Optimized Agroinfiltration and Virus-Induced Gene Silencing to Study Ve1-Mediated *Verticillium* Resistance in Tobacco

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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 at which 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 coexpression 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 a HR, and *N. glutinosa* was found to be resistant against race 1 *Verticillium 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.

Verticillium dahliae and *Verticillium albo-atrum* are among the world's most notorious plant pathogens, occurring in temperate and subtropical regions and 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 60 years. Isolates of *Verticillium* spp. 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; de Jonge et al. 2012; Paternotte and van Kesteren 1993). The *Ve* locus comprises two closely linked inversely oriented genes, *Ve1* and *Ve2*, which encode extracellular leucine-rich repeat (eLRR) receptor-like proteins (RLP)

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(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, 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, 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 RLP, candidate genes were tested for a role in Ve1-mediated *Verticillium* resistance in tomato, using virus-induced gene silencing (VIGS) (Fradin et al. 2009; 2011; Vossen et al. 2010). Despite the tools and resources that are available, research on tomato has major limitations when compared with other model plant species, such as the Solanaceous sister 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 RLP, the model plant *N. benthamiana* (Goodin et al. 2008) has frequently been used for *Agrobacterium* transient transformation assay (ATTA) and VIGS (Bar and Avni 2009; Fradin et al. 2009; Gabriëls et al. 2006; Rowland et al. 2005; Van der Hoorn et al. 2000; 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 (Faino et al. 2012; Gabriëls et al. 2006; Senthil-Kumar et al. 2007). 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

Coexpression of *Ave1* and *Ve1* induces a hypersensitive response (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 HR, rapid and localized cell death of plant tissue at the site at which 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 a HR can also be induced in tobacco, coexpression 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), generating expression construct *pFAST::Ave1* to establish *Ave1* expression driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter, and was transformed into *Agrobacterium tumefaciens* 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 was infiltrated into the leaves of *Nicotiana tabacum* cv. Petite Havana SR1. At 1 to 2 days postinfiltration (dpi), leaf tissue started to collapse and the infiltrated leaves developed clear necrosis by 5 dpi (Fig. 1A). In contrast, agroinfiltration of *pMOG800::Ve1* or *pFAST::Ave1* alone did not induce necrosis. Nevertheless, the HR that was induced upon coexpression of *Ve1* and *Ave1* was not as strong as HR induced upon coexpression of the corresponding tomato *Cf-9* and *C. fulvum Avr9* gene pair, for which the complete infiltrated sectors became fully necrotic (Fig. 1B).

It has been suggested that the accumulation of transiently expressed protein usually peaks between 1 and 3 dpi and rapidly decreases thereafter, due to post-transcriptional gene silencing (PTGS) in the host plant (Johansen et al. 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 coexpressed with silencing suppressor *p19* from *Tomato bushy stunt virus* (Voinnet et al. 2003) or with silencing suppressor *2b* from *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 coexpression of *Ave1* and *Ve1* (Fig. 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, coexpression of *Ave1* and *Ve1* did not result in any HR in *N. benthamiana*, even up to 14 dpi, while coexpression of tomato *Cf-9* with *C. fulvum Avr9* in a similar fashion induced clear HR within 4 dpi (Supplementary Fig. 1A).

Various Gateway-compatible binary vectors improve agroinfiltration efficiency.

Because *N. benthamiana* did not develop necrosis upon coexpression 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 overexpression 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 *A. tumefaciens* 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 5 dpi (Fig. 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 (Fig. 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, with these vectors, also, no necrosis was obtained in this species.

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

Fig. 1. Coexpression of tomato *Ve1* and *Verticillium dahliae Ave1* induces a hypersensitive response in *Nicotiana tabacum*. **A,** Co-agroinfiltration of constructs for constitutive expression of tomato immune receptor gene *Ve1* and the corresponding *V. dahliae* avirulence gene *Ave1* results in necrosis in *Nicotiana tabacum* at the injection sites. Whereas co-agroinfiltration of silencing suppressor *P19* induces necrosis under all conditions, silencing suppressor *2b* has no visible effect. **B,** Coexpression of avirulence gene *Avr9* from the tomato leaf mold fungus *Cladosporium fulvum* and the corresponding tomato receptor gene *Cf-9* results in strong necrosis in the absence of a silencing suppressor. All photos were taken at 5 days postinfiltration.

that was comparable to the strong HR that can be obtained with untagged Ve1 (Supplementary Fig. 2).

HR induced by coexpression of *Ve1* and *Ave1* is restricted to *N. tabacum* and *N. glutinosa*.

Because *N. tabacum* and *N. benthamiana* responded differentially to coexpression of *Ve1* and *Ave1*, we screened additional tobacco genotypes for their response to coexpression of this gene pair. In these assays, agroinfiltration of *Ve1-GFP* served as a marker for efficiency of agroinfiltration, while coexpression 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 coexpression of the *Cf-9/Avr9* gene pair resulted in a hypersensitive response in all of the *Nicotiana* species tested. In contrast, coexpression of the *Ve1/Ave1* gene pair did not result in specific necrosis in most *Nicotiana* species, although significant
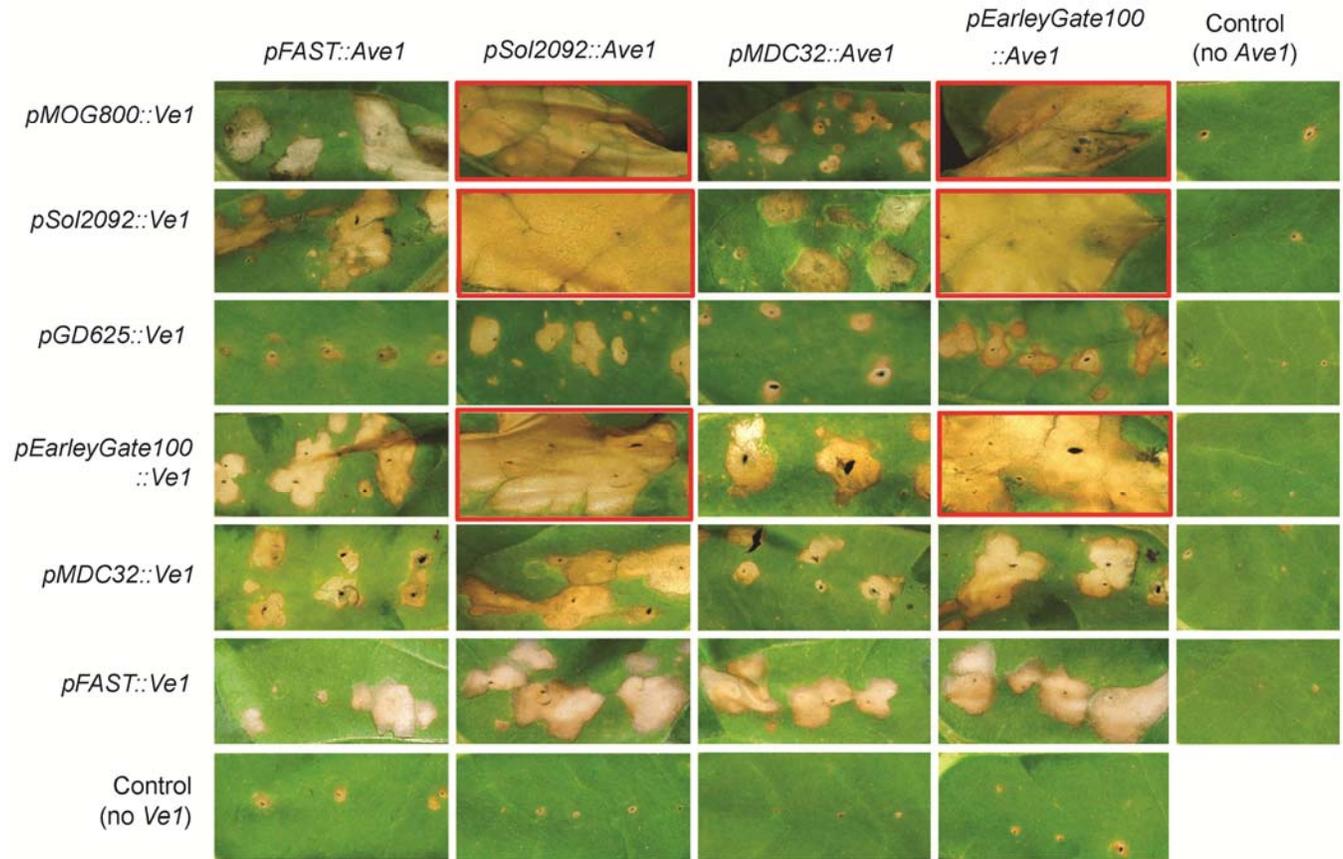
Fig. 2. Comparison of necrosis induced by coexpression of tomato *Ve1* and *Verticillium dahliae Ave1* in *Nicotiana tabacum* with various binary vectors. Strongest necrosis is observed upon coexpression of *Ve1* and *Ave1* using the pMOG800, pSol2092, and pEarleyGate100 vectors (red outline). All pictures were taken at 5 days postinfiltration.

Table 1. The hypersensitive response (HR) upon coexpression of *Ve1* and *Ave1* in various tobacco species

Species	Ve1/Ave1[a]	Cf-9/Avr9[a]	Ave1[a]	GFP[b]	PDS[c]	Viral symptoms[d]
N. benthamiana	−	+++	−	+	+++	+
N. tabacum cv. SR1	+++	+++	−	+	+	++
N. tabacum cv. White Burley	+++	+++	−	+	+	+++
N. tabacum cv. Xanthi	+++	+++	−	+	+	+
N. tabacum cv. Havana2000	+++	+++	−	+	+	++
N. tabacum cv. 2.1.1	+++	+++	−	+	+	++
N. tabacum cv. Samsun	+++	+++	−	+	+++	++
N. tabacum cv. Samsun NN	++	+++	−	+	+++	++
N. rustica	−	++	−	+	−	+
N. clevelandii	−	++	−	+	n.d.	n.d.
N. sylvestris	−	+++	−	+	++	++
N. glutinosa	+++	+++	++	+	−	++
N. debneyi	−	+++	−	+	+++	++
N. paniculata	−	++	−	n.d.	n.d.	n.d.
N. excelsior	−	++	−	n.d.	+++	++

[a] HR was monitored at 7 days postinfiltration (dpi). +++= strong HR over the entire infiltrated area; ++= moderate HR; += weak necrosis.
[b] Green flourescent protein (GFP) fluorescence derived from GFP-tagged Ve1 was examined with a UV microscope at 2 dpi. += clear GFP is visible; n.d.= not determined.
[c] Degree of photobleaching upon *phytoene desaturase* (*PDS*) gene silencing through virus-induced gene silencing. +++= 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.
[d] Viral symptoms upon agroinfiltration of *TRV::PDS*. +++= strong viral symptoms; ++= moderate viral symptoms; += weak viral symptoms.

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).

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 coexpression of *Ave1* with *Ve1*, while agroinfiltration of *Ve1* alone did not induce HR (Table 1; Fig. 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* JR2. At 2 weeks postinoculation, 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 (Fig. 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, cholorosis, and necrosis at 14 days postinoculation (Fig. 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* (Supplementary Fig. 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.

Tobacco rattle virus (TRV)-based VIGS 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 *phytoene desaturase* (*PDS*) gene was infiltrated into two cotyledons of five 2- to 3-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 3 weeks postinfiltration. However, *PDS* silencing was only consistently observed in the stems of the plants, as these were fully bleached
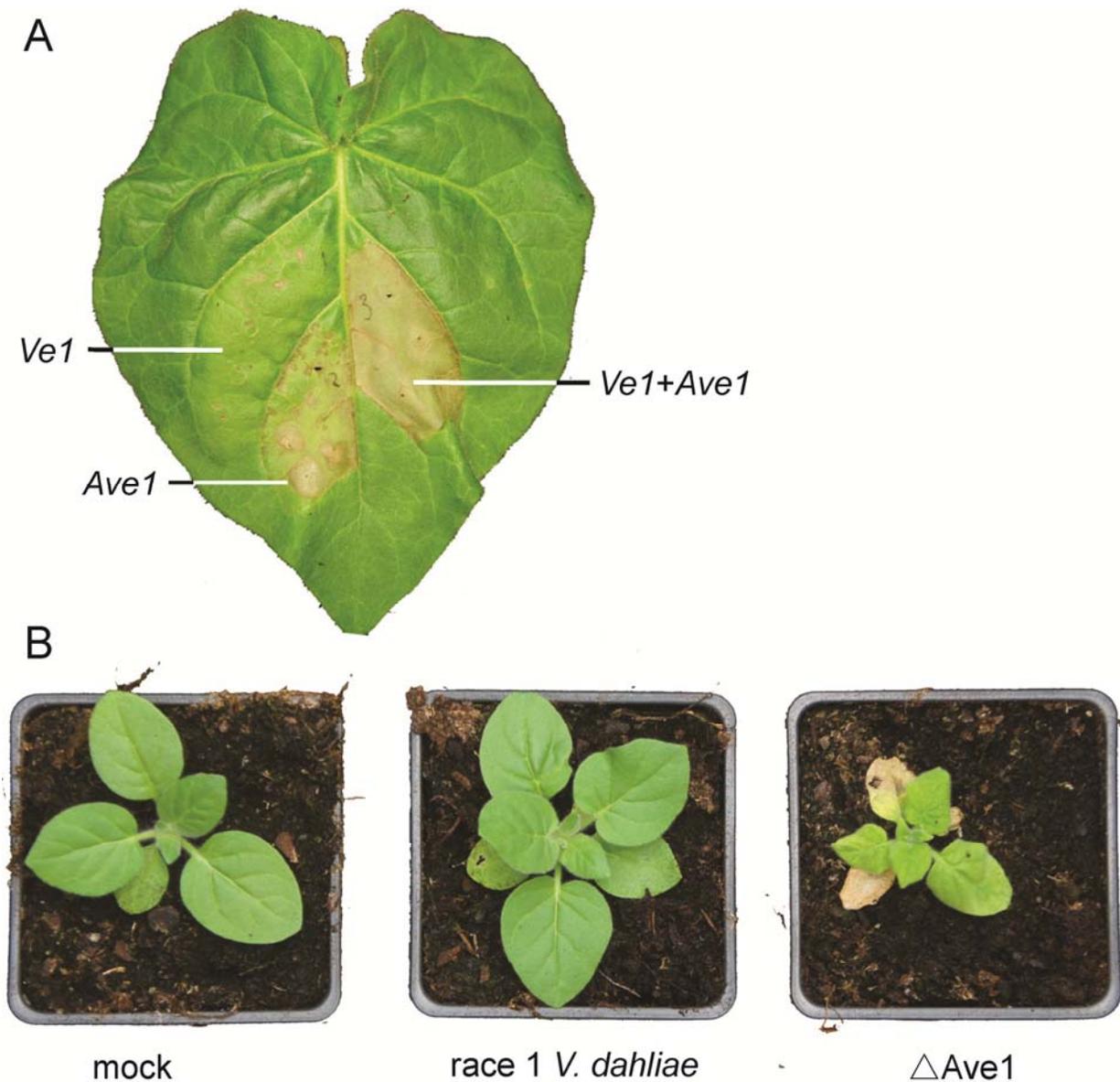
Fig. 3. *Nicotiana glutinosa* contains a functional *Ve1* ortholog. **A,** Agroinfiltration of *Ave1*, *Ve1*, and a combination thereof in *N. glutinosa*. In contrast to agroinfiltration of *Ve1* alone, agroinfiltration of *Ave1* alone in *N. glutinosa* induces a hypersensitive response. The picture was taken at 7 days postinfiltration. **B,** *N. glutinosa* is resistant against race 1 *V. dahlia*e but not against an *Ave1* deletion strain (ΔAve1). Photos were taken at 14 days postinoculation.

(Supplementary Fig. 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.

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 was limited in leaves (Table 1), highly efficient photobleaching was observed in leaves of the cultivars Samsun and Samsun NN (Table 1; Fig. 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* (Fradin et al. 2009; 2011; Hu et al. 2005), the role of this gene in the HR upon coexpression of *Ve1* and *Ave1* was assessed. At 3 weeks after TRV infection, TRV-inoculated plants were smaller than noninoculated plants. Coexpression 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 (Fig. 5A). As expected however, *N. tabacum* cv. Samsun plants inoculated with recombinant virus to target *EDS1* expression (*pTRV:EDS1*) showed a significantly compromised HR upon coexpression of *Ve1* and *Ave1*, confirming the involvement of EDS1 in Ve1-mediated resistance signaling (Fig. 5A). We also targeted expression of *NRC1* (a nucleotide binding–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 coexpression of *Ve1* and *Ave1* (Fig. 5B).

DISCUSSION

Coexpression 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 coexpression 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* and that carry *Avr4* and *Avr9*, respectively, which can be induced upon coexpression with these corresponding effector genes (Table 1) (Van der Hoorn et al. 2000). This suggests that most tobacco species lack at least one important signal transduction component that is required for *Ve1*-mediated immunity against *V. dahliae* 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 (Fradin et al. 2009, 2011; Vossen et al. 2010). 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, which are confined to tomato as the only known host of *C. fulvum* (Fradin et al. 2009; Thomma et al. 2005, 2011). 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 (Santos-Rosa et al. 2008; van der Gheynst et al. 2008; Van der Hoorn et al. 2000; Wroblewski et al. 2005). This allows the use of agroinfiltration to identify candidate functional *Ve1* orthologous genes by coexpression with *Ave1*. Since *V. dahliae* 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* (Simko et al. 2004), *StVe* cloned from *Solanum torvum* Swartz (Fei et al. 2004), *mVe1* cloned from *Mentha longifolia* (Vining and Davis
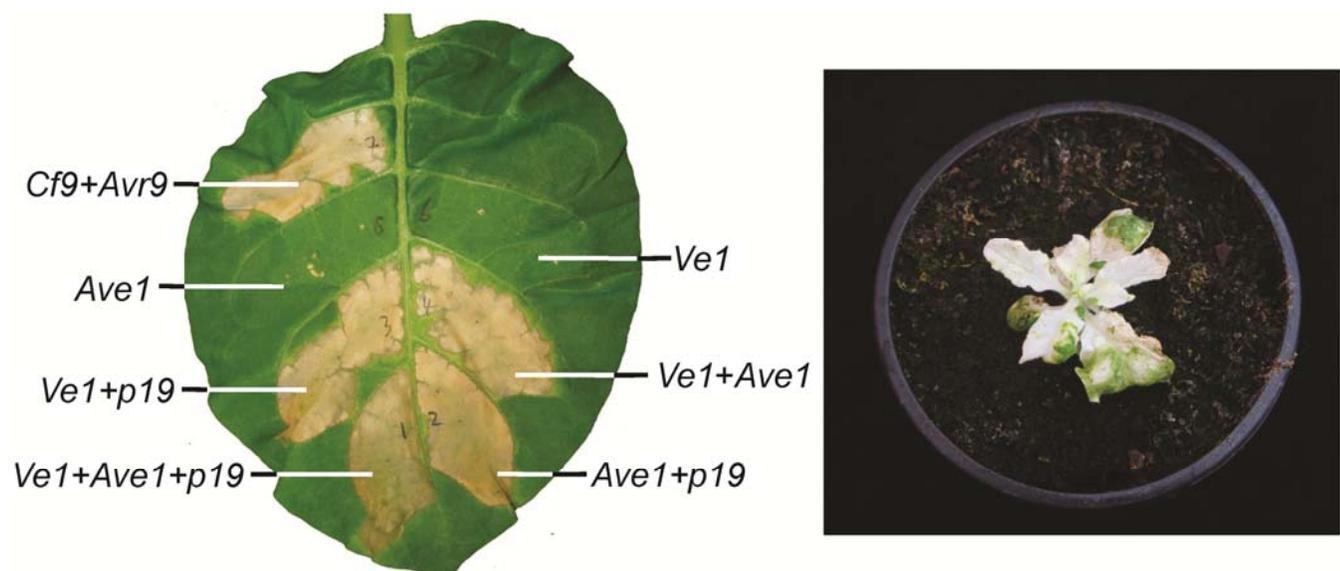
Fig. 4. Agroinfiltration and virus-induced gene silencing (VIGS) in *Nicotiana tabacum* cv. Samsun. On the left, co-infiltration of *Ve1* and *Ave1* induced a hypersensitive response in *N. tabacum* cv. Samsun. On the right, VIGS of the *phytoene desaturase* (*PDS*) gene leads to photobleaching in leaves of *N. tabacum* cv. Samsun.

2009; Vining et al. 2007), *GbVe* cloned from *Gossypium bar-badense* (Zhang et al. 2011), and the *StoVe1* gene cloned from *Solanum torvum* (Liu et al. 2012). 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 (Fradin et al. 2009; Kawchuk et al. 2001; Liu et al. 2012; 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 much work to get homo-

zygous transgenic *Arabidopsis* with those *Ve* orthologs. Our results indicate that coexpression of such a 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 coexpression 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
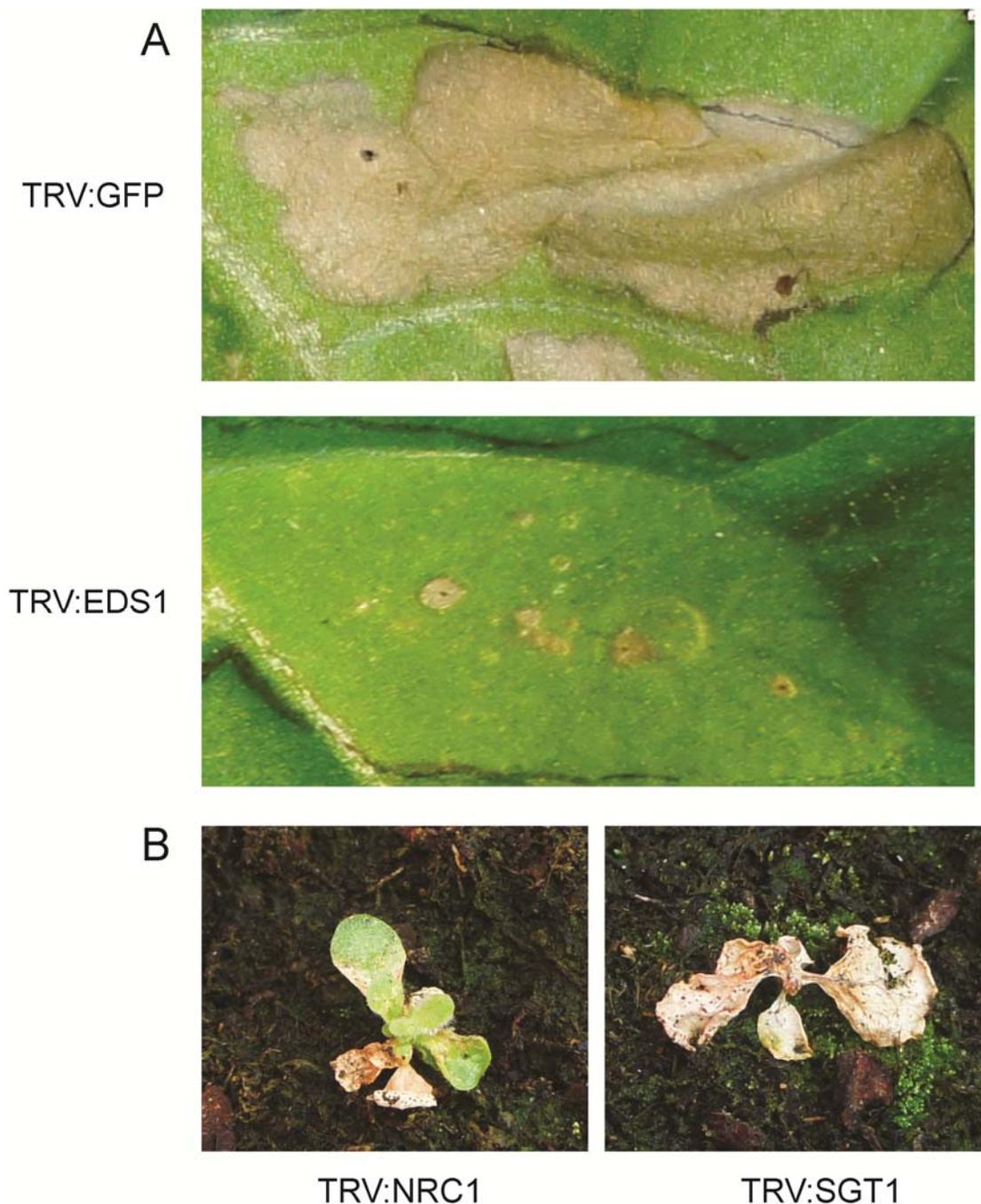
Fig. 5. Coexpression of *Ve1* and *Ave1* in *Nicotiana tabacum* cv. Samsun after virus-induced gene silencing. **A,** *N. tabacum* cv. Samsun plants were inoculated with a recombinant *Tobacco rattle virus* (TRV) targeting a green fluorescent protein gene as a control (*TRV:GFP*) or recombinant TRV targeting the *EDS1* gene (*TRV:EDS1*). At 5 days postinfiltration, compromised hyypersensitive response was observed in *EDS1*-silenced plants. **B,** *TRV:NRC1*-inoculated *N. tabacum* cv. Samsun plants show a strong developmental phenotype while *SGT1*-silencing results in lethality.

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, ample recombinant protein was detected in Western analysis upon agroinfiltration of *pSol2095::Ve1::GFP*, which activates strong HR. 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 *N. tabacum*.

Previously, by using TRV-based VIGS in tomato, we established a method for functional analysis of genes that are involved in *Verticillium* defense (Fradin et al. 2009). Several candidate genes were silenced in tomato, followed by inoculation with race 1 *V. dahliae* revealing a role in Ve1-mediated *Verticillium* defense for some of them (Fradin et al. 2009, 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 *N. benthamiana* and tomato, and consequently, many TRV-based VIGS vectors targeting potential defense components are available (Chen et al. 2009; Fradin et al. 2009; Gabriëls et al. 2006, 2007; Ho et al. 2009; Stulemeijer et al. 2007; 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 to be successfully used in tobacco and vice versa (Fradin et al. 2009; Gabriëls et al. 2006; Liebrand et al. 2012; Senthil-Kumar et al. 2007; Velasquez et al. 2009).

Recently, the development of VIGS in *Nicotiana tabacum* using *Tobacco curly shoot virus* was described (Huang et al. 2011). 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 *N. tabacum* (Ryu et al. 2004), we found it did not induce sufficient gene silencing in leaves of *N. tabacum* cv. SR1, which we used for HR induced by coexpression of *Ve1* and *Ave1*. The screening of *Nicotiana* species demonstrated that *N. tabacum* cv. Samsun is able to develop an HR upon coexpression of *Ve1* and *Ave1* and is amenable to TRV-based VIGS. By the silencing of *EDS1*, which is known to be required for Ve1-mediated resistance in tomato and *Arabidopsis* (Fradin et al. 2009, 2011; Hu et al. 2005), we demonstrated that ATTA 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: 16-h, 21°C day and 8-h, 19°C night periods, with 70% relative humidity, 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 polymerase chain reaction (PCR) were sequence-verified.

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′-GG GGACAAGTTTGTACAAAAAAGCAGGCTATGAAGCTTT CTACGCTT-3′ and Vd-R, 5′-GGGGACCACTTTGTACAAG AAAGCTGGGTTTATATCTGTCTAAATTC-3′. The resulting PCR product was cleaned from 1% agarose gel, using the QIAquick gel extraction kit (Qiagen, Valencia, CA, U.S.A.), and was transferred into donor vector pDONR207, using Gateway BP Clonase II enzyme mix (Invitrogen, Carlsbad, CA, U.S.A.) 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′-GGGGACAAGTTTGTACAAAAAAGCAGGCTAT GAAAATGATGGCAACTCT-3′ and attB-Ve1-R 5′-GGGGAC CACTTTGTACAAGAAAGCTGGGTAGTCTTTCTTGAAA ACCAAA -3′. The PCR product was cleaned from agarose gel and was 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) to generate expression constructs driven by the CaMV 35S promoter. The expression constructs were transformed into *Escherichia coli* and transformants were checked by colony-PCR analysis, using primers AttB1F 5′-ACAAGTTTGTACAAAAAAGCAGGCT-3′ and AttB2R 5′-ACCACTTTGTACAAGAAAGCTGGGT-3′. The expression constructs were subsequently transformed into *A. tumefaciens* GV3101 (Hellens et al. 2000) by electroporation. Finally, the plasmids were isolated from *A. tumefaciens* transformants and were sequenced.

Generation of constructs for Ve1-GFP fusion.

To generate Ve1 fused at the 3′ end to a GFP tag, the *Ve1* coding sequence lacking the stop codon was PCR-amplified using primers containing Gateway attB sites: attB-Ve1-F, 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAAT GATGGCAACTCT-3′ and attB-Ve1-R-SC, 5′-GGGGACCA CTTTGTACAAGAAAGCTGGGTACTTTCTTGAAAACCA AAG-3′. The PCR fragment was cloned into pDONR207 (Invitrogen) 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 et al. 2003), pGWB451 (Nakagawa et al. 2007), and pSol2095 to generate expression constructs driven by the constitutive CaMV 35S promoter and C-terminally tagged to GFP. Fusion constructs were transformed into *A. tumefaciens* GV3101 by electroporation.

***A. tumefaciens*–mediated transient expression.**

A. tumefaciens containing expression constructs was infiltrated into tobacco plants as described previously (Van der Hoorn et al. 2000). Briefly, an overnight culture of *A. tumefa-*

ciens cells was harvested at an optical density at 600 nm (OD_{600}) of 0.8 to 1 by centrifugation and was resuspended to a final OD_{600} of 2. *A. tumefaciens* cultures containing constructs to express *Ave1* and *Ve* proteins were mixed in a 1:1 ratio and were infiltrated into leaves of 5- to 6-week-old tobacco plants. After agroinfiltration, plants were grown in the climate room at 22°C and 19°C during 16-h day and 8-h night periods, respectively, with 70% relative humidity. At 5 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). First-strand cDNA was synthesized from 1 μg of total RNA, using the Super-Script III cDNA synthesis kit (Invitrogen) according to the manufacturers' instructions. The partial coding sequence of *N. glutinosa* was PCR-amplified from cDNA with *Pfu* high-fidelity enzyme (Promega, Madison, WI, U.S.A.), 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 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 60 s. The resulting 461-bp PCR product was cleaned from 1% agarose gel, was cloned into the pGEM-T Easy vector (Promega), and was sequenced using the M13F primer (5′-TGTAAAACGAC GGCCAGT-3′).

VIGS.

The TRV2 constructs *TRV:GFP*, *TRV:PDS*, *TRV:EDS1*, *TRV: SGT1*, and *TRV:NRC1* have been described before (Gabriëls et al. 2006). To establish VIGS in tobacco, cotyledons of 2- to 3-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 and associates (2000). Leaves were superficially wounded with a needle to improve infiltration. Photobleaching was observed at 3 weeks postinfiltration upon inoculation of *TRV:PDS*.

Verticillium inoculations.

Race 1 *V. dahliae* JR2 and the *Ave1* deletion strain (ΔAve1) (de Jonge et al. 2012) were grown on potato dextrose agar at 22°C. *V. dahliae* conidia were harvested from 7- to 14-day-old fungal plates and were washed with tap water. The conidia were suspended to a final concentration of 10^6 conidia/ml. For inoculation, 3-week-old *N. glutinosa* plants were gently up-rooted and the roots were rinsed in tap water. The roots were then dipped in the conidial suspension for 3 min. 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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