***Verticillium dahliae* Sge1 Differentially Regulates Expression of Candidate Effector Genes**

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The ascomycete fungus *Verticillium dahliae* causes vascular wilt diseases in hundreds of dicotyledonous plant species. However, thus far, only few *V. dahliae* effectors have been identified, and regulators of pathogenicity remain unknown. In this study, we investigated the role of the *V. dahliae* homolog of Sge1, a transcriptional regulator that was previously implicated in pathogenicity and effector gene expression in *Fusarium oxysporum*. We show that *V. dahliae* Sge1 (*VdSge1*) is required for radial growth and production of asexual conidiospores, because *VdSge1* deletion strains display reduced radial growth and reduced conidia production. Furthermore, we show that *VdSge1* deletion strains have lost pathogenicity on tomato. Remarkably, *VdSge1* is not required for induction of Ave1, the recently identified *V. dahliae* effector that activates resistance mediated by the Ve1 immune receptor in tomato. Further assessment of the role of *VdSge1* in the induction of the nine most highly in-plant-induced genes that encode putative effectors revealed differential activity. Although the expression of one putative effector gene in addition to Ave1 was not affected by *VdSge1* deletion, *VdSge1* appeared to be required for the expression of six putative effector genes, whereas two of the putative effectors genes were found to be negatively regulated by *VdSge1*. In conclusion, our data suggest that *VdSge1* differentially regulates *V. dahliae* effector gene expression.

All multicellular organisms have evolved immune systems to withstand microbial attack; therefore, immunity to infection by microbes that generally occur in their habitats is common. Nevertheless, particular microbes still developed into pathogens, which can be attributed to their deployment of effectors: secreted molecules that target the host's physiology such that it becomes susceptible to infection, often through direct suppression of immune responses (de Jonge et al. 2011). It can be anticipated that the expression of effector genes and other genes that are involved in pathogenicity or virulence are under tight regulation. Typically, fungal effectors are not, or lowly, expressed outside the host and are induced upon host penetration. Since it was found that some effectors are induced by nitrogen, it was proposed that nitrogen acts as an in planta trig-

ger of effector gene induction (van den Ackerveken et al. 1993). However, we now know that many effector genes do not respond to nitrogen starvation (Bolton and Thomma 2008). Thus, the in planta signals that trigger effector gene induction remain largely unknown. Unfortunately, regulators of effector gene expression also are generally unknown (de Jonge et al. 2011).

Recently, a transcriptional regulator that is important for early infection was identified in the root-invading tomato wilt fungus *Fusarium oxysporum* f. sp. *lycopersici* as Sge1. This transcriptional regulator was found to be localized in the nucleus, required for pathogenicity, and required for the in planta expression of various effector genes during conditions that mimic growth in planta (Michielse et al. 2009). Furthermore, *Sge1* mutants showed significantly reduced conidia production and an altered metabolic profile (Michielse et al. 2009). Interestingly, *Sge1* orthologs occur widely in fungi and family members are involved in morphological switching in dimorphic fungi. In the human pathogens *Candida albicans* and *Histoplasma capsulatum*, the transcriptional regulators Wor1 and Ryp1, respectively, were identified as major regulators of a morphological switch that is associated with disease-causing ability. Whereas *C. albicans* Wor1 regulates the switch from white to opaque cell type, *H. capsulatum* Ryp1 governs the transition from yeast to filamentous growth. Similar to *Sge1*, Wor1 and Ryp1 are localized in the nucleus and control expression of phase-specific genes (Huang et al. 2006; Nguyen and Sil 2008; Srikantha et al. 2006; Zordan et al. 2006).

After the functional characterization of *F. oxysporum* Sge1 (FoSge1) as a regulator of parasitic growth, homologs in other plant pathogens have been studied, revealing that these homologs also act as regulators of pathogenicity. In the necrotrophic plant pathogen *Botrytis cinerea*, the FoSge1 homolog *B. cinerea* Reg1 (*BcReg1*) is required for conidiation and pathogenicity on bean leaves. Although *BcReg1* mutants were able to penetrate host plant tissue, they were unable to establish necrotic lesions, possibly due to their inability to produce sesquiterpene and polyketide toxins. Similarly, the homolog Fgp1 of the wheat and barley pathogen *F. graminearum* regulates asexual and sexual spore formation as well as pathogenicity, which correlated with the lack of trichothecene mycotoxin production (Jonkers et al. 2012).

The ascomycete fungus *Verticillium dahliae* causes vascular wilt diseases in hundreds of dicotyledonous plant species (Fradin and Thomma 2006; Klosterman et al. 2009). The host range of *V. dahliae* also includes tomato, which is colonized in a similar fashion as by *F. oxysporum* f. sp. *lycopersici*. Triggered by root exudates, the melanized resting structures that reside in the soil germinate and penetrate roots of susceptible host plants. After crossing the cortex, hyphae of the fungus

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grow into the xylem vessels. The mycelium remains exclusively in these vessels and produces conidia which are transported acropetally with the water flow in the xylem throughout the plant. Once senescing, microsclerotia are produced that are released into the soil upon tissue decomposition. Compared with *F. oxysporum* f. sp. *lycopersici*, for which several effectors that are secreted in the xylem during host colonization have been identified as the so-called SIX proteins, only a few *V. dahliae* effectors have been identified. Among these are plant cell-wall-degrading enzymes, of which expression is regulated through the sucrose nonfermenting 1 protein (Tzima et al. 2010). Based on a query of the *V. dahliae* genome sequence, 127 (conserved) hypothetical proteins were designated as small (<400 amino acids), cysteine-rich (>4 cysteine residues) effector candidates but no homologs of any of the *F. oxysporum* SIX effectors were identified (Klosterman et al. 2011). The only bona fide effector that has recently been characterized is the Ave1 effector that activates Ve1-mediated immunity in race 1-resistant tomato plants (de Jonge et al. 2012). This Ave1 effector is a secreted, small (134 amino acids [aa]) protein with four cysteines that is required for full virulence on

tomato plants lacking *Ve1*, and the corresponding gene is strongly induced during host colonization, thus conforming to a typical effector protein (de Jonge et al. 2012). In this study, we investigated the role of the *V. dahliae* homolog of *F. oxysporum* Sge1 and assessed its role in pathogen virulence and regulation of effector gene expression.

RESULTS

Identification of *V. dahliae* Sge1.

To identify the *V. dahliae* ortholog of the *F. oxysporum* transcriptional regulator Sge1, the genome of *V. dahliae* strain VdLs17 was queried with BlastP. In this manner, a single homolog, VDAG_06298.1 (*E* value: 0), was identified which was tentatively called *V. dahliae* Sge1 (*VdSge1*). The *VdSge1* gene has an open reading frame of 1,533 nucleotides that is not interrupted by introns, as was verified by reverse-transcription polymerase chain reaction (PCR), and encodes a protein of 510 aa. The length of the various homologs that have been functionally analyzed varies considerably, because FOSge1 is 330 aa, *F. graminearum* Fgp1 is 342 aa, *H. capsulatum* Ryp1

Fig. 1. Alignment of *Verticillium dahliae* Sge1 (*VdSge1*) with homologs from other fungal species. **A**, Amino acid sequence alignment of the N-terminal region of *VdSge1* and of the homologs from *Histoplasma capsulatum* (Ryp1), *Fusarium oxysporum* (FoSge1), *F. graminearum* (Fgp1), *Botrytis cinerea* (BcReg1), *Candida albicans* (Wor1), and *Schizosaccharomyces pombe* (Gti1). Conserved residues are shaded. The box with the dashed line indicates the conserved phosphorylation site and the box with the solid line indicates the predicted nuclear localization signals. **B**, Phylogenetic tree of *VdSge1* with homologs from other fungal species. In addition to the homologs described above, homologs from *Aspergillus fumigatus*, *Colletotrichum higginsianum*, *Glomerella graminicola*, *Magnaporthe grisea*, *Mycosphaerella graminicola*, *Sclerotinia sclerotiorum*, and *Ustilago maydis* also are included. Bootstrap percentages over 60% are indicated at the nodes.

is 487 aa, BcReg1 is 506 aa, *Schizosaccharomyces pombe* Gti1 is 720 aa, and *C. albicans* Wor1 is 785 aa (Caspari 1997; Huang et al. 2006; Jonkers et al. 2012; Michielse et al. 2009, 2011; Nguyen and Sil 2008). Alignment of the N-terminal part, which is the most conserved part of the protein (Michielse et al. 2009, 2011), demonstrated that the TOS9 (COG5037) and Gti1_Pac2 family domain (Pfam09729) motifs which are found in all orthologs analyzed thus far are also present in VdSge1 (Fig. 1). In addition, the VdSge1 protein shares a putative protein kinase A phosphorylation site (+66 to +71; KRWTDG) and a nuclear localization signal (+94 to +100; PPGEKKR) with several other homologs (Fig. 1), suggesting that VdSge1 is nuclear localized.

VdSge1 is required for radial growth and conidia production.

Targeted replacement of *VdSge1* by a hygromycin resistance cassette through homologous recombination was pursued, and gene deletion was verified by PCR. Several independent *VdSge1* deletion mutants were obtained, of which two were used for further analysis in this study (Supplementary Fig. S1). It has previously been shown that the Sge1 orthologs from the plant pathogens *F. oxysporum*, *F. graminearum*, and *B. cinerea*

were not required for vegetative growth in vitro (Jonkers et al. 2012; Michielse et al. 2009, 2011). However, both orthologs were found to play a role in conidiation because deletion mutants in *F. oxysporum* and *F. graminearum* produced significantly reduced numbers of conidia, whereas deletion strains in *B. cinerea* did not produce conidia at all. Remarkably, the *VdSge1* deletion mutants showed clearly reduced radial growth on potato dextrose agar (PDA) medium when compared with the wild-type strain and ectopic transformants (Fig. 2A). Furthermore, the pigmentation of the mycelium also was affected by the *VdSge1* deletion, because the deletion mutants developed brown mycelium whereas the wild-type strain and ectopic transformants developed white mycelium (Fig. 2A). Furthermore, the effect of *VdSge1* deletion on pigmentation is evident from the agar medium on which the fungal strains were grown, because the medium surrounding the *VdSge1* deletion mutants turned brown whereas the medium surrounding the wild-type strain and ectopic transformants did not change color (Fig. 2A). In addition, the amount of conidia produced by the *VdSge1* deletion was significantly reduced when compared with the conidial production by the wild-type strain and ectopic transformants, although the appearance of conidia was phenotypically indistinguishable (Fig. 2B). Conidial germina-

Fig. 2. Targeted deletion of *Verticillium dahliae* *Sge1* (*VdSge1*) results in altered colony morphology and reduced conidiospore production. **A**, Colony morphology of wild-type *V. dahliae* (WT), two *VdSge1* deletion strains (Δ Sge1-1 and Δ Sge1-2), and two ectopic transformants (EC-1 and EC-2) after 7 days of incubation on potato dextrose agar (PDA) medium at 22°C. **B**, Average number of conidia produced after 7 days of growth on PDA medium based on two independent experiments. Different letters indicate significant differences at $P < 0.05$ as calculated with Student's *t* test. **C**, Representative picture of microscopic observation of conidial germination at 16 h after incubation in Czapek-Dox medium.

tion was found to be comparable in all strains, indicating that the conidia that are produced are fully viable (Fig. 2C). The altered colony morphology as well as the reduced conidia production were restored upon complementation of the *VdSge1* deletion strains with a genomic construct containing the wild-type *VdSge1* locus (Supplementary Fig. S2). Thus, *VdSge1* is involved in vegetative growth, mycelium pigmentation, and conidiation.

***VdSge1* is required for pathogenicity.**

We subsequently assessed the role of *VdSge1* in pathogenicity on tomato plants. To this end, it was determined that expression of *VdSge1* is induced in wild-type *V. dahliae* during infection of tomato plants in a time course harvested at regular intervals after inoculation (Supplementary Fig. S3). *VdSge1* deletion strains appeared to be nonpathogenic on tomato upon root-dip inoculation with conidiospores, whereas ectopic transformants were found to be as virulent as the wild-type strain (Fig. 3A). The plants that were inoculated with the *VdSge1* de-

letion strains remained devoid of any Verticillium wilt symptoms throughout the assay up to 21 days postinoculation (dpi), whereas the ectopic transformants and the wild-type strain induced severe wilting symptoms. Fungal outgrowth assays upon plating of stem sections harvested from the hypocotyls of the inoculated plants demonstrated that, although the ectopic transformants and the wild-type strain had extensively colonized the stems of the tomato plants, the *VdSge1* deletion strains were not able to colonize the xylem vessels of the inoculated tomato plants (Fig. 3B). Pathogenicity was restored upon complementation of the *VdSge1* deletion strains with a genomic construct containing the wild-type *VdSge1* locus (Supplementary Fig. S4). Thus, it can be concluded that *VdSge1* is required for pathogenicity on tomato.

***VdSge1* differentially regulates expression of effector genes.**

Whereas the *H. capsulatum* and *C. albicans* transcriptional regulators Ryp1 and Wor1 were previously demonstrated to regulate expression of phase-specific genes, FOSge1 was found

Fig. 3. *Verticillium dahliae Sge1* (*VdSge1*) is required for pathogenicity of *V. dahliae* on tomato. **A**, Side and top view of tomato ‘MoneyMaker’ plants inoculated with wild-type *V. dahliae* (WT), two *VdSge1* deletion strains ($\Delta Sge1-1$ and $\Delta Sge1-2$), and two ectopic transformants (EC-1 and EC-2) or mock inoculated at 21 days postinoculation. **B**, Fungal outgrowth at 7 days after plating of stem sections harvested at 21 days postinoculation. **C**, Average canopy area of six plants 21 days after inoculation with *V. dahliae* genotypes described above or mock inoculation. Different letters indicate significant differences at $P < 0.05$ as calculated with Student’s *t* test.

to regulate the expression of small in-plant-secreted SIX effector proteins during infection of the host. We similarly wanted to test whether *VdSge1* regulates the expression of effector genes in *V. dahliae*, using *Ave1* as target (de Jonge et al. 2012). Because the *VdSge1* deletion mutant is not able to infect and colonize tomato plants, assessment of *Ave1* gene expression during plant infection is impossible. Therefore, we tested whether the incubation of *V. dahliae* in a tomato cell suspension culture would induce *Ave1* expression. A suspension of MSK8 tomato cells was inoculated with *V. dahliae* conidia and, after 96 h, the cells were harvested. First, expression of *VdSge1* was assessed in wild-type *V. dahliae*, two *VdSge1* deletion lines, and two complementation lines. Real-time PCR analysis confirmed that *VdSge1* is induced in a suspension of MSK8 tomato cells, and no expression was observed in *VdSge1* deletion lines whereas expression was restored in the complementation lines (Fig. 4A). Next, expression of the *Ave1* gene was determined and compared with transcript levels of *V. dahliae* grown in Murashige-Skoog (MS) medium. As expected, *Ave1* expression was strongly induced upon incubation of the fungus with MSK8 cells (Fig. 4B). Surprisingly, although the expression level did not reach the level obtained in the wild type, *Ave1* expression was still also strongly (eight- to 10-fold) induced in the *VdSge1* deletion lines, demonstrating that *VdSge1* is not required for *Ave1* induction in planta.

To further investigate the role of *VdSge1* in the control of in planta expression of effector genes, we evaluated the role of *VdSge1* in the expression of the nine *V. dahliae* genes that, together with *Ave1*, belong to the most highly induced putative effector genes in planta (de Jonge et al. 2012; Faino et al. 2012). In addition to *Ave1*, *VdSge1* was also not required for induction of *Xloc_08811* (Fig. 4B). In contrast, *VdSge1* appeared to be required for the expression of six putative effector genes, of which the expression was (nearly) completely abolished in the two *VdSge1* deletion lines, whereas it was restored in the complementation lines (Fig. 4C). Interestingly, two of the effector genes appeared to be negatively regulated by *VdSge1*, because induction was enhanced in the two *VdSge1* deletion lines whereas it was restored in the complementation lines (Fig. 4D). These data strongly suggest that *VdSge1* differentially regulates effector gene expression in *V. dahliae*.

DISCUSSION

Research on pathogen effectors that are secreted during colonization has dominated research in the plant-microbe interactions over the recent years, as it has been recognized that effectors are crucial for the establishment of microbial infections (de Jonge et al. 2011; Lindeberg et al. 2012; Stassen and van den Ackerveken 2011). Effectors are typically not produced in vitro but their production is specifically induced upon host colonization. The identification of environmental cues that trigger effector gene expression and corresponding microbial regulators of effector gene expression remains a major goal in phytopathological research, because it may lead to novel strategies to combat microbial infections.

Because some effector genes are induced by nitrogen starvation in vitro, nitrogen limitation was proposed as an in planta trigger of their induction (van den Ackerveken et al. 1993). However, nitrogen availability may not be limited in planta, and many effector genes are not induced by nitrogen deprivation; therefore, nitrogen starvation cannot be considered to be a general trigger for effector gene induction in planta and, thus, nitrogen response factors likely are not general regulators of effector gene expression (Bolton and Thomma 2008; Thomma et al. 2006). More recently, the transcriptional regulator *Sge1* was identified in the root-invading tomato wilt fungus *F. oxysporum*

f. sp. *lycopersici*, and found to be localized in the nucleus, required for pathogenicity, and required for the in planta expression of various *SIX* effector genes during conditions that mimic in planta growth (Michielse et al. 2009). Several of these effector genes were demonstrated to contribute to *F. oxysporum* virulence (Houterman et al. 2009; Rep 2005; Takken and Rep 2010; Thatcher et al. 2012). Interestingly, *Sge1* orthologs occur widely in fungi and, presently, two homologs from other plant pathogens have similarly been characterized as regulators of pathogenicity; *Fgp1* from the related pathogen *F. graminearum* and *BcReg1* from *B. cinerea* (Jonkers et al. 2012; Michielse et al. 2011). However, a role on effector gene induction in these pathogens is unclear, because no effector proteins have been found to date that are required for infection by either of these pathogens (Amselem et al. 2011; Cuomo et al. 2007). Rather,

Fig. 4. Expression of *Verticillium dahliae* *Sge1* (*VdSge1*) and putative *V. dahliae* effector genes in *VdSge1* deletion mutants. Real-time polymerase chain reaction was used to measure gene expression levels in wild-type *V. dahliae* (WT), two *VdSge1* deletion mutants (KO-1 and KO-2), and two complementation strains (C-1 and C-2) grown for 4 days in Murashige-Skoog (MS) medium (black bars) and a tomato cell suspension (MSK8; gray bars) using the elongation factor 1- α gene as a reference. Expression in WT *V. dahliae* in MS medium was set to one, and bars represent averages with stranded deviation of two biological experiments.

these studies focused on the role of the *Sge1* homologs in the production of secondary metabolites that play a crucial role in pathogenicity (Jonkers et al. 2012; Michielse et al. 2011).

Similar to *F. oxysporum* f. sp. *lycopersici*, the ascomycete fungus *V. dahliae* causes vascular wilt disease in tomato, and both pathogens share important traits of their life styles (Klosterman et al. 2011). Although, for *F. oxysporum* f. sp. *lycopersici*, a considerable number of SIX effector proteins have been identified, until recently no *V. dahliae* effectors were characterized and no homologs of the SIX effectors were found (Klosterman et al. 2011). Recently, the *V. dahliae* effector that is recognized by the Ve1 immune receptor of tomato and that contributes to fungal virulence on susceptible plant genotypes was identified as Ave1 (de Jonge et al. 2012). Interestingly, Ave1 homologs were identified in a number of fungal pathogens, including *F. oxysporum* f. sp. *lycopersici* (de Jonge et al. 2012). Surprisingly, we found that induction of *V. dahliae* Ave1 under conditions that mimic in planta growth is not regulated by *Sge1*, despite the observation that *VdSge1* deletion strains are not pathogenic on tomato. To further assess the role of *VdSge1* on effector gene induction, we evaluated the induction of additional effector gene candidates under conditions that mimic in planta growth. These candidates are derived from a comparative population genomics study in *V. dahliae*, and have been selected based on patterns of selection pressure (R. de Jonge and B. P. H. J. Thomma, *unpublished data*) and their strong induction in planta (de Jonge et al. 2012). Although another effector candidate was identified of which the induction, like that of Ave1, is not regulated by *VdSge1*, induction of six other candidate effector genes was abolished in the *VdSge1* deletion strain. These data indicate a role of *VdSge1* as a positive regulator of at least a subset of candidate effector genes in planta, some of which were recently demonstrated to act as genuine virulence factors (R. de Jonge, G. C. M. van den Berg, and B. P. H. J. Thomma, *unpublished data*). Remarkably, however, deletion of *VdSge1* resulted in enhanced induction of two candidate effector genes, suggesting that *VdSge1* may also act as a negative regulator of particular effector genes in planta. Thus, we conclude that *VdSge1* differentially regulates *V. dahliae* candidate effector genes.

The question of whether the differential effect of *VdSge1* deletion on candidate effector gene regulation is responsible for the lack of pathogenicity in the *VdSge1* deletion strains presently remains unanswered. Not only was significantly reduced vegetative growth observed for the *VdSge1* deletion strains in vitro but also reduced conidiospore production was monitored under these conditions. Interestingly, whereas reduced spore production is found upon the deletion of the *Sge1* homologs in other plant-pathogenic species as well, effects on vegetative growth have not been observed previously (Jonkers et al. 2012; Michielse et al. 2009, 2011). Nevertheless, the effects of *VdSge1* deletion on vegetative growth as well as on conidiospore production may significantly affect pathogenicity, because the production and release of conidiospores is a crucial step in the colonization of xylem vessels of the host (Fradin and Thomma 2006). Furthermore, as previously mentioned, deletion of the *Sge1* homologs was found to affect secondary metabolite production in the other plant-pathogenic species (Jonkers et al. 2012; Michielse et al. 2009, 2011). The role of mycotoxins and other secondary metabolites in pathogenicity is particularly well documented for *F. graminearum* (Maier et al. 2006) but a role of such products in pathogenicity has also been established for *B. cinerea* (van Kan 2006). Although the role of secondary metabolites in pathogenicity of the vascular wilt pathogens *F. oxysporum* f. sp. *lycopersici* and *V. dahliae* is less clear, a significant effect on pathogenicity cannot be excluded. Moreover, although we have not characterized changes

in metabolite profiles upon targeted deletion of *VdSge1*, the significant change in pigmentation of the mycelium from white to brown and the secretion of brown pigment into the medium upon *VdSge1* deletion implies an altered metabolite production profile.

In summary, we conclude that *VdSge1* is crucial for pathogenicity of *V. dahliae* and affects vegetative growth and conidiospore production as well as secondary metabolite production. Furthermore, because *VdSge1* differentially regulates the expression of a collection of candidate effector genes, it is unlikely the central regulator of *V. dahliae* effector gene expression in planta.

MATERIALS AND METHODS

Phylogenetic analyses.

Phylogenetic analyses of *VdSge1* with homologs from other fungal species were conducted using the Phylogeny.fr web service. Sequences were aligned with MUSCLE and curated with Gblocks, and the phylogenetic tree was reconstructed using the *PhyML* program (maximum likelihood method). Statistical reliability of the tree was tested using bootstrap with 500 replications.

Fungal transformations.

To generate a *VdSge1* deletion construct, sequences flanking the *VdSge1* coding sequence were amplified from the genomic DNA of *V. dahliae* strain JR2 using the primers KO-*VdSge1*-LF with KO-*VdSge1*-LR to amplify the left border, and KO-*VdSge1*-RF with KO-*VdSge1*-RR to amplify the right border (Supplementary Table S1). The resulting amplicons were cloned into pRF-HU2 as described previously (Frandsen et al. 2008).

To generate a *VdSge1* complementation construct, a 2,699-bp *EcoRI/PacI* fragment containing the *VdSge1* coding sequence with a sequence 1,100 bp upstream and 66 bp downstream was amplified from *V. dahliae* strain JR2 genomic DNA, and cloned into binary vector pBT081 (Houterman et al. 2008).

Agrobacterium tumefaciens-mediated transformation of *V. dahliae* was performed as described previously (Santhanam 2012), and transformants were selected on PDA supplemented with cefotaxime (Duchefa, Haarlem, The Netherlands) at 200 µg/ml, hygromycin (Duchefa) at 50 µg/ml, or nourseothricin (Werner Bioagents, Jena, Germany) at 50 µg/ml. Homologous recombination was verified by PCR. Complementation transformants were selected on PDA supplemented with cefotaxime at 200 µg/ml and phleomycin at 100 µg/ml (InvivoGen, San Diego, CA, U.S.A.).

Pathogenicity assays.

Pathogenicity assays were performed on 10-day-old tomato ('MoneyMaker') seedlings using root dip inoculation as described previously (Fradin et al. 2009). Disease symptoms were scored up to 14 dpi, pictures were taken, and ImageJ was used to determine the canopy area. To determine in planta colonization, stem sections at the height of the first internode were taken, surface sterilized, sliced, placed on PDA supplemented with chloramphenicol at 50 µg/ml, and incubated at 22°C. Fungal outgrowth was monitored after 7 days. All pathogenicity assays were performed three times independently with six plants for each genotype, and plate assays were performed for two biological experiments in triplicate.

Growth, conidiogenesis, and germination assays.

Radial growth was monitored by placing a 2-µl droplet of a conidial suspension of 10⁶ conidia/ml in the center of a PDA or Czapek-Dox agar plate, incubating the suspension at 22°C,

and measuring the colony diameter after 10 days. For quantification of conidia production, agar plugs were taken from the fungal colony using a cork borer and shaken in water, and conidia were counted in a hemocytometer. Two biological experiments were performed in triplicate for each genotype. To determine the germination rate, 10^6 conidia/ml were inoculated in Czapek-Dox medium at 22°C for 24 h.

Gene expression analysis.

To assess *V. dahliae* gene expression, conidia were inoculated into 30 ml of MS medium supplemented with vitamins, 3% sucrose, 2,4-D at 1 mg/liter, and kinetin at 0.1 mg/liter or in 30 ml of a five times diluted 5-day-old tomato cell culture (MSK8), such that the final concentration was 10^7 conidia/ml. After 4 days of incubation at 25°C, cultures were harvested by centrifugation and freeze dried. Total RNA was isolated using the RNeasy mini kit (Qiagen, Venlo, The Netherlands), including on-column DNase treatment, following the manufacturer's instructions. Total RNA (2 µg) was used for reverse-transcription PCR with SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. The resulting cDNA was diluted 10-fold and used as template for expression profiling of *Ave1* and nine other highly in planta-expressed putative effector genes. Primers used for expression profiling are listed in Supplementary Table S1.

To determine the in planta expression of *VdSge1*, 10-day-old tomato seedlings were root inoculated with *V. dahliae*. Whole plants were harvested at 4, 8, 12, and 16 dpi and flash frozen in liquid nitrogen. After grinding, 100 mg of ground material was used for total RNA extraction (Qiagen) and cDNA synthesis (Invitrogen). Real-time PCR was performed to determine *VdSge1* expression. *VdGAPDH* was used as an endogenous control and reactions were performed in triplicate. Real-time PCR conditions consisted of an initial 95°C denaturation step for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

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