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Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*

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Sclerotinia sclerotiorum is a necrotrophic fungus that causes a devastating disease called white mould, infecting more than 450 plant species worldwide. Control of this disease with fungicides is limited, so host plant resistance is the preferred alternative for disease management. However, due to the nature of the disease, breeding programmes have had limited success. A potential alternative to developing necrotrophic fungal resistance is the use of host-induced gene silencing (HIGS) methods, which involves host expression of dsRNA-generating constructs directed against genes in the pathogen. In this study, the target gene chosen was chitin synthase (*chs*), which commands the synthesis of chitin, the polysaccharide that is a crucial structural component of the cell walls of many fungi. Tobacco plants were transformed with an interfering intron-containing hairpin RNA construct for silencing the fungal *chs* gene. Seventy-two hours after inoculation, five transgenic lines showed a reduction in disease severity ranging from 55·5 to 86·7% compared with the non-transgenic lines. The lesion area did not show extensive progress over this time (up to 120 h). Disease resistance and silencing of the fungal *chs* gene was positively correlated with the presence of detectable siRNA in the transgenic lines. It was demonstrated that expression of endogenous genes from the very aggressive necrotrophic fungus *S. sclerotiorum* could be prevented by host induced silencing. HIGS of the fungal chitin synthase gene can generate white mould-tolerant plants. From a biotechnological perspective, these results open new prospects for the development of transgenic plants resistant to necrotrophic fungal pathogens.

Keywords: fungus resistance, gene silencing, HIGS, *Sclerotinia sclerotiorum*, white mould

Introduction

Sclerotinia sclerotiorum is a necrotrophic ascomycete fungus that causes a worldwide and devastating disease called white mould. The fungus infects more than 450 plant species, including important crops such as canola, potato, cotton, tomato, sunflower, common bean and soybean (Bolton *et al.*, 2006).

Effective control of white mould with fungicides is difficult due to low penetration and uneven distribution (Schwartz & Singh, 2013). Additionally, the use of fungicides increases production costs and has environmental impacts. Therefore, fungicide control should be combined with disease management based on good agronomic practices, such as planting under tillage, use of lodging resistant varieties, wide-row planting, and rotation with non-host crops, irrigation water management and biological control. In favourable environmental conditions for pathogen development, these practices are not sufficient to control disease. In addition, the fungus is seed-transmitted and produces sclerotia (hardened dense mycelial bodies) that survive in soil for years, awaiting the appropriate conditions for germination (Schwartz & Steadman, 1989). Thus, use of adequate levels of host plant resistance is the best alternative for white mould management (Disi *et al.*, 2014). Due to the nature of the disease, breeding programmes have had limited success. Genetic resistance to *S. sclerotiorum* is complex, has low heritability and is restricted to a few lines that have shown only partial resistance (Disi *et al.*, 2014). In addition, the plant escape mechanisms and environmental conditions make white mould resistance evaluations difficult in the field. It may be difficult to discriminate between physiological resistance and plant architectural disease avoidance because these are easily confused under field conditions (Miklas & Grafton, 1992).

A potential alternative to developing white mould-resistant plants is the development of genetic engineering strategies by expressing or silencing genes involved in plant–*Sclerotinia* interactions. The main strategy used so far consists of the expression of genes encoding enzymes capable of degrading oxalic acid, such as oxalate oxidases and oxalate decarboxylases, which have produced enhanced resistance to *S. sclerotiorum* (Kesarwani *et al.*, 2000; Hu *et al.*, 2003; Dias *et al.*, 2006; Dong *et al.*, 2008; Cunha *et al.*, 2010). Another alternative is the use of host-induced gene silencing (HIGS) methods, which exploit the silencing effect of small interfering RNA

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(siRNA) signals in interacting organisms, and involves host expression of siRNA-generating constructs directed against genes in associated pathogens, parasites or symbionts (Knip *et al.*, 2014). It has been demonstrated that large and small RNA molecules are mobile within organisms, and several cases in which RNA-silencing signals travel between different organisms have been described (Kim *et al.*, 2014; for a review see Nunes & Dean, 2012). Cross-species transfer of RNA-silencing signals have been reported from plants to pathogenic and symbiotic microbes, from plants to nematodes (Lilley *et al.*, 2012), from fungal pathogens to plants (Nunes & Dean, 2012), and from plants to insects (Zhang *et al.*, 2013). Tinoco *et al.* (2010) demonstrated, for the first time, the *in vivo* interference phenomenon in the pathogenic fungus *Fusarium verticillioides*, by inoculating mycelial cells into transgenic tobacco plants engineered to express siRNAs from a dsRNA corresponding to a particular fungal transgene, so specifically abolishing expression of that transgene. HIGS strategies are now being explored to obtain stable and transient expression of siRNA transgenes in order to achieve resistance to biotrophic fungi, such as *Blumeria graminis* in barley and wheat (Nowara *et al.*, 2010), *Puccinia striiformis* in wheat (Yin *et al.*, 2011), *Puccinia triticina* in wheat (Panwar *et al.*, 2013), *Fusarium oxysporum* in banana (Ghag *et al.*, 2014) and *Bremia lactucae* in lettuce (Govindarajulu *et al.*, 2015).

The examples of HIGS reported so far corroborate the hypothesis that small RNA molecules are able to move from plants into fungal cells and effectively silence their target genes. Nevertheless, the pathogenic fungi tested so far have a long-term biotrophic phase, establishing conditions for a longer feeding relationship with a living host plant cell, allowing the nutrient-absorbing cells to take up double-stranded RNA molecules from their host. The aim of the present study was to investigate whether HIGS could be used to silence an endogenous gene in *S. sclerotiorum*, a necrotrophic plant pathogen, in which the virulence process proceeds faster to the transition from the initial symptomless phase to the necrotrophic phase. The target gene chosen was chitin synthase (*chs*), which commands the synthesis of chitin, the polysaccharide that is a crucial structural component of the cell walls of many fungi.

Materials and methods

RNAi construct and plant transformation

A fragment containing a partial sequence from the *chs* coding sequence from *F. verticillioides* was amplified by PCR. Primer pair ChirnaiF (5′-AGGGCCCGGTACCTTCAGCGTATGCC-3′) and ChirnaiR (5′-AGAGCTCTCTAGAGGCCAATCGAGGG-3′) (including the sites for *Xba*I/*Sac*I and *Kpn*I/*Apa*I (underlined), respectively) was used to amplify a 494 bp fragment (position 624–1117 in the GenBank accession number GU066261.1) from the *chs* gene. The *F. verticillioides* gene has 67% similarity with the *S. sclerotiorum* chitin synthase gene (*Sschs*) (GenBank accession number XM_001584969.1). PCR reactions were carried out in a thermocycler (MyCycler; Bio-Rad) in a 50 μL solution containing 40 ng cDNA, 60 mM Tris-SO_4 (pH 8·9), 18 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 250 nM each dNTP, 200 nM each primer, and 5 U Platinum *Taq* DNA polymerase (Invitrogen). The mixture was treated at 94°C (1 min) and subjected to 40 cycles of amplification (94°C for 1 min, 60°C for 1 min, and 68°C for 1 min), with a final elongation cycle of 7 min at 68°C. The 521 bp fragment (*chs* sequence plus enzyme sites) was cloned into the pGEMT-Easy vector (Promega Corp.) and sequenced by using universal M13 and T7 primers on an automatic sequencer (ABI Prism1 3700). The *chs* gene fragment was excised from pGEMT-Easy and inserted in sense (with *Xba*I and *Kpn*I) and antisense (with *Sac*I and *Apa*I) orientations into the vector pSIU (Tinoco *et al.*, 2010) to generate the plasmid pSIUSynchit2. The interfering cassette was removed with *Eco*RI and *Hin*dIII from pSIUSynchit2 and cloned into the vector pCAMBIA1301 (CAMBIA Company; GenBank accession number AF234297.1), generating pCAMBIASynchit2, which was used to transfect *Agrobacterium tumefaciens* LBA4404 by electroporation. The plasmid pCAMBIASynchit2 was used for tobacco transformation (*Nicotiana tabacum* 'Xanthi') as previously described by Horsch *et al.* (1985). The cassette containing the *chs* sequence hairpin will be referred to as Δ*chs* (Fig. 1a).

Screening of transgenic plants by PCR

DNA was isolated from leaf disks according to Edwards *et al.* (1991) and PCR was carried out according to Bonfim *et al.* (2007). The primer pair ChirnaiF/ChirnaiR within the Δ*chs* cassette was used to amplify a 521 bp sequence.

Progeny analysis

Seeds of the first generation (T_1) of self-pollinated plants were germinated and analysed for the presence of the Δ*chs* as

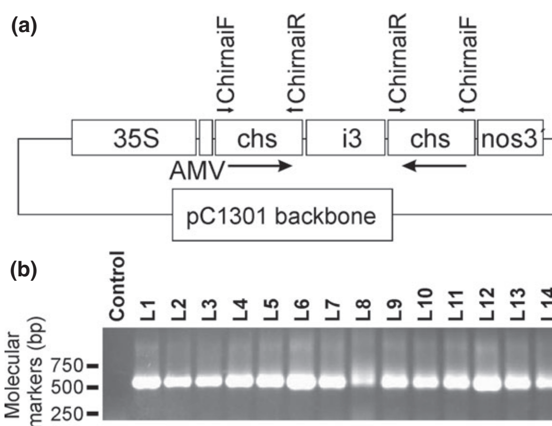

Figure 1 (a) Diagram representing the construct for expressing dsRNA in transgenic tobacco within the pCAMBIASynchit2 plasmid. The intron-hairpin interfering cassette (Δ*chs*) is under the control of the RNA35S promoter of *Cauliflower mosaic virus* (35S) and the enhancer sequence from *Alfalfa mosaic virus* (AMV). The 494 bp fragment of the chitin synthase gene coding sequence from *Fusarium verticillioides* (*chs*) was directionally cloned in order to generate sense and antisense arms flanking the malate synthase gene intron 3 from *Arabidopsis thaliana* (i3). The cassette was cloned into the pCAMBIA1301 vector (pC1301), generating pCAMBIASynchit2, which was used to transform tobacco. (b) PCR analysis of transformed lines (L1 to L14) for detection of the *chs* sequence (using the primer pair ChirnaiF/ChirnaiR). Control is a non-transgenic plant.

described above and also for GUS gene expression using the histochemical assay according to Jefferson *et al.* (1987). Chi-square analyses, using the correction factor of Yates (Steel & Torrie, 1980), were performed to determine whether or not the observed segregation ratio was consistent with a Mendelian ratio (3:1 or 15:1), with a 95% level of confidence.

Plant inoculation with *S. sclerotiorum* and symptom evaluation

The detached leaf assay was carried out according to Cunha *et al.* (2010) using plants from the T_1 generation. One or two mycelial agar plugs were applied to the surface of third or fourth fully expanded leaves. Non-transgenic plants and plants transformed with the vector pCAMBIA1301 (without the Δ*chs* cassette) were used as controls. Sclerotia were collected from an infected bean field located in Goiás (Brazil) and cultured on potato dextrose agar medium at room temperature to produce mycelia. Leaves were photographed at 48 and 72 h after fungus inoculation, and images were used to measure the infected area using QUANT v. 1.0.1 software (Vale *et al.*, 2003). The means of the lesion areas were grouped by the Scott & Knott (1974) test, at 5% probability (using R v. 3.1.2; www.R-project.org/). Experiments were repeated twice with four repetitions.

Northern blot analysis of siRNA

Total RNA was isolated from tobacco leaves (T_2 generation) according to Aragão *et al.* (2013). Northern blot analysis of siRNA was carried out according to Tinoco *et al.* (2010). Hybridization was carried out with a DNA probe, corresponding to the PCR fragment amplified using the primer pair ChirnaiF/ChirnaiR, labelled with [α^{32}P] dCTP using a random primer DNA labelling kit (Amersham Pharmacia Biotech), according to the manufacturer's instructions. Three oligomers (18, 24 and 44 nucleotides) were used as molecular size markers. The bands were visualized with a fluorescent image analyser (FLA-3000; Fujifilm).

Quantitative real-time PCR

The transcription levels of the endogenous *Sschs* gene in *S. sclerotiorum* mycelia interacting with transgenic leaves (T_2 generation) were quantified by quantitative reverse transcription (RT)-PCR. Leaves were inoculated with a mycelial disc as previously described. Ninety-six hours after inoculation, the growing mycelia were removed and total RNA was isolated by extraction with Trizol (Invitrogen), as recommended by the manufacturer. The remaining genomic DNA was eliminated by DNase digestion of the RNA samples. Total RNA (2 μg) was used to produce cDNA using the reverse transcriptase Superscript III (Promega), according to the protocol suggested by the manufacturer. PCR was performed in an Applied Biosystems 7300 real-time PCR system using SYBR Green (Stratagene), according to the manufacturer's instructions. The amplification programme was 94°C for 15 s, 58°C for 15 s and 72°C for 15 s. The primers for the endogenous *Sschs* (5′-TCGTATCTGGGTCGCTATCA-3′ and 5′-TGCGTACATC
TGGACGTTTC-3′; product size 95 bp; XM001584969.1) and *Ssα-tubulin* (5′-GTCGGTGAAGGTATGGAAGAAG-3′ and 5′-CCTCTTCAGCCTCAAAGCTATC-3′; product size 109 bp; XM001597572.1) genes were designed using the PRIMERQUEST tool (IDT Integrated DNA Technologies, Inc.). Products from the PCR reaction were separated by agarose gel electrophoresis and sequenced to confirm the identity of the products. The relative *Sschs* transcription levels in different RNA samples were normalized with respect to the internal standard *Ssα-tubulin* gene. Triplicate quantitative assays were performed on each

Table 1 Segregation analyses and disease progress of transgenic tobacco lines and controls after inoculation with a *Sclerotinia sclerotiorum* mycelial agar plug

Line	Lesion area (cm^2)[a]		T_1 generation[b]		Segregation ratio tested	χ^2	P[c]
	48 h	72 h	Positive	Negative			
L13	6·35 ± 3·12 a	20·00 ± 5·20 a	13	7	3:1	0·87	0·30
L8	5·15 ± 1·41 a	19·13 ± 4·03 a	18	2	15:1	0·13	0·49
L12	7·19 ± 3·01 a	19·04 ± 5·03 a	18	2	15:1	0·13	0·49
L9	5·31 ± 2·16 a	15·99 ± 2·66 a	16	4	3:1	0·47	0·61
L11	4·52 ± 0·34 a	14·13 ± 1·24 a	13	7	3:1	0·87	0·30
L10	3·92 ± 0·35 a	13·32 ± 2·17 a	14	6	3:1	0·20	0·61
L14	3·67 ± 0·32 a	12·71 ± 2·09 a	18	2	15:1	0·13	0·49
L2	2·88 ± 0·86 a	11·95 ± 1·08 a	6	14	3:1	20·47	0·00
L1	2·65 ± 0·07 a	11·20 ± 0·12 a	4	16	3:1	30·87	0·00
L4	2·22 ± 0·16 a	7·45 ± 1·84 b	6	14	3:1	20·47	0·00
L5	1·64 ± 0·85 a	3·49 ± 1·55 b	18	2	15:1	0·13	0·49
L6	1·10 ± 0·12 a	2·76 ± 0·21 b	16	4	3:1	0·47	0·61
L3	1·02 ± 0·07 a	2·65 ± 0·37 b	3	17	3:1	36·87	0·00
L7	1·39 ± 0·59 a	2·23 ± 0·51 b	18	2	15:1	0·13	0·49
82C[d]	4·02 ± 1·34 a	14·47 ± 1·87 a					
Control[e]	5·10 ± 2·37 a	16·76 ± 2·83 a					

[a]Means followed by identical letters, in the columns, do not differ by the Scott–Knott test, at 5% probability. $n = 4$.
[b]Data are based on PCR analyses for detection of the Δ*chs* cassette and a GUS histochemical assay.
[c]*P*, probability that observed ratios reflect expected segregation ratio of 3:1 or 15:1.
[d]Plants transformed with pCAMBIA1301 without the Δ*chs* cassette.
[e]Non-transgenic plants.

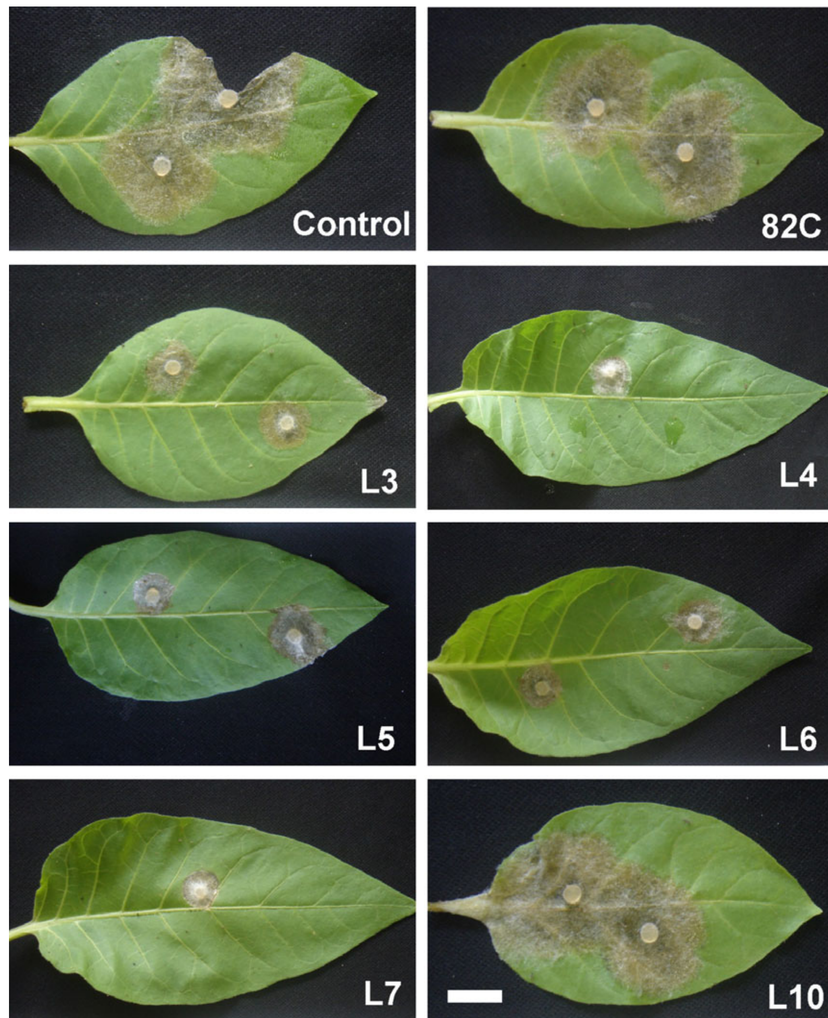

Figure 2 Resistance response of transgenic tobacco plants to *Sclerotinia sclerotiorum* inoculation. Symptoms observed on detached leaves 72 h after inoculation of transgenic and non-transgenic (control) lines with 5-mm mycelial agar plugs. The line 82C is transformed with pCAMBIA1301 without the Δ*chs* cassette. Bar = 2 cm.

cDNA sample. The relative level of expression was calculated using the Livak method (Livak & Schmittgen, 2001). Results were compared by one-way ANOVA with Dunnett's post hoc test ($\alpha = 0{\cdot}05$) to analyse the differences between isolates that interacted with each transgenic plant line event, compared to the control using R v. 3.1.2.

Results

Tobacco plants were transformed with an interfering intron-hairpin construct (Fig. 1a) to express dsRNA molecules corresponding to the chitin synthase gene. Plants were generated to verify if this construction would trigger RNA silencing of the gene in a necrotrophic fungus, resulting in disease severity reduction or suppression. PCR analyses revealed the presence of the Δ*chs* cassette in 14 regenerated lines (Fig. 1b) and these lines were rooted, acclimatized and presented normal phenotypes when compared with non-transgenic plants. All acclimatized transgenic lines (T_0 generation) were allowed to produce seeds and segregation analyses revealed that the transgenes segregated in a Mendelian ratio in 10 lines. Lines L6, L9, L10, L11 and L13 presented a segregation ratio of 3:1 (one locus) and the lines L5, L7, L8, L12 and L14 presented a segregation ratio of 15:1 (two loci). However, the lines L1, L2, L3 and L4 presented a non-Mendelian segregation (Table 1).

Detached leaves of Δ*chs*-positive plants from the T_1 generation were inoculated with *S. sclerotiorum* cultures and lesion area was recorded 48 and 72 h after inoculation. Results showed that five of the 14 transgenic lines presented a delay in symptom development compared with the non-transgenic lines. The infected area at 72 h after inoculation ranged from 2·2 to 20·0 cm^2 (mean 11·1 cm^2) in the transgenic lines, while in the control (non-transgenic plants) the infected area ranged from 13·1 to 23·1 cm^2 (mean 16·8 cm^2). No differences were observed between the non-transgenic plants and plants transformed with the pCAMBIA1301 vector without the Δ*chs* cassette (Table 1).

In the three transgenic lines that had the largest phenotypic alteration compared to the controls, the reduction in disease severity 48 h after inoculation was 86·7% (for line L7), 84·2% (for line L3) and 83·5% (for line L6).

However, the Scott–Knott test performed on lesion areas of all plants revealed no statistically significant difference between transgenic lines and the control (wild type) 48 h after inoculation. At 72 h, five lines (L3, L4, L5, L6, L7) revealed a significant reduction in the lesion area compared to the controls and the lesions did not progress extensively up to 120 h after inoculation (Table 1; Figs 2 & 3). In contrast, the lesion area continued to grow in the non-transgenic control, reaching the edge of the leaves by 120 h (Fig. 3). No correlation was observed between the number of transgenic loci and tolerance to *S. sclerotiorum* (Table 1).

Northern analyses were carried out to detect the siRNA in leaves of transgenic and non-transgenic plants. The analysis showed siRNA detectable bands of the expected size range in the lines L3, L4, L5, L6 and L7. No detectable signal was observed in the other transgenic plants or in the control lines (Fig. 4). These results showed a positive correlation between the presence of detectable siRNA and reduction in disease severity after inoculation with *S. sclerotiorum*.

A quantitative RT-PCR analysis was carried out to verify the cause–effect relationship between silencing of the *Sschs* gene induced by *Sschs*-specific siRNA produced by the host and impairment of fungal capacity to colonize host tissues. *Sclerotinia sclerotiorum* was inoculated on leaves of the best *Sschs*-RNAi expressing lines (L3, L4, L5, L6 and L7) and five lines in which it was not possible to detect the *Sschs*-RNAi (L8, L9, L10, L13, L44 and non-transgenic) and fungal transcripts were analysed 96 h after inoculation (Fig. 5). In addition, semiquantitative RT-PCR was carried out to demonstrate the specificity of the primers to distinguish cDNA of the fungus from possible contaminants from the plants. The amplified 95 bp fragment was sequenced and presented 100% identity with the *Sschs* gene. No signal was observed in plants not inoculated with the fungus (Fig. 5a). Results showed a reduction in the level of chitin synthase endogenous transcripts in *S. sclerotiorum* interacting with transgenic leaves. Compared with the control, the reduction ranged from 46·7 to 61·5% (Fig. 5b). A relationship between the *Sschs* expression level and lesion area was observed (Fig. 5c). These results suggest that the silencing signal was translocated from transgenic tobacco into fungal cells, partially suppressing expression of the endogenous chitin synthase

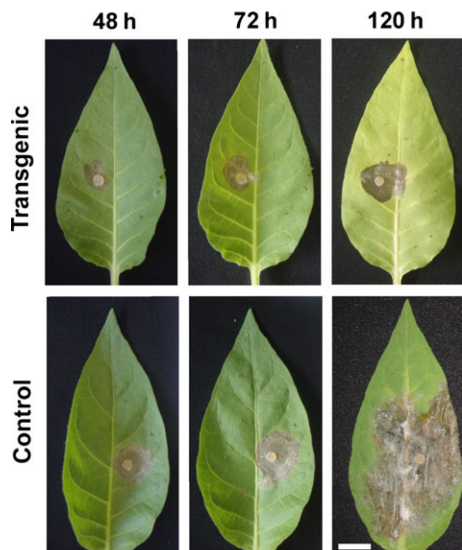

Figure 3 Detail of tobacco transgenic line L5 response to *Sclerotinia sclerotiorum* mycelium 48, 72 and 120 h after inoculation, compared to non-transgenic control plants, showing that the lesion area did not present extensive progress over this time (up to 120 h) when compared to the control plants. Bar = 2 cm.

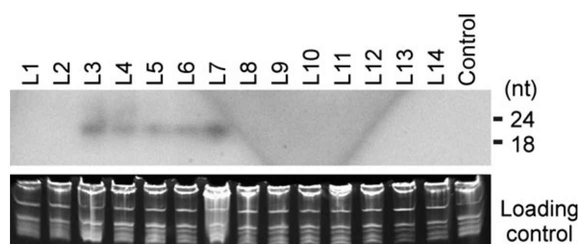

Figure 4 Northern blot analysis to detect the presence of siRNA corresponding to the *chs* sequence in transgenic and non-transgenic (control) tobacco lines. Ethidium bromide-stained RNA serves as the loading control (below). Molecular size markers are indicated on the right.

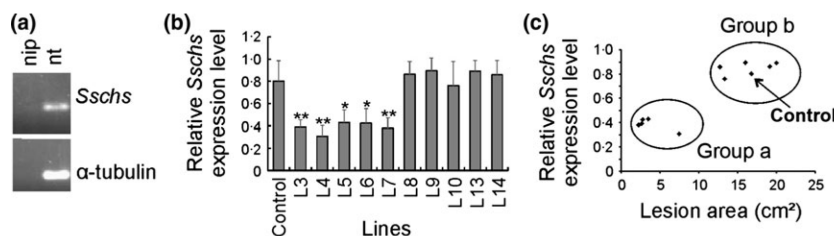

Figure 5 Expression of fungal genes in *Sclerotinia sclerotiorum* infecting transgenic and non-transgenic (control) tobacco plants. (a) Semiquantitative RT-PCR analysis for detection of *Sschs* and Ssα-tubulin transcripts from mycelium growing in infected non-transgenic plants (nt). A non-inoculated transgenic plant (nip) was also analysed to demonstrate that primers are specific to the fungal genes (b) Relative expression of *Sschs* 96 h after inoculation of non-transgenic (Control) and transgenic lines (L3, L4, L5, L6, L7, L8, L9, L10, L13 and L14), determined by quantitative real-time RT-PCR. Data represent means of three replications (±SD). *$P < 0{\cdot}05$; **$P < 0{\cdot}01$, $n = 9$, related to control. (c) Relation of *Sschs* expression versus lesion area in transgenic lines L3, L4, L5, L6 and L7 (group a), and L8, L9, L10, L13, L14 and control (non-transgenic line, arrowed) (group b).

gene and impairing fungal development and leaf infection.

Discussion

In previous work, the authors reported for the first time that a plant expressing dsRNA could cause the suppression of the corresponding target gene in fungal cells attached to plant tissues (Tinoco *et al.*, 2010). Since then, several reports have demonstrated the biotechnological potential of this approach for the development of fungus-resistant plants (Yin *et al.*, 2011; Nunes & Dean, 2012; Panwar *et al.*, 2013; Ghag *et al.*, 2014; Govindarajulu *et al.*, 2015). However, fungal species in which HIGS has been effective are biotrophic pathogens. These fungi establish a long-term feeding relationship with the living host cells but do not kill them. On the other hand, it is hypothesized that hemibiotrophic parasites absorb nutrients from host cells in early biotrophic stages and then kill them for nutrient acquisition. For biotrophic fungi, the long period of feeding activity allows the nutrient-absorbing fungal cells to take up dsRNA molecules from their host.

In contrast, necrotrophic pathogens kill host cells faster, often secreting toxins, plant cell wall-degrading enzymes, and proteinases to facilitate host cell death (Zhu *et al.*, 2013). However, recent evidence suggests a strong interaction of necrotrophic pathogens with their host before killing the cells and tissues (Zhu *et al.*, 2013; Kabbage *et al.*, 2015). *Sclerotinia sclerotiorum* is generally believed to be a typical necrotrophic fungus, being an aggressive pathogen secreting copious amounts of oxalic acid and associated with several cell wall-degrading enzymes that lead to the death of host cells and tissues (Kim *et al.*, 2008). However, recent analysis has suggested *Sclerotinia* is a hemibiotrophic pathogen, with fungal establishment prior to plant recognition that is more temporally and spatially complex than previously observed (Zhu *et al.*, 2013; Kabbage *et al.*, 2015).

In the present study, the hypothesis was tested that the short interaction period between necrotrophic pathogens and living host tissue would be sufficient to mobilize small RNA molecules from plant to fungal cells, silencing genes in the pathogen. Results have shown that transgenic lines, in which siRNA corresponding to the *chs* gene was found, presented a significant difference in the lesion area at 72 h. The lesion did not show extensive progress after 120 h, suggesting that fungal cells interacting with living cells at the edge of the lesion were gradually impaired.

As *S. sclerotiorum* is a very aggressive pathogen that develops speedily, there is a delay between acquirement of siRNA molecules and the effect of gene silencing, resulting in delayed symptoms. This corroborates the hypothesis that a hemibiotrophic stage exists in the interaction between *S. sclerotiorum* and the plant, and this allows siRNA molecules to be absorbed by fungal feeding cells and subsequent RNA interference of the *Sschs* gene.

The chitin synthase gene was chosen because chitin is an important structural component of morphologically distinct structures assembled during various phases of growth and development and is responsible for cell wall rigidity. Chitin biosynthesis is a target for antifungal drugs (Ruiz-Herrera & San-Blas, 2003) and is an attractive target for obtaining resistant plants via HIGS because chitin is absent in plants, which reduces the probability of any off-target effect of expressing dsRNA corresponding to *chs* genes. The *chs1* gene was inactivated in *Neurospora crassa* by repeat-induced point mutation (Yarden & Yanofsky, 1991). Because fungi contain several chitin synthases, mutated *N. crassa* showed a residual chitin synthase activity. Nevertheless, mutants produced slow-growing progeny that formed hyphae with morphologic abnormalities, and this phenotype was correlated with a significant reduction in chitin synthase activity (Yarden & Yanofsky, 1991). Single mutants with Δ*chs5* or Δ*chs7* and the Δ*chs5/7* double mutant of *F. verticillioides* grew poorly and exhibited small, hyperpigmented colonies with very few aerial mycelia compared to the wild type (Larson *et al.*, 2011). In *Botrytis cinerea*, a model organism for necrotrophic pathogens, the disruption of the *Bcchs1* gene reduced chitin synthase activity with a reduction in chitin content of 30%, leading to cell wall weakening and *in planta* fungal progression (Soulié *et al.*, 2003). Disruption of *Bcchs3a* altered host cell adhesion with reduction in virulence (Soulié *et al.*, 2006; Arbelet *et al.*, 2010). Single deletions of genes coding for distinct chitin synthases indicated that *Bcchs4* is not essential for development and pathogenicity of the fungus, and *Bcchs7* is required for pathogenicity in a host-dependent manner. In addition, *Bcchs6* disruption generated only heterokaryotic strains, showing that it represents a valuable antifungal target (Morcx *et al.*, 2013).

In the present work, it was demonstrated that endogenous genes from the very aggressive necrotrophic fungus *S. sclerotiorum* could be silenced by host induction. HIGS of the fungal chitin synthase gene can generate white mould-tolerant plants. As fungi have many *chs* genes, RNAi expression cassettes that simultaneously silence a group of chitin synthase-coding genes would provide more effective disease control. The progress made in sequencing fungal genomes and understanding fungus–plant interaction will help to identify more appropriate target genes for necrotrophic pathogens. From a biotechnological perspective, these results open new prospects for the development of transgenic plants resistant to necrotrophic fungal pathogens, which are among the most important challenges in plant breeding. Climate change, modification in pathogen populations and fungicide resistance issues have increased the immediacy of this task.

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