Genetic Dissection of *Verticillium* Wilt Resistance Mediated by Tomato Ve1[1][C][W][OA]

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Vascular wilt diseases caused by soil-borne pathogens are among the most devastating plant diseases worldwide. The *Verticillium* genus includes vascular wilt pathogens with a wide host range. Although *V. longisporum* infects various hosts belonging to the Cruciferaceae, *V. dahliae* and *V. albo-atrum* cause vascular wilt diseases in over 200 dicotyledonous species, including economically important crops. A locus responsible for resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* has been cloned from tomato (*Solanum lycopersicum*) only. This locus, known as *Ve*, comprises two closely linked inversely oriented genes, *Ve1* and *Ve2*, that encode cell surface receptor proteins of the extracellular leucine-rich repeat receptor-like protein class of disease resistance proteins. Here, we show that *Ve1*, but not *Ve2*, provides resistance in tomato against race 1 strains of *V. dahliae* and *V. albo-atrum* and not against race 2 strains. Using virus-induced gene silencing in tomato, the signaling cascade downstream of Ve1 is shown to require both EDS1 and NDR1. In addition, NRC1, ACIF, MEK2, and SERK3/BAK1 also act as positive regulators of Ve1 in tomato. In conclusion, Ve1-mediated resistance signaling only partially overlaps with signaling mediated by Cf proteins, type members of the receptor-like protein class of resistance proteins.

Vascular wilt diseases caused by soil-borne pathogens are among the most devastating plant diseases worldwide (Tjamos and Beckman, 1989). Vascular wilts are particularly notorious since, in the vascular system of host plants, the pathogens cannot be reached by many fungicides and few fungicides exist to cure plants once they are infected. Because of extremely persistent resting structures, such as microsclerotia, vascular wilt fungi survive in soil for many years, and the only effective control measure, soil fumigation, is expensive and has harmful environmental effects (Rowe et al., 1987; Fradin and Thomma, 2006). Their high economic impact, combined with the absence of curative treatments, justifies increased attention for vascular wilt diseases. However, to design novel control strategies, understanding the biology of vascular pathogens is of fundamental importance.

Four fungal genera, *Ceratocystis*, *Fusarium*, *Ophiostoma*, and *Verticillium*, contain the main vascular wilt pathogens (Agrios, 2005). Most vascular pathogens are characterized by narrow host ranges; the exceptions are fungi of the genus *Verticillium*. While *V. longisporum* infects various hosts that belong to the Cruciferaceae, including cabbage (*Brassica oleracea* var *capitata*), cauliflower (*Brassica oleracea*), and rapeseed (*Brassica napus*), *V. dahliae* and *V. albo-atrum* are responsible for monocyclic vascular wilt diseases in over 200 dicotyledonous species, including economically important crops (Pegg and Brady, 2002; Fradin and Thomma, 2006). Triggered by root exudates, microsclerotia in the soil germinate and penetrate the roots through the root tip or via wounds and sites of lateral root formation. After crossing the root endodermis, the fungus enters the xylem and produces conidia that are transported by the water stream throughout the plant. Once senescing, tissues become colonized and microsclerotia are produced that are released in the soil during decomposition of plant materials. Little is known about the genetics and molecular biology of *Verticillium*-host

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interactions. Recently, transcriptome profiling has been undertaken to study compatible, incompatible, and tolerant interactions to identify genes that play a crucial role in host defense (Robb et al., 2007; van Esse et al., 2009). Intriguingly, it was recently demonstrated that posttranscriptional gene silencing governs basal defense against *Verticillium* in Arabidopsis (*Arabidopsis thaliana*; Ellendorff et al., 2009).

In several plant species, including alfalfa (*Medicago sativa*), cotton (*Gossypium hirsutum*), potato (*Solanum tuberosum*), strawberry (*Fragaria vesca*), sunflower (*Helianthus annuus*), and tomato (*Solanum lycopersicum*), sources of genetic resistance to *Verticillium* have been described (Schaible et al., 1951; Lynch et al., 1997, Bae et al., 2008). However, a locus responsible for resistance against *Verticillium* has been cloned only from tomato (Kawchuk et al., 2001). This *Ve* locus governs resistance against race 1 strains of *V. dahliae* and *V. albo-atrum*, and strains that are not contained by this locus are assigned to race 2 (Schaible et al., 1951; Diwan et al., 1999). The *Ve* locus contains two closely linked inversely oriented genes, *Ve1* and *Ve2*, that, upon independent heterologous expression in potato, were shown to provide resistance against a race 1 *V. albo-atrum* strain (Kawchuk et al., 2001). Both *Ve1* and *Ve2* were found to encode cell surface receptor proteins that belong to the extracellular Leu-rich repeat (eLRR) receptor-like protein (RLP) class of disease resistance proteins (Kawchuk et al., 2001; Wang et al., 2008). The largest group of eLRR-containing cell surface receptors comprises the receptor-like kinases that contain an eLRR domain, a single-pass transmembrane domain, and a cytoplasmic kinase domain, with over 200 representatives in the Arabidopsis genome (Shiu and Bleecker, 2001). The second largest group of eLRR-containing cell surface receptors, represented by 57 members in the Arabidopsis genome, is formed by the RLPs, which differ from receptor-like kinases in that they lack a cytoplasmic kinase domain and carry only a short cytoplasmic tail that lacks obvious signaling motifs other than the putative endocytosis motif found in some members (Fritz-Laylin et al., 2005; Wang et al., 2008). This class of resistance protein was identified originally as Cf resistance proteins that provide resistance in tomato against the foliar leaf mold pathogen *Cladosporium fulvum* (Jones et al., 1994; Thomma et al., 2005) but also includes the apple (*Malus domestica*) HcrVf proteins that confer resistance to the scab fungus *Venturia inaequalis* (Vinatzer et al., 2001; Belfanti et al., 2004; Malnoy et al., 2008). In addition to race-specific resistance proteins, the RLP family harbors receptors that act in basal defense and nonhost resistance, including the tomato LeEIX genes that encode receptors for the ethylene-inducible xylanase produced by *Trichoderma* biocontrol fungi (Ron and Avni, 2004) and Arabidopsis AtRLP52 and AtRLP30 that provide resistance against the powdery mildew pathogen *Erysiphe cichoracearum* and nonhost resistance toward *Pseudomonas syringae* pv *phaseolicola*, respectively (Ramonell et al., 2005; Wang et al., 2008).

The interaction between tomato and *C. fulvum* has been the most extensively used model to study the molecular basis (and the evolution) of recognition specificity in RLP-type disease resistance proteins (Parniske et al., 1997; Thomas et al., 1997; Parniske and Jones, 1999; van der Hoorn et al., 2001a, 2001b, 2005; Wulff et al., 2001; Seear and Dixon, 2003; Kruijt et al., 2004; Thomma et al., 2005). Also, the genetics of RLP-mediated disease resistance signaling has been most extensively studied exploiting the tomato *Cf* genes. Using transcriptomics approaches based on AFLPs, the transcriptional response of tobacco (*Nicotiana tabacum*) suspension cells heterologously expressing the tomato resistance gene *Cf-9* was monitored upon addition of the *C. fulvum* effector Avr9 (Durrant et al., 2000). Similarly, the transcriptome of tomato *Cf-4* seedlings heterologously expressing *C. fulvum Avr4* was monitored (Gabriëls et al., 2006). Subsequent analysis of candidate genes has revealed several components that are required for the *Cf*-mediated hypersensitive response or resistance against *C. fulvum*. These include the thioredoxin CITRX (Rivas et al., 2004), the protein kinase ACIK1 (Rowland et al., 2005), the NB-LRR protein NRC1 (Gabriëls et al., 2006, 2007), the U-box protein CMPG1 (González-Lamothe et al., 2006), the mitogen-activated protein (MAP) kinases LeMPK1, LeMPK2, and LeMPK3 (Stulemeijer et al., 2007), and the F-box protein ACRE189/ACIF1 (van den Burg et al., 2008). Although the use of tomato has been successful so far, it may be anticipated that unraveling the genetics of RLP signaling would be facilitated by the use of the model plant Arabidopsis. However, despite significant efforts, so far no race-specific disease resistance proteins of the RLP class have been identified in Arabidopsis (Ellendorff et al., 2008; Wang et al., 2008).

Here, we describe the functional analysis of *Ve1* and *Ve2* in resistant and susceptible tomato plants. We show that *Ve1*, but not *Ve2*, provides resistance against race 1 strains of *V. dahliae* and *V. albo-atrum* and not against race 2 strains. Furthermore, the signaling cascade downstream of *Ve1* in tomato is shown to overlap only partially with the *Cf*-mediated signaling cascade.

RESULTS

Sequence Analysis of the *Ve* Locus in Resistant and Susceptible Tomato Genotypes

Verticillium resistance in most tomato cultivars is based on the introduction of the dominant *Ve* locus that was identified in the tomato accession Peru Wild in the 1930s (Schaible et al., 1951). To study the composition of the *Ve* locus in resistant and susceptible tomato genotypes, the coding sequences (CDSs) of *Ve1* and *Ve2* homologs were amplified from genomic DNA of the tomato cultivars MoneyMaker (LA2706), which is susceptible to race 1 strains of *Verticillium*, and Motelle (LA2823) and VFN8 (LA1022), which are

resistant to those strains. Furthermore, the homologs were also amplified from the isogenic lines Craigella GCR26 (LA3247) and Craigella GCR218 (LA3428), which are susceptible and resistant to race 1 *Verticillium* strains, respectively. The *Ve1* and *Ve2* CDSs, 3.1 and 3.4 kb, respectively, were amplified successfully from all genotypes, and the sequences were compared with the previously published *Ve* sequences (Kawchuk et al., 2001) for *Ve1* genomic DNA (accession no. AF272367; VFN8), *Ve1* cDNA (AF272366; Craigella), *Ve2* genomic DNA (AF365929; VFN8), and *Ve2* cDNA (AF365930; Craigella). Between the two published *Ve1* sequences (AF272366 and AF272367), five single nucleotide polymorphisms (SNPs) were identified in the coding region, four resulting in a single amino acid change, while one mutation was silent (Table I, positions 246, 610, 706, 1,548 and 1,888). Interestingly, in the *ve1* CDS amplified from the susceptible genotypes, these five SNPs also were found, suggesting that these SNPs are not causing the susceptibility of these genotypes. In addition, four SNPs were identified in the various *Ve1* alleles that all resulted in amino acid substitutions (Table I). Remarkably, two of these SNPs (Table I, positions 29 and 35) were identified in all sequenced genotypes, while a third SNP was found in the *Ve1* alleles from the resistant genotypes but was absent from the two published *Ve1* sequences (Table I, position 380). As these mutations do not discriminate the resistant from the susceptible genotypes, they are unlikely to be the basis of susceptibility in Craigella CGR26 or MoneyMaker. We finally identified a single nucleotide deletion at position 1,220 resulting in a predicted premature stop codon. As a consequence of this deletion, a truncated Ve1 protein of 407 amino acids is predicted in the susceptible cultivars, whereas the protein in resistant cultivars is 1,053 amino acids. Intriguingly, this mutation was present in all susceptible cultivars but not in the resistant cultivars.

For *Ve2*, eight SNPs were identified, of which six led to predicted amino acid substitutions while two were silent (Table I). Remarkably, two of these SNPs (Table I, positions 3,380 and 3,383) leading to a predicted amino acid change from two Phes into two Sers were identified in all of the sequenced genotypes. In addition to

Table I. *Sequence analysis of the Ve locus in various tomato genotypes*

Polymorphisms in the CDS of *Ve1* and *Ve2* isolated from the tomato isogenic lines Craigella GCR26 (*ve/ve*) and Craigella GCR218 (*Ve/Ve*) and the cultivars MoneyMaker (*ve/ve*), Motelle (*Ve/Ve*), and VFN8 (*Ve/Ve*).

				Ve1 Alleles[c]						
				Resistant Genotypes					Susceptible Genotypes	
SNP Position[a]	Nucleotide Sequence[b]	Mutation	Protein Mutation	VFN8 Genomic AF272367 (Kawchuk et al., 2001)	Craigella cDNA AF272366 (Kawchuk et al., 2001)	Craigella GCR218 (This Study)	Motelle (This Study)	VFN8 (This Study)	Craigella GCR26 (This Study)	MoneyMaker (This Study)
29/35	CCTATGGTT	CTATGGCTT	PMV>LWL	−	−	+	+	+	+	+
246	GTG	GTC	Silent	−	+	−	−	−	+	+
380	GAC	GCC	D>A	−	−	+	+	+	−	−
610	ACT	TCT	T>S	−	+	−	−	−	+	+
706	TCT	ACT	S>T	−	+	−	−	−	+	+
1,220	TCAGAG	TAGAG	SE>Stop	−	−	−	−	−	+	+
1,548	AAC	AAG	N>K[d]	−	+	−	−	−	+	+
1,888	GAC	AAC	D>N[d]	−	+	−	−	−	+	+

				Ve2 Alleles[c]						
				Resistant Genotypes					Susceptible Genotypes	
SNP Position[a]	Nucleotide Sequence[e]	Mutation	Protein Mutation	VFN8 Genomic AF365929 (Kawchuk et al., 2001)	Craigella cDNA AF365930 (Kawchuk et al., 2001)	Craigella GCR218 (This Study)	Motelle (This Study)	VFN8 (This Study)	Craigella GCR26 (This Study)	MoneyMaker (This Study)
1,811	GTA	GCA	V>A	−	−	+	+	+	−	−
2,761	GAC	AAC	D>N	−	−	−	−	−	−	+
2,771	AGA	ACA	R>T	−	−	+	+	+	−	−
2,893	TCA	CCA	S>P	−	−	+	+	+	−	−
2,934	CTC	CTT	Silent	−	−	+	+	+	−	−
3,243	GGT	GGG	Silent	−	−	+	+	+	−	−
3,380/ 3,383	TTTTTT	TCTTCT	FF>SS	−	−	+	+	+	+	+

[a]Position of the SNP relative to the ATG start codon. [b]As in GenBank accession AF272367. [c]+/− indicates presence/absence of a mutation. [d]Amino acid change does not occur in genotypes with *Ve1* mutation at position 1,219, resulting in a premature stop codon. [e]As in GenBank accession AF365929.

these two SNPs, five SNPs were identified in the *Ve2* alleles from the resistant genotypes that were absent from the two published *Ve2* sequences, while one SNP was identified only in MoneyMaker. We were not able to identify a single mutation for *Ve2* that discriminated between resistant and susceptible genotypes.

To further analyze the *Ve* locus, the intergenic region between *Ve1* and *Ve2* was amplified from the resistant tomato genotypes Motelle and Craigella GCR218 and the susceptible genotype Craigella GCR26. In addition to a number of SNPs, approximately in the middle of this intergenic region of 3.4 kb a significant deletion of 36 nucleotides was found in the susceptible Craigella genotype. Subsequently, the intergenic region of the three genotypes was analyzed using the PlantCARE software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Lescot et al., 2002) to identify putative cis-acting regulatory elements (Supplemental Table S1; Supplemental Fig. S1). In addition to TATA boxes, putative regulatory elements were identified, such as a Box-W1 domain with a putative function in fungal elicitor responsiveness and several TC-rich repeats that are involved in defense and stress responses. Furthermore, a putative ethylene-responsive element was identified in the resistant Craigella genotype but not in the resistant Motelle or susceptible Craigella genotypes. Most importantly, however, no differences in regulatory elements were observed between the resistant and susceptible genotypes.

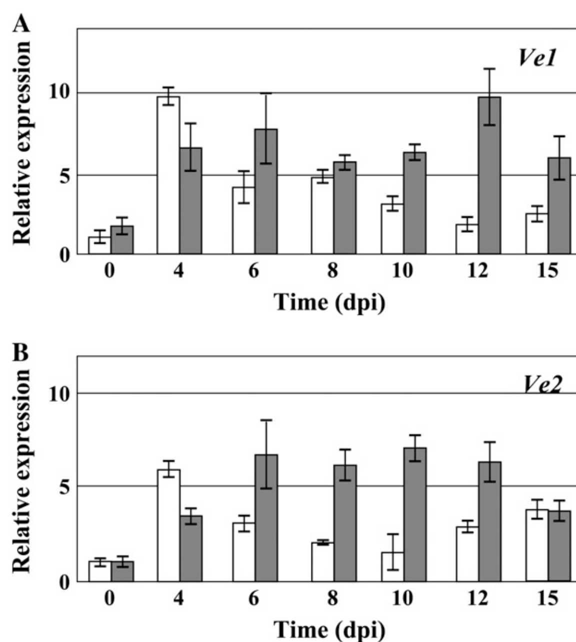

Figure 1. Real-time PCR of a time course of *Ve1* (A) and *Ve2* (B) expression in susceptible and resistant tomato isolines. Time courses are shown for *Ve1* and *Ve2* expression in stem tissue of *V. dahliae*-inoculated Craigella isolines GCR218 (resistant; white bars) and GCR26 (susceptible; gray bars), respectively. Bars represent relative levels of *Ve* transcripts relative to the transcript levels of tomato *actin* (for normalization) with SD of a sample of three pooled plants. A representative of three experiments with similar results is shown. dpi, Days after inoculation.

Ve Expression Analysis in Resistant and Susceptible Tomato Genotypes

The expression of the *Ve* genes in root, stem, and leaf tissues from susceptible and resistant tomato cultivars MoneyMaker and Motelle at 2 weeks after inoculation with a race 1 *V. dahliae* strain or mock inoculation was assessed with real-time PCR. Transcripts of the *Ve* genes were detected in all samples. In the compatible interaction on MoneyMaker plants, transcription of *ve1* and *ve2* was clearly increased by *V. dahliae* challenge. Also in the incompatible interaction, transcript accumulation of *Ve1* and *Ve2* was increased, albeit only moderately, which may reflect a rather localized response, because the fungus is halted at an early stage of the infection process (Fig. 1). Both genes follow a similar transcription pattern, although the level of *Ve2* expression is slightly lower than that of *Ve1* (Supplemental Fig. S2). Subsequently, expression of the *Ve* genes was assessed in the stems of the resistant and susceptible Craigella isogenic lines in time-course experiments (Fig. 1). This analysis demonstrated that the peak of induction for both genes occurred faster in the incompatible interaction than in the compatible interaction. Several studies show that *Verticillium* species enter the xylem vessels of the root and start sporulating after 2 to 5 d (Gold and Robb, 1995; Heinz et al., 1998; Chen et al., 2004). After 1 week, sporulation results in colonization of stem vessels coinciding with fungal elimination as a consequence of plant defense. In compatible interactions, the pathogen is able to overcome this elimination (Gold and Robb, 1995; Heinz et al., 1998; Chen et al., 2004; van Esse et al., 2009). Also in the Craigella lines, both genes follow a similar expression pattern, with a slightly higher level of *Ve1* transcription when compared with *Ve2* (Fig. 1). In any case, these results indicate that lack of *Ve* gene expression cannot explain *Verticillium* compatibility with susceptible tomato genotypes.

Silencing Reveals Differential Activity of *Ve1* and *Ve2*

Based on the sequence analysis and the expression study, it can be concluded that *Ve1* and *Ve2* expression is induced in resistant as well as susceptible tomato genotypes and that no single mutation in the CDS of *Ve2* discriminates resistant and susceptible tomato genotypes. However, a single point mutation in *Ve1*, resulting in a premature stop codon, was found in all susceptible genotypes and was absent in all resistant genotypes. This suggested that *Ve1*, but not *Ve2*, governs *Verticillium* resistance in tomato.

To investigate the role of *Ve1* and *Ve2* in *Verticillium* resistance, we used virus-induced gene silencing (VIGS). VIGS is a well-established method for gene functional analysis in interactions of plants with various foliar pathogens (Burch-Smith et al., 2004). How-

ever, VIGS has not been used so far to study interactions with vascular pathogens. Three recombinant tobacco rattle virus (TRV) vectors (Liu et al., 2002a) were designed to target *Ve* gene expression. While *TRV*:*Ve* was designed to target expression of *Ve1* and *Ve2* simultaneously, *TRV*:*Ve1* and *TRV*:*Ve2* were designed to target expression of *Ve1* and *Ve2* individually, respectively (Supplemental Fig. S3). As a control, an empty *TRV* construct (*TRV*:*00*) was used. Target specificity of the different constructs was verified by assessment of *Ve1* and *Ve2* silencing in tomato (Supplemental Fig. S4). Subsequently, the recombinant *TRV* vectors were inoculated with *Nicotiana benthamiana* leaf sap containing the recombinant virus (Brigneti et al., 2004) using at least 10 plants per construct of the resistant cultivars VFN8 and Motelle. One week later, half of the plants were inoculated with a race 1 *V. dahliae* strain, while the other half were mock inoculated. Two weeks after inoculation, *Verticillium* resistance was assessed by comparing the degree of stunting (height of the plant, length of the leaves, diameter of the stems) that has occurred in host plants, an indicator of disease progression. Upon *Verticillium* inoculation of *TRV*:*00*-treated plants, little stunting was observed when compared with mock-inoculated plants (Fig. 2A; Table II), indicating that *TRV* inoculation by itself does not compromise *Ve*-mediated *Verticillium* resistance in VFN8 or Motelle plants. In contrast, *Verticillium* inoculation of *TRV*:*Ve*-treated VFN8 and Motelle plants resulted in clear and consistent stunting that was not observed in mock-inoculated *TRV*:*Ve*-treated plants (Fig. 2A; Table II). This confirms that the *Ve* locus is responsible for *Verticillium* resistance and, importantly, that VIGS can be used as a tool to investigate gene function in resistance signaling against this vascular fungus. Selective targeting of only *Ve2* by means of the *TRV*:*Ve2* construct resulted in slight stunting after *Verticillium* inoculation, similar to that in *Verticillium*-inoculated *TRV*:*00*-treated plants (Fig. 2A; Table II). Interestingly, clearly compromised *Verticillium* resistance was observed after selective targeting of *Ve1* expression using *TRV*:*Ve1* (Fig. 2A; Table II). These findings were confirmed by fungal recovery from stem sections of the inoculated plants (Fig. 2B) and confirm the hypothesis that *Ve1*, but not *Ve2*, mediates *Verticillium* resistance in VFN8 and Motelle plants.

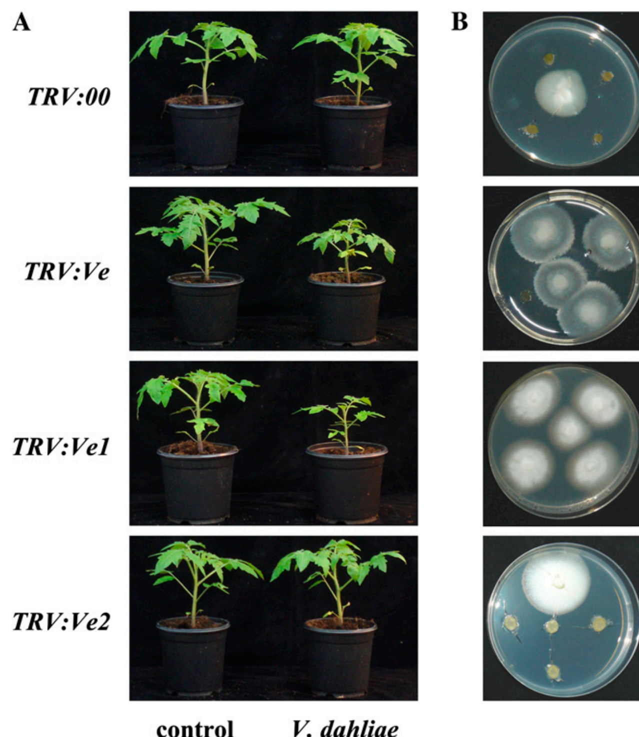

Figure 2. VIGS of *Ve1*, but not of *Ve2*, impairs *Verticillium* resistance. A, Motelle (*Ve*/*Ve*; resistant) plants were treated with an empty recombinant *TRV* vector (*TRV*:*00*), a *TRV* vector targeting a region shared by *Ve1* and *Ve2* (*TRV*:*Ve*), or a *TRV* vector specifically targeting *Ve1* (*TRV*:*Ve1*) or *Ve2* (*TRV*:*Ve2*). Two weeks after treatment, the plants were mock inoculated (control) or inoculated with a race 1 strain of *V. dahliae*. Photographs were taken at 14 d after *V. dahliae* inoculation, and compromised resistance is evident from a stunted appearance of the *V. dahliae*-inoculated plants when compared with mock-inoculated control plants. B, As a measure for fungal colonization, 2 weeks after *V. dahliae* inoculation stem sections were plated on agar medium, allowing the fungus to grow from sections. The number of stem sections from which the fungus grows is a measure of the extent of fungal colonization. Photographs were taken at 14 d after plating. [See online article for color version of this figure.]

Ve1, But Not *Ve2*, Provides *Verticillium* Resistance in Tomato

To confirm our finding that *Ve1*, but not *Ve2*, mediates *Verticillium* resistance in tomato, stable overexpression lines were generated in the susceptible tomato cultivar MoneyMaker expressing either *Ve1* or *Ve2* driven by the cauliflower mosaic virus (CaMV) 35S promoter (Supplemental Figs. S5 and S6). For *Ve1*, the Motelle/VFN8 allele was used (*P35S*:*Ve1*; Supplemental Fig. S6) because it was shown to provide resistance in our VIGS analysis and this genotype was used previously to engineer *Verticillium*-resistant potato (Kawchuk et al., 2001). For *Ve2*, the Craigella GCR26 allele was used (*P35S*:*Ve2*; Supplemental Fig. S3) that most closely matches the allele used to engineer *Verticillium*-resistant potato (Kawchuk et al., 2001). As shown in Table I, we have not been able to identify the exact same *Ve2* allele used by Kawchuk et al. (2001). However, the only polymorphism that is present in the Craigella GCR26 allele is present in all *Ve2* alleles analyzed. For each construct, at least 10 transgenic lines were obtained, of which, after determination of diploidy levels and the copy number of the transgene, lines with one- or two-copy inserts were chosen for further analysis. For each of the constructs, a minimum of five T2 plants of a minimum of two different lines were challenged with each of five different race 1 *Verticillium* isolates, three belonging to *V. dahliae* and two to *V. albo-atrum* (Table III). Intriguingly,

Table II. *VIGS analysis of candidate genes in resistant Motelle plants*

VIGS Construct	No. of Plants Challenged with *V. dahliae*	No. of Stunted Plants	Percentage of Stunted Plants[a]
TRV:00	6	1	16
TRV:Ve	5	5	100
TRV:Ve1	6	5	83
TRV:Ve2	6	1	16
TRV:Acif1	5	4	80
TRV:Acik1	6	1	16
TRV:Cmg1	4	0	0
TRV:Eds1	5	4	80
TRV:Fls2	6	1	16
TRV:Mek2	5	4	80
TRV:Mpk1	5	1	20
TRV:Mpk2	5	0	0
TRV:Mpk3	6	2	33
TRV:Ndr1	6	4	66
TRV:Npr1	6	2	33
TRV:Nrc1	6	4	66
TRV:Rar1	5	1	20
TRV:Serk2	5	1	20
TRV:Serk3-I	6	5	83
TRV:Serk3-II	6	6	100

[a]Data from one representative experiment out of three are shown.

while all plants carrying the *P35S:Ve1* transgenes were found to exhibit robust *Verticillium* resistance, all plants carrying *P35S:Ve2* transgenes were as susceptible as MoneyMaker plants toward these race 1 isolates, showing typical wilt symptoms that included stunting, chlorosis, wilting, and necrosis (Fig. 3A; Table III). Furthermore, when challenged with race 2 isolates belonging to *V. dahliae* and *V. albo-atrum*, all transgenic plants showed typical symptoms of *Verticillium* disease (Table III). All findings were confirmed in subsequent analyses using the T3 generation of the transgenic lines. Moreover, the disease phenotypes were corroborated by assessing *Verticillium* colonization of the transgenic plants through measurement of fungal recovery from stem sections (Fig. 3B).

In addition to the lines with constitutive *Ve* expression, stable transgenic MoneyMaker lines were generated expressing either the same *Ve1* or *Ve2* CDS but driven by the endogenous promoter isolated from Motelle (*PVe1:Ve1* and *PVe2:Ve2*, respectively; Supplemental Fig. S5). For *PVe2:Ve2*, the full intergenic region was used, while for *Ve1*, only half the intergenic region adjacent to the *Ve1* CDS was used (Supplemental Fig. S5). Subsequent *Verticillium* assays on transgenic lines in the T2 and T3 generations revealed that, when driven by the Motelle promoter, *Ve1* but not *Ve2* conferred resistance toward race 1 isolates of *V. dahliae* and *V. albo-atrum* but not toward race 2 isolates (Fig. 3A; Table III). These disease phenotypes were corroborated by assessment of *Verticillium* colonization of the transgenic plants through measurement of fungal recovery from stem sections (Fig. 3B).

Characterization of Ve-Mediated Signaling by VIGS

So far, little is known about the genetic requirements for *Ve* signaling. The only gene implicated in downstream signaling is the tomato homolog of Arabidopsis *Eds1* (for Enhanced Disease Susceptibility1; Aarts et al., 1998; Hu et al., 2005). To further identify genes required for *Ve*-mediated resistance, a set of candidate genes was selected, some of which have previously been implicated in RLP signaling mediated by the tomato *Cf* genes against *C. fulvum*. In addition to *Eds1*, this set included genes encoding the disease signaling components RAR1 (for Required for Mla12 Resistance1; Liu et al., 2002b), SGT1 (for Suppressor of the G2 allele of SKP1; Peart et al., 2002), NDR1 (for Non-race-specific Disease Resistance; Ekengren et al., 2003), and NPR1 (for Nonexpressor of Pathogenesis-Related genes1; Liu et al., 2002b) but also the Ser/Thr protein kinase ACIK1 (Rowland et al., 2005), the F-box protein ACIF1 (van den Burg et al., 2008), the U-box protein CMPG1 (González-Lamothe et al., 2006), the MAP kinase kinase MEK2 (Ekengren et al., 2003), the MAP kinases LeMPK1, LeMPK2, and LeMPK3 (Stratmann and Ryan, 1997; Stulemeijer et al., 2007),

Table III. *Ve1 but not Ve2 provides resistance against Verticillium race 1 isolates*

In each assay, a minimum of five plants were tested for each combination of plant line and *Verticillium* strain. For the transgenes, a minimum two independent lines were tested per construct. S indicates 80% to 100% diseased plants, while R indicates 80% to 100% plants without symptoms of disease.

Verticillium Isolate	Race	Tomato Genotype					
		MoneyMaker	Motelle	*P35S:Ve1*	*PVe1:Ve1*	*P35S:Ve2*	*PVe2:Ve2*
V. dahliae St14.01	1	S	R	R	R	S	S
V. dahliae JR2	1	S	R	R	R	S	S
V. dahliae CBS381.66	1	S	R	R	R	S	S
V. albo-atrum 5431	1	S	R	R	R	S	S
V. albo-atrum CBS385.91	1	S	R	R	R	S	S
V. dahliae CBS321.91	2	S	S	S	S	S	S
V. dahliae M050414	2	S	S	S	S	S	S
V. albo-atrum CBS451.88	2	S	S	S	S	S	S
V. albo-atrum VA1	2	S	S	S	S	S	S

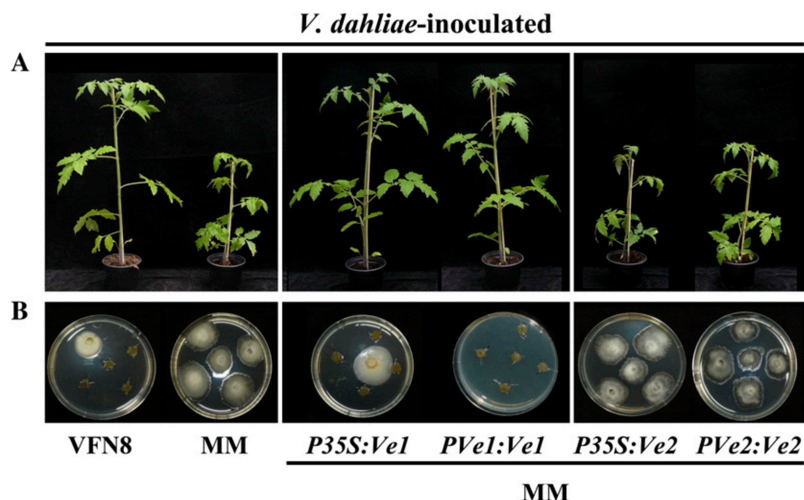

Figure 3. Transgenic expression of *Ve1*, but not of *Ve2*, triggers *Verticillium* resistance in susceptible tomato. A, Typical appearance of wild-type and transgenic tomato cultivars after inoculation with a race 1 strain of *V. dahliae*. Left, VFN8 (*Ve*/*Ve*; resistant) and MoneyMaker (MM; *ve*/*ve*; susceptible) after inoculation with a race 1 strain of *V. dahliae*. Middle, Transgenic MoneyMaker plants expressing the Motelle *Ve1* allele driven by the constitutive CaMV 35S promoter (*P35S*:*Ve1*) or the endogenous *Ve1* promoter (*PVe1*:*Ve1*) after inoculation with a race 1 strain of *V. dahliae*. Right, Transgenic MoneyMaker plants expressing the Craigella *Ve2* allele driven by the constitutive CaMV 35S promoter (*P35S*:*Ve2*) or the endogenous *Ve2* promoter (*PVe2*:*Ve2*) upon inoculation with a race 1 strain of *V. dahliae*. All photographs were taken at 28 d after inoculation. B, As a measure for fungal colonization, 2 weeks after *V. dahliae* inoculation stem sections were plated on agar medium, allowing the fungus to grow from sections. The number of stem sections from which the fungus grows is a measure of the extent of fungal colonization. Photographs were taken at 14 d after plating. [See online article for color version of this figure.]

and the NB-LRR protein required for hypersensitive response-associated cell death NRC1 (Gabriëls et al., 2007). All of these TRV constructs have been described and used previously for effective silencing in tomato (Peart et al., 2002; Ekengren et al., 2003; Rowland et al., 2005; Gabriëls et al., 2006; González-Lamothe et al., 2006; Stulemeijer et al., 2007; van den Burg et al., 2008).

As expected, silencing of *Eds1* resulted in a clear and consistent decrease of resistance in Motelle tomato plants (Fig. 4A; Table II), indicated by stunting (reduced plant height, leaf length, and stem diameter), confirming the previously described involvement of *Eds1* in *Ve*-mediated signaling (Hu et al., 2005). Clear and consistent loss of *Verticillium* resistance in Motelle plants also was observed upon treatments with recombinant viruses targeting *Mek2*, *Nrc1*, *Acif1*, and *Ndr1* (Fig. 4A; Table II), indicating their requirement for *Ve*-mediated disease resistance. In contrast, recombinant viruses targeting *Cmpg1*, *Mpk1*, *Mpk2*, and *Rar1* did not compromise *Verticillium* resistance in Motelle plants (Fig. 4B; Table II), while viruses targeting *Mpk3* and *Npr1* caused a slightly higher number of stunted plants when compared with the empty vector control, suggesting that these components could make a minor contribution to disease resistance (Table II).

It was shown recently that the Arabidopsis Somatic Embryogenesis Receptor Kinase3 (SERK3)/Brassinosteroid (BR)-Associated Kinase1 (BAK1) takes part in an elicitor-dependent complex with Flagellin Sensing2 (FLS2) to initiate a defense response upon elicitation with the bacterial pathogen-associated molecular pattern (PAMP) flagellin or its peptide derivative flg22 (Chinchilla et al., 2007; Heese et al., 2007). In *N. benthamiana*, *Serk3/Bak1* is also required for flagellin-triggered immunity (Heese et al., 2007). In addition, in Arabidopsis as well as *N. benthamiana*, *Serk3/Bak1* is required for full responses to unrelated PAMPs, basal defense, and restriction of pathogen infection (Heese et al., 2007; Kemmerling et al., 2007). Therefore, we attempted to silence the tomato gene encoding SERK3/BAK1 using two different TRV constructs to target different regions of *NbSerk3* (Heese et al., 2007). As a control, TRV constructs targeting *NbFls2* and *NbSerk2* (Colcombet et al., 2005; Heese et al., 2007) were included. Treatment of Motelle tomato plants with the two constructs targeting *Serk3/Bak1* or the construct targeting *Serk2* resulted in slight stunting and weakly distorted leaves. These results are consistent with the phenotype of *N. benthamiana* upon treatment with these constructs (Heese et al., 2007). Interestingly, treatment with the two different recombinant viruses targeting expression of *Serk3/Bak1*, but not with viruses targeting expression of *Fls2* or *Serk2*, clearly compromised *Verticillium* resistance (Fig. 4; Table II). This result suggests that, in addition to PAMP-triggered immunity, *Serk3/Bak1* also functions in race-specific disease resistance.

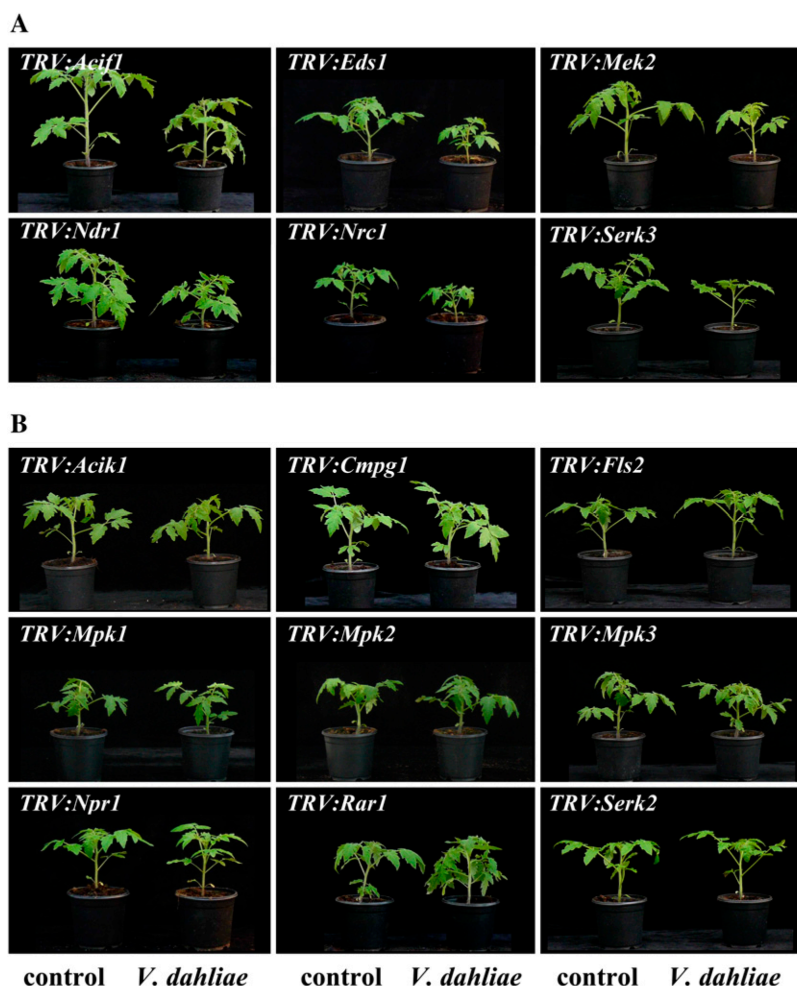

Figure 4. Characterization of *Ve*-mediated signaling in resistant tomato by VIGS. Motelle (*Ve*/*Ve*; resistant) plants were agroinfiltrated with recombinant *TRV* vector carrying a fragment of candidate disease signaling genes. Two weeks after agroinfiltration, the plants were mock inoculated (control) or inoculated with a race 1 strain of *V. dahliae* (JR2). Photographs were taken at 14 d after *V. dahliae* inoculation, and compromised resistance is evident from a stunted appearance of *V. dahliae*-inoculated plants when compared with control plants. A, VIGS of tomato genes required for *Ve*-mediated *Verticillium* resistance. B, VIGS of tomato genes that do not play a major role in *Ve*-mediated *Verticillium* resistance. [See online article for color version of this figure.]

DISCUSSION

Ve1, and Not *Ve2*, Is a Functional *Verticillium* Resistance Gene in Tomato

Many crop species contain genes for tolerance or partial resistance, but not complete resistance, to *Verticillium* species (Fradin and Thomma, 2006). Tomato is an exception in which resistance to race 1 *Verticillium* isolates is conferred by a single dominant locus that was introduced in cultivated varieties in the 1950s (Schaible et al., 1951; Diwan et al., 1999) and that is still carried by most commercial tomato varieties. This article describes the functional analysis of *Ve1* and *Ve2* in resistant and susceptible tomato plants. We were not able to identify a single mutation for *Ve2* that discriminated between resistant and susceptible genotypes. However, sequence analysis revealed that *Ve1* encodes a truncated protein in all susceptible genotypes that were analyzed. This suggested that solely *Ve1* determines the resistance of tomato toward race 1 strains of *Verticillium*. This hypothesis was verified through two lines of evidence. First, VIGS of *Ve1* but not of *Ve2* compromised *Verticillium* resistance in Motelle and VFN8 plants that harbor the *Ve* locus. Second, transgenic tomato plants expressing either *Ve1* or *Ve2* showed that *Ve1* expression, and not *Ve2* expression, resulted in resistance against race 1 strains of *V. dahliae* and *V. albo-atrum*, irrespective of whether expression was driven by the endogenous promoter or the constitutive CaMV 35S promoter.

Previously, the *Ve* locus was cloned from tomato and used for heterologous expression in susceptible potato (Kawchuk et al., 2001). Our study revealed a number of sequence differences for the *Ve1* and *Ve2* alleles that were sequenced by Kawchuk et al. (2001). Support for the veracity of the sequences from our study is provided by Acciarri et al. (2007), who similarly reported on the sequencing of *Ve1* and *Ve2* alleles from resistant and susceptible Italian tomato genotypes. That study confirms the polymorphisms found at positions 246, 380, 610, 706 and 1,220 in *Ve1* and at positions 1,811, 2,771, 2,893, and 2,934 in *Ve2* in our study. Remarkably, Kawchuk et al. (2001) reported that both *Ve1* and *Ve2* provided resistance against a race 1 strain of *V. albo-atrum*, irrespective of whether expression was driven by the endogenous promoter or the constitutive CaMV 35S promoter. Possibly, the Ve2 protein is no longer active in tomato while it is still able to connect to a

disease signaling cascade in potato, for instance, through the presence of auxiliary components in potato that confer functionality. Also, in contrast to Ve1, Ve2 contains a PEST motif that is typically observed in many rapidly degraded proteins (Hershko and Ciechanover, 1998). Therefore, the protein stability of Ve2 may be significantly reduced in tomato when compared with Ve1. Alternatively, the single race 1 *V. albo-atrum* strain that was used on potato contains an elicitor that is not generally carried by most race 1 isolates. Loci with active (demonstrated resistance specificities) and nonactive (unknown functions) homologs of *RLP*-type resistance genes are found commonly, not only in tomato (Dixon et al., 1996; Parniske et al., 1997) but also in apple (Malnoy et al., 2008). It has been speculated that members with unknown functions are a source to generate new recognition (*R* gene) specificities (Kruijt et al., 2005), which may also be true for the *Ve* locus.

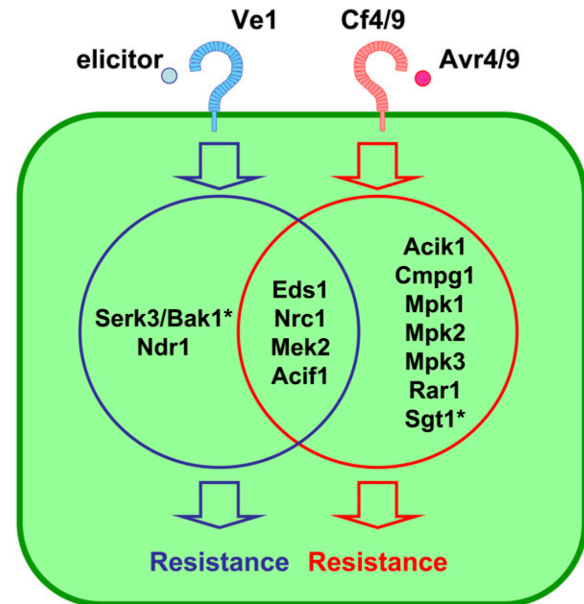

Figure 5. The Ve1 and Cf proteins differentially require downstream signaling components. Signaling components that are required for Ve1-mediated *Verticillium* resistance (in the blue circle) and those required for Cf-mediated *Cladosporium* resistance (in the red circle) are shown. The components in the overlap of the two circles are required for both resistance responses. The asterisks indicate that Serk3/Bak1 has not been tested for requirement in Cf signaling, while Sgt1 has not been tested for requirement in Ve1 signaling.

Genetic Analysis of Ve-Mediated Signaling in Tomato

Interestingly, VIGS using recombinant viruses that target *Ve1* expression resulted in compromised *V. dahliae* resistance, demonstrating that this transient assay can be used to investigate defense against a vascular pathogen. Apart from *Eds1* (Hu et al., 2005), little is known about the genetic requirements for *Ve* signaling. In Arabidopsis, a differential requirement for *Eds1* and *Ndr1* was shown, particularly for cytoplasmic disease resistance proteins of the NB-LRR class (Aarts et al., 1998). Although exceptions exist for this class of resistance proteins, *Eds1* generally mediates signaling initiated by the TIR-NB-LRR subclass, whereas *Ndr1* mediates signaling initiated by the CC-NB-LRR subclass (Century et al., 1995; Aarts et al., 1998). Previously, *Eds1* but not *Ndr1* was found to play a role in *Cf4*-mediated signaling (Gabriëls et al., 2007). Intriguingly, both *Eds1* and *Ndr1* are required for *Ve1* resistance, which represents, to our knowledge, the first example of a membrane-anchored resistance protein with extracellular LRRs that requires both of these genes that are more commonly associated with NB-LRR resistance.

In addition to *Eds1* and *Ndr1*, also the MAP kinase kinase gene *Mek2*, the NB-LRR protein encoding *Nrc1*, and the F-box protein encoding *Acif1* are required for *Ve* signaling, which was confirmed by performing fungal recovery assays from stem sections of the inoculated plants showing enhanced *Verticillium* outgrowth. These components have been implicated in *Cf*-mediated signaling as well (Gabriëls et al., 2007; van den Burg et al., 2008; Fig. 5). Furthermore, tomato *Mek2* has been implicated in tomato resistance against *Pseudomonas syringae* mediated by Pto, while the NB-LRR protein encoding *Nrc1* is required for the hypersensitive response induced by diverse R proteins, including LeEix, Pto, Rx, and Mi (Ekengren et al., 2003; Gabriëls et al., 2007). Recombinant TRV targeting expression of the U-box protein CMPG1, the MAP kinases MPK1 to MPK3, and the disease signaling components RAR1 (Liu et al., 2002b) and NPR1 (Liu et al., 2002b; Ekengren et al., 2003) did not consistently compromise *Verticillium* resistance in Motelle plants. Although we have not verified the VIGS efficiency of these genes, our results indicate that they do not play a major role in the resistance response. All of these, except NPR1, have been found to play a role in *Cf* signaling (González-Lamothe et al., 2006; Gabriëls et al., 2007; Stulemeijer et al., 2007), suggesting that the Ve1 and Cf proteins differ significantly in their requirements for downstream signaling components (Fig. 5). This is further substantiated by the recent observation that the *C. fulvum*-induced transcriptional changes in tomato show little overlap with those induced by *V. dahliae* in compatible as well as incompatible interactions (van Esse et al., 2009).

SERK3/BAK1 May Form a Receptor Complex with Ve1 in Tomato

For VIGS of all genes tested in this study, silencing constructs were employed that have been published previously and have been shown to be effective in silencing the tomato genes that were targeted. The only exception was the construct used to target the expression of *Serk3/Bak1* and the corresponding con-

trol constructs to target the related SERK family member *Serk2* and the expression of the receptor for the bacterial PAMP flagellin and its peptide derivative flg22 *Fls2*. In Arabidopsis, AtSERK3/BAK1 takes part in an elicitor-dependent complex with FLS2 (Chinchilla et al., 2007; Heese et al., 2007), and in *N. benthamiana*, *Serk3/Bak1* is also required for flagellin-triggered immunity (Heese et al., 2007). In addition, in Arabidopsis as well as in *N. benthamiana*, *Serk3/Bak1* is required for full responses to unrelated PAMPs, basal defense, and restriction of pathogen infection (Heese et al., 2007; Kemmerling et al., 2007). In our study, we observed weakly distorted leaves in tomato plants silenced with either of the *NbSerk3* constructs. These results are consistent with the leaf phenotypes upon silencing of *NbSerk3* in *N. benthamiana* plants and upon SERK3/BAK1 knockout in Arabidopsis, which were attributed to defects in brassinosteroid perception (Heese et al., 2007). This suggests that, indeed, the true tomato *Serk3/Bak1* homolog had been silenced. So far, tomato *Serk3/Bak1* has not been identified, but several studies have exploited *N. benthamiana* sequences to successfully target genes in the close relative tomato (Gabriëls et al., 2006, 2007). Interestingly, in our study, both of the *NbSerk3* constructs that target different regions of *Serk3*, but not the *Serk2* or the *Fls2* construct, clearly compromised *Verticillium* resistance. Since SERK3/BAK1 is a coreceptor that physically associates with BRI1 for BR-dependent signaling and with FLS2 for flagellin-induced immunity, this may indicate that tomato SERK3/BAK1 physically associates with the RLP Ve1 to initiate *Verticillium* immunity. Future experiments will be directed to investigate this possibility.

MATERIALS AND METHODS

All experiments have been performed a minimum of three times yielding similar results.

Plant Manipulations

Tomato (*Solanum lycopersicum*) was grown in soil in the greenhouse at 21°C/19°C during 16-h/8-h day/night periods, respectively, with 70% relative humidity and 100 W m^{-2} supplemental light when the intensity dropped below 150 W m^{-2}. For *Verticillium* inoculations, 10-d-old tomato plants were uprooted and the roots were rinsed in water. Subsequently, the roots were dipped for 3 min in a suspension of 10^6 conidia per mL of water and harvested from 1- to 2-week-old *Verticillium* cultures on potato dextrose agar (Oxoid). Control plants were treated similarly, but their roots were dipped in water without conidiospores. After replanting in fresh soil, disease development was monitored up to 28 d after inoculation. The following isolates were used: *V. dahliae* ST14.01, JR2, CBS381.66 (all race 1), CBS321.91, and M050414 (both race 2) and *V. albo-atrum* 5431, CBS385.91 (both race 1), CBS451.88, and VA1 (both race 2).

Cloning of *Ve* Sequences

To amplify the CDSs of *Ve1* and *Ve2*, the primer pairs Ve1F-Ve1R and Ve2F-Ve2R, respectively (Supplemental Table S2), were used with Expand High-Fidelity PCR system enzyme mix (Roche). PCR products were sequenced in both directions (Supplemental Table S2) and with overlapping sequencing products directly or, alternatively, cloned into a pBluescript variant with *Bam*HI and *Asc*I restriction sites, after which multiple clones from independent PCRs were sequenced (BaseClear). For constitutive expression, the *Ve1* and *Ve2* CDSs were cloned into a binary vector pmog800 variant (Honée et al., 1998), resulting in *P35S*:*Ve1* and *P35S*:*Ve2* (Supplemental Fig. S4).

The region between the inversely oriented *Ve* CDSs was PCR amplified (Supplemental Table S2) and sequenced (BaseClear). Furthermore, two constructs were designed (Supplemental Fig. S4): one containing the *Ve1* CDS and half the intergenic region (IR) adjacent to the *Ve1* CDS (*PVe1*:*Ve1*), and one containing the complete IR fused to the *Ve2* CDS (*PVe2*:*Ve2*). The primer pair Ve1ProRegF and VeProReg3R (Supplemental Table S2) was used with Expand High-Fidelity PCR system enzyme mix (Roche) to amplify half of the IR and part of *Ve1*, containing an endogenous *Pst*I restriction site. The PCR fragment was cloned into pGEM-T Easy (Promega) and sequenced (Supplemental Table S2). Subsequently, the IR fragment was excised using *Apa*I and *Pst*I and directionally cloned into the binary vector pGREEN (Hellens et al., 2000). Next, a *Pst*I-*Sma*I fragment of the *P35S*:*Ve1* construct containing the *Ve1* sequence and the terminator from the potato proteinase inhibitor II (*PiII*) gene was cloned into the vector, resulting in *PVe1*:*Ve1*. For *Ve2*, the complete IR was obtained using two PCR-amplified fragments. The first IR fragment was amplified with the primer combination *Ve*ProRegF and *Ve*ProReg3R (Supplemental Table S2) and partially overlapped with the second IR fragment that was amplified with the primer pair *Ve*ProReg3F and *Ve2*ProRegR (Supplemental Table S2), with an endogenous *Spe*I restriction site in the region of overlap. The second IR fragment also partially overlapped with part of the *Ve2* CDS, with an endogenous *Pst*I restriction site in the region of overlap. Both fragments were cloned into pGEM-T Easy, sequenced, and excised using *Apa*I, *Spe*I, and *Pst*I. Both fragments were cloned into *Apa*I- and *Pst*I-digested pGREEN. Subsequently, a *Pst*I-*Sma*I fragment of the *P35S*:*Ve2* construct containing the *Ve2* sequence and the *PiII* terminator was cloned into the vector, resulting in *PVe2*:*Ve2.* All constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation and used for tomato transformation.

Engineering of Transgenic Plants

Tomato transformation was performed as described previously (van Esse et al., 2008). The ploidy level of transgenic tomato plants was determined as described (Jacobs and Yoder, 1989). Subsequently, diploid plants were retained and the transgene copy number was determined by quantitative real-time PCR using the qPCR Core kit for SYBR Green I (Eurogentec) with genomic DNA (Supplemental Table S3; Ingham et al., 2001). The single-copy tomato gene encoding protein phosphatase 5 was used as a reference to determine the number of copies of the neomycin phosphotransferase II transgene selection marker (Supplemental Table S2). Real-time PCR conditions consisted of an initial denaturation step of 10 min at 95°C, followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 40 cycles. Only one- or two-copy transgenes were used in this study.

VIGS Experiments

For all VIGS experiments, the binary TRV constructs pTRV-RNA1 and pTRV-RNA2 were used (Liu et al., 2002a). The inserts to generate *TRV*:*Ve1* and *TRV*:*Ve* were amplified from the *P35S*:*Ve1* plasmid using the primer pairs Ve1F-Ve1VIGSspeR and VeVIGSF2-VeVIGSR1, respectively, while the insert for *TRV*:*Ve2* was amplified from the *P35S*:*Ve2* plasmid using the Ve2F-Ve2VIGSspeR primer pair (for primer sequences, see Supplemental Table S2). PCR fragments were cloned into pTRV:RNA2 (pYL156) using *Bam*HI and *Kpn*I. The constructs were transformed to *A. tumefaciens* GV3101 by electroporation.

TRV vectors were agroinfiltrated as described (Liu et al., 2002a) into cotyledons of 9-d-old Motelle (*Ve*/*Ve*) or VFN8 (*Ve*/*Ve*) plants, and after 2 weeks the plants were inoculated with race 1 *V. dahliae*. Alternatively, TRV vectors were agroinfiltrated into a leaf of 3- to 4-week-old *Nicotiana benthamiana* plants, and 3 to 6 d after agroinfiltration leaf sap was collected by grinding the agroinfiltrated leaves in 50 mM phosphate buffer (pH 7.2). Subsequently, 9-d-old Motelle plants were virus inoculated by rubbing the cotyledons with 6 to 12 μL of the leaf sap and inoculated with a race 1 strain of *V. dahliae* 1 week after treatment.

Expression Analyses

Target specificity of the constructs *TRV:Ve*, *TRV:Ve1*, and *TRV:Ve2* was determined in the MoneyMaker overexpression lines expressing either *Ve1* or *Ve2* driven by the CaMV 35S promoter. Two weeks after virus inoculation, RNA was isolated from whole plants using the RNeasy kit (Qiagen) and used for cDNA synthesis using an oligo(dT) primer (Supplemental Table S2) and the SuperScript III reverse transcriptase kit (Invitrogen), according to the manufacturers' instructions. To analyze expression of the *Ve* alleles, real-time PCR was conducted with *Ve*-specific primers (Ve1QPCRF2-Ve1QPCRR1 for *Ve1* and Ve2SeqF7-Ve2R for *Ve2*) with tomato actin as an internal standard (Supplemental Table S2) using the qPCR Core kit for SYBR Green I (Eurogentec). Real-time PCR conditions consisted of an initial denaturation step of 10 min at 95°C, followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 30 cycles.

Ve expression analyses in resistant and susceptible tomato genotypes as well as in *Ve* transgenic tomato lines were performed similarly.

Fungal Recovery Assay

Two weeks after *Verticillium* inoculation, a stem section immediately above the cotyledons was taken from three inoculated plants, surface sterilized for 15 min in 70% ethanol, followed by 15 min in 10% hypochlorite, rinsed three times with sterile water, and sliced. In total, for each plant, 10 slices were transferred onto potato dextrose agar supplemented with chloramphenicol (34 mg L^{-1}) and incubated at 22°C. The frequency of stem slices from which *Verticillium* grew out is a measure of the susceptibility of the plant.

Sequences described in this study have been deposited in GenBank with accession numbers FJ464553 to FJ464565.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Sequence alignment of the *Ve* locus intergenic region of a resistant and a susceptible isogenic tomato line.

Supplemental Figure S2. Real-time PCR of *Ve1* and *Ve2* expression in susceptible and resistant tomato cultivars.

Supplemental Figure S3. Alignment of fragments used to target the expression of *Ve* genes.

Supplemental Figure S4. Specificity of fragments used to target the expression of *Ve* genes.

Supplemental Figure S5. Constructs used for transgenic expression of *Ve1* and *Ve2*.

Supplemental Figure S6. Reverse transcription-PCR of *Ve1* and *Ve2* expression in transgenic tomato lines.

Supplemental Table S1. Putative regulatory elements identified in the intergenic region of the *Ve* locus of susceptible and resistant tomato genotypes.

Supplemental Table S2. Primers used in this study.

Supplemental Table S3. Copy number determination in transgenic tomato.

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