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RESEARCH PAPER

RNA silencing is required for *Arabidopsis* defence against *Verticillium* wilt disease

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Abstract

RNA silencing is a conserved mechanism in eukaryotes that plays an important role in various biological processes including regulation of gene expression. RNA silencing also plays a role in genome stability and protects plants against invading nucleic acids such as transgenes and viruses. Recently, RNA silencing has been found to play a role in defence against bacterial plant pathogens in *Arabidopsis* through modulating host defence responses. In this study, it is shown that gene silencing plays a role in plant defence against multicellular microbial pathogens; vascular fungi belonging to the *Verticillium* genus. Several components of RNA silencing pathways were tested, of which many were found to affect *Verticillium* defence. Remarkably, no altered defence towards other fungal pathogens that include *Alternaria brassicicola*, *Botrytis cinerea*, and *Plectosphaerella cucumerina*, but also the vascular pathogen *Fusarium oxysporum*, was recorded. Since the observed differences in *Verticillium* susceptibility cannot be explained by notable differences in root architecture, it is speculated that the gene silencing mechanisms affect regulation of *Verticillium*-specific defence responses.

Key words: Abiotic stress, post-transcriptional gene silencing (PTGS), suppressor of gene silencing (SGS), *Verticillium dahliae*, *V. albo-atrum*, *V. longisporum*.

Introduction

Plant defence against pathogens is activated through specific host signalling mechanisms (Chisholm *et al.*, 2006; Jones and Dangl, 2006). Microbial intruders can be recognized by extracellular receptor molecules that detect the presence of pathogen-associated molecular patterns (PAMPs) and subsequently activate PAMP-triggered immunity (PTI) as a basal defence response. Virulent pathogen strains are able to interfere with, or suppress, PTI by utilizing effector molecules (Bolton *et al.*, 2008; van Esse *et al.*, 2007, 2008). In turn, some plant genotypes have developed specific receptor molecules, the resistance proteins, to detect the presence of the pathogen effector molecules and activate effector-triggered immunity (ETI; Chisholm *et al.*, 2006; Jones and Dangl, 2006). Only in a few cases has direct interaction of the host resistance protein with the pathogen effector molecule been observed (Scofield *et al.*, 1996; Tang *et al.*, 1996; Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006; Burch-Smith *et al.*, 2007). More often, however, the resistance protein monitors the status of a host target of the pathogen effector molecule in compliance with the guard hypothesis (Dangl and Jones, 2001; Mackey *et al.*, 2002; Rooney *et al.*, 2005; Shao *et al.*, 2003).

Nearly 20 years ago, the phenomenon of RNA silencing was discovered in experiments with transgenic plants that showed silencing of a transgene and also, in a number of cases, of homologous endogenous genes (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). The gene silencing was found to result from the inhibition of gene transcription (transcriptional gene silencing, TGS) or from post-transcriptional degradation of RNA (post-transcriptional gene silencing, PTGS), and correlated with the

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accumulation of small double-stranded RNA segments of 20–27 nucleotides, so-called small RNAs (sRNAs). These corresponded to the promoter of the silenced gene, or to the degraded RNA in TGS and PTGS, respectively (Hamilton and Baulcombe, 1999; Mette *et al.*, 2000).

RNA silencing is now known as a conserved regulatory mechanism in most eukaryotic organisms that plays a determinant role in various biological processes, including the regulation of endogenous gene expression, genome stability, the taming of transposons, heterochromatin formation, and defence against viruses (Baulcombe, 2004; Brodersen and Voinnet, 2006; Vaucheret, 2006). The key characteristic of RNA silencing is the formation of the sRNAs that are produced by RNaseIII-like Dicer enzymes (Bernstein *et al.*, 2001). These sRNAs can be divided into two major types, the small interfering RNAs (siRNAs) and the micro RNAs (miRNAs), based on their origin and formation. Subsequently, a selected sRNA strand is incorporated into an effector complex that is targeted towards partially or fully complementary RNA or DNA stretches. This so-called RNA-induced silencing complex (RISC) contains an Argonaute (Ago) protein that has an sRNA-binding domain and an endonucleolytic activity to cleave target RNAs (Martinez *et al.*, 2002).

Several studies have shown that PTGS mechanisms are an RNA-based host defence system to control nucleic acid invaders of various natures through the action of *cis*-acting siRNAs that derive from, and target, the invaders (Vance and Vaucheret, 2001; Bartel, 2004; Baulcombe, 2004; Dunoyer and Voinnet, 2005). These invaders may be endogenous, such as transposons, or exogenous, such as transgenes and viral pathogens. Thus, RNA silencing has been implicated in pathogen defence through its role in viral defence. Upon virus infection, the accumulation of virus-derived sRNAs has been observed (Hamilton and Baulcombe, 1999). Moreover, plant mutants defective in PTGS are often hyper-susceptible to viral infection (Mourrain *et al.*, 2000; Dalmay *et al.*, 2001; Qu *et al.*, 2005; Schwach *et al.*, 2005).

Apart from viral defence, evidence accumulates for RNA silencing to play a role in interactions with other pathogen types, more specifically bacterial defence (Voinnet, 2008). The first example is a miRNA from *Arabidopsis* that contributes to basal defence against *Pseudomonas syringae* by regulating auxin signalling (Navarro *et al.*, 2006). The miRNA was induced upon perception of flg-22, a PAMP that is derived from bacterial flagellin, and negatively regulated transcripts of a number of F-box auxin receptors. In turn, repression of auxin signalling was shown to restrict growth of the bacterium *P. syringae* (Navarro *et al.*, 2006). Another example is an endogenous *Arabidopsis* siRNA that is specifically induced by avirulent *P. syringae* carrying *AvrRpt2* (Katiyar-Agarwal *et al.*, 2006). This siRNA contributes to RPS2-mediated disease resistance by repressing a putative negative regulator of the RPS2 resistance pathway. Recently, a novel class of small RNAs, long siRNAs (lsiRNAs that are 30–40 nt) that are induced by pathogen infection or under specific growth conditions, was identified. One of the lsiRNAs, AtlsiRNA-1, was specifically induced by avirulent *P. syringae* carrying *AvrRpt2* and induction of AtlsiRNA-1 was found to silence a RAP-domain protein that is involved in disease resistance (Katiyar-Agarwal *et al.*, 2007). Finally, in a forward genetics screen, an *Arabidopsis* mutant with enhanced disease susceptibility towards a compatible *P. syringae* strain, an incompatible strain carrying *AvrRpm1*, and non-adapted *P. syringae* pv. *tabaci* was isolated (Agorio and Vera, 2007). Positional cloning revealed a mutation in the *Argonaute* gene *AGO4*, that is associated with small interfering RNAs involved in RNA-directed DNA methylation (RdDM), showing that AGO4 plays a role in non-host resistance, basal defence, and effector-triggered immunity against bacterial pathogens (Agorio and Vera, 2007). In addition to *P. syringae*, it has been shown that RNA silencing mutants are hypersusceptible to the crown gall bacterium *Agrobacterium tumefaciens* (Dunoyer *et al.*, 2006). Finally, RNA silencing has been shown to be required for the development of nodule differentiation on *Medicago truncatula* roots in the interaction with the nitrogen fixing *Rhizobium* bacteria (Combier *et al.*, 2006; Boualem *et al.*, 2008).

Recently it has been demonstrated that miRNAs are key components of plant basal defence as miRNA-deficient *Arabidopsis* mutants sustained growth of a non-pathogenic, type III secretion-defective *P. syringae* mutant, non-pathogenic *P. fluorescens*, and *Escherichia coli* strains (Navarro *et al.*, 2008). Interestingly, *P. syringae* effectors were identified that suppressed the transcriptional activation or activity of several PAMP-responsive miRNAs, demonstrating that these bacteria suppress RNA silencing to cause disease (Navarro *et al.*, 2008).

In our research, *Arabidopsis thaliana* has been used as a host to investigate the biology of the vascular wilt pathogen *Verticillium dahliae* (Fradin and Thomma, 2006). To investigate the role of putative defence genes against *Verticillium* infection, transgenic over-expression in wild-type (Col-0) *Arabidopsis*, but also in the PTGS mutant *sgs2* (Butaye *et al.*, 2004), was used. Previously, it has been shown that the inter-transformant variability of transgene expression is reduced in *sgs* mutants, as the incidence of highly expressing transformants increased from 20% in Col-0 to 100% in *sgs* mutants (Butaye *et al.*, 2004). Intriguingly, it was observed in several of our experiments that non-transformed *sgs2* plants displayed significantly enhanced susceptibility towards *V. dahliae* when compared with the parental line Col-0. In this paper, the role of RNA silencing in *Arabidopsis* defence against a number of fungal pathogens, including *V. dahliae*, was investigated.

Materials and methods

Plant growth conditions

Soil-grown *Arabidopsis* plants were cultivated in a growth chamber at 22 °C, 72% relative humidity, and a 16 h photoperiod, or in a greenhouse at 21 °C for the 16 h day period and 19 °C for the 8 h night period at 72% relative

humidity. In the greenhouse, supplemental light (100 W m^{-2}) was used when the sunlight influx intensity was below 150 Wm^{-2}.

For *in vitro* growth of *Arabidopsis*, seeds were surface-sterilized and sown on MS medium (Duchefa, Haarlem, NL) solidified with 1.5% plant agar (Duchefa, Haarlem, NL). For phenotypic evaluations of root growth and development, *Arabidopsis* plants were grown on vertically oriented half-strength MS plates, supplemented with 1% sucrose and 0.5 g l^{-1} MES (2-(*N*-morpholino) ethane-sulphonic acid) (pH 5.8). After sowing, the plates were incubated at 4 °C in the dark for 3 d and subsequently transferred to the growth chamber.

Conditional phenotype assays

To assess susceptibility toward abiotic stress and responsiveness to hormones, *in vitro* assays were performed (Wang *et al.*, 2008; see Supplementary Table S1 at *JXB* online). For abiotic stress assays, seeds were sown on MS agar amended with 100 or 150 mM NaCl, 20 or 30 mM LiCl, 150 or 200 mM mannitol, and 3.3 or 6.7 mM H_2O_2 (see Supplementary Table S1 at *JXB* online) and evaluated for aberrant growth. To assay heavy metal resistance, plants were grown on vertically oriented half-strength MS plates amended with 2% (w/v) sucrose and 85 μM $CdCl_2$. To assay hormone responsiveness, the sterilized seeds were grown on vertically oriented half-strength MS plates containing different hormones (see Supplementary Table S1 at *JXB* online). All plates were incubated in the growth chamber. For hypocotyl length assays, plates were incubated in the dark.

Pathogen cultivation

Verticillium dahliae strains JR2 and ST12.01, *Verticillium longisporum* strain 43, *Verticillium albo-atrum* strains VA1 and CBS451.88, *Fusarium oxysporum* f.sp. *raphani* strain 815 (Diener and Ausubel, 2005), *Alternaria brassicicola* strain MUCL20297 (Mycotheque Université Catholique de Louvain, Louvain-la-Neuve, Belgium), and *Plectosphaerella cucumerina* were maintained on potato dextrose agar (PDA; Oxoid, Hampshire, UK). *Botrytis cinerea* (Brouwer *et al.*, 2003) was grown on half-strength PDA amended with 5 g l^{-1} agar and 150 g l^{-1} blended tomato leaves. All fungal cultures were grown at 22 °C. The bacterial strains of *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 with or without *avrRpt2*, *avrRpm1*, or *avrRps4*, was grown on King's B agar (King *et al.*, 1954) supplemented with the appropriate antibiotics (25 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin). All bacterial strains were grown overnight at 28 °C.

Pathogen inoculations

Inoculum of all fungi (except *F. oxysporum* f. sp. *raphani*) was prepared as previously described by Broekaert *et al.* (1990) and prepared as a suspension of 10^6 conidia ml^{-1} in water. For *Verticillium* inoculations, a minimum of eight 2-week-old *Arabidopsis* plants were up-rooted and the roots were incubated in the conidial suspension for 3 min. Subsequently, the plants were replanted into fresh soil. Inoculations with *F. oxysporum* f. sp. *raphani* were performed in a similar way to the *Verticillium* inoculations, except that the budcell-inoculum was prepared as described by Diener and Ausubel (2005). All other pathogens were inoculated onto a minimum of four approximately 4-week-old soil-grown plants with fully expanded rosette leaves. Inoculations with *A. brassicicola*, *B. cinerea*, and *P. cucumerina* were performed by placing 6 μl drops of the conidial suspensions on each expanded leaf (Thomma *et al.*, 1998, 2000; Brouwer *et al.*, 2003; O'Connell *et al.*, 2004).

For inoculations with *P. syringae*, bacteria were grown overnight at 28 °C in liquid King's B medium supplemented with the appropriate antibiotics. *Arabidopsis* plants were spray-inoculated with a bacterial suspension of OD_{600} 0.3 supplemented with 0.05% (v/v) Silwet L-77 (van Meeuwen Chemicals BV, Weesp, NL).

For all inoculations, except those with *F. oxysporum* f. sp. *raphani* and *Verticillium* spp., plants were kept in boxes with transparent lids at high relative humidity for the remainder of the experiment. All inoculations were performed a minimum of three times with similar results.

V. dahliae *biomass quantification* in planta

Two-week-old *Arabidopsis* plants were inoculated with *V. dahliae* strain JR2 as described above. After visible symptom development at 19–29 d post-inoculation, for each experiment and for each *Arabidopsis* genotype all above-ground tissues were harvested per plant and flash-frozen in liquid nitrogen. The samples were ground to a powder, of which an aliquot of approximately 100 mg was used for DNA isolation (Fulton *et al.*, 1995). Quantitative real-time PCR was conducted using an ABI7300 PCR machine (Applied Biosystems, Foster City, USA) with the qPCR Core kit for SYBR Green I (Eurogentec Nederland BV, Maastricht, NL). To measure *V. dahliae* biomass, the internal transcribed spacer region of the ribosomal DNA was targeted using the fungus-specific ITS1-F primer (AAAGTTTTAATGGTTCGCTAAGA; Gardes and Bruns, 1993) in combination with the *V. dahliae*-specific reverse primer ST-VE1-R (CTTGGTCATTTAGAGGAAGTAA; Lievens *et al.*, 2006), generating a 200 bp amplicon. For sample equilibration, the *Arabidopsis* large subunit of the RuBisCo gene was targeted using the primer set At-RuBisCo-F3 and -R3 (GCAAGTGTTGGGTTCAAAGC-TGGTG and CCAGGTTGAGGAGTTACTCGGAATG-CTG, respectively), generating a 120 bp amplicon. Real-time PCR conditions consisted of an initial 95 °C denaturation step for 4 min, followed by 30 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. The average fungal biomass was determined using at least four *Verticillium*-inoculated plants for each genotype.

Reverse transcription PCR

Total RNA was extracted from plant tissue frozen in liquid nitrogen using the RNeasy Plant Mini kit (Qiagen, Venlo,

the Netherlands). On-column DNaseI treatment was performed as described by the manufacturer using the RNase-free DNase Set (Qiagen, Venlo, the Netherlands). Approximately 1.5 μg of total RNA was used for cDNA synthesis using SuperScriptTM III Reverse Transcriptase and Oligo(dT)12–18 primers according to the manufacturer's protocol (Invitrogen, Breda, the Netherlands). PCR amplification of actin (with primer pair Actin2-F2 TAACTCTCCCGCTATGTATGTCGC, and Actin2-R2 GAGAGAAACCCTCGTAGATTGGC) and of PR-1 (with primer pair PR1-F1 AGGCTAACTACAACTACGCTGCG, and PR1-R1 GCTTCTCGTTCACATAATTCCCAC) consisted of an initial denaturing step at 94 °C for 5 min, followed by 30–35 cycles of 20 s at 94 °C, 20 s at 56 °C and 20 s at 72 °C, followed by a final extension step for 5 min at 72 °C. PCR products were visualized on ethidium bromide-stained 1% agarose gels.

Results

sgs *mutants display enhanced susceptibility towards* V. dahliae

Transgenic expression in the post-transcriptional gene silencing (PTGS) mutant *suppressor of gene silencing 2* (*sgs2*; Elmayan *et al.*, 1998; Mourrain *et al.*, 2000) reduces the inter-transformant variability of transgene expression (Butaye *et al.*, 2004). In several experiments to investigate putative defence genes against *V. dahliae* in *Arabidopsis*, transgenic overexpression in Col-0 as well as *sgs2-1* was performed. Remarkably, in subsequent disease susceptibility assays with *V. dahliae* strain JR2 it appeared that untransformed *sgs2-1* plants displayed more severe disease symptoms than Col-0 plants (Fig. 1A, B). While Col-0 plants displayed only mild disease symptoms upon *V. dahliae* inoculation as visualized by rather slight stunting resulting in a reduced rosette diameter at 3 weeks post-inoculation, inoculated *sgs2-1* plants showed severe stunting, wilting, anthocyanin accumulation, and tissue necrosis (Fig. 1A, B). The ratio of leaves displaying symptoms of disease was also significantly more for *sgs2-1* plants than for Col-0 plants (Fig. 1A, B)

In addition to *V. dahliae* strain JR2, our analysis was extended to include other *Verticillium* pathogens of *Arabidopsis* (Fradin and Thomma, 2006). These included *V. dahliae* strain ST12.01, the *V. albo-atrum* strains VA1 and CBS451.88, and *V. longisporum* strain Vl43. All these *Verticillium* strains caused more disease symptoms on *sgs2-1* plants when compared with Col-0 plants (see Supplementary Fig. S1 at *JXB* online), confirming that the enhanced susceptibility of the *sgs2-1* mutant broadly concerns plant pathogenic *Verticillium* species.

In addition to *sgs2-1*, reduced inter-transformant variability in transgene expression was similarly demonstrated in the non-allelic *sgs3-1* mutant (Butaye *et al.*, 2004). To investigate the role of PTGS in *Arabidopsis* defence against *Verticillium* further, the two additional non-allelic PTGS mutants; *sgs1-1* and *sgs3-1* (Elmayan *et al.*, 1998; Mourrain *et al.*, 2000), were tested for their susceptibility towards *V. dahliae* strain JR2.

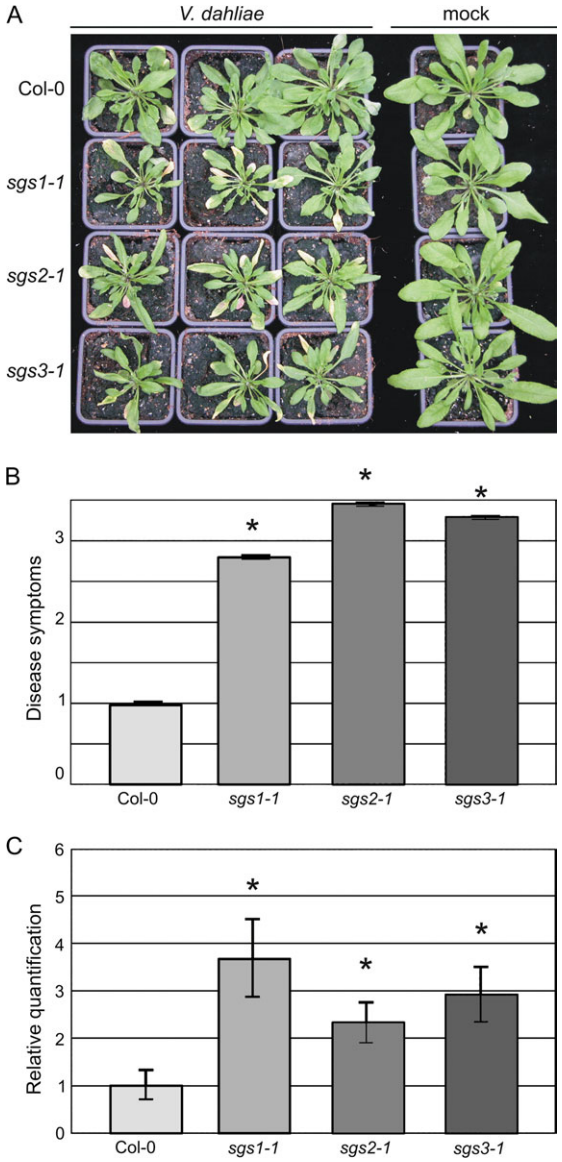

Fig. 1. *Arabidopsis sgs* mutants display enhanced susceptibility towards *Verticillium dahliae*. (A) Typical symptoms of *V. dahliae* on *Arabidopsis sgs* mutants. The mutants *sgs1-1*, *sgs2-1*, *sgs3-1*, and the corresponding wild type Col-0 were inoculated with *V. dahliae* strain JR2 or mock-inoculated. *V. dahliae*-inoculated *sgs* mutants show enhanced symptom development, including more severe stunting, wilting, anthocyanin accumulation, and tissue necrosis, when compared with Col-0 plants at 19 d post-inoculation. (B) Quantification of symptom development at 19 d post-inoculation shown as a ratio of diseased rosette leaves with standard deviation. The ratio of diseased rosette leaves for Col-0 is set to one. Asterisks indicate significant differences when compared with the wild type Col-0 (P <0.05). (C) Quantitative real-time PCR of fungal colonization by comparing *V. dahliae* internal transcribed spacer (ITS) transcript levels (as a measure for fungal biomass) relative to *Arabidopsis* Rubisco transcript levels (for equilibration) at 19 d post-inoculation. The mutants *sgs1-1*, *sgs2-1*, *sgs3-1*, and the corresponding wild type Col-0 were inoculated with *V. dahliae* strain JR2 and the relative average fungal biomass is shown with standard errors. Asterisks indicate significant differences when compared with colonization of the wild type Col-0.

Similar to the *sgs2-1* plants, *sgs1-1* and *sgs3-1* plants also consistently displayed enhanced disease development upon *V. dahliae* inoculation (Fig. 1A, B).

To quantify *V. dahliae* colonization in the different *Arabidopsis* genotypes, the fungal biomass was measured with real-time PCR. Determination of the average fungal biomass revealed significantly enhanced fungal colonization in *V. dahliae*-inoculated *sgs1-1*, *sgs2-1*, and *sgs3-1* plants when compared with the inoculated Col-0 plants (Fig. 1C), since at least double the amount of fungal biomass was detected in these mutants at 3 weeks post-inoculation.

sgs *mutants do not display enhanced susceptibility towards other pathogens*

To investigate whether the enhanced pathogen susceptibility phenotype of the *sgs* mutants extended to other pathogens in addition to *Verticillium* species, the susceptibility of the *sgs1-1*, *sgs2-1*, and *sgs3-1* mutants towards the vascular fungus *F. oxysporum* f.sp. *raphani* (Diener and Ausubel, 2005) was tested. However, disease development on the three *sgs* mutants did not differ from disease development on Col-0 plants upon inoculation with this pathogen (Fig. 2). Furthermore, a number of additional fungal and bacterial pathogens was tested on the *sgs* mutants (see Supplementary Table S1 at *JXB* online; Wang *et al.*, 2008). These comprised the foliar fungal pathogens *Botrytis cinerea*, *Alternaria brassicicola*, and *Plectosphaerella cucumerina*, and virulent and avirulent strains of the bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000. However, for none of these pathogens was altered susceptibility observed in the *sgs* mutants when compared with Col-0 (data not shown). Thus, the enhanced susceptibility of the *sgs* mutants is specific for *Verticillium* pathogens and does not extend to other pathogens.

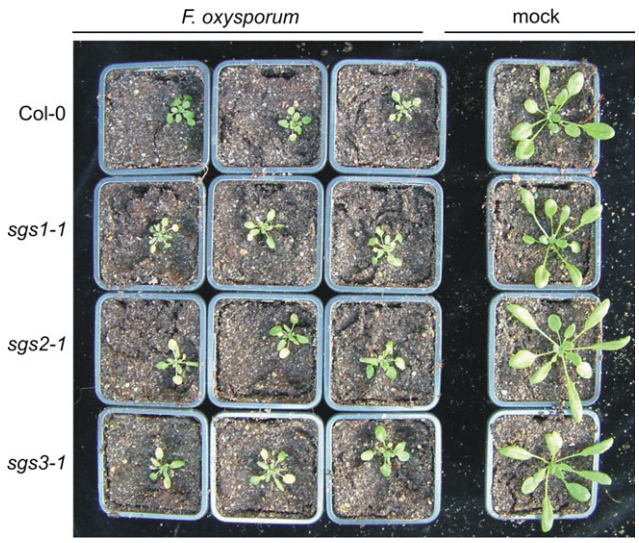

Fig. 2. Typical symptoms caused by *F. oxysporum* on *Arabidopsis sgs* mutants. The mutants *sgs1-1*, *sgs2-1*, *sgs3-1*, and the corresponding wild type Col-0 were inoculated with *F. oxysporum* f.sp. *raphani*, or mock-inoculated. The picture was taken at 12 d post-inoculation.

sgs *mutants do not display altered sensitivity towards abiotic stress*

RNA silencing has also been implicated in abiotic stress resistance (Borsani *et al.*, 2005; Sunkar *et al.*, 2007). Therefore, the *sgs* mutants were screened for their responses towards treatment with different hormones (abscisic acid, auxin, brassinolide, cytokinin, ethylene, gibberellic acid, and jasmonate) and sensitivity towards salt, heavy metal, reactive oxygen, and osmotic stress (see Supplementary Table S1 at *JXB* online; Wang *et al.*, 2008). However, none of the *sgs* mutants showed significantly altered phenotypes towards these treatments when compared with Col-0 plants (data not shown).

Assessment of Verticillium *susceptibility in additional gene-silencing mutants*

The enhanced susceptibility phenotype of the *sgs* mutants upon *Verticillium* inoculation directed us to assess susceptibility towards this pathogen in additional gene-silencing mutants. These comprised additional mutant alleles of *SGS2* (also known as *RDR6*), namely *rdr6-11* and *rdr6-15*, and for *SGS3*, namely *sgs3-11*. Furthermore, mutants of other components of RNA-silencing pathways were also included (Table 1). These included mutants of genes that encode different enzyme families, such as the argonautes AGO1 and AGO7, the dicers DCL2 and DCL4, the methyltransferase HEN1, the putative sRNA transporter HST, the DNA-dependent RNA polymerase NRPD1a, the RNA-dependent RNA polymerase RDR2, and the RNA helicase SDE3 that all have been implicated in different RNA-silencing pathways (Table 1; Voinnet, 2008). All mutants, derived from a Col-0 parental line, were challenged with *V. dahliae* strain JR2. As expected, additional mutant alleles of *SGS2* and *SGS3* (*rdr6-11, rdr6-15*, and *sgs3-11*) were more susceptible than Col-0 plants upon *V. dahliae* inoculation (Fig. 3A), thus confirming the enhanced susceptibility observed in the *sgs2-1* and *sgs3-1* mutants. The other PTGS mutants could be divided into three classes, based on the phenotypes after *V. dahliae* inoculation; those displaying enhanced susceptibility (Fig. 3A), mutants displaying enhanced resistance (Fig. 3B), and mutants displaying similar disease phenotypes as *Verticillium*-inoculated Col-0 plants (Fig. 3C). The mutants *ago7-2*, *dcl4-2, nrpd1a-3*, and *rdr2-4* were found to be more susceptible to *V. dahliae* challenge by showing more severe stunting and necrosis when compared with inoculated Col-0 plants (Fig. 3A; see Supplementary Fig. S2 at *JXB* online). By contrast, the mutants *ago1-25*, *ago1-27*, *hen1-6*, and *hst-1* were found to be more resistant because they displayed less necrosis and no anthocyanin production when compared with Col-0 plants upon *V. dahliae* inoculation (Fig. 3B; see Supplementary Fig. S2 at *JXB* online). Finally, the mutants *dcl2-1*, *sde3-4*, and *sde3-5* showed a disease susceptibility phenotype that was similar to that of Col-0 with respect to

Table 1. Arabidopsis mutants used in this study

Gene name	AGI code	Protein function	Mutant allele	Reference
AGO1	At1g48410	slicer in RISC	*ago1-25*	Morel *et al.*, 2002
			ago1-27	Morel *et al.*, 2002
AGO7	At1g69440	slicer in RISC	*ago7-2*	SALK_095997[a]
DCL2	At3g03300	dicer	*dcl2-1*	Xie *et al.*, 2004
DCL4	At5g20320	dicer	*dcl4-2*	Yoshikawa *et al.*, 2005
HEN1	At4g20910	methyltransferase	*hen1-6*	Li *et al.*, 2005
HST	At3g05040	transporter	*hst-1*	Telfer and Poethig, 1998
NRPD1a/SDE4	At1g63020	polymerase	*nrpd1a-3*	Herr *et al.*, 2005
RDR2	At4g11130	RDR	*rdr2-4*	Smith *et al.*, 2007
RDR6/SDE1/SGS2	At3g49500	RDR	*sgs2-1*	Elmayan *et al.*, 1998
			rdr6-11	Peragine *et al.*, 2004
			rdr6-15	Allen *et al.*, 2004
SDE3	At1g05460	RNA helicase	*sde3-4*	Vazquez *et al.*, 2004*b*
			sde3-5	SALK_003347[a]
SGS1	Unknown	Unknown	*sgs1-1*	Elmayan *et al.*, 1998
SGS3/SDE2	At5g23570	CC-domain	*sgs3-1*	Mourrain *et al.*, 2000
		protein	*sgs3-11*	Peragine *et al.*, 2004

[a] SALK T-DNA insertion mutant (Alonso *et al.*, 2003).

severity of stunting, necrosis, and anthocyanin production (Fig. 3C; see Supplementary Fig. S2 at *JXB* online).

Quantification of V. dahliae *biomass* in planta

To quantify *V. dahliae* colonization in the different *Arabidopsis* genotypes, the fungal biomass was measured in individual plants with real-time PCR. For each of the genes tested, the average fungal colonization of at least one mutant allele was quantified with real-time PCR. This analysis demonstrated that the altered susceptibility phenotypes correlated with the degree of fungal colonization when compared with inoculated Col-0 plants (Table 2). The mutants displaying enhanced symptoms upon *Verticillium* inoculation (*sgs1-1*, *sgs2-1*, *sgs3-1*, *ago7-2*, *dcl4-2, nrpd1a-3, rdr2-4*, and *rdr6-15*) accumulated significantly more fungal biomass when compared with inoculated Col-0 plants, while the mutants that showed reduced symptom development (*ago1-27*, *hen1-6*, and *hst-1*) accumulated significantly less fungal biomass. By contrast, fungal biomass accumulation in *Verticillium*-inoculated *dcl2-1* and *sde3-4* plants was not significant different from that of inoculated Col-0 plants (Table 2).

Assessment of root development and architecture

Being a root pathogen, differences in *Verticillium* susceptibility of the different mutants may be explained by differences in root architecture, the tissues that are inoculated. Although no obvious differences in root architecture were observed during uprooting and inoculation of the mutants, except for the *ago* mutants that had developed shorter roots, root development and architecture was assessed upon *in vitro* growth on MS medium. However, apart from rather slight growth differences, no notable differences in root development and architecture were observed for the RNA-silencing mutants that correlated with the differences in *Verticillium* susceptibility (Fig. 4). For all mutants, development of the primary, dominant, root was followed by production of lateral roots in a later stage.

Assessment of basal defence responses

To investigate whether the altered *Verticillium* susceptibility phenotypes of the various PTGS mutants can be explained by defects in basal defence signalling pathways, the expression of molecular markers for salicylic acid- (SA) and jasmonic acid- (JA) mediated defence response pathways was assessed. Expression of the SA marker gene *PR-1* (Uknes *et al.*, 1992) was clearly induced in Col-0 plants as well as in all PTGS mutants at 24 h after drop-inoculation with 2 mM SA (see Supplementary Fig. 3 at *JXB* online). In non-treated plants, little or no *PR-1* expression was monitored in these genotypes (data not shown). Thus, the altered susceptibility phenotypes could not be correlated to changes in SA-mediated defence responses. Similarly, the expression patterns of the JA-marker *PDF1.2* (Penninckx *et al.*, 1996; Thomma *et al.*, 1998) and the chitin elicitor-responsive marker *MPK3* (Wan *et al.*, 2008) could not be correlated to the altered susceptibility phenotypes (data not shown).

Discussion

Recent evidence indicates that, apart from defence against viruses, RNA silencing plays a role in defence against bacterial pathogens (Voinnet, 2008), and that, similar to viruses, bacteria have also developed mechanisms to suppress RNA silencing in order to cause disease (Navarro *et al.*, 2008). It is shown here that RNA silencing is also important for defence against multicellular, eukaryotic,

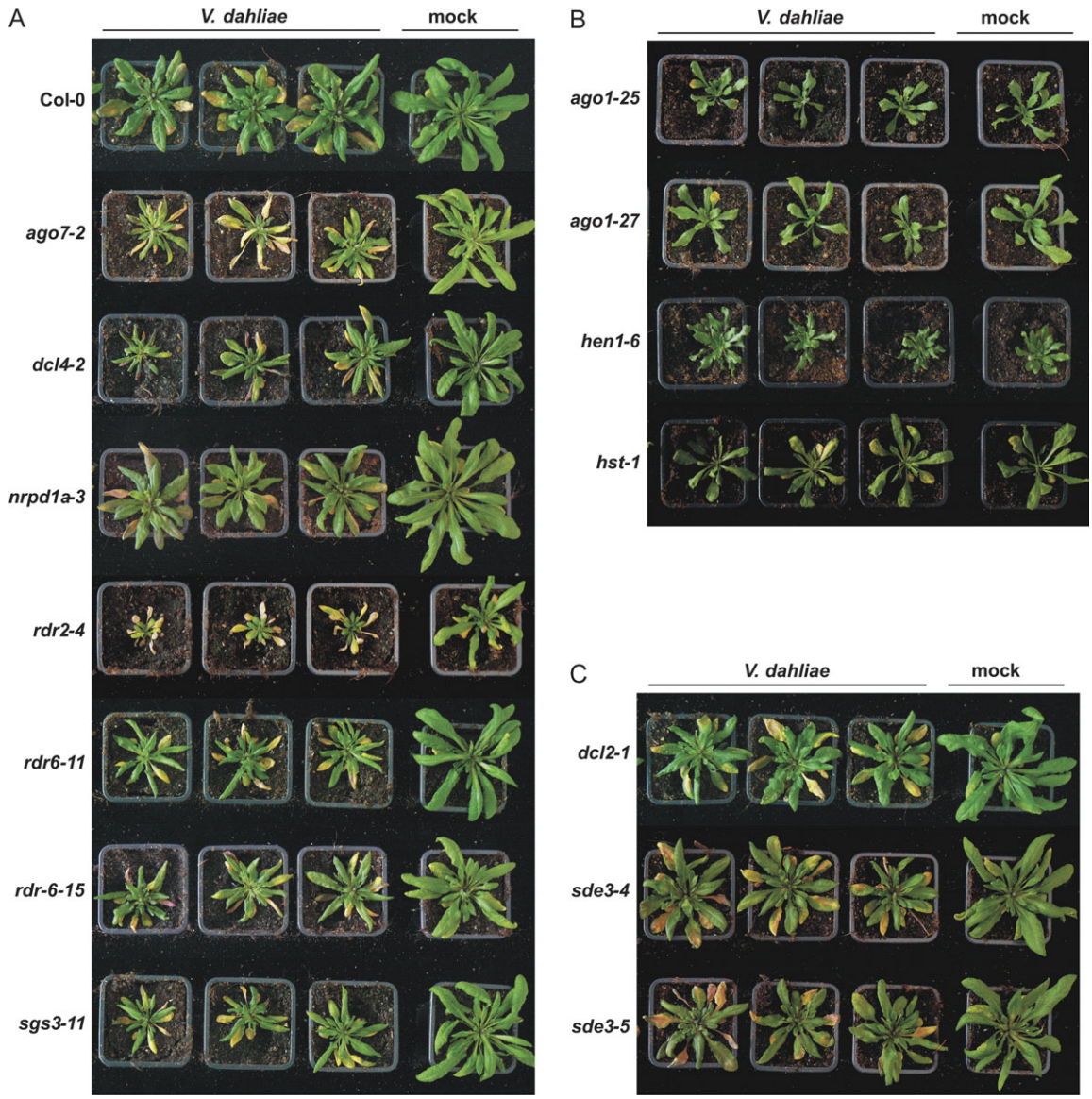

Fig. 3. Typical symptoms caused by *V. dahliae* on various *Arabidopsis* silencing mutants. *Arabidopsis* gene-silencing mutants and the corresponding wild type Col-0 were inoculated with *V. dahliae* strain JR2, or mock-inoculated. (A) *V. dahliae*-inoculated *ago7-2*, *dcl4-2, rdr6-11, rdr6-15,* and *sgs3-11* plants show enhanced symptom development, including more severe stunting, wilting, anthocyanin accumulation, and tissue necrosis, compared with inoculated Col-0 plants at 20 d post-inoculation. (B). *V. dahliae*-inoculated *ago1-25*, *ago1-27*, *hen1-6*, and *hst-1* mutants develop fewer symptoms than inoculated Col-0 plants (A) at 25 d post-inoculation. (C) *V. dahliae*-inoculated *dcl2-1*, *sde3-4*, and *sde3-5* mutants show similar disease symptoms as inoculated Col-0 plants (A) at 20 d post-inoculation.

microbial pathogens, namely vascular fungi of the *Verticillium* genus. These include strains of the species *V. dahliae*, *V. albo-atrum*, and *V. longisporum* that are all pathogenic on *Arabidopsis* (Fradin and Thomma, 2006). Various components of RNA-silencing pathways were tested and most of them were found to affect *Verticillium* resistance, some positively and others negatively. Furthermore, our results show that PTGS is truly affecting *Verticillium* resistance and not merely symptom development or display, since altered symptom development of the *Verticillium* inoculated RNA-silencing mutants correlated with altered *Verticillium* colonization in these mutants as shown by real-time PCR-based fungal biomass quantification (Table 2).

The altered susceptibility phenotypes of the RNA-silencing mutants is specific for *Verticillium* defence as shown for the *sgs* mutants. Inoculation of the *sgs* mutants with strains belonging to different pathogenic species of the *Verticillium* genus all resulted in a similar increased susceptibility phenotype. Inoculations with other pathogens that use different colonization and feeding styles did not show altered susceptibility phenotypes. This suggests that the enhanced susceptibility is not due to defects in any of the well-known basal defence signalling pathways (Thomma *et al.*, 2001*a*). Indeed, in our analysis it was not possible to correlate altered susceptibility to SA or JA signalling. However, this is not surprising because alterations in these

Table 2. Quantification of *Verticillium dahliae* biomass in *Arabidopsis* gene-silencing mutants by real-time PCR comparison of *V. dahliae* internal transcribed spacer (ITS) transcript levels (as a measure for fungal biomass) relative to *Arabidopsis* RuBisCo transcript levels (for equilibration) at 19–29 d post-inoculation with *V. dahliae* strain JR2

Gene name	Genotype	Symptom display[a]	Biomass fold change[b]	Significance[c]
	Col-0	–	1	–
AGO1	*ago1-27*	Reduced	0.007	*P* <0.1
AGO7	*ago7-2*	Enhanced	3.174	*P* <0.2
DCL2	*dcl2-1*	Similar	0.829	No
DCL4	*dcl4-2*	Enhanced	2.422	*P* <0.05
HEN1	*hen1-6*	Reduced	0.045	*P* <0.1
HST	*hst1-1*	Reduced	0.039	*P* <0.05
NRPD1a/SDE4	*nrpd1a-3*	Enhanced	1.816	*P* <0.2
RDR2	*rdr2-4*	Enhanced	2.701	*P* <0.05
RDR6/SDE1/SGS2	*sgs2-1*	Enhanced	2.279	*P* <0.05
	rdr6-15	Enhanced	3.286	*P* <0.05
SDE3	*sde3-4*	Similar	1.674	No
SGS1	*sgs1-1*	Enhanced	3.729	*P* <0.05
SGS3/SDE2	*sgs3-1*	Enhanced	2.938	*P* <0.05

[a] Symptom display upon *V. dahliae* inoculation when compared with Col-0 (also see Fig. 3).
[b] The relative average fungal biomass is indicated as relative fold-change when compared with fungal biomass in *V. dahliae*-inoculated Col-0 plants of which the average fungal biomass was set to one.
[c] Statistically significant differences are given as *P*-values according to a Student's *t* test with a 95% to an 80% confidence interval (*P* <0.05–0.2).

Fig. 4. Typical root architecture of *in vitro*-grown *Arabidopsis* gene silencing mutants. Roots were grown on vertically oriented MS plates and pictures were taken 10 d after sowing.

basal defence responses would most likely be reflected in altered susceptibility towards some of the other pathogens that were tested. For instance, altered SA signalling would most likely lead to altered susceptibility towards *P. syringae* and *P. cucumerina*, while altered JA-signalling would be reflected in *A. brassicicola* and *B. cinerea* resistance (Thomma *et al.*, 1998, 2001*a*, *b*). Our assays also included the vascular fungal pathogen *F. oxysporum* f sp. *raphani* that displays a similar life-style to *Verticillium* spp. Both *F. oxysporum* and *Verticillium* spp infect plants through the roots and enter the xylem where they release conidia that spread upwards through the vessels with the transpiration stream (Di Pietro *et al.*, 2001; Fradin and Thomma, 2006; Berrocal-Lobo and Molina, 2008). Despite these similarities in host colonization, the susceptibility of the RNA silencing mutants is specific towards *Verticillium* spp, suggesting that a highly specific disease mechanism is affected in these mutants. Since the different RNA-silencing mutants did not show obvious alterations in root development or architecture that correlated with the altered susceptibility phenotypes, this mechanism could not be linked to root development.

In contrast to *SGS1*, both *SGS2* (also known as *RDR6* and *SDE1*) and *SGS3* were cloned and found to encode an RNA-dependent RNA polymerase (RDR) and a protein of unknown function, respectively. SGS2 and SGS3 are required for the synthesis of dsRNA in different RNA-silencing pathways (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Brodersen and Voinnet, 2006; Vaucheret, 2006). Furthermore, our analysis comprised mutants for the argonautes AGO1 and AGO7, the dicers DCL2 and DCL4, the methyltransferase HEN1, the putative sRNA transporter HST, the DNA-dependent RNA polymerase NRPD1a, the RNA-dependent RNA polymerase RDR2, and the RNA helicase SDE3, all of which have been implicated in different RNA-silencing pathways and regulate processes including TGS, PTGS, antiviral defence,

plant development, hormone signalling, and abiotic and biotic stress tolerance (Brodersen and Voinnet, 2006; Vaucheret, 2006; Voinnet, 2008). While HEN1 methylates small RNA species and thus protects these sRNAs from degradation and polyuridylation (Chen *et al.*, 2002; Li *et al.*, 2005; Yu *et al.*, 2005), HST possibly mediates the transport of miRNAs from the nucleus to the cytoplasm (Mallory and Vaucheret, 2006; Sunkar *et al.*, 2007). SDE3 acts as an RNA helicase and may facilitate the synthesis of dsRNA by SGS2/RDR6/SDE1 (Dalmay *et al.*, 2001). Although its precise function is unclear, NRPD1a is suggested to be a silencing-specific polymerase (Herr *et al.*, 2005). In this study, as many as ten different RNA-silencing components, namely AGO7, DCL4, NRPD1a, RDR2, SGS1, SGS2/RDR6/SDE1, SGS3, AGO1, HEN1, and HST were all shown to affect *Verticillium* defence.

The combination of RNA-silencing components that is involved in altered *Verticillium* susceptibility does not comply with one single RNA-silencing pathway among those that are currently discriminated. However, the identification and full characterization of such pathways is still in its infancy. Defence against *Verticillium* might trigger a novel RNA-silencing pathway that is similar to the natural *cis*-antisense transcript-derived siRNA (nat-siRNAs) pathway that is induced upon stresses including bacterial infection (Borsani *et al.*, 2005; Wang *et al.*, 2005; Katiyar-Agarwal *et al.*, 2006). In this case siRNAs might be specifically produced upon induction of NATs by the action of RDR6/SGS2/SDE1, SGS3 NRPD1a3, RDR2, and DCL4 and incorporated in AGO7 to trigger a defence response by repression of AGO1, HEN1, and HST. Alternatively, the observed phenomena may be the result of the cross-interaction of multiple RNA-silencing pathways that influence the defence response. Furthermore, the presence of ten AGOs, four DCLs and six RDRs in *Arabidopsis* (Morel *et al.*, 2002; Schauer *et al.*, 2002; Yu *et al.*, 2003) may reflect the versatility of these components in RNA-silencing pathways.

Whatever the exact pathway that is involved, it is likely that RNA silencing is involved either in a highly specific defence response against *Verticillium* pathogens or, alternatively, is involved in a developmental cue that is of particular importance for *Verticillium* infections. Interestingly, it was recently demonstrated that inoculation of *Arabidopsis* with non-pathogenic *P. syringae* that triggers a robust basal defence response in *Arabidopsis* leads to altered accumulation of several microRNAs, including those targeting multiple components of auxin signalling pathways (Fahlgren *et al.*, 2007). Furthermore, it was recently suggested that the transcriptional regulation of resistance gene loci may be under the control of RNA silencing, as demonstrated for the *RPP5*-locus for recognition of the oomycete downy mildew pathogen *Peronospora parasitica* (Yi and Richards, 2007). This demonstrates that RNA silencing may affect diverse pathogens by regulating various modulators of host defence (Voinnet, 2008). Relatively little is known about the biology of vascular wilt diseases, and processes that are involved in defence against these pathogens (Fradin and Thomma, 2006). This makes it difficult to identify the physiological process that is affected in the RNA-silencing mutants and that explains the observed disease phenotypes. It is possible that microarray analyses on inoculated wild-type plants and RNA-silencing mutants will facilitate the identification of this process. However, the main challenge will be to identify the small RNAs that are the basis of the altered *Verticillium* susceptibility in these mutants.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. Conditional phenotype assays for *sgs1-1*, *sgs2-1*, and *sgs3-1* mutants.

Supplementary Fig. S1. Typical symptoms of *Arabidopsis sgs2-1* mutants upon inoculation with plant pathogenic *Verticillium* species.

Supplementary Fig. S2. Quantification of symptom development at 20 dpi shown as the ratio of diseased rosette leaves with standard deviation.

Supplementary Fig. S3. Salicylic acid-induced *PR-1* expression in *Arabidopsis* gene silencing mutants.

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