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

ZHAO ZHANG[1,2], H. PETER VAN ESSE[1], MIREILLE VAN DAMME[1], EMILIE F. FRADIN[1], CHUN-MING LIU[2] AND BART P. H. J. THOMMA[1,3,*]

[1]*Laboratory of Phytopathology, Wageningen University, 6708 PB Wageningen, the Netherlands*
[2]*Key Laboratory of Plant Molecular Physiology, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China*
[3]*Centre for BioSystems Genomics, 6700 AB Wageningen, the Netherlands*

SUMMARY

The 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 have demonstrated previously that the recognition of the *Verticillium dahliae* effector protein Ave1 by the tomato immune receptor Ve1 triggers an HR in tomato and tobacco. Furthermore, we have demonstrated that tomato Ve1 provides *Verticillium* resistance in Arabidopsis upon Ave1 recognition. In this study, we investigated whether the co-expression of *Ve1* and *Ave1* in Arabidopsis results in an HR, which could facilitate a forward genetics screen. Surprisingly, we found that the co-expression of *Ve1* and *Ave1* does not induce an HR in Arabidopsis. These results suggest that an HR may occur as a consequence of Ve1/Ave1-induced immune signalling 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 (Boller and Felix, 2009; Thomma *et al*., 2011). The recognition of such ligands by immune receptors results in the activation of defence 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 and Thomma, 2006; Fradin *et al*., 2009). In this model, immunity against *V. dahliae* is governed by the interaction between the tomato gene *Ve1* and the *V. dahliae* gene *Ave1* (de Jonge *et al*., 2012; Fradin *et al*., 2009). *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 signalling (Fradin and Thomma, 2006; Fradin *et al*., 2009; Kawchuk *et al*., 2001). *Ave1* encodes an effector protein that has a high degree of homology to plant natriuretic peptides and 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 signalling 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 (Bar *et al*., 2010; Gabriels *et al*., 2006, 2007; van der Hoorn *et al*., 2000; Ron and Avni, 2004; Vossen *et al*., 2010). One of the tools that has been exploited is the progeny of a cross of *Cf-4* tomato with transgenic tomato lines expressing the cognate *Cladosporium fulvum* effector gene *Avr4*, which results in *Cf-4*/*Avr4* offspring that display lethality at the seedling stage, but can be rescued on incubation at 33 °C (Cai *et al*., 2001; de Jong *et al*., 2002; Thomas *et al*., 1997). On transfer to 20 °C, a synchronous systemic HR is activated, which has been employed successfully to study Cf-4 signalling (de Jong *et al*., 2002; Gabriels *et al*., 2006; Stulemeijer *et al*., 2007).

As a result of the lack of RLPs that have been implicated in the immune signalling 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, 2010). Interestingly, it has been demonstrated recently 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 (somatic embryogenesis receptor-like kinase) family members for Ve1 resistance has been investigated in Arabidopsis, demonstrating a critical role for SERK1 in addition to SERK3/BAK1 (brassinosteroid-associated kinase 1). With virus-induced gene silencing, the requirement of SERK1 for Ve1-mediated resistance

**Correspondence*: Email: bart.thomma@wur.nl

was subsequently confirmed in tomato, demonstrating that Arabidopsis can be used to characterize Ve1 signalling (Fradin *et al*., 2011).

We have demonstrated recently that the *Potato virus X* (PVX)-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 induces HR (de Jonge *et al*., 2012; Zhang *et al*., 2012). In this study, we investigated whether the co-expression of *Ve1* and *Ave1* in Arabidopsis results in an HR that can be used as read-out to investigate RLP signalling mediated by Ve1. Surprisingly, we found that, although *Ave1* is able to trigger an HR in resistant tomato and *Nicotiana* plants, such an 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 the constitutive expression of VdNLP2 was infiltrated into Arabidopsis leaves. It has been shown recently that the VdNLP2 protein from *V. dahliae* exhibits cytotoxic activity on infiltration into Arabidopsis leaves (Santhanam *et al*., 2013; Zhou *et al*., 2012). Indeed, agroinfiltration of VdNLP2 resulted in clear necrosis within 7 days (Fig. 1A). Next, *A. tumefaciens* carrying a construct for the constitutive expression of C-terminally green fluorescent protein (GFP)-tagged Ve1 was infiltrated into the leaves of 3-week-old Arabidopsis plants. As a control, *A. tumefaciens* carrying a construct for the constitutive expression of untagged Ve1 was infiltrated. At 2 days post-infiltration (dpi), GFP fluorescence was detected in leaves infiltrated with *A. tumefaciens* carrying a construct for the expression of GFP-tagged Ve1, which was not observed on expression of untagged Ve1 (Fig. 1B). Closer inspection of the localization of GFP-tagged Ve1 in *N. benthamiana* revealed that Ve1 localizes to the plasma membrane (Fig. S1, see Supporting Information), as predicted previously (Fradin *et al*., 2009; Kawchuk *et al*., 2001). Collectively, these results confirm that agroinfiltration can be used for the 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 7 dpi, no signs of necrosis could be observed in agroinfiltrated Arabidopsis plants (Fig. 1C). Similarly, the co-expression of *Ave1* and *Ve1* in wild-type Arabidopsis did not lead to HR (Fig. 1C). In contrast, the co-expression of *Ave1* and *Ve1* in *Nicotiana tabacum* cv. Petite Havana SR1 resulted in clear HR within 5 dpi (Fig. 1D; Zhang *et al*., 2012).

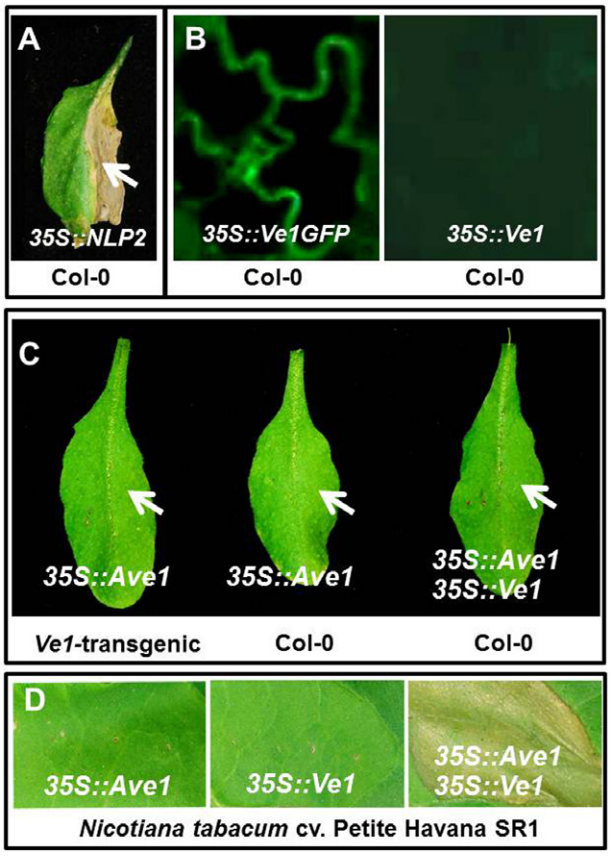


Fig. 1 Co-expression of *Ave1* and *Ve1* in Arabidopsis leaves does not induce a hypersensitive response (HR). (A) Infiltration of *Agrobacterium tumefaciens* carrying a construct for constitutive VdNLP2 expression into Arabidopsis leaves results in clear necrosis at 7 days post-infiltration (dpi). (B) Leaves of wild-type Arabidopsis Col-0 plants were infiltrated with *A. tumefaciens* cultures carrying *35S::Ve1GFP* or *35S::Ve1*. Green fluorescent protein (GFP) fluorescence was only detected in the leaf infiltrated with *A. tumefaciens* carrying a construct for the expression of GFP-tagged Ve1 at 2 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.

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 the binary vector pFAST-R02 (Shimada *et al*., 2010) to

generate the expression construct pFAST::*Ave1*, in which *Ave1* expression was driven by the cauliflower mosaic virus (CaMV) *35S* promoter. This construct contains a nondestructive red fluorescent protein (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 *Ave1* genes, were grown on Murashige and Skoog (MS) medium in a growth chamber at 22 °C, or on soil in the glasshouse, alongside *Ve1*-transgenic, *Ave1*-transgenic and nontransgenic control plants. No phenotypic alterations were observed in plants that co-expressed *Ve1* and *Ave1* when compared with plants that expressed either of the transgenes alone or nontransgenic controls (Fig. 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 (Fig. 2A). Reverse transcription-polymerase chain reaction (RT-PCR) was performed to confirm the simultaneous expression of *Ve1* and *Ave1* in these lines (Fig. 2B).

We further examined the potential occurrence of micro-HR-like lesions microscopically on trypan blue staining of *Ve1/Ave1-1*, *Ve1/Ave1-2* and *Ve1/Ave1-3* plants. No micro-HR-like lesions were observed in the three independent transgenic lines that co-expressed *Ve1* and *Ave1* when compared with plants that expressed either of the transgenes alone or nontransgenic controls (Fig. 3). These results further confirm that HR is not induced in Arabidopsis on co-expression of *Ve1* and *Ave1*.

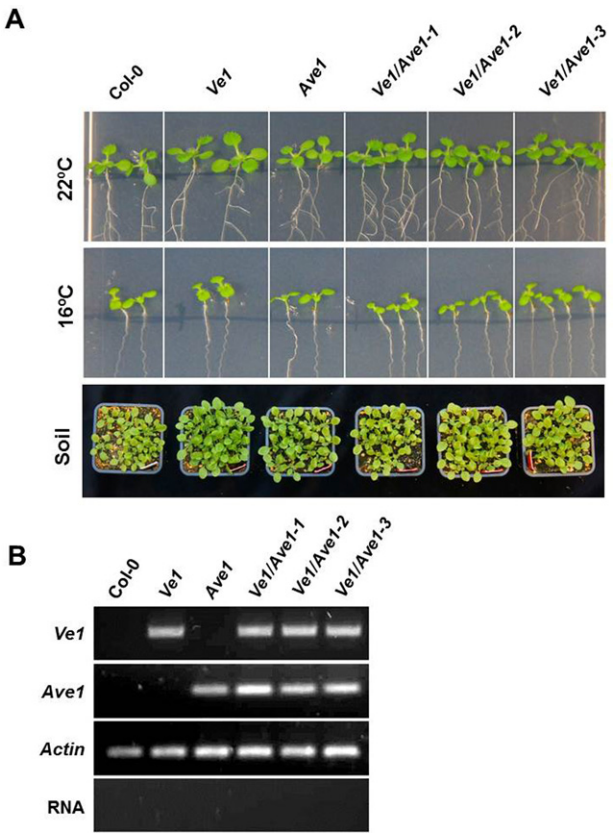


Fig. 2 Stable co-expression of *Ve1* and *Ave1* does not affect Arabidopsis viability. (A) No phenotypical alterations were observed in plants that co-expressed *Ve1* and *Ave1* when compared with plants that expressed either of the transgenes alone or nontransgenic control plants. Arabidopsis plants were grown on Murashige and Skoog (MS) medium in a growth chamber at 22 °C or 16 °C, or on soil in the glasshouse. (B) Reverse transcription-polymerase chain reaction (RT-PCR) was performed to confirm the expression of *Ve1* and *Ave1* in the transgenic lines.

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

Previously, we have shown that transient expression of *Ave1* by PVX specifically induces HR in resistant tomato carrying *Ve1* (de Jonge *et al*., 2012). In addition, HR can also be induced in tobacco on co-expression of *Ave1* and *Ve1* by agroinfiltration (Zhang *et al*., 2012). These experiments demonstrate that *in planta*-expressed *Ave1* is able to activate *Ve1*-mediated HR. However, as both transient and stable expression of Ave1 did not induce HR in *Ve1*-transgenic Arabidopsis, we investigated whether the Ave1 protein produced in Arabidopsis 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 AF obtained was subsequently infiltrated into the leaves of *N. tabacum* transiently expressing *Ve1* or its nonfunctional homologue *Ve2* (Zhang *et al*., 2012). By 3 dpi, *Ve1*-expressing leaves developed clear necrosis when AF of *Ave1*-transgenic Arabidopsis was infiltrated (Fig. 4). 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* (Fig. 4). These data demonstrate that the Ave1 protein expressed in transgenic Arabidopsis potentially can activate Ve1-mediated HR.

Inoculation of Ve1/Ave1-transgenic Arabidopsis

We hypothesized that the co-expression of *Ve1* with *Ave1* may result in the constitutive activation of plant immunity in Arabidopsis in the absence of HR. However, considering the absence of a visible phenotype, such as dwarfing, which is typically observed in Arabidopsis constitutive defence mutants, such as *cpr*, *cim* and other constitutive PR expression mutants (Bowling *et al*., 1994; Cheng *et al*., 2011; Gou *et al*., 2009; Maleck *et al*., 2002), strongly

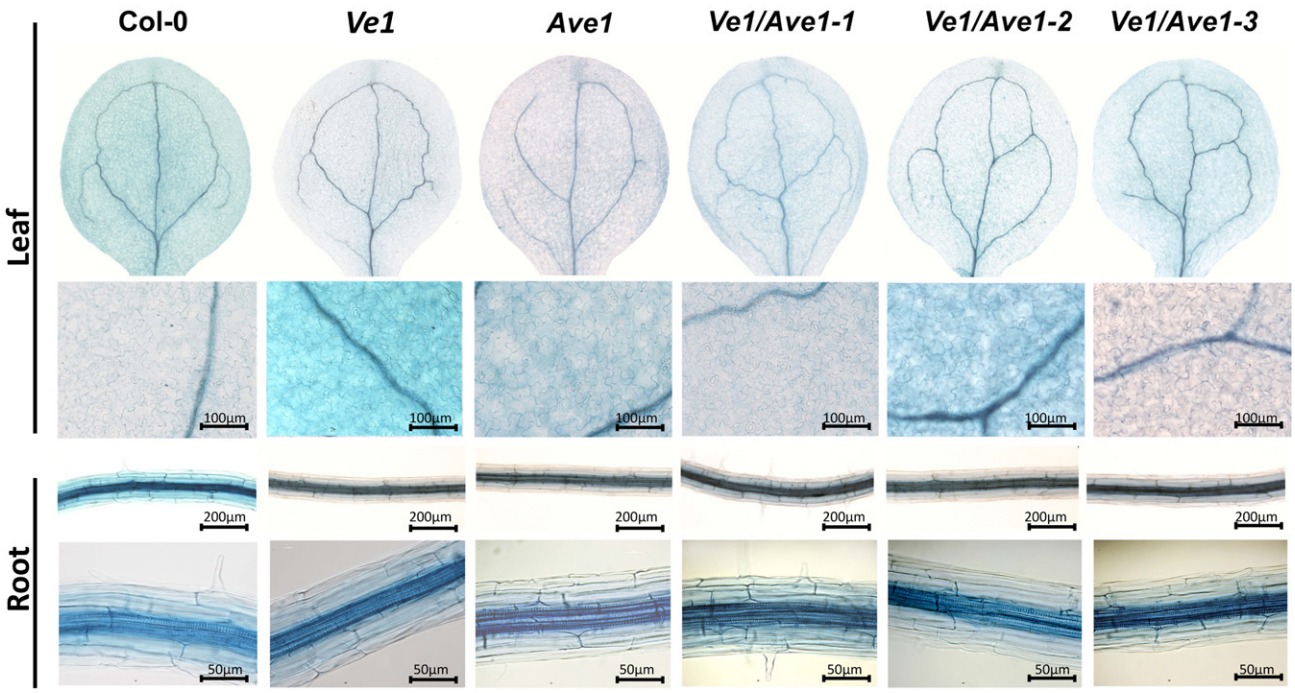


Fig. 3 Microscopic examination of trypan blue-stained Arabidopsis seedlings co-expressing *Ve1* and *Ave1*. No micro-hypersensitive response (HR)-like lesions were observed in plants that co-expressed *Ve1* and *Ave1* or in plants that expressed either of the transgenes alone or in nontransgenic control plants.

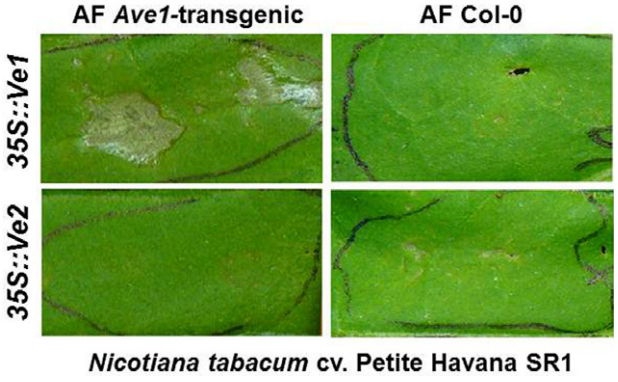


Fig. 4 *In planta*-expressed Ave1 triggers a Ve1-mediated hypersensitive response (HR) in *Nicotiana tabacum*. Apoplastic fluid (AF) extracted from *Ave1*-transgenic Arabidopsis induced HR in *Ve1*-expressing, but not in *Ve2*-expressing, *N. tabacum* leaves. AF from wild-type Arabidopsis (Col-0) did not induce HR.

elevated defence 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, nontransgenic and *Ave1*-transgenic plants displayed typical *Verticillium* wilt symptoms on *V. dahliae* inoculation, including wilting, stunting, chlorosis and necrosis (Fig. 5A). In contrast, *Ve1*-expressing plants, as well as plants that co-expressed *Ve1* and *Ave1*, showed clear resistance against *V. dahliae* (Fig. 5). Next, we inoculated *Ave1* deletion mutants of *V. dahliae* strain JR2 (de Jonge *et al*., 2012) on the various genotypes. Nontransgenic 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 (Fig. 5; de Jonge *et al*., 2012). Surprisingly, however, also plants that co-expressed *Ve1* and *Ave1* were susceptible to the *Ave1* deletion mutants (Fig. 5). These data suggest that the co-expression of *Ve1* and *Ave1* does not activate basal defence against fungal infection.

To corroborate that the co-expression of *Ve1* and *Ave1* does not activate basal defence, 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-expressed *Ve1* and *Ave1* (Fig. 6), confirming that basal defence is not activated through the co-expression of *Ve1* and *Ave1*.

DISCUSSION

Recently, the *V. dahliae* effector that activates immunity in *Ve1* tomato plants has been 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 has been demonstrated that the combination of Ve1 and Ave1 induces HR (de Jonge *et al*., 2012). Furthermore, it has been shown recently that *Ve1*-transgenic Arabidopsis is resistant to race 1 strains of *V. dahl-*

iae and *V. albo-atrum*, demonstrating that Ve1 remains fully functional after interfamily transfer into Arabidopsis (Fradin *et al*., 2011). Tomato crosses, in which the cross involves plant lines that express a pathogen effector and corresponding RLP immune receptor, set seeds normally, but develop seedling lethality on germination of the seeds (Cai *et al*., 2001; de Jong *et al*., 2002; Thomas *et al*., 1997). With this knowledge, we aimed to develop a similar 'dying seedling' in Arabidopsis. The mutagenesis of such seeds would allow for a simple forward genetics screen, as seeds that survive after germination are probably affected in signalling components downstream of the immune receptor if they are not mutagenized in either the *Ave1* or *Ve1* transgene. Unexpectedly, however, we failed to identify the HR on co-expression of *Ve1* and *Ave1* in transient assays and on stable transformation in Arabidopsis. Moreover, the progeny of a cross of Arabidopsis plants constitutively expressing *Ve1* and *Ave1* resulted in progeny that did not show any obvious phenotypical differences when compared with nontransgenic wild-type plants.

Traditionally, the HR was considered as a defence mechanism that prevents pathogen growth directly (Spoel and Dong, 2012). However, a growing number of examples have reported on immunity in the 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 examples include *Rx1*- and *Rx2*-mediated resistance to PVX in potato (Bendahmane *et al*., 1999, 2000), *Mla1*-mediated resistance against the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* in barley (Bieri *et al*., 2004), *Rrs1*-mediated resistance against the scald pathogen *Rhynchosporium secalis* (Lehnackers and Knogge, 1990; Rohe *et al*., 1995), *Rdg2a*-mediated resistance to the leaf stripe pathogen *Pyrenophora graminea* in barley (Bulgarelli *et al*., 2010) and *RPS4*-mediated resistance to *Pseudomonas syringae* in Arabidopsis (Gassmann, 2005). In Arabidopsis, a genetic separation of disease resistance and the HR was first described for the *dnd1* mutant (*defence, no death 1*; Clough *et al*., 2000), which shows resistance to *Pseudomonas syringae* bacteria expressing the avirulence genes *avrRpt2*, *avrB*, *avrRpm1* and *avrRps4* in the absence of an HR. Similarly, the Arabidopsis mutant *hlm1* (*HR-like lesion mimic*; Balagué *et al*., 2003) and its allelic mutant *dnd2* (Jurkowski *et al*., 2004) were found to display resistance in the absence of HR. In addition to NBS-LRR-type immune receptors, also for Cf-4, an LRR-RLP-type receptor, HR and resistance to *C. fulvum* on Avr4 recognition could be separated (Stulemeijer *et al*., 2007). Our data suggest that *Ve1*-mediated *Verticillium* resistance in Arabidopsis also does not involve an HR. Presently, we cannot rule out the possibility that Ave1 needs to be processed in order to be recognized by Ve1, and that the lack of HR in Arabidopsis is caused by a lack of an enzyme in the apoplast of Arabidopsis that is required for the maturation of Ave1. However, the observation that *Ve1*-expressing Arabidopsis plants are resistant to *Verticillium* infection, which is based on the recognition of Ave1 (de Jonge *et al*., 2012), suggests that Ave1 perception by Ve1 is functional in Arabidopsis, even if this requires Ave1 processing by host enzymes.

To date, several types of immune receptor have been identified, which 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). Although many of these receptors activate an HR on 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* or 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 signalling on Ave1 recognition in tomato and tobacco. The mechanism by which plants actually stop *V. dahliae* infection still requires further investigation.

EXPERIMENTAL PROCEDURES

Plant materials

Arabidopsis plants were grown in the glasshouse or climate chamber with the following settings: 22/19 °C (unless mentioned otherwise) with 70% relative humidity and 16-h/8-h day/night periods. Supplemental light (100 W/m^2) was supplied in the glasshouse when the light intensity dropped below 150 W/m^2.

Generation of the constructs

To generate *Ve1* fused at the 3′ end to GFP, the *Ve1* coding sequence lacking the stop codon was PCR amplified using the primers attB-Ve1-F (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGAAAATGATGGCAACTC T-3′) and attB-Ve1-R-SC (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT ACTTTCTTGAAAACCAAAG-3′). The PCR fragment was cloned into pDONR207 (Invitrogen, Carlsbad, CA, USA) 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*., 2012) to generate an expression construct for GFP-tagged Ve1 driven by the constitutive CaMV *35S* promoter. The fusion construct was transformed into *A. tumefaciens* strain GV3101 by electroporation. For the agroinfiltration of untagged Ve1, construct pMOG800::*Ve1* was used (Fradin *et al*., 2009). pFAST::*Ave1* has been described by Zhang *et al*. (2012).

Agrobacterium tumefaciens-mediated transient expression

Agrobacterium tumefaciens-containing expression constructs were infiltrated into Arabidopsis plants as described previously (van der Hoorn

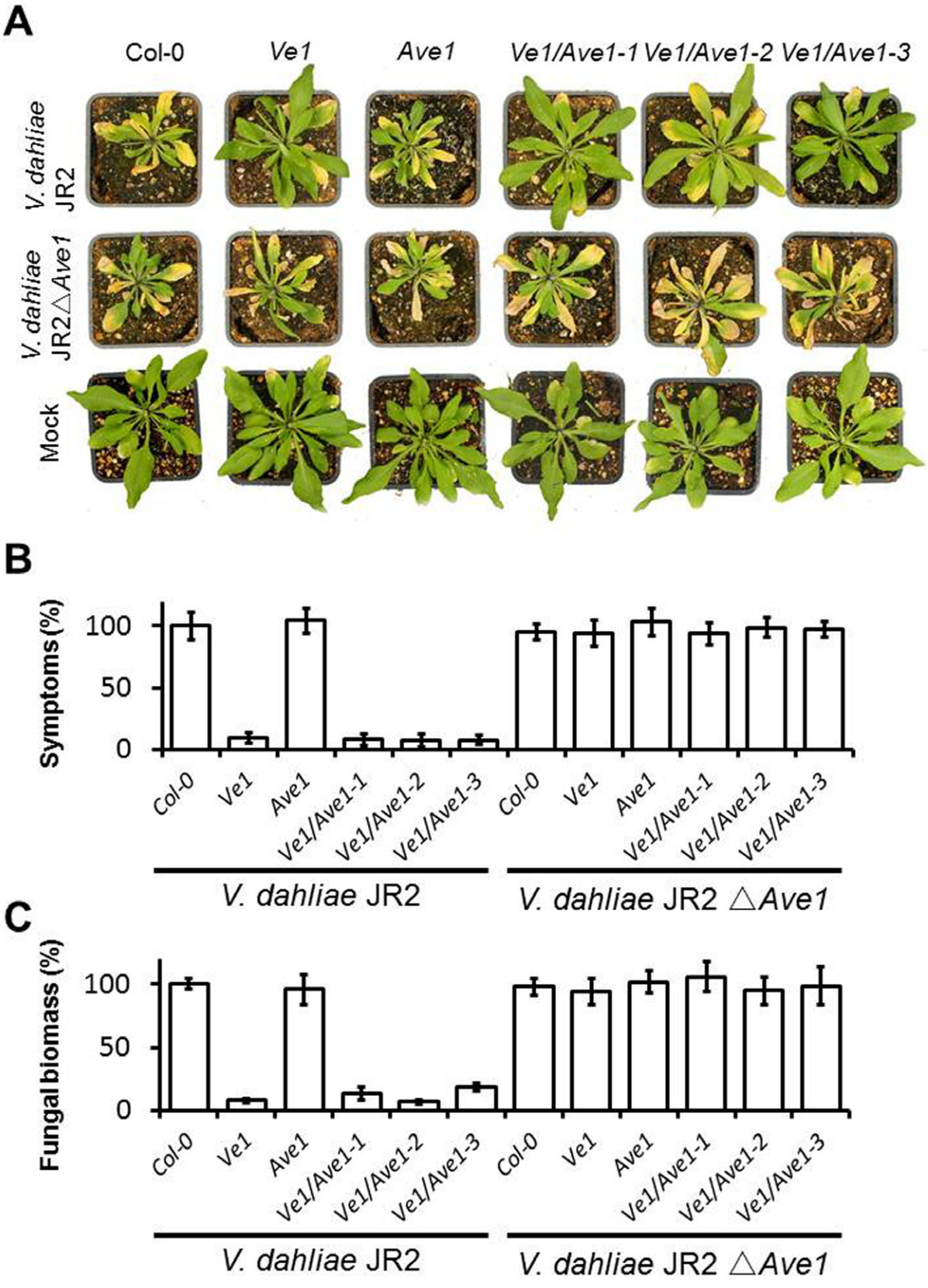
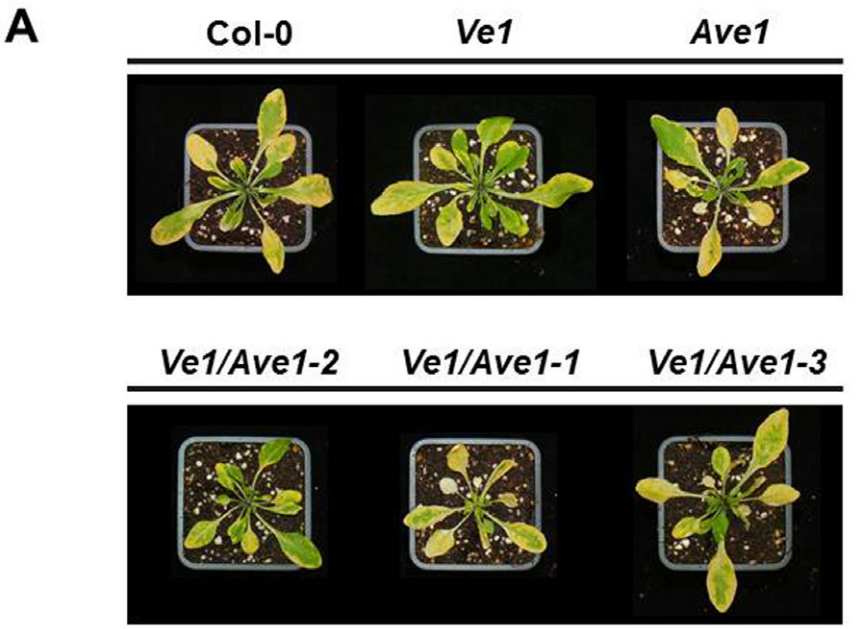


Fig. 5 Inoculation of Ve1/Ave1-transgenic Arabidopsis with *Verticillium dahliae*. (A) Typical appearance of nontransgenic and transgenic Arabidopsis lines on mock inoculation or inoculation with *V. dahliae* race 1 isolate JR2 or the *Ave1* deletion strain (JR2Δ*Ave1*). (B) Quantification of *Verticillium* wilt symptoms in wild-type and transgenic Arabidopsis. Bars represent quantification of symptom development shown as the percentage of diseased rosette leaves. Symptoms on Col-0 are set to 100%. (C) Fungal biomass determined by real-time polymerase chain reaction (PCR) in wild-type Arabidopsis and transgenic lines. Bars represent *Verticillium* internal transcribed spacer (ITS) transcript levels relative to Arabidopsis RuBisCo transcript levels (for equilibration). The fungal biomass in Col-0 is set to 100%. Data from a representative experiment are shown.



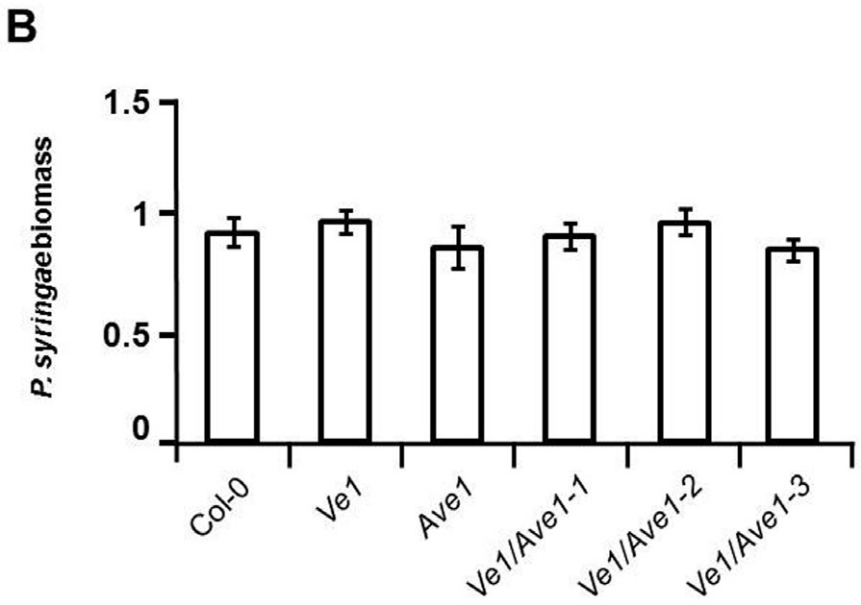


Fig. 6 Inoculation of *Ve1/Ave1*-transgenic Arabidopsis with *Pseudomonas syringae* pv. *tomato* strain DC3000. (A) Typical appearance of nontransgenic and transgenic Arabidopsis lines on mock inoculation or inoculation with *P. syringae*. (B) Bacterial biomass determined by real-time polymerase chain reaction (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).

et al., 2000). Briefly, an overnight culture of *A. tumefaciens* cells was harvested at an optical density at 600 nm (OD_{600}) of 0.8–1 by centrifugation and resuspended to a final OD of 2. *Agrobacterium tumefaciens* cultures containing constructs to express *Ave1* or *Ve1* were infiltrated into leaves of 3-/4-week-old Arabidopsis plants.

RT-PCR

Arabidopsis seedlings were collected and total RNA was extracted using the QIAGEN RNeasy extraction kit (Qiagen, Valencia, CA, USA). First-strand

cDNA was synthesized from 1 μg of total RNA, using the SuperScript™ III cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. PCRs were performed in a total volume of 25 μL with 17.9 μL water, 5 μL 5 × PCR buffer, 0.5 μL deoxynucleoside triphosphates (dNTPs), 0.5 μL of each primer, 0.1 μL GoTaq polymerase (Promega, Madison, WI, USA) and 1 μL of first-strand cDNA. PCR was performed for 30 cycles, with denaturation at 95 °C for 15 s, annealing at 55 °C for 45 s and elongation at 72 °C for 60 s. The generated PCR products were evaluated by agarose gel electrophoresis. RT-PCR was conducted with *Ve1*-specific primers Ve1F3 (5′-GGAACAATTTACTCAGCGGGAGC-3′) and Ve1R4 (5′-CCATGACT GATTCTTGAGATCGG-3′), or *Ave1*-specific primers Ave1F (5′-CACTGGTC ACTGCCGATCTA-3′) and Ave1R (5′-CTTGCAGGACCCTCTAGCAC-3′). As an endogenous control, AtRub-F3 (5′-GCAAGTGTTGGGTTCAAAGCTGGTG-3′) and AtRub-R3 (5′-CCAGGTTGAGGAGTTACTCGGAATGCTG-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.

Trypan blue staining of Arabidopsis seedlings

Two-week-old Arabidopsis seedlings were stained with trypan blue. To this end, whole seedlings were collected in a 1.5-mL centrifuge tube. An adequate volume of lactophenol (1:1:1:1 volume of lactic acid : glycerol- : phenol : water) with trypan blue (1 mg/mL) was added. The tubes were placed in a boiling water bath for 1–2 min. Seedlings were de-stained in chloral hydrate and placed in 50% glycerol. The tubes were placed in a speed-vacuum infiltrator for 1 min to remove air bubbles from the seedlings, and seedlings were mounted on microscope slides in 50% glycerol. Cell death was monitored by differential interference contrast microscopy.

Verticillium inoculations

Verticillium dahliae race 1 strain JR2 and the corresponding *Ave1* deletion strain (Δ*Ave1*) were grown on potato dextrose agar (PDA) at 22 °C. *Verticillium dahliae* conidia were harvested from 7–14-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 min. 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 the wilting of leaves at 21 dpi. The quantification of *Verticillium* biomass was performed as described previously (Ellendorff *et al*., 2009).

Pseudomonas 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 colony-forming units/mL in 10 mM $MgCl_2$ and 0.05% Silwet L-77 (Lehle Seeds) was sprayed onto the leaves until droplet runoff. Plants were incubated at 100% relative humidity for 1 h, followed by incubation at 24 °C, 60% relative humidity and a 16-h/8-h light/dark regime. Disease progression was scored at 4 days after inoculation.

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, GCAAGTGTTGGGTTCAAAGCT GGTG; AtRub-R3, CCAGGTTGAGGAGTTACTCGGAATGCTG) and primers amplifying the *P. syringae Oprf* gene (OWB575, AACTGAAAAACACCTT GGGC; OWB576, CCTGGGTTGTTGAAGTGGTA). Real-time PCR was conducted using an ABI7300 PCR machine (Applied Biosystems, Foster City, CA, USA) in combination with the qPCR SensiMix kit (BioLine, Taunton, MA, USA). Real-time PCR conditions were as follows: an initial 95 °C hot start activation step for 10 min was 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.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Ve1 localizes to the plasma membrane. (A) Localization of untagged Ve1 in *Nicotiana benthamiana* leaf epidermis with fluorescence microscopy at 30 h after agroinfiltration. (B) Localization of green fluorescent protein (GFP)-tagged Ve1 in *Nicotiana benthamiana* leaf epidermis with fluorescence microscopy at 30 h after agroinfiltration. (C) Localization of GFP-tagged Ve1 in *Nicotiana benthamiana* leaf epidermis on plasmolysis by incubation in 750 mM mannitol using confocal microscopy. (D) Bright field image of (C). (E) Overlay of (C) and (D). The arrows indicate plasma membrane detached from the cell wall. (F) Localization of GFP-tagged Ve1 in protoplasts of *Nicotiana benthamiana* leaf epidermis.