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RESEARCH PAPER

Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a *Trichoderma* α-1,3-glucanase

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Abstract

The leaf surface of a very large number of plant species are covered by trichomes. Non-glandular trichomes are specialized unicellular or multicellular structures that occur in many different plant species and function in xenobiotic detoxification and protecting the plant against pest attack. By analysing the susceptibility of trichome mutants, evidence is provided that indicates the influence of leaf trichomes on foliar fungal infections in *Arabidopsis thaliana*, probably by facilitating the adhesion of the fungal spores/hyphae to the leaf surface. A decreased trichome number in the hairless *Arabidopsis* mutant *gl1* enhances tolerance against the necrotrophic fungus *Botrytis cinerea*. By contrast, the *try* mutant shows an increased susceptibility to both fungal infection and accumulation. Trichome density does not influence infection by the soil-borne pathogen *Rhizoctonia solani*. In addition, the influence of trichomes on foliar infection is supported by targeting the high-level expression of the *Trichoderma harzianum* α-1,3-glucanase protein to the specialized cell structures. Trichome expression of this anti-fungal hydrolase shows a significant resistance to infection by the foliar pathogen *Botrytis cinerea*. Resistance to this fungus is not dependent on the constitutive induction of the salicylic or jasmonic defence signalling pathways, but the presence of the α-1,3-glucanase protein in trichomes.

Key words: Antifungal activity, phyllosphere, plant defence, *Rhizoctonia solani*, *Trichoderma harzianum*.

Introduction

Trichomes are specialized unicellular or multicellular structures derived from the epidermal cell layer. Multicellular trichomes occur in many different species and often form glands that secrete various compounds including organic acids, polysaccharides, terpenes, nectar, or salt (Werker, 2000). In *Arabidopsis thaliana*, the unicellular non-glandular trichomes originate from a single epidermal cell that differentiates to form a characteristic, three-branched structure with an unknown function. Leaf trichomes form on stem and leaf surfaces to an extent that depends on the ecotype (Hülskamp and Kirik, 2000; Werker, 2000). Multiple mutants have been isolated in *Arabidopsis* with altered trichome number or development. The earliest stages of trichome morphogenesis are blocked by mutations in the *GLABROUS1* (*GL1*) and *TRANSPARENT TESTA GLABRA* (*TTG1*) loci. Trichomes are not found in the *gl1* leaves, and the epidermal cells are uniform in size and shape. The *ttg1* mutants also show the same hairless phenotype and both mutations are epistatic to all the remaining trichome mutations, suggesting that GL1 and TTG1 are required for the specification of the trichome cell (Hülskamp *et al*., 1994). However, the *ttg1* mutant has several pleiotropic defects, one of which is the lack of anthocyanin biosynthesis (Walker *et al*., 1999). Mutations at the *TRYPTYCHON* (*TRY*) locus also affect the number of trichomes and produce a unique phenotype, where nests of up to four trichomes occur in the place of a single trichome (Hülskamp and Kirik, 2000).

Generally, the non-glandular trichomes, such as those present in *Arabidopsis*, are believed to be physical defensive structures, and it has been demonstrated that natural enemies impose selection for increased trichome density

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(Eisner *et al*., 1998; Mauricio and Rausher, 1997). Therefore, a direct plant defence mechanism against herbivore attack is the production of thorns and trichomes in order to avoid wounding by the predator, thus acting as a physical barrier. In the herb plant *Mentzelia pumila*, leaves and stems are covered with hooked trichomes that are able to entrap and kill several insect species (Eisner *et al*., 1998). There is also evidence that *Arabidopsis* trichomes play additional or alternative roles in response to abiotic stress conditions such as the detoxification of heavy metals (Ager *et al*., 2003; Domínguez-Solís *et al*., 2004). In cadmium-contaminated soil, leaf trichomes accumulate the toxic metal at a concentration of up to 10-fold higher compared with epidermal cells (Gotor *et al*., 2003). In addition, high levels of glutathione biosynthesis, a process related to the accumulation of xenobiotic molecules, were measured in *Arabidopsis* trichome cells (Gutiérrez-Alcalá *et al*., 2000). The function of trichomes as sinks for toxic molecules may also decrease the nutritional value of the plant and therefore deter herbivore feeding. Although there are many reports that link glandular and non-glandular trichomes with herbivore attacks, information is lacking as to whether trichomes serve an additional function against microbial infection. Trichomes do, however, increase the capture and retention rate of airborne particles such as pollen and fungal spores on leaf surfaces (Roda *et al*., 2003).

Evidence is provided here that indicates the influence of leaf trichomes on foliar fungal infection in *Arabidopsis thaliana*. Based on these initial observations, a regulatory DNA fragment was then used that is able to confer high levels of expression in trichomes (Gutiérrez-Alcalá *et al*., 2005), in order to direct exo-α-1,3-glucanase (EC 3.2.1.84) protein production in the specialized cell structures. This enzyme was isolated from the mycoparasitic fungus *Trichoderma harzianum* which has been described as an antagonist fungus against a broad range of phytopathogenic fungi (Papavizas, 1985). With this approach, a phenotype with significant resistance to infection by the foliar pathogen *Botrytis cinerea* was obtained.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana gl1 mutants in Landsberg *erecta* and Columbia-0 backgrounds were provided by the Nottingham *Arabidopsis* Stock Centre (NASC), and the *try* mutant in the Landsberg *erecta* background was provided by Dr Martin Hülskamp. Wild-type, transgenic, and mutant plants were grown on moist vermiculite supplemented with Hoagland medium or in soil media at 20/18 °C light/dark, under a 16/8 h light/dark photoperiod as described by Domínguez-Solís *et al*. (2001). For infectivity assays and PR expression analysis, plants were grown on soil media under a 10/14 h 22/20 °C light/dark cycle.

Fungus infections

Botrytis cinerea strain ME4 was grown in solid strawberry broth for 12 d, and spore suspensions were prepared at a concentration of 5×10^5 spores ml^{-1} in 12 g l^{-1} Potato-Dextrose-Broth (PDB). For needle-wound assays, three leaves per plant were punctured in two positions per leaf and a 5 µl drop of the spore suspension was placed over the wound. Plants were covered with a transparent film in order to keep 100% humidity. After a 4 d incubation period in the growth chamber, lesions were scored. For sprayed assays, plants randomly localized in flat trays were pulverized with a Preval sprayer with 5×10^5 spore suspension. Around 300 µl of spore suspension per plant was used. Plants were covered with a transparent film and incubated as described above. Symptoms were observed after 4 d.

Rhizoctonia solani, strain CBS 326.84 was grown on potato agar plates at room temperature. Two-week-old *Arabidopsis* plants were inoculated with *Rhizoctonia* by placing a 1 cm diameter agar plug of mycelium on the soil in the centre of the pot. Each pot containing 45–50 plants homogeneously distributed was covered with a plastic bag after inoculation and transferred to the growth chamber as described. Infected plants were evaluated 7 d post-inoculation.

Statistical analysis of the results was done with one-way ANOVA.

DNA cloning and plasmid construction

The plasmid pATP-AGN13.1, containing the gene *agn13.1* coding for an exo-α-1,3-glucanase from the fungus *Trichoderma harzianum* (GenBank/EBI accession no. AJ243799) (Ait-Lahsen *et al*., 2001), was under control of the *OASA1* gene promoter (Gotor *et al*., 1997; Gutiérrez-Alcalá *et al*., 2005) and constructed by PCR amplification using the proofreading *Platinum Pfx* DNA polymerase (Invitrogen). Forward and reverse primers used for amplification of the *agn13.1* cDNA were AGN13F, AATGGATCCATGCGGTCTCTCGA GGAA (*Bam*HI restriction site underlined) and AGN13R, TCA-GAGCTCGATTGATCCCTCCTA GCA (*Sst*I restriction site underlined), respectively. These primers amplified a DNA fragment of 1848 bp coding for the mature AGN13.1 protein beginning at amino acid 28 and therefore lacking the transit peptide. The PCR amplified DNA fragment was cut with *Bam*HI and *Sst*I and ligated to a derivative of pBI121 plasmid (Clontech) containing the *OASA1* gene promoter instead of the CaMV 35S promoter (Gutiérrez-Alcalá *et al*., 2005). This promoter spans from −1435 to +375 of the genomic *OASA1* gene sequence (At4g14880) and corresponds to nucleotides 8521846–8520037 of *Arabidopsis thaliana* chromosome 4.

Transformation of Arabidopsis *plants*

For plant transformation, the chimeric gene was transformed into the *Agrobacterium tumefaciens* strain C58pMP90. *Arabidopsis thaliana* (ecotype Columbia) was transformed by dipping developing floral tissue into a solution containing the *Agrobacterium* strain, 5% sucrose and 0.02% (v/v) of surfactant Silwet L-77 as described by Clough and Bent (1998). Transgenic plants were recovered by selecting seeds on solid MS medium containing 50 mg l^{-1} kanamycin, and copy number assessed by monitoring the segregation of resistance to kanamycin. T_2 or subsequent generations of each line were used for experiments.

RT-PCR

Arabidopsis RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA (2 µg) was retro-transcribed by the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). An aliquot of the cDNA (2 µl) was amplified in subsequent PCR reactions using the following oligonucleotides: AGN13F, 5′-AATGGATC-CATGCGGTCTCTCGAGGAA-3′ and AGN13R, 5′-TCA-GAGCTCGATTGATCCCTCCTAGCA-3′ for the *agn13.1* gene; PR1F, 5′-GTAGGTGCTCTTGTTCTTCCC-3′ and PR1R, 5′-CACATAATTCCCACGAGGATC-3′ for the *pr1* gene; PDF1.2F, 5′-AATGAGCTCTCATGGCTAAGTTTGCTTCC-3′ and PDF1.2R, 5′-AATCCATGGAATACACACGATTTAGCACC-3′

for the *pdf1.2* gene. PCR conditions were as follows: a denaturation cycle of 2 min at 94 °C, 15 (for *pr1* and *pdf1.2*) or 35 (for *agn13.1*) amplification cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C, and an extension cycle of 3 min at 72 °C. In each case, a control of the constitutive *ubq10* gene was made in parallel with the primers UBQ10F, 5′-GATCTTTGCCGGAAAACAATTG-GAGGATGGT-3′ and UBQ10R, 5′-CGACTTGTCATTAGAAA-GAAAGA GATAACAGG-3′.

Quantitative PCR (qPCR)

DNA from infected plants was extracted following the described protocol of Specht *et al*. (1982). qPCR was carried out using a qPCR Core Kit for SYBR Green I-No ROX (Eurogentec) following the manufacturer's instructions. Two or three dilutions of each DNA sample were used and at least three independent experiments were quantified. DNA from the *B. cinerea creA* gene (Tudzynski *et al*., 2000) was amplified using the oligonucleotides creABOTF (5′-TCCTGCTCTTGCACCCATGGA-3′) and creABOTR (5′-CCGGAGCTTGAGGAACTGGT-3′). As an internal standard to normalize the qPCR, *A. thaliana ubq10* DNA was amplified using the oligonucleotides UBQ10F and UBQ10R described above. Relative quantifications were performed according to described mathematical models (Pfaffl, 2001).

Microarray slide treatment

Superamine Telechem slides containing more than 28 000 spots corresponding to the *Arabidopsis thaliana* oligo set from Qiagen-Operon were obtained from David Galbraith (Arizona University). More information about printing and the oligo sets can be found at the following web site (http://ag.arizona.edu/ microarray/). Printed slides were rehydrated over a 65 °C water bath for 10 s and dried on a 65 °C heating block for 10 s. The hydration step was repeated three times. Oligonucleotides were fixed by UV radiation at 120 mJ. Slides were washed in 1% SDS for 5 min, water for 5 min and in absolute ethanol for 30 s. Finally, slides were dried by centrifugation at 141 *g* for 3 min.

Prehybridization was performed at 42 °C for 30–45 min in 6× SSC, 0.5% SDS, and 1% BSA. Slides were rinsed five times with distilled water. Fragmented Cy5- and Cy3-amplified RNA (aRNA) probes were mixed (200 pmol of each label) with 20 μg of PolyA (Sigma) and 20 μg of yeast tRNA (Sigma) in a final volume of 90 μl hybridization buffer (50% formamide, 6× SSC, 0.5% SDS, 5× Denhardt's). The probe was denatured at 95 °C for 5 min and applied to the slide using a LifterSlip (Erie Scientific). Slides were then incubated at 37 °C for 16 h in hybridization chambers (Array-It). After incubation, slides were washed twice with 0.5× SSC, 0.1% SDS for 5 min each, twice with 0.5× SSC for 5 min and finally in 0.05× SSC for 5 min. Slides were dried by centrifugation at 563 *g* for 1 min.

Images from Cy3 and Cy5 channels were equilibrated and captured with a GenePix 4000B (Axon) and spots quantified using GenPix software (Axon). The data from each scanned-slide were first escalated and normalized using the Lowess method and were then log-transformed. The mean for log-ratio intensities and standard deviation among replicates was generated. Two statistical approaches were used to identify differentially-regulated genes; the *t* test (Smyth *et al*., 2002) and a *z*-score (Quackenbush, 2002). Only genes with a *P*-value of less than 0.05 and a *z*-score greater than two were considered differentially expressed.

RNA amplification and labelling for microarray analysis

Total RNA (1 μg of each) was amplified and aminoallyl-labelled using MessageAmp™ II aRNA kit (Ambion) and 5-(3-aminoallyl)-2′deoxyuridine-5′-triphosphate (aa-dUTP, Ambion), following the manufacturer's instructions. Approximately 40–50 μg of aRNA was obtained. For each sample, 7.5 μg of aminoallyl-labelled aRNA was resuspended in 0.1 M Na_2CO_3 (pH 9.0) and labelled with either Cy3 or Cy5 Mono NHS Ester (Cy™Dye Post-labeling Reactive Dye Pack, Amersham). The samples were purified following manufacturer's instructions for Megaclear™ (Ambion). Cy3 and Cy5 incorporation was measured using 1 μl of the probe in a Nanodrop spectrophotometer (Nanodrop Technologies Inc.). For each hybridization, 200 pmol of Cy3 and Cy5 probes were mixed, dried in a Speed-Vac, and resuspended in 9 μl of RNase-free water. Labelled aRNA was fragmented by adding 1 μl of 10× Fragmentation buffer (Ambion) and incubating at 70 °C for 15 min. The reaction was stopped with 1 μl of Stop solution (Ambion). Integrity and average size of total RNA, aRNA, and fragmented aRNA were evaluated using a Bioanalyser 2100 (Agilent). The average size of aRNAs was about 1000 nucleotides and of fragmented aRNAs about 100 nucleotides. The final volume of the probe was diluted to 100 μl in hybridization solution.

Determination of α-1,3-glucanase activity

Crude extracts were prepared from wild-type and transgenic plant leaves by grinding in liquid nitrogen. The extracts were dialysed against 50 mM potassium acetate buffer, pH 5.5, to eliminate reducing sugars from the extracts before the enzyme activity measurements. α-1,3-Glucanase activity was assayed by measuring the reducing sugars produced in the enzyme reaction as previously described by Ait-Lahsen *et al*. (2001). The substrate of the enzyme reaction was mutan, which is composed of α-1,3-glucan with some α-1,6-glucan side chains, and was prepared from *Streptococcus* mutants CECT 4034, as previously described by Robert *et al*. (1993).

Preparation of trichome protein extracts

Tichomes were isolated from leaves by chemical extraction with EGTA (ethylene glycol-*bis*[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetra-acetic acid) based on a previously described method (Zhang and Oppenheimer, 2004). Ten grams of mature leaves were submerged in 100 ml of PMT (25 mM PIPES, 0.5 mM $MgSO_4$, 0.05% (v/v) Triton X-100, pH 7.2) supplemented with 50 mM EGTA, vacuum infiltrated for 20 min, and incubated 16 h at 4 °C. The solution was filtrated through a Miracloth (Calbiochem) to collect the isolated trichomes, which were then rinsed with 500 μl of PMT and centrifuged at 1500 *g* for 10 min. The resulting pellet containing the trichomes was resuspended in 200 μl of 50 mM TRIS–HCl, pH 7.5. To the trichome suspension, 150 mg of wet glass beads (425–600 μm diameter) was added and the mixture was vortexed for 4–5 min in periods of 1 min with intervals of 1 min at 4 °C. The suspension was then centrifuged at 16 000 *g* for 20 min at 4 °C and the supernatant collected. To the remaining pellet, another 200 μl of 50 mM TRIS–HCl, pH 7.5, was added, vortexed, and centrifuged as described above. The resulting supernatant was mixed with the previous one and both constituted the final trichome protein extract. The amount of soluble protein was determined using the Bio-Rad dye reagent and the yield routinely was 2–4 μg total protein.

Immunoprecipitation of the α-1,3-glucanase protein and SDS-PAGE

Trichome extracts containing 2–4 μg total protein were incubated with rabbit polyclonal antibodies raised against the purified AGN13.1 protein (Ait-Lahsen *et al*., 2001) at 4 °C overnight with gentle shaking. The immunoprecipitates were collected by centrifugation at 16 000 *g* for 10 min, washed four times with 50 mM TRIS–HCl, pH 7.5, 50 mM NaCl, 2% Triton X-100, and twice with 10 mM TRIS–HCl, pH 7.5. The washed immunoprecipitates were then subjected to SDS-PAGE with 12% acrylamide separating gel.

Results

Influence of trichomes on foliar fungal infection

In an attempt to determine if trichomes have any effect on the infection by pathogens attacking the leaf surface, *Arabidopsis* wild-type leaves were challenged with a spore suspension of *Botrytis cinerea*. Figure 1 clearly shows the progress of the *B. cinerea* infection 4 d post-inoculation and the colonization of leaf trichomes by the wrapping of fungal hyphae. These micrographs may suggest that the presence of trichomes facilitates the fungal infection, acting as physical adhesion points of the hyphae.

To confirm that trichomes affect plant infections by foliar pathogenic fungi, a challenge was made with two different fungi, the foliar pathogen *B. cinerea* and the soil-borne pathogen *Rhizoctonia solani*, on plant mutants with different trichome numbers (Fig. 2). It was observed that the *Arabidopsis* L*er-gl1* mutant which is devoid of trichomes shows enhanced tolerance to the fungus *B. cinerea* when compared with the susceptibility of the wild-type ecotype Landsberg *erecta* and the trichome over-producer L*er-try* mutant (Fig. 2A, B). Several tests were carried out for leaf infection and, in all cases, the *gl1* mutant plants were less susceptible to infection by *B. cinerea*. *Botrytis* is a foliar necrotrophic fungal pathogen that causes necrotic lesions in the inoculated leaves and is able to spread to non-inoculated tissues. A well-established standard inoculation assay was initially performed on wound leaves to facilitate the initiation of the infection. In this needle-wound test, drops of spore suspension were layered over 0.8 mm needle-prick wounds made on the leaves, and the production of lesions were examined after 4 d of inoculation. With this assay, the contribution of trichomes on the spreading of the infection, after the initial infection had been established on the surface of the leaf, was determined. In a second test, a non-disruptive assay was used to ensure that the trichomes and the leaf surface were not damaged during the inoculation. Therefore, plants were sprayed with the spore suspension and were examined 4 d post-treatment for the appearance of lesions. With this assay, the contribution of trichomes on spore attachment, initiation, and infection spreading was mainly measured. Brown necrotic and chlorotic lesions on the leaf were considered positive infections and the HR (hypersensitive response)-like lesions were considered non-positive infections. After challenging with the fungi, *gl1* had up to 14% fewer infections than the wild-type plants in the needle-wound test and this number increased to 24% fewer infections in the spray test (Fig. 2A). The differences observed between both assays clearly indicate that trichome number is as important in the establishment as it is in the spreading of the infection.

The wild-type and *gl1* plants in the needle-wound test showed a similar 26% of HR-like lesions, yet the number of non-infected inoculations was significantly higher in the *gl1* line (data not shown). Similar tests for leaf infection performed to compare *gl1* and wild type in Col-0 background showed the same level of resistance to *B. cinerea* in both L*er-gl1* and Col-*gl1* mutants (data not shown). Although the *try* mutant was more susceptible to *B. cinerea* infection, the high variability of the data voided the results as statistically significant (Fig. 2A).

Fig. 1. Imaging of an *Arabidopsis* leaf challenged with *Botrytis cinerea* spore suspension. (A) Transmitted light imaging showing the growth of *B. cinerea* hyphae 4 d after fungal inoculation, stained with trypan blue. (B) Magnification 2× of the image in (A), showing the *B. cinerea* hyphae wrapped around a trichome. (C, D) Images of two other trichomes wrapped by *B. cinerea* hyphae: hs, hyphae suspension; t, trichome.

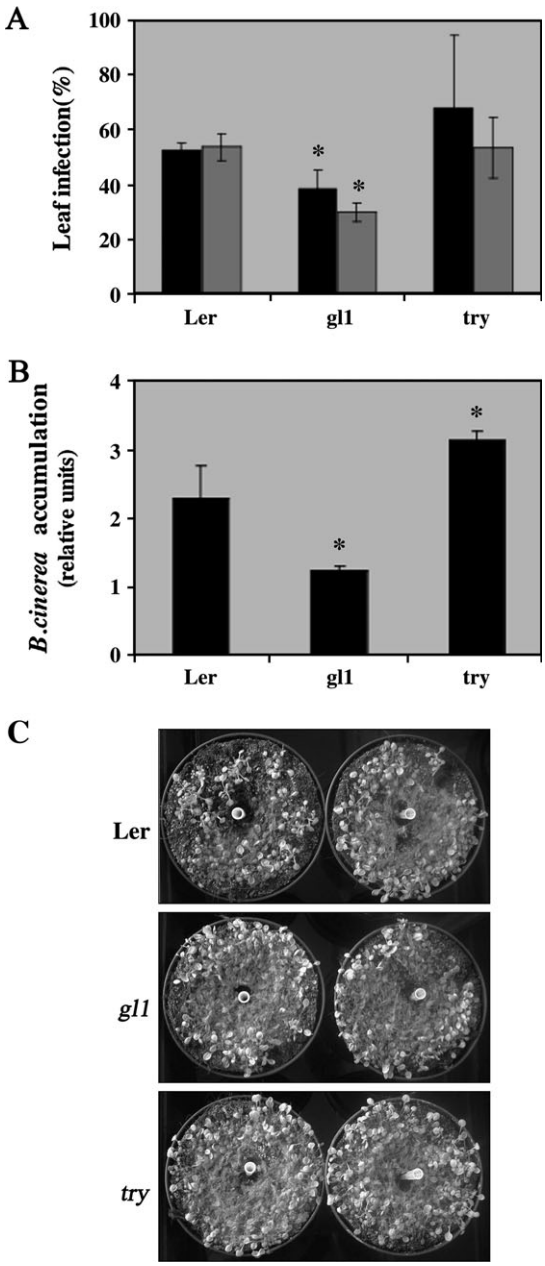

Fig. 2. Susceptibility of different *Arabidopsis* mutant leaves to fungus infection. (A) Quantification of the *Botrytis cinerea* infections. *Arabidopsis* wild-type, *gl1* and *try* mutant lines were challenged with spores of *B. cinerea* in needle-wounded (black boxes) or sprayed (grey boxes) assays. Four days after fungal inoculation, infectivity lesions were counted and represented as a percentage of the total number of inoculated leaves. (B) Detection and quantification of *B. cinerea* growth in plants by quantitative PCR. Whole rosette leaves from pathogen-inoculated plants were used for DNA isolation and qPCR amplification of the *B. cinerea creA* gene, normalized against the *Arabidopsis ubq10* gene. Measurements were taken 4 d after spore inoculation of 20–30 independent plants. Error bars represent the standard deviation from measurements of at least three independent experiments. An asterisk denotes significant differences (P <0.05) from wild-type plants. (C) Infection of *Arabidopsis* mutants with *Rhizoctonia solani*. *Arabidopsis* wild-type, *gl1* and *try* mutant lines were challenged with a plug of *R. solani* mycelium placed in the centre of the pot. Image was captured after 7 d of post-inoculation.

To validate these results, quantitative PCR (qPCR) analysis of the *creA* gene from *B. cinerea* in the infected leaves was carried out; this test also served as a quantitative determination of fungal biomass. Real-time fluorescence PCR has been proven to be an accurate technique to evaluate disease susceptibility phenotypes for several microbial pathogens (Brouwer *et al.*, 2003; Van Wees *et al.*, 2003). In this experiment, leaves were first challenged with fungi using the needle-wound test and DNA from entire leaves was isolated after 4 d of treatment. The needle-wound test was selected over the spray test because the latter gave very high background amplification even after an exhaustive wash of the leaves prior to DNA extraction. The data confirmed that the *gl1* mutant was significantly less susceptible to *B. cinerea*, with almost 50% less fungal accumulation than the wild type (Fig. 2B). In addition, this quantitative analysis revealed a statistically significant 40% more fungal biomass in the *try* mutant than the wild-type plant. This result may suggest that the *try* mutant had an enhanced susceptibility to *B. cinerea*, probably due to a higher attachment of the fungal hyphae in the *try* mutant than in wild-type plants.

The soil-borne pathogen *Rhizoctonia solani* attacks plants at the soil surface and infects the roots and the collar. Infection tests performed with *R. solani* in both wild type and trichome mutants did not show any significant difference in plant susceptibility to the fungus (Fig. 2C). This observation suggests that the increased resistance to *B. cinerea* observed in the *gl1* mutant is not a general effect but only affects fungal infection through the leaves, and confirms the influence of trichomes for an efficient infection.

Transcriptomic analysis of the gl1 *mutant*

The *gl1* mutant does not show any additional phenotypic differences compared with wild type under standard *Arabidopsis* growth conditions, and therefore, there are no apparent pleiotropic changes in the *gl1* mutant. However, to ascertain whether the *gl1* mutation may activate plant defence responses, whole-genome expression profiles of wild-type plants and *gl1* mutants were compared under the experimental conditions used here. Following standard statistical criteria (P <0.05 and z-score >2; see Materials and methods), 35 differentially-expressed genes were found between both genotypes, 18 of them induced and 17 repressed in the *gl1* mutant (Fig. 3; see supplementary material, Tables I and II, at *JXB* online). These genes were categorized with the MapMan software (Thimm *et al.*, 2004) and all of them appeared in 10 functional categories, six of them only containing one gene. Most of the differentially expressed genes were of unknown function (48%) and only five of them may be related to biotic or abiotic stress. However, it is clear that activation of systemic acquired resistance or of salicylic-, jasmonic- or ethylene-dependent defence signalling

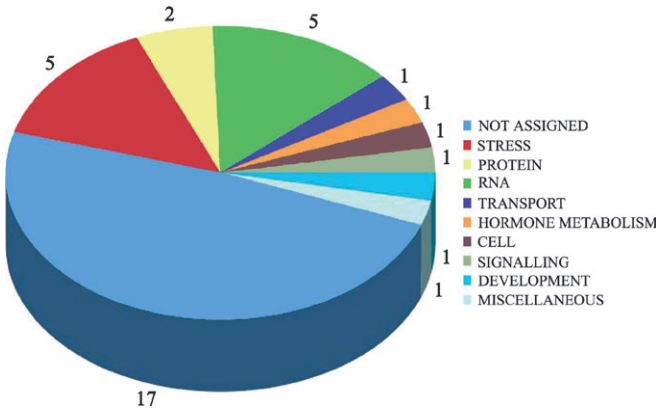

Fig. 3. Functional distribution of differentially expressed genes between *Arabidopsis* wild type and *gl1* mutant.

pathways does not occur. Changes on the surface of the *gl1* mutant were also excluded since none of the differentially expressed genes were categorized within the cell wall metabolism group or were involved in stomatal differentiation or cuticle biosynthesis.

Trichome overexpression of the Trichoderma harzianum agn13.1 *gene enhances tolerance to* Botrytis cinerea

The observed effect of trichomes facilitating the foliar fungal infection prompted the question as to whether the localization of proteins with anti-fungal activity to the trichomes could increase the defence machinery of the plant against *B. cinerea* infection. In addition, it was hypothesized that the increased resistance of the engineered plants could be used as additional evidence of a trichome effect on fungal infection. For this purpose, a well-characterized promoter that confers high expression levels in trichomes (Gotor *et al*., 1997; Gutiérrez-Alcalá *et al*., 2005) was fused to the *agn13.1* gene, coding for an exo α-1,3-glucanase from *Trichoderma harzianum* (Ait-Lahsen *et al*., 2001); this produced the pATP-AGN13 construct. The α-1,3-glucanase enzyme possesses both lytic and anti-fungal activity against fungal plant pathogens (Ait-Lahsen *et al*., 2001). *Arabidopsis* plants were transformed with pATP-AGN13 and six independent homozygous lines were selected for further analysis. All six transgenic lines showed significant expression of the *agn13.1* gene as analysed by RT-PCR (Fig. 4A). The expression of the *agn13.1* gene, under control of the ATP promoter, did not alter the growth of the transgenic plants and showed an identical phenotype to that of the wild-type plants grown under standard conditions.

The activity of α-1,3-glucanase was also measured in concentrated total leaf crude extracts and was detectable only at very low levels in all the transgenic lines (data not shown). The low activity levels in the leaf extracts was expected as the main location of the enzyme was the trichome cells whose contribution of cell proteins represent less than 0.01% of the entire pool of leaf proteins. In an attempt to detect the AGN13.1 protein, trichomes were isolated from several transgenic lines using a chemical treatment previously described by Zhang and Oppenheimer (2004) and trichome protein extracts were prepared for immunoprecipitation. Polyclonal antibodies raised against the purified AGN13.1 protein (Ait-Lahsen *et al*., 2001) were able to immunoprecipitate a single protein with the estimated molecular weight of AGN13.1 from trichome extracts prepared from transgenic leaves, whereas no AGN13.1 protein band was observed in a wild-type extract (Fig. 4B). Curiously, the line AGN13-18 that showed the highest resistance to fungus attack (see below) displayed the most intense AGN13.1 protein band.

Leaves of the ATP-AGN13 transgenic lines were challenged with spores of *B. cinerea* and their susceptibility to disease was then quantified. Brown chlorotic lesions smaller than 3 mm in diameter were distinguished, as well as brown necrotic and chlorotic lesions greater than 3 mm, often expanded over the whole leaf. Both these types of lesion were quantified as positive leaf infections and the HR-like lesions were counted with the non-infections (Fig. 5A). All six transgenic lines displayed a lower susceptibility to a *B. cinerea* infection than the wild-type plants, both in the needle-wounded and the sprayed assays. Statistically significant differences when compared with wild type were observed for lines AGN13-18, -21, -26, and -44. The most significant results seen were from the line AGN13-18; up to 20% fewer total leaf infections were observed. It was apparent that disease severity was significantly inhibited in the transgenic lines compared with the wild type.

To assess the growth of *B. cinerea* in the leaves of the transgenic lines, fungal biomass was determined by qPCR as previously described. Fungal growth was inhibited in all transgenic lines when compared with wild type. Fungal accumulation was significantly inhibited in lines AGN13-8, AGN13-18, AGN13-26, and AGN13-31 by up to 60% compared with wild type (Fig. 5B). Lines AGN13-21 and AGN13-44 also showed less content of fungal biomass, but the data were not statistically significant due to the high standard deviations.

Although the expression of the *agn13.1* gene in trichomes clearly decreases fungal infection and growth within the plant, these effects may also be due, in part, to a constitutive induction of the plant defence machinery as a consequence of the expression of the transgene. To determine this, the expression of the *pr1* and *pdf1.2* genes in the transgenic ATP-AGN13 lines were quantified by RT-PCR. PR1 is a pathogenesis-related protein whose gene (*pr1*) expression is regulated by salicylic acid (SA) and is used as a reporter for SA-dependent defence mechanisms. Similarly, the plant *defensin1.2* (*pdf1.2*) gene, encoding a pathogenesis-related protein, is regulated by ethylene and jasmonic acid (JA) and is used as a reporter of JA-dependent defence responses (Traw and

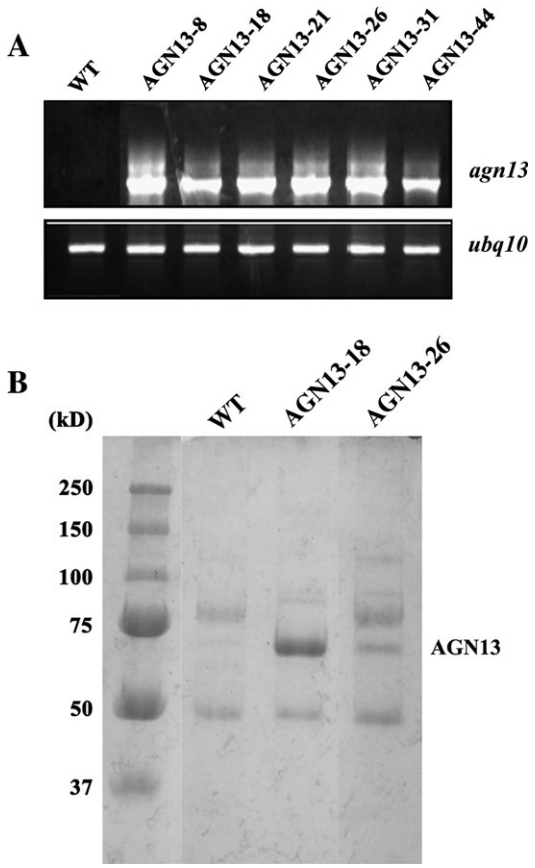

Fig. 4. Characterization of the transgenic *Arabidopsis* lines ATP-AGN13. (A) RT-PCR analysis of transgenic *Arabidopsis* lines expressing the *agn13.1* gene. *Arabidopsis* RNA was isolated from wild-type and ATP-AGN13 transgenic lines. Two µg of RNA were retro-transcribed and the resulting cDNA was used for PCR amplification and detection of the heterologous *agn13.1* gene. The *Arabidopsis ubq10* gene was used as a control for the quality and quantity of the RNA. (B) Immunoprecipitation of the AGN13 protein from trichome protein extracts. Coomassie blue staining of SDS–PAGE of immunoprecipitates from trichome proteins isolated from wild-type (WT), and the transgenic lines ATP-AGN13-18 (18) and ATP-AGN13-26 (26). See experimental procedures for details. Protein molecular markers are shown.

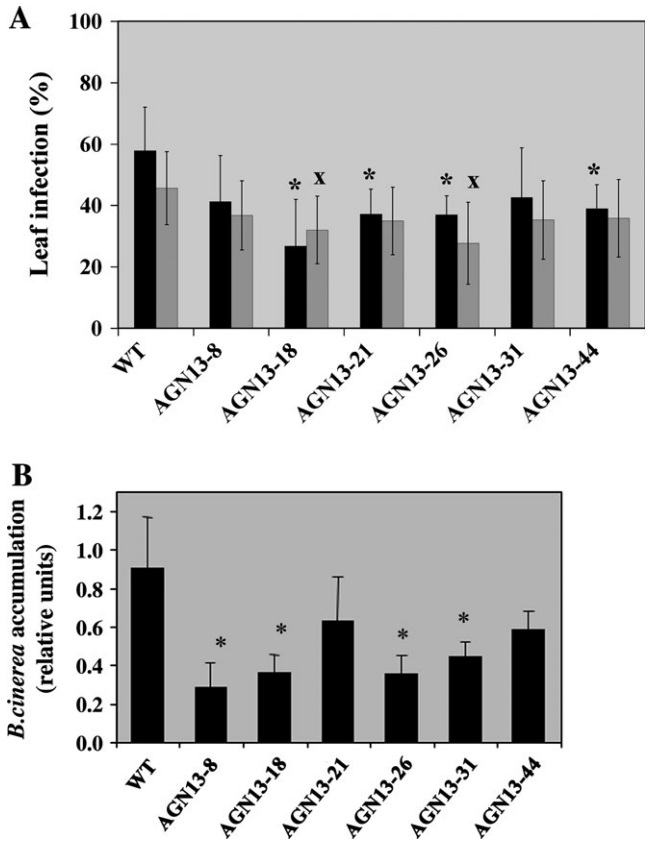

Fig. 5. Susceptibility of transgenic *Arabidopsis* lines to *Botrytis cinerea* infection. (A) Quantification of the positive infections. ATP-AGN13 transgenic lines were challenged with *B. cinerea* spore suspensions in needle-wounded (black boxes) or sprayed (grey boxes) assays. Four days after fungal inoculation, infectivity lesions were counted and represented as a percentage of the total number of inoculated leaves. Measurements were taken of 20–30 independent plants. (B) Quantification of *Botrytis cinerea* growth in transgenic *Arabidopsis* leaves by quantitative PCR. Whole rosette leaves from pathogen-inoculated plants were used for DNA isolation and qPCR amplification of the *B. cinerea creA* gene normalized against the *Arabidopsis ubq10* gene. Measurements were taken 4 d after spore inoculation of 20–30 independent plants. Error bars represent the standard deviation from measurements taken from at least three independent experiments. The asterisks and crosses denote significant differences from wild-type plants with $P < 0.05$ and $P < 0.1$, respectively.

Bergelson, 2003). Wild-type plants were also sprayed with SA or JA, or challenged with *B. cinerea* as positive controls of *pr1* or *pdf1.2* expression. The analysis by RT-PCR revealed that the transgenic lines as well as the wild type do not constitutively express the *pr1* or *pdf1.2* genes as was observed in the positive controls. Thus, the SA- or JA-dependent defence responses are not activated in the ATP-AGN13 transgenic lines (Fig. 6).

Discussion

Decreased trichome number in Arabidopsis *enhance tolerance against the necrotrophic fungus* Botrytis cinerea

A plant's defence against a pathogen attack involves a complex process with many local and systemic responses, including the production of antimicrobial metabolites and pathogenesis-related proteins. These defence mechanisms against viral, bacterial, and fungal infections are so sophisticated that plant diseases rarely occur. However, a successful infection of the phyllosphere by bacteria and fungi is influenced by climatological and environmental factors such as moisture, light, temperature, or nutrient availability (Duggar, 2002; Lindow and Brandl, 2003; Yuste, 1999). The internal anatomy and surface features of the leaves also determine plant resistance towards insect herbivores, and there is an opposite correlation between trichome density and insect feeding (Mauricio and Rausher, 1997; Peeters, 2002). Although these factors have been widely studied in insect–plant interaction, the

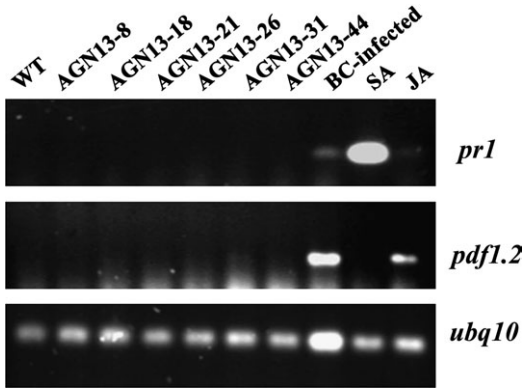

Fig. 6. RT-PCR analysis of the *pr1* and *pdf1.2* gene expressions in transgenic *Arabidopsis* lines. RNA from mature (4 weeks) wild-type and ATP-AGN13 transgenic lines was isolated, retro-transcribed and analysed by PCR with *pr1* and *pdf1.2* specific primers. As positive controls, plants treated for 48 h with sprayed jasmonic acid (100 μM), sprayed salicylic acid (5 mM) or challenged with *B. cinerea* for 4 d were included. The *Arabidopsis ubq10* gene was used as a control for the quality and quantity of the RNA.

anatomical properties of the host have rarely been studied in relation to fungal infections. However, adhesion of the fungal spore to the host surface is the first step of pathogenesis and is therefore critical in determining a successful infection (Vidhyasekaran, 2004).

In the current study, it was found that trichome density on the leaf surface affects foliar fungal infections, trichomes facilitating the adhesion of the fungal spores/hyphae to the leaf surface. A decrease in trichome number in the hairless *Arabidopsis* mutant *gl1* enhanced the tolerance against the necrotrophic fungus *Botrytis cinerea*. By contrast, the trichome over-producer *try* mutant showed an increased susceptibility to fungal infection and accumulation. Therefore, an opposite correlation between trichome number and leaf infection was clearly observed. This conclusion was reached from two different experimental approaches to evaluate plant susceptibility. In the needle-wound test, drops of spore suspension were layered over needle-prick wounds made on the leaves to facilitate the infection on damaged tissue. Therefore, trichome number should not contribute to the initial step of the infection but rather to the spreading of the hyphae throughout the leaf surface. However, in the spray test, since no damage was produced on the leaves, the effect of trichome number on spore retention and initial establishment of the infection can also be determined. The *gl1* mutant was less susceptible than the wild type to fungal infection based on both inoculation tests; however, as expected, the differences were less significant if fungal spores were placed on damaged portions of the leaves.

The fact that the observed effect is general to other phytopathogens, such as *R. solani* that penetrates the plant through the roots, was excluded. In this case, no difference was observed in the susceptibility of the wild type and the trichome mutant lines to be infected by the pathogen. Besides, it was possible to observe microscopically the progression of the fungal hyphae on the surface of the leaves through the attachment to the trichomes. In apple trees, varieties with trichome-rich leaves show more fungal spores and pollen on leaf surfaces as well as an increased density of predatory mites that use them as an alternative food supply (Chamberlain, 1975; Kunkel and Brooks, 2002).

Thus, it seems clear that phyllosphere properties influence the retention of fungal spores but also determine the survival, attachment, and penetration of the hyphae. There is documentation that fungal proteins named hydrophobins are essential for attachment of the hyphae to hydrophobic surfaces and the development of appressoria of fungi (Wosten, 2001). Trichome surfaces are covered by a highly hydrophobic and thick cuticular layer involved in water impermeability and protection against physical damage (Aharoni *et al*., 2004). After a few days of spore application, skeins of hyphae that developed around the trichomes were observed, suggesting that they are excellent counterparts for recognition and attachment of fungal hydrophobins. The presence of trichomes on the leaf surface should therefore determine the attachment of the spores and further development of the hyphae. These results clearly correlate the presence of trichomes with an enhanced susceptibility to *B. cinerea*, probably due to a greater attachment of the fungal spores/hyphae in the leaf. Further, trichomes apparently retain water on the plant surface and provide nutrients for microbial growth (Lindow and Brandl, 2003; Monier and Lindow, 2003). Therefore, in addition to the attachment of the spores, trichome structures may also provide a protected microenvironment for fungal growth.

Transcriptional profile analysis of the *gl1* mutant indicated very little change in gene expression; 18 genes were up-regulated and 17 genes were down-regulated. Three of the activated genes coded for a putative resistance protein that functions quite upstream of the defence activation signalling pathway. However, this analysis excludes the possibility that mutations in the *gl1* locus activate the pathogen defence response of the plant since activation of the pathogenesis-related genes (Kunkel and Brooks, 2002) could not be detected. In addition, changes have not been detected in the transcript profile of genes involved in cell wall biosynthesis or phenotypic changes in the surface of the leaf. Therefore, it is unlikely that the observed effect of enhanced fungal tolerance can be due to pleiotropic changes in the *gl1* mutant.

Engineering tolerance by expressing an α-1,3-glucanase gene in trichomes

If trichomes are one of the preferential ports for plant colonization by fungi, it can be predicted that targeting antifungal agents in trichomes must inhibit or at least decrease fungal infection. This hypothesis is supported by the results obtained by targeting proteins with anti-fungal

activity in the trichomes. The *Trichoderma* α-1,3-glucanase protein, AGN13.1, is considered a cell wall-degrading enzyme that binds and hydrolyses cell walls of various phytopathogenic fungi, including *B. cinerea*. The lytic activity of this enzyme has anti-fungal effects through the inhibition of hyphal growth and spore germination (Ait-Lahsen *et al*., 2001). By using the ATP promoter that confers high expression levels in trichome cells, the *agn13.1* gene was expressed in *Arabidopsis*, as demonstrated by RT-PCR and enzyme activity measurements taken from crude extracts. Moreover, the AGN13.1 protein was localized in the trichomes of the over-expressing lines, demonstrating the proper function of the promoter. The overexpression of the *agn13.1* gene in trichomes increased the resistance of the plant to infection by *B. cinerea* and inhibited the accumulation of the fungal pathogen in leaf tissues. This effect is due to a direct contact between the fungus and the α-1,3-glucanase enzyme and not to the activation of the systemic resistance after plant transformation since *pr1* and *pdf1.2* gene transcripts are not present in the transgenic lines grown under standard conditions.

Similar approaches have been used previously by expressing the *Trichoderma ThEn-42* gene under control of the cauliflower mosaic virus 35S subunit (CaMV 35S) promoter. The constitutive expression of the endochitinase-encoded gene in tobacco and potato confers resistance to several pathogens such as *Alternaria alternata*, *A. solani, B. cinerea*, and *R. solani* (Lorito *et al*., 1998). Therefore, the isolation of genes from the bio-control agent *T. harzianum*, coding for anti-fungal proteins and their further transfer to a plant genome, can result in a significant improvement in plant defence against fungal pathogens. In addition, trichome overexpression of these genes is enough to confer an increased resistance, and therefore, it is possible to avoid gene expression in edible parts (fruits) or other tissues suitable for airborne spread, such as pollen or seeds.

From the results obtained, it can be concluded that trichomes should affect fungal infection through the attachment of the fungal spore/hyphae to the surface of the trichome. Penetration through the trichome cell can be used for further plant colonization. This last step can be inhibited in our engineered plants by the contact of the hyphae with the antifungal α-1,3-glucanase protein targeted to the trichome.

Supplementary data

Supplementary data can be found at JXB online.

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