-expression lines (*OE-5* and *OE-9*) and of the *AHL19* knockout line (*ahl19-2*) by comparing levels of the *V. dahliae* internal transcribed spacer region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RuBisCo* gene (for equilibration) at 22 dpi. Bars represent averages with standard deviation of four technical replicates. A representative of three independent experiments is shown.

Transcriptional regulation of *AHL19*

Since constitutive activation of *AHL19* is causal to the enhanced Verticillium resistance in the A1 mutant, we assessed *AHL19* expression in the A2, A3 and A4 mutant in absence of pathogen challenge. As expected, this analysis showed that the level of *AHL19* transcripts in these mutants was not elevated when compared with wild type plants, and thus constitutive activation of *AHL19* cannot explain the enhanced Verticillium resistance in the A2, A3 and A4 mutants (Figure S11). Subsequently, the expression level of *AHL19* was assessed in roots and shoots of non-inoculated Ws and A1 mutant plants. Whereas in wild type plants slight *AHL19* expression was detected in the roots and no expression was detected in the shoots, A1 plants showed slightly elevated *AHL19* expression in the roots and strong expression in the shoots (Figure 6A), consistent with the finding that *Verticillium* colonization is contained in the shoots of A1 plants. Furthermore, *AHL19* expression was assessed in a time course of *V. dahliae*-inoculated wild type (Ws) plants with real-time PCR in the shoots. This analysis showed that *AHL19* is strongly induced upon *V. dahliae* inoculation, as the level of *AHL19* transcripts is nearly 10-fold induced upon 5 minute incubation in a suspension of *V. dahliae* conidiospores when compared with mock-inoculation (Figure 6B). Also foliar inoculation with *B. cinerea* conidiospores resulted in a fast induction of *AHL19* transcription (Figure S12A). By 7 days post *V. dahliae* inoculation, *AHL19* transcription was ~3-fold induced and by 14 dpi the level of *AHL19* transcripts was reduced to basal levels (Figure 6B). A similar repression of *AHL19* transcription was observed in *V. dahliae* inoculated Col-0 plants (Figure S12B).

To further assess the transcriptional regulation of *AHL19* in Arabidopsis, the Bio-Array Resource Expression Angler (Toufighi et al., 2005) was queried for *AHL19* expression in various micro-array studies. This analysis showed that *AHL19* is induced in a transient fashion upon inoculation with various pathogens (*B. cinerea*, *Erysiphe orontii*, *P. syringae*, *P. infestans*) and the pathogen-associated molecular pattern flg22. Furthermore, *AHL19* expression is induced upon treatment with IAA, but not with other plant hormones including abscisic acid, methyl jasmonate, salicylic

acid, gibberellic acid or brassinosteroids (Figure S13). Finally, while osmotic and salt stress slightly down-regulate *AHL19* expression, the gene appears slightly induced upon drought and oxidative stress (Figure S13).

The Arabidopsis genome encodes 29 AHLs of which AHL15 and AHL20 are most closely related to AHL19 (Fujimoto et al., 2004). Query of the Bio-Array Resource Expression Angler revealed that *AHL15* and *AHL20* do not respond as extensively to pathogen attack as *AHL19* (Figure S14). Whereas >2-fold induction of *AHL19* expression can be observed upon challenge inoculation with all of the pathogens or flg22, expression of *AHL15* and *AHL20* is only little induced and not by all of the pathogens (Figure S14).

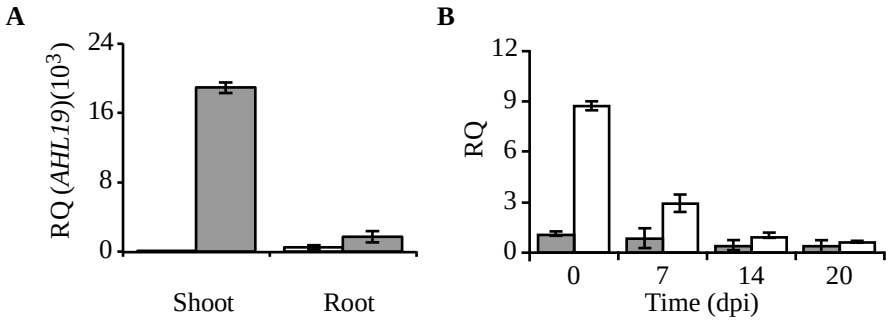

Figure 6. Expression of *AHL19* upon *V. dahliae* inoculation. (A) Relative quantification of *AHL19* transcription in roots and shoots of non-inoculated wild type (Ws) (white bars) and A1 mutant (grey bars) plants. The *AHL19* transcript level in the shoots of Ws is set at one and used for calibration. A representative of two independent biological replications is shown and bars indicate averages of three technical replicates with standard deviation. (B) The relative quantification (RQ) of *AHL19* transcription in wild type (Ws) plants immediately upon inoculation (0) and at 7, 14 and 20 days post *V. dahliae*- (white bars) or mock-inoculation (grey bars) is shown. The *AHL19* transcript level of mock-inoculation at 0 dpi is set at one and used for calibration. Bars represent averages with standard deviation of three independent biological replicates.

Expression of defence marker genes in the A1 mutant

The expression of typical defence markers genes was analysed in the Ws and A1 mutant plants before and after *Verticillium* inoculation. The expression of salicylic acid marker gene *PR1* was low in non-inoculated plants and showed a significant induction by 8 dpi in Ws (Figure S15 A). In the A1 mutant no increased *PR1* expression was observed after inoculation, which may be attributed by the absence of fungal colonization. Interestingly, expression of the jasmonic acid marker genes *PDF1.2* and *VSP2* was enhanced in the non-inoculated A1 mutant and expression decreased

after inoculation (Figure S15B, C). In Ws plants, *VSP2* expression follows a similar pattern while *PDF1.2* expression is induced by *Verticillium* inoculation (Figure S15B, C). In addition, we investigated the expression of three flg22-induced genes *NHO1*, *FRK1* and *WRKY22* in Ws and A1 mutant plants. *Verticillium* inoculation did not significantly affect *NHO1* expression in wild type and A1 mutant plants (Figure S15D). In contrast, *FRK1* expression was suppressed in non-inoculated A1 mutant plants when compared with non-inoculated Ws plants and not influenced by *Verticillium* inoculation (Figure S15E). Finally, *WRKK22* was suppressed in the A1 mutant at 4 dpi and again induced by 8 dpi in the A1 mutant (Figure S15F). Overall, the expression analysis does not reveal constitutive defence gene expression that can explain the enhanced resistance phenotype towards *Verticillium* spp. of the A1 mutant.

Discussion

In a phenotypic screening for altered susceptibility to *V. dahliae*, we identified four gain-of-function mutants which showed enhanced Verticillium resistance. Interestingly, in all mutants the enhanced resistance phenotype concerned the three pathogenic *Verticillium* spp. that are able to infect Arabidopsis; *V. dahliae*, *V. albo-atrum* and *V. longisporum*. Furthermore, it was found that the enhanced Verticillium resistance was combined with enhanced resistance towards the vascular bacterial pathogen *R. solanacearum* in the A2 mutant, and with enhanced susceptibility towards the foliar bacterial pathogen *P. syringae* in the A1 and A3 mutants. Remarkably, in none of the mutants enhanced resistance towards the vascular fungus *F. oxysporum* was obtained, despite the fact that *F. oxysporum* shares important features with respect to biology and infection style with *Verticillium* spp. (Klosterman et al., 2011).

In this study, we pursued cloning of the activation tag insertion site in the A1 mutant. Interestingly, of the 14 genes that were identified in a 30 kb window surrounding the activation tag insertion site, only two genes were induced in the A1 mutant while three genes were repressed when compared with the expression in wild type plants. Analysis of knock-out alleles of nearly all genes showed that an insertion in only one of the induced genes, *AHL19*, resulted in enhanced Verticillium susceptibility. Thus, the enhanced resistance can be attributed to over-expression of the *AHL19* gene. In none of the other activation tagging mutants, A2, A3 and A4, activation of *AHL19* was observed. Altogether, these findings demonstrate that the four mutants are not allelic, and the cloning of the insertion sites in the other mutations will be reported in future manuscripts.

AHL19 encodes a predicted AT-hook DNA binding protein. The AT-hook

is a positively charged 13 amino acid motif centred on a glycin-arginine-proline (GRP) tripeptide that is necessary and sufficient to bind DNA and that is flanked by basic residues (Reeves and Nissen, 1990; Huth et al., 1997; Aravind and Landsman, 1998). The motif was initially described in the high mobility group non-histone chromosomal proteins (HMG-I/Y), but later widely identified in single or multiple copies in DNA-binding proteins, many of which are transcription factors or components of chromatin remodelling complexes, from various organisms (Reeves and Nissen, 1990; Aravind and Landsman, 1998). In plants, the AT-hook motif was found in nuclear matrix proteins that bind to the so-called matrix attachment regions of chromosomal DNA (Morisawa et al., 2000; Fujimoto et al., 2004). In general, the AT-hook motif binds to the minor groove of DNA, changes the DNA architecture and alters gene expression (Aravind and Landsman 1998; Fujimoto et al. 2004; Lim et al. 2007; Matsushita et al. 2007; Xiao et al. 2009).

The Arabidopsis genome encodes 29 AT-hook motif proteins (AHLs for **A**T-**H**ook motif nuclear **L**ocalized proteins) that, in addition to the AT-hook motif, contain a conserved hydrophobic PPC (**P**lants and **P**rokaryotes **C**onserved) domain which is responsible for nuclear localization (Fujimoto et al. 2004). Interestingly, several AHL proteins have been reported to regulate plant development. Whereas ESC/AHL27, SOB3/AHL29 and AHL22 all negatively regulate hypocotyl growth (Street et al. 2008; Lim et al. 2007; Xiao et al. 2009), over-expression of *AHL27* delays senescence and increases the post-harvest storage life of plants (Lim et al. 2007), over-expression of *SOB3/AHL29* increased leaf and flower size, and over-expression of *AHL22* causes late flowering (Xiao et al. 2009). As observed in our study, also *AHL19* over-expressing plants gained similar developmental characteristics including increased rosette leaf size and delayed maturity. This suggests that AHL19, like other members of AT-hook DNA binding protein family, regulates plant development.

AHL19 regulates Verticillium resistance

We have shown that over-expression of *AHL19* in Ws and Col-0 plants resulted in robust Verticillium resistance. Furthermore, the T-DNA insertion line in Col-0 (*ahl19-1*) displayed clearly enhanced Verticillium susceptibility. The observation that a T-DNA insertion in the Ws allele (*ahl19-2*) did not result in enhanced susceptibility may be explained by the fact that the susceptibility of the Ws ecotype is already high when compared to the Col-0 ecotype, and thus a further enhancement of susceptibility may remain unnoticed.

Interestingly, our data show that resistance to *Verticillium* is not obtained in the roots, but rather in the shoots of the A1 mutant. Fungal presence was clearly

detected in above-ground plant organs, revealing that the fungus is able to enter the xylem vessels and is not contained in the roots. Moreover, *AHL19* appeared to be especially over-expressed in the shoots of the A1 mutant. The finding that Verticillium resistance is only established once the fungus has entered the xylem vessels is typical for the physiology of Verticillium resistance in other plant species as well. Also in tomato lines that harbour the *Ve1* resistance gene incompatible fungal strains enter the vascular system after root penetration, and resistance is only established after the fungus has started to colonize the plant, leading to elimination of the fungus (Gold and Robb, 1995; Heinz et al., 1998; Chen et al., 2004; Fradin et al., 2009; Fradin et al., 2011).

To study how AHL19 is regulated during *Verticillium* infection, we analysed the expression of *AHL19* in the wild type plant after inoculation with *Verticillium.* The real-time PCR analyses of time-course experiments suggested that the fungus attempts to suppress *AHL19* expression. *AHL19* transcription is activated immediately upon *V. dahliae* inoculation, as the level of *AHL19* transcripts is nearly 10-fold induced upon 5 minutes incubation in a suspension of *V. dahliae* conidiospores when compared with mock-inoculation. The immediate activation of *AHL19* upon pathogen presence, and targeting of its expression by the pathogen further substantiates a crucial role for *AHL19* in the activation of host defence against pathogens.

Until recently, AHL proteins have not been implicated in plant defence against pathogens. In chili pepper (*Capsicum annuum*) the AT-hook DNA binding protein-encoding gene *CaATL1* was specifically induced upon pathogen inoculation. Transgenic tomato plants over-expressing *CaATL1* showed enhanced resistance against *P. syringae* and *Phytophthora capsici* (Kim et al., 2007). Furthermore whereas in this study we characterized AHL19 as a positive regulator of fungal defence, recently the close relative AHL20 was characterized as a negative regulator of plant immunity. Over-expression of *AHL20* in Arabidopsis followed by treatment with the flg22 peptide that is derived from bacterial flagellin was found to suppress expression of *NHO1*, a gene that encodes a glycerol kinase that is required for bacterial immunity (Lu et al., 2001; Kang et al., 2003), and resulted in enhanced susceptibility towards *Pseudomonas syringae* (Lu et al., 2010). Furthermore, transient expression of *AHL20* in protoplasts abolished flg22-induced expression of the flagellin-induced receptor kinase FRK1 (Asai et al., 2002; Lu et al., 2010). However, these studies solely relied on over-expression and, a detailed genetic analysis including knock-out alleles has not been performed (Kim et al., 2007; Lu et al., 2010). Here, we have provided a thorough genetic and phytopathological study to implicate *AHL19* as a positive regulator of defence against *Verticillium* infection. The expression analysis suggests that *AHL19* and *AHL20* do not act redundantly, as

AHL19 is generally induced by pathogen attack, while *AHL20* and also the closely related *AHL15* are only moderately pathogen induced. The major challenge will now be to determine how *AHL19* regulates pathogen defence. Although expression of the jasmonate signalling marker genes *PDF1.2* and *VSP2* was found to be enhanced in non-inoculated A1 plants, it is unlikely that constitutive jasmonate-mediated defences can account for the enhanced resistance in the A1 mutant. Firstly, as suggested by the marker gene expression levels, and by the lack of enhanced resistance towards the foliar necrotrophic pathogens *B. cinerea* and *P. cucumerina* that are typically contained by jasmonate-mediated defences (Thomma et al., 1998; 2001), the level of enhanced jasmonate-mediated defences is likely too low to achieve actually enhanced pathogen resistance. Secondly, several studies have indicated that basal defence towards *Verticillium* in Arabidopsis is not governed by jasmonate, or by salicylic acid signalling (Veronese et al., 2003; Johansson et al., 2006; Pantelides et al., 2010; Fradin et al., 2011). Finally, other than by IAA, *AHL19* expression does not appear to be induced by defence-related hormones.

Materials and methods

Cultivation of plants and microorganisms

Arabidopsis thaliana plants were soil-grown either in the greenhouse or in a growth chamber. In the greenhouse, the conditions were 21°C/19°C during the 16-h-day/8-h-night period, 70% RH, and 100 W/m^2 supplemental light when the intensity dropped below 150 W/m^2. In the climate chamber, the conditions were 21°C/19°C during the 14-h-day/10-h-night period, 70% RH, and a light intensity of 150 W/m^2.

Verticillium spp. and *A. brassicicola* were cultivated on potato dextrose agar (PDA), *B. cinerea* and *P. cucumerina* on malt extract agar (MEA) and *Fusarium oxysporum* f.sp. *raphani* on Czapek-Dox agar (CzD), all at room temperature. *P. syringae* pv. *tomato* strain DC3000 and *R. solanacearum* strains were cultivated as described (van Esse et al., 2008; Deslandes et al., 1998).

Pathogen inoculations

Verticillium inoculations were performed as previously described (van Esse et al., 2008, Ellendorff et al., 2009) with the modification that roots were dipped in the conidial suspension for five minutes. *F. oxysporum* f.sp. *raphani* budcell inoculum was prepared as described (Diener and Ausubel, 2005), and inoculation was performed similarly as with *Verticillium*. Inoculations with *B. cinerea*, *P. cucumerina* (both at

10^6 conidia/mL) and *Pst* DC3000 were performed as previously described (van Esse et al., 2008). *A. brassicicola* was inoculated similarly as with *P. cucumerina*. At 3 and 5 dpi, pictures were taken of all inoculated plants and lesion diameters were measured using ImageJ software. Inoculation with *R. solanacearum* was performed as described (Deslandes et al., 1998).

Assessment of root development

Arabidopsis was grown *in vitro* on half-strength Murashige and Skoog (MS) medium supplemented with 0.5% sucrose and solidified with 1.2% plant agar. Prior to sowing, seeds were surface sterilized using 40% sodium hypochlorite containing 3 mL hydrochloric acid (37%) for 4 hr. After sowing, plates were incubated at 4 ºC for 4 days and subsequently transferred to the growth chamber and incubated vertically. At two weeks post germination, roots of the wild type and activation-tagged mutants were examined using a microscope.

For microscopic analysis, two weeks after transfer to the growth chamber root pieces (~1 cm) were fixed in 0.1 M phosphate buffer (pH 7.2), 2.5% glutaraldehyde and 4% paraformaldehyde for 1 hr. After rinsing in 0.1M phosphate buffer (15 min) and distilled water (15 min), the root pieces were dehydrated by successive incubation for 20 min in 10, 30, 50, 70, 90 and 100% ethanol. Subsequently, the pieces were successively incubated for 1 hr in 25, 50 and 75% pre-solution A (100 mL technovit 7100 (Heraeaus Kulzer, Wehrheim, Germany), a packet of Hardener I and 2.5 mL polyethylene glycol 400) in ethanol, followed by overnight incubation in 100% pre-solution A at room temperature. Subsequently, the root pieces were embedded in mould using polymerization solution (pre-solution A and Hardener II, 15:1 v/v), glued to a block with technovit 3040 and sectioned using a microtome. The section was stained with toluidine blue and observed under microscope.

For the Confocal laser scanning microscopy, the roots were fixed and stained as previously described (Truernit et al., 2008). A Zeiss LSM confocor 2 microscope was used. The samples were imaged through a C-Apochromat 40xW. The pinhole was adjusted to a Z-depth of 1.5 µm. The excitation wavelength for the propidium iodide stained samples was 543 nm using HeNe laser and the fluorescence was filtered by 560 nm long pass.

Determination of the activation tag insertion site

TAIL-PCR was performed as described (Terauchi and Kahl, 2000) with a combination of nested primers (Marsch-Martinez et al. 2002) and 10-mer random

primers (Terauchi and Kahl, 2000). The secondary and tertiary TAIL-PCRs were separated on 1.2% agarose gel, stained with ethidium bromide, and visualized using the ChemiDoc XRS system (Bio-Rad). Specific product, judged based on the size differences generated by the nested primers, was excised, cleaned using the QIAquick Gel Extraction Kit (QIAGEN), cloned into the pGEM-T Easy Vector (Invitrogen), and sequenced. BlastN search of the TAIR database was used to identify the genomic insertion site. Based on the putative insertion site in *AHL19*, primers DBPR-F1 and DBPR-R1 (Table S1) were designed to amplify the flanking region. By sequencing this region in the wild type and the mutant the exact position of the activation tag insertion was identified.

AHL19 over-expression

The *AHL19* CDS was amplified with primers AHL19-BaF and AHL19-AsR (Table S1) that include *Bam*HI and *Asc*I restriction sites, respectively, using *Pfu* DNA polymerase (Promega). The amplicon was cloned into the *Bam*HI-, *Asc*I-digested binary vector pB7k40, a variant of vector pB7WG2 (Karimi et al., 2002) where the sequences between *Kpn*I and *Sac*I (which include the 35S promoter and terminator) were replaced with sequences between *Kpn*I and *Sac*I (35S promoter, multiple cloning site and terminator from potato proteinase inhibitor II-PiII) of a pmog800 variant (Fradin et al., 2009). The resulting vector was checked by sequencing. Subsequently, the construct was transformed into *A. tumefaciens* strain GV3101 and transformed into Arabidopsis (Clough and Bent, 1998). All assays were performed with T2 generation transgenes, and transgene presence was confirmed for each individual plant by PCR. *AHL19* expression was assessed with real-time using an ABI7300 PCR machine (Applied Biosystems) in combination with the qPCR Core kit for SYBR Green I (Eurogentec) and analyzed using the 7300 System SDS software (Applied Biosystems). The Arabidopsis *actin 2* gene primer pair, Act2-F and Act2-R, was used as endogenous control and the primer pair DBP-F1 and DBP-R1 was used for quantification of *AHL19* transcript levels. Real-time PCR conditions consisted of 2 min incubation at 50ºC and 10 min at 95ºC followed by 40 cycles of 95ºC for 15 sec. and 60ºC for 1 min.

Expression of defence markers

For expression analysis of defence marker genes, mock-and *V. dahliae*-inoculated Ws and A1 mutant plants were harvested prior to inoculation, and at 4 and 8 days post inoculation. Subsequently, total RNA was extracted using the NucleoSpin RNA

Plant kit (Machery-Nagel) and cDNA was synthesised as previously described (Ellendorff et al., 2009). Real-time PCR was performed using target gene primers (Table S1) and the Arabidopsis *actin 2* gene primer as endogenous control. Real-time PCR conditions consisted of 2 min incubation at 50ºC and 10 min at 95ºC followed by 40 cycles of 95ºC for 15 sec. and 60ºC for 1 min.

Pathogen quantification *in planta*

Real-time PCR on genomic DNA was used for quantification of pathogen colonization *in planta* using an ABI7300 PCR machine (Applied Biosystems) in combination with the qPCR Core kit for SYBR Green I (Eurogentec) and analyzed using the 7300 System SDS software (Applied Biosystems). Unless described otherwise, the primer pair AtRub-F4 and AtRub-R4 (Table S1) targeting the gene encoding the large subunit of RuBisCo was used as endogenous control. *Verticillium* colonization was assessed as described in Ellendorff et al. (2009). For *P. syringae* and *R. solanacearum* colonization, at least three inoculated plants were harvested at 3 and 5 dpi and DNA was isolated (Fulton et al., 1995). The primer pair Pstq-F and Pstq-R was used to target *P. syringae*, while the primer pair R.solF and R.solR was used to target *R. solanacearum* (Table S1; Brouwer et al., 2003; Huang et al., 2009).

Acknowledgements

We thank Asaph Aharoni, Sarah van Broekhoven, Mulatu W. Jalata, Nayelli Marsch-Martinez and Norbert de Ruijter for technical assistance and Henk Smid and Bert Essenstam for excellent plant care.

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Supplementary data

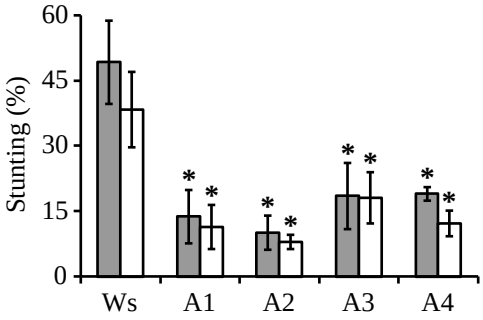


Figure S1. *Verticillium*-induced stunting of wild type (Ws) and activation-tagged mutant plants (A1-A4) at 21 days post inoculation (dpi).Plants were inoculated with *V. dahliae* (grey bars) and *V. albo-atrum* (white bars) and rosette diameters were compared with those of mock-inoculated plants. The reduction in rosette diameter (stunting) is lower for the activation-tagged mutants than for wild type plants. In each experiment, rosette diameters from at least 5 mock- and 8 *Verticillium*-inoculated plants were measured. The bars represent averages of three independent experiments with standard deviation and the asterisks indicate significant differences (Dunnett t-test at *P=0.01*) when compared with the wild type Ws.

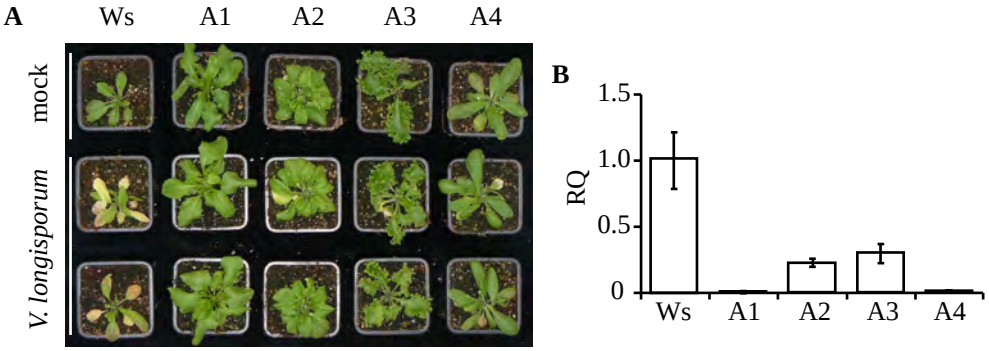


Figure S2. Activation-tagged Arabidopsis mutants are resistant to *V. longisporum*. (A) Typical symptoms of *V. longisporum* on wild type (Ws) and activation-tagged mutant plants (A1-A4) at 21 days post inoculation (dpi). A representative of three independent experiments is shown. (B) Relative quantification (RQ) by real-time PCR on genomic DNA of *Verticillium* colonization by comparing levels of the *V. longisporum* internal transcribed spacer region of the ribosomal DNA (as measure for fungal biomass) relative to the large subunit of the Arabidopsis *RuBisCo* gene (for normalization) at 21 dpi. Bars represent averages with standard deviation of three independent biological replicates.

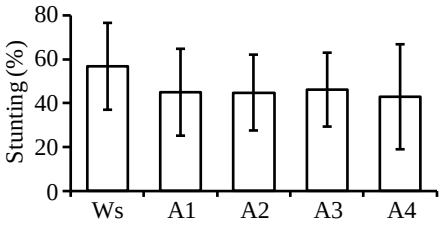


Figure S3. *F. oxysporum*-induced stunting of wild type (Ws) and activation-tagged mutant plants (A1-A4) at 21 days post inoculation (dpi). Rosette diameters of plants inoculated with *F. oxysporum* f.sp. *raphani* were compared with those of mock-inoculated plants, showing that the reduction in rosette diameter (stunting) is lower for the activation-tagged mutants than for wild type plants. In each experiment, rosette diameters from at least 5 mock- and 8 Fusarium-inoculated plants were measured. The bars represent averages of three independent experiments with standard deviation and no significant differences were observed between the different plant lines (Dunnett t-test at *P=0.01*).

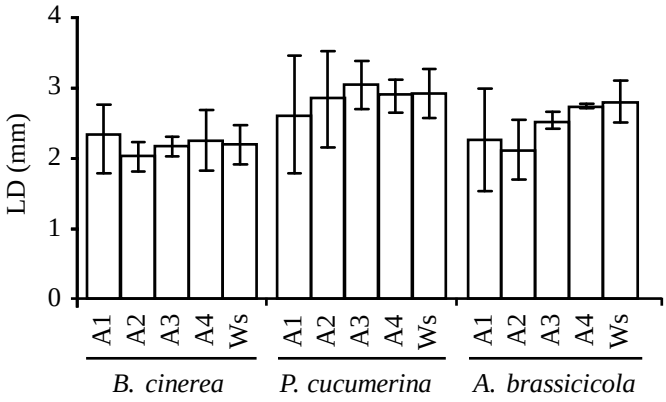


Figure S4. Lesion diameter on activation-tagged Arabidopsis mutants upon inoculation with necrotrophic foliar pathogens. Lesion diameters (LD) were measured on wild type (Ws) and activation-tagged mutant plants (A1-A4) at 5 days post inoculation with the fungi *Botrytis cinerea*, *Plectosphaerella cucumerina* and *Alternaria brassicicola*. Bars indicate average lesion diameters with standard deviation measured on all inoculated rosette leaves of six plants.

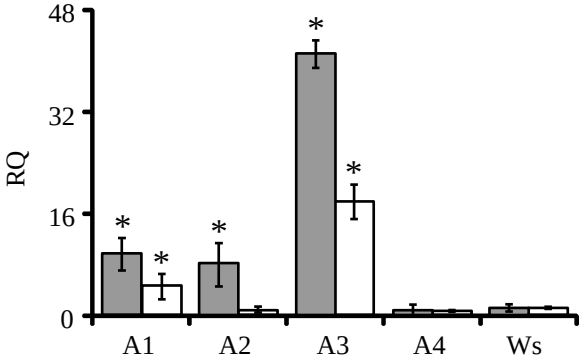


Figure S5. Quantification of *Pseudomonas syringae* colonization *in planta*. Relative quantification (RQ) on wild type (Ws) and activation-tagged mutant plants (A1-A4) by real-time PCR of *Pseudomonas syringae* DNA relative to the amount of DNA of large subunit of the Arabidopsis *RuBisCo* gene (for equilibration) at 3 (grey bars) and 5 (white bars) days post inoculation. Bars represent averages with standard deviation of four independent biological replicates and asterisks indicate significance difference (Dunnett t *p=0.05*) when compared to the wild type (Ws) at each respective time points.

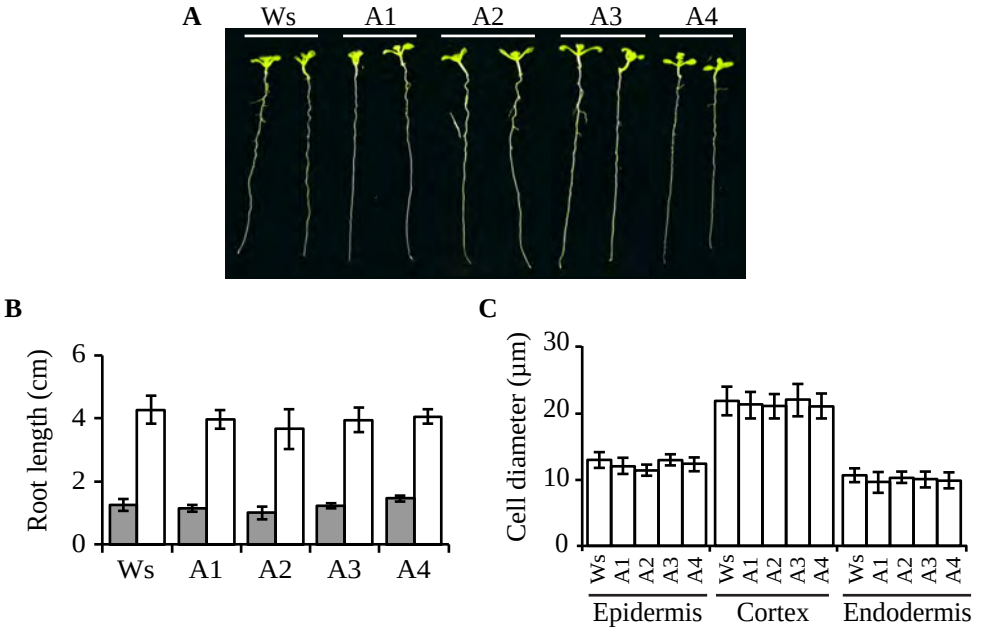


Figure S6. Root development and architecture of *in vitro*-grown Verticillium wilt resistant mutants. (A) Typical phenotype of 14-day-old *in vitro*-grown roots of the wild type (Ws) and activation-tagged mutant (A1-A4) plants. (B) The primary root length of *in vitro* grown wild type (Ws) and activation-tagged mutants (A1-A4) measured at one- (grey bars) and two- (white bars)-weeks post germination. (C) The diameter of the epidermal, cortical and endodermal cells of an *in vitro* grown wild type and activation tagged mutant plants. No significance differences in the diameter of the cells between the wild type and activation tagged mutant plants were observed (Dunnett t test $p=0.05$, n=12)

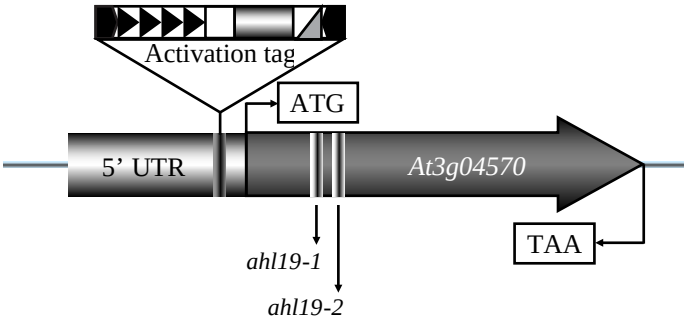


Figure S7. Schematic representation showing the position of the activation-tag insertion site in the *AHL19*gene (*At3g04570*) in mutant A1, and the position of the two T-DNA insertion sites in *ahl19-1* and *ahl19-2* (SALK and FLAG, respectively). The positions of translational start (ATG) and stop (TAA) codons are indicated.

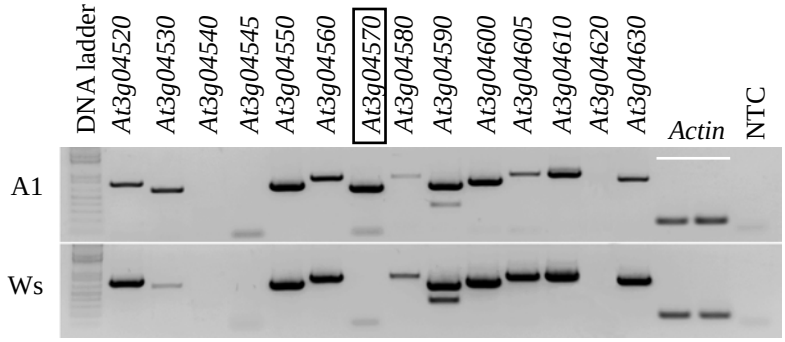


Figure S8. RT-PCR based expression analysis of genes flanking the activation tag insertion site in the A1 mutant when compared to wild type (Ws) plants in absence of *Verticillium*. The gene encoding *AHL19* is boxed (*At3g04570*). Reactions to amplify the *Actin 2* gene and a non-template control (NTC) were included as controls.

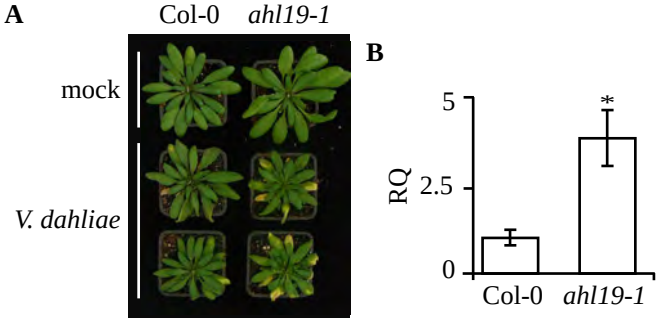


Figure S9. Knocking-out of *AHL19* in Arabidopsis enhances susceptibility to *V. dahliae.* (A) Typical symptoms of *V. Dahliae* on the wild type (Col-0) plants and plants of the *AHL19* knock-out (*ahl19-1*) plants when compared with mock-inoculated plants at 22 days post inoculation (dpi). A representative of three independent experiments is shown. (B) Relative quantification (RQ) by real-time PCR on genomic DNA of *V. dahliae* colonization in wild type (Col-0) and *ahl19-1*plants by comparing levels of the *V. dahliae* internal transcribed spacer region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RuBisCo* gene (for equilibration) at 22 dpi. Bars represent averages with standard deviation of four technical replicates. A representative of three independent experiments is shown.

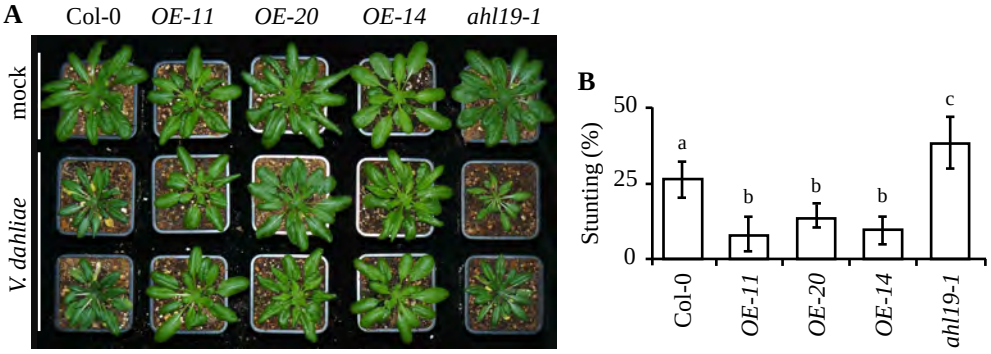


Figure S10. Over-expression of *AHL19* enhances *V. dahliae* resistance. (A) Typical symptoms upon *V. dahliae* inoculation of wild type (Col-0) plants, three independent *AHL19* over-expressing plants (*OE-11*, *OE-14* and *OE-20*) and of the *AHL19* knockout line (*ahl19-1*), all in Col-0 background, when compared with mock-inoculated plants at 21 days post inoculation (dpi). A representative of three independent experiments is shown. (B) *Verticillium*-induced stunting of wild type (Col-0) plants, three independent *AHL19* over-expressing plants (*OE-11*, *OE-20* and *OE-14*) and of the *AHL19* knockout line (*ahl19-1*) at 21 days post inoculation (dpi). Rosette diameters of inoculated plants were compared with those of mock-inoculated plants. The bars represent averages of three independent experiments with standard deviation and different letters indicate significant differences (Dunnett t-test at *P=0.05*) when compared to the wild type (Col-0).

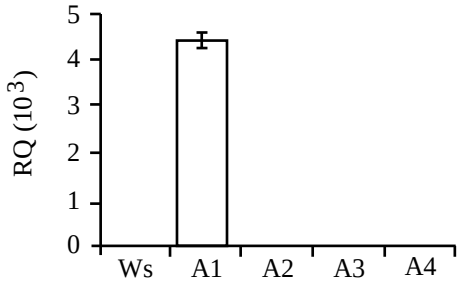


Figure S11. Relative quantification (RQ) of *AHL19* transcription level in 4-week-old wild type (Ws) and activation-tagged mutant plants (A1-A4). Bars represent averages with standard deviation of three independent biological replicates.

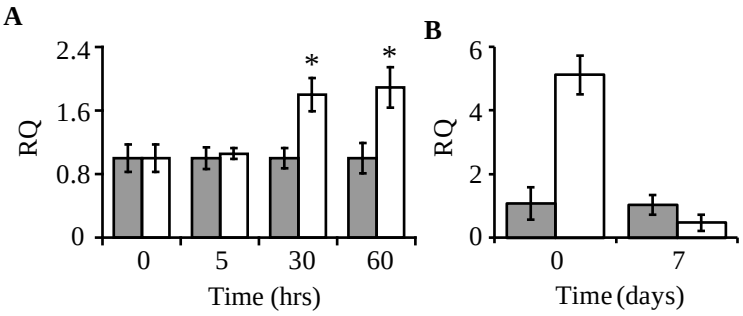


Figure S12. Expression of *AHL19* upon pathogen inoculation. (A) Relative quantification (RQ) of *AHL19* transcription level in wild type (Ws) plants immediately upon inoculation (0) and at 5, 30 and 60 minutes post *B. cinerea*- (white bars) or mock-inoculation (grey bars).The *AHL19* transcript level of mock-inoculation at 0 dpi is set at one and used for calibration. Bars represent averages with standard deviation of three biological replicates and asterisks indicate statistically significant differences (Dunnett t-test at *p=0.05*). (B) Relative quantification (RQ) of *AHL19* transcription in mock-inoculated wild type (Col-0) (grey bars) and plants

immediately upon inoculation (0) and at 7 days post *V. dahliae*-inoculation (white bars).The *AHL19* transcript level in mock-inoculated plants at 0 dpi is set to one and used for calibration. Bars represent averages with standard deviation of three independent biological replicates.

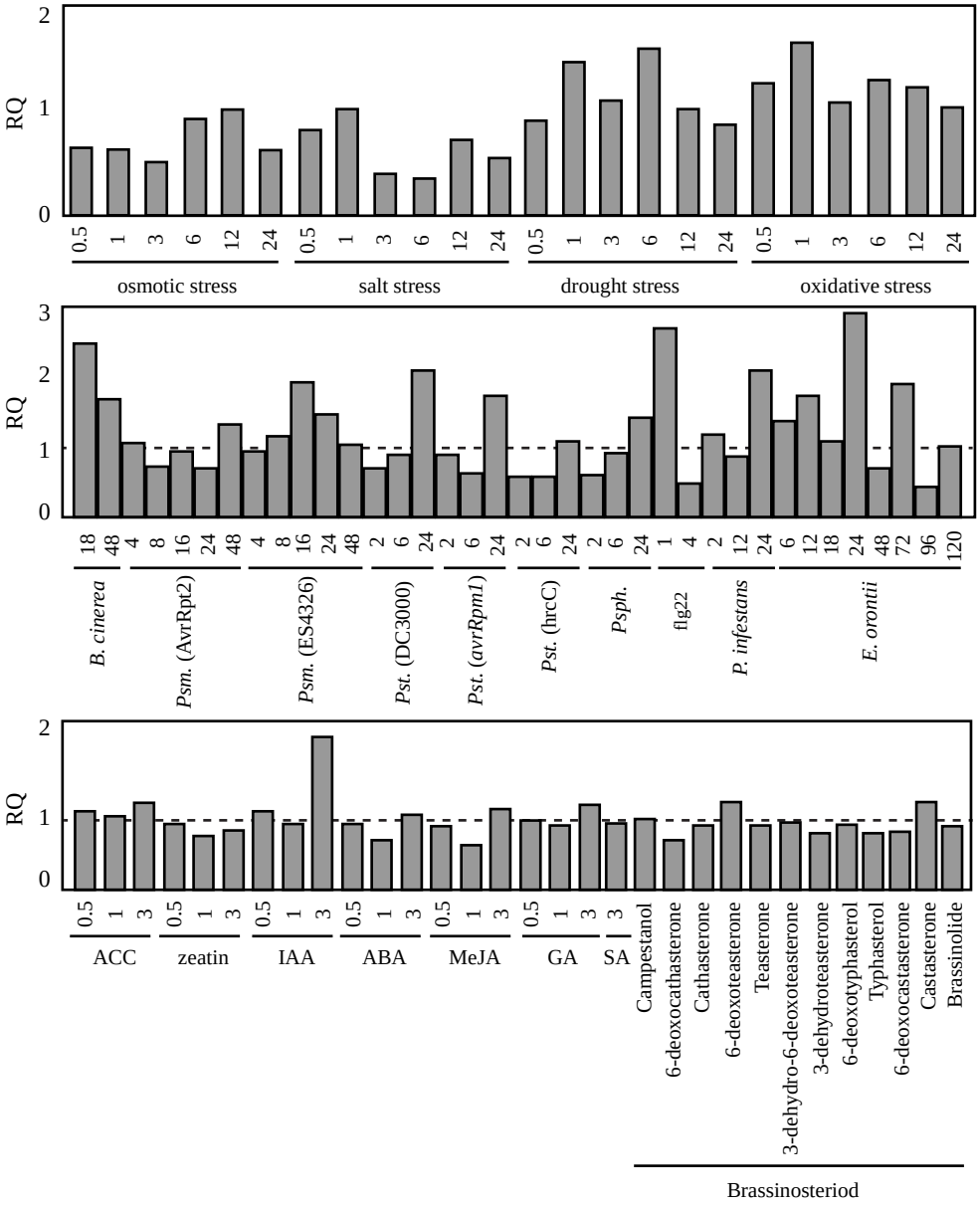


Figure S13. Expression of *AHL19* extracted from publically available microarray data (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The fold-change in expression level (RQ) relative to the control that is set to one for each of the individual treatments is shown. Below the bars, the duration of the respective treatments is indicated in hours. Osmotic, salt and oxidative stress was induced by treatment with 300 mM mannitol, 150 mM sodium chloride and 10 µM methyl viologen, respectively. Drought stress was induced by air stream exposure for 15 minutes. The pathogens include *Botrytis cinerea*,

Erysiph eorontii, *Phytophthora infestans*, *Pseudomonas syringae* pv. *phaseolicola* (*Psph*; avirulent), *Pseudomonas syringae pv. maculicola (Psm)* strain ES4326 (virulent) and ES4326 that carries *AvrRpt2* (avirulent), and the *P. syringae* pv. *tomato* (*Pst*) strains DC3000 (virulent), DC3000 that carries *AvrRpm1* (avirulent), DC3000 *hrcC* mutant (avirulent), and the bacterial derived elicitor flg22. For the hormone treatments, 10 mM of 1-aminocyclopropane-1-carboxylic acid (ACC), methyl jasmonate (MeJA), or abscisic acid (ABA), and 1 µM of zeatin, indole-3-acetic acid (IAA), gibberellic acid (GA-3) or 10 µM of salicylic acid (SA) was used. Furthermore, the brassinosteriods campestanol (10 µM), 6-deoxocathasterone (1 µM), cathasterone (1 µM), 6-deoxoteasterone (1 µM), teasterone (1 µM), 3-dehydro-6-deoxoteasterone (1 µM), 3-dehydroteasterone (1 µM), 6-deoxotyphasterol (1 µM), typhasterol (1 µM), 6-deoxocastasterone (1 µM), castasterone (100 nM), and brassinolide (100 nM) were used.

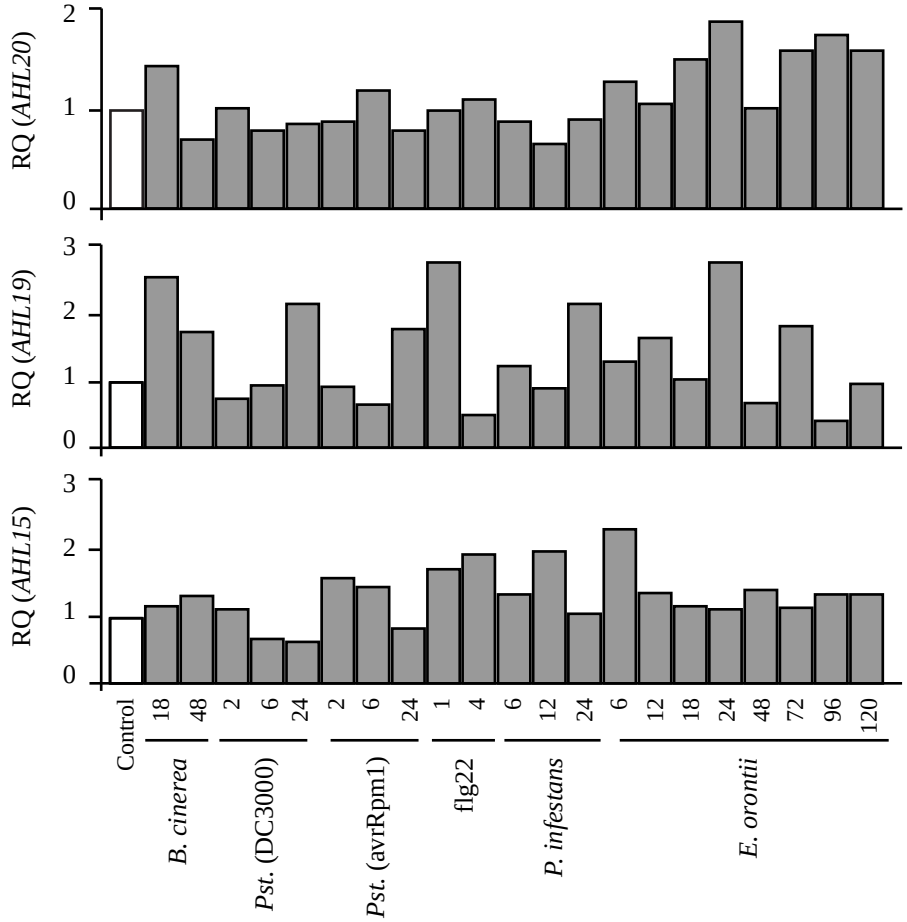


Figure S14. Transcriptional changes of *AHL19* and its close homologues *AHL15* and *AHL20* upon challenge with diverse pathogens extracted from publically available microarray data. The fold-change in expression level (RQ) relative to the control (grey bar), which is set to one, for each of the individual treatments is shown. Below the bars, the duration of the respective treatments is indicated in hours. The

pathogens include *Botrytis cinerea*, *Erysiphe orontii*, *Phytophthora infestans*, and the *P. syringae* pv. *tomato* (*Pst*) strains DC3000 (virulent) and DC3000 that carries *AvrRpm1* (avirulent). Also the bacterial flagellin-derived PAMP flg22 is included.

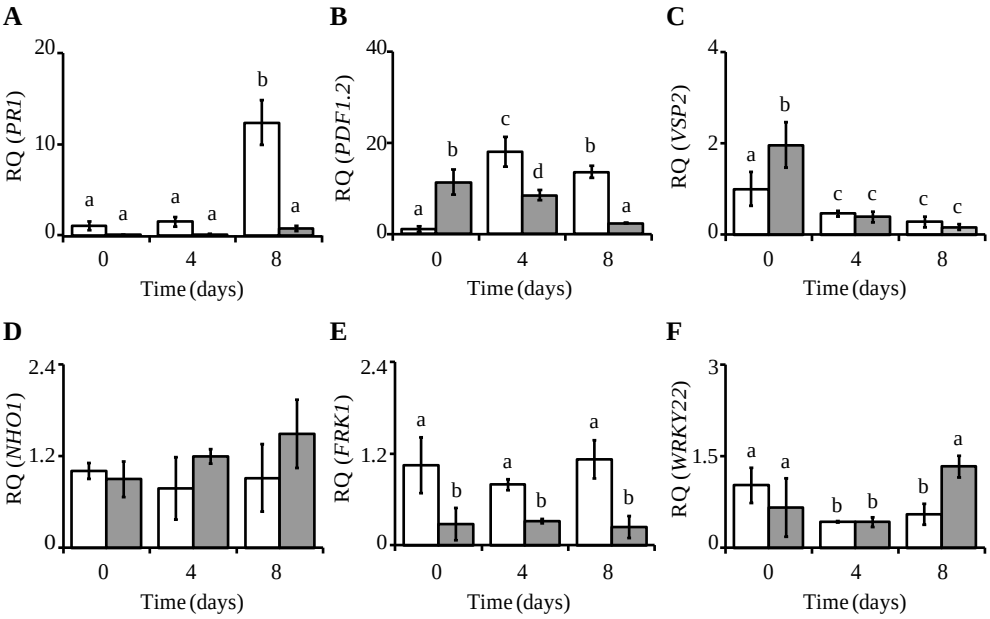


Figure S15: Expression of defence marker genes in wild type (Ws) and A1 mutant plants upon *V. dahliae* challenge. Expression analysis by real-time PCR of the salicylic acid marker gene *PR1* (A), jasmonic acid marker genes *PDF1*.2 (B) and *VSP2* (C) and the flg22-induced marker genes *NHO1* (D), *FRK1* (E) and *WRKY22* (F) before inoculation (0) and at 4 and 8 days post *V. dahliae* inoculation in wild type (white bars) and A1 mutant (grey bars) plants. The experiment was repeated two times and a representative experiment is shown. The bars represent averages of three technical replicates with standard deviations.

Supplemental Table 1: Primers used in this study

Primer name	Sequence (5' - 3')	Purpose
DBPR-F1	TGTGTGTACGTCTTATGGGC	Confirmation of activation tag isertion in A1 mutant
DBPR-R1	TTAACTGAGAGGAACCAGGC	Confirmation of activation tag isertion in A1 mutant
AHL19-BaF	GGATCCATGGCGAATCCATGGTGGAC	AHL19 overexpression (*Bam*HI underlined)
AHL19-AsR	GGCGCGCCTTAAAATCCT-GACCTAGCTTGAGCC	AHL19 overexpression (*Asc*I underlined)
AtRub-F4	GCAAGTGTTGGGTTCAAAGCTGG	RuBisCo

Primer name	Sequence (5' - 3')	Purpose
AtRub-R4	AACGGGCTCGATGTG GTAGC	RuBisCo
ITS1-F	AAAGTTTTAATGGTTCGCTAAGA	Quantification *V. dahliae*
ST-VE1-R	CTTGGTCATTTAGAGGAAGTAA	Quantification *V. dahliae*
Pstq-F	AACTGAAAAACACCTTGGGC	Quantification *P. syringae*
Pstq-R	CCTGGGTTGTTGAAGTGGTA	Quantification *P. syringae*
R.solF	CCGACACCACGACCCTGAA	Quantification *R. solanacearum*
R.solR	GCGGACGGATAGATGTAGTTGC	Quantification *R. solanacearum*
Act2-F2	TAACTCTCCCGCTATGTATGTCGC	Arabidopsis Actin
Act2-R2	GAGAGAAACCCTCGTAGATTGGC	Arabidopsis Actin
DBP-F1	TGGCGAATCCATGGTGGACAGG	*AHL19* expression analysis
DBP-R1	TTCTACGGCTCCTTCACGTGGC	*AHL19* expression analysis
Oligo(dT)	TTTTTTTTTTTTTTTTTTTTTTTTT(A,G, or C)	cDNA synthesis
FRK1-F	CAGGATGTTGATGCCATGAG	*FRK1* expression analysis
FRK1-R	AAGGTTGGAGAAGGCTGGAT	*FRK1* expression analysis
NHO1-F	TAACGGGAAAGGGGAGTTCT	*NHO1* expression analysis
NHO1-R	TTCCTTCCAGAATCCCACAG	*NHO1* expression analysis
VSP2-F	CTTTCACTTCTCTTGCTCTTGGCC	*VSP2* expression analysis
VSP2-R	GGTCTCAACACCAAGGTGCC	*VSP2* expression analysis
PR1-F	TCGTCTTTGTAGCTCTTGTAGGTGC	*PR1* expression analysis
PR1-R	ACCCCAGGCTAAGTTTTCCC	*PR1* expression analysis
PDF1.2-F	CACCCTTATCTTCGCTGCTC	*PDF1*.2 expression analysis
PDF1.2-R	GTTGCATGATCCATGTTTGG	*PDF1*.2 expression analysis
AtWRKY22-F1	GTAAGCTCATCAGCTACTACGAC	*WRKY22* expression analysis
AtWRKY22-R1	ACCGCTAGATGATCCTCAACAG	*WRKY22* expression analysis

Chapter 3

EVR1 provides resistance to vascular wilt pathogens

Koste A. Yadeta, Mathieu Hanemian, Yves Marco, Bart P.H.J. Thomma
Patent pending, Manuscript in preparation

Abstract

Soil-borne vascular wilt diseases caused by *Verticillium* spp. in a wide range of plant species are among the most destructive diseases worldwide. The most effective means of controlling Verticillium wilt diseases is the use of genetic resistance. We have previously reported the identification of four activation-tagged Arabidopsis mutants (A1-A4) which showed enhanced resistance to Verticillium wilt. Among these, mutant A2 also showed enhanced resistance to *Ralstonia solanacearum*, a bacterial vascular wilt pathogen. Determination of the activation tag insertion site in the genome of the A2 mutant showed that the activation tag is inserted upstream of gene *At3g13437* which we designated as *AtEVR1* (for *Arabidopsis thaliana Enhancer of Verticillium Resistance 1*). *AtEVR1* encodes a putatively secreted protein of unknown function. While *AtEVR1* over-expression enhances Arabidopsis resistance to three vascular wilt pathogens; *V. dahliae, R. solanacearum* and *Fusarium oxysporum* f.sp. *raphani*, gene knock-out enhances susceptibility to *V. dahliae* and *R. solanacearum*. The search for homologs in publicly available sequence databases only identified homologs within the *Brassicaceae* family. We subsequently cloned the homolog from *Brassica oleracea* (*BoEVR1*) for over-expression in Arabidopsis. Interestingly, *BoEVR1* over-expression in Arabidopsis also resulted in resistance to *V. dahliae*. Furthermore, preliminary results show that over-expression of *AtEVR1* and *BoEVR1* in *N. benthamiana*, a member of the Solanaceae family, results in *V. dahliae* resistance. These results suggest that Brassicaceae-specific *EVR1* homologs can be used to engineer Verticillium wilt resistance in crops that belong to other plant families.

Introduction

Verticillium species belong to the phylum Ascomycota, which comprises many fungal pathogens. The genus *Verticillium* contains three major plant pathogenic species: *V. dahliae*, *V. albo-atrum*, and *V. longisporum* (Fradin and Thomma, 2006; Klosterman et al., 2009). While *V. dahliae* and *V. albo-atrum* infect over 200 plant species, *V. longisporum* is pathogenic mainly on *Brassicaceae*. The pathogens cause vascular wilt, which is a devastating disease on many economically important crop species such as tomato, potato, cotton, and lettuce, but also on ornamental plants (Agrios, 2005; Fradin and Thomma, 2006; Klosterman et al., 2009). Controlling Verticillium wilt disease is difficult for several reasons: *Verticillium* produces resting structures that can survive in the soil for many years (Rowe and Powelson, 2002), it has a broad host range, and the fungus is difficult to be reached by fungicides once it has entered the xylem tissue. A commonly used control option, crop rotation, is mostly

ineffective for controlling Verticillium wilt disease. Although soil fumigation is effective to control Verticillium wilt disease, use of soil fumigation is not appreciated due to the detrimental effects of the chemicals on public health and the environment. Soil fumigation is also not a preferred method for large scale field application. As a consequence, the preferred method to control Verticillium wilt disease is the use of genetic resistance.

Two distinct races (race 1 and race 2) have been described for *V. dahliae* and *V. albo-atrum* in tomato and lettuce (Fradin and Thomma, 2006; Klosterman et al., 2009). While resistance against race 1 strains has been identified in these two plant species (Schaible, 1951;Fradin et al., 2009; Hayes et al., 2011), no resistance against race 2 has been identified so far. Genetic resistance against Verticillium wilt diseases has also been reported for several other economically important crop species (Pegg, 2002). However, so far the only Verticillium wilt resistance locus that has been cloned and functionally characterized is the tomato *Ve* locus that contains the *Ve1* gene that provides resistance in tomato against race 1 isolates of *V. dahliae* and *V. albo-atrum* (Kawchuk et al., 2001; Fradin et al., 2009).

Recently, it has been shown that transgenic expression of *Ve1* in Arabidopsis provides resistance against *Verticillium* race 1 isolates (Fradin et al., 2011). Over the years, Arabidopsis has increasingly been used as a model host for studying *Verticillium*-host interactions (Veronese et al., 2003; Tjamos et al., 2005; Fradin and Thomma, 2006; Johansson et al., 2006; Ellendorff et al., 2009; Pantelides et al., 2010b). In addition to screening germplasm for resistance (Schaible, 1951; Veronese et al., 2003), mutagenesis followed by screening for enhanced resistance with a pathogen of interest is a means to identify novel resistance traits. Several molecular techniques have been used to generate random mutants in Arabidopsis, such as EMS- and radiation-induced mutation, and transposon and activation tagging. Activation tagging involves the random integration of promoter or enhancer sequences in a genome, using either a T-DNA or a transposon, generally leading to enhanced expression of genes near the integration site and generating gain-of-function mutations (Weigel et al., 2000; Ayliffe and Pryor, 2007; Pereira and Marsch-Martínez, 2011). Insertion of enhancer sequences in the genome may positively regulate gene expression, even when inserted at a considerable distance to the target gene (Lewin, 2008). Some of the advantages of activation tagging over knock-out strategies include that activation tagging generates dominant instead of recessive mutations, it generates viable mutants for those genes where knock-outs would lead to lethal phenotypes and it is also applicable to dissect phenotypes of redundant genes (Pereira and Marsch-Martínez, 2011).

Transposon-based activation tagging has been successfully employed in various plant species to identify novel genes involved in various physiological

processes (Ayliffe and Pryor, 2007), including pathogen defence (Grant et al., 2003; Xia et al., 2004; Aboul-Soud et al., 2009). In an attempt to identify sources of Verticillium wilt resistance using the model plant Arabidopsis, we have screened an activation-tagged Arabidopsis mutant collection with *V. dahliae*. Previously, we have reported the identification of four mutants with enhanced resistance to Verticillium wilt disease (Yadeta et al., 2011). In this chapter, we pursued functional characterization of one of the mutants, A2, and demonstrate that the activation of the *At3g13437* gene, which encodes a protein of unknown function, is responsible for the enhanced Verticillium wilt resistance phenotype. This gene is designated as *Enhancer of Verticillium Resistance 1* (*EVR1)*.

Results

Identification of the *Enhancer of Verticillium Resistance 1*

Previously, we have reported the identification of four activation-tagged Arabidopsis mutants, A1 to A4, that displayed enhanced resistance to Verticillium wilt disease (Yadeta et al., 2011, Chapter 2). Mutant A2 not only displayed resistance to *V. dahliae* (Figure 1), but also to the bacterial vascular wilt pathogen *Ralstonia solanacearum* (Yadeta et al., 2011). In this chapter we investigated this mutant further and determined the position of the activation tag insertion site using thermal asymmetric interlaced PCR (TAIL-PCR) (Liu et al., 1995). The tag was found to be inserted on chromosome 3 at a position 376 bp upstream of the translational start codon of gene *At3g13435*. To identify the gene responsible for the enhanced Verticillium wilt resistance of the A2 mutant, we analysed the expression of genes flanking the T-DNA insertion site to detect transcriptional changes. The analysis of expression of 11 genes spanning a region of ~14 kb upstream to ~17 kb downstream of the activation tag insertion site showed that four of these genes, namely *At3g13432*, *At3g13435*, *At3g13437* and *At3g13445*, were induced in the A2 mutant when compared to wild-type plants (Table 1, Figure S1). Simultaneously, we analysed homozygous knock-out alleles of all genes flanking the activation tag insertion site for Verticillium susceptibility. Interestingly, the knock-out allele of *At3g13437* showed clearly enhanced susceptibility to *V. dahliae* (Figure 2A), whereas none of the other knock-out alleles showed enhanced susceptibility to Verticillium wilt when compared to the Col-0 wild-type (Table 1). Therefore, we tentatively named *At3g13437 EVR1,* for *Enhancer of Verticillium Resistance 1* (*AtEVR1* for *Arabidopsis thaliana Enhancer of Verticillium Resistance 1*).

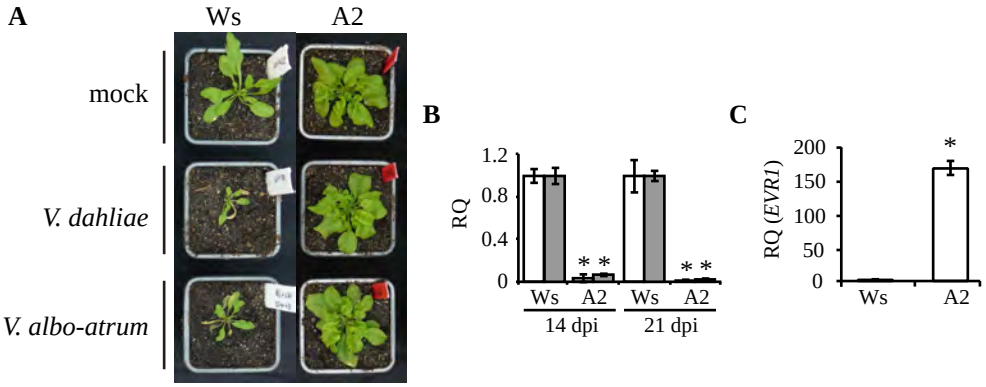


Figure 1: The activation-tagged Arabidopsis mutant A2 is more resistant to *V. dahliae* and *V. albo-atrum*. (A) Typical symptoms of *Verticillium* on the wild-type (Ws) and the activation-tagged mutant A2. Picture was taken at 21 days post inoculation (dpi) and a representative of three independent experimental replicates is shown. (B) Relative quantification (RQ) by real-time PCR of *Verticillium* colonization by comparing levels of the *V. dahliae* (white bars) and *V. albo-atrum* (grey bars) internal transcribed spacer (ITS) region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RubisCo* gene (for equilibration) at 14 and 21 dpi. Bars represent averages with standard deviation of four technical replicates. A representative of three independent experiments is shown. (C) Relative quantification (RQ) of *EVR1* transcription level in the wild-type Ws and the activation-tagged mutant A2. The bar represents the average of three independent experiments and standard deviation of the means and asterisks indicate significant differences (Dunnett t-test at *P=0.01*) compared to the wild-type Ws.

To validate the enhanced susceptibility of *evr1*, we quantified the ratio of rosette leaves showing Verticillium wilt symptoms at 14 and 20 dpi, showing that the percentage of diseased rosette leaves of the *evr1* mutant is significantly higher when compared with wild-type plants (Figure 2B). We further validated the enhanced susceptibility of *evr1* by quantifying *Verticillium* colonization using real-time PCR. As expected, more fungal DNA was detected in *evr1* plants when compared with wild-type plants (Figure 2C). Overall, the gene expression data, combined with the Verticillium wilt phenotyping, strongly suggests that enhanced expression of *EVR1* causes the enhanced Verticillium wilt resistance phenotype of the activation-tagged mutant A2.

We have previously shown that the induction of *AHL19*, which encodes an AT-hook DNA binding protein, is causal to the enhanced Verticillium wilt resistance in the A1 mutant (Yadeta et al., 2011, Chapter 2). To investigate whether *EVR1* over-expression can explain the enhanced Verticillium wilt resistance of the A3 and A4 mutants, we assessed *EVR1* expression in these mutants in absence of pathogen challenge. This analysis showed that *EVR1* is not over-expressed in these mutants (Figure S2), showing that constitutive activation of *EVR1* cannot explain the enhanced Verticillium wilt resistance in the A3 and A4 mutants.

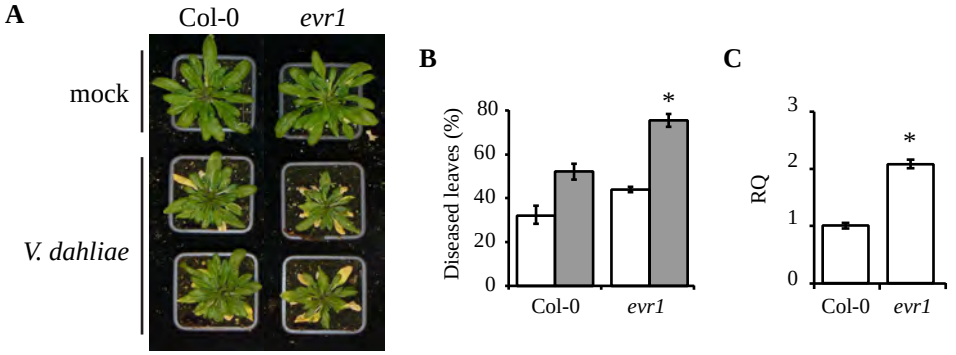


Figure 2: Knocking out *EVR1* enhances Arabidopsis susceptibility to Verticillium wilt. (A) Typical symptoms of *V. dahliae* on the wild-type (Col-0) and *EVR1* knock out (*evr1*) plants. Picture was taken at 21 days post inoculation (dpi) and a representative of three independent experimental replicates is shown. (B) Disease severity score for the wild-type (Col-0) and *evr1* at 14 (white bar) and 21 (grey bar) days post inoculation (dpi). The total number of rosette leaves and the number of rosette leaves that showed Verticillium wilt symptoms were counted at least from eight plants and percentage of the diseased leaves were calculated as an indication of disease severity. The bars represent averages of three independent experiments with standard deviation and asterisks indicate significance differences (Dunnett t-test at *P=0.05*). (C) Relative quantification (RQ) by real-time PCR of *Verticillium* colonization by comparing levels of the *V. dahliae* internal transcribed spacer (ITS) region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RubisCo* gene (for equilibration) at 21 dpi. Bars represent averages with standard deviation of four technical replicates. A representative of three independent experiments is shown.

Table 1. Analysis of the genes flanking the activation-tag insertion site in mutant A2

Gene	**Annotation**	**Knock-out allele**	**Expression[1]**	***Verticillium* phenotype[2]**
At3g13405/03	MicroRNA	SALK_113174C	Not tested	Similar
At3g13410	Unknown protein	None available	Similar	Similar
At3g13420	Zinc finger family	SALK_041147C	Similar	Similar
At3g13430	Zinc finger family	SALK_135697	Similar	Similar
At3g13432	Unknown protein	None available	Similar	Similar
At3g13435	Unknown protein	SALK_091102	Induced in A2 mutant	Similar
At3g13437	**Unknown protein**	**SALK_139498C**	Induced in A2 mutant	**Enhanced susceptibility**
At3g13440	Methyltransferase/ nucleic acid binding protein	SALK_020621	Similar	Similar
At3g13445	TATA binding protein	SALK_084279C	Induced in A2 mutant	Similar
At3g13450	Alpha-keto acid dehydrogenase E1	SALK_042796C	Similar	Similar
At3g13460	ECT2 like (Physically interacts with CIPK1)	SALK_002225C	Similar	Similar

[1] Gene expression in mutant A2 relative to the expression in wild-type.
[2] Phenotype of knock-out alleles upon *V. dahliae* inoculation when compared to wild-type plants.

EVR1 over-expression provides resistance to Verticillium wilt

To corroborate whether the enhanced expression of *EVR1* is causal to the enhanced Verticillium wilt resistance of mutant A2, we generated *EVR1* over-expressing lines in Arabidopsis ecotype Col-0. Similar to the activation-tagged mutant A2, which displays compact and rounded rosette leaves with short petioles (Yadeta et al., 2011, Chapter 2), also *EVR1* over-expressing plants displayed altered plant morphology (Figure 3A, S3A). *EVR1* over-expressing plants show compact, dark green and slightly thicker leaves than wild-type plants with short petioles, and shorter and fewer inflorescences (Figure S4).

Three independent *EVR1* over-expressing lines of the Col-0 ecotype (*EVR1-1*, *EVR1-2*, and *EVR1-3*) were challenged with *V. dahliae* along with *evr1* and wild-type plants. While Col-0 and *evr1* showed clear wilting, chlorosis and stunting symptoms at 14 dpi that were significantly increased by 21 dpi, *EVR1*-expressing plants showed only few symptoms at these time points (Figure 3A; S3A). Furthermore, quantification of fungal colonization *in planta* using real-time PCR showed only little *V. dahliae* biomass in *EVR1*-transgenic lines (Figure 3C). Similar phenotypes were observed on *EVR1* over-expressing lines of the Ws ecotype (Figure S3A, B). These data confirm that enhanced *EVR1* expression is causal to the enhanced Verticillium wilt resistance of the A2 mutant.

Transcriptional regulation of *EVR1*

We showed that constitutive over-expression of *EVR1* enhances Arabidopsis resistance to Verticillium wilt disease (Figure 1A, 3A, S3A). To understand how *EVR1* is regulated at the transcriptional level during the course of the *Verticillium*-Arabidopsis interaction, we performed a time course experiment where we challenged the wild-type Col-0 and Ws plants with *V. dahliae*. Subsequently, we assessed transcription of *EVR1* using real-time PCR. This analysis showed that *EVR1* expression is transiently induced upon *V. dahliae* inoculation in both Col-0 and in Ws ecotypes (Figure S5). Subsequently, the expression level of *EVR1* was assessed in roots and shoots of non-inoculated Ws, A2 mutant and *EVR1-5* mutant plants. Except for *EVR1-5*, which showed slight induction, *EVR1* expression was hardly detected in roots of Ws and mutant A2 whereas in shoots *EVR1* was strongly expressed in mutant A2 and *EVR1-5* when compared with wild-type plants (Figure S6).

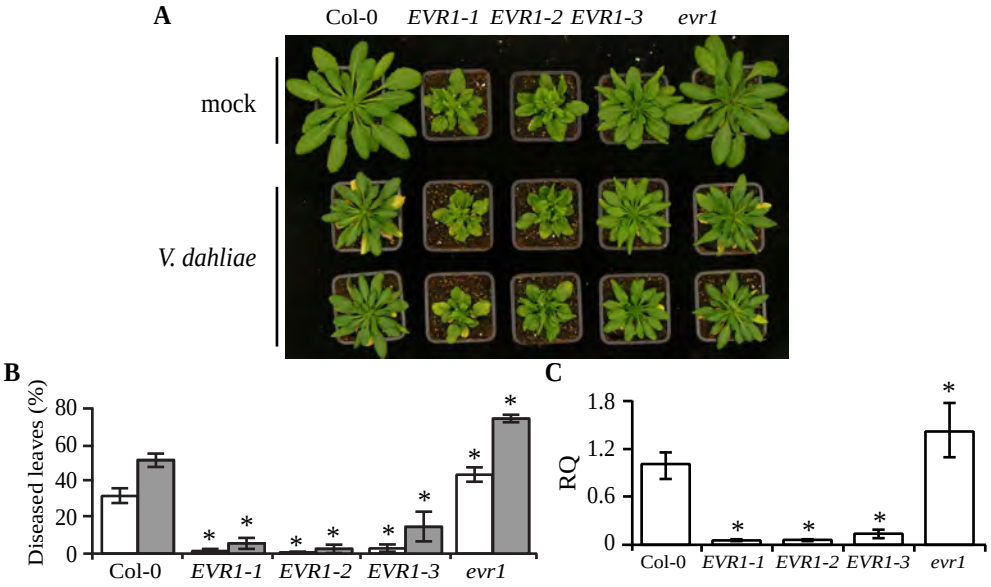
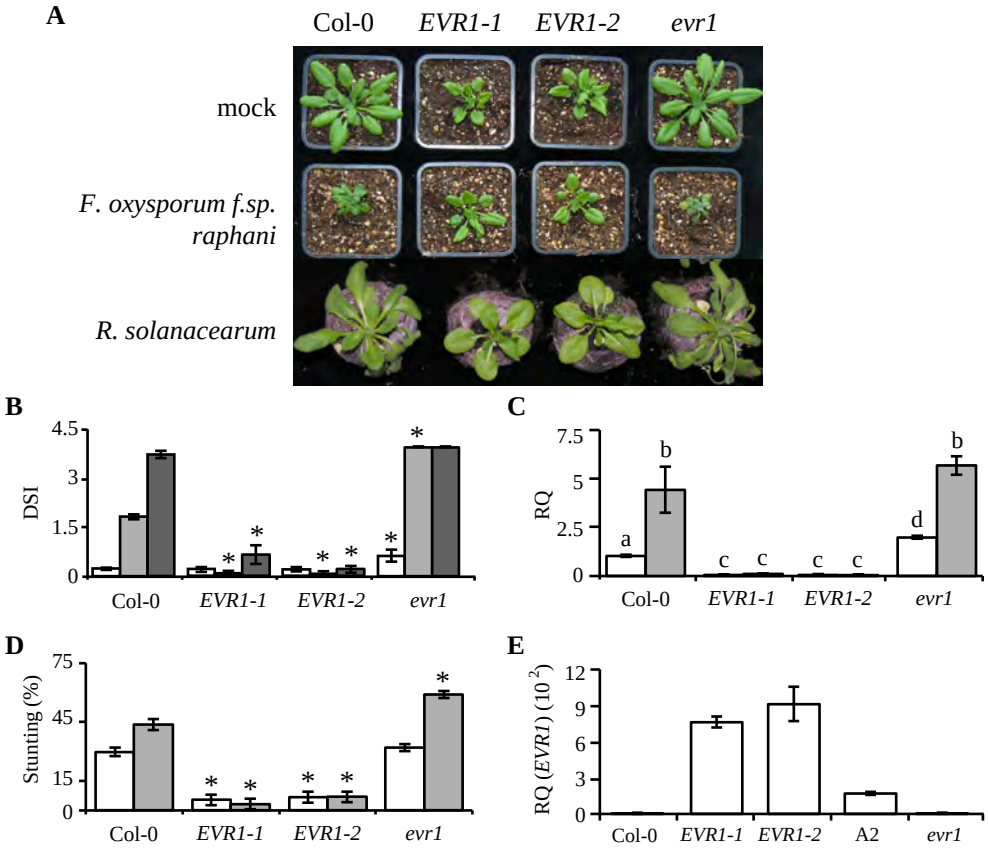


Figure 3: *AtEVR1* over-expressing Arabidopsis plants are resistant to *V. dahliae*. (A) Typical symptoms of *V. dahliae* on the wild-type (Col-0), three *AtEVR1* expressing lines (*EVR1-1*, *EVR1-2*, and *EVR1-3*) and *AtEVR1* knock out line (*evr1*). Picture was taken at 21 days post inoculation and a representative of three experimental replicates is shown. (B) Disease severity score for the wild-type (Col-0), the three *EVR1* expressing lines (*EVR1-1*, *EVR1-2*, and *EVR1-3*) and *AtEVR1* knock out line (*evr1*) at 14 (white bar) and 21 (grey bar) days post inoculation (dpi). The total number of rosette leaves and the number of rosette leaves that showed *Verticillium* symptoms were counted at least from eight plants and percentage of the diseased leaves were calculated as an indication of disease severity. The bars represent the average of three independent experiments with standard deviation and asterisks indicate significance differences (Dunnett t-test at $P=0.05$). (C) Relative quantification (RQ) by real-time PCR of *Verticillium* colonization by comparing levels of the *V. dahliae* internal transcribed spacer (ITS) region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RubisCo* gene (for equilibration) at 21 dpi. Bars represent averages with standard deviation of four technical replicates. A representative of three independent experiments is shown.

Figure 4 (right page): *AtEVR1* over-expression provides resistance to other vascular wilt pathogens. (A). typical disease symptoms caused by *Fusarium oxysporum* f.sp. *raphani* (FOR) and *R. solanacearum* on the wild-type (Col-0), two *AtEVR1* expressing plants (*EVR1-1* and *EVR1-2*) and the *AtEVR1* knock out line (*evr1*) at 12 (*F. oxysporum*) and 5 (*R. solanacearum*) days post inoculation (dpi). The experiment was repeated at least three times and representative of the three replications is shown. (B) Disease severity index (DSI) scores upon inoculation of at least 21 plants with *R. solanacearum* on a scale of 0 (no infection) to 4 (all rosette leaves diseased) at 3 (white bar), 6 (light grey bar) and 10 (dark grey bar) dpi. Bars represent averages with standard deviation of three independent biological replicates and asterisks indicate significant differences ($p = 0.05$). (C) Relative quantification (RQ) by real-time PCR of *R. solanacearum* colonization in wild-type (Col-0), two independent *AtEVR1* over-expressing lines (*EVR1-1* and *EVR1-2*), and of the *AtEVR1* knockout line (*evr1*) by comparing levels of the *R. solanacearum* endoglucanase gene (as measure for Ralstonia biomass) relative to levels of the large



subunit of the Arabidopsis *RubisCo* gene (for equilibration) at 3 and 5 dpi. Bars represent averages with standard deviation of four technical replicates and a representative of three independent experiments is shown. (D) *Fusarium*-induced stunting of wild-type (Col-0) plants, two independent *AtEVR1* over-expression lines (*EVR1-1* and *EVR1-2*) and of the *AtEVR1* knockout line (*evr1*) at 10 and 14 dpi. Rosette diameters of inoculated plants were compared with those of mock-inoculated plants. The bars represent averages of two independent experiments with standard deviation and asterisks indicate significant differences (Dunnett t-test at *P=0.05*). (E) Relative quantification (RQ) of *AtEVR1* transcription in wild-type (Col-0) plants, two independent *AtEVR1* over-expressing plants (*EVR1-1* and *EVR1-2*), the A2 mutant, and of the *AtEVR1* knock out line (*evr1*). Bars represent averages with standard deviation of three biological replicates.

EVR1 provides resistance to other vascular wilt pathogens

To investigate whether the enhanced *Ralstonia* resistance in the A2 mutant can similarly be attributed to *EVR1* over-expression, we challenged two *EVR1* over-expressing lines (*EVR1-1* and *EVR1-2*), along with the corresponding wild-type Col-0 and the *evr1* mutant with *R. solanacearum* strain GMI1000. While Col-0

plants showed mild disease symptoms at 3 dpi which aggravated by 6 dpi (Figure 4 A, B), resulting in death of the inoculated plants by 10 dpi, most rosette leaves of GMI1000-inoculated *evr1* plants showed clear wilting at 3 dpi and completely collapsed by 6 dpi, indicating that *evr1* plants show enhanced susceptibility to *R. solanacearum*. Conversely, *EVR1* over-expressing plants were completely resistant to *R. solanacearum* and did not show any disease symptoms throughout the assay up to 10 dpi (Figure 4A, B). With real-time PCR it was confirmed that the amount of disease symptoms observed on the various genotypes correlates with the degree of *R. solanacearum* colonization (Figure 4C). Hardly any bacterial DNA was monitored in extracts of *EVR1-1* and *EVR1-2* plants, indicating that *EVR1* over-expression provides a high level of *R. solanacearum* resistance.

We have previously shown that the activation-tagged mutant A2 displayed wild-type levels of susceptibility to *Fusarium oxysporum* f.sp. *raphani* (Yadeta et al., 2011, Chapter 2). In this study, we have challenged the *EVR1* over-expressing plants with *F. oxysporum*. While wild-type plants that were inoculated with *F. oxysporum* showed clear wilting of rosette leaves and overall stunting of the plants at 10 dpi which led to a complete collapse of the plants by 14 dpi, *EVR1* over-expressing plants showed enhanced resistance to this pathogen (Figure 4A, D). Inoculated *EVR1-1* and *EVR1-2* plants hardly showed any symptoms of disease throughout the assay. To explain the discrepancy in disease phenotypes between the A2 mutant and the *EVR1* over-expressing lines, we analysed the *EVR1* expression levels in these plants, showing that *EVR1* expression is significantly higher in the over-expression lines when compared to the A2 mutant (Figure 4E).

EVR1 encodes a protein of unknown function

The full-length genomic DNA sequence of *EVR1* consists of 599 bp containing two exons of 97 and 116 bp separated by an intron of 87 bp (Figure 5A). The ORF encodes a protein of 70 amino acids with a predicted molecular mass of 7.93 kDa which is annotated as unknown. With SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Emanuelsson et al., 2007) it is strongly predicted that EVR1 contains a signal peptide of 21 amino acids. Searching for recognizable protein domains or signatures of conserved motifs in the EVR1 protein sequence in publicly available databases did not result in any significant hits.

A *B. oleracea EVR1* homolog provides Verticillium wilt resistance in Arabidopsis

A tblastx search using the nucleotide sequence of *AtEVR1* in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified only three significant hits, all within the Brassicaceae family. The hits were with *Arabidopsis lyrata* (E-value = 1e-15), *Sisymbrium irio* clone SIR-40E09 (E-value= 4e-11) and *Brassica rapa* subsp. *pekinensis* clone KBrH040N18 (E-value = 9e-08) (Figure 5B). No *EVR1* homologs were identified in other plant species, suggesting that *EVR1* is a Brassicaceae-specific gene.

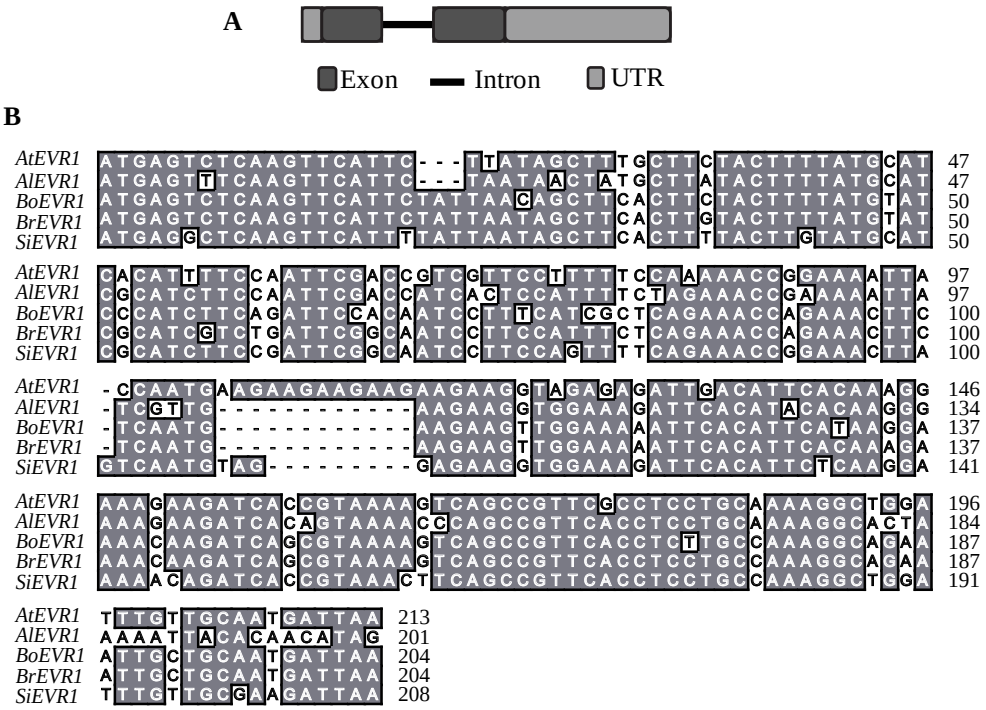


Figure 5: *EVR1* is highly conserved in *Brassicaceae* family. (A) Schematic representation of the full-length genomic DNA sequence of *AtEVR1* gene. (B) Nucleotide sequence alignment of *AtEVR1* and its homologs from *Arabidopsis lyrata* (*AlEVR1*), *Brassica oleracea* var. *gemmifera* (*BoEVR1*), and *Sisymbrium irio* (*SiEVR1*).

Based on the sequence conservation, we designed primers to amplify the *EVR1* homolog from *B. oleracea*, designated *BoEVR1* (for *B. oleraceae Enhancer of Verticillium Resistance 1*), using genomic DNA. We next aimed to test whether *BoEVR1* expression in Arabidopsis provides Verticillium wilt resistance. To this end, *BoEVR1* was amplified from *B. oleracea* cDNA and constitutively expressed

in the Arabidopsis Col-0 and Ws ecotypes. As expected, *BoEVR1* expressing plants displayed similar leaf morphology as *AtEVR1* expressing plants (Figure 6A). Subsequently, *BoEVR1* expressing plants were challenged with *V. dahliae*, revealing enhanced resistance to Verticillium wilt when compared with wild-type plants (Figure 6A, B). Real-time PCR analysis confirmed reduced *Verticillium* colonization on *BoEVR1* expressing plants when compared with wild-type plants (Figure 6C). These data show that the *EVR1* homologs of *Arabidopsis thaliana* and *Brassica oleracea* are functional homologs with respect to their role in providing Verticillium wilt resistance.

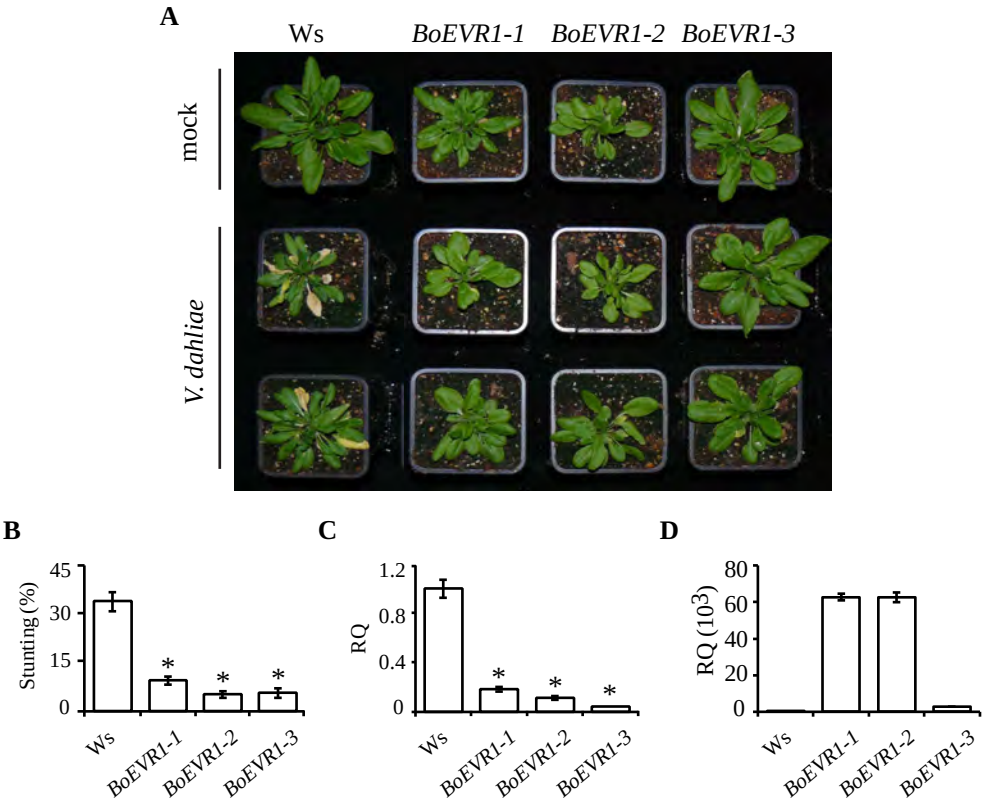


Figure 6: *BoEVR1* over-expression enhances Arabidopsis resistance to Verticillium wilt. (A). Typical disease symptoms caused by *V. dahliae* on the wild-type (Ws) and three independent *BoEVR1* over-expressing plants (*BoEVR1-1*, *BoEVR1-2* and *BoEVR1-3*) at 21 days post inoculation (dpi). The experiment was repeated at least three times and representative of the three independent biological replications is shown. (B) *Verticillium*-induced stunting of wild-type (Ws), three independent *BoEVR1* over-expressing plants (*BoEVR1-1*, *BoEVR1-2* and *BoEVR1-3*) at 21 dpi. Rosette diameters of inoculated plants were compared with those of mock-inoculated plants. The bars represent averages of three

independent experiments with standard deviation and asterisks indicate significant differences (Dunnett t-test at *P=0.05*). (C) Relative quantification (RQ) by real-time PCR of *Verticillium* colonization by comparing levels of the *V. dahliae* internal transcribed spacer (ITS) region of the ribosomal DNA (as measure for fungal biomass) relative to levels of the large subunit of the Arabidopsis *RubisCo* gene (for equilibration) at 21 dpi. Bars represent averages with standard deviation of four technical replicates. A representative of three independent experiments is shown. (D) Relative quantification (RQ) of *EVR1* transcription in wild-type (Ws) and three independent *BoEVR1* over-expressing plants (*BoEVR1-1* and *BoEVR1-2*, and *BoEVR1-3*). Bars represent averages with standard deviation of three biological replicates.

AtEVR1 or *BoEVR1* over-expression in *N. benthamiana* confers Verticillium wilt resistance

Thus far it appears that *AtEVR1* homologs only occur in Brassicaceae plant species. To investigate whether *EVR1* over-expression results in Verticillium wilt resistance in plant species that does not belong to the Brassicaceae, we over-expressed *AtEVR1* and *BoEVR1* in the Australian tobacco species *Nicotiana benthamiana*. This Solanaceous plant species has been used as a model system to study interactions with various plant pathogens (Goodin et al., 2008). Unlike in Arabidopsis, *AtEVR1* or *BoEVR1* over-expression did not cause any obvious changes in the morphology of *N. benthamiana* plants when compared to non-transgenic control plants (Figure 7A, B). We subsequently challenged three randomly chosen *AtEVR1* (*AtEVR1*-a, *AtEVR1-b*, and *AtEVR1-c*) and *BoEVR1* (*BoEVR1-a*, *BoEVR1-b*, and *BoEVR1-c*) T2 lines with *V. dahliae*. Interestingly, in this assay, both *AtEVR1* and *BoEVR1* over-expressing *N. benthamiana* plants showed reduced Verticillium wilt symptoms when compared to inoculated wild-type plants (Figure 7A; B). While the wild-type plants showed severe wilting, stunting, and chlorosis symptoms at 7 dpi, and leaves are completely collapsed by 10 dpi, *AtEVR1* as well as *BoEVR1* over-expressing plants showed only mild wilting symptoms, mainly on the older, lower leaves at 10 dpi (Figure 7A; B). When we counted the number of plants that showed any symptoms of Verticillium wilt disease at 10 dpi, irrespective of the severity of the symptoms, 100% of the wild-type plants showed signs of infection, whereas only 40 to 60% of the *AtEVR1* and *BoEVR1* over-expressing plants displayed *Verticillium* symptoms (Figure 7C). This indicates that *EVR1* homologs from Brassicaceae species can be used to establish Verticillium wilt resistance in non-Brassicaceae plant species.

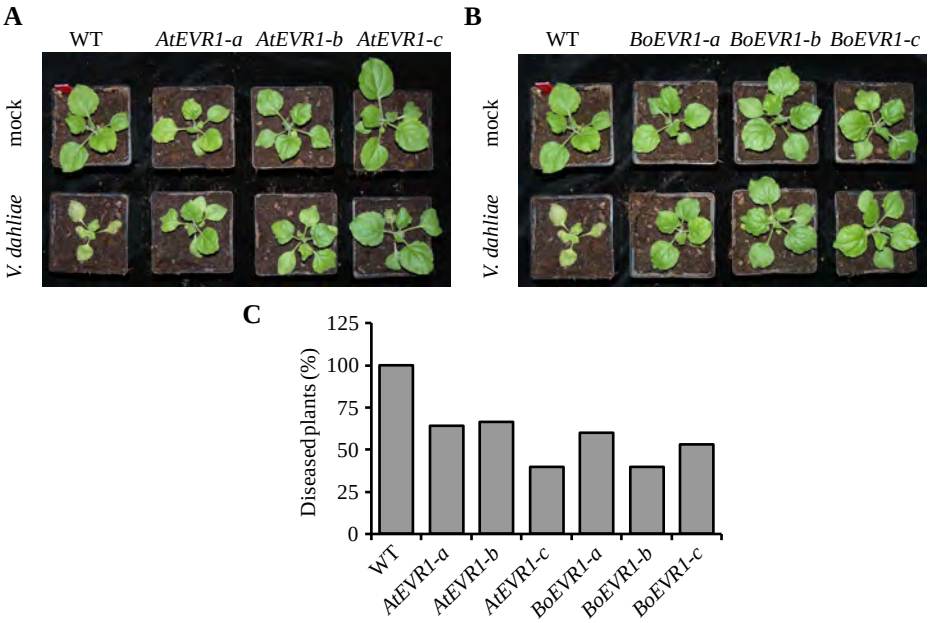


Figure 7: *AtEVR1* or *BoEVR1* over-expression in *N. benthamiana* results in resistance to *V. dahliae*. Typical symptoms of *V. dahliae* on the wild-type (WT), *AtEVR1* (*AtEVR1-a, b, c*) (A) and *BoEVR1* (*BoEVR1-a, b, c*) (B) over-expressing *N. benthamiana* plants. Pictures were made at 10 dpi and the upper and lower rows indicate mock- and *V. dahliae*-inoculated plants, respectively. (C) Percentage of plants (n = 20) that showed clear *Verticillium* symptoms at 10 dpi.

EVR1 over-expression enhances drought tolerance

In addition to enhanced Verticillium wilt resistance, we investigated whether over-expression of *AtEVR1* plays a role in drought stress tolerance. To this end, we tested the drought stress resistance of *EVR1-2*, the Col-0 wild-type and the *evr1* mutant. After 3 weeks of growth with regular watering, we stopped watering the plants and evaluated the response of the lines to drought stress. The assay showed that Col-0 plants and *evr1* mutants started to show wilting symptoms 10 days after the last watering, while the rosette leaves were collapsed after 14 days. In contrast, *AtEVR1* expressing plants did not show any drought symptoms up to 14 days after the last watering (Figure 8). We similarly evaluated the drought stress resistance of two *AtEVR1* over-expressing lines in Ws background along with the Ws wild-type and the activation-tagged mutant A2 and found similar results. *AtEVR1* over-expressing plants and A2 mutant showed enhanced drought tolerance when compared to the wild-type Ws. This indicates that *AtEVR1* over-expression in Arabidopsis enhances drought stress tolerance.

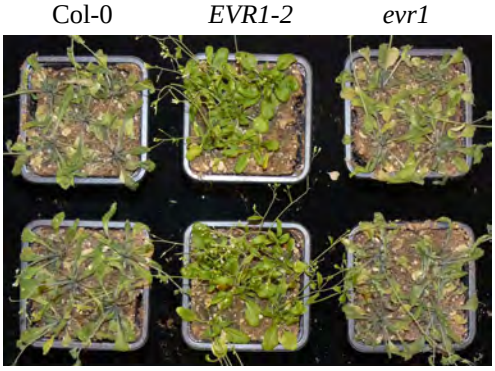


Figure 8: *AtEVR1* over-expressing plants are tolerant to drought stress. Three weeks-old wild-type Col-0, *AtEVR1* expressing line (*EVR1-2*) and *AtEVR1* knock out line (*evr1*) plants were exposed to drought stress and picture was taken at 14 days post drought treatment. The assay was repeated three times and a representative of the replicates is shown.

Discussion

Arabidopsis has increasingly been used as a model host for the identification of Verticillium wilt resistance sources and studying the molecular mechanisms of *Verticillium*-host interactions (Tjamos et al., 2005; Johansson et al., 2006; Ellendorff et al., 2009; Pantelides et al., 2010b; Fradin et al., 2011). In a phenotypic screening for gain-of-function mutants, we have previously reported the identification of four activation-tagged Arabidopsis mutants (A1-A4) which showed enhanced Verticillium wilt resistance (Yadeta et al., 2011, Chapter 2). We have also reported that the specific activation of the gene encoding the AT-hook DNA binding protein AHL19 causes enhanced Verticillium wilt resistance in the A1 mutant (Yadeta et al., 2011, Chapter 2). Here, we cloned the activation-tag insertion site in the A2 mutant that showed enhanced resistance not only to *Verticillium* spp., but also to *R. solanacearum*. Among the 11 genes found within a 31 Kb window spanning the activation tag insertion site, four genes showed induced expression in the A2 mutant, of which *At3g13437* (*AtEVR1*) showed the strongest over-expression when compared to wild-type plants. Intriguingly, only the KO allele of *AtEVR1* showed increased Verticillium susceptibility when compared to wild-type, whereas over-expression of *AtEVR1* in wild-type Arabidopsis provided resistance to *V. dahliae*, *F. oxysporum* and *R. solanacearum*. Interestingly, mutant A2 shows wild-type susceptibility to the necrotrophic foliar pathogens *B. cinerea*, and *P. cucumerina* and to the bacterial foliar pathogen *P. syringae* (Yadeta et al., 2011, Chapter 2). This suggests that *EVR1* over-expression does not lead to overall enhanced plant defence against a wide range of pathogens.

Soil-borne vascular wilt pathogens share several important features with respect to their biology and infection style. The pathogens enter their hosts via

the roots, invade xylem vessels and spread rapidly to the aerial part of the plants (Agrios, 2005; Fradin and Thomma, 2006; Klosterman et al., 2009). Thus, any plant resistance mechanism that prevents root penetration, xylem colonization or systemic spread could potentially contribute to resistance towards vascular wilt pathogens. We previously showed that the A2 mutant has similar root morphology as wild-type plants (Yadeta et al., 2011, Chapter 2), suggesting that *EVR1*-mediated pathogen resistance is not due to altered root morphology. Moreover, the stronger *AtEVR1* induction in shoots than in roots of the A2 mutant suggests that *EVR1*-mediated resistance occurs in shoots rather than in roots. Previous studies in Arabidopsis as well as in tomato have shown that Verticillium resistance is established once the fungus has entered and colonized the xylem vessels (Chen et al., 2004; Fradin et al., 2009; Fradin et al., 2011; Yadeta et al., 2011).

AtEVR1 encodes a mature protein of 49 amino acids with unknown function. Homologs are only found in *Brassicaceae* species, showing high sequence conservation at the C-termini and more diversity at the N-termini. The C-termini of the *B. rapa*, *B. oleracea* and *S. irio* homologs, but not of the *A. lyrata* homolog, contain two adjacent cysteine residues. Although cysteine residues are often implicated in disulphide bond formation to enhance protein stability, the adjacent localization makes intramolecular disulphide bond formation unlikely. However, possibly the cysteine residues might be involved in EVR1 homodimerization.

The search for recognizable protein domains in EVR1 did not result in any significant hits in publicly available databases. The absence of a functional annotation and any known motif or domain in EVR1 complicates the prediction of EVR1 function. The presence of an N-terminal signal peptide, an overall net positive charged (+2), and a relatively high number of hydrophobic amino acids (28%) are typical features that are shared with many antimicrobial peptides (AMPs) (Thomma et al., 2002; Wang and Wang, 2004; Brown and Hancock, 2006). AMPs are found in all living organisms (Hancock and Diamond, 2000; Wang and Wang, 2004). In plants, six different AMPs families have been described, comprising thionins, defensins, lipid transfer proteins, knottins, heveins, and snakins, of which defensins are the largest group and best characterised (Hancock and Diamond, 2000; Thomma et al., 2002; Wang and Wang, 2004; Brown and Hancock, 2006). In Arabidopsis, 825 small cysteine-rich proteins with typical features of antimicrobial peptides have been predicted (Silverstein et al., 2007). Several lines of evidence indicate that AMPs play role in plant defence against viral, bacterial and fungal pathogens (Hancock and Diamond, 2000; Thomma et al., 2002; Wang and Wang, 2004; Brown and Hancock, 2006; Hancock and Sahl, 2006). AMPs are expressed in plants both constitutively and in response to pathogen attack (García-Olmedo et al., 1998; Thomma et al.,

2002). It has been shown that constitutive over-expression of AMPs increases plant defence against bacterial and fungal pathogens. For instance, the constitutive over-expression of the alfalfa defensin (*alfAFP*) in potato provides resistance against *V. dahliae* (Gao et al., 2000). Similarly, constitutive expression of the radish defensin in tobaco and tomato, provides resistance against *Alternaria longipes* and *Alternaria solani*, respectively (Thomma et al., 2002). To know whether EVR1 functions as an AMP, *in vitro* antimicrobial activity can be tested once the protein is obtained in sufficient amounts. Furthermore, determination of the subcellular localization and also interacting partners of EVR1 may give insight into its function.

Vascular wilt symptoms such as wilting, stunting, chlorosis and leaf defoliation are similar to those symptoms caused by drought stress. Indeed, the physical presence of vascular wilt pathogens in the xylem vessels, enzymes secreted by the fungus or plant defence responses may interfere with water transport in the xylem (Cirulli et al., 2010). In potato, it has been shown that Verticillium wilt resistant potato cultivars also show drought stress tolerance (Arbogast et al., 1999). We observed that *AtEVR1* over-expressing plants similarly show drought stress tolerance. Leaf morphology such as size, thickness, and shape has direct implication on water loss through transpiration (Khurana et al., 2008; Yang et al., 2011). *EVR1* over-expressing plants have a smaller leaf size; have thicker and curly leaves than wild-type plants, which all can contribute to the amount of water loss through transpiration. Determining the effect of *EVR1* over-expression on the number of open stomata and the amount of water loss through transpiration in *EVR1* over-expressing plants when compared to the wild-type may provide insight in how *EVR1* regulates drought stress resistance.

Future application

Genetic resistance is nowadays regarded as the preferred method for disease control. For years, it has been a major focus for plant breeders to identify effective and durable resistance to a wide range of pathogens. However, most of the resistance genes identified so far are either race- or species-specific and thus provide resistance to a limited number of pathogens. Thus, to obtain effective and durable resistance, it requires the transfer of multiple resistant genes into a cultivar. Since most AMPs have both antibacterial and antifungal activities and can be used across eukaryotic kingdoms (Terras et al., 1992; Gao et al., 2000; Thomma et al., 2002; Schaefer et al., 2005; Aerts et al., 2007; Thevissen et al., 2007), they can potentially be used for developing effective resistance in plants against a broad spectrum of pathogens. Here we identified an Arabidopsis gene, *AtEVR1*, possibly encoding an AMP, which is effective at least against three vascular wilt pathogens. Moreover, heterologous

expression of *AtEVR1* and *BoEVR1* in *N. benthamiana*, a plant species that belongs to the Solanaceae, confers Verticillium wilt resistance, making *EVR1* a potential gene to control vascular wilt pathogens in Brassicaceae and non-Brassicaceae plant species. However, it needs to be noted that only a single assay was performed on three randomly chosen *AtEVR1* and *BoEVR1* lines of the T2 generation that were not yet evaluated for transgene expression efficiencies. Further characterization of these and additional transgenic lines will have to reveal the robustness of the preliminary phenotypes.

A possible concern with respect to the use of *EVR1* to establish resistance could be the developmental phenotype that *EVR1* over-expression causes in Arabidopsis. One possible way of overcoming this developmental phenotype could be by the use of a pathogen-inducible promoter (Gurr and Rushton, 2005). This will avoid the constitutive expression of the resistance gene, which may negatively affect plant development. The second possibility could be the use of a weak promoter. Indeed we have observed the correlation of *EVR1* expression level with the severity of the developmental phenotype. *EVR1* expression is about 4- and 5-fold higher in *EVR1-1* and *EVR1*-2 plants, respectively, when compared to mutant A2. Similarly, *EVR1-1 and EVR1-2* also show overall smaller plant sizes when compared to mutant A2. Therefore, it would be interesting to determine the *EVR1* expression level at which resistance is still obtained but no, or reduced, developmental phenotypes are observed. The third possibility is to express *EVR1* where and when it is required. Our result so far indicated that *EVR1*-mediated resistance is specific to vascular wilt pathogens. Therefore, targeting *EVR1* either to the roots, where the infection begins, or to vascular tissue might reduce the impact of *EVR1* on shoot development. An encouraging finding with respect to the effect of EVR1 expression on plant development is the preliminary observation that *AtEVR1* and *BoEVR1* expression in *N. benthamiana* resulted in resistance to *V. dahliae* in absence of any developmental aberrations.

Materials and methods

Plant inoculations

Arabidopsis plants and the microbial pathogens *V. dahliae* (isolates JR2, Dvd S26), *V. albo-atrum* (isolate #5431), *V. longisporum* (V10), *F. oxysporum* f.sp. *raphani* (strain #815), *P. syringae* p.v. *tomato* (strain DC3000), and *R. solanacearum* (strain GMI1000 and RD-15) were cultivated and inoculated as reported previously (Yadeta et al., 2011).

Determination of the activation-tag insertion site

The activation-tag insertion site in mutant A2 was determined using thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995). The PCR was performed with a combination of nested primers (Marsch-Martinez et al., 2002) and 10-mer random primers (Terauchi and Kahl, 2000). The secondary and tertiary TAIL-PCRs were separated on 1.2% agarose gel, stained with ethidium bromide, and visualized using the ChemiDoc XRS system (Bio-Rad). Specific product, judged based on the size differences generated by the nested primers, was excised, cleaned using the QIAquick Gel Extraction Kit (QIAGEN), cloned into the pGEM-T Easy Vector (Invitrogen), and sequenced. Blastn search of the TAIR database using the PCR sequences was performed to identify the genomic insertion site. Based on the putative insertion site, the primer pair MPR15F and MPR15R were designed and used to amplify the flanking genomic region. By sequencing this region in the wild-type and the mutant A2, the exact insertion site was determined.

AtEVR1 over-expression

The *AtEVR1* CDS was amplified with the primer pair dMRP15-F1 and dMRP15-R1 that contain *Bam*HI and *Asc*I restriction sites, respectively, using *Pfu* DNA polymerase (Promega). The amplicon was cloned into the *Bam*HI-and *Asc*I-pre-digested binary vector pmk40, a variant of the vector pmog800 (Honée et al., 1998; Fradin et al., 2009). The resulting *P35S::AtEVR1* vector construct was transformed into *A. tumefaciens* strain GV3101 and eventually in to Ws and Col-0 Arabidopsis ecotypes using the floral dip technique (Clough and Bent, 1998).

Cloning of *AtEVR1* homologs

Primer pair EVR1H-BrF0 and EVR1H-BrR1 was used to amplify *BoEVR1* from genomic DNA (gDNA) of *Brassica oleracea* (Brussels sprout). The PCR product was excised from the gel, cleaned (GE Healthcare) and cloned into the pGMET-easy vector (Promega) and sequenced. Based on the sequence alignment of the PCR sequence and the *B. rapa* sequence in the database, primer EVR1H-BrR3 was designed and used in combination with EVR1H-BrF0 to amplify the predicted full length CDS of *BoEVR1* from *B. oleracea* cDNA. As a control, the same primer combination was used to amplify *BoEVR1* from gDNA. The PCR fragments were sequenced to confirm the full length CDS. To generate an *BoEVR1* over-expression construct, the full length CDS of *BoEVR1* was amplified from cDNA using

primer pair EVR1H-BaF1 and EVR1H-AsR1 containing *BamH*I and *Asc*I custom restriction sites, respectively, and cloned into *BamH*I and *Asc*I pre-digested binary vector pB7K40 (Yadeta et al., 2011). Subsequently, the binary vector construct was transformed into *A. tumefaciens* (strain GV3101) and eventually into Arabidopsis ecotypes Ws and Col-0.

Expression of *EVR1* homologs in *N. benthamiana*

In order to test whether expression of *AtEVR1* and *BoEVR1* results in Verticillium wilt resistance in non-Brassicaceae plants as well, the binary vectors containing *AtEVR1* or *BoEVR1* (described above) were transformed into *N. benthamiana*, a Solanaceae family member, following a standard *N. benthamiana* transformation protocol (Wang, 2006). *AtEVR1* and *BoEVR1* transformed calli were selected on Kanamycin (50 µg/ml) and ammonium glufosinate (Basta = 25µg/ml) plates, respectively. After root generation, about 20 independent transformants per constructs were transferred to a soil for seed production. Subsequently, transformants were tested with PCR for transgene using Kanamycin and Basta specific primers, respectively. T2 seeds were harvested and three PCR-positive lines were selected and used in the preliminary *Verticillium* assay. Before inoculation, both the wild-type, *AtEVR1*, and *BoEVR1* over-expressing *N. benthamiana* plants were grown for four weeks in a greenhouse. Subsequently, plants were carefully uprooted, the roots were rinsed in water, and eventually inoculation was performed by root-dipping method as described for tomato and Arabidopsis (Fradin et.al., 2009, Yadeta et al., 2011).

Pathogen quantification *in planta*

Real-time PCR was used for quantification of pathogen colonization *in planta* using an ABI7300 PCR machine (Applied Biosystems) in combination with the qPCR Core kit for SYBR Green I (Eurogentec) and analyzed using the 7300 System SDS software (Applied Biosystems). Unless described otherwise, the primer pair AtRub-F4 and AtRub-R4 targeting the gene encoding the large subunit of *RuBisCo* was used as endogenous control. *Verticillium* colonization was assessed as previously described (Ellendorff et al., 2009; Yadeta et al., 2011).

Expression analysis

Both reverse transcription PCR and real-time PCR were used to analyze gene expression. Unless described otherwise, the primer pair Act2-F2 and Act2-R2

targeting the Arabidopsis *Actin 2* gene was used as endogenous control. A list of primers used in this study and their targets is presented in Table S1. The real-time PCR conditions consisted of 2 min incubation at 50 ºC and 10 min at 95 ºC followed by 40 cycles of 95 ºC for 15 sec. and 60 ºC for 1 min.

Acknowledgements

This research is supported by the Dutch Technology Foundation STW, which is the applied science division of NWO, and the Technology Programme of the Ministry of Economic Affairs. BPHJT is supported by a Vidi grant of the Research Council for Earth and Life sciences (ALW) of the Netherlands Organization for Scientific Research (NWO). We acknowledge the Van Gogh programme for supporting the collaboration with CNRS-INRA in Toulouse, France. We thank Bert Essenstam and Henk Smid for taking care of the plants.

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Supplementary data

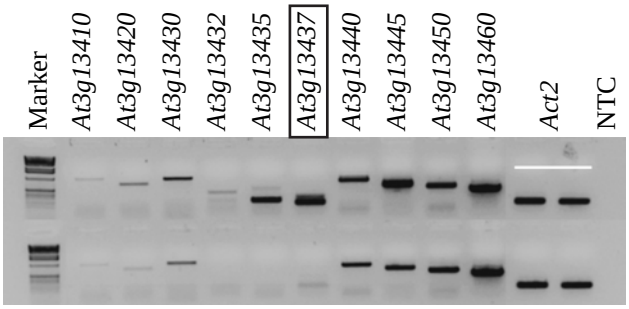

Figure S1. Expression analysis of genes flanking the insertion site in the A2 mutant when compared to wild-type (Ws) plants in absence of pathogen inoculation. The gene encoding *AtEVR1* is boxed (*At3g13437*). Reactions to amplify the *Actin 2* gene and a non-template control (NTC) were included as controls.

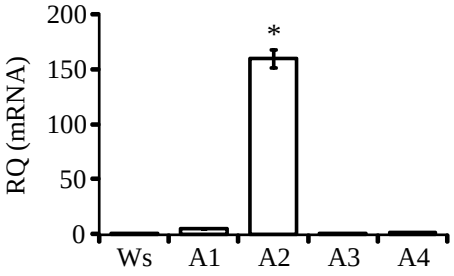

Figure S2. Relative quantification (RQ) of *AtEVR1* transcription in wild-type (Ws) and activation-tagged mutants (A1-A4). Bars represent averages with standard deviation of three biological replicates.

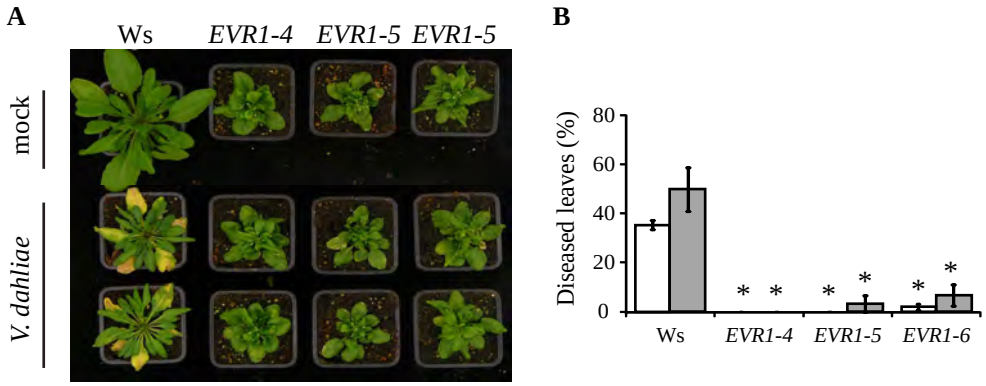

Figure S3. *AtEVR1* over-expressing Arabidopsis plants are resistant to *V. dahliae*. (A) Typical symptoms of *V. dahliae* on the wild-type (Ws) and three independent *AtEVR1* over-expressing lines in Ws background (*EVR1-4*, *EVR1-5*, and *EVR1-6*) at 21 days post inoculation (dpi). Representative of three experimental replicates is shown. (B) Disease severity score for the wild-type (Ws) and three independent *AtEVR1* over-expressing lines in Ws background (*EVR1-4*, *EVR1-5*, and *EVR1-6*) at 14

(white bar) and 21 (grey bar) dpi. The total number of rosette leaves and number of rosette leaves that showed *Verticillium* symptoms was counted at least from eight plants and percentage of the disease leaves were calculated as an indication of disease severity. The bars represent averages of three independent experiments with standard deviation and asterisks indicate significance differences (Dunnett t-test at $P=0.05$).

Figure S4. *AtEVR1* over-expression alters Arabidopsis leaf morphology when compared to the wild-type (Col-0).

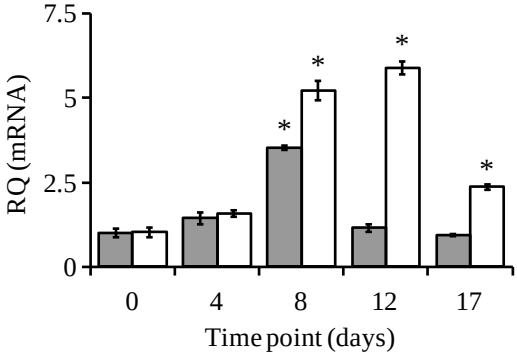

Figure S5. Transcriptional regulation of *AtEVR1* gene during *Verticillium* infection. Relative quantification of *AtEVR1* transcription levels in the wild-type Ws (white bar) and Col-0 (grey bar) plants at 0 (before inoculation), 4, 8, 12, and 17 days post *Verticillium* inoculation. The bars represent average and standard deviation of three technical replicates. Representative of three independent experimental replicates is shown.

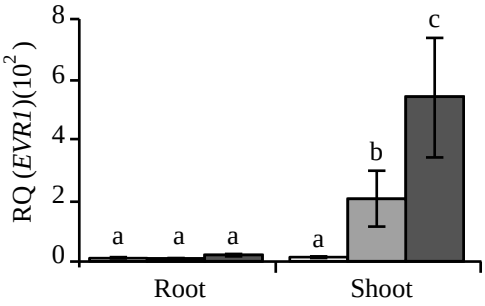

Figure S6. Relative quantification of *AtEVR1* transcription in the root and shoot of non-inoculated wild-type (Ws) (white bar), the activation-tagged mutant A2 (light grey bar) and *AtEVR1* over-expressing line (*EVR1-4*) (dark grey bar). The *AtEVR1* transcript level in the shoot of Ws is set at one and used for calibration. A representative of two independent biological replications is shown and bar indicates average of three technical replicates and standard deviation.

Table S1: Primers used in this study

Primer code	sequence (5' to 3')	purpose
MPR15F	ACCTTGTCTTTTGTATTCACTG	Confirmation of activation tag insertion site
MPR15R	AAGTTTGGAACGAGGCAG	Confirmation of activation tag insertion site
MPR15-F1	GGAGTTTTGTACTTTGCGACG	Confirmation of activation tag insertion site
MPR15-R1	AGTTTGGAACGAGGCAGC	Confirmation of activation tag insertion site
dMRP15-2F1	GCATCACATTTTCCAATTCGAC	*AtEVR1* expression analysis (RT-PCR)
dMRP15-2R1	CATTGCAACAAATCCAGC	*AtEVR1* expression analysis (RT-PCR)
dMRP15-F1	**GGATCC**ATGAGTCTCAAGTTCATTC	*AtEVR1* over-expression construct (*BamH*I underlined)
dMRP15-R1	**GGCGCGCC**TTAATCATTGCAACAAATCC	*AtEVR1* over-expression construct (*Asc*I underlined)
ITS1-F	AAAGTTTTAATGGTTCGCTAAGA	*Verticillium* quantification (Ellendorf et al., 2009)
St-Ve1-R	CTTGGTCATTTAGAGGAAGTAA	*Verticillium* quantification (Ellendorf et al., 2009)
AtRub-F4	GCAAGTGTTGGGTTCAAAGCTGG	*Verticillium* quantification (Yadeta et al., 2011)
AtRub-R4	AACGGGCTCGATGTGGTAGC	*Verticillium* quantification (Yadeta et al., 2011)
EVR1-F1	GTATCACACCAACTGTAATGAGAACG	T-DNA insertion check
EVR1-R1	TTAATCATTGCAACAAATCCAG	T-DNA insertion check
EVR1H-BrF0	ATGAGTCTCAAGTTCATT	Cloning *BsEVR1*
EVR1H-BrR1	CAGAGCTTCTTTTAATCATTGC	Cloning *BsEVR1*
EVR1H-BrR3	TTAATCATTGCAGCAATT	Cloning *BsEVR1*

Primer code	sequence (5' to 3')	purpose
EVR1H-BaF1	**GCAGGATCC**ATGAGTCTCAAGTTCATT	Making *BsEVR1* over-expression construct
EVR1H-AsR1	**ACTGGCGCGCC**TTAATCATTGCAGCAATT	Making *BsEVR1* over-expression construct
Act2-F2	TAACTCTCCCGCTATGTATGTCGC	Arabidopsis *act2* gene (Endogenous control)
Act2-R2	GAGAGAAACCCTCGTAGATTGGC	Arabidopsis *act2* gene (Endogenous control)
dMRP15-1F1	GAATTGGAAGTTGGTTTTGC	Expression analysis
dMRP15-1R1	AGAAATGATCTTCGGTGG	Expression analysis
dMRP15-2F1	GCATCACATTTTCCAATTCGAC	Expression analysis
dMRP15-2R1	CATTGCAACAAATCCAGC	Expression analysis
dMRP15-3F1	AGAGAGTAATCCAATGGACC	Expression analysis
dMRP15-3R1	GATGTCTCTTTGTCCTGG	Expression analysis
dMRP15-4F1	GATTGGAAGGGAGTAATCC	Expression analysis
dMRP15-4R1	TCTGAATTCCGAGAGCAC	Expression analysis
uMRP15-1F1	GTTCTGTTTGATTGCTTCCC	Expression analysis
uMRP15-1R1	CTGAATTTGGACTTGCGG	Expression analysis
uMRP15-2F1	CATCAGAGACTAGCTACTGG	Expression analysis
uMRP15-2R1	GTTCGAACTTGAGTCTGG	Expression analysis
uMRP15-3F1	GCTTTGTGTTTCGTTACG	Expression analysis
uMRP15-3R1	AAGACCTGTGTTGCATTG	Expression analysis
uMRP15-4F1	GTGTTTCTATCTGTGGCC	Expression analysis
uMRP15-4R1	GAATCTTGAGGAGTCTCG	Expression analysis

Chapter 4

Identification and characterization of *Verticillium* race 2 resistance in wild tomato accessions

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Manuscript in preparation

Abstract

Soil-borne vascular wilt diseases caused by species of the *Verticillium* genus are among the most destructive diseases that cause substantial losses in a wide range of economically important crops. Controlling Verticillium wilt disease is difficult because of the long-term persistence of the over-wintering structures in the soil, its broad host range, and inaccessibility of the fungus for most fungicides once it has entered the xylem. Therefore, use of resistant cultivars is the most favourable strategy to combat Verticillium wilt disease. Moreover, genetic resistance is also more effective, economically more attractive and has limited environmental impact when compared to other control options. The only resistance gene identified and functionally characterized so far in tomato against Verticillium wilt disease is the *Ve* locus, which was genetically identified about 60 years ago in wild Peruvian tomato. A few years after the *Ve* locus was introgressed into cultivated tomato cultivars, a *Verticillium* isolate was identified that broke *Ve*-mediated resistance, and since then race 2 isolates are a major threat for tomato production. To identify resistance sources against *Verticillium* race 2 isolates, we have screened 57 wild tomato accessions. From this screen, we found six accessions that showed strong resistance to *Verticillium* race 2 isolates. In contrast, the six accessions showed wild type susceptibility to *V. dahliae* race 1 isolates, indicating that the resistance is race 2- specific. Furthermore, using virus-induced gene silencing we showed that the signalling pathway leading to race 2 resistance in the wild accession is distinct from that providing *Ve1*-mediated race 1 resistance.

Introduction

Vascular wilt diseases caused by species of the *Verticillium* genus are among the most destructive that cause substantial losses in a wide range of economically important crops (Fradin and Thomma, 2006; Klosterman et al., 2009). In some crops, like potato, Verticillium wilt disease causes up to 50% yield loss while in lettuce up to 100% yield loss has been reported (Klosterman et al., 2009). Verticillium wilt is also a major threat for the production of alfalfa, cotton, cucurbits, eggplant, mint, tomato, strawberry, oilseed rape, sunflower and trees (Agrios, 2005; Happstadius et al., 2003).

The genus *Verticillium* consists of four plant pathogenic species: *V. dahliae*, *V. albo-atrum*, *V. longisporum* and *V. tricorpus* (Klosterman et al., 2009). While *V. longisporum* is particularly pathogenic on cruciferous host plants, *V. dahliae* and *V. albo-atrum* infect over 200 different plant species and are often described as the

major plant pathogenic species of the genus (Fradin and Thomma, 2006;Klosterman et al., 2009). *V. tricorpus* is also able to infect several plant species including potato, artichokes, lettuce, snapdragon, potato, *Antirrhinum* spp., mint, cantaloupe, cotton, and various weed species (Qin et al., 2008; Usami et al., 2011; Cirulli et al., 2010). However, *V. tricorpus* causes mild foliar wilt symptoms and is considered a weak plant pathogen. Because of its low plant pathogenic potential, pre-or co-inoculation of *V. tricorpus* with either *V. dahliae* or *V. albo-atrum* reduces the severity of the disease caused by the two aggressive species (Klosterman et al., 2009; Cirulli et al., 2010).

As a soil-borne fungus, *Verticillium* enters its hosts through the roots and colonizes the xylem tissues. Although is not well known how the wilting symptoms are caused by *Verticillium*, it has been reported that the proliferation of the fungus in the xylem and the defence response induced in the host plant to the fungus, such as secretion of gums, gels and tyloses, causes the obstruction of the xylem vessels that eventually interferes with water transport, thereby causing wilting symptoms (Fradin and Thomma, 2006; Báidez et al., 2007). Stunting, chlorosis, necrosis and vascular browning are other typical symptoms of Verticillium wilt disease (Agrios, 2005).

Controlling Verticillium wilt disease is difficult for several reasons: the long-term persistence of *Verticillium* survival structures in the soil, the broad host range of the fungus, and inaccessibility of the fungus for most fungicides once the fungus has entered the xylem. The use of the most effective control measure, soil fumigation, is severely restricted because of its ecologically adverse effects, whereas other agronomic measures such as crop rotation are ineffective due to the broad host range of the pathogen and the persistence of its resting structures in soil for long period of time (Rowe and Powelson, 2002). The development of resistant cultivars is the most favourable strategy to combat plant pathogens in general, and Verticillium wilt disease in particular, because of its efficiency, low cost and limited environmental impact. Genetic resistance against Verticillium wilt has been reported in several crop species such as alfalfa, cotton, potato, tomato, strawberry, sunflower, oilseed rape, lettuce, wild olive and others (Fradin and Thomma, 2006; Klosterman et al., 2009). However, only from tomato a Verticillium wilt resistance locus, the *Ve* locus, has been cloned and functional characterized (Kawchuk et al., 2001; Fradin et al., 2009). The tomato *Ve* locus has been discovered about 60 years ago in the Peruvian wild tomato accession (Schaible, 1951), and comprises two genes, *Ve1* and *Ve2*. Although it was initially reported that both *Ve1* and *Ve2* confer resistance when expressed in potato (Kawchuk et al. 2001), it was subsequently shown that only *Ve1* provides resistance in tomato (Fradin et al., 2009).

After its discovery, the *Ve* locus has been introduced into most commercial

tomato cultivars. However, already a few years later a *Verticillium* isolate that could overcome *Ve* resistance was reported (Robinson et al., 1957). This finding differentiates *Verticillium* isolates into two races based on their avirulence or virulence on tomato containing the *Ve* locus (Bender and Shoemaker, 1984). From this time onwards, *Verticillium* race 2 isolates became the major cause of Verticillium wilt, causing up to 25% yield losses in tomato (Klosterman et al., 2009). No resistance against *Verticillium* race 2 isolates has been reported so far.

In this study, we aimed to identify resistance against *Verticillium* race 2 isolates in wild tomato accessions. To this end, 57 accessions of 9 wild tomato species have been screened, resulting in the identification of six accessions that displayed resistance to race 2 *Verticillium* isolates. These accessions all showed susceptibility towards a race 1 *Verticillium* isolate, suggesting that the resistance traits identified are specific to race 2.

Results

Screening of wild tomato accessions for race 2 resistance

Wild relatives of cultivated plant species are potential sources of resistance against plant pathogens. To explore such sources, we screened a collection of 57 wild tomato accessions with a race 2 *V. dahliae* isolate (Table 1). The accessions belonged to 10 different species: *Solanum pimpinellifolium* (29 accessions), *S. hirsutum* (10 accessions), *S. pennellii* (4 accessions), *S. cheesimanii* (4 accessions), *S. parviflorum* (3 accessions), *S. habrochaites* (2 accessions), *S. chilense* (2 accessions), *S. peruvianum* (1 accession), *S. lycopersicum* (1 accession), and *S. glabratum* (1 accession). Initially, all accessions were challenged with a race 2 *V. dahliae* isolate (Dvd S26) and symptom development was evaluated at different time points up to four weeks post inoculation. As susceptible controls, the race 2-susceptible cultivars Moneymaker and Motelle were included.

Monitoring the inoculated plants at 10 days post inoculation (dpi) revealed that the cotyledons and primary leaves of Moneymaker and Motelle plants showed clear wilting and chlorosis. Furthermore, severe wilting and chlorosis of primary and secondary leaves and also stunting were observed at 14 and 21 dpi. Similar observations were made for 31 wild accessions that showed equal or sometimes even higher susceptibility to the race 2 *V. dahliae* isolate than the susceptible controls. However, 27 accessions showed a varying degree of resistance against the race 2 isolate, with six accessions displaying strong resistance (Table 1). These 27 accessions were sown again, and challenged with the same race 2 isolate. The six

accessions that displayed the strongest resistance in the first screening, namely VG-20, VG-21, VG-51, VG-57, VG-58, and VG-63, again displayed strong resistance when compared with the susceptible control plants, Moneymaker and Motelle (Figure 1A). Of these, VG-20 accession belongs to *S. cheesmanii*, while all others belong to *S. pimpinellifolium*.

Table 1: Wild tomato species screened for resistance to Verticillium race 2 isolate Dvd-S26

Accession	Species	Phenotype*	Accession	Species	Phenotype*
VG-39	*S. pimpinellifolium*	-[a]	VG-38	*S. cheesmanii*	-
VG-40	*S. pimpinellifolium*	-	VG-19	*S. cheesmanii*	-
VG-41	*S. pimpinellifolium*	-	VG-20	*S. cheesmanii*	+++
VG-42	*S. pimpinellifolium*	-	VG-23	*S. cheesmanii*	+
VG-43	*S. pimpinellifolium*	-	VG-17	*S. chilense*	-
VG-44	*S. pimpinellifolium*	-	VG-18	*S. chilense*	-
VG-45	*S. pimpinellifolium*	-	VG-4	*S. glabratum*	-
VG-46	*S. pimpinellifolium*	-	VG-7	*S. habrochaites*	-
VG-47	*S. pimpinellifolium*	-	VG-5	*S. habrochaites*	-
VG-48	*S. pimpinellifolium*	-	VG-13	*S. hirsutum*	-
VG-49	*S. pimpinellifolium*	-	VG-14	*S. hirsutum*	-
VG-50	*S. pimpinellifolium*	-	VG-26	*S. hirsutum*	-
VG-51	*S. pimpinellifolium*	+++[d]	VG-27	*S. hirsutum*	-
VG-52	*S. pimpinellifolium*	-	VG-28	*S. hirsutum*	-
VG-53	*S. pimpinellifolium*	-	VG-29	*S. hirsutum*	-
VG-54	*S. pimpinellifolium*	-	VG-30	*S. hirsutum*	-
VG-55	*S. pimpinellifolium*	-	VG-31	*S. hirsutum*	-
VG-56	*S. pimpinellifolium*	+[b]	VG-32	*S. hirsutum*	-
VG-57	*S. pimpinellifolium*	++[c]	VG-33	*S. hirsutum*	-
VG-58	*S. pimpinellifolium*	+++	VG-6	*S. lycopersicum*	-

Accession	Species	Phenotype*	Accession	Species	Phenotype*
VG-59	*S. pimpinellifolium*	-	VG-10	*S. parviflorum*	-
VG-60	*S. pimpinellifolium*	-	VG-24	*S. parviflorum*	-
VG-61	*S. pimpinellifolium*	-	VG-25	*S. parviflorum*	+
VG-62	*S. pimpinellifolium*	-	VG-35	*S. pennellii*	-
VG-63	*S. pimpinellifolium*	++	VG-36	*S. pennellii*	-
VG-64	*S. pimpinellifolium*	-	VG-8	*S. pennellii*	-
VG-3	*S. pimpinellifolium*	-	VG-15	*S. pennellii*	-
VG-21	*S. pimpinellifolium*	+++	VG-16	*S. peruvianum*	-
VG-22	*S. pimpinellifolium*	-			

[a] the accession is susceptible to *V. dahliae* race 2 isolate
[b, c, d] indicate mild, moderate and strong resistance, respectively
* *V. dahliae* race 2 phenotypes

To further validate the resistance phenotype, 10 plants of each of the six resistant accessions were inoculated with the same race 2 isolate once more and the canopy diameter of the *Verticillium*- and mock-inoculated plants were measured at 14 and 21 dpi. The *Verticillium*-inoculated Moneymaker and Motelle control plants showed on average 40% and 44% stunting when compared with mock-inoculated plants, respectively (Figure 1C). In contrast, five of the wild accessions showed less than 20% reduction in canopy diameter when compared with mock-inoculated plants, while VG-63 showed 26% reduction (Figure 1C). This further confirms the resistance of the wild accessions to the race 2 isolate when compared with Moneymaker and Motelle plants.

Subsequently, the six accessions were challenged with another race 2 *V. dahliae* isolate, Dvd-3. Interestingly, all six accessions also showed enhanced resistance to isolate Dvd-3 when compared to the susceptible control plants Moneymaker and Motelle (Figure 2A).

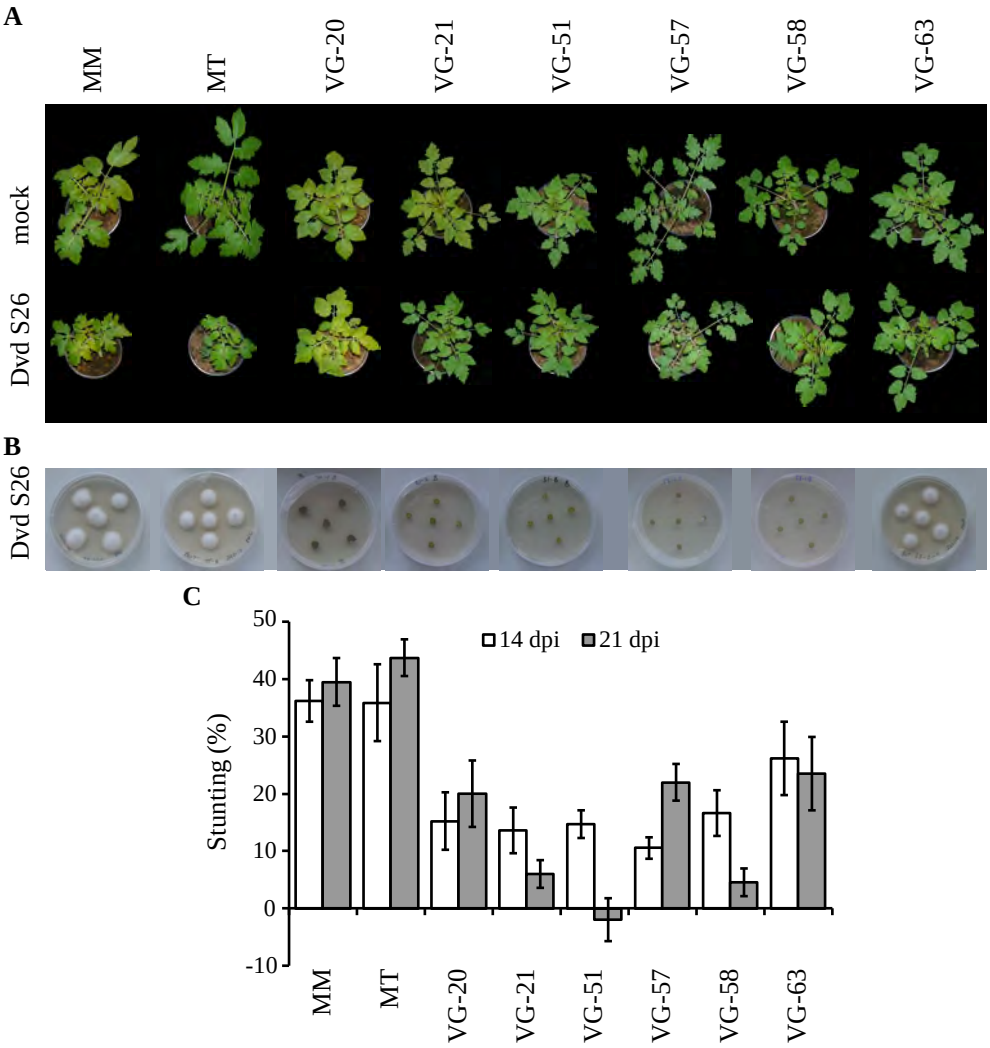

Figure 1. Wild tomato accessions showing enhanced resistance to race 2 *V. dahliae.* (A) Representative pictures of mock- and race 2 (Dvd S26) *V. dahliae*-inoculated Moneymaker (MM), Motelle (MT), VG-20, VG-21, VG-51, VG-57, VG-58, and VG-63 plants taken at 21 days post inoculation (dpi). (B) As a measure of *Verticillium* colonization, 21 dpi stem sections were plated allowing the fungus to grow out of the stem sections. The number of the stem sections from which the fungal outgrowth was observed is a measure of the extent of *Verticillium* colonization. Pictures were taken at 10 days post plating. (C) As a measure for stunting, leaf canopy diameter of mock- and *V. dahliae* race 2-inoculated plants was measured at 14 (white bars) and 21 (grey bars) dpi and the percentage of stunting was calculated relative to mock-inoculated plants. Bars indicate averages of three independent biological replicates with standard errors.

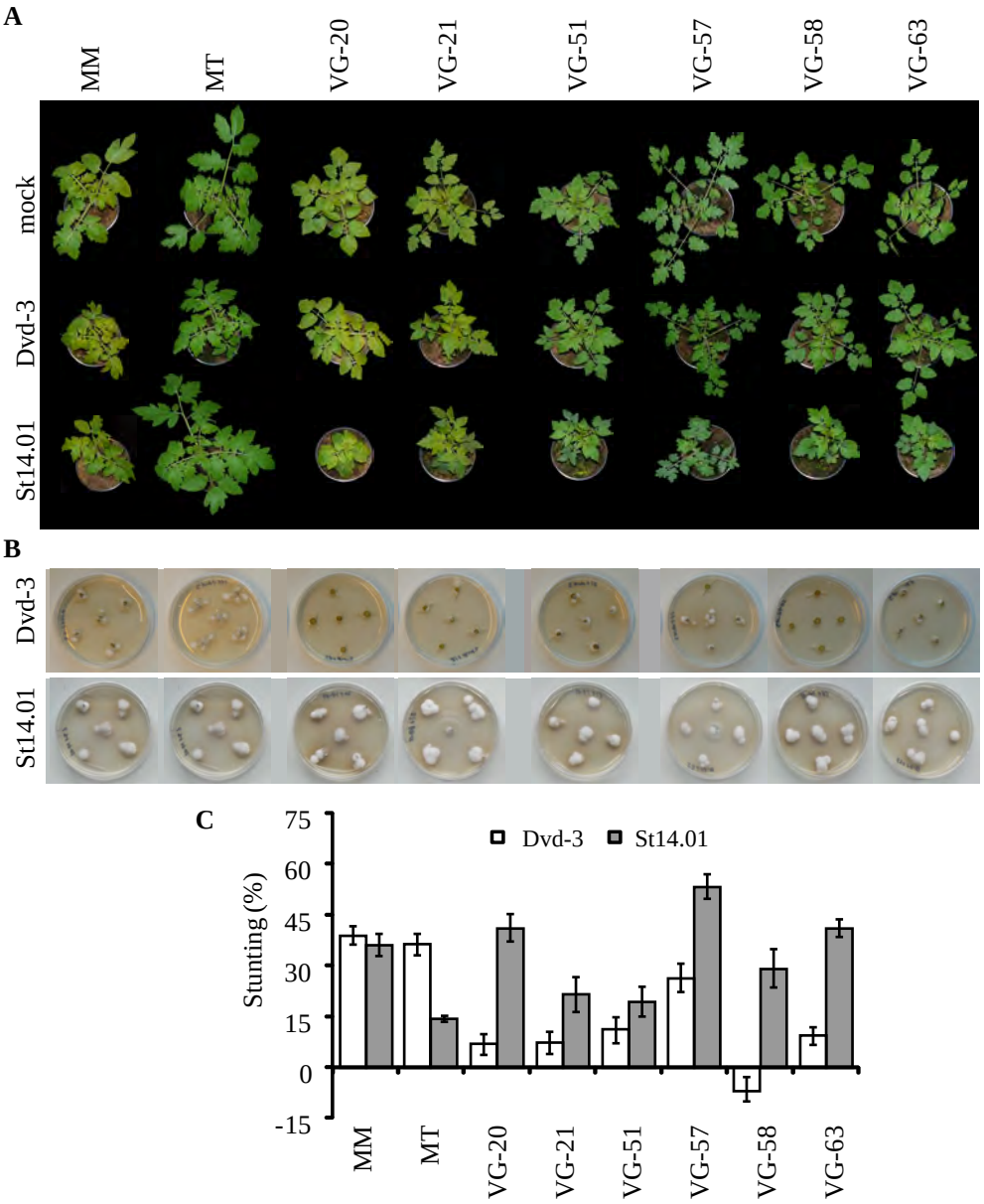

Figure 2. Wild tomato accessions show race-specific resistance. (A) Representative pictures of mock (upper row)-, race 2 (Dvd-3, middle row) - and race 1 (St14.01, bottom row)-inoculated Moneymaker (MM), Motelle (MT), VG-20, VG-21, VG-51, VG-57, VG-58 and VG-63 at 21 days post inoculation (dpi). (B) As a measure of *Verticillium* colonization, 21 days post inoculation (dpi) sections were plated allowing the fungus to grow out of the stem sections. The number of the stem sections from which the fungal outgrowth was observed is a measure of the extent of *Verticillium* colonization and pictures were taken at 10 days post plating. (C) As a measure for stunting, leaf canopy diameter of Dvd-3

(white bar) and St14.01 (grey bar)-inoculated plants were measured and the percentage of stunting was calculated relative to the mock-inoculated plants. Bars indicate the averages of three independent biological replicates with standard error.

Recovery of fungal biomass

To investigate whether the resistance phenotype of wild accessions correlates with reduced fungal colonization, a fungal recovery assay was performed (Fradin et al., 2009). To this end, stem sections of three *Verticillium*-inoculated plants for each of the accessions were harvested, surface sterilized, sliced and placed on PDA plates. While *Verticillium* grew out of most of the stem sections of Moneymaker and Motelle plants, the fungus was also recovered from most stem sections of VG-63 plants (Figure 1B). In contrast, little to no fungal growth out of the stem sections from plants of VG-20, VG-21, VG-51, VG-57 and VG-58 accessions was observed (Figure 1B). The fungal recovery assay was also performed for plants inoculated with the race 2 Dvd-3 isolate and similar results were obtained (Figure 2B). Little to no fungus was recovered from the stem sections of the wild accessions while *Verticillium* grew out from most stem sections of Moneymaker and Motelle plants (Figure 2B). Overall, these data show that the accessions VG-20, VG-21, VG-51, VG-57 and VG-58 show reproducible resistance towards race 2 *V. dahliae*.

Resistance is race 2-specific

To further investigate the Verticillium resistance in the six wild tomato accessions, we challenged these accessions also with a race 1 isolate (JR2) of *V. dahliae*, including the race 1-susceptible cultivar Moneymaker and the race 1-resistant cultivar Motelle as controls. As expected, Motelle plants did not show *Verticillium* symptoms, while Moneymaker plants were severely stunted and showed wilting and chlorosis of the lower leaves (Figure 3A). All six race 2-resistant accessions showed susceptibility to race 1 *Verticillium* when compared with Moneymaker and Motelle plants (Figure 3A), which was confirmed by measurements of the canopy diameter at 10 and 16 dpi (Figure 3C). Furthermore, the fungal recovery assay showed similar recovery from Moneymaker and the wild accessions (Figure 3B). In contrast, little to no fungus was recovered from race 1-inoculated Motelle plants.

To validate race 1-susceptibility of the wild accessions, we challenged these accessions with another race 1 *V. dahliae* isolate, St14.01. All the accessions showed wild type levels of susceptibility to this isolate as well (Figure 2A, bottom row). The fungal recovery assay revealed similar fungal recovery from Moneymaker plants

and the wild accessions, whereas no to little recovery of fungus was observed from Motelle plants (Figure 2B). This shows that the resistance of the wild accessions is indeed race 2-specific.

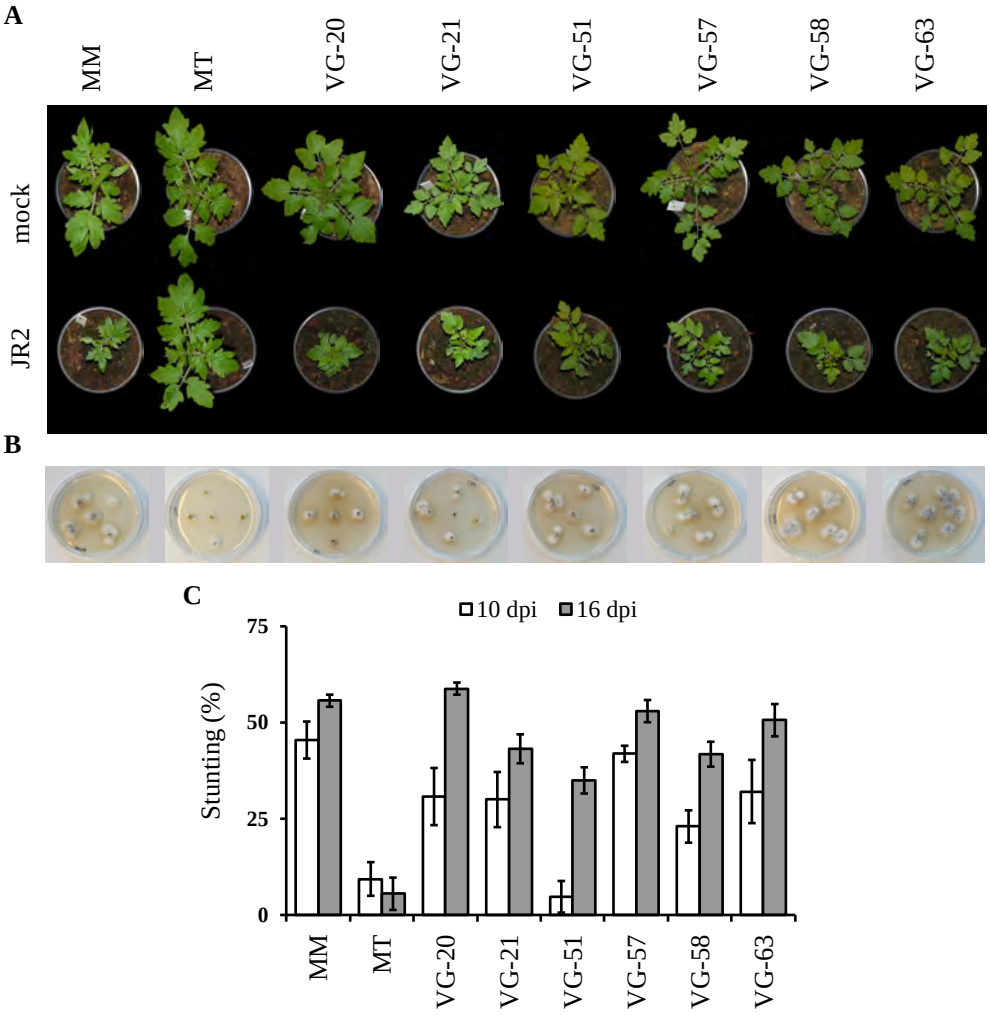

Figure 3. Wild tomato accessions are susceptible to race 1 *V. dahliae*. (A) Representative pictures of mock- and race 1 (JR2) *V. dahliae*-inoculated Moneymaker (MM), Motelle (MT), VG-20, VG-21, VG-51, VG-57, VG-58 and VG-63 plants taken at 16 days post inoculation (dpi). (B) As a measure of *Verticillium* colonization, 16 dpi stem sections were plated allowing the fungus to grow out of the stem sections. The number of the stem sections from which fungal outgrowth was observed is a measure of the extent of *Verticillium* colonization and pictures were taken 10 days post plating. (C) As a measure for stunting, leaf canopy diameter of mock- and *V. dahliae* race 1-inoculated plants was measured at 10 (white bar) and 16 (grey bar) dpi and the percentage of stunting was calculated relative to mock-inoculated plants. Bars indicate averages of three independent biological replicates with standard error.

Ve1-mediated resistance signalling is distinct from race 2 resistance signalling

Recently, a number of genes required for *Ve1*-mediated resistance against race 1 *Verticillium* isolates in tomato has been identified (Fradin et al., 2009). We have selected some of these signalling genes to assess their involvement in race 2 resistance signalling. These include: *NDR1* (Non-race-specific resistance 1), *MEK2* (MAP kinase kinase 2), *NRC1* (NB-LRR protein required for hypersensitive response-associated cell death 1), *EDS1* (Enhanced Disease Susceptibility 1) and *BAK1* (Brassinosteroid-Associated Kinase 1). We furthermore included two newly identified genes that play a role in *Ve1*-mediated resistance; TDF_1-3-1 and TDF_V10-1 (S. Rehman and B.P.H.J. Thomma, unpublished results).

Ten-day-old cotyledons of accession VG-58 were infiltrated with *Agrobacterium tumefaciens* carrying binary TRV constructs to target expression of the candidate genes and two weeks later the plants were inoculated with *V. dahliae* race 2 isolate Dvd S26. Plants treated with recombinant TRV targeting *EDS1*, *NRC1*, *MEK2*, and *TDF_1-3-1* showed clear stunting when compared with mock-inoculated plants, indicating that silencing of these genes compromised race 2 resistance (Figure 4). In contrast, silencing of *NDR1*, *BAK1*, and *TDF_V10-1* did not compromise race 2 resistance. This shows that the signalling pathway that governs race 2 resistance in accession VG-58 is distinct from *Ve1*-mediated resistance signalling.

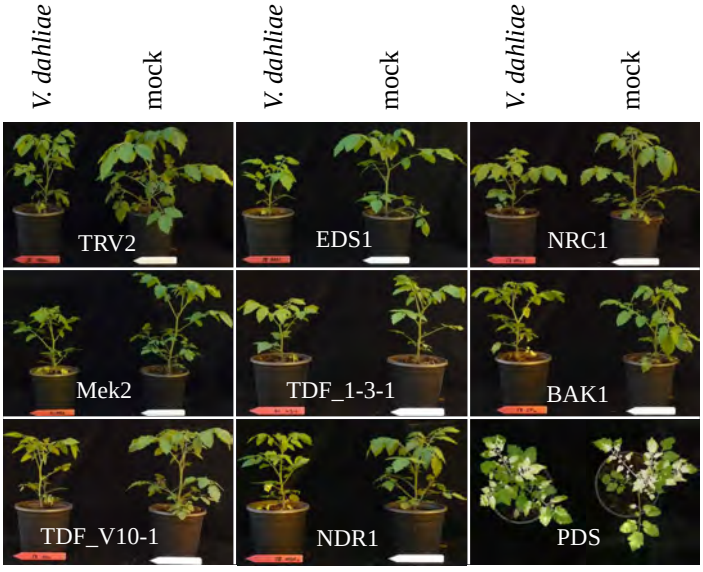

Figure 4. Characterization of race 2 resistance signalling in the wild accessions using VIGS. The *Verticillium* race 2 resistant accession VG-58 was agroinfiltrated with recombinant TRV vectors to target expression of *NRC1*, *NDR1*, *EDS1*, *MEK2*, *BAK1*, *TDF_1-3-1*, *TDF_V10-1* and *PDS*. Two weeks post agroinfiltration, half of the plants were inoculated with a *V. dahliae* race 2 strain (Dvd S26) and the other half was mock inoculated. Two weeks after Dvd S26 inoculation plants were evaluated for stunting and representative pictures were taken. The experiment was repeated three times with similar results.

Discussion

Tomato is one of the most important vegetable crops grown worldwide and used as fresh vegetable or processed product. Vascular wilt fungi, caused by species of the genus *Verticillium*, are among the most devastating diseases of tomato. Although the *Ve* resistance locus, which was identified in the Peruvian wild tomato species, has been transferred to most commercial cultivars and protects these against race 1 isolates, race 2 *Verticillium* isolates have become a major threat to tomato production as yet no resistance against these isolates is present in commercially grown tomato. Various strategies can be exploited to obtain resistance against race 2 *V. dahliae* strains in tomato eventually. In chapters 2 and 3, we have described the identification of resistance sources against Verticillium wilt in the model plant *Arabidopsis thaliana*. In chapter 3 we have furthermore shown that genes encoding such resistance may be transferred to other plant families in order to engineer Verticillium wilt resistance, exemplified by the transfer of *EVR1* into *N. benthamiana*, a member of the family of *Solanaceae* to which also tomato belongs.

In this chapter we describe the screening of wild tomato accessions for *Verticillium* race 2 resistance, leading to the identification of six accessions with a high level of resistance. Except for some slight wilting of the cotyledons and primary leaves of VG-63 at three weeks post inoculation, no symptoms were observed on the remaining accessions, when compared with the susceptible controls (Moneymaker and Motelle) that showed severe wilting symptoms. Plant stunting, which includes reduced plant height and stem thickness and overall reduced leaf canopy diameter, is the most conspicious symptom of Verticillium wilt on tomato. After inoculation with the race 2 isolate, the six wild accessions showed significantly less reduction in overall canopy diameter when compared to Moneymaker and Motelle. We also demonstrated that the reduced symptom development on the wild accessions upon inoculation with race 2 strains is correlated with reduced colonization of the fungus, providing evidence for enhanced resistance in these accessions.

Race 2-specific resistance

For *Verticillium*-host interactions, race-specific resistance has been previously reported in tomato and lettuce (Schaible, 1951; Kawchuk et al., 2001; Fradin and Thomma, 2006; Vallad et al., 2006). In tomato, the *Ve* locus that contains two closely linked genes, *Ve1* and *Ve2*, provides race 1-specific resistance (Kawchuk et al., 2001; Fradin et al., 2009). Similarly, Vallad and colleagues identified race-specific interaction in lettuce (Vallad et al., 2006). Initially, the accessions that are described

in this study were identified by screening for *Verticillium* race 2 resistance. To study race-specificity of the race 2-resistant wild accessions, we challenged the identified six accessions with an aggressive (JR2) and mildly aggressive (St14.01) *Verticillium* race 1 isolates. In contrast to the race 2 isolates, all six accessions showed severe wilting and stunting similar to the susceptible control (Moneymaker) upon inoculation with both race 1 isolates. We confirmed the susceptibility of the accessions to race 1 *Verticillium* by fungal recovery assays. All these data showed that the resistance in wild accessions is specific to race 2 isolates of *Verticillium*. The observed specificity of resistance against race 1 and race 2 strains is highly unexpected. Typically, race-specific resistance is established by distinct plant immune receptors that recognize the presence or activity of particular pathogen effectors (Thomma et al., 2011). In turn, pathogens may exploit several means of avoiding or overcoming recognition by the host, including alteration of the gene that encodes the effector that is recognized by point mutations, frameshifts, gene deletions, and transposon insertions in the coding sequences of effector genes (Stergiopoulos and de Wit, 2009). However, this will only occur in case selection pressure is imposed on the pathogen to overcome resistance upon introduction of genes encoding immune receptors that recognize these effectors. In pathosystems that are characterized by extensive gene-for-gene relationships, such as the interaction between the leaf mould fungus *Cladosporium fulvum* and tomato (Thomma et al., 2005), single pathogen strains typically carry multiple effectors (Avrs and Ecps) that may be recognized by distinct plant immune receptors (Stergiopoulos and de Wit, 2009). As a consequence, resistance against a particular pathogen strain may be established by multiple immune receptors. However, whereas Avrs may carry mutations to avoid recognition, and consequently not all strains are recognized by immune receptors that recognize single Avrs, all strains are still recognized by Cf-Ecp immune receptors as these have not yet been deployed in commercial tomato lines (Laugé et al., 1998; Stergiopoulos et al., 2007). Although the number of strains that is analyzed in this study is limited, the finding that the two race 2 strains are contained on the six wild accessions and are virulent on Ve1 plants, while the two race 1 strains are virulent on the six wild accessions and contained on Ve1 plants is surprising, especially when considering that the race 2 resistance of the wild accessions has, to our knowledge, not yet been deployed in tomato cultivation. Possibly, a genetic interaction between the two effectors exists, preventing simultaneous presence of the two effector genes in a single *V. dahliae* isolate. Alternatively, presence of the race 1 effector is required to overcome the resistance that is provided by the wild accessions. This can be tested by expression of the race 1 effector gene in race 2 strains, which should then lead to loss of race 2 resistance in the wild accessions. The recent cloning of the race 1 effector gene,

named *Ave1* (for *A*virulence on *Ve1* tomato plants; de Jonge et al., 2012), allows performing such an experiment.

Race 2 resistance signalling

We have used VIGS to characterize the mechanism of race 2 resistance signalling in the wild tomato accession VG-58. Previously, a similar approach has been used to identify genes involved in *Ve1*-mediated *Verticillium* race 1 resistance signalling (Fradin et al., 2009). We have selected a number of the defence signalling genes that are required for *Ve1* resistance, namely *EDS1*, *NRC1*, *NDR1*, *MEK2*, *BAK1*, as well as *TDF_1-3-1* and *TDF_V10-1* that have presently unknown functions, and tested their requirement for race 2 resistance signalling. Whereas the Brassinosteroid (BR)-Associated Kinase1 (BAK1) was previously reported to be involved in *Ve1*-mediated resistance signalling (Fradin et al., 2009; 2011), silencing of *BAK1* did not affect race 2 resistance. BAK1 is a receptor-like kinase that has been shown to participate in several immune receptor complexes that are involved in the perception of pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin (Chinchilla et al., 2007; Heese et al., 2007; Kemmerling et al., 2007). Over recent years, BAK1 has been characterized as a master positive regulator of innate immunity mediated by LRR-RLK and LRR-RLP type immune receptors (Monaghan and Zipfel, 2012). Whereas physical interaction between BAK1 and Ve1 has not been demonstrated, BAK1 was found to be required for *Ve1*-mediated resistance in tomato as well as in *Ve1*-transgenic Arabidopsis plants (Fradin et al., 2009; 2011). Nevertheless, apparently race 2 resistance in the wild accessions does not require BAK1, suggesting a different composition of the receptor complex at the plasma membrane. In addition to *BAK1*, also *NDR1* and *TDF-V10-1* did not have an effect on race 2 resistance while they were previously found to be involved in *Ve1*-mediated resistance signalling (Fradin et al., 2009), indicating the differential requirement of signalling components mediating race 1 and race 2 resistance.

We also observed overlapping involvement of signalling components in *Ve1*-mediated resistance and race 2 resistance signalling in accession VG-58. Inoculation of race 2 *V. dahliae* on *EDS1*-silenced VG-58 plants resulted in compromised resistance. In Arabidopsis, it has been shown that EDS1 and NDR1 are differentially required for resistance mediated by different types of R proteins. While EDS1 is required for *TIR-NB-LRR*-mediated resistance, NDR1 is required for *CC-NB-LRR/ LZ-NB-LRR*-mediated resistance (Aarts et al., 1998; Martin et al., 2003;Hu et al., 2005; Wiermer et al., 2005). While EDS1 is involved in *Cf-4*- and *Cf-9*-mediated resistance to *C. fulvum* in tomato, NDR1 is not required for *Cf*-mediated resistance

signalling (Gabriëls et al., 2007). Both EDS1 and NDR1 are required for *Ve1*-mediated resistance signalling in tomato and in *Ve1*-transgenic Arabidopsis plants, which was unexpected since such dual requirement of both components was unprecedented in immune signalling (Fradin et al., 2009; 2011). The finding that EDS1, but not NDR1, is required for race 2 resistance signalling in VG-58 conforms to the generally observed differential requirement of these signalling components for immune signalling, and further confirms the differential requirement of downstream signalling components between *Ve1*-mediated race 1 resistance signalling and the race 2 resistance signalling in the wild accessions. In addition to EDS1, also NRC1, MEK2 and TDF_1-3-1 were found to be involved in *Ve1*-mediated resistance as well as race 2 resistance signalling.

Future perspectives

When compared with Arabidopsis, cloning and functional characterization of novel Verticillium wilt resistance sources from tomato is much more difficult and time consuming. This is due to the longer generation time (a few months for tomato versus a few weeks for Arabidopsis), the considerably larger genome (950 Mb for tomato versus 125 Mb for Arabidopsis), and the more limited availability of genetic tools (mutants, markers, etc.) for tomato as compared with Arabidopsis. VIGS is a well-established method for gene functional analysis in various plant species (Burch-Smith et al., 2004), and has been optimized to determine the role of tomato genes in the interaction with *V. dahliae* (Fradin et al., 2009). With the VIGS experiments described in this chapter, we have shown that race 1 resistance signalling mediated by Ve1 and race 2 resistance in the VG-58 accession differ. Although it is highly likely that the resistance in the six identified wild accessions is mediated by allelic genes, genetic analyses should be performed to prove this hypothesis by analyzing the progeny of reciprocal crosses. Genetic analyses should also reveal the genetic nature of the resistance, and ultimately, cloning of the genes responsible for the resistance will reveal the underlying mechanism of race 2-specific Verticillium wilt resistance. Due to time limitations these analyses have not yet been initiated. However, in order to be employed in commercial practice, the currently identified race 2 resistance can be introgressed into cultivated tomato via conventional breeding.

Ultimately, cloning of the genes that are responsible for the resistance of the wild accessions will help to unveal the mechanism underlying the race 2 resistance. Nowadays, there are several methods for cloning of *R* genes. One of the most commonly used approaches is map-based cloning (Martin et al., 1993;Sharma et al., 2009). However, this approach is often considered expensive, labour intensive,

and time consuming, since it needs determination of tightly linked markers and fine mapping in a large mapping population. Other approaches recently used to clone *R* genes include nucleotide binding site (NBS) profiling (Van Der Linden et al., 2004; Sharma et al., 2009), bulked segregant analysis (BSA) (Michelmore et al., 1991), and the target region amplification polymorphism (TRAP) (Hu and Vick, 2003). These approaches can potentially be used, either separately or in combination, to clone (the) *Verticillium* race 2 resistance gene(s) from the identified wild accessions. Presently, molecular technologies are advancing at an unprecedented rate. Next generation sequencing is among the recently emerged technologies that can generate large genomic and transcriptomic data much more efficiently and also more cost-effective than the traditional sequencing technologies (Varshney et al., 2009). The fast declining costs of genome sequencing will enable to sequence individual genotypes and compare these to identify traits of interest (e.g. disease resistance genes). Furthermore, generation of large genomic and transcriptomic data (RNA-seq) can also greatly increase the accuracy of positioning molecular markers on the genetic and physical maps (Varshney et al., 2009). Obviously, the recently released tomato genome sequence (Tomato Genome Consortium, 2012) will greatly facilitate cloning of the responsible gene(s).

Materials and methods

Plant materials and inoculations

A collection of 57 accessions belonging to 9 wild tomato species were screened for resistance to a race 2 *V. dahliae* isolate. All plants were grown in the greenhouse under previously described conditions (van Esse et al., 2008; Fradin et al, 2009). The *V. dahliae* race 1 isolates JR2 and St14.01, and the race 2 isolates Dvd S26 and Dvd 3 were used. The isolates were cultivated on potato dextrose agar (PDA) for at least one week prior to harvesting of conidiospores. For inoculation, 10-day-old seedlings were carefully uprooted, the roots were rinsed in water and dipped in *V. dahliae* inoculum (10^6 conidia/mL) for 5 minutes. Subsequently, the plants were transferred to fresh soil. Canopy diameter, plant height and stem diameter just above the cotyledon leaves were measured and representative pictures were taken at regular intervals (10, 14/16, 21 days post inoculation; dpi).

For all measurements, canopy diameter, plant height, and stem diameter, four representative *V. dahliae*-inoculated and two mock-inoculated plants were used. An ordinary ruler and Mitutoyo Digimatic Caliper (Thread check inc.) were used to measure the plant height and stem diameter immediately above the cotyledon

leaves, respectively. For measuring the canopy diameter, top-view pictures were taken from *V. dahliae*-inoculated and mock-inoculated plants and measured using imageJ software.

Quantification of fungal biomass

Fungal colonization *in planta* was assessed at 21 dpi with race 2 *V. dahliae* and at 16 dpi with race 1 *V. dahliae*. To this end, stems of three representative plants were harvested and surface-sterilized by sequential treatment for 15 minutes with 70% ethanol and with 10% sodium hypochlorite. After three 5-minute washing steps in sterile water, five stem disks of about 4 mm thick were cut from bottom and top parts and placed on potato dextrose agar (PDA) supplemented with 34 µg/mL chloramphenicol. The plates were incubated at room temperature and pictures of fungal outgrowth were taken after 10 days.

VIGS experiments

All VIGS experiments were performed as described previously (Fradin et al., 2009).

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Chapter 5

General discussion

Introduction

Verticillium wilt diseases cause tremendous losses of crops grown in greenhouses and open fields. So far, genetic resistance is the most preferred strategy to control Verticillium wilt diseases, not only due to its effectiveness and ease of application, but also because of the lack of negative impact on human health and environment. Although genetic resistance against Verticillium wilt diseases has been reported for various plant species, presently the tomato *Ve* locus remains the only resistance locus that has been cloned and functionally characterized (Kawchuk et al., 2001; Fradin et al., 2009; Fradin et al., 2011). The *Ve* locus contains two genes, *Ve1* and *Ve2*, of which only *Ve1* provides resistance against Verticillium wilt disease that is caused by race 1 isolates of *V. dahliae* and *V albo-atrum* (Fradin et al., 2009). Recently, the corresponding *Verticillium* effector that is recognized by the Ve1 protein was identified through comparative genomics of race 1 and race 2 strains (de Jonge et al., 2012). This effector, named Ave1, is a small (134 amino acids), secreted protein that was shown to be required for full virulence on tomato plants lacking the *Ve1* resistance gene (de Jonge et al. 2012). Intriguingly, Ave1 homologs are found in a few plant pathogenic fungi and some of these homologs, including the one of the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici*, are recognized by Ve1. Consequently, *Ve1* was also found to mediate resistance toward *F. oxysporum* in tomato and act as a dual resistance gene (de Jonge et al. 2012).

Considering the wide host range of *Verticillium*, little genetic resistance is available, as in many crops no resistance has been identified at all (Fradin and Thomma, 2006). The tomato *Ve* locus has been reported in 1951 (Schaible et al., 1951), and has widely been used by tomato breeders ever since. However, as early as in 1957, *Verticillium* race 2 isolates have appeared that overcome recognition by Ve1 because they lost the *Ave1* gene (Bender and Shoemaker, 1984; de Jonge et al. 2012). These isolates presently remain the major cause of Verticillium wilt disease in tomato production. For a very long time, no high level resistance, analogous *Ve1*-mediated resistance against race 1 strains, has been reported against race 2 strains (Baergen et al., 1993). A number of years ago the identification of resistance against race 2 *V. dahliae* strains was claimed, but its robustness presently remains unknown and it is not yet available in cultivars (Stamova, 2004).

In order to identify novel sources of resistance against pathogens that can be applied in crop cultivars, several general strategies can be exploited. These include the screening of (wild) relatives of the crop of interest for novel resistance sources, the identification of resistance sources in more distantly related species, or induction of resistance through mutagenesis. In this PhD research, a combination of these

approaches was exploited as mutagenesis in Arabidopsis (with the advantage of relatively easy identification of causal genes once resistance is identified; described in Chapters 2 and 3) was combined with a screening for resistance in wild tomato accessions (with the advantage of relatively easy transfer into cultivars once resistance is identified; described in Chapter 4).

Strategy I: Arabidopsis as a model system to identify novel sources of Verticillium wilt resistance

Arabidopsis has widely been used by plant scientists to investigate various biological, physiological, and genetic processes in plants, including defence against pathogens. The advantages of using Arabidopsis as a model system for molecular studies include the availability of a wealth of omics data, the availability of knock-out mutant collections covering nearly all Arabidopsis genes, straightforward techniques for genetic transformation, the short life cycle and high seed production rate, and the availability of well-advanced bioinformatics and molecular tools that can be exploited for instance for the genetic mapping of particular traits. Arabidopsis has broadly been used to investigate the molecular and genetic bases of the interaction between plants and pathogens (Quirino and Bent, 2003; Gheysen and Fenoll, 2011; Trontin et al., 2011). Nowadays, several genes that are involved in Arabidopsis defence against pathogens have been identified and functionally characterized (Chinchilla et al., 2007; Zipfel, 2009; Wulff et al., 2011). In general, Arabidopsis can be used as a genetic resource to identify resistance genes that can potentially be applied in crop species (Lacombe et al., 2010; Parkhi et al., 2010b; Parkhi et al., 2010a; Wulff et al., 2011), or as a model host to characterize the (molecular) mechanisms underlying immune responses (Gayoso et al., 2010; Pantelides et al., 2010; Fradin et al., 2011; Zhang et al., 2011). So far, Arabidopsis has mainly been used in combination with model pathogens, including the bacterium *Pseudomonas syringae*, the oomycete *Hyaloperonospora arabidopsidis*, and powdery mildew fungi belonging to the *Golovinomyces* genus. For a long time, a model to study vascular wilt diseases in Arabidopsis has been lacking. In the last decade, several research groups have used Arabidopsis to investigate the interaction with *Verticillium* spp. (Johansson et al., 2006; Ellendorff et al., 2009; Häffner et al., 2010; Pantelides et al., 2010; Fradin et al., 2011). Building on convincing data showing that Arabidopsis is a true host for *Verticillium* spp. that can colonize the vascular tissues of this model plant (Ellendorff et al., 2009; Fradin et al., 2011), we have screened a collection of Arabidopsis gain-of-function mutants to identify resistance traits that can contribute to Verticillium wilt resistance. Four mutants (A1 to A4) that

display enhanced resistance to *V. dahliae*, *V. albo-atrum* and *V. longisporum* were identified (Chapter 2, Yadeta et al., 2011). From mutants A1 and A2 the *AHL19* and *EVR1* gene was cloned, respectively, and over-expression of these genes in wild-type Arabidopsis was found to confer robust Verticillium wilt resistance (Chapter 2; Chapter 3; Yadeta et al., 2011). Importantly, expression of *EVR1* in the Solanaceous model plant *Nicotiana benthamiana*, a relative of tomato, was similarly found to confer Verticillium wilt resistance, demonstrating that the strategy of identifying resistance in Arabidopsis that can subsequently be transferred in order to establish resistance in crops can be succesful (Chapter 3). Previously, Veronese and colleagues have identified *VET1*, a gene that confers tolerance to *V. longisporum* by screening various Arabidopsis ecotypes (Veronese et al., 2003). And a similar screening on a segregating Arabidopsis population resulted in the identification of four quantitative trait loci (QTLs) that interfere with the systemic spreading of *V. longisporum*, thereby contributing to Verticillium wilt resistance (Häffner et al., 2010). However, the transfer of these traits to crops is less favourable, as tolerance is not desirable as it still leads to the accumulation of pathogen propagules, and the transfer of resistance based on multi-gene encoded traits across plant families may be complicated.

Although interfamily transfer of especially race-specific *R* genes across species boundaries has generally met little success (Stuiver and Custers, 2001; Hammond-Kosack and Parker, 2003; Gurr and Rushton, 2005; Gust et al., 2010; Wulff et al., 2011), the transfer of genes that determine pathogen resistance across family boundaries is not unprecedented. The transfer of the Arabidopsis immune receptors EFR for bacterial EF-Tu and FLS2 for bacterial flagellin from Arabidopsis to *N. benthamiana* and tomato resulted in resistance to bacterial infections in the recipient hosts (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006; Zipfel et al., 2006; Lacombe et al., 2010). Specifically for *Verticillium* it has been shown that transfer of gene encoding the Ve1 immune receptor from tomato to Arabidopsis resulted in resistance in the latter species (Fradin et al., 2011).

The future of strategy I: Further identification of Verticillium wilt resistance in Arabidopsis

Despite the fact that Arabidopsis is a model organism for which many genetic tools are available, gene cloning is still not straightforward in all cases. The mutants A3 and A4 were shown to display enhanced Verticillium wilt resistance (Chapter 2), but the genes that are responsible for the enhanced resistance in these mutants remain unknown. Several attempts have been made to clone the activation-tag insertion site in these mutants using TAIL-PCR. However, none of the sequences of the fragments

obtained by TAIL-PCR allowed us to determine the exact location of the activation tag insertion site in either of the mutants. Sequences of various TAIL-PCR fragments obtained from mutant A3 showed hits with different transposable elements such as *At4g06574*, *At2g09820*, *At1g37405*, *At5g39060*, *At2g12260*, and *At2g47895*. However, subsequent attempts to confirm the insertion site by amplification and sequencing the flanking chromosomal regions were not successful, which may be due to the repetitive nature of the nucleotide sequences of transposon elements. For mutant A4, TAIL-PCR fragment sequences suggested T-DNA insertion in the *At3g03380* gene on chromosome 3, but also in this case we failed to confirm the insertion by sequencing the chromosomal region flanking the putative insertion site. As it has been demonstrated that neither *AHL19* expression nor *EVR1* expression is affected in the A3 and A4 mutant (Chapters 2 and 3), the enhanced resistance of these mutants must be based on other yet uncharacterized sources, implying that further sources of resistance in addition to *AHL19* and *EVR1* can be identified in this model plant species. Thus, cloning of the genes that are responsible for the enhanced resistance in mutants A3 and A4 is worthwhile, but different strategies than the one employed in this PhD research should be undertaken. Such alternative strategy could be a traditional map-based cloning approach, or whole genome sequencing of the A3 and A4 mutants with next-generation sequencing technologies.

Strategy II: The screening of wild species for novel resistance sources

Wild germplasm is another source of genetic variation that can be used for improving crops for yield, quality, and resistance to biotic and abiotic stresses. More than 80% of the traits obtained from wild species are used in disease and pest resistance breeding (Hajjar and Hodgkin, 2007), indicating that wild germplasms are a rich source for these traits. So far in tomato only, over 40 resistance genes have been identified from wild tomato species (Hajjar and Hodgkin, 2007). To identify resistance against *Verticillium* race 2 strains that can be used in tomato breeding, we have also screened wild tomato germplasm (Chapter 4). Six accessions from the wild tomato species *S. cheesmanii*, and *S. pimpinellifolium* were found to be resistant to race 2 *Verticillium* isolates, while they were susceptible to race 1 *Verticillium* isolates (Chapter 4). Presenly, the genetics of the resistance in these wild tomato accessions remains unknown. In comparison to Arabidopsis, genetic characterization in tomato is more complex and considerably more time comsuming, as less genetic tools are available, and generation times as considerably longer. As a first genetic characterization of the resistance, we have chosen to roughly characterize the signalling pathway that leads

to race 2 resistance by testing the role of candidate genes that have been implicated in race 1 resistance. Whereas in Arabidopsis such analysis would be performed by analysing the progeny of crosses with signalling mutants, only few mutants are available in tomato and crossing takes considerable time. However, VIGS is a well-established transient method for gene functional analysis in tomato (Burch-Smith et al., 2004), and has been optimized for gene functional analysis in the interaction with *V. dahliae* (Fradin et al., 2009). The analysis demonstrated that the signaling pathway used in race 1 and race 2 resistance signaling overlaps only partially (Chapter 4). Unfortunately, due to time limitations, we have not determined the genetic inheritance of the race 2 resistance yet, which requires analyzing the F1 and F2 progenies of crosses of the wild accessions with accessions that are susceptible to race 2 *V. dahliae* strains. Obviously, this information is required to determine the strategy how to introduce the race 2 resistance into cultivars. However, perhaps even more important at this stage is to determine the robustness of the race 2 resistance under field conditions. Eventually, if the resistance is robust under field conditions the genetic inheritance will likely not obstruct introduction into cultivars.

What have we learned about Verticillium resistance in plants?

Arabidopsis can be used not only as a genetic resource to identify sources of resistance against Verticillium wilt disease, but also as a model system to dissect molecular mechanisms of plant resistance, as well as to characterize signalling components involved in the interaction with *Verticillium* (Fradin et al., 2011). It is generally accepted that perception of vascular wilt pathogens occurs when the fungus is in the xylem vessels, likely by the surrounding parenchyma cells that activate physical and chemcical defense responses. These responses are aimed at arresting pathogens in the xylem vessel, preventing spread to adjacent vessels, and eventually elimination of the pathogen or inhibition of its growth. In this PhD research we show that *AHL19* and *EVR1* contribute to xylem defense, as the activity of both genes is observed only after the pathogen has entered the xylem vessel (Chapters 2 and 3). *Verticillium* was determined to be able to colonize both *AHL19* and *EVR1* over-expressing plants, as fungal biomass was detected also in the shoots, demonstrating that both genes do not establish resistance at the level of penetration of either the roots or the xylem. Furthermore, both genes were found to be highly expressed in the shoots of A1 and A2 mutants, respectively, while expression in the roots was low. Also for *Ve1*-mediated resistance it has been found that defense against *Verticillium* is only activated once the fungus has entered the xylem tissue (Chen et al., 2004; Fradin et al., 2009; Fradin et al., 2011). This suggests that *Verticillium* either efficiently avoids or suppresses

its recognition in plant roots, or that recognition of, and defense against, *Verticillium* in plant roots is not very well developed. Intriguingly, the occasional outgrowth of *Verticillium* from stem sections of plants of the six identified race 2-resistant wild accessions after inoculation with race 2 strains suggests that also race 2 resistance occurs at the point of xylem colonization.

Further research is required to determine how the resistance is mediated by the AHL19 and EVR1 proteins. It would be interesting to investigate whether the typical xylem defense responses such as tylose formation, vascular coating, vascular HR or any changes in xylem structure are observed after *Verticillium* infection. The root growth and vascular structure of non-inoculated mutants were not different than of wild-type plants, suggesting that the resistance is not of physical nature. To investigate how the AT-hook DNA binding protein AHL19 establishes *Verticillium* resistance a micro-array analysis was initiated, showing that hundreds of genes that could be assigned to diverse physiological processes, including plant defence against pathogens, were differentially regulated. However, due to the enormous variation in transcriptional regulatuion between biological repeats, no firm conclusions could be drawn from this analysis (unpublished data). Whereas it is more or less obvious that AHL19 will affect the regulation of gene expression, providing a starting point for functional analysis, a lead towards the function of EVR1 is less obvious. Identifying the function of a small protein with no homology to known proteins can be a true quest, as has been experienced by researchers that try for decades to assign biological functions to pathogen effectors; small proteins with little to no homology (de Jonge et al., 2011).

Knowledge transfer: from models to crops

Arabidopsis is unarguably the best studied species of the higher plants. It has widely been used as a model to investigate the biology, physiology, genetics as well as the morphological phenotypes of plant species. Arabidopsis is the first plant species whose genome was fully sequenced (The Arabidopsis Genome Initiative, 2000). Thus, it is also an important resource for functional and comparative genomics studies. Given the increasing world population, and thus the need for more food production, it is very important to apply knowledge generated in Arabidopsis into crop species in order to improve crop performance and eventually increase food production.

Biotic and abiotic stresses are the major factors that cause tremendous crop losses. Today, various Arabidopsis genes that can significantly contribute to both biotic and abiotic stress resistance or tolerance have been identified and functionally

characterized (Jones, 2001; Meyers et al., 2005; Kant et al., 2007; Hirayama and Shinozaki, 2010; Nishimura and Dangl, 2010; Medina et al., 2011). However, so far little of this knowledge has been applied in crops to enhance resistance against plant pathogens, or to increase tolerance to abiotic stresses such as drought, salt, cold and others, to reduce production losses. Various factors might contribute to this slow knowledge transfer from Arabidopsis to crops. These include genetic barriers that complicate transferring traits of interest discovered in Arabidopsis into crop species through conventional breeding. Nowadays, this can be overcome by using transgenic or cisgenic approaches. However, use of genetically modified (GM) crops has raised concerns in society associated with its potential impact on public health and environment (eg. gene flow into natural ecosystems). The effect of GM crops on human health is still controversial although so far there is no clear evidence that show a negative impact of GM crops on human health (Domingo and Giné Bordonaba, 2011). Secondly, biological processes identified in Arabidopsis might function differently in crops species. Nevertheless, recent reports have shown that several biotic and abiotic stress resistance and tolerance genes remain fully functional when transferred between plant families (Karaba et al., 2007; Lacombe et al., 2010; Fradin et al., 2011). Regardless of these limitations, however, there are few reports on traits discovered in Arabidopsis being transferred into crop plants. For instance, *WRR4* is a TIR-NB-LRR class resistance gene that confers resistance to white blister rust disease caused by *Albugo candida* in Arabidopsis. *A. candida* is also a major threat for the production of three important *Brassica* spp.: *Brassica juncea*, *Brassica rapa*, and *Brassica oleracea* (Cooper et al., 2008; Borhan et al., 2010). Interestingly, transgenic expression of *WRR4* in *A. candida*-susceptible *B. juncea* and *B. napus* cultivars confers resistance to this oomycete (Borhan et al., 2010), indicating a potential use of Arabidopsis genes in crops to increase disease resistance. Similarly, various abiotic stress resistance genes discovered in Arabidopsis have successfully been transferred into crops and have shown to improve crop abiotic stress tolerance (Gilmour et al., 2000; Karaba et al., 2007; Zhang et al., 2007; Zhang et al., 2008). The *HARDY* (*HRD*) gene was identified in Arabidopsis via screening of a gain-of-function mutant library and over-expression of *HRD* confers drought and salt tolerance in Arabidopsis (Karaba et al., 2007). Similarly, over-expression of the *HRD* gene in rice increases the drought stress tolerance, water-use efficiency, and the rice biomass production under both stressed and non-stressed conditions (Karaba et al., 2007); also over-expression of an Arabidopsis vacuolar pyrophosphatase *(AVP1*) gene in cotton increases its drought and salt tolerance as well as fibre yield (Pasapula et al., 2011). Hopefully, succesful transfers of traits from Arabidopsis to crops will be identified more often in future as this might contribute to an increase in crop yield,

disease resistance, and the tolerance of crop species to various biotic and abiotic stresses (Silverstone and Sun, 2000; Karaba et al., 2007; Zhang et al., 2007; Zhang et al., 2008; Borhan et al., 2010).

The search for functional homologs of traits discovered in Arabidopsis in target crop species is an alternative strategy to exploit knowledge obtained by the study of Arabidopsis to crops. This approach has been widely employed, and nowadays several functional homologs of Arabidopsis genes have been identified in various crop plant species (Hu et al., 2005; Robatzek et al., 2007; Takai et al., 2008; Le Henanff et al., 2009; Li et al., 2009; Zipfel, 2009). As described in Chapter 3, *BoEVR1* has been identified in *B. oleracea* via searching for homologues using the Arabidopsis *AtEVR1* gene sequence. *BoEVR1* is fully functional in both Arabidopsis and *N. benthamiana*, which supports the suggestion that homologs of resistance genes identified in model plants can potentially be used to engineer resistance in other plant species.

In spite of all the basic knowledge generated in Arabidopsis, so far little of it has been transferred into crops to boost agricultural productivity. Furthermore, knowledge generated in Arabidopsis can also be employed to industrial crops to increase biomass production that is important for instance in biofuel production. Thus, more attention should be devoted to transfer knowledge generated in Arabidopsis into the economically as well as environmentally important plant species.

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Summaries

Summary

Vascular wilt pathogens, which comprise bacteria, fungi and oomycetes, are among the most destructive plant pathogens that affect annual crops as well as woody perennials, thus not only impacting world food and feed production but also natural ecosystems. Vascular wilt pathogens colonize the xylem vessels of their host plants and interfere with the normal transportation of water and nutrients from the roots to upper parts of the plant, thus causing wilting symptoms. The structure and composition of xylem vessels has a significant impact on the colonization of host plants by these pathogens. Presently, genetic resistance is the most preferred control strategy against this group of plant pathogens.

Verticillium wilt disease, which is caused by the vascular fungal pathogen *Verticillium* spp., is among the major diseases in various horticultural crops in tropical, subtropical, and temperate agro-ecological regions. The genus *Verticilllium* comprises of three major plant pathogenic species; *V. dahliae, V. albo-atrum*, and *V. longisporum*. While *V. dahliae* and *V. albo-atrum* are characterized with the ability to infect broad host range, *V. longisporum* has relatively limited host range infecting mainly crucifers family. *V. dahliae* and *V. albo-atrum* isolates are categorized into race 1 and race 2 based on their ability to infect tomato plants containing a *Ve1* resistance gene. On tomato, while race 1 isolates are contained by *Ve1* resistance gene, race 2 isolates overcome *Ve1*-mediated resistance.

Chapter 1 is the introduction to the thesis that describes xylem defence responses that are directed against vascular wilt pathogens. Plants recognize xylem-invading vascular wilt pathogens by using extracellular or intracellular receptors. Pathogen recognition activates innate immune responses that include physical and chemical defense responses in the xylem vessels and the surrounding parenchyma cells. While physical defense responses often halt pathogen movement between vessels, chemical defense responses can eliminate the pathogen or inhibit its growth, thereby leading to resistance.

In order to identify novel sources of Verticillium wilt resistance, a collection of activation-tagged Arabidopsis mutants was screened for plants that displayed enhanced Verticillium wilt resistance. **Chapter 2** describes four mutants (A1 to A4) that showed enhanced resistance to not only *V. dahliae*, but also to *V. albo-atrum*, and the Brassicaceae pathogen *V. longisporum*. Further characterization of resistance in these mutants against other vascular wilt pathogens, *Ralstonia solanacearum* and *Fusarium oxysporum* f. sp. *Raphani*, and the foliar pathogens such *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Alternaria brassicicola*, and *Pseudomonas syringae* pv. *tomato*, is presented in this chapter. Except for mutant A2, that showed enhanced resistance to *R. solanacearum*, and mutants A1 and A3, that showed enhanced

susceptibility to *P. syringae*, all the mutants responded similar as wild-type plants to these pathogens. In chapter 2, we furthermore describe the cloning and functional characterization of the gene encoding the AT-hook DNA-binding protein AHL19 that is responsible for the enhanced resistance of the A1 mutant to Verticillium wilt disease. The Arabidopsis genome contains 29 AHL proteins (Fujimoto et al., 2004) some of which have been implicated in various biological processes including plant development (Lim et al., 2007; Xiao et al., 2009) and defense (Kim et al., 2007; Lu et al., 2010). *AHL19* provides Verticillium wilt resistance upon over-expression, whereas knock-out enhances susceptibility, indicating that *AHL19* positively regulates Verticillium wilt resistance. *AHL19* not only regulates Verticillium wilt resistance, but also affects plant development, as *AHL19* over-expressing plants showed larger leaf size, delayed maturity, and low seed production (Yadeta et al., 2011).

Chapter 3 describes the cloning and functional characterization of *EVR1* (for *Enhanced Verticillium Resistance 1*), the gene that is responsible for the enhanced Verticillium wilt resistance in mutant A2. Mutant A2 furthermore confers resistance to the bacterial vascular wilt pathogen *R. solanacearum* (Yadeta et al., 2011). While *EVR1* over-expression enhances Arabidopsis resistance to three vascular wilt pathogens: *V. dahliae*, *R. solanacearum*, and *F. oxysporum*, knock-out enhances susceptibility to *V. dahliae* and *R. solanacearum*. Furthermore, *EVR1* appears to regulate drought stress resistance. *EVR1* is a single copy gene that encodes a protein of unknown function, and *EVR1* homologs are only found in Brassicaceae species thus far. Interestingly, over-expression of the *B. oleraceae EVR1* homolog in Arabidopsis conferred Verticillium wilt resistance. Moreover, over-expression of the Arabidopsis and *B. oleraceae AtEVR1* and *BoEVR1* in the Solanaceous species *N. benthamiana* enhanced Verticillium wilt resistance. This suggests that the Brassicaceae-specific *EVR1* gene can be used to engineer Verticillium wilt resistance in other plant families.

Whereas chapters 2 and 3 focus on the identification of novel sources of Verticillium wilt resistance by screening a collection of Arabidopsis gain-of-function mutants, **Chapter 4** describes the identification of novel Verticillium wilt resistance in wild tomato accessions. Six wild accessions were identified that displayed enhanced resistance to race 2 isolates. Surprisingly, however, these accessions did not show enhanced resistance to race 1 isolates. Using virus-induced gene silencing, the resistance signalling leading to race 2 resistance in the wild accessions was investigated, showing that the resistance signalling in the wild accessions is distinct from the signalling pathway employed by the resistance protein Ve1.

Finally in **chapter 5**, the highlights of this thesis are discussed and placed in a broader perspective.

Samenvatting

Vaatpathogenen, die zowel bacteriën, schimmels als Oömyceten omvatten, behoren tot de schadelijkste ziekteverwekkers van planten die niet alleen eenjarigen, maar ook meerjarige houtige gewassen aantasten. Daarmee beïnvloeden ze wereldwijd niet alleen de productie van voeding voor mens en dier, maar ook natuurlijke ecosystemen. Vaatpathogenen koloniseren de houtvaten van hun waardplanten en verstoren het transport van water en voedingsstoffen uit de wortels naar de bovenste delen van de plant, waardoor verwelkingssymptomen ontstaan. De structuur en samenstelling van de houtvaten heeft een aanzienlijke invloed op de kolonisatie van waardplanten door deze ziekteverwekkers. Genetische resistentie geniet de voorkeur als methode om vaatpathogenen te bestrijden. Verwelkingsziekten veroorzaakt door de schimmel *Verticillium* behoren tot de belangrijkste ziekten in diverse tuinbouwgewassen in tropische, subtropische en gematigde agro-ecologische regio's. Het geslacht *Verticillium* bestaat uit drie belangrijke plant-pathogene soorten; *V. dahliae*, *V. albo-atrum*, en *V. longisporum*. Terwijl *V. dahliae* en *V. albo-atrum* worden gekenmerkt door de mogelijkheid om een breed gastheerbereik infecteren, heeft *V. longisporum* een beperkt gastheerbereik dat vooral uit kruisbloemigen bestaat. *V. dahliae* en *V. albo-atrum* isolaten worden onderverdeeld in fysio 1 en fysio 2 op basis van hun vermogen om tomatenplanten met het *Ve1* resistentiegen te infecteren. Terwijl fysio 1 isolaten niet in staat zijn ziekte te veroorzaken op tomatenplanten met het *Ve1* resistentiegen, zijn fysio 2 isolaten in staat Ve1-gemedieerde resistentie te doorbreken.

Hoofdstuk 1 is de inleiding tot het proefschrift die de verdedigingsreacties die gericht zijn tegen vaatpathogenen in het xyleem beschrijft. Planten herkennen vaatpathogenen die het xyleem binnendringen door gebruik te maken extracellulaire en intracellulaire receptoren. Herkenning van de ziekteverwekker activeert aangeboren immuunreacties die fysische en chemische reacties in de verdediging van de houtvaten en de omringende parenchymcellen aanschakelen. Terwijl de fysische verdedigingsreacties vaak voorkomen dat het pathogeen nieuwe xyleemvaten binnendringt, kunnen chemische verdediging reacties het pathogeen remmen of verwijderen, en zo resistentie tot stand brengen.

Met het oog op identificatie van nieuwe resistentiebronnen tegen Verticillium verwelkingsziekte, werd een verzameling van zogenaamde “activation-tagged” Arabidopsis mutanten gescreend voor planten die een verhoogde weerstand tegen verwelkingsziekte vertoonden. **Hoofdstuk 2** beschrijft vier mutanten (A1 tot A4) die een verhoogde weerstand hebben, niet alleen tegen *V. dahliae*, maar ook tegen *V. albo-atrum* en *V. longisporum*. Verdere karakterisering van de resistentie in deze

mutanten tegen andere vaatpathogenen pathogenen, *Ralstonia solanacearum* en *Fusarium oxysporum* f. sp. *raphani*, maar ook tegen bladpathogenen zoals *Botrytis cinerea*, *Plectosphaerella cucumerina*, *Alternaria brassicicola*, en *Pseudomonas syringae* pv. *tomato*, wordt beschreven in dit hoofdstuk. Behalve mutant A2, die een verhoogde weerstand tegen *R. solanacearum* vertoont, en mutanten A1 en A3, die een verhoogde gevoeligheid voor *P. syringae* vertonen, reageren alle mutanten op dezelfde wijze als wild-type planten. Hoofdstuk 2 beschrijft bovendien de klonering en functionele karakterisering van het gen dat codeert voor het AT-haak DNA-bindend eiwit AHL19 dat verantwoordelijk is voor de verhoogde weerstand van de A1 mutant tegen Verticillium verwelkingsziekte. Het genoom van Arabidopsis bevat 29 AHL eiwitten (Fujimoto et al., 2004), waarvan een aantal betrokken zijn bij diverse biologische processen, waaronder plantontwikkeling (Lim et al., 2007; Xiao et al, 2009) en afweer (Kim et al., 2007; Lu et al., 2010). Overexpressie van *AHL19* leidt tot resistentie tegen verwelkingsziekte, terwijl uitschakeling van *AHL19* tot verhoogde vatbaarheid leidt. Dit geeft aan dat *AHL19* de weerstand tegen Verticillium verwelkingsziekte positief reguleert. *AHL19* reguleert niet alleen weerstand tegen Verticillium verwelkingsziekte, maar is ook van invloed op plantontwikkeling. Over-expressie van *AHL19* resulteert in grotere bladeren, vertraagde volwassenheid, en lagere zaadproductie (Yadeta et al., 2011).

Hoofdstuk 3 beschrijft de klonering en functionele karakterisering van *EVR1* (voor Enhanced *Verticillium* Resistance 1), het gen dat verantwoordelijk is voor de verhoogde weerstand tegen Verticillium verwelkingsziekte in mutant A2. Mutant A2 vertoont bovendien verhoogde weerstand tegen de bacteriële vaatpathogeen *R. solanacearum* (Yadeta et al., 2011). Terwijl overexpressie van *EVR1* de weerstand van Arabidopsis tegen drie vaatpathogenen verbetert: *V. dahliae*, *R. solanacearum*, en *F. oxysporum*, verhoogt uitschakeling de gevoeligheid voor *V. dahliae* en *R. solanacearum*. Daarnaast blijkt *EVR1* ook weerstand tegen droogtestress te reguleren. Het *EVR1* gen codeert voor een eiwit met onbekende functie. Tot nu toe zijn *EVR1* homologen alleen te vinden in Brassicaceae soorten. Overexpressie van de *EVR1* homoloog uit *B. oleraceae* in Arabidopsis zorgt voor verhoogde weerstand tegen Verticillium verwelkingsziekte, terwijl overexpressie van Arabidopsis en *B. oleraceae EVR1* en *BoEVR1* in *N. benthamiana* ook de weerstand tegen Verticillium verwelkingsziekte verhoogt. Dit suggereert dat het Brassicaceae-specifieke *EVR1* gen kan worden gebruikt om weerstand tegen Verticillium verwelkingsziekte tot stand te brengen in andere plantenfamilies.

Hoofdstuk 4 beschrijft de identificatie van nieuwe bronnen voor resistentie tegen Verticillium verwelkingsziekte in wilde tomatensoorten. Zes wilde accessies zijn geïdentificeerd met verbeterde weerstand tegen fysio 2 isolaten. Echter, deze

accessies tonen geen verbeterde weerstand tegen fysio 1 isolaten. Met behulp van virus-geïnduceerde gen silencing is de signalering die leidt tot weerstand tegen fysio 2 isolaten in de wilde accessies onderzocht, waaruit blijkt dat de signalering verschilt van de signaleringsroute die aangeschakeld wordt door het Ve1 resistentie eiwit.

Tot slot worden in **Hoofdstuk 5** de hoogtepunten van dit proefschrift besproken en in een breder perspectief geplaatst.

Acknowledgements

It would have not been possible to accomplish this PhD thesis without the extraordinary support of a number of people. My first sincere gratitude goes to my daily supervisor and co-promoter, Dr. Ir. Bart Thomma, for his tireless guidance, encouragement, and support all the way through, from the beginning of the project until the end of the thesis defence. Bart, beside your encouragement and educative advice, you also gave me an opportunity to be in “real” science, and I would like to say thank you for bringing me into this world of science. I am also grateful for the complete freedom you gave me in my PhD project, of course with its challenges, and being there whenever I was in need of your assistance, not only for scientific matters but also for my personal life. I really appreciate the personal experiences and knowledge you have shared with me, which have been helpful so far, and will certainly be helpful in my future life and career. Bart, *hedduu galatoomi.*

I would also like to thank my promoter, prof. Pierre de Wit, for his valuable suggestions and comments during the proofreading of my thesis. I am very grateful, especially for the time you took to go through most of my thesis chapters while you were on sabbatical leave in New Zealand.

I am indebted to Sarah van Broekhoven, Mulatu W. Jalata, Patrick Wijten, and Jojanne Peters for their help with my experiments during their MSc thesis research. Besides your hard work, I have also gained a lot of experience and knowledge by working with you. Most of you are currently PhD students, and I wish all of you great successes in your research and future careers.

The Phytopathology department is where I started my career in molecular biology research, meaning that what I know today was acquired in this department, and particularly from the Verticillium group. In this respect, I am very grateful to Peter van Esse, who taught me about the basics of molecular biology during my MSc thesis research and who continued to do so during my PhD research. I also want to express my gratitude to Sajid Rehman. As we have been working on the same project, we had a lot of scientific discussions about the project and even performed a number of experiments together, such as the screening of the wild tomato accessions for *Verticillium* resistance, which is reported in a chapter in this thesis. Sajid, thank you for all the valuable discussions and help in my experiments, as well as for the advice you have given to me about the general social life in the Netherlands, and in particular about the Dutch language course.

Verticillium group members, I must say I was very lucky to be with you guys in the lab. Without you, my stay in the lab for over four years would have not been

that pleasant. I take this moment to say thank you very much for the unforgettable time I had in Wageningen, and particularly in the lab. My special thanks goes to Anja Kombrink, Parthasarathy Santhanam, Andrea Sanchez Vallet, and Daniela Sueldo for the nice diners at your place.

My sincere acknowledgement also goes to all members of the Phytopathology department who, at some point directly or indirectly, were involved in my PhD project as well as in my personal life. Here, I would like to particularly thank Patrick Smit, for his willingness to help me over the weekend with the confocal microscope at a very critical time of my manuscript preparation, which eventually resulted in a publication. I would also like to express my sincere gratitude to Bert Essenstam and Henk Smid for taking great care of my experiments in the greenhouse, and for being so friendly to me.

Emilie Fradin, your involvement in my work starting from the day I stepped into the Phytopathology department. Firstly, as a supervisor of my internship, you taught me how to work with Verticillium, which I have been using during my entire PhD study. Secondly, beside all those scientific discussions and your assistance while you were still in the department, you also offered to help me with the lay-out of my thesis. In short, without your help my thesis would not have been finished in time. Thus, I would like to say thank you very much for bringing all my chapters together during your busiest time and become a thesis. I am so grateful to you and your families and wish you all the best in your future life.

Ronnie de Jonge, we share a lot of memories as colleagues in the scientific field, as well as friends in social matters. Beside here in Wageningen, we had a remarkable and joyful time in Toulouse (France) and in Oromia (Ethiopia). Indeed, your friendship did not end after we were together either in The Netherlands, France, or Oromia, but still continues today when I am across the Atlantic Ocean. Without you, it would have not been so easy to arrange all the administrative stuff for my PhD defence. I appreciate the way handle all my matters like your own, from setting the date for my defence to printing, binding, and submission of my thesis to the promotion office. In fact, you handled all these things for me, while you were extremely busy finishing your own thesis. Ronnie, hartelijk bedankt. I would also like to thank Manon de Jonge for being nice to me, and for all those nice dinners I had at your place. I wish you guys a successful future life and career.

I know there are many more people out there who made my stay in The Netherland so enjoyable and made me feel at home at some point. I am thankful to my friend Ebba Ulfata and his family, Sehani Terefe, and Bontu Akassa. I am also grateful to my fellow friends who are pursuing their PhD or MSc studies at Wageningen University for the special time and dinners we had together. I would

like also to extend my gratitude to all my friends back home, namely Tashome Takele, Meskele Takele, Waqbeka Nagasa and his family, Tashome Oljira and his family, Mengistu Tulu and his family, and Negase Adugna for their encouragement, and moral support. I am especially indebted to Hailu Gudeta and his girlfriend Yeshimebet Milkessa for their uninterrupted friendship and moral support during my study (*Hedduu galatooma*).

Lastly, but not least, I would like to thank my family; my mother, my brothers, and sisters for their encouragements and moral support. Special thanks to my brother Dereje Abdissa for his courage and financial support, starting with my early college studies, till I studied overseas. I am also grateful to my nephew Akassa Dereje for your being a great link between me and my family as well as for all the responsibilities you have been bearing on behalf of me.

I always recall my father telling me that his dream was to send all his children to school and see them graduating from University. Though your dream is accomplished, it is unfortunate that he left us so early without being able to enjoy the outcome of his hard work. This thesis is dedicated to the memories of my father Abdissa Yadeta.

Curriculum vitae

Koste Abdissa Yadeta was born on the 8th of January 1978 in Wollega, Oromia, Ethiopia. After completing his high school studies in 1997, he joined Hawassa University, Hawassa, Ethiopia, for his BSC study. He obtained his BSc degree in Agricultural science, with specialization in Plant production and dry land management, in 2001. Subsequently, he was employed by the Ethiopian Agriculture Research Organization and assigned to the Bako National Maize research project as an assistant researcher in the maize breeding section. In August 2005, he received a scholarship offered by NUFFIC (Netherlands organization for international cooperation in higher education) and joined Wageningen University for his MSc study on Plant Sciences. He obtained his MSc degree in Plant Biotechnology with specialization in plant pathology and pest control. Koste performed his MSc thesis in the department of Phytopathology under the supervision of Dr. Ir. Bart Thomma on the topic 'The role of the effector protein Avr2 in *Cladosporium fulvum* virulence', a fungal plant pathogen that causes tomato leaf mould. Subsequently, Koste performed his internship in the same department on the topic 'Pathogenic and molecular characterization of a collection of *Verticillium* isolates', a fungal species that causes vascular wilt disease and topic of this PhD thesis.

After finishing his MSc, Koste performed his PhD study from September 2007 to December 2011 in the department of Phytopathology of the Wageningen University on the project entitled 'Identification of traits conferring resistance against *Verticillium* species using Arabidopsis and tomato as a model plant' that was funded by Technology Foundation STW.

Since December 2011, Koste is working as a postdoctoral researcher in the laboratory of Dr. Gitta Coaker at the department of Plant Pathology of the University of California, Davis, USA.

Koste A. Yadeta,
October 15, 2012

Publications

Van Esse, H.P., Van't Klooster, J.W., Bolton, M.D., Yadeta, K.A., Van Baarlen, P., Boeren, S., Vervoort, J., de Wit, P.J.G.M., Thomma, B.P.H.J. (2008). The *Cladosporium fulvum* virulence protein Avr2 inhibits host proteases required for basal defense. *Plant cell* **20**, 1948-1963.

Yadeta, K.A., Hanemian, M., Smit, P., Hiemstra, J.A., Pereira, A., Marco, Y., Thomma, B.P.H.J. (2011). The *Arabidopsis thaliana* DNA-binding protein AHL19 mediates Verticillium wilt resistance. *Molecular plant-microbe interactions* **24**, 1582-1591.

Denancé, N., Philippe, R., Nicolas, O., Xavier, B., Develey-Rivière, M.P., Yadeta, K.A., Laurent, H., Perreau, F., Gilles, C., Alessandra, M.G., van den Berg, G., Savelli, B., Fournier, S., Aubert, Y., Pelletier, S., Thomma, B.P.H.J., Molina, A., Jouanin, L., Marco, Y., Goffner, D. (2012). Arabidopsis *wat1* (*walls are thin1*)-mediated resistance to the bacterial vascular pathogen, *Ralstonia solanacearum*, is accompanied by cross-regulation of salicylic acid and indole metabolism. *In press.*

de Jonge, R., Bolton, M.D., Kombrink, A., Yadeta, K.A., van den Berg, G.C.M., Thomma, B.P.H.J. (2012). Chromosome plasticity drives asexual genome evolution; birth of pathogen effector genes. *Manuscript submitted.*

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Koste A. Yadeta
Date: 15 October 2012
Group: Phytopathology, Wageningen University & Research Centre

1) Start-up phase	*date*
▸ **First presentation of your project**	
Identification of traits confering resistance against race 2 isolates of *Verticillium* spp. using Arabidopsis and tomato	Dec 04, 2007
▸ **Writing or rewriting a project proposal**	
▸ **Writing a review or book chapter**	
▸ **MSc courses**	
▸ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1,5 credits**
2) Scientific Exposure	*date*
▸ **EPS PhD Student Days**	
EPS PhD student day, Wageningen University	Sep 13, 2007
EPS PhD student day, Naturalis Museum, Leiden	Feb 26, 2009
EPS International Retreat of PhD Students in Experimental Plant Sciences, Cologne (Germany)	Apr 15-17, 2010
▸ **EPS theme symposia**	
EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten day, Utrecht University	Jan 22, 2009
EPS Theme 2: Interactions between Plants and Biotic Agents & Willie Commelin Scholten Day, University of Amsterdam	Feb 03, 2011
▸ **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW Platform Molecular Genetics annual meeting	Sep 18-19, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW Platform Molecular Genetics annual meeting	Oct 15-16, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
▸ **Seminars (series), workshops and symposia**	
Seminar of Dr. Anne Obourm, John Innes Center, UK : 'The evolution of metabolic diversity in plants'	Sep 12, 2007
Seminar of Dr. Nicholas Provart, University of Tronoto: Raising the BAR for Arabidopsis Research: Using Large-scale Data Sets for Hypothesis Generation	Jun 13, 2008
Seminar of Dr. Henk-jan Schoonbeek, Universite Fribourg, Switzerland: Attack and defence in Botrytis cinerea pathogenicity: ABC transporters, camalexin and oxalic acid.	Sep 03, 2008
Seminars of Dr. Richard Oliver, Murdoch University in Murdoch, Western Australia: Genomics of Stagonospora nodorum; Genes, Genomes and Growers	Oct 16, 2008
Seminars of Dr. James M. Bradeen, University of Minnesota : Potato Pathology and Genomics Potato Pathology and Genomics	Oct 16, 2008
Seminars of Prof.dr. T. Nürnberger, University of Tübingen, ZMBP - Plant Biochemistry, Germany: Patterns and receptors in plant immunity	Dec 17, 2008
Seminar of Dr. Pieter van West, University of Aberdeen: Saprolegnia parasitica an oomycete with a fishy appetite, new challenges for an old problem	Feb 03, 2009
Seminar of Dr. Rays H.Y. Jiang, Broad Institute: Host-pathogen interaction drives genome plasticity in animal and plant pathogens	Jun 10, 2009
Seminar by Dr. Prof.dr. Ton Bisseling, Molecular biology and Prof.dr. Harro Bouwmeester, plant physiology, Wageningen university: Plant Sciences Seminar.	Sep 08, 2009
Plant Sciences Seminar by Prof. Olaf van Kooten and Prof. Jack Leunissen, wageningen university, wageningen	Oct 13, 2009
Plant Sciences Seminar by Prof.dr.ir. Pierre de Wit and Prof.dr. Fred van Eeuwijk, wageningen university, wageningen	Nov 10, 2009
Seminar Prof. Richard Oliver, Director of the Australian Centre for Necrotrophic Fungal Pathogens, Murdoch University, Perth, Australia. Genomic, proteomic and transcriptomic analyses of Stagonospora nodorum and other necrotrophic fungal pathogens in the pleosporales'	Nov 20, 2010
Edith Lammerts van Bueren of Plant Breeding: 'The challenges and focus of the endowed chair Organic Plant Breeding at Wageningen UR'	Mar 16, 2010
Plant Sciences Seminar by Prof. Holger Meinke (Crop & Wees Ecology) and Prof. Paul Struik (Crop Physiology): wageningen university, wageningen	Apr 13, 2010
Plant Sciences Seminar by Louise Vet, Entomology / NIOO and Just Vlak, Virology: wageningen university, wageningen	May 11, 2010
Seminar of Dr. Brigitte Mauch-Mani: Grapevine and downy mildew. Wine is not the only difference between grapevine and Arabidopsis	May 31, 2010
Seminar of Prof. Felix Mauch: Old fashioned secondary metabolites save Arabidopsis from Phytophthora brassicae, University of Fribourg, CH-1700	May 31, 2010
Seminar of Prof. Naoto Shibuya : PAMP receptor (CBEIC and CERK1)	Sep 09, 2010
Mini-symposium "How to write a world-class article: Wageningen university	Oct 26, 2010
▸ **International symposia and congresses**	
XIV international MPMI congress meeting, Quebec , Canada	Jul 19-24, 2009
The 26th Fungal Genetics Conference: Asilomar, California, USA	Mar 15-20, 2011
▸ **Presentations**	
poster: Identification of resistance against *Verticillium* spp. using Arabidopsis, Wageningen	Jun 18-20, 2008
poster: Activation tagging in Arabidopsis to identify resistance against *Verticillium* spp., MPMI, Quebec	July 19-23, 2009
poster: Identification of *Verticillium* virulence factors using proteomics: Asilomar, Califomia, USA	Mar 15-20, 2011
oral: Activation tagging in Arabidopsis to identify resistance against *Verticillium* spp., ALW Genetics	Oct 15-16, 2009
oral: Search for resistant traits in Arabidopsis against *Verticillium* ; INRA, Toulouse	Dec 2009
oral:Verticillium wilt resistance in Arabidopsis: identification and functional characterization	Feb 03, 2011
▸ **IAB interview**	Feb 17, 2011
▸ **Excursions**	
Subtotal Scientific Exposure	*17,4 credits**
3) In-Depth Studies	*date*
▸ **EPS courses or other PhD courses**	
Ph.D Summerschool 'The evolution of Plant Pathogen Interactions: from Principles to Practice', Wageningen	Jun 18-20, 2008
PhD course: Systems Biology: statistical analysis of ~omics data	Dec 13-17, 2010
▸ **Journal club**	
Participate in literature discussion of *Verticillium* group: Phytopathology	2007-2010
▸ **Individual research training**	
Training INRA Toulouse	Dec 01-30, 2009
Subtotal In-Depth Studies	*8,5 credits**
4) Personal development	*date*
▸ **Skill training courses**	
Dutch langauage course for buitenlanders, wageningen university, ROC12, wageningen	2008-2010
Advanced course: guide to scientific artwork	Nov 04-05, 2010
Course: Project- and Time Management	Jan 11, 25,Feb 22, 2011
▸ **Organisation of PhD students day, course or conference**	
▸ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4,6 credits**
TOTAL NUMBER OF CREDIT POINTS*	**32**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

This work was performed at the Laboratory of Phytopathology, Wageningen University, and financially supported by the Dutch Technology Foundation STW, which is the applied science division of NWO, and the Technology Programme of the Ministry of Economic Affairs.

The printing of the thesis was financially supported by the J.E. Jurriaanse Stichting.

Cover:

Front: Genetic diversity generated in *Arabidopsis thaliana* through artificial mutagenesis and being screened for resistance to Verticillium wilt disease. Typical Verticillium wilt resistant plants have green and healthy leaves while susceptible plants are stunted and have chlorotic leaves.

Back: *Arabidopsis thaliana* plant of the Columbia-0 ecotype (left) and plant of the same ecotype over-expressing *EVR1* (*Enhancer of Verticillium Resistance 1*) gene that provides resistance to vascular wilt diseases (right).

Printed by Wöhrmann Print Service, Zutphen, The Netherlands.