FUNCTIONAL ANALYSIS OF TOMATO IMMUNE RECEPTOR VE1 AND RECOGNITION OF VERTICILLIUM EFFECTOR AVE1

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Functional analysis of tomato immune receptor Ve1

and recognition of *Verticillium* effector Ave1

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Thesis

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With summaries in English and Dutch

This thesis is dedicated to my parents.
And in memory of my paternal grandparents.

谨以此书，献给我亲爱的爸爸妈妈和爷爷奶奶。
毋意，毋必，毋固，毋我。
《论语 子罕第九》

No foregone conclusions, no arbitrary predeterminations, no obstinacy, and no egoism.
(Holy Analects of Confucius: The Book of Tsze Han, Chapter IX)
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Chapter 1

Structure-function aspects of extracellular leucine-rich repeat-containing cell surface receptors in plants

Zhao Zhang and Bart P.H.J. Thomma

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ABSTRACT

Plants exploit several types of cell surface receptors for perception of extracellular signals, of which the extracellular leucine-rich repeat (eLRR)-containing receptors form the major class. Although the function of most plant eLRR receptors remains unclear, an increasing number of these receptors are shown to play roles in innate immunity and a wide variety of developmental processes. Recent efforts using domain swaps, gene shuffling analyses, site-directed mutagenesis, interaction studies, and crystallographic analyses resulted in the current knowledge on ligand binding and the mechanism of activation of plant eLRR receptors. This chapter provides an overview of the eLRR receptor research, specifically summarizing the recent understanding of interactions among plant eLRR receptors, their co-receptors and corresponding ligands. The functions of distinct eLRR receptor domains, their role in receptor structure, ligand perception and multimeric complex formation are discussed.
I. INTRODUCTION

Both plants and animals exploit cell-surface receptors for the perception of extracellular signals that are either from oneself (e.g. endogenous signaling molecules) or nonself (e.g. pathogen-derived molecules). Often, these receptors contain extracellular leucine-rich repeats (eLRRs). In animals, eLRR-containing Toll-like innate immune receptors (TLRs) recognize a wide variety of pathogen-associated molecular patterns (PAMPs) and play a central role in innate immune responses. To date, ten receptors have been assigned to the human TLR family (Chang et al., 2012). For example, TLR4 forms a complex with the MYELOID DIFFERENTIATION PROTEIN 2 (MD-2) and detects lipopolysaccharide (LPS) from gram-negative bacteria (Kim et al., 2007; Park et al., 2009), while TLR1 and TLR6 serve as co-receptors that form heterodimers with TLR2 to perceive bacterial lipoproteins (Jin et al., 2007; Kang et al., 2009) and TLR5 recognizes bacterial flagellin (Hayashi et al., 2001). TLRs are composed of an extracellular domain with 20-26 eLRRs which are considered to be responsible for ligand binding, a single-pass transmembrane domain and an intracellular signaling Toll-IL-1R (TIR) domain (Bell et al., 2003).

Two groups of eLRR-containing cell-surface receptors have been described in plants. The first group consists of the receptor-like kinases (eLRR-RLKs) that are composed of an eLRR domain shielded by N-terminal and C-terminal flanking regions, a single-pass transmembrane domain and a cytoplasmic kinase domain. Over 200 eLRR-RLKs were identified in the Arabidopsis genome (Shiu and Bleecker, 2003). The second group of eLRR-containing cell surface receptors is composed by the so-called receptor-like proteins (eLRR-RLPs) that differ from eLRR-RLKs because they lack the cytoplasmic kinase domain and only carry a short cytoplasmic tail, with 57 members in the Arabidopsis genome (Wang et al., 2008a). RLKs and RLPs were shown to play roles in development and innate immunity in several plant species (Wang et al., 2010a; De Smet et al., 2009). One of the best studied eLRR-RLK receptors is Arabidopsis BRASSINOSTEROID INSENSITIVE 1 (BRI1), a receptor for brassinosteroid hormones (Li and
The tomato (*Solanum peruvianum*) BRI1 homolog tBRI1/SR160 does not only perceive brassinosteroid hormones but also the 18 amino acid peptide hormone systemin (Montoya et al., 2002; Scheer and Ryan, 2002). Systemin and other peptide hormones have been shown to regulate diverse processes in plant development and immunity. The CLE (CLAVATA3/EMBRYO SURROUNDING REGION) peptides, which are involved in meristem maintenance and vascular development, belong to the best understood family of peptide hormones with 83 members in Arabidopsis (Matsubayashi and Sakagami, 2006; Oelkers et al., 2008; Qiang et al., 2013). Arabidopsis CLAVATA1 (CLV1) perceives the CLE peptide hormone and promotes stem cell differentiation (Clark et al., 1997; Ogawa et al., 2008). BARELY ANY MERISTEM 1 (BAM1), BAM2 and BAM3 also act as receptors for CLE peptide hormone and are similarly required for stem cell maintenance (DeYoung et al., 2006). Phytosulfokine is a sulfated peptide hormone that contains only five amino acids and is perceived by the PHOTOSULFOKINE RECEPTOR (PSKR), an RLK that promotes plant growth and differentiation (Matsubayashi et al., 1997; Matsubayashi et al., 2002). In addition to plant development, eLRR-RLKs also play vital roles in innate immunity. For example, rice XA21 has been reported to recognize the sulphated 17 amino acid peptide axYs22, which is processed from the bacterial *Xanthomonas oryzae pv oryzae* (Xoo) elicitor Ax21 (Lee et al., 2009). Similarly, FLAGELLIN SENSING 2 (FLS2) perceives a 22 amino acid peptide sequence derived from bacterial flagellin (Zipfel et al., 2004), while the Arabidopsis EF-Tu receptor (EFR) recognizes ELF18 derived from bacterial elongation factor Tu protein (Zipfel et al., 2006).

Similar to eLRR-RLKs, also eLRR-RLPs play roles in both development and immunity. Arabidopsis CLAVATA2 (CLV2) perceives CLE peptide hormones in the process of meristem maintenance (Kayes and Clark, 1998). In tomato, Cf-2, Cf-4, Cf-4E, Cf-5, Cf-9 and Cf-9B provide resistance against *Cladosporium fulvum* strains that carry the matching avirulence (*Avr*) effectors. Tomato Ve1 confers race specific resistance to race 1 strains of *Verticillium* spp. that produce the Ave1 elicitor (de Jonge et al., 2012; Fradin
et al., 2009). And tomato LeEIX2 provides recognition of ethylene-inducing xylanase (EIX) from biocontrol fungus *Trichoderma viride* (Ron and Avni, 2004). As a final example, apple (*Malus domestica*) HcrVf-2 has been implicated in resistance to the apple scab fungus *Venturia inaequalis* (Belfanti et al., 2004).

**II. STRUCTURAL ANALYSIS OF THE eLRR DOMAIN**

In animals, structure-function aspects of plasma membrane localized TLRs have been well studied through crystallographic analysis (Nagai et al., 2012; Chang et al., 2012; Kim et al., 2007; Jin et al., 2007; Kang et al., 2009; Yoon et al., 2011; Ferrao et al., 2012; Choe et al., 2005; Liu et al., 2008; Park et al., 2009). In contrast, BRI1 is the only plant eLRR-containing cell surface receptor for which a crystal structure has been released (She et al., 2011; Hothorn et al., 2011). Due to the lack of crystallographic analyses, most of our understanding of eLRR-containing plant cell surface receptors is based on site-directed mutagenesis, domain swaps and gene shuffling analyses.

**General aspects of the eLRR structure**

The leucine-rich repeat was first identified in the human leucine-rich α2-glycoprotein (LRG; Takahashi et al., 1985), of which the exact function presently still remains unknown (Nakajima et al., 2011). LRG consists of eight repeating consensus sequences, each of which consists of 24 amino acid residues that exhibit a periodic pattern with leucine, asparagine and proline. This consensus sequence was termed the leucine-rich repeat (LRR). In plants, LRR sequences were first described for a bean polygalacturonase-inhibiting protein (PGIP) (Toubart et al., 1992), and the Arabidopsis TRANSMEMBRANE KINASE 1 (TMK1) (Chang et al., 1992). PGIP is a secreted eLRR protein that interacts with fungal polygalacturonases, which degrade polygalacturonans in the cell walls of host plants, to inhibit their enzymatic activity and protect against fungal invasion (Leckie et al., 1999; Di Matteo et al., 2003). TMK1 encodes an eLRR-RLK (Chang et al., 1992) that responds to various biotic and abiotic stresses (Cho and Pai,
A typical plant eLRR consensus motif has 20-29 amino acids that are rich in leucine, with the consensus sequence \( xxLxxLxxLxxNxLt/sGxIP \), where \( x \) represents any amino acid, and leucine is sometimes substituted by other hydrophobic residues such as valine or isoleucine. Plant eLRR-containing cell surface receptors contain tandem eLRR copies. The eLRR region is regularly separated into two domains, C1 and C3, being interrupted by a non-LRR loop-out region which is referred to as the C2 or island domain (Wang et al., 2010a).

A crystal structure of an LRR protein was first determined for the cytoplasmic porcine ribonuclease inhibitor (Kobe and Deisenhofer, 1993), while the first plant eLRR crystal structure was determined for PGIP only a decade later (Di Matteo et al., 2003). More recently, the crystal structure of the BRI1 eLRR domain has been resolved in presence and absence of the brassinosteroid ligand (She et al., 2011; Hothorn et al., 2011). The high-resolution structure of this plant eLRR protein revealed a similar shape as the porcine ribonuclease inhibitor, revealing that the \( \beta \)-strand and helices of successive eLRRs align in parallel and form a spring or coil that is curved like a horseshoe and slightly twisted in the three-dimensional structure. The parallel core \( \beta \)-strands (\( xxLxLxx \)) form a concave (inner) side of the protein, whereas the convex (outer) surface is irregularly decorated with various secondary structures, including \( \alpha \)-helices, \( 3_{10} \)-helices (a rare type of helix structure), short \( \beta \)-strands and additional connecting residues (Kobe and Kajava, 2001; Di Matteo et al., 2003; She et al., 2011; Hothorn et al., 2011). In addition, when compared with canonical horseshoe-shaped structures which are essentially flat, one notable feature of the plant PGIP and BRI1 structure is that they are exceptionally twisted, and display decreased curvature of the \( \beta \)-sheet surface (She et al., 2011; Hothorn et al., 2011; Di Matteo et al., 2003).

**The role of the concave face of the eLRR domain**

The concave side of the eLRR that is formed by parallel \( \beta \)-strands (\( xxLxLxx \)) is thought to serve for ligand binding (Kobe and Kajava, 2001). Whereas the hydrophobic (L) residues in the \( \beta \)-strands are involved in the overall
framework of protein, the five variable solvent exposed residues (x) determine specificity of ligand binding. The crystal structure of the cytoplasmic porcine ribonuclease inhibitor revealed that the interaction between the ribonuclease inhibitor and ribonuclease occurs through solvent-exposed residues at the concave β-sheet surface (Kobe and Deisenhofer, 1996). Similar to the porcine ribonuclease inhibitor, also the crystal structure of the cell surface-localized TLR4–MD-2–LPS complex demonstrated a key role for the concave β-sheet surface of TLR4 eLRRs in the interaction with cofactor MD-2 (Kim et al., 2007; Park et al., 2009).

In plants, domain-swaps and site-directed mutagenesis showed that the concave β-sheet surface of the PGIP eLRRs contains residues that determine specificity towards different polygalacturonases (Leckie et al., 1999; Casasoli et al., 2009). Further crystallographic analysis of PGIP revealed that a cluster of negatively charged residues on the concave surface of protein is involved in polygalacturonase binding (Di Matteo et al., 2003; Benedetti et al., 2011). Furthermore, domain swaps between tomato Cf-4 and Cf-9 and between Cf-9 and Cf-9B revealed that residues exposed at the concave surface of the central LRRs determine Cf-4 and Cf-9 specificity, respectively (van der Hoorn et al., 2001; Wulff et al., 2009; Chakrabarti et al., 2009). Moreover, site-directed mutagenesis across the LRR domain of FLS2 identified solvent-exposed concave residues in LRRs 9 to 15 that are critical for flagellin perception (Dunning et al., 2007). Interestingly, the majority of studied examples revealed that ligand specificity is determined by a number of clustered (i.e. neighboring) eLRR repeats on the concave surface (Table 1).

The role of convex face of the eLRR domain

The elements in the convex (outer) surface of the eLRRs are thought to be important for correct folding of the protein. Crystallographic analysis of human TLR4 and the TLR-like receptor RP105 revealed that the RP105 eLRRs displayed a more closed, circular shape because of the presence of an α-helix in the convex face of eLRR21 that required more space than the loops and 3_10-helix segments present in the corresponding region of TLR4 (Kim et
<table>
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Table 1. LRR recognition of eLRR-containing cell surface receptors.
al., 2007; Ohto et al., 2007; Yoon et al., 2011). Similarly, the porcine ribonuclease inhibitor exhibits a more closed shape than TLR3 because the convex surface of porcine ribonuclease inhibitor is composed of repeating α-helices rather than loops that occur in TLR3 (Choe et al., 2005). In plants, the twisted assemblies of PGIP and BRI1 are caused by noncanonical, plant-specific β-sheets which are present in the convex surface of their eLRR domains (Di Matteo et al., 2003; Hothorn et al., 2011; She et al., 2011). On the convex side of BRI1, disulphide bonds are regularly formed between two consecutive repeats (between eLRRs 2 and 3, 5 and 6, 7 and 8, 10 and 11, and 14 and 15) to stabilize the protein structure (She et al., 2011).

In addition to protein folding, the convex face of the eLRRs might serve as the interaction surface for receptor dimerization, as has been shown in the crystal structure of the TLR4–MD-2–LPS complex (Park et al., 2009; Kim et al., 2007). Presently, no examples of ligand perception at the convex face of the eLRR domain of cell surface receptor have been reported.

The role of the non-eLRR C2 or island domain

The non-eLRR island (C2) domain that separates the C1 and C3 eLRR domains has been proposed to act as a flexible hinge region for protein folding into the regular LRR structure, which favors ligand binding and subsequent receptor activation. (Fritz-Laylin et al., 2005; Jones and Jones, 1997). Tritium-labeled brassinosteroid was cross-linked to the BRI1 receptor, and mass spectrometry was used to subsequently identify the BRI1 ligand binding site as a 70 amino acid fragment within the island domain and the flanking eLRR22 that belongs to the C3 domain (Kinoshita et al., 2005). The recently released BRI1 crystal structure confirmed that brassinosteroid binds to a hydrophobic groove in between the island domain and the concave β-sheet surface (She et al., 2011; Hothorn et al., 2011). Similarly, a radiolabeled PSK analog was cross-linked to a 15 amino acid fragment within the island domain of PSKR, demonstrating that the island domain of PSKR contains its ligand-binding site (Shinohara et al., 2007). Nevertheless, in other eLRR receptors the island domain is not involved in ligand
specificity or dispensable for receptor activity. Many eLRR receptors lack an island domain, which is the case for twelve of the 57 eLRR-RLPs found in Arabidopsis (Wang et al., 2008a). Absence of the island domain has also been shown in functionally characterized eLRR-RLKs, such as Xa21, ERECTA, CLV1, FLS2, and BAM1, BAM2 and BAM3 (Song et al., 1995; Torii et al., 1996; Clark et al., 1997; Gómez-Gómez and Boller, 2000; DeYoung et al., 2006). Furthermore, the sequence-unrelated effectors Avr4 and Avr9 from the fungal pathogen *C. fulvum* are perceived by Cf-4 and Cf-9, respectively, which share identical island regions, showing that these regions are not involved in ligand specificity (van der Hoorn et al., 2001). Finally, deletion of the island domain from CLV2 does not affect its functionality in plant development (Wang et al., 2010b). Overall, these examples demonstrate the differential requirement of the island domain for eLRR receptor functionality.

**III. FUNCTIONS OF OTHER DOMAINS IN PLANT eLRR RECEPTORS**

**N-terminal and C-terminal flanking regions**

Most eLRR proteins contain flanking regions at the N- and C-termini of the eLRR domain. These eLRR-flanking regions are an integral part of the eLRR domain that cap exposed edges of the horseshoe-like eLRR structure (Kobe and Kajava, 2001). The capping prevents the hydrophobic core of the first and last LRR to be exposed to the solvent, and may therefore contribute to the stability of the protein. In addition, crystallographic analyses of human TLR3 revealed β-strands belonging to the N- and C-terminal flanking regions to align to the concave surface, and possibly take part in interaction with its double-stranded RNA (dsRNA) ligand (Choe et al., 2005). Indeed, a subsequent crystallographic study demonstrated that His39 located on the N-terminal flanking region of TLR3 is one of the interaction sites in the dsRNA-TLR3 complex (Liu et al., 2008). Alignments of plant eLRR proteins distinguished several conserved motifs in the eLRR-flanking regions (van der Hoorn et al., 2005), and mutagenesis of these motifs in Cf-9 revealed the importance of the eLRR-flanking regions for Cf-9 function (van der Hoorn et al., 2005). In eLRR proteins, cysteines are frequently found in eLRR-flanking
regions that may be involved in the formation of disulfide bridges (Kobe and Kajava, 2001). These cysteine residues are functionally important for plant eLRR proteins. Mutagenesis of the six cysteine residues in both N- and C-terminal flanking regions of Cf-9 significantly compromised Cf-9 function (van der Hoorn et al., 2005). Furthermore, recent studies demonstrated that the cysteine pair in the N-terminal flanking region of the FLS2 eLRRs is required for protein stability and function, while disruption of the cysteine pair in C-terminal flanking region does not block FLS2 function (Sun et al., 2012). Crystallographic studies revealed the presence of disulfide bridges located in both N- and the C-terminal flanking regions of PGIP that may contribute to protect the hydrophobic core of the protein solenoid (Di Matteo et al., 2003). Similarly, upon crystallographic analyses two disulfide bonds were identified in the N- and C-terminal flanking regions of Leucine Rich Protein (LRP), a secreted protein from tomato that contains five eLRRs, that protect the eLRR domain from proteolysis in vitro (Kolade et al., 2006).

**The transmembrane domain**

The transmembrane domain is mainly composed of helices that associate within the plasma membrane by electrostatic and van der Waals interactions. Structurally, the transmembrane domain plays a critical role in localization and orientation of cell surface receptors, allowing ligand perception and signal transduction. Furthermore, the TM has also emerged as an important region for dimerization of cell surface proteins.

The transmembrane domain from several mammalian receptor tyrosine kinases were shown to play a role in homo- or heterodimerization (Stokes and Rao, 2010). More specifically, a GxxxG was identified as sequence motif that mediates interactions between transmembrane domains (Curran and Engelman, 2003). The GxxxG motif was first defined as the dimeric interface in the transmembrane domain of glycophorin A (GpA) on the surface of erythrocytes (Lemmon et al., 1992), which was subsequently confirmed by NMR studies (MacKenzie et al., 1997). Since then, the GxxxG dimerization motif has been shown to mediate protein-protein interactions in other
proteins as well (Senes et al., 2000; Russ and Engelman, 2000; Deber et al., 1993; Melnyk et al., 2002). The GxxxG motif is present the transmembrane domain of many eLRR cell surface receptors, including in Cf-2, Cf-4, Cf-9, EFR, HrcVf-2, and Ve1. Loss-of-function mutations in AtRLP51 of Arabidopsis caused enhanced susceptibility towards the phytopathogenic bacterium *Pseudomonas syringae pv tomato* DC3000 (Zhang et al., 2010b). Interestingly, arginine substitution of the second glycine of the GxxxG motif in both AtRLP51 and its close homolog AtRLP55 resulted in constitutively activated defense, which may be due to disruption of the interaction with an unidentified negative regulator (Zhang et al., 2010b). In addition, a similar mutation in the GxxxG motif abolished Cf-9 function (Wulff et al., 2004).

**The cytoplasmic domain**

The perception of ligands by eLRR receptors, either direct or indirect, triggers an intracellular signaling cascade. For eLRR-RLKs this signal transduction often involves phosphorylation or dephosphorylation of its kinase domain and the kinase domain of co-receptors. The kinase domain of eLRR-RLKs may determine interaction with co-receptors and cytoplasmic components. For example, the BRI1-ASSOCIATED KINASE1 (BAK1) eLRR-RLK was identified as co-receptor of BRI1, and their interaction was reported to be largely mediated by their kinase domains (Wang et al., 2008b; Li et al., 2002; Nam and Li, 2002). Furthermore, the BOTRYTIS INDUCED KINASE1 (BIK1) forms a constitutive complex with the kinase domain of FLS2 and EFR to positively regulate defense signaling (Zhang et al., 2010a; Lu et al., 2010). Interestingly, cytoplasmic domains have also been reported to interact with negative regulators. For example, in absence of brassinosteroid, the BRI1 kinase domain is kept in a resting state through association with the inhibitor protein BKI1 (BRI1 KINASE INHIBITOR1) (Wang and Chory, 2006). Brassinosteroid binding to the eLRR domain of BRI1 leads to autophosphorylation of the BRI1 kinase domain and trans-phosphorylation of the inhibitor BKI1. BKI1 then dissociates from the BRI1 kinase domain, and allows formation of the BRI1-BAK1 complex.
through their kinase domains to activate downstream signaling (Wang and Chory, 2006). Thus, BKI1 acts as a negative regulator of BRI1. The kinase domain of XA21 is similarly inactivated prior to ligand binding through interaction with the ATPase XB24, which dissociates from XA21 after binding of axY-22 to XA21 (Chen et al., 2010). Using the cytoplasmic tail of tomato Cf-9 as bait, yeast two hybrid has been exploited to identify a Cf-9-interacting thioredoxin (CITRX) that negatively regulates Cf-9/Avr9-mediated immune responses (Rivas et al., 2004).

Similar to mammalian TLRs (Husebye et al., 2006), ligand-induced endocytosis has been intensively studied for plant eLRR receptors BRI1 (Russinova et al., 2004; Robert et al., 2008), FLS2 (Salomon and Robatzek, 2006; Robatzek et al., 2006; Beck et al., 2012) and LeEIX2 (Bar and Avni, 2009). In plants and animals, endocytosis is often associated with presence of Yxxφ and E/DxxxLφ consensus motifs within cytoplasmic domains, where φ is a hydrophobic residue and x is any amino acid (Murphy et al., 2005; Geldner and Robatzek, 2008). In addition, endocytosis can also involve a so-called PEST endocytosis motif in cytoplasmic domains (peptide sequence rich in proline, glutamic acid, serine and threonine; Beck et al., 2010). Many eLRR-RLKs, such as BRI1, carry the putative Yxxφ endocytosis motif at a conserved position in the kinase domain (Geldner and Robatzek, 2008). Co-localization of fluorescently labeled BRI1 and endocytic tracer FM4-64 confirmed that BRI1 vesicles constitutively recycle between the plasma membrane and endocytic compartments (Russinova et al., 2004). Similarly, the Yxxφ endocytosis motif is also present in the cytoplasmic tail of LeEIX2, and is essential for EIX-triggered signaling (Ron and Avni, 2004). Further studies demonstrated that LeEIX2 localizes exclusively on the plasma membrane, while LeEIX2 rapidly accumulates in mobile intracellular vesicles after EIX stimulation (Bar and Avni, 2009). The endocytosis inhibitory protein AtEHD2 was demonstrated to bind to the cytoplasmic tail of LeEIX2 and inhibit its internalization, thus negatively regulating LeEIX2 signaling (Bar and Avni, 2009). GFP-tagged FLS2 internalize into motile vesicles upon flg22 treatment, suggesting a ligand-induce endocytosis process (Robatzek
et al., 2006). FLS2 carries a PEST motif in the cytosolic domain, and a mutation of this motif abolished FLS2 endocytosis (Geldner and Robatzek, 2008). Interestingly, unlike many eLRR receptors, FLS2 does not contain an Yxxφ motif in its cytoplasmic domain. However, sequence alignment of FLS2 homologs revealed the presence of three non-canonical Yxxxφ motifs, which has been suggested facilitate endocytosis in animals (Beck et al., 2012). In addition, the vesicle-associated protein VAP27 was shown to interact with the Cf-9 in yeast two-hybrid screening. As VAP27 is involved in protein trafficking and vesicle movement, it has been speculated to play a role in endocytosis of the Cf-9 receptor (Laurent et al., 2000).

IV. OUTLINE OF THE THESIS
The fungal vascular wilt pathogen Verticillium dahliae causes wilt diseases in over 200 herbaceous and woody plant species worldwide. The tomato Ve1 gene encodes an eLRR-containing cell surface receptor, and acts as a resistance gene against race 1 Verticillium strains. Recently, the corresponding Verticillium effector Ave1 (for Avirulence on Ve1 tomato) that activates Ve1-mediated immunity was identified through comparative genomics of race 1 and race 2 strains.

To examine the role of various domains of the Ve1 protein and study perception of the Ave1 effector in more detail, a transient gene expression and gene silencing model system was developed for the Ve1 resistance and Ave1 effector gene. Chapter 2 describes a robust Agrobacterium tumefaciens transient expression assay (agroinfiltration) and virus-induced gene silencing (VIGS) in Nicotiana tabacum plants. The model allows investigating signaling components involved in Ve1-mediated resistance. In addition, by agroinfiltration and pathogen assays, we demonstrate that Nicotiana glutinosa plants potentially contain a functional Ve1 ortholog that mediates Verticillium resistance.

Although co-expression of Ve1 and Ave1 activates a hypersensitive response (HR) in tomato and tobacco, in Chapter 3 we demonstrate that co-expression of Ve1 and Ave1 does not lead to HR in Arabidopsis, despite
the fact that Ve1 expression results in robust *Verticillium* resistance. This finding suggests that the HR is a consequence of resistance rather than that it directly contributes to *Verticillium* resistance.

The agroinfiltration assay is an excellent system for functional analysis of Ve1 domains. In Chapter 4, agroinfiltration as well as Arabidopsis transformation was used to determine the contribution of particular regions of Ve1 to immune signaling. By swapping domains of Ve1 with its non-functional homolog Ve2, chimeric Ve proteins were generated. Analysis of the chimeras suggest that Ve2 may still interact with the Ave1 effector in the eLRR C1 domain, but that its C3 domain and C-terminus are not able to activate defense signaling. We speculate that the conserved C3 domain maybe involved in co-receptor interaction.

To further determine specific ligand determinants in the eLRR domain of Ve1, we generated alanine scanning mutants in the solvent exposed residues across the convex eLRR domain in Chapter 5. By analysis of the Ve1 mutants through agroinfiltration and stable Arabidopsis transformation, eLRR regions potentially required for ligand specificity and for co-receptor interaction were identified. In addition, alanine substitution was also employed to functionally analyse putative protein-protein interaction and endocytosis motifs in the Ve1 protein.

To identify the surface epitope of the *V. dahliae* effector Ave1, various Ave1 homologs were assessed for their capability to activate the Ve1-mediated HR in Chapter 6. It was demonstrated that the C-terminal of Ave1 is functionally important to activate the HR. Further, domain deletions and swaps between homologs were performed to show that Ve1-mediated immunity can be activated by a nine amino acid sequence derived from the C-terminus of Ave1 that is required and sufficient for recognition by Ve1.

Finally, in Chapter 7 the experimental data presented in the previous chapters are discussed in a broader context.
REFERENCES


Chapter 2

Optimized agroinfiltration and virus-induced gene silencing to study Ve1-mediated *Verticillium* resistance in tobacco

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ABSTRACT

Recognition of pathogen effectors by plant immune receptors often leads to the activation of a hypersensitive response (HR), which is a rapid and localized cell death of plant tissue surrounding the site where recognition occurs. Due to its particular amenability to transient assays for functional genetics, tobacco is a model for immune signaling in the Solanaceae plant family. Here, we show that co-expression of the tomato (Solanum lycopersicum) immune receptor Ve1 and the corresponding Verticillium effector protein Ave1 leads to HR only in particular tobacco species. Whereas HR is obtained in Nicotiana tabacum, no such response is obtained in N. benthamiana. Furthermore, our analysis revealed an endogenous Ve1 ortholog in Nicotiana glutinosa, as expression of Ave1 in absence of Ve1 induced an HR, and N. glutinosa was found to be resistant against race 1 V. dahliae. We furthermore report the establishment of virus-induced gene silencing in N. tabacum for functional analysis of Ve1 signaling. Collectively, our data show that N. tabacum can be used as a model plant to study Ve1-mediated immune signaling.
INTRODUCTION

Verticillium dahliae and Verticillium albo-atrum are among the world’s most notorious plant pathogens that occur in temperate and subtropical regions, causing vascular wilt diseases in over 200 herbaceous and woody plant species (Fradin and Thomma, 2006; Klosterman et al., 2009). Genetic resistance has been identified in several plant species, and a single dominant locus that confers Verticillium resistance, named Ve, has been used by tomato (Solanum lycopersicum) breeders for over sixty years. Isolates of Verticillium that are contained by the Ve locus are assigned to race 1, while all others are designated as race 2 (Fradin and Thomma, 2006). However, race 2 strains are typically not as aggressive as race 1 strains (Armen and Shoemaker, 1985; Paternotte and van Kesteren, 1993; de Jonge et al., 2012). The Ve locus comprises two closely linked inversely oriented genes, Ve1 and Ve2, which encode extracellular leucine-rich repeat (eLRR) receptor-like proteins (RLPs) (Kawchuk et al., 2001). These are cell surface receptors that typically comprise an eLRR domain, a single-pass transmembrane domain and a short cytoplasmatic tail that lacks obvious motifs for intracellular signaling. More recent research has demonstrated that only Ve1 acts as a functional Verticillium resistance gene in tomato, while no functionality could be assigned to Ve2 (Fradin et al., 2009; Fradin et al., 2011).

Genes encoding RLP-type immune receptors were found to act in pathogen defense in several plant species (Fritz-Laylin et al., 2005; Wang et al., 2008; Wang et al., 2010). The interaction between Cladosporium fulvum and tomato that carries Cf resistance genes has been exploited extensively to study RLP-mediated resistance signaling (Thomma et al., 2005; Wulff et al., 2009). Based on their involvement in defense responses mediated by other RLPs, candidate genes were tested for a role in Ve1-mediated Verticillium resistance in tomato using virus-induced gene silencing (VIGS) (Fradin et al., 2009; Fradin et al., 2011; Vossen et al., 2010). Despite the tools and resources that are available, research on tomato has major limitations when compared to other model plant species such as the Solanaceous sister
Ve1-mediated Ave1 recognition in tobacco

species *Nicotiana benthamiana* and the Brassicaceous model plant *Arabidopsis thaliana* which are also genuine hosts for *Verticillium* infection. For instance, VIGS efficiency in tomato is not as good as in *N. benthamiana* (Liu et al., 2002). Interestingly, tomato Ve1 remains fully functional after transfer to Arabidopsis, suggesting that this model plant can be used to unravel the genetics of resistance signaling mediated by Ve1. Indeed, after discovering that functionality of the receptor-like kinase SERK1 is required for Ve1 signaling in Arabidopsis, the requirement of SERK1 for Ve1-mediated resistance was confirmed in tomato as well (Fradin et al., 2011).

To study tomato RLPs, the model plant *N. benthamiana* (Goodin et al., 2008) has frequently been used for *Agrobacterium tumefaciens*-mediated transient transformation assays (agroinfiltration) and VIGS (van der Hoorn et al., 2000; Rowland et al., 2005; Gabriëls et al., 2006; Bar and Avni, 2009; Fradin et al., 2009; Vossen et al., 2010). Since tomato and *N. benthamiana* are close relatives they share a high degree of coding sequence homology, allowing the use of tomato sequences to target homologs in *N. benthamiana* and vice versa (Gabriëls et al., 2006; Senthil-Kumar et al., 2007; Faino et al., 2012). So far, however, full exploitation of these assays for the investigation of Ve1-mediated signaling was hampered by the unknown identity of the *Verticillium* avirulence molecule that activates the Ve1 receptor. Recently, through comparative genomics of race 1 and race 2 strains this molecule was identified, and named Ave1 (for Avirulence on Ve1 tomato) (de Jonge et al., 2012). The availability of the tomato resistance gene (Ve1) and the corresponding *V. dahliae* avirulence gene (Ave1) facilitates studies into the genetics of Ve1-mediated *Verticillium* resistance. Here, we describe the development of protocols to investigate Ve1-mediated defense signaling in tobacco.
RESULTS

Co-expression of Ave1 and Ve1 induces HR in *Nicotiana tabacum*, but not in *Nicotiana benthamiana*.

Often, recognition of pathogen avirulence molecules by plant immune receptors leads to the activation of a hypersensitive response (HR); rapid and localized cell death of plant tissue at the site where recognition occurs. For Ave1 it was shown that transient expression mediated by Potato virus X (PVX) (Chapman et al., 1992) specifically induced HR on tomato carrying Ve1 (de Jonge et al., 2012). To test whether an HR can also be induced in tobacco, co-expression of Ave1 and Ve1 by agroinfiltration was pursued. To this end, the coding sequence of *Verticillium dahliae* Ave1 was cloned into the Gateway-compatible vector *pFAST-R02* (Shimada et al., 2010) to generate expression construct *pFAST::Ave1* to establish Ave1 expression driven by the CaMV 35S promoter, and transformed into *A. tumefaciens* strain GV3101. The resulting strain was mixed in a 1:1 ratio with *A. tumefaciens* GV3101 carrying *pMOG800::Ve1* to establish Ve1 expression similarly driven by the CaMV 35S promoter (Fradin et al., 2009), and infiltrated into the leaves of *Nicotiana tabacum* cv. Petite Havana SR1. At one to two days post infiltration (dpi), leaf tissue started to collapse, and the infiltrated leaves developed clear necrosis by five dpi (Figure 1A). In contrast, agroinfiltration of *pMOG800::Ve1* or *pFAST::Ave1* alone did not induce necrosis. Nevertheless, the HR that was induced upon co-expression of Ve1 and Ave1 was not as strong as HR induced upon co-expression of the corresponding tomato *Cf-9* and *C. fulvum Avr9* gene pair, for which the complete infiltrated sectors became fully necrotic (Figure 1B).

It has been suggested that the accumulation of transiently expressed protein usually peaks between one and three dpi and rapidly decreases thereafter due to post-transcriptional gene silencing (PTGS) in the host plant (Johansen and Carrington, 2001; van der Hoorn et al., 2003; Voinnet et al., 2003). Thus, weak HR induced upon co-infiltration of *pMOG800::Ve1* and *pFAST::Ave1* may be caused by PTGS. In an attempt to overcome this, *Ve1 and*
Ave1 were co-expressed with silencing suppressor p19 from the tomato bushy stunt virus (Voinnet et al., 2003), or with silencing suppressor 2b from the cucumber mosaic virus (Wang et al., 2004). Unfortunately, expression of p19 induced necrosis in N. tabacum by itself, while expression of 2b did not enhance necrosis induced by co-expression of Ave1 and Ve1 (Figure 1A).

To bypass the weak necrosis in N. tabacum, we attempted agroinfiltration of pMOG800::Ve1 and pFAST::Ave1 together with silencing suppressor p19 (1:1:1 ratio) in leaves of N. benthamiana. Surprisingly, however, co-expression of Ave1 and Ve1 did not result in any HR in N. benthamiana, even up to 14 days post infiltration, while co-expression of tomato Cf-9 with C. fulvum Avr9 in a similar fashion induced clear HR within 4 dpi (Supplemental Figure 1A).

Various Gateway-compatible binary vectors improve agroinfiltration efficiency.

Because N. benthamiana did not develop necrosis upon co-expression of Ve1 and Ave1, and the silencing suppressors p19 and 2b could not improve the
results of the agroinfiltration in *N. tabacum*, we tested various over-expression vectors to improve our results. To this end, the coding sequences of *V. dahliae Ave1* and *tomato Ve1* were cloned into various expression vectors that carry the CaMV 35S promoter for transgene expression. All constructs were transformed into *Agrobacterium* strain GV3101 and a 1:1 mixture of *Agrobacterium* carrying 35S::Ve1 and 35S::Ave1 was infiltrated into *N. tabacum* plants. Leaves were examined for necrosis at five dpi (Figure 2). Interestingly, various expression vectors strongly affected necrosis development, as expression vectors *pMOG800*, *pSol2092* and *pEarleyGate100* promoted strong necrosis in which the entire infiltrated area became necrotic (Figure 2).

Based on these results obtained in *N. tabacum*, we revisited the agroinfiltration in *N. benthamiana* and infiltrated leaves with a 1:1:1 mixture of *Agrobacterium* cultures carrying *pMOG800::Ve1*, *pSol2092::Ave1* and silencing suppressor *p19*. Nevertheless, also with these vectors no necrosis was obtained in this species (Supplemental Figure 1B).

**C-terminally tagged Ve1 is functional in *N. tabacum***.

Protein tagging with the green fluorescent protein (GFP) is frequently used for protein localization studies and also for affinity purification (Heese et al., 2007). We cloned the Ve1 coding sequence without stop codon into various Gateway-compatible binary vectors that establish a C-terminal fusion to GFP. Only the construct in *pSol2095* developed a strong HR that was comparable to the strong HR that can be obtained with untagged Ve1 (Supplemental Figure 2).

**HR induced by co-expression of Ve1 and Ave1 is restricted to *N. tabacum* and *N. glutinosa***.

Because *N. tabacum* and *N. benthamiana* responded differentially to co-expression of Ve1 and Ave1, we screened additional tobacco genotypes
for their response to co-expression of this gene pair. In these assays, agroinfiltration of Ve1-GFP served as a marker for efficiency of agroinfiltration, while co-expression of the Cf-9/Avr9 gene pair was used as a positive control for the HR. The results of the agroinfiltration are shown in Table 1. Transient co-expression of the Cf-9/Avr9 gene pair resulted in a hypersensitive response in all of the Nicotiana species tested. In contrast, co-expression of the Ve1/Ave1 gene pair did not result in specific necrosis in most Nicotiana species, although significant GFP fluorescence was observed upon expression of Ve1-GFP, confirming that the agroinfiltration worked. The Ve1/Ave1-induced HR could only be observed in N. tabacum cultivars and in Nicotiana glutinosa (Table 1).
Table 1. The hypersensitive response upon co-expression of Ve1 and Ave1 in various tobacco species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Ve1/Ave1</th>
<th>Cf-9/Avr9</th>
<th>Ave1</th>
<th>GFP</th>
<th>PDS</th>
<th>Viral symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. benthamiana</em></td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. SR1</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. White Burley</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. Xanthi</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. Havana2000</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. 2.1.1</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. Samsun</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>N. tabacum</em> cv. Samsun NN</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>N. rustica</em></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>N. clevelandii</em></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td><em>N. sylvestris</em></td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>N. glutinosa</em></td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td><em>N. debneyi</em></td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><em>N. paniculata</em></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>n.d.</td>
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<tr>
<td><em>N. excelsior</em></td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>n.d.</td>
<td>+++</td>
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</tr>
</tbody>
</table>

1 HR was monitored at seven days post infiltration; +++= strong HR over the entire infiltrated area; ++= moderate HR; += weak necrosis.
2 GFP fluorescence derived from GFP-tagged Ve1 was examined with a UV microscope at two days post infiltration; += clear GFP is visible; n.d.= not determined.
3 Degree of photobleaching upon PDS silencing through VIGS; +++= strong photobleaching with the entire leaf turning white; ++= moderate photobleaching with more than 50% of leaf area turning white; += weak photobleaching of leaf; -= no photobleaching was observed; n.d.= not determined.
4 Viral symptoms upon agroinfiltration of TRV::PDS; +++= strong viral symptoms; ++= moderate viral symptoms; += weak viral symptoms.
N. glutinosa contains a functional Ve1 ortholog

Intriguingly, agroinfiltration of only Ave1 into Nicotiana glutinosa leaves already induced HR, albeit not as strong as the HR induced by co-expression of Ave1 with Ve1, while agroinfiltration of Ve1 alone did not induce HR (Table 1; Figure 3A). This suggests that N. glutinosa contains a functional Ve1 ortholog. To confirm the presence of a functional Ve1 ortholog in N. glutinosa, we tested the resistance of N. glutinosa to race 1 V. dahliae by inoculating 3-week-old N. glutinosa plants with race 1 V. dahliae strain JR2. At two weeks post inoculation, it was clearly observed that the inoculated N. glutinosa plants were resistant to the V. dahliae infection as plants were completely devoid of wilt disease symptoms (Figure 3B). Importantly, when inoculated with an Ave1 deletion strain (de Jonge et al., 2012), the N. glutinosa plants displayed clear symptoms of Verticillium wilt disease, including stunting, wilting, chlorosis, and necrosis at 14 days post inoculation (Figure 3B). We subsequently attempted to clone the Ve1 homolog from N. glutinosa making use of primers designed on the tomato Ve1 sequence. Although cloning of complete coding sequence failed so far, a 461 bp product was amplified from N. glutinosa cDNA showing a high degree of homology to tomato Ve1 (Supplemental Figure 4). Collectively, these experiments demonstrate that N. glutinosa contains a functional Ve1 ortholog that provides resistance against race 1 V. dahliae through recognition of Ave1.

TRV-based virus-induced gene silencing in N. tabacum.

In an attempt to establish VIGS in N. tabacum, a 1:1 mixture of A. tumefaciens cultures carrying pTRV1 and pTRV2::PDS to target the PDS gene was infiltrated into two cotyledons of five two- to three-week-old plants of the N. tabacum cultivar SR1. Photobleaching symptoms were observed in all of the five agroinfiltrated N. tabacum cv. SR1 plants at three weeks post infiltration. However, PDS silencing was only consistently observed in the stems of the plants, as these were fully bleached (Supplemental Figure 3). In the leaves, photobleaching was found to be restricted to a limited area immediately
adjacent to some of the veins, suggesting that the TRV-based VIGS is not efficient in *N. tabacum* under the conditions used (Supplemental Figure 3).

Next, we tested in which of the tobacco species TRV could induce efficient gene silencing. The degree of photobleaching varied among the species from no bleaching in *N. rustica* and *N. glutinosa*, to very strong bleaching in *N. benthamiana*, *N. debneyi* and *N. excelsior*. Interestingly, while in most *N. tabacum* cultivars photobleaching occurred only weakly or limited in leaves (Table 1), highly efficient photobleaching was observed in leaves of

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**Figure 3.** *Nicotiana glutinosa* contains a functional Ve1 ortholog. (A) Agroinfiltration of Ave1, Ve1, and a combination thereof in *N. glutinosa*. In contrast to agroinfiltration of Ve1 alone, agroinfiltration of Ave1 alone in *N. glutinosa* induces a hypersensitive response. The picture was taken at seven days post infiltration. (B) *N. glutinosa* is resistant against race 1 *V. dahliae* but not against an Ave1 deletion strain (∆Ave1). Pictures were taken at 14 days post inoculation.
Ve1-mediated Ave1 recognition in tobacco

the cultivars Samsun and Samsun NN (Table 1; Figure 4). As it has been reported that the EDS1 (Enhanced Disease Susceptibility 1) gene is required for Ve1-mediated Verticillium resistance in tomato and Arabidopsis (Hu et al., 2005; Fradin et al., 2009; Fradin et al., 2011), the role of this gene in the HR upon co-expression of Ve1 and Ave1 was assessed. At three weeks post TRV infection, TRV-inoculated plants were smaller than non-inoculated plants. Co-expression of Ve1 and Ave1 in N. tabacum cv. Samsun plants inoculated with recombinant virus to target GFP expression as a control (pTRV:GFP) resulted in a clear HR within 2 days (Figure 5A). However, as expected, N. tabacum cv. Samsun plants inoculated with recombinant virus to target EDS1 expression (pTRV:EDS1) showed a significantly compromised HR upon co-expression of Ve1 and Ave1, confirming the involvement of EDS1 in Ve1-mediated resistance signaling (Figure 5A). We also targeted expression of NRC1 (NB-LRR protein required for HR-associated cell death 1), which has similarly been reported to be involved in Ve1-mediated resistance in tomato (Fradin et al., 2009). However, similar to SGT1, inoculation of N. tabacum cv. Samsun with pTRV:NRC1 strongly affected plant development, and plants could not be used for co-expression of Ve1 and Ave1 (Figure 5B).
DISCUSSION

Co-expression of Ve1 and Ave1 induces HR only in particular Nicotiana species

In this study, we investigated the occurrence of HR in leaves of various Nicotiana species upon co-expression of tomato Ve1 and V. dahliae Ave1, revealing that HR can only be obtained in N. tabacum and N. glutinosa. Intriguingly, HR was not observed in the widely used tobacco model species.
N. benthamiana. This is in contrast to the HR that is induced by the tomato Cf-4 and Cf-9 immune receptors that belong to the same RLP family as Ve1, and that provide resistance against strains of the foliar pathogen C. fulvum that carry Avr4 and Avr9, respectively, which can be induced upon co-expression with these corresponding effector genes (Table 1) (Gabriëls et al., 2007). This suggests that most tobacco species lack at least one important signal transduction component that is required for Ve1-mediated immunity against Verticillium and further confirms the earlier finding that Cf and Ve1 signaling is distinct despite the fact that both immune receptors belong to the same RLP family (Vossen et al., 2010; Fradin et al., 2009; Fradin et al., 2011). The failure of Ve1 to mediate HR in most tobacco species was rather unexpected, considering that Ve1 orthologs that have been implicated in resistance against the broad host range pathogen V. dahliae are more widespread than Cf orthologs that are confined to tomato as the only known host of C. fulvum (Thomma et al., 2005; Thomma et al., 2011; Fradin et al., 2009). This finding is even more surprising considering that a functional Ve1 ortholog was identified even within the Nicotiana family, in N. glutinosa, and that heterologous expression of tomato Ve1 in Arabidopsis results in resistance against race 1 Verticillium strains.

Agroinfiltration as a method to identify functional Ve1 orthologs
Agroinfiltration has been demonstrated to be effective for transient expression in many plant species, including tobacco, lettuce, Arabidopsis, radish, pea, lupine, flax, tomato, grapevine and switchgrass (van der Hoorn et al., 2000; Wroblewski et al., 2005; Santos-Rosa et al., 2008; van der Gheynst et al., 2008). This allows the use of agroinfiltration to identify candidate functional Ve1 orthologous genes by co-expression with Ave1. Since Verticillium is an economically important pathogen, many efforts have been made to identify Ve1 orthologs from various plant species, within and outside the Solanaceae family, including SlVe1 cloned from Solanum lycopersicoides (Chai et al., 2003), StVe1 cloned from Solanum tuberosum
Functionality of the putative Ve1 orthologs was assessed by subsequent transformation into susceptible plants followed by *Verticillium* inoculation which, depending on the plant species, is laborious (Liu et al., 2012; Kawchuk et al., 2001; Fradin et al., 2009; Vining and Davis, 2009). We have previously shown that transfer of tomato Ve1 to Arabidopsis resulted in resistance against race 1 *Verticillium* strains (Fradin et al., 2011), providing a relatively fast method to assess functionality of Ve1 orthologs (Zhang et al., 2011). Nevertheless, the engineering of stable Arabidopsis transgenes still requires a couple of months and a lot of works to get homozygous transgenic Arabidopsis with those Ve orthologs. Our results indicate that co-expression of such candidate with Ave1 in *N. tabacum* may be a much more rapid (matter of days) method to identify functional Ve1 orthologs.

**Expression vectors differentially affect Ave1-Ve1-induced necrosis in tobacco**

Our results show that co-expression of the secreted *Verticillium* effector Ave1 and the corresponding tomato immune receptor Ve1 by agroinfiltration induces HR in *N. tabacum*, but not in *N. benthamiana*. Since silencing suppressors could not improve the strength of the HR in *N. tabacum*, we tested various Gateway-compatible binary vectors that mediate transgene expression driven by the CaMV 35S promoter. These vectors differentially affected the level of necrosis induced by Ve1-mediated recognition of Ave1 in tobacco. These differences may be caused by different transformation efficiencies of the various vectors, or by different expression rates of these vectors. Whereas recombinant protein could not be detected in western analysis upon agroinfiltration of *pK7FWG2::Ve1::GFP*, which only triggers weak HR (Supplemental Figure 2), ample recombinant protein was detected
in western analysis upon agroinfiltration of \textit{pSol2095::Ve1::GFP}, which activates strong HR (Supplemental Figure 2). However, as it was our aim to set up an appropriate expression system for Ve1/Ave-induced HR, we did not further invest to discover the technical reasons for differences in vector efficiencies.

**TRV-based VIGS for functional analysis in \textit{N. tabacum}.**

Previously, by using TRV-based VIGS in tomato, we established a method for functional analysis of genes that are involved in \textit{Verticillium} defense (Fradin et al., 2009). Several candidate genes were silenced in tomato, followed by inoculation with race 1 \textit{V. dahliae} revealing a role in Ve1-mediated \textit{Verticillium} defense for some of them (Fradin et al., 2009; Fradin et al., 2011; Vossen et al., 2010). As TRV induces only mild viral symptoms and infects large areas of adjacent cells, TRV-mediated VIGS has been used extensively in \textit{N. benthamiana} and tomato, and consequently many TRV-based VIGS vectors targeting potential defense components are available (Gabriëls et al., 2007; Gabriëls et al., 2006; Stulemeijer et al., 2007; Chen et al., 2009; Ho et al., 2009; Fradin et al., 2009; Vossen et al., 2010). Since tomato and tobacco are close relatives they share a high degree of coding sequence homology between orthologs, allowing TRV constructs targeting tomato sequences to be successfully used in tobacco and vice versa (Gabriëls et al., 2006; Senthil-Kumar et al., 2007; Fradin et al., 2009; Velasquez et al., 2009; Liebrand et al., 2012).

Recently, the development of VIGS in \textit{Nicotiana tabacum} using Tobacco curly shoot virus was described (Huang et al., 2009). However, many TRV-based VIGS vectors targeting potential defense components are available. Therefore, we tried to establish TRV-based VIGS in combination with agroinfiltration in tobacco to study Ve1-signaling. And although TRV-based VIGS has been reported in \textit{N. tabacum} (Ryu et al., 2004), we found it did not induce sufficient gene silencing in leaves of \textit{N. tabacum} cv. SR1, which we used for HR induced by co-expression of \textit{Ve1} and \textit{Ave1}. The
screening of *Nicotiana* species demonstrated that *N. tabacum* cv. Samsun is able to develop an HR upon co-expression of *Ve1* and *Ave1*, and is amenable to TRV-based VIGS. By silencing of *EDS1*, which is known to be required for *Ve1*-mediated resistance in tomato and Arabidopsis (Hu et al., 2005; Fradin et al., 2011; Fradin et al., 2009) we demonstrated that agroinfiltration in combination with VIGS can be employed to unravel *Ve1*-mediated defense signaling using tobacco.

**MATERIALS AND METHODS**

**Plant materials**

Tobacco plants were grown in soil in the greenhouse with the following settings: 21/19°C with 70% relative humidity 16/8 hour day/night periods, respectively, and 100 W·m⁻² supplemental light when the light intensity dropped below 150 W·m⁻².

**Expression vectors pSol2092 and pSol2095**

To construct pSol2092 and pSol2095, first a backbone was constructed that contains the NPTIII, trfA, oriV, T-DNA left border and part of the nptII plant selection marker from pEAQ-HT (Sainsbury et al., 2009), the aadA gene and the pBR322 origin of replication from pPZP200 (Hajdukiewicz et al., 1994), the T-DNA right border and part of the nptII plant selection marker derived from pMOG800 (Knoester et al., 1998). Subsequently, the expression cassettes from pB7WG2 and pK7FWG2 (Karimi et al., 2002) were inserted in between the right border of the T-DNA region and the NPTII plant selection marker to create pSol2092 and pSol2095, respectively. During the process a number of restriction sites were added and removed. DNA Fragments that were created by PCR were sequence verified. The vector sequences are deposited in the NCBI nucleotide database.

**Generation of constructs for over-expression of *Ve1* and *Ave1***

The coding sequence of *V. dahliae Ave1* (de Jonge et al., 2012) was PCR amplified using primers containing AttB1 and AttB2 sites for Gateway-compatible cloning; Vd-F 5’-GGGGACAAAGTTGACAAAAAGCAGG
CTATGAAGCTTCTACGCTT-3' and Vd-R 5'-GGGACCACCTTTGTACAAGAAAAGCTGGT'TATATCTGTCTAAATTC-3'. The resulting PCR product was cleaned from 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, California) and transferred into donor vector pDONR207 using Gateway BP Clonase II enzyme mix (Invitrogen, Carlsbad, California) to generate entry vector pDONR207::Ave1. Similarly, the tomato Ve1 coding sequence was PCR amplified from pMOG800::Ve1 (Fradin et al., 2009) using primers, attB-Ve1-F 5'- GGGGACAAGTTTGTACAAAAAGCAGGCTATGAAAATGATGGCAACTCT-3' and attB-Ve1-R 5'- GGGGACCACCTTTGTACAAGAAAGCTGGGTACTTTCTTGAAAACCAAAG-3'. The PCR product was cleaned from agarose gel and transferred into Gateway donor vector pDONR207 to generate entry vector pDONR207::Ve1. The entry constructs pDONR207::Ave1 and pDONR207::Ve1 were subsequently cloned into Gateway destination vector pFAST-R02 (Shimada et al., 2010), pMDC32 (Curtis and Grossniklaus, 2003), pEarleyGate100 (Earley et al., 2006), pGD625 (Dekkers et al., 2008) and pSol2092 using Gateway LR Clonase II enzyme mix (Invitrogen, Carlsbad, California) to generate expression constructs driven by the CaMV35S promoter. The expression constructs were transformed into E. coli and transformants were checked by colony PCR analysis using primers AttB1F 5'-ACAAGTTTGTACAAAAAGCAGGCT-3' and AttB2R 5'-ACCACTTTGTACAAGAAAGCTGGG-3'. The expression constructs were subsequently transformed into Agrobacterium tumefaciens strain GV3101 (Hellens et al., 2000), by electroporation. Finally, the plasmids were isolated from A. tumefaciens transformants and sequenced.

**Generation of constructs for Ve1-GFP fusion**

To generate Ve1 fused at the 3’ end to a Green fluorescent protein (GFP) tag, the Ve1 coding sequence lacking the stop codon was PCR amplified using primers containing Gateway attB sites; attB-Ve1-F 5'- GGGGACAAGTTTGTACAAGAAAAGCAGGCTATGAAAATGATGGCAACTCT-3' and attB-Ve1-R-SC 5'-GGGACCTTTGTACAAGAAAGCTGGTACTTTCTTGAAAACCAAAG-3'. The PCR fragment was cloned into pDONR207 (Invitrogen, Carlsbad, California)
through a Gateway BP reaction to generate entry vector pDONR207::Ve1-SC. Subsequently, pDONR207::Ve1-SC was transferred into the Gateway-compatible destination vectors pK7FWG2 (Karimi et al., 2002), pMDC83 (Curtis and Grossniklaus, 2003), pGWB451 (Nakagawa et al., 2007) and pSol2095 to generate expression constructs driven by the constitutive CaMV35S promoter and C-terminally tagged to GFP. Fusion constructs were transformed into *A. tumefaciens* strain GV3101 by electroporation.

**A. tumefaciens-mediated transient expression**

*A. tumefaciens* containing expression constructs were infiltrated into tobacco plants as described previously (van der Hoorn et al., 2000). Briefly, an overnight culture of *A. tumefaciens* cells was harvested at OD<sub>600</sub> of 0.8 to 1 by centrifugation and resuspended to a final OD of 2. *A. tumefaciens* cultures containing constructs to express Ave1 and Ve proteins were mixed in a 1:1 ratio and infiltrated into leaves of five- to six-week-old tobacco plants. After agroinfiltration, plants were grown in the climate room at 22°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity. At five days post infiltration (dpi), leaves were examined for necrosis.

**Amplification of a Ve1 homolog from *N. glutinosa***

Total RNA of *N. glutinosa* seedlings was extracted using the QIAGEN RNeasy extraction kit (Qiagen, Valencia, California). First-strand cDNA was synthesized from 1 μg of total RNA, using the SuperScript™ III cDNA synthesis kit (Invitrogen, Carlsbad, California) according to the manufacturers’ instructions. The partial coding sequence of *N. glutinosa* was PCR amplified from cDNA with *Pfu* high-fidelity enzyme (Promega, Madison, Wisconsin) using primer Ve1-F4 5’-GGTCTCACCCACCTGAATCTTTC-3’ and Ve1-R5 5’-CCTTGTAAGTTATTCGCACTGA-3’. PCR was performed for 30 cycles, with denaturing at 95°C for 30s, annealing at 55°C for 30s, and elongation at 72°C for 60s. The resulting 461bp PCR product was cleaned from 1% agarose gel, cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin), and sequenced using the M13F primer 5’-TGTAAAACGACGGCCAGT-3’.
VIGS
The TRV2 constructs \textit{TRV:GFP}, \textit{TRV:PDS}, \textit{TRV:EDS1}, \textit{TRV:SGT1} and \textit{TRV:NRC1} have been described before (Gabriëls et al., 2006). To establish VIGS in tobacco, cotyledons of two- to three-week-old tobacco seedlings were infiltrated with 1:1 mixtures of pTRV1 and pTRV2 constructs, using a needleless 1 mL syringe as described by van der Hoorn et al (2000). Leaves were superficially wounded with a needle to improve infiltration. Photobleaching was observed at three weeks post infiltration upon inoculation of \textit{TRV:PDS}.

\textit{Verticillium} inoculations
Race 1 \textit{V. dahliae} strain JR2 and the \textit{Ave1} deletion strain (\(\Delta\textit{Ave1}\); de Jonge et al, 2012) were grown on potato dextrose agar (PDA) at 22°C. \textit{V. dahliae} conidia were harvested from 7- to 14-day-old fungal plates and washed with tap water. The conidia were suspended to a final concentration of 10\(^6\) conidia/mL. For inoculation, 3-week-old \textit{N. glutinosa} plants were gently uprooted and the roots were rinsed in tap water. The roots were then dipped in the conidial suspension for 3 minutes. As a control, plants were mock-inoculated in tap water. After (mock-) inoculation, plants were transferred to soil. The inoculated plants were evaluated by observing disease symptoms such as stunting of plant growth, wilting, chlorosis and necrosis at 14 dpi.

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Ve1-mediated Ave1 recognition in tobacco


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SUPPLEMENTAL DATA

Supplemental Figure 1. Co-expression of tomato Ve1 and Verticillium dahliae Ave1 does not induce a hypersensitive response in Nicotiana benthamiana. Agroinfiltration of constructs for constitutive expression of tomato immune receptor gene Ve1 and the corresponding V. dahliae avirulence gene Ave1 do not result in necrosis. In contrast, necrosis is triggered in the same leaf upon co-expression of avirulence gene Avr9 from the tomato leaf mold fungus Cladosporium fulvum in combination with the corresponding tomato receptor gene Cf-9. In all cases silencing suppressor p19 was included. (A) Co-infiltration of pMOG800::Ve1 and pFAST::Ave1. (B) Co-infiltration of pMOG800::Ve1 and pSol2092::Ave1. All pictures were taken at seven days post infiltration.

Supplemental Figure 2. Co-expression of C-terminally GFP-tagged Ve1 with Verticillium dahliae Ave1 triggers necrosis in Nicotiana tabacum. Strongest necrosis is observed upon co-expression of GFP-tagged Ve1 and Ave1 using the pSol2092 vector. No expression is observed when only GFP-tagged Ve1 is expressed (control). All pictures were taken at five days post infiltration.
Supplemental Figure 3. Virus-induced gene silencing induces bleaching of stems but not of leaves in *N. tabacum* cv. SR1 plants. Photobleaching symptoms on stems and leaves of *N. tabacum* cv. SR1 plants upon agroinfiltration of constructs containing recombinant tobacco rattle virus with a fragment of the *phytoene desaturase* (*PDS*) gene, shown at 21 days post infiltration.

Supplemental Figure 4. Alignment of part of tomato *Ve1* with a partial coding sequence from a putative *Nicotiana glutinosa* homolog. Identical amino acids are indicated in yellow.
Chapter 3

Ve1-mediated resistance against *Verticillium* does not involve a hypersensitive response in *Arabidopsis*

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ABSTRACT
Recognition of pathogen effectors by plant immune receptors leads to the activation of immune responses that often include a hypersensitive response (HR); rapid and localized host cell death surrounding the site of attempted pathogen ingress. We previously demonstrated that recognition of the *Verticillium dahliae* effector protein Ave1 by the tomato immune receptor Ve1 triggers an HR in tomato and tobacco. We furthermore demonstrated that tomato Ve1 provides *Verticillium* resistance in Arabidopsis upon Ave1 recognition. In this study, we investigated whether co-expression of Ve1 and Ave1 in Arabidopsis results in an HR, which could facilitate a forward genetics screen. Surprisingly, we found that co-expression of Ve1 and Ave1 does not induce an HR in Arabidopsis. These results suggested that an HR may occur as a consequence of Ve1/Ave1-induced immune signaling in tomato and tobacco, but is not absolutely required for *Verticillium* resistance.
INTRODUCTION

Immunity in plants against pathogens is generally governed by immune receptors that detect pathogen (–induced) ligands of various nature (Thomma et al., 2011; Boller and Felix, 2009). Recognition of such ligands by immune receptors results in the activation of defense responses, which are often accompanied by a hypersensitive response (HR) in which necrosis of plant tissue surrounding the site of attempted penetration is activated to stop further pathogen colonization.

Verticillium spp. are economically important pathogens that cause vascular wilt diseases in a wide range of plant species worldwide, with V. dahliae and V. albo-atrum as the main pathogenic species (Fradin and Thomma, 2006; Klosterman et al., 2009). The interaction between tomato and V. dahliae has been established as a model to study the interaction between plants and vascular pathogens (Fradin et al., 2009; Fradin and Thomma, 2006). In this model, immunity against V. dahliae is governed by the interaction between the tomato gene Ve1 and the V. dahliae gene Ave1 (Fradin et al., 2009; de Jonge et al., 2012). Ve1 encodes a receptor-like protein (RLP)-type immune receptor that carries extracellular leucine rich repeats (eLRRs), a single-pass transmembrane (TM) domain and a short cytoplasmic tail that lacks obvious motifs for intracellular signaling (Fradin et al., 2009; Fradin and Thomma, 2006; Kawchuk et al., 2001). Ave1 encodes an effector protein that has a high degree of homology to plant natriuretic peptides and that is secreted by Verticillium during host colonization (de Jonge et al., 2012).

Various RLPs have been shown to play roles in plant development or in pathogen resistance in several plant species (Wang et al., 2010). However, the genetics of RLP-mediated disease resistance signaling has been most extensively studied in Solanaceous plants, making use of the tomato Cf, Ve and LeEix proteins, and also exploiting tobacco as a heterologous model species (Gabriëls et al., 2006; Gabriëls et al., 2007; Vossen et al., 2010; van der Hoorn et al., 2000; Bar et al., 2010; Ron and Avni, 2004). One of the tools that has been exploited is the progeny of a cross of Cf-4 tomato with
transgenic tomato lines expressing the corresponding *Cladosporium fulvum* effector gene *Avr4*, which results in *Cf-4/Avr4* offspring that displays lethality at the seedling stage, but can be rescued upon incubation at 33°C (Cai et al., 2001; de Jong et al., 2002; Thomas et al., 1997). Upon transfer to 20°C, a synchronous systemic HR is activated, which has successfully been employed to study Cf-4 signaling (de Jong et al., 2002; Gabriëls et al., 2006; Stulemeijer et al., 2007).

Due to the lack of RLPs that have been implicated in immune signaling of *Arabidopsis thaliana*, the many resources that are available for this model species have only been exploited to a limited extent thus far (Wang et al., 2008; Wang et al., 2010). Interestingly, it was recently demonstrated that interfamily transfer of tomato *Ve1* into Arabidopsis results in resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* (Fradin et al., 2011). Moreover, based on mutant analysis the requirement of SERK family members for Ve1 resistance was investigated in Arabidopsis, demonstrating a critical role for SERK1 in addition to SERK3/BAK1. With virus-induced gene silencing, the requirement of SERK1 for Ve1-mediated resistance was subsequently confirmed in tomato, demonstrating that Arabidopsis can be used to characterize Ve1 signaling (Fradin et al., 2011).

We recently demonstrated that potato virus X-mediated transient expression of *Ave1* specifically triggers HR on tomato carrying *Ve1* (de Jonge et al., 2012). In addition, transient co-expression of *Ave1* and *Ve1* through Agrobacterium *tumefaciens*-mediated transient transformation (Agroinfiltration) in *Nicotiana tabacum* and *N. glutinosa* similarly induced HR (de Jonge et al., 2012; Zhang et al., 2013). In this study, we investigated whether co-expression of *Ve1* and *Ave1* in Arabidopsis results in an HR that can be used as read-out to investigate RLP signaling mediated by Ve1. Surprisingly, we found that although *Ave1* is able to trigger an HR in resistant tomato and *Nicotiana* plants, such HR does not occur in Arabidopsis. However, our results show that the HR is not required for Ve1-mediated resistance in this species.
RESULTS

Agroinfiltration in Arabidopsis leaves

Previously, it has been demonstrated that agroinfiltration can be employed to study immune receptor-mediated HR in Arabidopsis (Lee and Yang, 2006). To investigate whether agroinfiltration can similarly be exploited in Arabidopsis, *A. tumefaciens* carrying a construct for constitutive expression of VdNLP2 was infiltrated into Arabidopsis leaves. It has recently been shown that the VdNLP2 protein from *V. dahliae* exhibits cytotoxic activity upon infiltration into Arabidopsis leaves (Zhou et al., 2012; Santhanam et al., 2013). Indeed, agroinfiltration of VdNLP2 resulted in clear necrosis within seven days (Figure 1A). Next, *A. tumefaciens* carrying a construct for constitutive expression of C-terminally GFP-tagged Ve1 was infiltrated into leaves of three-week-old Arabidopsis plants. As a control, *A. tumefaciens* carrying a construct for constitutive expression of untagged Ve1 was infiltrated. At two days post infiltration (dpi), GFP fluorescence was detected in leaves infiltrated with *A. tumefaciens* carrying a construct for expression of GFP-tagged Ve1 which was not observed upon expression of untagged Ve1 (Figure 1B). Collectively, these results corroborate that agroinfiltration can be used for transgenic expression of Ve1 in Arabidopsis.

Co-expression of Ave1 and Ve1 in Arabidopsis does not induce HR

To test whether Ve1-mediated recognition of Ave1 results in HR in Arabidopsis, transient expression of Ave1 by agroinfiltration was pursued in Ve1-transgenic Arabidopsis. However, up to seven dpi, no signs of necrosis could be observed in agroinfiltrated Arabidopsis plants (Figure 1C). Similarly,
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infiltration (dpi). (C) Leaves of wild type or Ve1-expressing Arabidopsis plants were infiltrated with *A. tumefaciens* carrying 35S::Ave1. In addition, leaves of wild type Arabidopsis were co-infiltrated with *A. tumefaciens* carrying 35S::Ave1 and 35S::Ve1. No necrosis was observed in infiltrated Arabidopsis leaves. (D) Co-expression of Ve1 and Ave1 in *Nicotiana tabacum* cv. Petite Havana SR1 results in HR.
co-expression of Ave1 and Ve1 in wild type Arabidopsis did not lead to HR (Figure 1C). In contrast, co-expression of Ave1 and Ve1 in Nicotiana tabacum cv. Petite Havana SR1 results in clear HR with in five dpi (Figure 1D; Zhang et al., 2013).

To further investigate whether Ve1-mediated recognition of Ave1 induces HR in Arabidopsis, stable co-expression of Ave1 and Ve1 was pursued. To this end, the Ave1 coding sequence was cloned into binary vector pFAST-R02 (Shimada et al., 2010) to generate expression construct pFAST::Ave1 in which Ave1 expression was driven by the CaMV 35S promoter. This construct contains a non-destructive RFP marker to identify transgenic seeds by UV microscopy (Shimada et al., 2010). The pFAST::Ave1 construct was subsequently transformed into Ve1-transgenic and wild type Arabidopsis (Clough and Bent, 1998), and Ave1-transgenic seeds were selected. Unexpectedly, the seeds germinated, and seedlings developed into mature plants that successfully set seeds. To evaluate the growth in more detail, three independent transgenic lines (named Ve1/Ave1-1, Ve1/Ave1-2 and Ve1/Ave1-3) which carry both the Ve1 and the Ave1 gene were grown on MS medium in a growth chamber at 22°C, or on soil in the greenhouse, alongside Ve1-transgenic, Ave1-transgenic and non-transgenic control plants. No phenotypic alterations were observed in plants that co-express Ve1 and Ave1 when compared with plants that express either of the transgenes alone or non-transgenic controls (Figure 2A). As it has been demonstrated for tomato that the HR can be suppressed by elevated temperature (de Jong et al., 2002), we also grew the plants at 16°C. However, also under these conditions, no necrosis or growth inhibition was observed (Figure 2A). RT-PCR was performed to confirm simultaneous expression of Ve1 and Ave1 in these lines (Figure 2B).

**In planta expressed Ave1 activates Ve1-mediated HR in N. tabacum**

We previously showed that transient expression of Ave1 by Potato virus X (PVX) specifically induced HR in resistant tomato carrying Ve1 (de Jonge et
Figure 2. Stable co-expression of Ve1 and Ave1 does not affect Arabidopsis viability. (A) No phenotypical alterations were observed in plants that co-express Ve1 and Ave1 when compared with plants that express either of the transgenes alone or non-transgenic control plants. Arabidopsis plants were grown on MS medium in a growth chamber at 22°C or 16°C, or on soil in the greenhouse. (B) RT-PCR was performed to confirm expression of Ve1 and Ave1 in the transgenic lines.
In addition, HR can also be induced in tobacco upon co-expression of Ave1 and Ve1 by agroinfiltration (Zhang et al., 2013). These experiments demonstrate that in planta expressed Ave1 is able to activate Ve1-mediated HR. However, since both transient and stable expression of Ave1 did not induce HR in Ve1-transgenic Arabidopsis, we investigated whether the Ave1 protein produced in Arabidopsis is can be recognized by Ve1. To this end, apoplastic fluid (AF) was extracted from leaf tissue of Ave1-transgenic and wild type Arabidopsis by the vacuum infiltration-centrifugation technique (Joosten, 2012). The obtained AF was subsequently infiltrated into the leaves of N. tabacum transiently expressing Ve1 or its non-functional homolog Ve2 (Zhang et al., 2013). By three dpi, Ve1-expressing leaves developed clear necrosis when AF of Ave1-transgenic Arabidopsis was infiltrated (Figure 3). In contrast, AF of wild type Arabidopsis did not induce necrosis in Ve1-expressing N. tabacum. Furthermore, AF of neither Ave1-transgenic nor wild type Arabidopsis induced HR in N. tabacum leaves expressing Ve2 (Figure 3). These data demonstrate that the Ave1 protein expressed in

**Figure 3. In planta expressed Ave1 triggers Ve1-mediated HR in N. tabacum.** Apoplastic fluid (AF) extracted from Ave1-transgenic Arabidopsis induced HR in Ve1-expressing, but not in Ve2-expressing, N. tabacum leaves. AF from wild type Arabidopsis (Col-0) does not induce HR.
transgenic Arabidopsis potentially can activate Ve1-mediated HR.

**Inoculation of Ve1/Ave1-transgenic Arabidopsis**

We hypothesized that co-expression of *Ve1* with *Ave1* may result in constitutive activation of plant immunity in Arabidopsis in the absence of HR. However, considering the absence of a visible phenotype, such as dwarfing that is typically observed in Arabidopsis constitutive defense mutants such as *cpr*, *cim*, and other constitutive PR expression mutants (Gou et al., 2009; Cheng et al., 2011; Maleck et al., 2002; Bowling et al., 1994), strongly elevated defense is not expected in the lines that co-express *Ve1* and *Ave1*. We challenged all transgenic lines and wild type plants with the *V. dahliae* race 1 strain JR2. As expected, non-transgenic and *Ave1*-transgenic plants displayed typical Verticillium wilt symptoms upon *V. dahliae* inoculation, including wilting, stunting, chlorosis and necrosis (Figure 4A). In contrast, *Ve1*-expressing plants, as well as plants that co-express *Ve1* and *Ave1*, showed clear resistance against *V. dahliae* (Figure 4). Next, we inoculated *Ave1* deletion mutants of *V. dahliae* strain JR2 (de Jonge et al., 2012) on the various genotypes. Non-transgenic and *Ave1*-transgenic plants displayed similar symptoms when compared with inoculation with the wild type fungal strain and, as shown previously, *Ve1*-expressing plants were not able to provide resistance against *Ave1* deletion mutants (Figure 4; de Jonge et al., 2012). Surprisingly, however, also plants that co-express *Ve1* and *Ave1* were susceptible to the *Ave1* deletion mutants (Figure 4). These data suggest that co-expression of *Ve1* and *Ave1* does not activate basal defense against fungal infection.

To corroborate that co-expression of *Ve1* and *Ave1* does not activate basal defense, inoculation of the transgenic lines with the bacterial pathogen *Pseudomonas syringae* pv. tomato strain DC3000 was performed. Also in this case, no increased resistance was observed in plants that co-express *Ve1* and *Ave1* (Figure 5), confirming that basal defense is not activated though co-expression of *Ve1* and *Ave1*. 
Figure 4. Inoculation of Ve1/Ave1-transgenic Arabidopsis with *V. dahliae*. (A) Typical appearance of non-transgenic and transgenic Arabidopsis lines upon mock-inoculation or inoculation with *V. dahliae* race 1 isolate JR2 or the Ave1 deletion strain (JR2ΔAve1). (B) Quantification of Verticillium wilt symptoms in wild type and transgenic Arabidopsis. Bars represent quantification of symptom development shown as percentage of diseased rosette.
DISCUSSION

Recently, the *V. dahliae* effector that activates immunity in *Ve1* tomato plants was identified through population genomics as Ave1, a protein that has homology to plant natriuretic peptides (de Jonge et al., 2012). When expressed through PVX in *Ve1*-carrying tomato, and when co-expressed with *Ve1* through agroinfiltration in *N. tabacum* and *N. glutinosa*, it was demonstrated that the combination of *Ve1* and Ave1 induces HR (de Jonge et al., 2012). Furthermore, it was recently shown that *Ve1*-transgenic Arabidopsis is resistant to race 1 strains of *V. dahliae* and *V. albo-atrum*, demonstrating that *Ve1* remains fully resistance functional after interfamily transfer into Arabidopsis (Fradin et al., 2011). Tomato crosses, in which the cross of plants lines that express a pathogen effector and corresponding RLP immune receptor set seeds normally, but develop seedling lethality upon germination of the seeds (Cai et al., 2001; Thomas et al., 1997; de Jong et al., 2002). With this knowledge, we aimed to develop a similar “dying seedling” in Arabidopsis. Mutagenesis of such seeds would allow for a simple forward genetics screen, as seeds that survive after germination are likely affected in signaling components downstream of the immune receptor if they are not mutagenised in either the *Ave1* or *Ve1* transgene. Unexpectedly, however, we failed to identify the HR upon co-expression of *Ve1* and Ave1 in transient assays and upon stable transformation in Arabidopsis. Moreover, the progeny of a cross of constitutively expressing *Ve1* and Ave1 Arabidopsis plants resulted in progeny that did not show any obvious phenotypical differences when compared with non-transgenic wild type plants.

Traditionally, the HR was considered as a defense mechanism that directly prevents pathogen growth (Spoel and Dong, 2012). However, a
Figure 5. Inoculation of Ve1/Ave1-transgenic Arabidopsis with *P. syringae* pv. tomato strain DC3000. (A) Typical appearance of non-transgenic and transgenic Arabidopsis lines upon mock-inoculation or inoculation with *P. syringae*. (B) Bacterial biomass determined by real-time PCR in wild type Arabidopsis and transgenic lines. Bars represent levels of the *P. syringae Oprf* gene relative to Arabidopsis Rubisco transcript levels (for equilibration).
growing number of examples report on immunity in absence of HR mediated by nucleotide binding site leucine-rich repeat (NBS-LRR) type immune receptors, suggesting that HR and immunity to infection are genetically separable. These example include \textit{Rx1}- and \textit{Rx2}-mediated resistance to PVX in potato (Bendahmane et al., 1999; Bendahmane et al., 2000), \textit{Mla1}-mediated resistance against the powdery mildew pathogen \textit{Blumeria graminis} f. sp. \textit{hordei} in barley (Bieri et al., 2004), \textit{Rrs1}-mediated resistance against the scald pathogen \textit{Rhynchosporium secalis} (Lehnackers and Knogge, 1990; Rohe et al., 1995), \textit{Rdg2a}-mediated resistance to the leaf stripe pathogen \textit{Pyrenophora graminea} in barley (Bulgarelli et al., 2010), and \textit{RPS4}-mediated resistance to \textit{Pseudomonas syringae} in Arabidopsis (Gassmann, 2005). In Arabidopsis, a genetic separation of disease resistance and the HR was first described for the \textit{dnd1} mutant (\textit{defense, no death 1}; Clough et al., 2000), which shows resistance to \textit{Pseudomonas syringae} bacteria expressing the avirulence genes \textit{avrRpt2}, \textit{avrB}, \textit{avrRpm1}, and \textit{avrRps4} in absence of an HR. Similarly, the Arabidopsis mutant \textit{hlm1} (\textit{HR-like lesion mimic}) (Balagué et al., 2003) and its allelic mutant \textit{dnd2} (Jurkowski et al., 2004) were found to display resistance in absence of HR. In addition to NBS-LRR-type immune receptors, also for Cf-4, an LRR-RLP-type receptor, HR and resistance to \textit{C. fulvum} upon Avr4 recognition could be separated (Stulemeijer et al., 2007). Our data suggest that \textit{Ve1}-mediated \textit{Verticillium} resistance in Arabidopsis does not involve an HR as well, although it cannot be excluded that local, small-scale HR occurs that is not visible to the naked eye.

To date, several types of immune receptors have been identified that can be divided into extracellular receptors and cytoplasmic receptors. Both perceive pathogen-derived ligands or ligands that are released as a consequence of pathogen colonization to activate immune responses (Boller and Felix, 2009). While many of these receptors activate an HR upon ligand perception, others generally do not (Thomma et al., 2011). Here, we demonstrate that the occurrence of the HR may be determined by the plant
species in which the receptor is expressed, as treatment with Ave1 leads to HR in tomato and tobacco plants that express Ve1, but not in *N. benthamiana*, nor in Arabidopsis (de Jonge et al., 2012; Zhang et al., 2012; this study). Nevertheless, *Ve1*-expressing Arabidopsis is resistant to race 1 *Verticillium* strains (Fradin et al., 2011). These data suggest that the HR is not absolutely required for *Verticillium* wilt resistance, and may occur as a consequence of escalated signaling upon Ave1 recognition in tomato and tobacco. The mechanism by which plants actually stop *V. dahliae* infection still requires further investigation.

**MATERIALS AND METHODS**

**Plant materials**

Arabidopsis plants were grown in the greenhouse or in the climate chamber with the following settings: 22/19°C (unless mentioned otherwise) with 70% relative humidity and 16/8 hour day/night periods, respectively. 100 W·m⁻² supplemental light was supplied in the greenhouse when the light intensity dropped below 150 W·m⁻².

**Generation of constructs**

To generate *Ve1* fused at the 3’ end to the green fluorescent protein (GFP), the *Ve1* coding sequence lacking the stop codon was PCR amplified using primers attB-Ve1-F 5’- GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAA TGATGGCAACTCT-3’ and attB-Ve1-R-S 5’- GGGGACCACTTTGTACAAGAAAG CTGGGTACTTTCTGAAAAACCAAG-3’. The PCR fragment was cloned into pDONR207 (Invitrogen, Carlsbad, California) through a Gateway BP reaction to generate entry vector *pDONR207::Ve1-SC*. Subsequently, *pDONR207::Ve1-SC* was recombined with the Gateway-compatible destination vector pSol2095 (Zhang et al., 2013) to generate an expression construct for GFP-tagged Ve1 driven by the constitutive CaMV35S promoter. The fusion construct was transformed into *A. tumefaciens* strain GV3101 by electroporation. For agroinfiltration of untagged Ve1, construct
Absence of Ve1-mediated HR in Arabidopsis

*pMOG800::Ve1* was used (Fradin et al., 2009). The *pFAST::Ave1* has been described by Zhang et al (2013).

**A. tumefaciens-mediated transient expression**

*A. tumefaciens* containing expression constructs were infiltrated into Arabidopsis plants as described previously (van der Hoorn et al., 2000). Briefly, an overnight culture of *A. tumefaciens* cells was harvested at OD<sub>600</sub> of 0.8 to 1 by centrifugation and re-suspended to a final OD of 2. *A. tumefaciens* cultures containing constructs to express *Ave1* or *Ve1* were infiltrated into leaves of three/four-week-old Arabidopsis plants.

**RT-PCR**

Arabidopsis seedlings were collected and total RNA was extracted using the QIAGEN RNeasy extraction kit (Qiagen, Valencia, California). First-strand cDNA was synthesized from 1 µg of total RNA, using the SuperScript™III cDNA synthesis kit (Invitrogen, Carlsbad, California) according to the manufacturers’ instructions. PCR reactions were performed in a total volume of 25 µL with 17.9 µL water, 5 µL 5x PCR buffer, 0.5 µL dNTPs, 0.5 µL of each primer and 0.1 µL GoTaq polymerase (Promega, Madison, Wisconsin) and 1 µL of first-strand cDNA. PCR was performed for 30 cycles, denaturing at 95°C for 15s, annealing at 55°C for 45s, and elongation at 72°C for 60s. The generated PCR products were evaluated by agarose gel electrophoresis. RT-PCR was conducted with *Ve1*-specific primers *Ve1F3* 5’-GGAACAATTTA CTCAGCGGGAGC-3’ and *Ve1R4* 5’-CCATGACTGATTCTTGAGATCGG-3’, or *Ave1*-specific primers *Ave1F* 5’-CACTGGTCACTGCCATCGG-3’ and *Ave1R* 5’-CTTGCAAGGACCCCTCTAGCAC-3’. As an endogenous control, *AtRub-F3* 5’-GCAAGTGTTGGGTTCAAAGCTGGTG-3’ and *AtRub-R3* 5’-CCAGGGTAGGGTTACTCAGATGCTG-3’ were used to amplify a fragment of the Arabidopsis RuBisCo gene from cDNA and from 1 µL of total RNA as control for DNA contamination.
Verticillium inoculations

*V. dahliae* race 1 strain JR2 and the corresponding Ave1 deletion strain (ΔAve1) were grown on potato dextrose agar (PDA) at 22°C. *V. dahliae* conidia were harvested from 7- to 14-day-old fungal plates and washed with tap water. The resuspended conidia were adjusted to a final concentration of 10^6 conidia/mL. For inoculation, the plants were gently uprooted and rinsed in tap water. Subsequently, the roots were dipped in the conidial suspension for 3 minutes. As a control, plants were mock-inoculated in tap water. After inoculation, plants were immediately transplanted to new pots. The inoculated plants were evaluated by observing wilting of leaves at 21 dpi. Quantification of *Verticillium* biomass was performed as described previously (Ellendorff et al., 2009).

P. syringae inoculations

*Pseudomonas syringae* pv. tomato strain DC3000 was cultured on King’s B medium containing 200 μg/mL rifampicin. Inoculation was performed as described previously (van Esse et al., 2008). Briefly a bacterial suspension of 5 × 10^8 cfu/mL in 10 mM MgCl2 and 0.05% Silwet L-77 (Lehle Seeds) was sprayed onto the leaves until droplet runoff. Plants were incubated at 100% RH for 1h, followed by incubation at 24°C, 60% RH, and a 16-h/8-h light/dark regime. Disease progression was scored at 4 DAI.

Bacterial quantification in infected Arabidopsis plants was performed with real-time PCR as described previously (Brouwer et al., 2003). Briefly, real-time PCR was conducted on DNA isolated from *P. syringae* infected Arabidopsis with primers amplifying the Arabidopsis RuBisCo gene as endogenous loading control (AtRub-F3: GCAAGTGTTGGTTCAAGCTGGTG and AtRub-R3: CCAGGTTGAGAGCTCCGGAATGCTG) and primers amplifying the *P. syringae* Oprf gene (OWB575: AACTGAAAAACACCTTGGG and OWB576: CCTGGGGTTGTTGAAGTGTA). Real-time PCR was conducted using an ABI7300 PCR machine (Applied Biosystems) in combination with the qPCR SensiMix kit (BioLine). Real-time PCR conditions were as follows:
an initial 95°C hot start activation step for 10 min was followed by
denaturation for 15s at 95°C, annealing for 30 s at 60°C and extension for
30s at 72°C for 40 cycles.

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Absence of Ve1-mediated HR in Arabidopsis


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Chapter 4

Functional analysis of the tomato immune receptor Ve1 through domain swaps with its non-functional homolog Ve2

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Submitted
ABSTRACT
Resistance in tomato against race 1 strains of the fungal vascular wilt pathogens *Verticillium dahliae* and *V. albo-atrum* is mediated by the Ve locus. This locus comprises two closely linked inversely oriented genes, Ve1 and Ve2, that encode cell surface receptors of the extracellular leucine-rich repeat receptor-like protein (eLRR-RLP) type. While Ve1 mediates *Verticillium* resistance through monitoring the presence of the recently identified *V. dahliae* Ave1 effector, no functionality for Ve2 has been demonstrated in tomato. Ve1 and Ve2 contain 37 eLRRs and share 84% amino acid identity, facilitating investigation of Ve protein functionality through domain swapping. In this study it is shown that Ve chimeras in which the first thirty eLRRs of Ve1 were replaced by those of Ve2 remain able to induce HR and activate *Verticillium* resistance. However, deletion of these thirty eLRRs in Ve1 resulted in loss of functionality, suggesting that these LRRs detect the pathogen-derived Ave1 effector in Ve1 as well as in the non-functional homolog Ve2. It is furthermore shown that the C-terminus of Ve2 fails to activate immune signaling. Nevertheless, both Ve1 and Ve2 were found to interact through their C-termini with the eLRR-containing receptor-like kinase (eLRR-RLK) interactor SOBIR1 that was recently identified as an interactor of eLRR-RLP (immune) receptors. Collectively, these results suggest that Ve2 still interacts with the Ave1 effector, but fails to subsequently activate immune signaling. The finding that Ve2 still interacts with SOBIR1 suggests that this eLRR-RLK does not directly mediate Ve1-dependent immune signaling.
INTRODUCTION

Immunity in plants against pathogen attack is governed by immune receptors that detect appropriate ligands to activate defense. These ligands can either be microbial structures or ligands that occur as a consequence of plant-manipulating activities of microbial effectors (Thomma et al., 2011; Boller and Felix, 2009). The host immune receptors activate various defence responses, often including a hypersensitive response (HR), which is necrosis of plant tissue surrounding the infection site that restricts further growth of the invading pathogen (Hammond-Kosack and Jones, 1996).

Verticillium wilt, caused by species of the soil borne fungal pathogen genus *Verticillium*, has been reported on over 200 dicotyledonous plant species (Fradin and Thomma, 2006; Pegg and Brady, 2002). From tomato (*Solanum lycopersicum*) a locus providing *Verticillium* resistance has been cloned (Kawchuk et al., 2001). This *Ve* locus controls *V. dahliae* and *V. albo-atrum* strains belonging to race 1, while strains that are not controlled are assigned to race 2 (Pegg, 1974). The *Ve* locus is composed of two genes, *Ve1* and *Ve2*, that are highly homologous and that both encode extracellular leucine-rich repeat containing cell surface receptors of the receptor-like protein (eLRR-RLP) class (Kawchuk et al., 2001; Wang et al., 2010a). *Ve1* and *Ve2* are predicted to contain a signal peptide, an eLRR domain composed of two eLRR regions that are separated by a non-LRR island domain (also referred as C1, C3 and C2, respectively), a transmembrane domain, and a short cytoplasmic tail that lacks obvious signaling motifs besides putative homologs of mammalian endocytosis motifs (Kawchuk et al., 2001). Although *Ve1* and *Ve2* share 84% amino acid identity (Kawchuk et al., 2001), only *Ve1* mediates resistance against race 1 *Verticillium* strains in tomato (Fradin et al., 2009). However, it is presently unknown which domains of *Ve1* are required to mediate resistance, and why *Ve2* is not a functional resistance protein. For other eLRR-containing receptors, the eLRRs have been implicated in recognition specificity (Kobe and Deisenhofer, 1996; Kobe and Kajava, 2001; Kinoshita et al., 2005; Hothorn et al., 2011; She et al,
Several tomato eLRR-RLP-type immune receptors, referred to as Cf-proteins, that provide resistance against particular strains of the leaf mold fungus *Cladosporium fulvum* have been cloned (Jones et al., 1994; Dixon et al., 1996; Thomas et al., 1997; Dixon et al., 1998; Takken et al., 1999; Panter et al., 2002). Through domain swaps and gene shuffling analyses, these Cf proteins were scrupulously dissected to identify specificity determining amino acids in their eLRR domains (Chakrabarti et al., 2009; Seear and Dixon, 2003; van der Hoorn et al., 2001; Wulff et al., 2001; Wulff et al., 2009). Overall, these studies demonstrated that specificity of the Cf proteins is determined by the number of eLRRs and specific amino acid residues that can either be clustered or scattered along the eLRR region (Chakrabarti et al., 2009; van der Hoorn et al., 2001; Wulff et al., 2001; Wulff et al., 2009). Furthermore, it was shown that specificity of the Cf proteins can be altered such that they are able to recognize other *C. fulvum* effectors (Chakrabarti et al., 2009; Seear and Dixon, 2003; van der Hoorn et al., 2001; Wulff et al., 2001; Wulff et al., 2009).

Recently, through a population genomics approach in which we compared whole genome sequences of race 1 and race 2 strains, the effector of *Verticillium* race 1 strains that activates Ve1-mediated resistance was identified, designated Ave1. Transient expression of Ave1 by potato virus X (PVX) induced an HR in tomato carrying the Ve1 gene (de Jonge et al., 2012). Furthermore, simultaneous expression of Ve1 and Ave1 through *Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration) in *Nicotiana tabacum* similarly induced an HR (de Jonge et al., 2012; Zhang et al., 2013). Recently, it was demonstrated that functionality and specificity of tomato Ve1 is maintained when it is expressed in Arabidopsis (*Arabidopsis thaliana*) plants, as Ve1-transgenic plants are resistant to race 1 strains of *V. dahliae* as well as *V. albo-atrum*, while race 2 strains remain virulent on these plants (Fradin et al., 2011; Fradin et al., 2009). The use of Arabidopsis thus allows testing the functionality of chimeric Ve proteins in resistance
against race 1 *Verticillium* strains. In this manuscript, we report on domain swaps between Ve1 and Ve2 that were expressed in *N. tabacum* and Arabidopsis to investigate functionality of the chimeric Ve proteins.

**RESULTS**

**Co-expression of Ave1 with HA-tagged Ve1 induces HR in tobacco**

To screen for functionality of constructs encoding domain swaps between Ve1 and Ve2, the coding sequence (CDS) of *V. dahliae* Ave1 was cloned behind the *cauliflower mosaic virus* (CaMV) 35S promoter to generate expression construct Ave1. The CDSs of Ve1 (FJ464556) and Ve2 (FJ464558), fused to the CDS for an HA epitope tag, were cloned behind the CaMV 35S promoter to the generate expression constructs Ve1HA and Ve2HA, respectively (Figure 1A). When tobacco leaves were co-infiltrated with a 1:1 mixture of *A. tumefaciens* cultures carrying Ave1 and Ve1HA respectively, HR was observed (Figure 1B). In contrast, co-expression of Ave1 with Ve2HA in tobacco did not induce an HR (Figure 1B). Finally, stability of the HA-tagged Ve proteins was verified by immunoblotting (Figure 1C).

**Ve1 provides resistance against Verticillium in sgs2 plants**

The Arabidopsis posttranscriptional gene silencing (PTGS) mutant sgs2 (Dalmay et al., 2000; Mourrain et al., 2000) typically shows little variation in transgene expression between individual transformants, and thus reduced numbers of transgenes need to be analysed (Butaye et al., 2004). Furthermore, we have previously demonstrated that the sgs2 mutant displays enhanced *Verticillium* susceptibility when compared with wild type plants (Ellendorff et al., 2009). To assess the functionality of HA-tagged Ve proteins, sgs2 plants were transformed with Ve1HA or Ve2HA and the resulting transgenic lines were challenged with the *V. dahliae* race 1 strain JR2. As expected, Ve2HA-expressing plants were as diseased as non-transgenic plants and displayed typical Verticillium wilt symptoms including stunting, wilting, anthocyanin accumulation, chlorosis, and
Figure 1. *Ve1*, but not of *Ve2*, provides resistance against *V. dahliae* race 1. (A) Schematic representation of the transgenically expressed *Ve1* (*Ve1HA*) and *Ve2* (*Ve2HA*) proteins. (B) Co-expression of *Ve1HA*, but not *Ve2HA*, with *Ave1* in tobacco results in a HR. (C) HA-tagged *Ve* proteins were detected using HA antibody (α-HA). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading. (D) Typical appearance of non-transgenic *sgs2* (Co.) and transgenic Arabidopsis *sgs2* lines that constitutively express *Ve1* or *Ve2* (*Ve1HA* and *Ve2HA*, respectively) upon mock-inoculation or inoculation with *V. dahliae* race 1. (E) Quantification of Verticillium wilt symptoms (Sympt.) in Co. and transgenic lines. Bars represent quantification of symptom development shown as percentage of diseased rosette leaves with standard deviation. Co. is set to 100%. Asterisks indicate significant differences when compared with Co. (P<0.001). For each construct two transgenic lines are shown (1, 2).
necrosis (Figure 1D). In contrast, *Ve1HA*-expressing plants displayed clear *Verticillium* resistance as only few, if any, symptoms were observed on the inoculated plants (Figure 1D; 1E). These data show that HA-tagged Ve1 was able to provide *Verticillium* resistance, while HA-tagged Ve2 did not. Collectively, these results demonstrate that PTGS, which is affected in the *sgs2* mutant and is required for basal defence against *Verticillium* (Ellendorff et al., 2009), is not required for Ve1-mediated resistance in Arabidopsis, and that HA-tagging of Ve1 does not affect its functionality.

**Ve1 and Ve2 comparison**

Ve1 and Ve2 contain 37 imperfect eLRRs and share 84% amino acid identity (Figure 2). Of the 174 amino acid differences between Ve1 and Ve2, 117 are in the eLRRs and non-eLRR island domain. Furthermore, the Ve1 cytoplasmic tail is 104 amino acids shorter than the cytoplasmic tail of Ve2 (Figure 2). Remarkably, the region between eLRR19 and eLRR24 in the C1 domain is characterized by only a few amino acid differences. To identify regions that are required for Ve protein functionality, a domain swap strategy was designed, allowing to exchange eLRRs between Ve1 and Ve2. The exact locations for the domain swaps between Ve1 and Ve2 were selected based on the presence of conserved endogenous restriction sites in the coding sequences of the two proteins (Figure 2).

*Figure 2. Protein sequence alignment of Ve1 and Ve2.* Columns from Left to Right, I: Alignment of Ve1 (red) and Ve2 (blue) divided into: N-terminal signal peptide (A), leucine-rich repeat (eLRR) domains with each of the 37 eLRRs separated by a dashed line (B and D), non-LRR island domain (C), extracytoplasmic domain (E), transmembrane domain (F), and cytoplasmic domain (G). Conserved amino acid residues between Ve1 and Ve2 are highlighted. The underlined amino acid residues in eLRR8, eLRR14-15, eLRR21, eLRR30, and eLRR35 indicate positions that were used for domain swaps. II: Number of different amino acids between Ve1 and Ve2. III: Schematic representations of Ve1 and Ve2. Red and turquoise boxes represent the 37 eLRR domains of Ve1 and Ve2, respectively. Yellow and dark blue boxes represent the non-LRR island domains of Ve1 and Ve2, respectively. Green and mauve boxes represent the extracytoplasmic, transmembrane, and cytoplasmic domains of Ve1 and Ve2, respectively. IV: Restriction enzyme recognition site in eLRR8, eLRR14-15, eLRR21, eLRR30, and eLRR35 that were used for domain swaps.
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Chimeras containing the C-terminus of Ve2 do not provide Verticillium resistance

To investigate whether Ve2 can be engineered to provide Verticillium resistance, we generated five chimeric Ve proteins; Ve1[8]Ve2, Ve1[14]Ve2, Ve1[21]Ve2, Ve1[30]Ve2, and Ve1[35]Ve2, in which the first 8, 14, 21, 30 or 35 eLRRs of Ve2 were replaced by those of Ve1, respectively (Figure 3A). Expression of none of the constructs resulted in HR upon coinfiltration with Ave1 by agroinfiltration in tobacco (Figure 3B). Stability of the chimeric Ve proteins was confirmed by immunoblotting (Figure 3C). To further investigate the functionality of the chimeric Ve proteins, Arabidopsis sgs2 plants were transformed with the domain swap constructs and the transgenic lines were challenged with race 1 V. dahliae. As expected, all transgenic lines were as diseased as wild type plants (Figure 3D-3E).

eLRR30 to eLRR35 are required for Ve1 functionality

To identify eLRRs that are required for Ve1 protein functionality, five Ve chimeric proteins were engineered; Ve2[8]Ve1, Ve2[14]Ve1, Ve2[21]Ve1, Ve2[30]Ve1, and Ve2[35]Ve1 in which the first 8, 14, 21, 30 or 35 eLRRs of Ve1 were replaced with those of Ve2, respectively (Figure 4A). Intriguingly, co-expression of Ave1 in combination with Ve2[8]Ve1, Ve2[14]Ve1, Ve2[21]Ve1, and Ve2[30]Ve1 resulted in HR in tobacco (Figure 4B). In contrast, tobacco leaves expressing the Ve chimera in which eLRR1 to eLRR35 of Ve1 were replaced with those of Ve2 did not show HR upon co-expression with Ave1 (Figure 4B). Again, stability of the chimeric Ve

Figure 3. Functional characterization of Ve chimeric proteins that contain the C-terminus of Ve2. (A) Schematic representations of transgenically expressed Ve1 (Ve1HA) and Ve2 (Ve2HA) and the proteins encoded by the chimeric genes Ve1[8]Ve2, Ve1[14]Ve2, Ve1[21]Ve2, Ve1[30]Ve2, and Ve1[35]Ve2. The numbers indicate the eLRR at the site of the swap. (B) Chimeras containing the Ve2 C-terminus do not induce HR upon coinfiltration with Ave1. (C) Stability of chimeric Ve proteins is shown by immunoblotting using HA antibody (α-HA). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples
confirm equal loading. (D) Typical appearance of non-transgenic *sgs2* (Co.) and transgenic Arabidopsis *sgs2* lines upon mock-inoculation or inoculation with *V. dahliae* race 1. (E) Quantification of Verticillium wilt symptoms (Sympt.) in Co. and transgenic lines. Bars represent quantification of symptoms presented as percentage of diseased rosette leaves with standard deviation. Co. is set to 100%. No significant differences were monitored when compared with Co. (P<0.001). For each construct two transgenic lines are shown (1, 2).
proteins was confirmed by immunoblotting (Figure 4C). To further investigate the chimeras, sgs2 plants were transformed with Ve2[8]Ve1, Ve2[14]Ve1, Ve2[21]Ve1, Ve2[30]Ve1 and Ve2[35]Ve1, and the resulting transgenic lines were challenged with race 1 V. dahliae. As expected based on the occurrence of HR in tobacco, expression of Ve2[8]Ve1, Ve2[14]Ve1, Ve2[21]Ve1 and Ve2[30]Ve1 in Arabidopsis resulted in Verticillium resistance, as the transgenes showed few to no symptoms (Figure 4D-4E). In contrast, plants carrying Ve2[35]Ve1 displayed Verticillium wilt symptoms that were comparable to those of inoculated wild type plants (Figure 4D-4E). Collectively, these results suggest that the region between eLRR30 and eLRR35 is required for Ve1-mediated resistance, and that this region is not functional in Ve2.

To further investigate the requirement of eLRR30 to eLRR35 for Ve1-mediated resistance, we generated Ve1[21]Ve2[35]Ve1 and Ve1[30]Ve2[35]Ve1, in which eLRR21 to eLRR35 and eLRR30 to eLRR35 of Ve1 were replaced with the corresponding eLRRs of Ve2, respectively (Figure 5A). Tobacco leaves expressing these Ve chimeras did not show HR upon co-expression with Ave1 (Figure 5B), while immunodetection confirmed stability of the chimeric proteins (Figure 5C). Arabidopsis plants expressing the constructs Ve1[21]Ve2[35]Ve1 and Ve1[30]Ve2[35]Ve1 displayed typical Verticillium wilt symptoms that were comparable to those of inoculated wild type plants and Ve2[35]Ve1-expressing plants (Figure 5D-5E). Collectively, these results confirm that the region between eLRR30 and eLRR35 is required for Ve1-mediated resistance, and is not functional in Ve2.

**Figure 4. Functional characterization of Ve chimeric proteins that contain the C-terminus of Ve1.** (A) Schematic representations of transgenically expressed Ve1 (Ve1HA) and Ve2 (Ve2HA) and the proteins encoded by the chimeric genes Ve2[8]Ve1, Ve2[14]Ve1, Ve2[21]Ve1, Ve2[30]Ve1, and Ve2[35]Ve1. The numbers indicate the eLRR at the site of the swap. (B) Typical appearance of tobacco leaves coinfiltrated with chimeric genes and Ave1. (C) Stability of chimeric Ve proteins is shown by immunoblotting using HA antibody (α-HA). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal
Functional analysis of Ve1

D) Typical appearance of non-transgenic sgs2 (Co.) and transgenic Arabidopsis sgs2 lines upon mock-inoculation or inoculation with V. dahliae race 1. (E) Quantification of Verticillium wilt symptoms (Sympt.) in Co. and transgenic lines. Bars represent quantification of symptoms presented as percentage of diseased rosette leaves with standard deviation. Co. is set to 100%. Asterisks indicate significant differences when compared with Co. (P<0.001). For each construct two transgenic lines are shown (1, 2).
Deletion of eLRR1 to eLRR30 compromises Ve1 functionality

The observation that the region carrying eLRR1 to eLRR30 of Ve1 can be replaced by the corresponding region of Ve2 without compromising Ve1-mediated resistance suggests that this region is not required for Ve1 functionality or, alternatively, that this region is equally functional in both receptors. To investigate whether the region between eLRR1 and eLRR30 is required for Ve1 protein functionality, a truncated version of Ve1 was generated in which the first 30 eLRRs were deleted (Δ[30]Ve1; Figure 6A). Co-expression of Δ[30]Ve1 with Ave1 in tobacco did not induce HR (Figure 6B), while immunoblotting confirmed the stability of the truncated protein (Figure 6D). These data suggest that the region between eLRR1 and eLRR30 is indeed required for Ve1 protein functionality, and can functionally be replaced by the corresponding region of Ve2.

Both Ve proteins interact with the receptor-like kinase SOBIR1

It was recently shown that the eLRR-RLK SOBIR1 constitutively interacts in planta with a broad range of eLRR-RLPs that act in development or in immunity, including Ve1 (Liebrand et al., 2013). In addition, SOBIR1 was found to be required for the Ve1-mediated hypersensitive response and immunity against Verticillium wilt in Arabidopsis and tomato (Liebrand et al., 2013). Since SOBIR1 constitutively interacts with eLRR-RLPs that act either in development or in immunity, it was proposed that this protein functions as regulatory eLRR-RLK for eLRR-RLP-type of cell surface receptors (Liebrand et al., 2013). To investigate whether perhaps absence of interaction of Ve2 with SOBIR1 could explain non-functionality of Ve2, co-immunoprecipitations were performed to test the interaction of Ve1 and Ve2 with tomato SOBIR1 (SlSOBIR1) both in N. tabacum and N. benthamiana. Interestingly, these assays revealed that Ve1 as well as Ve2 interacts with SlSOBIR1 (Figure 6C). Thus, it can be concluded that lack of Ve2 functionality cannot be attributed to the absence of interaction with the putative regulatory eLRR-RLK SOBIR1.
Figure 5. Analysis of the requirement of Ve1 eLRR30 to eLRR35 for mediating resistance against V. dahliae race 1. (A) Schematic representations of transgenically expressed Ve1 (Ve1HA) and Ve2 (Ve2HA) and the proteins encoded by the chimeric genes Ve1[21]Ve2[35]Ve1 and Ve1[30]Ve2[35]Ve1. The numbers indicate the eLRR at the site of the swap. (B) Typical appearance of tobacco leaves coinfiltrated with chimeric genes and Ave1. (C) Stability of truncated and chimeric Ve proteins is shown by immunoblotting using HA antibody (α-HA). Coomassie-stained blots (CBS) showing the 50 kDa Rubisco band present in the input samples confirm equal loading. (D) Typical appearance of non-transgenic sgs2 (Co.) and transgenic Arabidopsis sgs2 lines upon mock-inoculation or inoculation with V. dahliae race 1. (E) Quantification of Verticillium wilt symptoms (Sympt.) in Co. and transgenic lines. Bars represent quantification of symptoms presented as percentage of diseased rosette leaves with standard deviation. Co. is set to 100%. No significant differences were monitored when compared with Co. (P<0.001). For each construct two transgenic lines are shown (1, 2).
eLRR1 to eLRR30 are not required for SOBIR1 interaction

Involvement of the eLRR domain in assembly of cell surface receptor complexes has recently been demonstrated (Li, 2011; Jaillais et al., 2011). To investigate whether the region between eLRR1 and eLRR30 contributes to the interaction between Ve1 and SOBIR1, co-immunoprecipitations were performed using Δ[30]Ve1 and SOBIR1. Interestingly, these assays revealed that Δ[30]Ve1 still interacts with SOBIR1 (Figure 6D), suggesting that eLRR1...
to eLRR30 of Ve1 do not contribute to the interaction with SOBIR1, and that this interaction is established through the C-terminus of the receptor.

**DISCUSSION**

In this manuscript we describe the analysis of a set of domain swaps between the eLRR-RLP-type cell surface receptor Ve1 and its close homolog Ve2. We show that the C-terminus and the region between eLRR30 to eLRR35 of Ve1 are crucial for resistance against *Verticillium* infection, and that these regions appear to be non-functional in Ve2. The finding that the first 30 eLRRs of Ve1 cannot be deleted without loss of Ve1 functionality suggests that the N-terminus is crucial for Ve1 function. Moreover, the observation that this region can be functionally replaced by the first 30 eLRRs of Ve2 suggests that this region is not impaired in Ve2.

**The C-terminus of Ve2 is not functional**

All chimeric proteins in which eLRRs of Ve2 were replaced with those of Ve1 did not mediate HR upon co-expression of Ave1. Moreover, *sgs2* plants expressing Ve1[8]Ve2, Ve1[14]Ve2, Ve1[21] Ve2, Ve1[30]Ve2, and Ve1[35]Ve2, respectively, were susceptible towards *Verticillium*. These results show that the C-terminus of Ve2 is not functional. eLRR-RLPs typically have a short cytoplasmic tail of 20-30 amino acids lacking obvious signaling motifs, apart from motifs homologous to mammalian endocytosis motifs (Wang et al., 2010a). The C-terminus of Ve2 is a rather atypical cytoplasmic tail for an eLRR-RLP, as it is exceptionally long with 121 amino acids. In addition to the dileucine E/DXXXLφ and tyrosine YXXφ signal sequences that are thought to stimulate receptor-mediated endocytosis of mammalian receptors, the Ve2 C-terminus contains a PEST-like sequence that may induce protein degradation, and a KKF motif that has been suggested to promote endoplasmic reticulum retention (Kawchuk et al., 2001). Of these, Ve1 only contains the dileucine E/DXXXLφ and tyrosine YXXφ sequences, although their functionality remains unclear. Although the Ve1 C-terminus lacks other signaling domains, it may interact with
additional proteins which contribute to signal transduction. However, the recently identified regulatory eLRR-RLK SOBIR1, that broadly interacts with eLRR-RLP-type cell surface receptors, interacts with both Ve1 and Ve2, and therefore cannot explain the differential functionality of these proteins. Furthermore, our data suggest that SOBIR1 does not directly mediate Ve1-triggered immune signaling.

**eLRRs 30 to 35 are required for resistance, and are non-functional in Ve2.**

Intriguingly, the chimeras Ve2[8]Ve1, Ve2[14]Ve1, Ve2[21]Ve1, Ve2[30]Ve1 are able to trigger HR upon co-expression with Ave1 in tobacco. Furthermore, Arabidopsis sgs2 plants expressing these chimeras were resistant against *Verticillium*, showing that the region containing the first 30 eLRRs of Ve2 is functional. This region includes the signal peptide (A-domain) and the major part of the C1 domain. The chimeric protein Ve2[35]Ve1, in which the first 35 eLRRs of Ve1 were replaced with those of Ve2, was not able to activate HR, and Ve2[35]Ve1 transgenic sgs2 remained susceptible towards *Verticillium*, suggesting that eLRRs 30 to 35 of Ve1 are required for *Verticillium* resistance, and are not functional in Ve2. This region includes two eLRRs from the C1 domain (eLRR30 and eLRR31), the island domain, and four eLRRs of the C3 domain (eLRR32 to eLRR35). Domain swap experiments between the eLRR-RLP receptor pairs Cf-4/Cf-9, Cf-2/Cf-5, Cf-9/Cf-9B demonstrated that ligand specificity is determined by the eLRR domain, specifically by the C1 domain (Chakrabarti et al., 2009; Seear and Dixon, 2003; van der Hoorn et al., 2001; Wulff et al., 2001; Wulff et al., 2009). So far, the role of the C3 domain remains unclear.

A comparison of tomato RLPs Cf-2, Cf-4, Cf-9, EIX2, Ve1 and Ve2 shows that the C3 domain is more conserved (31.2% identical in amino acids) than the C1 domain (8.8% identical in amino acids). Moreover, in the C3 domain a number of highly conserved amino acids were observed, whereas the C3 domain of Cf-4 and Cf-9 is identical (Figure 7; van der Hoorn et al., 2001). Previous comparison of RLP sequences of Arabidopsis and rice has similarly
shown that the C3 domains along with the extracytoplasmic and transmembrane domains are highly conserved (Fritz-Laylin et al., 2005; Wang et al., 2010a). Domain-swaps between CLV2 and AtRLP38 (a CLV2-like RLP) demonstrated that the region from C3 to the C-terminus of AtRLP38 could substitute that of CLV2 without affecting CLV2 functionality (Wang et al., 2010b). The relatively high conservation of the C3 domain suggests that this region could be involved in interaction with co-receptors and other proteins that may form part of a receptor complex. The interaction of eLRR-containing cell surface receptors with other transmembrane receptors may be regulated by the transmembrane domain (Zhang et al., 2010; Wulff et al., 2004) or even by the cytoplasmic domain (Wang et al., 2008). Recent studies also revealed a crucial role for the eLRR domain as a platform for receptor interactions (Jaillais et al., 2011; Li, 2011). Since we demonstrated that a truncated Ve1 protein that lacks the first 30 eLRRs still interacts with SOBIR1, we can hypothesize that this interaction is mediated by the C-terminus of the Ve1 protein, containing the remaining C3 domain the transmembrane domain and the cytoplasmic tail. In this light it is worthwhile to note that SOBIR1 only carries a short extracellular domain with only five eLRRs (Gao et al., 2009; Leslie et al., 2010).

In addition to C1 and C3 eLRRs, eLRR30 to eLRR35 encompass the non-LRR island domain (C2) which differs by three amino acids between Ve1 and Ve2. The C2 domain has been proposed to act as a flexible hinge region that facilitates the eLRR structure formation between the C1 and C3 regions (Jones and Jones, 1997). However, requirement and function of the C2
domain has been shown to vary from one receptor protein to another (Wang et al., 2010a). For instance, not all eLRR-RLPs and eLRR-RLKs contain a C2 domain (Matsushima et al., 2009). Furthermore, the C2 domains of Cf-4 and Cf-9 are identical, suggesting that these regions are not involved in ligand specificity (van der Hoorn et al., 2001; Wulff et al., 2009). Deletion of the C2 domain in CLV2 does not affect its functionality in plant development (Wang et al., 2010b), whereas the C2 domains of BRI1 (Li and Chory, 1997) and PSKR1 (Matsubayashi et al., 2002) are essential for functionality as they are directly involved in binding the ligands brassinolide and phyto sulfokine, respectively (Kinoshita et al., 2005; Shinohara et al., 2007).

Overall, our results show that the C-terminus and the eLRR region from eLRR30 to eLRR35 are not functional in Ve2. However, the region carrying eLRR1 to eLRR30 is required for Ve1 functionality, and Ve chimeras in which eLRR1 to eLRR30 of Ve1 were replaced with those of Ve2 remained able to induce HR and provide resistance against race 1 Verticillium. Because for all known eLRR-RLPs the C1 domain determines ligand specificity, this may similarly be true for the Ve proteins. Thus, Ve2 may still interact with the Ave1 elicitor through the eLRRs of the C1 domain, but the C3 domain and the C-terminus of Ve2, which appear to be required for the interaction with co-receptors or downstream signaling components, may not be able to activate successful defense signaling.

**MATERIALS AND METHODS**

**Plant materials and manipulations**

Plants were grown in soil in the greenhouse or in the climate chamber at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity and 100 W·m⁻² supplemental light when the light intensity dropped below 150 W·m⁻². Arabidopsis transformations were performed as described previously (Clough and Bent, 1998) and single insertion T2 lines were selected by analyzing the segregation of glufosinateammonium resistance (Basta herbicide, Bayer CropScience). For each construct, at least
two independent transgenic lines were used that showed no developmental aberrations. Inoculations with race 1 *V. dahliae* strain JR2 were performed as described previously (Fradin et al., 2011).

**Generation C-terminal HA-tag fusions of Ve1 and Ve2**

*pGEM-TdsVe1HA* was engineered to contain the tomato *Ve1* CDS (FJ464556) fused at the 3' end to a CDS for the triple hemagglutinin (HA) epitope tag. To this end, the 392 bp fragment upstream of the *Ve1* stop codon was amplified from *P35S:Ve1* (Fradin et al., 2009) with the Expand High-Fidelity PCR system enzyme mix (Roche) using primer pair *Ve1SeqF6* and *Ve1HAtagR* (Supplementary Table 1). The PCR fragment was cloned into *pGEM-T Easy* (Promega), sequenced using M13F and M13R (Supplementary Table 1), and excised using *NciI* and *Ascl*. In addition, construct *P35S:Ve1* was excised with *BamHI* and *NciI* to obtain the first 2791 nucleotides of *Ve1*. Both fragments were cloned into *BamHI*- and *Ascl*-digested *pGEM-Tds* (a modified *pGEM-T Easy* vector that was engineered to contain a *BamHI* and *Ascl* restriction site, Supplementary Table 1), resulting in *pGEM-TdsVe1HA*. Similarly, *pGEM-TdsVe2HA* was engineered to encode tomato *Ve2* (FJ464558) fused at the 3' end to the triple HA tag. The 860 bp fragment upstream of the *Ve2* stop codon was amplified from *P35S:Ve2* (Fradin et al., 2009) using primer pair *Ve2SeqF6* and *Ve2HAtagR* (Supplementary Table 1), cloned into *pGEM-T Easy*, sequenced, and excised with *NciI* and *Ascl*. The first 2785 nucleotides of *Ve2* were excised from *P35S:Ve2* using *BamHI* and *NciI*. Subsequently, both fragments were cloned into *pGEM-Tds*, resulting in *pGEM-TdsVe2HA*.

For *in planta* expression of the Ve chimeras a variant of the Gateway vector *pB7WG2* (Karimi et al., 2002) was engineered. To this end, the expression cassette between the restriction enzymes *KpnI* and *SacI* of *pB7WG2* was excised and replaced by the expression cassette present between the *KpnI* and *SacI* restriction sites of a binary vector *pMOG800* variant (Honée et al., 1998; Fradin et al., 2009). This resulted in the construct *pB7K40*, which contains the constitutive CaMV35S promoter, unique *BamHI* and *Ascl* restriction sites, and the terminator of the potato proteinase
inhibitor II (Pil II) gene. Finally, the CDS encoding HA-tagged Ve1 and Ve2 were excised from \( pGEM-TdsVe1HA \) and \( pGEM-TdsVe2HA \), respectively, and cloned into \( BamHI \) and \( Ascl \)-digested \( pB7K40 \), resulting in \( Ve1HA \) and \( Ve2HA \), respectively.

**Generation of constructs encoding Ve chimeras**

The endogenous restriction sites \( HindIII \), \( XbaI \), \( SspI \), \( Hhal \), and \( NciI \) that are conserved between \( Ve1 \) and \( Ve2 \) (Figure 2) were used to generate the domain-swaps. To generate the construct encoding a chimeric Ve protein that contains the first eight eLRRs of \( Ve1 \) and the remainder of the protein of \( Ve2 \) (\( pGVe1[8]Ve2 \)), the \( Ve1 \) fragment between \( BamHI \) (in the multiple cloning site) and \( HindIII \) (conserved in the Ve proteins) was excised from \( pGEM-TdsVe1HA \) and cloned into \( BamHI\)- and \( HindIII \)-digested \( pGEM-TdsVe2HA \), resulting in \( pGVe1[8]Ve2 \). Similarly, to generate the construct encoding a chimeric Ve protein that contains the first 14 eLRRs of \( Ve1 \) and the remainder of the protein of \( Ve2 \) (\( pGVe1[14]Ve2 \)), the \( Ve1 \) fragment between \( BamHI \) and \( XbaI \) was excised from \( pGEM-TdsVe1HA \) and cloned into \( BamHI \)- and \( XbaI \)-digested \( pGEM-TdsVe2HA \). To generate the construct encoding a chimeric Ve protein that contains the first 21 eLRRs of \( Ve1 \) and the remainder of the protein of \( Ve2 \) (\( pGVe1[21]Ve2 \)), the \( Ve1 \) and \( Ve2 \) fragments between \( XbaI \) and \( SspI \), and between \( SspI \) and \( Ascl \), respectively, were excised from \( pGEM-TdsVe1HA \) and \( pGEM-TdsVe2HA \), respectively. The excised fragments were then cloned into \( XbaI \)- and \( Ascl \)-digested \( pGEM-TdsVe1HA \). To generate the construct encoding a chimeric Ve protein that contains the first 30 eLRRs of \( Ve1 \) and the remainder of the protein of \( Ve2 \) (\( pGVe1[30]Ve2 \)), the \( Ve1 \) and \( Ve2 \) fragments between \( BamHI \) and \( Hhal \), and between \( Hhal \) and \( Ascl \), respectively, were excised from \( pGEM-TdsVe1HA \) and \( pGEM-TdsVe2HA \). The excised fragments were then cloned into \( BamHI \)- and \( Ascl \)-digested \( pGEM-TdsVe1HA \). To generate the construct encoding a chimeric Ve protein that contains the first 35 eLRRs of \( Ve1 \) and the remainder of the protein of \( Ve2 \) (\( pGVe1[35]Ve2 \)), the \( Ve1 \) and
Ve2 fragments between BamHI and NcI, and between NcI and Ascl, respectively, were excised from pGEM-TdsVe1HA and pGEM-TdsVe2HA, respectively. The excised fragments were then cloned into BamHI- and Ascl-digested pGEM-Tds. Reciprocal constructs pGVe2[8]Ve1, pGVe2[14]Ve1, pGVe2[30]Ve1, and pGVe2[35]Ve1 were generated following a similar cloning strategy as described above. For pGVe2[21]Ve1, the Ve2 and Ve1 fragments between BamHI and SspI, and between SspI and Ascl, respectively, were excised from pGEM-TdsVe2HA and pGEM-TdsVe1HA, respectively. The excised fragments were then cloned into BamHI- and Ascl-digested pGEM-Tds.

To generate pGVe1[21]Ve2[35]Ve1, a chimeric Ve CDS encoding LRR1 to LRR21 of Ve1, LRR21 to LRR35 of Ve2 and LRR35 to the C-terminus of Ve1, the chimeric fragment between SspI and Ascl was excised from pGVe2[35]Ve1, and the Ve1 fragment between XbaI and SspI was excised from pGEM-TdsVe1HA. The excised fragments were cloned into XbaI- and Ascl-digested pGEM-TdsVe1HA, resulting in pGVe1[21]Ve2[35]Ve1. To generate pGVe1[30]Ve2[35]Ve1, a chimeric Ve CDS encoding LRR1 to LRR30 of Ve1, LRR30 to LRR35 of Ve2 and LRR35 to the C-terminus of Ve1, the chimeric fragment between HhaI and Ascl was excised from pGVe2[35]Ve1, and the Ve1 fragment between BamHI and HhaI was excised from pGEM-TdsVe1HA. The excised fragments were then cloned into BamHI- and Ascl-digested pGEM-Tds, resulting in pGVe1[30]Ve2[35]Ve1.


To generate truncation construct Δ[30]Ve1, the Ve1 coding sequence was PCR amplified from 35S:Ve1 (Fradin et al., 2009) using primers Δ[30]Ve1-F2 and C3R. A signal peptide sequence was added by subsequent PCR using primers SP-F and Ve1HAtagR. The product from the second PCR
was cloned into the pENTR™/D TOPO vector according to manufacturer’s instructions (Invitrogen, Carlsbad, California) to obtain entry vector pENTR::Δ[30]Ve1. pENTR::Δ[30]Ve1 was subsequently cloned into Gateway destination vector pSol2092 (Zhang et al., 2013) using Gateway LR Clonase II enzyme mix (Invitrogen, Carlsbad, California) to generate expression construct Δ[30]Ve1 driven by the CaMV35S promoter.

**A. tumefaciens-mediated transient expression**

The coding sequence of *V. dahliae* Ave1 was cloned into Gateway destination vector pFAST_R02 (Shimada et al., 2010) to generate an expression construct driven by the CaMV35S promoter. To generate Ve2 with a C-terminal GFP tag, the Ve2 CDS was cloned into Gateway destination vector pSol2095 (Zhang et al., 2013). The expression constructs for GFP-tagged Ve1, SFLS2 and Myc-tagged SOBIR1 were described previously (Zhang et al., 2013; Liebrand et al., 2013). The construct was transformed into *A. tumefaciens* strain GV3101 and infiltrated into tobacco plants (*N. tabacum cv. Petite Havana SR1*) as described previously (van der Hoorn et al., 2000). Briefly, an overnight culture of *A. tumefaciens* cells was harvested at OD_{600} of 0.8 to 1 by centrifugation and resuspended to a final OD of 2. *A. tumefaciens* cultures containing constructs to express Ave1 and chimeric Ve protein were mixed in a 1:1 ratio and infiltrated into leaves of five- to six-week-old tobacco plants. At five days post infiltration (dpi), leaves were examined for necrosis.

**Protein extraction, co-immunoprecipitation and immunoblotting**

For detection of HA-tagged Ve chimeras, *A. tumefaciens* containing the relevant expression constructs was infiltrated into tobacco plants as described previously (van der Hoorn et al., 2000). Two days post infiltration, leaves were frozen in liquid nitrogen and ground to a fine powder. Proteins were dissolved in extraction buffer (150 mM Tris-HCL pH 7.5, 150 mM NaCl, 10 mM DTT, 10 % glycerol, 10 mM EDTA, 0.5 % polyvinylpyrrolidone [PVPP], 1 % IGEPAL CA-630 [NP-40] and 1 % protease inhibitor cocktail [Roche]).
Samples were then centrifuged at 4°C for 15 min at 5000g and the supernatant was passed through a 0.45μm filter. The immunopurifications and immunoblotting were done as described previously (Liebrand et al., 2012).

For the co-immunoprecipitation of SlSOBIR1-Myc with the different Ve fusion proteins, constructs were agroinfiltrated in a 1:1 ratio into tobacco plants. Infiltrated leaves were harvested after one day and ground to a fine powder. The protein extraction, immunopurifications and immunoblotting were performed as described previously (Liebrand et al., 2012).

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We thank Bert Essenstam at Unifarm for excellent plant care; Inge Veldkamp and Yin Song for technical assistance.

REFERENCES


References


**SUPPLEMENTAL DATA**

**Supplementary Table 1.** Primers used in this study

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aRestriction site in bold
bThe type of experiment for which the primers were used is indicated in brackets (C: cloning; S: sequencing)
Chapter 5

Mutational analysis of the Ve1 immune receptor that mediates \textit{Verticillium} resistance in tomato

Zhao Zhang, Yin Song, Chun-Ming Liu and Bart P.H.J. Thomma

To be submitted for publication
ABSTRACT
Pathogenic *Verticillium* species are economically important plant pathogens that cause vascular wilt diseases in hundreds of plant species. The *Ve1* gene of tomato confers resistance against race 1 strains of *Verticillium dahliae* and *V. albo-atrum*. *Ve1* encodes an extracellular leucine-rich repeat (eLRR) receptor-like protein (RLP) that serves as a cell surface receptor for recognition of the recently identified secreted *Verticillium* effector Ave1. To investigate recognition of Ave1 by Ve1, alanine scanning was performed on the solvent exposed β-strand/β-turn residues across the eLRR domain of Ve1. In addition, alanine scanning was also employed to functionally characterize motifs that putatively mediate protein-protein interactions and endocytosis in the transmembrane domain and the cytoplasmic tail of the Ve1 protein. Functionality of the mutant proteins was assessed by screening for the occurrence of a hypersensitive response upon co-expression with Ave1 by *Agrobacterium tumefaciens*-mediated transient expression (agroinfiltration). In order to confirm results obtained with agroinfiltration, the constructs encoding the *Ve1* mutants were transformed into Arabidopsis and the transgenes were challenged with race 1 *Verticillium*. Our analyses identified several regions of the *Ve1* protein that are required for functionality.
INTRODUCTION

In order to activate immune responses that ward off invading microorganisms, plants utilize various types of receptors that recognize pathogen (-induced) ligands of various nature (Boller and Felix, 2009; Thomma et al., 2011). Appropriate recognition of these ligands by immune receptors is crucial for the activation of immune responses. These immune receptors are either cell surface receptors that detect (conserved) pathogen-associated molecular patterns (PAMPs) or damage-associated modified self-patterns, or cytoplasmic receptors that recognize highly specific pathogen effectors either directly, or indirectly through recognition of their activities (Jones and Dangl, 2006). Both types of receptors may activate a hypersensitive response (HR), which is a rapid cell death surrounding the infection site that is thought to prevent further pathogen invasion (Hammond-Kosack and Jones, 1996).

The *Verticillium* genus comprises vascular pathogens that cause *Verticillium* wilt diseases in over 200 plant species worldwide (Fradin and Thomma, 2006; Klosterman et al., 2009). In tomato, immunity against *Verticillium* wilt is governed by the immune receptor Ve1 that recognizes the secreted *Verticillium* effector Ave1 (de Jonge et al., 2012; Fradin et al., 2009). Ve1 encodes a putative plasma membrane-localized extracellular leucine-rich repeat (eLRR)-containing cell surface receptor of the receptor-like protein (RLP) class (Kawchuk et al., 2001; Wang et al., 2010a). Typically, the amino acid sequence of RLPs is composed of a signal peptide (SP), an eLRR domain that is shielded by N-terminal and C-terminal eLRR-caps, a single-pass transmembrane (TM) domain, and a short cytoplasmic tail that lacks obvious motifs for intracellular signaling. In some cases, an acidic domain is present between the eLRR domain and the TM domain. Furthermore, the eLRR domain can be subdivided into three domains in which a non-eLRR island or C2 domain interrupts the C1 and C3 eLRR regions (Jones and Jones, 1997). As RLPs lack an obvious domain for intracellular signaling, they presumably form a complex with other proteins, such as receptor-like kinases, to respond
to ligand binding and initiate an immune response (Wang et al., 2010; Liebrand et al., 2013).

It is conceivable that the eLRR domain of cell surface receptors acts as ligand sensor (Kobe and Kajava, 2001). This similarly holds true for the eLRRs of Toll-like receptors (TLRs) that act in animal innate immunity (Chang et al., 2012). The typical plant eLRR consensus motif comprises 24 amino acids, xxLxxLxxLxxLxxNxxLtx/sGxIP, where (x) represents any amino acid and (L) is sometimes substituted by other hydrophobic residues. For plants, the first eLRR protein crystal structures were resolved for a polygalacturonase-inhibiting protein (PGIP) (Di Matteo et al., 2003) and the brassinosteroid receptor brassinosteroid-insensitive 1 (BRI1) (Hothorn et al., 2011; She et al., 2011). These studies revealed that successive eLRRs align in parallel to form a curved, slightly twisted “horseshoe-like” structure, in which parallel core β-strands (xxLxLxx) form the concave (inner) side of the protein and various helices, short β-strands and additional connecting residues form the convex (outer) side (Kobe and Kajava, 2001). The concave side of the eLRR is thought to serve for ligand binding, where the hydrophobic (L) residues in the β-sheet (xxLxLxx) are involved in the framework that determines the overall shape of the protein, and the five variable, solvent exposed residues (x) of the β-strands determine ligand binding specificity (Kobe and Kajava, 2001). Crystallographic analysis of PGIP demonstrated that the solvent exposed residues on the concave β-sheet surface determine the interaction with polygalacturonases (Di Matteo et al. 2003). Furthermore, the recently released crystal structure of BRI1 showed that the brassinosteroid hormone binds to a groove in between the concave β-sheet surface and the island domain (She et al., 2011, Hothorn et al., 2011).

In the majority of studied eLRR receptors, ligand specificity is determined by the C1 domain (Chapter 1). We previously carried out domain swaps between Ve1 and its non-functional homolog Ve2, and demonstrated that the chimeras in which the first thirty eLRRs of Ve1 were replaced with those of Ve2 remained able to activate Verticillium resistance (Chapter 4). However, the C3 domain and C-terminus of Ve2 appeared not to be
Potentially, the non-functional Ve2 receptor still interacts with the Ave1 elicitor in the C1 domain, but fails to activate immune signaling due to a non-functional C3 domain and C-terminus. To further determine the role of eLRRs of Ve1 in ligand specificity and signal transduction, we employed a high-throughput alanine scanning mutagenesis strategy to mutate solvent exposed residues in concave surface of each eLRR repeat.

In addition to the eLRR domain, the domain swaps between Ve1 and Ve2 also pointed towards a function of the transmembrane region and cytoplasmic tail of Ve1 (Chapter 4). A GxxxG motif that has been implicated in protein-protein interactions is found in the transmembrane domain of many membrane proteins (Senes et al., 2000; Curran and Engelman, 2003), including Ve1 and other eLRR-containing cell surface receptors such as Cf-2, Cf-4, Cf-9, EFR and HrcVf (Chapter 1). Interestingly, a mutation in the second glycine of GxxxG motif abolished the function of Cf-9, which may be due to disruption of the interaction with a co-receptor that associates through the GxxxG motif (Wulff et al., 2004). Similar mutations in Arabidopsis AtRLP51 and AtRLP55 resulted in constitutively activated defense (Zhang et al., 2010). Furthermore, endocytosis of membrane proteins is often associated with presence of a Yxxφ or E/DxxxLφ consensus motif in the cytoplasmic domains of such proteins, where φ is a hydrophobic residue and x is any amino acid (Murphy et al., 2005; Geldner and Robatzek, 2008). Both Yxxφ and E/DxxxLφ motifs are present in the cytoplasmic domain of Ve1. To

**Figure 1. Primary structure of the Ve1 protein. Alignment of the amino acid sequence of Ve1 with a schematic representation of the protein structure.** Ve1 is composed of a signal peptide (SP), eLRR region C1 (C1), island domain (IS), eLRR region C3 (C3), acidic domain (AC), transmembrane domain (TM) and cytoplasmic tail (CT). Double alanine scanning was performed on the solvent exposed β-strand residues across the Ve1 eLRR domain. The putative parallel β-strands (xLxLxL) on the concave surface are boxed, and the conserved hydrophobic residues on the concave β-sheet surface are indicated with black shading. Triangles represent solvent-exposed amino acid residues (x) subjected to alanine substitution for each of the repeats. Only one eLRR was mutated per mutant allele. The putative GxxxG motif and endocytosis signals are underlined.
evaluate role of the GxxxG, E/DxxxLφ and Yxxφ motifs of Ve1, alanine scanning mutagenesis of these motifs was pursued in this study.

RESULTS

Alanine scanning of the concave side of the Ve1 eLRR domain

Considering the large size of the Ve1 eLRR domain and avoiding the potential inefficiency of random mutagenesis, a site-directed mutagenesis strategy was performed to identify functional regions of the Ve1 eLRR domain which contains 37 imperfect eLRRs. To this end, solvent exposed residues in the β-strand of each eLRR repeat were mutated. In total, 37 mutant Ve1 alleles were engineered, named M1-M37 respectively, in which two of the five variable solvent exposed residues in the xxLxLxx consensus of a single eLRR were mutated such that they were substituted into alanines (Figure 1). To generate mutant alleles, the Ve1 coding sequence was cloned into pDONR207 (Invitrogen, Carlsbad, California) through a Gateway BP reaction to generate entry vector pDONR207::Ve1. Using pDONR207::Ve1 as template, inverse PCR was performed to establish alanine substitutions by changing wild type codons in the primer sequence. The mutated Ve1 was sequenced and subsequently cloned into an expression construct driven by the constitutive CaMV35S promoter.

C1 domain eLRRs 1 to 8 and 20 to 23 are required for Ve1 functionality

We previously suggested that ligand recognition is determined by the Ve1 eLRRs 1 to 30 (Chapter 4). To determine which eLRRs of the C1 domain are required for Ve1 functionality in more detail, tobacco leaves were

Figure 2. Double alanine scanning reveals eLRRs required for Ve1 functionality. Left: Schematic representations of Ve1 eLRR domain and summary of Ve1 functionality of the double alanine scanning mutant alleles. The gray boxes indicate the mutant alleles which compromise Ve1 function. Middle: Occurrence of HR upon co-expression of Ve1 double alanine scanning mutant alleles with Ave1. +++, HR similar to wild type Ve1; ++, HR reduced when compared with wild-type Ve1; +, limited HR; -, no detectable HR. Right: Quantification of Verticillium wilt symptoms in wild type (WT) and transgenic lines. Bars represent quantification of symptoms presented as percentage of diseased rosette leaves with standard deviation with WT set to 100%. Asterisks indicate significant differences when compared with WT (P<0.001).
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co-infiltrated with 1:1 mixture of *Agrobacterium tumefaciens* cultures carrying *Ave1* and *Ve1* alleles that encode mutants in the C1 domain (M1-M31). Intriguingly, agroinfiltration in at least three independent experiments revealed that expression of mutant alleles M1, M3 to M8, and M20 to M23 together with *Ave1* showed significantly compromised HR at five days post infiltration (dpi; Figure 2; Figure 3A). To further assess the functionality of the mutant alleles, all mutant constructs were transformed into Arabidopsis (Fradin et al., 2011). For each mutant, three independent transformants were challenged with race 1 *V. dahliae*. As expected based on the occurrence of HR in tobacco, transgenic plants carrying the non-functional mutant alleles M1, M3-M8 and M20-M23 displayed *Verticillium* wilt symptoms that were comparable to those on inoculated non-transgenic control plants (Figure 2; Figure 4). In contrast, expression of functional mutant alleles M2, M9-M19, M24-M31 in Arabidopsis resulted in complete *Verticillium* resistance, as the transgenes showed few to no symptoms upon inoculation when compared to non-transgenic control plants (Figure 2; Figure 4). Collectively, these results show that the LRR region between eLRR1 and eLRR8, as well as between eLRR20 and eLRR23, is required for *Ve1*-mediated resistance, possibly because these regions are involved in ligand binding.

**The island (C2) domain is required for Ve1 function**

To test the contribution of the island domain to *Ve1* function, two alanine substitutions were introduced into the predicted island domain to engineer mutant allele MIS (Figure 1). Agroinfiltration revealed that the mutant allele can still activate an HR upon co-expression with Ave1, as the complete infiltrated sectors became fully necrotic (Figure 2; Figure 3A). Similarly, expression of the mutant allele in Arabidopsis resulted in *Verticillium* resistance, as the transgenes showed few to no symptoms of disease upon inoculation with race 1 *V. dahliae* when compared with wild-type plants (Figure 2; Figure 4). Previously, Wang et al. (2010b) demonstrated that deletion of the island domain from CLV2 does not affect its functionality in
plant development. We thus designed the deletion construct Ve1_ΔIS, in which the complete island domain of Ve1 was removed. In contrast to mutant allele MIS, co-expression of the deletion construct with Ave1 did not induce an HR in tobacco (Figure 3B), suggesting that the island domain is required for Ve1 functionality.
Figure 4. Typical appearance of non-transgenic Arabidopsis (WT) and transgenic Arabidopsis carrying Ve1 double alanine scanning mutant alleles, upon mock-inoculation or inoculation with *V. dahliae* race 1. Pictures were taken 21 days post inoculation and are representative of three independent experiments.
Alanine scanning reveals functionally important solvent-exposed residues in the β-strands of the C3 domain

Based on the domain swaps between Ve1 and Ve2, we previously demonstrated that the C3 domain and C-terminus of Ve2 are not able to activate immune signaling (Chapter 4). To further determine the role of solvent exposed residues in the β-strands of the C3 domain, tobacco leaves were co-infiltrated with *A. tumefaciens* cultures carrying mutant Ve1 alleles in the region that encodes the C3 domain (M32-M37) and Ave1. Intriguingly, five of six Ve1 mutants in the C3 domain resulted in abolished or significantly compromised HR in tobacco leaves at five dpi, as only mutant (M36) still activated full HR (Figure 2; Figure 3A). As expected based on the agroinfiltration results, expression of M36 resulted in *Verticillium* resistance in Arabidopsis, while plants expressing the other C3 domain mutant alleles displayed typical *Verticillium* wilt symptoms that were comparable to wild type plants (Figure 2; Figure 4). Collectively, as expected based on the domain swaps experiments, these alanine scanning assays confirm that the C3 region (eLRR32-eLRR37) is required for Ve1 functionality.

The C3 domain of Cf-9 is required for functionality

Previous comparison of eLRR-RLP sequences of Arabidopsis, rice and tomato has shown that the C3 domains of these proteins are relatively conserved (Fritz-Laylin et al., 2005; Wang et al., 2010; Chapter 4). Based on this finding it was suggested that the conserved C3 region may be involved in interaction with common factors, such as (a) co-receptor(s). To prove that the C3 domain of Cf-9 is also functionally important, we performed site-directed mutagenesis on the C3 domain of Cf-9, which has four eLRRs. The alanine substitutions are made at the same sites of the concave surface that were used for the mutagenesis of Ve1 (Figure 5). Intriguingly, co-expression of Avr9 with Cf-9 mutants M24, M25 and M27 resulted in compromised HR, whereas co-expression with mutant M26 did not show compromised HR. Collectively, these results demonstrate that the C3 region is required for Cf-9 function, as was similarly demonstrated for Ve1.
The putative transmembrane GxxxG motif is not required for Ve functionality

Considering the lack of a kinase domain for downstream signaling in Ve1, the GxxxG motif potentially mediates intermolecular interactions with other proteins such as receptor-like kinases (Figure 1). All five residues in the Ve1 putative GxxxG domain were selected for mutagenesis and subjected to alanine substitution (G1 to G5; Figure 6A). Co-expression of the mutants with Ave1 in tobacco showed that the mutations did not effect Ve1 functionality, as full HR was still observed (Figure 6A). Next, Arabidopsis plants were transformed with the mutant alleles, and the resulting transgenes were challenged with V. dahliae. As expected, all mutant Ve1 alleles still mediated Verticillium resistance as the transgenic plants showed few to no symptoms upon inoculation when compared with non-transgenic control plants (Figure 6C; Figure 7).

Figure 5. The C3 domain of Cf-9 is required for functionality. A sequence alignment of the C3 domain of Cf-9 and Ve1 is shown, with identical and similar residues indicated with black shading. The putative parallel β-strands (xxLxx) on the concave surface are boxed. Triangles represent solvent-exposed amino acid residues (x) subjected to alanine substitution. Functional characterization of the mutants is shown on the right. Photographs illustrate typical appearance of tobacco leaves upon co-expression of Cf-9 mutants with Avr9, or Ve1 mutants with Ave1. Pictures were taken 5 days post infiltration and are representative of at least three independent experiments.
Putative C-terminal endocytosis motifs are not required for Ve1 functionality

To investigate whether the putative C-terminal E/DXXXLφ endocytosis motif is involved in Ve1 functionality, we generated six Ve1 mutant alleles, E1 to E6, in which each amino acid of the E/DXXXLφ motif was replaced by an alanine (Figure 6B). Expression of none of the mutant alleles resulted in reduced HR upon co-expression with Ave1 by agroinfiltration in tobacco (Figure 6B). Also in this case, Arabidopsis transgenes expressing the mutant alleles were
resistant against *Verticillium* (Figure 6C; Figure 7). Similarly, we generated alanine substitution construct Y4 in which the conserved Tyr1032 of the putative Yxxφ endocytosis motif was mutated. However, co-infiltration with Ave1 showed that also this mutation does not affect Ve1 functionality (Figure 6B). Collectively, these data show that the two putative endocytosis motifs in the Ve1 C-terminus are not required for Ve1 functionality.

**DISCUSSION**

The plant eLRR-containing cell surface receptors encompass many members that were shown to play important roles in either development or pathogen immunity. Since structures of receptor-ligand co-crystals often are not readily available, thus far, knowledge about the functioning of plant eLRR receptors is mainly based on domain swaps, domain deletions, gene shuffling
analyses and site-directed mutagenesis. We previously swapped domains of Ve1 with homologous domains of its non-functional homolog Ve2, and analysis of the chimeras suggested that Ve2 may still detect the activity of the Ave1 effector in the C1 eLRR domain, but that its C3 domain and C-terminus are not able to activate defense signaling. Here, we employed a site-directed mutagenesis strategy to further dissect functional determinants of Ve1.

Previously, site-directed mutagenesis has been employed for functional analysis of eLRR-containing cell surface receptors. For example, van der Hoorn et al (2005) analyzed a number of site-directed mutants of Cf-9 and demonstrated that conserved Trp and Cys residues present in the N- and C-terminal eLRR flanking regions are important for Cf-9 activity. Similarly, recently reported site-directed mutations proved that the Cys residues in the N-terminal flanking region of the FLS2 eLRRs are required for protein stability and function (Sun et al., 2012). However, as these Trp or Cys residues are conserved in many other plant eLRR proteins as well, they likely contribute to the conformation and stability of the protein rather than to ligand specificity. In addition, another site-directed mutagenesis strategy focused on putative N-linked glycosylation sites, which frequently occur in the eLRR domain of cell surface receptors. Through Asn to Asp substitution, van der Hoorn et al (2005) demonstrated that four glycosylation sites contribute to Cf-9 functionality. These four sites are located in putative α-helixes that are exposed at the convex surface of the Cf-9 eLRR domain and are also conserved in many plant eLRR proteins (van der Hoorn et al., 2005). Glycosylation may contribute to protein conformation, facilitate interactions with the cell wall (Leconte et al., 1994), or protect proteins from degradation (Gahring et al., 2001). However, it seems unlikely that these putative glycosylation sites contribute to ligand specificity of Cf-9 (van der Hoorn et al., 2005). Most of the Ve1 glycosylation sites are located at convex face of the eLRR domain (18 of 21 for Cf-9 and 15 of 18 for Ve1), and thus they were not specifically targeted in our study. To the best of our knowledge, no examples
of ligand perception at convex side of the eLRR domain have been reported (Chapter 1). Moreover, N-linked glycosylation was determined to make only subtle quantitative contributions to FLS2 functionality (Sun et al., 2012). In contrast, alanine scanning mutagenesis on the concave β-sheet surface across the Arabidopsis FLS2 eLRR domain identified eLRR9-eLRR15 as contributors to flagellin perception (Dunning et al., 2007). To identify eLRRs that are required for Ve1 ligand recognition, we focused our attention on the concave β-sheet surface and evaded conserved hydrophobic leucine residues in β-sheets that are likely involved in framework of protein. A double-alanine scanning was performed in which two of the five variable, solvent exposed residues in a single eLRR repeat were mutated. Mutagenesis of two non-adjacent amino acids increases the chance of substituting functionally important residues.

In this study, we showed that mutant alleles that reveal compromised Ve1 function are restricted to three eLRR regions, eLRR1-eLRR8, eLRR20-eLRR23 and eLRR32-eLRR37. This is consistent with previous studies, in which eLRR function was found to be determined by solvent-exposed residues in clustered LRRs of the concave β-sheet surface. For example, domain swaps of tomato Cfs revealed that eLRR13-eLRR16 of Cf-4 contribute to ligand specificity (van der Hoorn et al., 2001), while ligand specificity of Cf-9 is determined by eLRR10-eLRR16 (Wulff et al., 2009). Similarly, eLRR9-eLRR15 of Arabidopsis immune receptor FLS2 is involved in flg22 peptide binding (Dunning et al., 2007). In addition, photoaffinity labeling showed that BAM1 directly interacts with the small peptide ligand CLE9 at the eLRR6–eLRR8 region (Shinohara et al., 2012). Finally, the crystal structure of PGIP showed that the concave surface of eLRR4-eLRR8 is involved in polygalacturonase binding (Di Matteo et al., 2003). Similarly, crystallographic studies revealed that brassinosteroid binds to a hydrophobic groove of BRI1 in between the island domain and the concave β-sheet surface of eLRR20-eLRR25 (She et al., 2011; Hothorn et al., 2011). This similarly holds true for the eLRR domain of mammalian TLRs, for
example, the crystal structure of the TLR4–MD-2–LPS complex demonstrated that the TLR4 interaction with cofactor MD-2 is restricted in concave β-sheet surface of two eLRR clusters, eLRR2-eLRR5 and eLRR8-eLRR10 (Kim et al., 2007).

Because ligand specificity is often determined by the C1 domain, we previously suggested that this may similarly be true for Ve1 (Chapter 4). Therefore, the two regions eLRR1-eLRR8 and eLRR20-eLRR23 are proposed to contribute to ligand binding. However, most of the mutant alleles in the C3 domain (eLRR32-eLRR37) also abolished Ve1 function. This finding is consistent with previous domain swap experiments between Ve1 and Ve2, which demonstrated that the C3 domain of Ve2 is not able to activate successful immune signaling (Chapter 4). Similar to Ve1, alanine scanning of the C3 domain of Cf-9, which is rather conserved when compared with the C3 domain of Ve1, compromised its functionality. This is also consistent with previous mutagenesis studies on Cf-9, where Wulff et al (2004) showed that a Ser675Leu mutation in the solvent-exposed resides of the concave side of the Cf-9 eLRR24 (C3 domain) abolished functionality. Similarly, van der Hoorn et al (2005) proved that Cf-9 function is compromised upon Asp substitution of Asn697, which is located on the concave side of eLRR25. In addition, a Glu662Val mutation in Cf-4 similarly showed the importance of the concave side of the eLRR C3 domain (Wulff et al., 2004). It has previously been demonstrated that the C3 domains of the Cf-4 and Cf-9 receptors, that perceive the sequence-unrelated effector proteins Avr4 and Avr9, respectively, is identical, supporting a role in immune signaling rather than in ligand perception (van der Hoorn et al., 2001). The eLRR domain has recently been shown to be involved in hetero-dimerization of receptor molecules (Jaillais et al., 2011; Li, 2011). Possibly, the relatively conserved C3 domain (Fritz-Laylin et al., 2005; Wang et al., 2010) is involved in the interaction with downstream signaling partners such as (a) common co-receptor(s) (Chapter 4). Such a common co-receptor for both Ve1 and Cf proteins has recently been identified as SUPPRESSOR OF BIR1 (SOBIR1) and
SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1), which both encode an eLRR-RLK with a short eLRR domain (Gao et al., 2009; Leslie et al., 2010; Li, 2010; Liebrand et al., 2013). It has been demonstrated that tomato SOBIR1 interacts with various eLRR-RLPs, including Cf-9, Cf-4 and Ve1, in a ligand-independent manner (Chapter 4; Liebrand et al., 2013). Similarly, SERK1 was shown to be genetically required for both Ve1- and Cf-4-mediated immune signaling (Fradin et al., 2009; Fradin et al., 2011). Although it remains unknown how various eLRR-RLPs interact with SOBIR1 and SERK1, the relatively high conservation of the C3 domain suggests that this region may be involved.

Overall, this study identified exposed concave β-sheet surfaces with a functional role in Ve1-mediated resistance. This extensive analysis of Ve1 provides fuel for our understanding of eLRR protein function and brings novel leads for further research on eLRR protein function in plants.

MATERIALS AND METHODS

Plant materials

Tobacco (Nicotiana tabacum cv. Petite Havana SR1) and Arabidopsis (Arabidopsis thaliana) plants were grown in the greenhouse at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity and 100 W•m⁻² supplemental light when the light intensity dropped below 150 W•m⁻². After agroinfiltration, plants were grown in the climate room at 22°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity. Arabidopsis transformations were performed as described (Clough and Bent, 1998). Homozygous single insert transgenic lines were selected by analyzing the segregation of antibiotic resistance.

Generation of constructs for over-expression of Ve1 and Cf-9

The tomato Ve1 coding sequence was PCR amplified from pMOG800::Ve1 (Fradin et al., 2009) using primers attB-Ve1-F and attB-Ve1-R containing
AttB1 and AttB2 sites for Gateway-compatible cloning. The tomato Cf-9 coding sequence was PCR amplified from \textit{pCf-9} (van der Hoorn et al., 2000) using primers attB-Cf9-F and attB-Cf9-R. The resulting PCR product was cleaned from 1% agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, California) and transferred into donor vector \textit{pDONR207} using Gateway BP Clonase II enzyme mix (Invitrogen, Carlsbad, California) to generate entry vector \textit{pDONR207::Ve1} and \textit{pDONR207::Cf-9}, respectively. The entry constructs \textit{pDONR207::Ve1} and \textit{pDONR207::Cf-9} were subsequently cloned into Gateway destination vector using Gateway LR Clonase II enzyme mix (Invitrogen, Carlsbad, California) to generate expression constructs driven by the CaMV35S promoter. The expression constructs were transformed into \textit{E. coli} and transformants were checked by colony PCR analysis using primers AttB1F and AttB2R. The expression constructs were subsequently sequenced and transformed into \textit{Agrobacterium tumefaciens} strain GV3101 by electroporation.

**Alanine scanning mutagenesis**

For the alanine scanning mutagenesis, inverse PCR was performed to introduce alanine substitutions. Primers to introduce mutations (Supplemental Table 1) were designed according to user manual of GeneTailor™ site-directed mutagenesis kit (Invitrogen, Carlsbad, California). PCR reactions were performed in a total volume of 30 µL with 23 µL water, 3 µL 10x PCR buffer, 1 µL dNTPs, 1 µL of each primer, 1 µL \textit{Pfu} DNA polymerase (Promega, Madison, Wisconsin) and 1 µL of \textit{pDONR207::Ve1} or \textit{pDONR207::Cf-9}. The PCR consisted of an initial denaturation step of 5 minutes at 95°C, followed by denaturation for 30 sec at 95°C, annealing for 30 sec at 45°C to 55°C, and extension for 14 min at 72°C for 20 cycles, and then a final extension for 20 min at 72°C. The product was purified by QIAquick PCR Purification Kit (Qiagen, Valencia, California), treated with \textit{DpnI} endonuclease (New England Biolabs, Ipswich, UK), and transformed into DH5α chemically competent cells. Mutant plasmid DNA was extracted
and sequenced to verify the mutations, and recombined with the Gateway-compatible destination vector to generate an expression construct driven by the constitutive CaMV35S promoter.

**Agrobacterium tumefaciens-mediated transient expression**

*Agrobacterium tumefaciens* containing expression constructs were infiltrated into tobacco plants as described previously (van der Hoorn et al., 2000; Zhang et al., 2013). Briefly, an overnight culture of *A. tumefaciens* cells was harvested at OD600 of 0.8 to 1 by centrifugation and resuspended to a final OD of 2. *A. tumefaciens* cultures containing constructs to express Ave1 and mutated Ve1 proteins were mixed in a 1:1 ratio and infiltrated into leaves of five- to six-week-old tobacco plants. At five days post infiltration (dpi), leaves were examined for necrosis.

**Verticillium inoculations**

Race 1 *V. dahliae* strain JR2 was grown on potato dextrose agar (PDA) at 22°C. *V. dahliae* conidia were harvested from 7- to 14-day-old fungal plates and washed with tap water. The conidia were suspended to a final concentration of $10^6$ conidia per milliliter in potato dextrose broth (PDB). For inoculation, 2- to 3-week-old Arabidopsis plants were uprooted, and subsequently the roots were dipped in the conidial suspension for 3 min. As a control, plants were mock-inoculated in PDB without conidia. After inoculation, plants were immediately transplanted to new pots, and disease development was evaluated at 21 days post inoculation (dpi) as described earlier (Fradin et al., 2011).

**ACKNOWLEDGEMENTS**

We thank Bert Essenstam at Unifarm for excellent plant care and Inge Veldkamp for technical assistance.
REFERENCES


## SUPPLEMENTAL DATA

### Supplementary Table 1. Primers used in this study

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Chapter 6
A nine amino acid epitope of *Verticillium dahliae* effector Ave1 determines recognition by tomato immune receptor Ve1

Zhao Zhang, Yin Song, Jernej Jakše, Karunakaran Maruthachalam, Chun-Ming Liu, Krishna V. Subbarao, Branka Javornik and Bart P.H.J. Thomma
Submitted
ABSTRACT

Effectors are molecules secreted by plant pathogens to facilitate infection, often by deregulation of plant immune responses. During infection, race 1 strains of the soil borne vascular wilt fungus *Verticillium dahliae* secrete the effector protein Ave1 (for Avirulence on Ve1 tomato) that is recognized by the Ve1 immune receptor of race 1-resistant tomato genotypes. Interestingly, homologs of *V. dahliae* Ave1 (VdAve1), have been identified in the bacterial pathogen *Xanthomonas axonopodis* pv. *citri* as XacPNP, and in the fungal pathogens *Fusarium oxysporum*, *Colletotrichum higginsianum* and *Cercospora beticola*, as FoAve1, ChAve1 and CbAve1, respectively. In this study, we identified a novel Ave1 homolog from *V. nubilum* (VnAve1), which is mainly known as a saprophyte and only a weak pathogen. Interestingly, the Ave1 homologs are differentially recognized by Ve1, with the most efficient recognition of VdAve1 and VnAve1, and little to no recognition of ChAve1 and XacPNP. Interestingly, C-terminal GFP tagging of VdAve1 resulted in loss of recognition by Ve1, suggesting that exposure of the C-terminus of VdAve1 is essential for recognition by Ve1. This was confirmed by truncations of VdAve1 and domain swaps between VdAve1 and XacPNP. We furthermore show that Ve1-mediated immunity can be activated by a nine amino acid sequence derived from the C-terminus of VdAve1, proving that a nine amino acid epitope is required and sufficient for recognition by Ve1.
INTRODUCTION

In nature, plants are threatened by diverse groups of potential microbial pathogens. However, only few of these potential pathogens actually cause disease, as plants have developed innate immunity to protect themselves against microbial attack (Dodds and Rathjen, 2010; Thomma et al., 2011). In its simplest form, plant immunity against pathogen attack is governed by immune receptors that sense pathogen-derived ligands to activate defense. Originally, the interaction between plant immune receptors and pathogen ligands was described in a “gene-for-gene” model, stating that the products of plant resistance (R) genes induce race-specific resistance upon recognition of the products of corresponding pathogen avirulence (Avr) genes (Flor, 1971). Recently, an updated view of plant innate immunity has been introduced as the “zigzag” model (Jones and Dangl, 2006). In this model, the first line of defense is governed by plasma membrane-localized pattern recognition receptors (PRRs) that detect conserved pathogen-associated molecular patterns (PAMPs) and active PAMP-triggered immunity (PTI). A successful pathogen utilizes highly specific effectors that contribute to pathogen virulence by deregulation of PTI. However, in a second line of plant defense these effectors are recognized by corresponding resistance proteins (R proteins), resulting in effector-triggered immunity (ETI). In contrast to plasma membrane-localized PPRs, the majority of R proteins are cytoplasmically localized nucleotide binding site-leucine-rich repeat (NBS-LRR) type receptors. PAMPs that trigger PTI are generally considered as highly conserved molecules within a class of pathogens that contribute to their fitness or survival, whereas effectors that induce ETI are pathogen strain or race specific. However, presently it is increasingly recognized that the delineation between PTI and ETI is not strict, but is rather a continuum (Thomma et al., 2011). The diverse defense responses that are activated by diverse host immune receptors often include a hypersensitive response (HR), which is rapid cell death surrounding the infection site that is thought to restrict further growth of the invading pathogen (Hammond-Kosack and
Jones, 1996). In this manner, plant resistance is determined by immune receptors that recognize appropriate ligands to activate defense, the amplitude of which is determined by the level required for effective immunity (Thomma et al., 2011).

*Verticillium dahliae* is a xylem invading fungal pathogen that causes *Verticillium* wilt diseases in a wide range of plant species worldwide (Fradin and Thomma, 2006). *Verticillium* species persist in the soil and enter plants through their roots. Once inside the root, the fungi grow intercellularly and invade the xylem vessels. Typical symptoms of *Verticillium* infection include plant stunting, wilting, chlorosis, and necrosis (Fradin and Thomma, 2006). In tomato, a single dominant locus that confers *Verticillium* resistance has been identified that controls isolates that are assigned to race 1 (Schaible et al., 1951). The locus comprises two genes, *Ve1* and *Ve2*, of which only *Ve1* acts as a functional *Verticillium* resistance gene in tomato (Fradin et al., 2009; Fradin et al., 2011). *Ve1* encodes an extracellular leucine-rich repeat (eLRR) receptor-like protein (RLP) (Fradin et al., 2009; Kawchuk et al., 2001). Typically, the amino acid sequences of RLPs contain a putative signal peptide, an eLRR domain, a transmembrane domain and a short cytoplasmic region (Wang et al., 2010). RLPs are anchored in the plasma membrane, presumably forming a complex with other proteins to detect their ligands and initiate signal transduction (Wang et al., 2010). Recently, the *V. dahliae* effector protein that is recognized by the Ve1 immune receptor of tomato was identified as Ave1 (for Avirulence on *Ve1* tomato) (de Jonge et al., 2012; Fradin et al., 2009). Although the intrinsic function of *V. dahliae* Ave1 (VdAve1) is not yet demonstrated, it is clear that Ave1 contributes to fungal virulence on susceptible plant genotypes (de Jonge et al., 2012). Interestingly, Ave1 homologs were identified in a number of fungal pathogens, including the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* (FoAve1), the sugar beet pathogen *Cercospora beticola* (CbAve1) and the brassica pathogen *Colletotrichum higginsianum* (ChAve1). However, most Ave1 homologs were found in plants, with the most closely related homologs derived from tomato
Chapter 6

(Solanum lycopersicum; SlPNP) and grape (Vitis vinifera; VvPNP). Finally, a more distantly related homolog was identified in the plant pathogenic bacterium X. axonopodis pv. citri (XacPNP) (Nembaware et al., 2004; de Jonge et al., 2012). Co-expression of SlPNP, FoAve1, and CbAve1 with tomato Ve1 in tobacco triggers an HR, whereas co-expression of ChAve1 with tomato Ve1 did not lead to HR (de Jonge et al., 2012). Consequently, Ve1 was found to mediate resistance towards F. oxysporum in tomato, demonstrating involvement of this immune receptor in resistance against multiple fungal pathogens (de Jonge et al., 2012).

It has previously been demonstrated that eLRR-containing cell-surface immune receptors recognize peptide sequences as epitopes of their pathogen ligands. For example, a sulphated 17-amino acid peptide axY22, derived from the 194-amino acid bacterial Xanthomonas oryzae pv. oryzae (Xoo) elicitor Ax21, was reported to be sufficient to activate immune signaling by the rice RLK XA21 (Lee et al., 2009). Similarly, flg22 is the 22-amino acid peptide derived from bacterial flagellin that is perceived by the Arabidopsis EFR RLK-type immune receptor FLS2 (Zipfel et al., 2004), while the Arabidopsis EFR RLK-type immune receptor was shown to recognize elf18, an 18-amino acid peptide derived from bacterial EF-Tu (Zipfel et al., 2006). Finally, a surface-exposed pentapeptide TKLGE of the 22-kDa ethylene-inducing xylanase (EIX) from the biocontrol fungus Trichoderma viride determines recognition by the tomato RLP-type receptor LeEIX2 (Rotblat et al., 2002; Ron and Avni, 2004). In addition to pathogen-derived peptide epitopes, also endogenous peptide epitopes have been identified for eLRR-containing cell-surface receptors. Shoot apical meristem maintenance during development requires the cooperative activity of several receptors, including the RLK receptor CLV1 which directly recognizes a 12 amino acid motif of the CLV3 peptide ligand (Kondo et al., 2006; Ogawa et al., 2008). Furthermore, phytosulfokine (PSK) is a sulfated peptide hormone of only five amino acids that is perceived by RLK-type receptor PSKR (Matsubayashi et al., 2002). Finally, perception of the 18 amino acid peptide systemin is
mediated by the tomato RLK SR160/tBRI1 (Montoya et al., 2002).

In the present study, we identified the epitope of VdAve1 that determines recognition by tomato immune receptor Ve1. Our approach was based on epitope prediction by the alignment of differentially recognized Ave1 homologs, followed by mapping of the epitope by a combination of deletions and domain swaps with other Ave1 homologs.

RESULTS
Conservation among Ave1 homologs
Previously, we reported the cloning of Ave1 from V. dahliae, and described absence of allelic variation among 85 Ave1 alleles of Verticillium strains (de Jonge et al., 2012). The Ave1 alleles were derived from V. dahliae and V. albo-atrum, whereas Ave1 alleles were not identified in any of the race 2 strains analyzed, nor in the V. dahliae and V. albo-atrum strains that are not pathogenic on tomato or in V. longisporum and V. tricorpus (de Jonge et al., 2012). To further explore Ave1 diversity, we assessed Ave1 presence in a collection of 97 Verticillium strains isolated from various host plants and different geographical locations, resulting in the identification of 17 novel Ave1 alleles (Supplemental Table 1). No allelic variation was found among the newly identified Ave1 alleles amplified from V. dahliae and V. albo-atrum. However, in addition to these two species, an Ave1 homolog was identified in four isolates of V. nubilum (VnAve1), a species that is mainly known as a saprophytic and only weakly pathogenic (Isaac, 1953). The four VnAve1 alleles were found to be identical to each other and the predicted protein sequence displays 13 amino acid polymorphisms when compared with VdAve1 (Figure 1).

VdAve1 is 134 amino acids long, and is predicted to have an N-terminal secretory signal peptide followed by a cleavage site between the 18th and 19th amino acid residue (Figure 1). Alignment of the amino acid sequences of VdAve1 with the homologs from plants (SIPNP and VvPNP), and plant pathogens (VnAve1, FoAve1, CbAve1, ChAve1 and XacPNP) reveals blocks of
highly conserved amino acids that are alternated with more variable regions. From the alignment it is apparent that XacPNP is the most divergent, while all other homologs are relatively comparable (Figure 1). Nevertheless, the four cysteine residues that are present in VdAve1 are conserved among all homologous proteins (Figure 1). In silico analysis using DISULFIND (Ceroni et al., 2006) suggests the formation of disulphide bridges between Cys35 and Cys63, as well as between Cys71 and Cys79. Based on prediction by SignalP
4.0 (Petersen et al., 2011), all Ave1 homologs contain N-terminal signal peptides that direct secretion of the molecules into the extracellular space (Figure 1; D-cutoff score>0.6).

**Comparison of necrosis-inducing activity of Ave1 homologs**

It was previously demonstrated that Ve1 recognizes not only VdAve1, but also SIPNP, FoAve1 and CbAve1 (de Jonge et al., 2012). We now also tested the HR-inducing capacity of VnAve1, VvPNP and XacPNP that were isolated from *V. nubilum, V. vinifera* and *X. axonopodis*, respectively. Whereas expression of VnAve1 or VvPNP together with Ve1 in *N. tabacum* resulted in strong HR, co-expression of XacPNP and Ve1 triggered little to no necrosis (Figure 2A). To compare the HR induced by the various Ave1 homologs, they were co-expressed with Ve1 in *N. tabacum* and HR development was measured at five days post infiltration by quantification of the leaf area that developed necrosis (Zhang et al., 2013) (Figure 2B). Importantly, none of the Ave1 homologs induced necrosis in absence of Ve1 (Figure 2A). Whereas agroinfiltration of VdAve1 and VnAve1 resulted in complete necrosis of the infiltrated leaf areas, agroinfiltration of FoAve1 resulted in large necrotic spots in the infiltrated leaf area, although no complete collapse of the infiltrated area was observed. Upon agroinfiltration of CbAve1, spreading of smaller and larger necrotic spots was observed in all infiltrated areas, but the infiltrated leaf area did not turn completely necrotic. For ChAve1 and XacPNP, at most only small necrotic spots were observed in the infiltrated area, whereas occasionally no necrosis was observed at all in the infiltrated sector (Figure 2A). Upon agroinfiltration of the tomato and grape homologs, SIPNP and VvPNP, most of infiltrated leaf area developed necrosis, occasionally affecting the complete infiltrated leaf sector.

**The C-terminus of VdAve1 determines recognition by Ve1**

Although most of Ave1 homologs can be favorably recognized by Ve1, the epitope that determines recognition is unclear. For further functional analyses, constructs encoding C- and N-terminal protein fusions of VdAve1...
to GFP were generated. Interestingly, C-terminal fusion of a GFP tag to Ave1 resulted in loss of recognition by Ve1, while the N-terminal fusion did not affect its recognition (Figure 3). This finding suggests that accessibility of the C-terminus of VdAve1 is important for recognition by Ve1.
To further investigate the role of the C-terminal domain in recognition of Ave1 by Ve1, a number of C-terminal truncations was generated (Figure 4). Deletion of 42 amino acids of the C-terminus, from Lys93 to Ile134 (VdAve1_M1), resulted in loss of recognition, and subsequent analysis of smaller truncations revealed that a C-terminal deletion of nine amino acids already resulted in loss of recognition by Ve1 (Figure 4).
Since the C-terminal nine amino acids are essential for VdAve1 recognition, and the bacterial homolog XacPNP that is not recognized by Ve1 (Figure 2) is significantly divergent in this region (Figure 1), an expression construct for a chimeric Ave1 protein was engineered in which the C-terminal nine amino acids of VdAve1 were replaced by those of XacPNP (Vd_Xac9AA; Figure 5). As expected, co-expression of the Vd_Xac9AA chimera with Ve1 in N. tabacum resulted in weak recognition only, as only small necrotic spots were observed and occasionally no necrosis was observed at all in the infiltrated leaf (Figure 5). Conversely, a chimeric Xac_Vd9AA protein was constructed, in which the last nine amino acids of XacPNP were replaced with those of VdAve1 (Figure 5). Co-expression of Xac_Vd9AA with Ve1 in N. tabacum resulted in a relatively strong HR. Although full necrosis was not observed in the infiltrated leaf area, large necrotic spots were monitored (Figure 5). Based on these domain swaps, it is confirmed that the C-terminal nine amino acids are required for recognition of VdAve1 by Ve1.
The epitope of Ave1

The C-terminal nine amino acids of VdAve1 induce HR by co-expression with Ve1

Our analysis of the VdAve1 deletion mutations and the domain swaps between VdAve1 and XacPNP confirm the requirement of the C-terminal nine amino acids for Ve1-mediated Ave1 recognition. To determine whether the C-terminal nine amino acids are sufficient to trigger Ve1-mediated recognition, we generated a construct encoding the C-terminal nine amino acids of VdAve1 fused to the VdAve1 signal peptide (SP). SP_GFP_Vd9AA encodes GFP that is C-terminally tagged with Vd9AA, and N-terminally fused to the VdAve1 signal peptide (SP) to establish extracellular targeting, while SP_GFP lacks the C-terminal Vd9AA fusion. Furthermore, a chemically synthetized peptide encompassing Vd9AA (AVNIEFRQI) was used. Pictures were taken at five days post infiltration.

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The C-terminal nine amino acids of VdAve1 are sufficient to active Ve1-mediated immunity. Occurrence of the hypersensitive response in N. tabacum upon introduction of the C-terminal nine amino acids of VdAve1 with Ve1. Construct SP_9AA encodes the C-terminal nine amino acids of VdAve1 (Vd9AA) fused to the VdAve1 signal peptide (SP). SP_GFP_Vd9AA encodes GFP that is C-terminally tagged with Vd9AA, and N-terminally fused to the VdAve1 signal peptide (SP) to establish extracellular targeting, while SP_GFP lacks the C-terminal Vd9AA fusion. Furthermore, a chemically synthetized peptide encompassing Vd9AA (AVNIEFRQI) was used. Pictures were taken at five days post infiltration.

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The C-terminal nine amino acids of VdAve1 induce HR by co-expression with Ve1

Our analysis of the VdAve1 deletion mutations and the domain swaps between VdAve1 and XacPNP confirm the requirement of the C-terminal nine amino acids for Ve1-mediated Ave1 recognition. To determine whether the C-terminal nine amino acids are sufficient to trigger Ve1-mediated recognition, we generated a construct encoding the C-terminal nine amino acids of VdAve1 fused to the VdAve1 signal peptide (SP_9AA) (Figure 6). This construct was co-expressed with Ve1 in N. tabacum, but no necrosis was observed in the infiltrated leaf (Figure 6). Furthermore, also infiltration of a chemically synthetized peptide encompassing the C-terminal nine amino acids of VdAve1 was not able to trigger HR in Ve1-expressing tobacco up to a concentration of 1 mg/mL (Figure 6). We speculated that the nine amino acid peptide is not stable upon secretion in the apoplast, irrespective whether it is in planta expressed or injected upon chemical synthesis. In an attempt to overcome this, we generated a construct in which the coding sequence of GFP was N-terminally fused to the VdAve1 signal peptide and
C-terminally fused to the C-terminal nine amino acids of VdAve1 (SP_GFP_Vd9AA; Figure 6). As a negative control, we generated a construct in which the coding sequence of GFP was N-terminally fused to the VdAve1 signal peptide without the C-terminal nine amino acids of VdAve1 (SP_GFP; Figure 6). Interestingly, co-expression of SP_GFP_Vd9AA with Ve1 induced specific HR in *N. tabacum*, whereas co-expression of SP_GFP with Ve1 did not induce necrosis. Although the HR that was induced upon co-expression of Ve1 and SP_GFP_9AA was not as strong as HR induced upon co-expression of VdAve1 and Ve1, these results show that the C-terminal nine amino acids of VdAve1 are sufficient to activate Ve1-mediated recognition (Figure 6).

**The C-terminal nine amino acids of ChAve1 are recognized by Ve1**

ChAve1 is closely related to VdAve1, and six of the nine C-terminal amino acids are identical. Nevertheless, only weak HR is detected upon co-expression of ChAve1 and Ve1 in *N. tabacum* (Figure 2). To further investigate recognition of the C-terminal nine amino acids of ChAve1, an expression construct for a chimeric Ave1 protein was engineered in which the C-terminal nine amino acids of VdAve1 were replaced by those of ChAve1 (Vd_Ch9AA). Intriguingly, expression of Vd_Ch9AA resulted in complete necrosis of the infiltrated leaf area upon co-expression with Ve1 in *N. tabacum* (Figure 7). Intriguingly, the reciprocal swap in which the C-terminal nine amino acids of ChAve1 were replaced by those of VdAve1 (Ch_Vd9AA) was not recognized by Ve1 (Figure 7). These data suggest that the folding of ChAve1 does not allow the C-terminal nine amino acids to be exposed in such manner that recognition can occur. To verify that the C-terminal nine amino acids of ChAve1 are indeed recognized by Ve1, a construct in which the coding sequence of GFP was N-terminally fused to the signal peptide of VdAve1 and C-terminally fused to the C-terminal nine amino acids of ChAve1 was generated (SP_GFP_Ch9AA; Figure 6). Indeed, the GFP tagged C-terminal nine amino acids from ChAve1 were found to activate Ve1-mediated immunity (Figure 7).
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**DISCUSSION**

**Recognition of the C-terminal nine amino acid epitope**

We have previously shown that the VdAve1 effector of *V. dahliae* is recognized by the tomato immune receptor Ve1, and identified several homologs in other species that are differentially recognized by Ve1 (de Jonge et al., 2012). In this study, we demonstrate that the C-terminal nine amino acids of VdAve1 act as the epitope that is required and sufficient for recognition by Ve1. The sequence of the C-terminal nine amino acids of the Ave1 homologs that are recognized (VdAve1, VnAve1, SLPNP, VvPNP, FoAve1, CbAve1 and also ChAve1) only share Asn128 and Gln133 that are completely conserved (Figure 8). However, also the other amino acids in this region are characterized by a high degree of conservation. A similar sequence variation in peptide epitopes, allowing substitutions by amino acids with similar chemical properties has previously been observed, for instance in flagellin, CLV3 and AtPep1 (Naito et al., 2008; Song et al., 2012; Pearce et al., 2008;
The C-terminal nine amino acids from XacPNP, which share little similarity with the corresponding region of VdAve1, were unable to trigger Ve1-mediated HR. A sequence alignment suggests that the amino acids that compose the C-terminal 9 amino acids of the various homologs can be classified into three categories, depending on their degree of conservation (Figure 8). The first category includes Asn128 and Gln133 which are identical among the Ave1 homologs that are recognized by Ve1, and are different in XacPNP. This suggests that these are amino acids are crucial for recognition by Ve1. The second category is represented by Val127, Ile129 and Glu130 of VdAve1 that are expected to play an important role in recognition by Ve1. Whereas they are substituted by other amino acids with similar chemical properties the homologs that are recognized by Ve1, they are substituted by non-similar amino acids in XacPNP. The last category comprises residues Ala126, Phe131, Arg132 and Ile134 that appear not to determine recognition by Ve1. On the one hand, while Ala126 is conserved in the non-recognized XacPNP, it is replaced by non-similar amino acids in various homologs that are

Figure 8. Amino acid sequence alignment of the C-terminal nine amino acids of various Ave1 homologs. Identical and highly similar residues are indicated with black shading whereas weakly similar residues are indicated with grey shading. All sequences are recognized by Ve1, except for the C-terminal nine amino acids of XacPNP.
recognized by Ve1, eliminating this position as a crucial determinant for Ve1 recognition. Similarly, Arg132 is replaced by non-similar amino acids in various homologs that are recognized by Ve1. On the other hand, Phe131 is replaced by similar amino acids in all homologs, including XacPNP, suggesting that also this position does not determine recognition. Finally, Ile134 is conserved in all homologs, including XacPNP, except for FoAve1 where this amino acid is substituted by the non-similar amino acid Arg, again disqualifying this position as a determinant of Ve1 recognition. Site-directed mutagenesis should be employed in future experiments to confirm the contribution of individual amino acids to recognition by Ve1.

**Involvement of C-terminal nine amino acids in Ave1 virulence**

Ave1 has been characterized as ortholog of plant natriuretic peptides (PNPs) (de Jonge et al., 2012). Natriuretic peptides were originally identified in animals where they are associated with cardiac and osmoregulatory homeostasis (Potter et al., 2009). It has been demonstrated that *X. axonopodis* pv. *citri* uses its PNP homolog, XacPNP, to modulate the regulation of host plant homeostasis to establish infection (Gottig et al., 2008). The high homology between tomato SIPNP and *Verticillium* VdAve1, likely due to acquisition of VdAve1 by *Verticillium* from plants through horizontal gene transfer (HGT), in combination with the fact that VdAve1 acts as a virulence factor of *Verticillium* in susceptible tomato plants, suggests that *Verticillium* uses VdAve1 to modulate host plant responses to generate conditions that favour colonization.

Absence of allelic variation in Ave1 alleles from *V. dahliae* and *V. albo-atrum* suggests that identical alleles are required for maximum virulence (de Jonge et al., 2012). However, the importance of the C-terminal amino acids for the function of VdAve1 related to virulence function is unknown. Mutational analysis within Pep-13, a surface-exposed fragment of a calcium-dependent cell wall transglutaminase (TGase) from the oomycete plant pathogen *Phytophthora sojae* that activates plant immune responses,
identified the same amino acids indispensable for both TGase activity and immune activation (Brunner et al., 2002). Similarly, amino acid residues that are required for immunity-inducing activity of the flagellin-derived peptide flg22 were found to be important for the intrinsic flagellar function, as mutant flagella were unstable and impaired in flagellar motility (Naito et al., 2008). However, in contrast, the surface exposed pentapeptide TKLGE epitope of the *Trichoderma viride* ethylene-inducing xylanase (EIX) that is essential for recognition by the eLRR receptor LeEIX2 is not involved in xylanase enzymatic activity (Rotblat et al., 2002; Ron and Avni, 2004). Further research, involving the complementation of VdAve1 deletion strains in *V. dahliae* with truncated versions of VdAve1, will reveal the particular involvement of the C-terminal nine amino acids in VdAve1 function.

**Folding and potential processing of VdAve1**

We noticed that expression of ChAve1 does not trigger the activation of HR although its C-terminal nine amino acids have the potential to be recognized, as was shown by recognition of the chimera containing the C-terminal nine amino acids of ChAve1. Likely, the nine amino acid epitope of ChAve1 is not properly exposed on the protein surface to interact with Ve1 or the molecular host component that is guarded by this receptor. However, obviously it needs to be realized that Ve1 evolved in tomato to recognize attempted host colonization by *V. dahliae*, while *C. higginsiamun* is not a tomato pathogen and thus Ve1 did not have the chance to co-evolve with ChAve1. Potentially, processing of the VdAve1 protein into smaller peptides occurs during infection. Animal atrial natriuretic peptides (ANPs) are polypeptide hormones that are secreted by heart muscle cells and that are involved in the homeostatic control of salt and water balances and blood pressure (Potter et al., 2009). The biologically active peptide is a C-terminally derived 28 amino acid peptide that is cleaved from the 126 amino acid ANP precursor (Potter et al., 2009). Potentially, ChAve1 is not accurately cleaved in tobacco, and may therefore not be recognized. The
importance of precursor maturation has been demonstrated for the recognition of the CLV3 peptide by the eLRR-containing cell-surface receptor CLV2. CLV2 directly recognizes a 12 amino acid CLE motif of the Arabidopsis CLV3 peptide, which is also conserved in the nematode CLE-like GrCLE peptide, to modulate shoot apical meristem maintenance during development (Ni et al., 2011; Guo et al., 2011; Wang et al., 2010). Both types of CLE peptides are cleaved from their larger precursor proteins. Many studies have suggested that maturation of the CLE peptide is essential for its function (Fiers et al., 2006; Fiers et al., 2005; Guo et al., 2011; Ni and Clark, 2006). In addition, elongation of chemically synthesized CLE peptides was found to decreases its activity (Kondo et al., 2008; Kondo et al., 2006).

**Survival of Ve1-carrying tomato**
The C-terminal nine amino acids of SLPNP from tomato are highly homologous to those of VdAve1, and SLPNP is able to activate Ve1-mediated HR. The survival of tomato plants that carry Ve1 can likely be explained by the assumption that SLPNP and Ve1 cannot be co-expressed, either in time or in location. In tomato, expression of SLPNP has remained elusive thus far. However, Arabidopsis PNP is mainly expressed in leaf mesophyll cells (Wang et al., 2011), and significantly up-regulated under abiotic and biotic stress conditions (Meier et al., 2008). Tomato Ve1 is mainly thought to be expressed in vascular tissue (Ermis Yanes Paz, unpublished data), and transcription of Ve1 is clearly increased upon *Verticillium* challenge (Fradin et al., 2009). However, an alternative hypothesis could be that the genuine interactor of SLPNP in the biological process in which it plays its role has a much higher affinity than Ve1 for SLPNP binding, possibly combined with a much higher abundance, leading to the depletion of SLPNP such that the Ve1 receptor is not activated. The quantitative difference in strength of the HR response that is activated by VdAve1 and by SLPNP may support this hypothesis.
MATERIALS AND METHODS

Plant materials

Tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) plants were grown in the greenhouse at 21°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity and 100 W•m⁻² supplemental light when the light intensity dropped below 150 W•m⁻². After agroinfiltration, plants were grown in the climate room at 22°C/19°C during 16/8 hours day/night periods, respectively, with 70% relative humidity.

Generation of expression constructs for Ave1 homologs

To generate constructs for constitutive expression of the Ave1 homologs, the coding sequences of the homologs were cloned into pDONR207 (Invitrogen, Carlsbad, California) through a Gateway BP reaction, and subsequently transferred into the Gateway-compatible destination vector pSol2092 (Zhang et al., 2013) to generate expression constructs driven by the constitutive CaMV35S promoter. *VdAve1*, *VnAve1* and *FoAve1* were amplified from cDNA using the primers attB-Vd-F and attB-Vd-R, attB-Vn-F and attB-Vn-R, attB-Fo-F and attB-Fo-R, respectively. *ChAve1*, *CbAve1*, *VvPNP*, *SlPNP* and *XacPNP* were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany).

Generation of expression constructs for GFP-VdAve1 fusion

To generate *VdAve1* fused at the C-terminus to the green fluorescent protein (GFP), the *VdAve1* coding sequence without stop codon was PCR amplified using primers containing Gateway attB sites (attB-Vd-F and attB-Vd-R-SC). The PCR fragment was cloned into pDONR207 (Invitrogen, Carlsbad, California) through a Gateway BP reaction to generate entry vector pDONR207::VdAve1-SC. Subsequently, pDONR207::VdAve1-SC was transferred into the Gateway-compatible destination vector pSol2095 (Zhang et al., 2013) to generate an expression construct driven by the constitutive CaMV35S promoter and C-terminally tagged to GFP. To generate
VdAve1 fused at the N-terminus to a GFP tag, the GFP coding sequence lacking the stop codon was fused at the N-terminus to the sequence encoding the signal peptide of VdAve1 to ensure extracellular targeting, and at the C-terminus to the VdAve1 coding sequence (without signal peptide). This construct was obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany), and subsequently cloned into Gateway destination vector pSol2092. Fusion constructs were transformed into *A. tumefaciens* strain GV3101 by electroporation.

**Generation of deletion and domain swapping constructs for Ave1**

Deletion constructs VdAve_M1, VdAve_M2, VdAve_M3 and VdAve_M4 were amplified from cDNA using the forward primers attB-Vd-F in combination with reverse primers attB-VdM1-R, attB-VdM2-R, attB-VdM3-R, and attB-VdM4-R, respectively. Vd_Ch9AA, Ch_Vd9AA, SP_GFP-Vd9AA and SP_GFP_Ch9AA were amplified from cDNA using the primers attB-Vd-F and attB-VdCh9AA-R, attB-Ch-F and attB-ChVd9AA-R, attB-Vd-F and attB-GFP-Vd9AA-R, attB-Vd-F and attB-GFP-Ch9AA-R respectively. Vd_Xac9AA, Xac_Vd9AA and SP_Vd9AA were obtained by gene synthesis (Eurofins MWG Operon, Ebersberg, Germany). The AVNIEFRQI peptides were synthesized by GenScript company (Piscataway, USA; Purity>75%). The deletion and domain swapping constructs were cloned into pDONR207 and subsequently transferred into pSol2092 to generate expression constructs.

**A. tumefaciens-mediated transient expression**

Overnight cultures of *A. tumefaciens* strain GV3101 containing expression constructs were harvested at OD600 of 0.8 to 1 by centrifugation and resuspended to a final OD of 2 in infiltration medium as described previously (Zhang et al., 2013). *A. tumefaciens* cultures containing constructs to express Ave1 and Ve1 proteins were mixed in a 1:1 ratio and infiltrated into leaves of five- to six-week-old tobacco plants. At five days post infiltration (dpi),
necrosis was examined and quantified by measuring the area of necrosis as percentage of the total infiltrated area.

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REFERENCES


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SUPPLEMENTAL DATA

Supplemental Table 1. Verticillium strains analysed for presence of Ave1 homologs

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### Supplementary Table 2. Primers used in this study

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Chapter 7

General discussion

Zhao Zhang and Bart P.H.J. Thomma

Part of this chapter has been published in J Integr Plant Biol, in press.
Over twenty years ago, Charles Janeway proposed that an innate immune mechanism, characterized by the constitutive presence of innate immune receptors that recognize conserved molecular structures from pathogens, provides the first line of defense in animals (Janeway, 1989). This hypothesis was validated seven years later when Jules Hoffman and his group showed that the extracellular leucine-rich repeat (eLRR)-containing cell surface receptor “Toll” was essential for Drosophila to activate an immune response against fungal attack (Lemaitre et al., 1996). In 1997, Janeway’s lab identified a human homologue of Drosophila Toll (Medzhitov et al., 1997), which is now known as TOLL-LIKE RECEPTOR 4 (TLR4). In 1998, Bruce Beutler and his colleagues showed that TLR4 is the receptor for bacterial lipopolysaccharide (LPS) (Poltorak et al., 1998). Charles Janeway passed away in 2003. In 2011, the Nobel Prize for physiology or medicine was partially awarded jointly to Jules Hoffmann and Bruce Beutler for discovering the sensors of innate immunity.

Plants have an innate immune mechanism that is highly similar to that of animals, which similarly employs cell surface immune receptors. Even before the Drosophila Toll receptor was identified as first animal innate immune receptor (Lemaitre et al., 1996), two plant immune receptors were cloned that encode cell surface receptors that are crucial for innate immunity. These were tomato Cf-9 (Jones et al., 1994) and rice XA21 (Song et al., 1995), that were later found to specifically recognize Avr9 from the tomato leaf mold fungus *Cladosporium fulvum* (van Kan et al., 1991) and bacterial axY'22 from the rice bacterial blight pathogen *Xanthomonas oryzae pv oryzae* (Lee et al., 2009), respectively. Structurally, XA21 and Cf-9 resemble animal Toll receptors and Toll-like receptors (TLRs) as they are composed of an eLRR domain, a single-pass transmembrane domain and a cytoplasmic domain. The eLRR-containing cell surface receptor family contains hundreds of members in plants (Shiu and Bleecker, 2003; Andolfo et al., 2013; Wang et al., 2008a). In addition to innate immunity, these plant eLRR-containing cell-surface receptors also play crucial roles in
developmental processes. The eLRR-containing cell-surface receptors can be divided into two groups based on their cytoplasmic domain, the receptor-like kinases (eLRR-RLKs) that carry a cytoplasmic kinase domain, and receptor-like proteins (eLRR-RLPs) that largely lack a cytoplasmic domain. XA21 belongs to the first group, while Cf-9 is an eLRR-RLP that only carries a short cytoplasmic tail that lacks obvious motifs for intracellular signaling.

The fungal genus *Verticillium* contains devastating fungal pathogens that cause Verticillium wilt diseases in over 200 plant species, including many economically important crops such as cotton, potato, tomato, pepper, eggplant and so on (Fradin and Thomma, 2006). Tomato eLRR-RLP type immune receptor Ve1 confers *Verticillium* resistance by recognition of the *Verticillium* effector Ave1 (de Jonge et al., 2012). Ave1 is an effector protein that is secreted by *Verticillium* during host colonization, and that has a high degree of homology to plant natriuretic peptides (de Jonge et al., 2012). In this thesis, by using Ve1-Ave1 as a model system, we studied ligand perception by eLRR-RLPs and subsequent activation of innate immune signaling.

**Dimerization of eLRR-containing cell surface receptors**

eLRR-containing cell surface receptors are often thought to be activated upon heteromerization of ligand-bound receptors with non ligand-binding co-receptors. Dimerization of eLRR-RLK type receptors and co-receptors likely facilitates subsequent transphosphorylation of their kinase domains. In addition, cellular signaling of eLRR-RLPs that lack obvious cytoplasmic signaling domains may be activated through the heteromerization with co-receptors carrying a cytoplasmic kinase domain.

Several co-receptors of eLRR-containing cell surface receptors have been characterized. The Arabidopsis SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) family encompasses five eLRR-RLKs that have only a short eLRR domain containing five repeats (Li, 2010). The SERK3
receptor, also known as BRI1-ASSOCIATED KINASE1 (BAK1), is the most well-known member of this family and involved in both development and innate immunity. BAK1 was originally identified as co-receptor of BRI1 in brassinosteroid perception (Nam and Li, 2002; Li et al., 2002; Karlova et al., 2006). It was later found that BAK1 is also required for innate immunity as co-receptor for FLS2 and EFR (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008). BAK1 seems not directly involved in ligand binding, but plays a role in downstream signaling upon its recruitment by ligand-bound BRI1, FLS2 and EFR (Li, 2011). Furthermore, both BAK1 and SERK1 were shown to be genetically required for Ve1-mediated *Verticillium* resistance (Fradin et al., 2009; Fradin et al., 2011).

The membrane-associated receptor kinase CORYNE (CRN) differs from typical eLRR-RLKs because it lacks an eLRR domain and only carries a short extracellular domain (Muller et al., 2008). CRN constitutively interacts with the eLRR-RLP CLV2 and plays a key role in the development of shoot and root apical meristems. It has been proposed that CLV3 is perceived by a tetrameric CLV2/CRN complex and a CLV1 homodimer that localize to the plasma membrane and can interact via CRN. (Zhu et al., 2010; Bleckmann et al., 2010).

Whereas FLS2 heterodimerizes with BAK1 upon flagellin recognition, the BAK1-interacting receptor-like kinase (BIR1) was identified as a negative regulator of immune signaling (Gao et al., 2009). Knock-out of *BIR1* leads to constitutive defense responses including cell death, which is reverted in the *suppressor of bir1-1 (sobir1-1)* mutant. SOBIR1 encodes a RLK with a short eLRR domain (Gao et al., 2009). It has recently been demonstrated that the tomato ortholog of SOBIR1 constitutively interacts with a broad range of eLRR-RLPs, including Ve1, thus acting as a co-receptor for eLRR-RLPs (Liebrand et al., 2013). In Chapter 4 of this thesis, we described that a truncated Ve1 protein that lacks the N-terminal 30 eLRRs still interacts with SOBIR1. This result demonstrates that Ve1-SOBIR1 dimerization is mediated through the C-terminus of the Ve1 that
encompasses the remaining C3 eLRR domain, the transmembrane domain and the cytoplasmic domain.

Involvement of the eLRR domain in assembly of cell surface receptor complexes has been recently demonstrated (Jaillais et al., 2011; Li, 2011). Jaillais et al (2011) described a mutant allele of BAK1 (bak1elg) that has an Asp122Asn mutation in the solvent exposed resides at the concave side of the third LRR of BAK1, that failed to bind FLS2 and therefore is impaired flagellin signaling. Interestingly, in contrast, the bak1elg mutant shows enhanced binding affinity to BRI1 and is hypersensitive to exogenous brassinosteroid treatment. These results point towards a crucial role for the eLRR domain in formation of the receptor complex.

BAK1 has only five eLRRs, and also many other co-receptors comprise only a short eLRR domain, such as members of SERK family (Li, 2010), SOBIR1 and SOBIR1-like (Gao et al., 2009; Leslie et al., 2010; Liebrand et al., 2013). Furthermore, CRN lacks an eLRR domain, but its short extracellular domain (28 amino acids) was shown to be required for interaction with CLV2 (Bleckmann et al., 2010). The short extracellular domain of these co-receptors is expected to bind to the membrane-proximal eLRR-domain of the ligand-binding receptor, more particularly the C3 domain (Jaillais et al., 2011). This suggestion is furthermore supported by the observation that the C3 domain of eLRR-containing cell surface receptors is significantly more conserved than the C1 domain that is often implicated in ligand specificity (Wang et al., 2008a; Fritz-Laylin et al., 2005) (Chapter 4).

Based on the hypothesis that the C3 domain of ligand-binding receptors may serve for interaction with co-receptors, and is therefore functionally important, site-directed mutagenesis on the C3 domain of both Ve1 and Cf-9 was performed (Chapter 5). The alanine substitutions were made at the same positions in the concave surface in Ve1 and Cf-9. Most of mutations abolished Ve1 and Cf-9 function. Interestingly, eLRR35 and eLRR36 of Ve1 share a high degree of homology to eLRR26 (50%) and eLRR27 (54%) of Cf-9, respectively. Alanine substitutions in eLRR35 of Ve1 resulted in
compromised Ve1 activity, while the same mutations in eLRR26 of Cf-9 did not compromise its functionality. Conversely, mutagenesis suggested that eLRR27 of Cf-9 is involved in functionality while LRR36 of Ve1 did not compromise functionality (Chapter 5). Thus, although the C3 domain of Ve1 and Cf-9 are relatively conserved, different eLRRs appear to contribute to immune signaling. In this light, it is interesting to note that differential binding affinity of FLS2 and BRI1 to the mutated co-receptor bak1<sup>del</sup> was observed (Jaillais et al., 2011).

In addition to the eLRR domain, also the kinase domain of RLKs can be involved in ligand-induced dimerization with co-receptors, since a kinase-dead BRI1 mutant was found not to interact with BAK1 <i>in planta</i> (Wang et al., 2008b). Therefore, it was recently suggested that receptor/co-receptor heterodimerization occurs through a “double-lock” model, in which both the eLRR domain and the kinase domains participate (Jaillais et al., 2011; Li, 2011).

Finally, the transmembrane domain appears to be crucial for dimerization of CRN and CLV2 (Bleckmann et al., 2010). Ve1 carries a GxxxG sequence which was identified as motif that mediates interactions between transmembrane proteins (Curran and Engelman, 2003). However, alanine substitution revealed that this putative transmembrane GxxxG motif is not required for Ve1 functionality (Chapter 5).

**eLRR-containing cell-surface receptors recognize peptide epitopes**

In this thesis, we demonstrated that a nine amino acid epitope derived from the C-terminus of Ave1 is required and sufficient to trigger Ve1-mediated HR (Chapter 6). Plant eLRR-containing immune receptors often recognize short peptide motifs on the surface of their ligand molecules. For example, flg22 is the 22-amino acid peptide derived from bacterial flagellin that activates FLS2-mediated immune signaling in Arabidopsis (Zipfel et al. 2004). Similarly, Arabidopsis EFR recognizes ELF18, an 18-amino acid peptide derived from bacterial elongation factor EF-Tu (Zipfel et al. 2006). The
surface-exposed pentapeptide TKLGE of the ethylene-inducing xylanase determines its recognition by the tomato LeEIX2 (Rotblat et al. 2002; Ron et al. 2004).

Short peptide sequences as epitope have not yet been reported as ligands for cytoplasmic LRR receptors, suggesting that cytoplasmic immune receptors recognize their ligands in a more sophisticated manner, perhaps by recognition of several surface epitopes simultaneously. For example, the cytoplasmic effector AVR3aKI of *Phytophthora infestans* is recognized by the nucleotide-binding site (NBS)-LRR type resistance protein R3a. AVR3aKI encodes a protein of 147 amino acids, and at least 75 amino acids in the C-terminal half of AVR3aKI are required for activation of R3a-mediated cell death (Bos et al., 2006).

Many ligands of eLRR-containing cell surface receptors are processed into (smaller) peptides. These peptides are cleaved from a larger precursor protein, and may be modified by sulfonation and hydroxylation. It has been demonstrated that processing of the CLV3 precursor protein into smaller CLE peptides by plant proteases is essential for functionality of Arabidopsis CLV3 (Fiers et al., 2006; Fiers et al., 2005; Ni and Clark, 2006). Moreover, a 204 amino acid GrCLE1 precursor protein secreted by the potato cyst nematode *Globodera rostochiensis* is processed into GrCLE1 peptides by host plant proteases (Guo et al., 2011; Gao and Guo, 2012). Mature GrCLE1 peptides of 12 amino acids mimic plant CLE peptides, and bind directly to the plant CLE receptors CLV2, BAM1, and BAM2 to modulate root developmental programs to facilitate infection, whereas the unprocessed GrCLE1 precursor protein is not functional (Guo et al., 2011). In addition, elongation of CLE peptides decreases its activity (Kondo et al., 2008; Kondo et al., 2006; Guo et al., 2011). Possibly, the epitope is not correctly exposed on surface of longer peptides and therefore not accessible for receptors. In a similar fashion, the sulphated 17-amino acid peptide aXY22, derived from the bacterial *X. oryzae pv oryzae* elicitor Ax21 (a 194-amino acid protein),
was shown to be sufficient to activate immune signaling by the rice XA21 (Lee et al. 2009).

*Verticillium Ave1* has been characterized as homolog of plant natriuretic peptides (de Jonge et al., 2012). Natriuretic peptides were originally identified in vertebrates where they are involved in the maintenance of osmotic and cardiovascular homeostasis (Potter et al., 2009). The biologically active peptide is a C-terminally derived 28 amino acid peptide that is cleaved from a 126 amino acid precursor (Potter et al., 2009). Plant natriuretic peptides are secreted in the apoplast and play an important role in the regulation of water and ion homeostasis (Gehring, 1999; Gehring and Irving, 2003; Ruzvidzo et al., 2011). It is still unknown whether *Verticillium Ave1* and plant natriuretic peptides are cleaved into smaller peptides that act as mobile signaling molecules. Possibly, *Verticillium Ave1* may be cleaved into smaller peptides, similar to GrCLE, which could explain why Ave1 has never been found in xylem sap of *Verticillium*-inoculated tomato plants using mass spectrometry, while this xylem sap was able to induce a hypersensitive response in *Ve1*-expressing tobacco (Peter van Esse and Zhao Zhang, unpublished data).

**Direct or indirect interaction?**

It has not yet been determined whether Ave1 directly interacts with Ve1, or requires an intermediate component. Direct interaction between peptide hormones and their eLRR-containing cell surface receptors has been demonstrated by *in vitro* assays in all studied cases, including CLE9-BAM1 (Shinohara et al., 2012), CLV3-CLV1(Ogawa et al., 2008), PSK-DcPSKR1 (Shinohara et al., 2007; Matsubayashi et al., 2002), AtPep1-PEPR1 (Yamaguchi et al., 2006), the CLE14/CLE20-CLAVATA2/CORYNE complex (Meng and Feldman, 2010), and Systemin-tBRI1/SR160 (Montoya et al., 2002). In addition, brassinosteroid directly binds to BRI1 (Kinoshita et al., 2005; She et al., 2011; Hothorn et al., 2011).
Direct binding of pathogen-derived ligands has also been shown for eLRR-containing immune receptors, such as EIX-EIX2 (Ron and Avni, 2004), flg22-FLS2 (Chinchilla et al., 2006), axYs22-XA21 (Lee et al., 2009), as well as ELF18-EFR (Albert et al., 2010). However, the C. fulvum effector Avr2 indirectly interacts with immune receptor Cf-2, as it binds and inhibits the tomato cysteine protease Rcr3 which then triggers Cf-2 mediated immunity (Rooney et al., 2005). Similarly, the immune receptor Cf-9 does not directly bind Avr9 (Luderer et al., 2001), but requires a high-affinity binding site (Kooman-Gersmann et al., 1998).

**Concluding remarks**

Using the interaction between tomato Ve1 and Verticillium Ave1 as model, experimental data presented in this thesis provide novel insight in the molecular basis of plant innate immunity. Future studies should be devoted to verify and extend the proposed model for eLRR-containing cell surface receptors in ligand perception and receptor complex formation. For instance, the analysis of the crystal structure of the Ve1 immune receptor with its ligand Ave1 will provide a better understanding of the basis of receptor ligand specificity, and potentially of the receptor activation mechanism. Furthermore, details of the role of Ave1 in pathogenicity of Verticillium may help to understand the role of the Ve1 immune receptor in the physiology of the tomato host. Overall, the concepts and technological methods that were developed in this thesis research will facilitate future investigations concerning Ve1 functionality.
REFERENCES


SUMMARY

Similar to the animal innate immune system, plants employ extracellular leucine-rich repeat (eLRR)-containing cell surface receptors to recognize conserved molecular structures that are derived from microbial pathogens. A number of these immune receptors, as well as the corresponding pathogen ligands, have been characterized. The interaction between the tomato Ve1 immune receptor and the Ave1 effector from the pathogenic fungus *Verticillium* serves as a model system for the study of plant innate immunity. The research described in this thesis was aimed at a further understanding of how the eLRR-containing cell surface receptor Ve1 confers recognition of the Ave1 ligand and how it activates downstream immune signaling.

It has been shown that eLRR-containing cell surface receptors play important roles in development and innate immunity in various plant species. Chapter 1 gives an overview of the current status of research on eLRR-containing cell surface receptors, their co-receptors and corresponding ligands, with emphasis on structural aspects. The functions of distinct eLRR receptor domains, their role in structural conformation, ligand perception, signal transduction and receptor complex formation are extensively discussed.

To facilitate studies on the Ve1-Ave1 model system, we describe the establishment of protocols to investigate Ve1-mediated recognition of Ave1 and immune signaling in tobacco in Chapter 2. We optimized an *Agrobacterium tumefaciens* transient expression assay (agroinfiltration) by testing various over-expression vectors, and found that co-expression of Ve1 and Ave1 leads to hypersensitive response (HR) only in particular tobacco species. We further report on virus-induced gene silencing (VIGS) in *Nicotiana tabacum* cv. Samsun that allows investigating signaling components involved in Ve1-mediated resistance. Collectively, we established *N. tabacum* as a model plant to study Ve1-mediated immunity.

In Chapter 3, we further investigated whether co-expression of Ve1 and Ave1 leads to an HR in Arabidopsis, which may potentially be used as a
straightforward screening method upon a random mutagenesis. However, although Ave1 is able to trigger an HR in resistant tomato and tobacco plants, co-expression of Ve1 and Ave1 did not activate an HR in Arabidopsis. These results suggest that the HR occurs as a consequence of Ve1-mediated resistance signaling, and it is not absolutely required for *Verticillium* resistance.

In **Chapter 4** we investigated the contribution of particular regions of Ve1 to the activation of immune signaling through domain swaps between Ve1 with its non-functional homolog Ve2. Agroinfiltration, as well as stable Arabidopsis transformation, revealed that chimeras in which the first thirty eLRRs of Ve1 were replaced with those of Ve2 remain able to induce HR and activate *Verticillium* resistance. However, a truncated Ve1 protein that lacks the first 30 eLRRs is no longer functional. We speculate that the non-functional Ve2 receptor may still interact with the Ave1 effector in the eLRR domain, but fails to activate immune signaling due to a non-functional C-terminus.

In **Chapter 5**, site-directed mutagenesis was employed to further investigate the eLRR domain of Ve1. We designed alanine scanning mutants in the solvent-exposed residues across the convex surface of the eLRR domain. In each mutant, two of the five solvent-exposed residues in β-sheet of a single eLRR were substituted into alanines. Functionality of the mutants through agroinfiltration and stable transformation of Arabidopsis revealed three eLRR regions (eLRR1-eLRR8, eLRR20-eLRR23 and eLRR32-eLRR37) that are potentially required for ligand specificity and for co-receptor interaction. In addition, alanine substitution was employed to evaluate role of putative protein-protein interaction and endocytosis motifs in the transmembrane domain and the cytoplasmic tail of the Ve1 protein. However, no requirement of these domains for Ve1 functionality could be demonstrated.

It has been demonstrated that eLRR-containing cell-surface immune receptors often recognize short peptide sequence stretches as epitopes of
their ligands. In Chapter 6, we aimed to identify the surface epitope of the Verticillium effector Ave1 that is recognized by Ve1. Firstly, we assessed whether various Ave1 homologs are recognized by Ve1. Since we found that C-terminal fusion of a GFP tag to Ave1 compromised its recognition, we hypothesized that accessibility of the Ave1 C-terminus is essential for Ve1-mediated recognition. Ave1 truncations and domain swaps with Ave1 homologs that are not recognized by Ve1 showed that a nine amino acid sequence derived from the C-terminus of Ave1 is essential for recognition by Ve1. This nine amino acid epitope is sufficient to activate Ve1-mediated immunity.

In Chapter 7 the highlights of the thesis are discussed and placed in a broader perspective. The current understanding of eLRR-containing cell surface receptors is discussed, taking the findings of this thesis into account, with specific emphasis on ligand perception and receptor complex formation. In addition, future perspectives on the future are sketched, and novel research questions are posed aimed to obtain further insights into how Ve1 may form complexes with various co-receptors and how Ave1 contributes to Verticillium pathogenicity.
SAMENVATTING

Net als het aangeboren immuunsysteem van dieren, gebruiken planten extracellulaire leucine-rijke repeat (eLRR)-bevattende receptoren op het celopervlak om geconserveerde moleculaire structuren van microbiële ziekteverwekkers te herkennen. Een aantal van deze immune receptoren zijn gekarakteriseerd, met de bijbehorende liganden van de ziekteverwekker. De interactie tussen de Ve1 immuun receptor van tomaat en de Ave1 effector van de pathogene schimmel Verticillium dient als een modelsysteem voor de studie aan aangeboren immunité van planten. Het onderzoek beschreven in dit proefschrift was gericht op een beter begrip van de manier waarop de eLRR-bevattende receptor Ve1 het Ave1 ligand herkent op het celopervlak, en hoe deze het immuunsysteem activeert.

In verschillende plantensoorten spelen eLRR-bevattende receptoren op het celopervlak een belangrijke rol in ontwikkeling en aangeboren immunité. Hoofdstuk 1 geeft een overzicht van de huidige status van het onderzoek aan eLRR-bevattende receptoren op het celopervlak, hun co-receptoren en bijbehorende liganden, met de nadruk op de structurele aspecten. De functies van verschillende eLRR receptor domeinen, hun rol in de structurele conformatie, ligand perceptie, signaaltransductie en vorming van het receptorcomplex worden uitgebreid besproken.

Om studies aan het Ve1-Ave1 modelsysteem te vergemakkelijken, beschrijven we in hoofdstuk 2 de totstandkoming van protocollen om Ve1-gemedieerde herkenning van Ave1 te onderzoeken. We optimaliseerden een Agrobacterium tumefaciens transiënte expressie toets (agroinfiltration) door verschillende over-expressievectorst te testen, en vonden dat co-expressie van Ve1 en Ave1 leidt tot een overgevoeligheidsreactie (HR) in bepaalde tabakssoorten. Verder rapporteren we dat virus-geïnduceerde gen-silencing (VIGS) in Nicotiana tabacum cv. Samsun gebruikt kan worden om signaleringscomponenten betrokken bij Ve1-gemedieerde resistentie te onderzoeken. Samenvattend hebben we N. tabacum beschreven als modelplant om Ve1-gemedieerde immunité bestuderen.
In hoofdstuk 3 werd onderzocht of co-expressie van Ve1 en Ave1 in Arabidopsis leidt tot een HR die mogelijk kan worden gebruikt als een eenvoudige screeningsmethode na mutagenese. Echter, hoewel herkenning van Ave1 leidt tot een HR in tomaat en tabak leidt co-expressie van Ve1 en Ave1 niet tot HR in Arabidopsis. Deze resultaten suggereren dat de HR optreedt als gevolg van Ve1-gemedieerde signalering, maar niet absoluut vereist is voor resistentie tegen Verticillium.

In hoofdstuk 4 onderzochten we de bijdrage van bepaalde regio’s van Ve1 in de activering van het immuunsysteem door middel van domeinuitwisseling tussen Ve1 en de niet-functionele homoloog Ve2. Met behulp van agroinfiltratie en stabiele Arabidopsis transformatie is aangetoond dat chimeren waarin de eerste dertig eLRRs van Ve1 werden vervangen door die van Ve2 nog steeds de HR activeren Verticillium resistentie induceren. Echter een afgekort Ve1 eiwit dat de eerste 30 eLRRs mist is niet meer functioneel. We speculeren dat de niet-functionele receptor Ve2 nog steeds interacteert met de Ave1 effector in het eLRR domein, maar het immuunsysteem niet kan activeren door een niet-functionele C-terminus.

In hoofdstuk 5 werd gerichte mutagenese gebruikt om het eLRR domein van Ve1 verder te onderzoeken. Alanine scanning mutanten werden gemaakt in de blootgestelde residuen over het convexe oppervlak van het eLRR domein. In elke mutant werden twee van de vijf blootgestelde residuen in de β-sheet van een eLRR vervangen door alanines. Functionaliteit van de mutanten werd getest door agroinfiltratie en stabiele transformatie van Arabidopsis, en onthulde drie eLRR regio’s (eLRR1-eLRR8, eLRR20-eLRR23 en eLRR32-eLRR37) die mogelijk noodzakelijk zijn voor ligand specificiteit en co-receptor interactie. Bovendien werd vervanging door alanine toegepast om de vermeende rol in eiwit-eiwit interactie en endocytose te testen voor motieven in het transmembraan domein en de cytoplasmatische staart van Ve1. Echter, een rol van deze domeinen in Ve1 kon niet worden aangetoond.
Het is aangetoond dat eLRR bevattende celoppervlak receptoren vaak korte peptides in hun ligand herkennen. In hoofdstuk 6 hebben we geprobeerd om het epitoot op het oppervlakte van de *Verticillium* effector Ave1 te identificeren dat wordt herkend door Ve1. Ten eerste hebben we onderzocht of verschillende Ave1 homologen worden herkend door Ve1. Aangezien de C-terminale fusie van GFP aan Ave1 de herkenning verhinderde, veronderstelden we dat de toegankelijkheid van de C-terminus van Ave1 van essentieel belang is voor Ve1-gemedieerde herkenning. Afkortingen van Ave1 afknottingen uitwisseling van domeinen met Ave1 homologen die niet worden herkend door Ve1 toonden aan dat een aminozuursequentie van negen aminozuren van de C-terminus van Ave1 essentieel is voor herkenning door Ve1. Deze epitoot van negen aminozuren is voldoende om Ve1-gemedieerde immuniteit te activeren.

In hoofdstuk 7 van de hoogtepunten van het proefschrift worden besproken en geplaatst in een breder perspectief. De huidige kennis van eLRR-bevattende receptoren op het celoppervlak wordt besproken, het nemen van de bevindingen van dit proefschrift in aanmerking genomen, met specifieke nadruk op ligand perceptie en receptor complexvorming. Bovendien worden toekomstperspectieven de toekomst geschetst en nieuwe onderzoek vragen gesteld gericht op verdere inzicht te krijgen in hoe Ve1 kunnen complexen vormen met verschillende co-receptoren en hoe Ave1 bijdraagt aan *Verticillium* pathogeniciteit.
ACKNOWLEDGEMENTS

Looking back, it seems like only a very short period in The Netherlands, although my stay started almost ten years ago on the 14th of August 2003. The beginning was difficult, but later on I adapted more and more to my new environment and lifestyle. Sometimes I even have the hallucination that Wageningen is my hometown. However, I spent the first twenty years of my life in Datong city, where I was born. During my stay in The Netherlands, there were times of disappointment and anger, and there were times of joy and excitement. Through all these times, many friends have been there to share them with me, and I am deeply grateful for that.

First of all, I gratefully acknowledge my promoter and daily supervisor Prof. Bart Thomma. Dear Bart, thanks for your extraordinary guidance. You spent so much time and effort to help me work out the details of my experiments and to improve our manuscripts and my thesis in every aspect. You shaped my scientific career and guided me in growing up from a young student into an experienced researcher ever since I started my BSc thesis under your supervision at the beginning of 2005 (when you were still a postdoc in the group of Prof. Pierre de Wit). You have also set a perfect example of a successful scientist for me. I witnessed the devotion to your work, your super-efficiency and the progress in your research program over the past eight years. Therefore, it was no surprise to me when I recently heard that you became head of the Laboratory of Phytopathology. With your experience, knowledge, and management skills you will surely be able to fulfill the responsibilities that are associated with this important position.

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A special acknowledgement goes to Prof. Shipeng Li (李师鹏), Dr. Si-Jun Zheng (郑泗军), Dr. Guodong Wang (王国栋) and Yongfeng Fan (樊永峰), for their friendship, continuous suggestions, support and inspiration during the past ten years, especially when I was facing the most difficult moments of my PhD research.

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All chapters of this thesis deal with plants. Bert Essenstam, and other staff of Unifarm, thank you for the excellent plant care. My research would not have been possible without your efforts.

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蓼蓼者莪，匪莪伊蒿。哀哀父母，生我劬劳。亲爱的爸爸妈妈，儿乘桴
海外，违离膝下，倏然已十易春秋。十年逝水流光，儿由稚子渐步中年，每从睡乡惊醒，梦里依稀故园；偶尔对镜一瞥，镜中不是我的脸，仿佛是记忆中你们的青春。十年天涯两隔，聚少离多，而相暌既远，相念益深。慈母之线，游子之衣，你们心中时时装满了对我的牵挂：你们为我的进步而欣喜，你们因我的挫折而忧心，你们给与我无尽的关怀、鼓励和支持。生死肉骨，岂过今恩，负山戴岳，未足胜荷。此春晖寸草之情，昊天罔极而难报万一。儿久出乡关，愧不能长守膝前之欢，谨以此书献给你们和已故的爷爷奶奶，作为十年负笈的一点总结。

Zhao Zhang 张钊

Wageningen,
29. April. 2013
LIST OF PUBLICATIONS


Fradin EF*, Zhang Z*, Rövenich H, Liebrand TWH, Masini L, van den Berg GCM, Joosten MHAJ and Thomma BPHJ (2013) Functional analysis of the tomato immune receptor Ve1 through domain swaps with its non-functional homolog Ve2. (Submitted)


Liebrand TWH*, Kombrink A*, Zhang Z, Sklenar J, Jones AM, Robatzek S, Thomma BPHJ and Joosten MHAJ (2013) Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to *Verticillium*. (Submitted)


*Co-First authorship
Zhao ZHANG (张钊) was born on the 24th of December 1981 in Datong, Shanxi province, China. He grew up and finished secondary school in Datong before he studied Plant Biotechnology at the China Agricultural University (CAU) in Beijing. Subsequently, Zhao was enrolled in the Hogeschool Larenstein in Velp in The Netherlands, and obtained his Bachelor degree in Plant Biotechnology in 2005. During his undergraduate studies, he worked in the group of dr. ir. Bart Thomma at the Laboratory of Phytopathology of the Wageningen University on the role of Receptor-Like Proteins in Arabidopsis immunity. The following years, from 2005 to 2007, Zhao continued as an MSc student Plant Biotechnology at Wageningen University. His first MSc thesis was supervised by dr. Si-Jun Zheng and Prof. dr. ir. Marcel Dicke at the Laboratory of Entomology, where Zhao worked on information-mediated indirect defense of the host plant (*Brassica oleracea*) against a herbivore (*Pieris* spp.), involving a parasitoid (*Cotesia* spp.). The second thesis focused on the signaling cascade in tomato defense against the vascular wilt fungus *Verticillium dahliae*, in the Laboratory of Phytopathology under supervision of dr. ir. Bart Thomma. In September 2007, Zhao started his PhD research in the Laboratory of Phytopathology of the Wageningen University, where he worked on the project described in this thesis.
### Education Statement of the Graduate School

**Experimental Plant Sciences**

**Issued to:** Zhao Zhang  
**Date:** 5 June 2013  
**Group:** Phytopathology, Wageningen University & Research Centre

#### 1) Start-up phase

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**Subtotal Start-up Phase:** 7.5 credits*

#### 2) Scientific Exposure

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#### 3) In-Depth Studies

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</table>

**Subtotal In-Depth Studies:** 4.8 credits*

#### 4) Personal development

<table>
<thead>
<tr>
<th>Activity</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skill training courses</td>
<td>Sep-Oct 2012</td>
</tr>
<tr>
<td>Organisation of PhD students day, course or conference</td>
<td></td>
</tr>
<tr>
<td>Membership of Board, Committee or PhD council</td>
<td></td>
</tr>
</tbody>
</table>

**Subtotal Personal Development:** 3.0 credits*

**TOTAL NUMBER OF CREDIT POINTS***

33.2

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