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Elucidation of defence responses and signalling pathways induced in *Arabidopsis thaliana* following challenge with *Phytophthora cinnamomi*

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ABSTRACT

Arabidopsis thaliana (Arabidopsis) Col-0 was inoculated with *Phytophthora cinnamomi* to assess the interaction and defence responses involved. Pathogen ingress and asexual reproduction occurred on root tissue but not leaf tissue. The colonisation of root tissue did not cause disease symptoms or plant death, indicating that Arabidopsis Col-0 was tolerant of the infection. The induction of several plant defence responses including the expression of defence-related genes were found, with differences displayed between inoculated root and leaf tissue. Arabidopsis defence-related gene mutant/over-expressing lines were also inoculated with *P. cinnamomi* but none of the lines tested exhibited a marked increase in susceptibility to the pathogen.

1. Introduction

The soil borne oomycete *Phytophthora cinnamomi* Rands causes disease in a multitude of plant species in agriculture and native ecosystems worldwide. In Australia, the threat that it poses to the natural environment is considerable and has been recognised as a key threatening process by the government (*Environment Protection and Biodiversity Act 1999*) [1]. The pathogen exhibits a large host range and a key question as to what mechanisms enable some plant species to survive infestation remains unanswered. Research into plant defence against *P. cinnamomi* has been limited in comparison to many other plant pathogens due a variety of factors, including the lack of established model systems and the technical difficulties that arise during experimentation of root–pathogen interactions. As no gene-for-gene interactions have been established with *P. cinnamomi*, resistance appears to be polygenic [2] and with few documented 'fully resistant' plants [3] the factors that influence the development of resistance may be complex.

Most of the research conducted into the interactions of Australian native plants with *P. cinnamomi* has focused upon the impact of the disease in the field. We still know relatively little about the cellular and molecular aspects of the interactions. *Arabidopsis thaliana* (Arabidopsis) has become a widely used model in the study of plant–pathogen interactions (e.g., Refs. [4–6]) due to it being the first plant to undergo complete genome sequencing, the availability of a multitude of mutant lines and its ability to be easily genetically transformed. Considerable similarity between defence responses of Arabidopsis and other plant species has been found, although there are also many instances where there is divergence in the defence responses triggered between species [7]. To date, there has been only a few instances of the use of Arabidopsis to investigate plant–pathogen interactions with *Phytophthora* species, such as in interactions with *Phytophthora infestans* [8], *Phytophthora brassicae* (formerly *Phytophthora porri*) [9], *Phytophthora palmivora* [10], *Phytophthora sojae* [11] and *P. cinnamomi* [12]. The latter study investigated the variability of defence responses in Arabidopsis ecotypes following inoculation with *P. cinnamomi* zoospores.

A variety of plant defence responses against *Phytophthora* species have been reported and include the early triggering of ion fluxes across the plasma membrane, the production of reactive oxygen species (ROS), involvement of defence signalling pathways, regulation by plant hormones and activation of secondary metabolic pathways (such as the phenylpropanoid pathway) [3,13]. The most commonly described defence response linked to the development of plant resistance against *Phytophthora* spp. is, however, that of rapid localised cell death commonly referred to as the hypersensitive response (HR) which is generally regarded as a form of programmed cell death [14]. Rapid localised cell death is present in various host and non-host interactions but it is currently unclear whether non-host HR is controlled by the same regulators of cell death responsible for host HR [15], although it was recently demonstrated that programmed cell death is triggered in *Pinus pinaster* suspension cells when challenged by the non-host pathogen *Botrytis cinerea* [16]. Non-host interactions are, therefore, often referred to as displaying 'non-host HR' or 'HR-like' cell death [15,17]. The magnitude of HR/HR-like cell death is dependent upon

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the particular plant–*Phytophthora* interaction, where non-host interactions usually result in HR-like cell death being limited to individual cells, through to large congregations of cells that can display HR-cell death in race/cultivar resistance [18]. For example, the non-host interaction between Arabidopsis and *P. infestans* displays HR-like cell death limited to cells penetrated by the pathogen [8]. This HR-like cell death was identified by the presence of granulated cytoplasm, condensed nuclei and cellular autofluorescence under ultraviolet light.

In many plant–pathogen interactions, the plant defence hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have been shown to be highly influential in the development of resistance. In general terms it is thought that SA-signalling is important for defence against biotrophic pathogens, while JA and/or ET-signalling is involved in defence against necrotrophic pathogens [19], although this is not the case for all plant–pathogen interactions. Some headway has been made in understanding the involvement of these pathways in responses against *Phytophthora* spp. In the non-host interaction between Arabidopsis and *P. infestans*, gene microarray analysis showed a strong similarity to the gene induction exhibited by JA-treated plants [8]. Direct analysis of SA and JA hormone levels in a resistant interaction between *Capsicum annuum* and *Phytophthora capsici* indicated that both JA and SA were produced, although the timing was different, with JA peaking within several hours of challenge and SA levels peaking at later time points [20]. Characterisation of the interaction between Arabidopsis and *P. brassicae* found that defence signalling pathways involving SA, JA or ET have minimal influence on the interaction, however, *pad2* mutants (recently shown to be defective in γ-glutamylcysteine synthetase) display elevated susceptibility [9,21]. Similarly, Smart et al. [22] found that tomato plants defective in either SA, JA or ET-signalling displayed no variation in *P. infestans* infection levels when compared to wild-type.

Treatment of Arabidopsis plants with elicitors from several *Phytophthora* species has also provided some insight into the defence hormones and responses involved in defence. Introduction of a cell wall glycoprotein named cellulose-binding elicitor lectin (CBEL) from *Phytophthora parasitica* var. *nicotianae* to Arabidopsis was shown to cause HR-like cell death and it was suggested that all three defence signalling pathways (SA, JA and ET) were involved in the response [23]. Similar results were produced by Fellbrich et al. [24] who showed that treatment of Arabidopsis with the cell wall glycoprotein necrosis-inducing *Phytophthora* protein 1 (NPP1) from *P. parasitica* resulted in the induction of HR-like cell death, ethylene, reactive oxygen species (ROS), callose biosynthesis and SA-related *PR* gene expression. Interestingly, elicitins from *P. cinnamomi* [25], *Phytophthora cryptogea* or *P. parasitica* var. *nicotianae* [26,27] which have been shown to induce HR-like cell death in other plant species do not cause HR-like cell death in Arabidopsis. While some parallels can be drawn in the involvement of defence hormones in responses against *Phytophthora* species, it is clear that some variability exists between the *Phytophthora*–plant interactions studied to date and generalisations cannot be easily made.

This study was conducted to characterise the Arabidopsis ecotype Columbia-0 (Col-0)–*P. cinnamomi* interaction and elucidate the defence responses involved. Our results indicate that although *P. cinnamomi* was able to colonise root tissue, the plant was able to tolerate the infection. Defence responses were differentially induced in inoculated leaf and root tissue and screening of Arabidopsis defence-related mutant/over-expressor lines suggests that the resistance/tolerance displayed towards the pathogen was not reliant on any of the defence responses/signalling pathways tested. This study also provides to our knowledge, the first report of the use of a model plant to understand interactions with *P. cinnamomi* at the gene level.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis Col-0 wild-type seeds were purchased from Lehle Seeds (Round Rock, Texas, USA). Gene mutant lines and over-expressing lines (as described in Table 1) were originally obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, USA), except for *NahG* (encoding salicylate hydroxylase) which was kindly provided by Dr. Robert Dietrich (Syngenta Biotechnology, North Carolina, USA) and the gene promoter–reporter gene line *PALGFP* (At2g37040, *phenylalanine ammonia-lyase 1* promoter-green fluorescent protein) which was previously constructed [5]. All lines were in the Col-0 background. Seeds were sterilised and germinated on agar plates containing MS basal medium (Sigma–Aldrich, New South Wales, Australia), 1% (w/v) sucrose and 0.8% (w/v) agar, pH 5.7. Seeded agar plates were stratified at 4 °C for 48 h, then placed in a growth cabinet (Thermoline Scientific, NSW, Australia) under a 12 h–12 h light–dark cycle (100 $\mu E\, m^{-2}\, s^{-1}$ cool white fluorescent illumination) at 21 °C. Seedlings were grown on agar plates for 14 days and were either planted in a soil medium [peat moss, sand and vermiculite (3:3:4) supplemented with 5 g/L slow release fertiliser (Osmocote Plus: pots, planters and indoors, Scotts, New South Wales, Austalia)] or within soil-free root observation trays [28]. Root observation trays consisted of two black polycarbonate squares (each 200 mm × 200 mm, 3 mm thick) clamped together with a 3 mm polycarbonate strip down the vertical edges of one of the squares to act as a spacer. One square had the top 30 mm bent at a 45° angle to provide light and space for aerial tissue growth, while the other was lined on the internal surface with Whatman No.1 filter paper (Crown Scientific, New South Wales, Australia). A 10 mm wide strip of cotton wool was placed across the tray at the base of the 45° bend to support the seedlings and the two squares were clamped together. The root observation trays were vertically stacked in black polycarbonate boxes to prevent the roots from being exposed to light and the trays were held in place within slits cut into the upper surface of the boxes. A further description of these soil-free root observation trays is provided in Gunning and Cahill [28]. Nutrient

Table 1
Summary of Arabidopsis defence-related gene mutant/over-expressing lines tested

Name	Locus	Genetic alteration	Trait/phenotype	References
agb1	At4g34460	T-DNA knockout	Lacking heterotrimeric G protein β-subunit mutant	[55]
coi1	At2g39940	T-DNA knockout	Insensitive to jasmonic acid	[45,56]
ein2	At5g03280	EMS mutant	Insensitive to ethylene	[57]
Erf1	At3g23240	Over-expression	Elevated resistance to some pathogens	[46,54]
jar1	At2g46370	EMS mutant	Reduced sensitivity to jasmonic acid	[58]
NahG	N/A	Introduced gene	Contains salicylate hydroxylase, no SA accumulation	[45,59]
npr1	At1g64280	EMS mutant	Defective in a regulator of SA mediated resistance	[45]
pad2	At5g66140	EMS mutant	Reduced glutathione biosynthesis	[9,21,60]
pad3	At3g26830	EMS mutant	Defective in camalexin biosynthesis	[60]
pad4	At3g52430	EMS mutant	Lacking protein involved in SA defence responses	[45,60]
pen3	At1g59870	T-DNA knockout	Putative ATP binding cassette transporter mutant	[36,44]
pmr4	At4g03550	EMS mutant	Defective in stress-related callose formation	[31,41,42]
sid2	At1g74710	T-DNA knockout	Defective in isochorismate-derived SA biosynthesis	[41,45]

All lines are in the Col-0 background. Locus information can be accessed at the Arabidopsis Information Resource (www.arabidopsis.org). References provide examples of the use of these lines in other plant–pathogen studies.

solution (1 ml L^{-1} Total Horticultural Concentrate, Excel Distributors, Victoria, Australia) was added to the boxes to a depth of 20 mm, which allowed the whole filter paper to be saturated with nutrient solution. The nutrient solution was replaced weekly.

2.2. Pathogen isolates and growth conditions

P. cinnamomi isolates DU28, DU54 and DU67 from the Deakin University culture collection were previously collected from various locations within Victoria, Australia. Isolates were confirmed to be *P. cinnamomi* through morphological identification and were further verified using RFLP analysis as described by Drenth et al. [29]. Each isolate was grown on V8 agar medium [10% (v/v) V8 juice (Campbells Soups, New South Wales, Australia), 0.1% (w/v) calcium carbonate and 1.5% (w/v) bacteriological agar (Oxoid, South Australia), pH 6.5]. Calcium carbonate was stirred in V8 juice for 2 h prior to media preparation and the resultant mixture was clarified by centrifugation at 2000*g* for 5 min, followed by filtration (Whatman No. 1 filter paper). V8 liquid broth was prepared in the same manner except that only 5% (v/v) V8 juice was added and agar was omitted. Zoospores were prepared based on the method of Byrt and Grant [30].

2.3. Plant inoculation procedures

Arabidopsis leaf and root tissues were inoculated with *P. cinnamomi* zoospores by pipetting a small volume (2–10 μl) directly onto root or leaf tissue. Following root inoculation, plants were kept stationary under low light for 1 h. Root inoculation of soil-grown plants was conducted by pipetting droplets of zoospores within soil that surrounded roots at the base of the plant. Root inoculation of MS agar plate-grown plants was conducted following careful removal of seedlings from the agar medium, immersion of roots in zoospore inoculum for 1 h and then the seedlings transferred to moist filter paper under sterile conditions or planted into soil. Soil and root observation tray-grown plants were inoculated at 3.5 weeks of age, while plants from MS agar plates were inoculated at 2.5 weeks of age. Zoospore densities were determined using a haemocytometer and inoculations were conducted at 5×10^4 zoospores ml^{-1} unless otherwise specified. Mock inoculations were conducted with distilled water (dH_20).

Virulence of *P. cinnamomi* zoospores was confirmed by control inoculations of the susceptible host plant *Lupinus angustifolius*. All three isolates displayed similar rates of infection of *L. angustifolius*. In interactions with Arabidopsis Col-0, no differences in infection characteristics or plant defence responses induced were observed between the three isolates. Data presented were produced following inoculations with isolate DU28.

2.4. Analysis of P. cinnamomi in infected tissues and cellular responses

To enable visualisation of *P. cinnamomi* within infected tissues, whole roots and leaves were stained with lactic acid–phenol–trypan blue (LPTB) following the method of Vogel and Somerville [31]. Callose was visualised in infected tissue by first clearing tissue in 80% (v/v) ethanol, incubation in 0.07 M sodium phosphate buffer for 1 h and then transferred to buffer containing 0.005% (w/v) aniline blue (ProSciTech, Queensland, Australia) for 2 h for staining. Following staining the tissues were quickly rinsed in fresh sodium phosphate buffer and mounted on microscope slides in 50% (v/v) glycerol. Aniline blue-stained callose was visualised with epifluorescence microscopy performed with a ultraviolet light filter set (365 nm excitation, 420 nm emission, Carl Zeiss, New South Wales, Australia). Detection of hydrogen peroxide used 3,3′-diaminobenzidine (DAB, 1 mg ml^{-1}, Sigma–Aldrich) as described by Robinson and Cahill [12], and lignin was detected with phloroglucinol–hydrochloric acid [32]. *PALGFP* tissue was mounted in dH_20 on microscope slides and the green fluorescent protein was visualised using an ultraviolet light filter set. All microscopy was performed using an Axioskop II Plus microscope (Carl Zeiss).

2.5. Northern blot analysis

For RNA expression analysis, leaf tissue was collected from soil-grown plants, while root tissue was obtained from plants grown in root observation trays. Tissue was collected from plants following zoospore (1×10^5 zoospores ml^{-1}) or mock inoculations across a time course of 0, 12, 24, 48 and 72 h and a minimum of 12 plants were sampled for each group and time point. The 0 h time point was taken within 15 min of completion of the mock-inoculation procedure. RNA was extracted following the protocol described by Etheridge et al. [33]. Northern blotting was carried out with 10 μg total RNA per sample using standard procedures [34]. DNA probes of the genes *PDF1.2* (At5g44420, encoding a plant defensin), *PR1* (At2g14610 encoding pathogenesis related protein 1) and *GST1* (At1g02930, encoding glutathione S-transferase) were radioactively labelled using the Amersham Rediprime II ^{32}P labelling system (GE Healthcare, Australia). Membranes were hybridised overnight in Church buffer [35] at 65 °C then washed in a solution containing 2 × SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7) and 0.1% (w/v) SDS at room temperature for 15 min, followed by a second wash at 55 °C for 15 min and a final wash in 0.2 × SSC, 0.1% SDS at 55 °C for 15 min. Membranes were then exposed to X-ray film for image capture.

3. Results

3.1. Characterisation of infection and defence responses of Arabidopsis Col-0 with P. cinnamomi zoospores

Inoculation with *P. cinnamomi* zoospores directly to the surface of Arabidopsis Col-0 leaves resulted in localised containment of the pathogen with no formation of macroscopic lesions or the development of disease symptoms within leaves (Fig. 1a). The pathogen remained contained for 21 days following inoculation, by which stage the plants had formed siliques and were senescing. Microscopic examination of leaves did, however, show pathogen growth and the induction of plant defence responses. *P. cinnamomi* growth and Arabidopsis HR-like cell death was determined following staining with lactic acid–phenol–trypan blue (LPTB). Identification of other defence-related responses was made with 3,3′-diaminobenzidine (DAB) to detect hydrogen peroxide, aniline blue for callose and phloroglucinol–hydrochloric acid to detect lignin. Cysts of *P. cinnamomi* had germinated and growth of germ tubes occurred on the leaf epidermis within 6 h. Approximately half of the germ tubes developed obvious appressorium-like structures (Fig. 1b, Table 2) which are associated with cellular penetration or attempted penetration [13]. Although appressorium-like swellings were evident, the entry of *P. cinnamomi* into cells and subsequent growth was highly restricted in leaf tissue, with intra- or intercellular hyphal growth rarely observed. Concurring with this result was a low level of HR-like cell death (Table 2), which appeared only to be triggered if penetration occurred, as contact with a germ tube or appressorium-like structure alone did not induce cell death. HR-like cell death was observed when a single cell or small group of cells were under challenge by several germ tubes (Fig. 1c). Accumulation of the ROS hydrogen peroxide, an early defence response associated with the HR was found in single challenged cells and small cell clumps within 6 h (Fig. 1d). Hydrogen peroxide was only produced in cells in direct contact with *P. cinnamomi* structures, although contact with the pathogen did not always cause

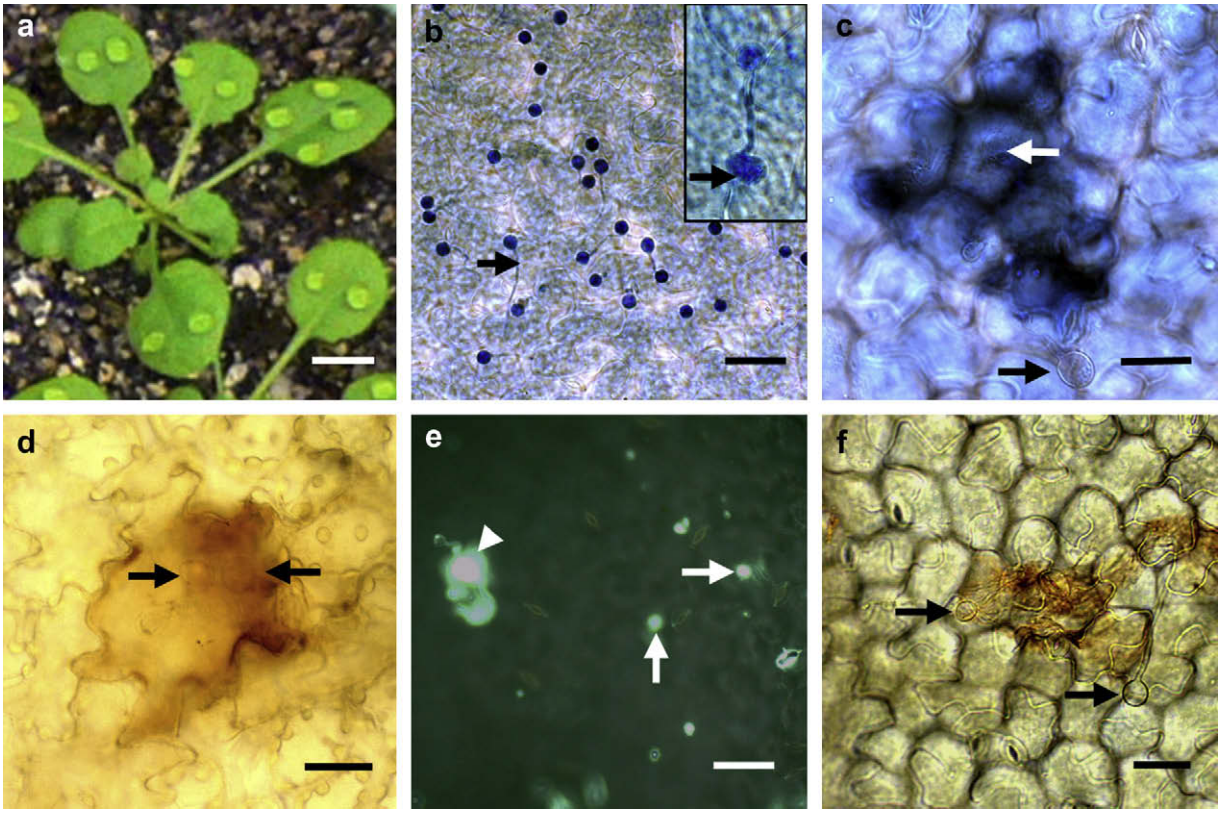

Fig. 1. Infection and defence responses of Arabidopsis Col-0 leaves following inoculation with *P. cinnamomi* zoospores. (a) Leaves following inoculation with zoospores, 5 days p.i. No macroscopic symptoms developed at leaf inoculation sites, scale bar = 5 mm. (b) Zoospores encysted on leaf epidermis and developed hyphae (arrow) stained with LPTB, 48 h p.i., scale bar = 50 μm. Inserted section displays close-up of encysted zoospore with germ tube and appressorium-like swelling at the hyphal tip (arrow). (c) Highly confined HR-like cell death (stained dark blue following LPTB staining) in leaf cells adjacent to *P. cinnamomi* cysts (arrows), 48 h p.i., scale bar = 20 μm. (d) Localised production of hydrogen peroxide (orange/brown colour, produced by DAB staining) in leaf tissue cells under attack from cysts (arrows), 6 h p.i., scale bar = 20 μm. (e) Zoospore-inoculated leaf tissue stained with aniline blue to detect callose (fluorescent blue–green under ultraviolet light), 48 h p.i. Callose papillae were produced at challenge sites within cells (arrows) and callose was also produced in whole cells under attack (arrowhead), scale bar = 50 μm. (f) Lignification of challenged leaf cells 48 h p.i., scale bar = 25 μm. Lignified cells stained orange in the presence of phloroglucinol–hydrochloric acid in close proximity to cysts (arrows) and germ tubes. Experiments were independently repeated a minimum of three times.

accumulation. Callose (β-1,3-D-glucan) production in the form of tightly formed papillae within cells at sites of pathogen challenge was evident by 48 h post-inoculation (p.i.) (Fig. 1e). Accumulation of callose was also observed across the whole area of some challenged epidermal cells but this occurred less frequently than the callose-rich papillae. The accumulation of another structural barrier, lignin, was also present by 48 h p.i. (Fig. 1f). The tight and controlled induction of these defence-related responses and containment of the pathogen demonstrates that Arabidopsis Col-0 leaf tissue was highly resistant to challenge by *P. cinnamomi*. The absence of asexual *P. cinnamomi* sporangia or chlamydospore production on the leaf tissue was further testament to a resistant interaction.

Table 2
Tissue-specific characteristics of the interaction between Arabidopsis Col-0 and *P. cinnamomi* zoospores

Parameter	Tissue type	
	Leaf	Root
Appressorium-like structures (%)	44.6 ± 9.3	++
Cellular penetration, no HR-like cell death (%)	<1	+++
HR-like cell death (%)	6.9 ± 1.6	–
Chlamydospores	–	18.2 ± 13.3
Sporangia	–	4.1 ± 3.5
Callose	+++	+++
Hydrogen peroxide	+	–
Lignin	+	–

Percentage data refers to the mean number of germinated cysts (±SE) displaying or causing a parameter, collected from three independent experiments with a minimum of 100 cysts counted across six plants in each experimental group. Chlamydospore and sporangia counts are the mean number of structures following root-dip inoculations (1×10^4 zoospores ml^{-1}) from three independent experiments with six plants in each experiment. Where statistical data was not available; – indicates parameter did not occur, + = 1–20% of germinated cysts displaying or causing a parameter, ++ = 21–40%, and +++ = greater than or equal to 41%. Hydrogen peroxide data provided was assessed at 6 h p.i., chlamydospore and sporangia counts were performed at 5 days p.i., while all other data were collected 48 h p.i. All data and assessments were based on information collected through microscopic analysis.

Three root inoculation methods were examined, (1) root inoculation of plants growing in soil, (2) root dipping of plate-grown plants in a zoospore solution and (3) direct inoculation of roots growing on filter paper within root observation trays, to determine whether different plant growth and inoculation procedures influenced the interaction of Arabidopsis Col-0 with *P. cinnamomi*. All three inoculation methods produced similar results with no macroscopic lesions or symptoms displayed in roots or in aerial tissues (Fig. 2a). Plants were observed for 21 days following inoculation and as found in leaf tissue, no disease symptoms developed over this time course. Microscopic examination of root tissues showed growth and proliferation of *P. cinnamomi* at inoculation sites (Fig. 2b). Appressorium-like structures were present on the root epidermis but the swellings varied considerably in size and were not easily defined. Hyphal growth was observed to be both inter- and intracellular with the later displaying hyphal swelling within cells (Fig. 2b). This penetration of root tissue did not cause the induction of HR-like cell death. Asexual reproductive structures

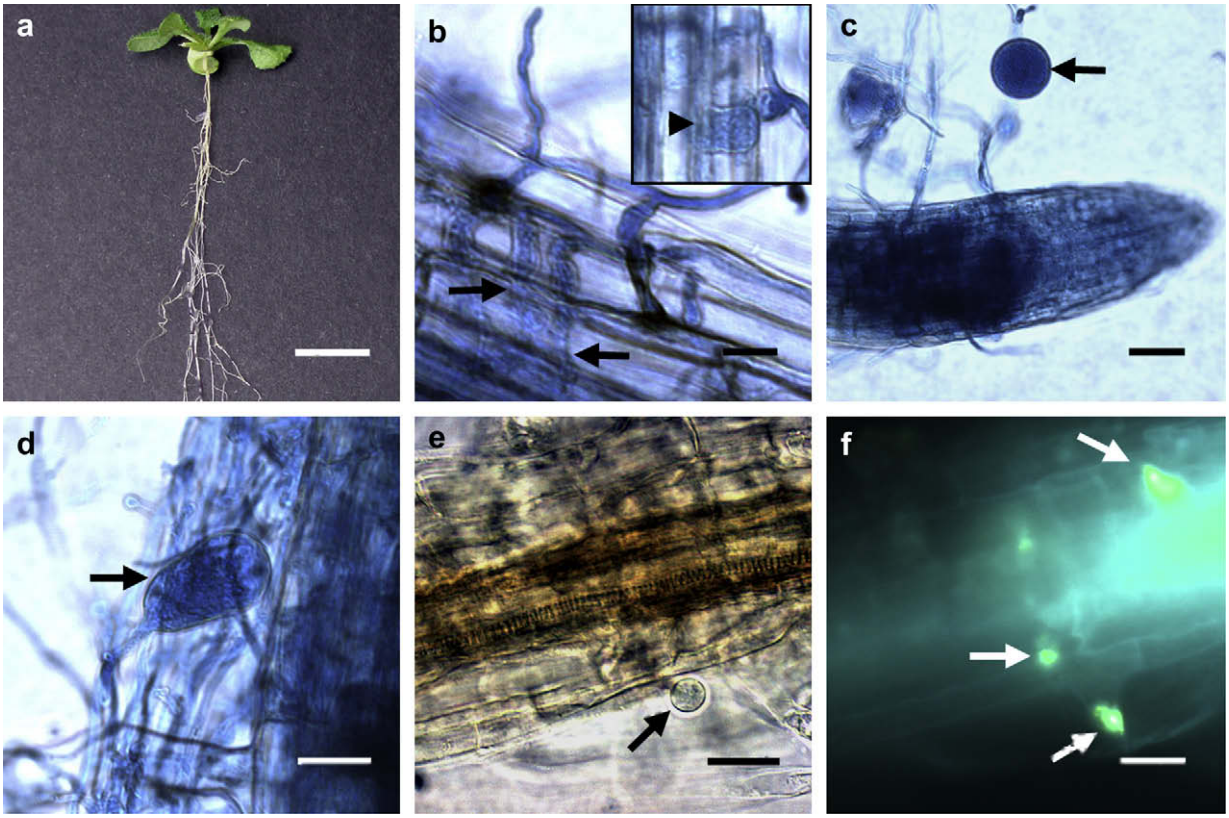

Fig. 2. Infection and defence responses of Arabidopsis Col-0 roots following inoculation with *P. cinnamomi* zoospores. (a) Root tissue following inoculation with zoospores, 5 days p.i. No macroscopic symptoms were evident, scale bar = 10 mm. (b) *P. cinnamomi* infected root tissue displaying a proliferation of hyphae stained with LPTB, 48 h p.i., scale bar = 10 μm. Both intracellular (arrows) and intercellular growth (arrowhead in inserted section) were present. (c) Root displaying hyphal infection and chlamydospore production (arrow) at the zone of elongation, 48 h p.i., scale bar = 40 μm. (d) Production of sporangia (arrow) on root tissue, 48 h p.i., scale bar = 50 μm. (e) Root tissue under attack from cysts and hyphae, 48 h p.i., scale bar = 20 μm. (f) Same root section as displayed in '(e)', but viewed under ultraviolet light to detect aniline blue-stained callose (arrows indicate pathogen-associated callose papillae), scale bar = 20 μm. Experiments were independently repeated a minimum of three times.

in the form of chlamydospores (Fig. 2c) and sporangia (Fig. 2d) were produced on roots within 48 h of inoculation. Although the production of asexual structures was variable between roots and individual plants, chlamydospores were more prevalent (Table 2). Staining for hydrogen peroxide or lignin did not demonstrate accumulation in challenged root cells, although lignin staining was evident in the root stele of both control and inoculated tissue (data not shown). Callose production was, however, associated with sites of pathogen challenge in root tissue with tightly formed callose-containing papillae (Fig. 2e,f, Table 2).

Roots that were 'dip' inoculated in zoospores displayed new pathogen-free root growth adjacent to infected areas within 5 days of inoculation (data not shown). Whilst colonisation of root tissue was observed during experimentation, the absence of disease symptoms and a lack of lesion development indicated that Arabidopsis Col-0 root tissue was tolerant of infection with *P. cinnamomi*.

3.2. *Assessment of* P. cinnamomi *infection in Arabidopsis defence-related gene mutant/over-expressor lines*

To determine whether specific signalling pathways or defence responses were influential in the interaction between Arabidopsis Col-0 and *P. cinnamomi*, 13 gene mutant/over-expressor lines were examined following inoculation with zoospores. These gene mutant/over-expressor lines were chosen due to their ability to affect the mounting of defence in Arabidopsis against other pathogens through previously described defence pathways (such as ET, JA and SA pathways) or responses (see Table 1 for further information and references of their use). No macroscopic symptoms of disease were present in any of the mutant lines following either leaf or root inoculations (all three root inoculation techniques were trialled), as was observed in the Col-0 wild-type. To examine whether infection of root tissue with *P. cinnamomi* influenced plant growth and development, Col-0 and gene mutant/over-expressor lines grown in root observation trays were inoculated and the weight of plants measured 14 days later. Plants were measured using fresh weight [36] as the individual weight of plants was low (typically 20–30 mg). Measurements were taken using one mutant/over-expressor line for each signalling pathway or defence response (a total of 10 mutant/over-expressor lines were measured). No disease symptoms were observed in inoculated plants within the 14-day experiment (Fig. 3a,b), similar to previous findings. There was a slight reduction in the fresh weight of inoculated Col-0 plants, as the weight of *P. cinnamomi*-inoculated plants was 88.4% ± 10.3 of those which were mock-inoculated (Fig. 3c). A comparison of the percentages of inoculated compared to mock-inoculated fresh weights of the mutant/over-expressor lines examined showed no significant differences ($P > 0.05$) from the fresh weight percentage displayed by Col-0. *P. cinnamomi* colonised the root tissue of all lines tested with hyphal growth, chlamydospore and sporangia development displayed evenly across all gene mutant/over-expressor lines (data not shown). The pathogen was not isolated from leaves of root observation tray-grown/root-inoculated plants 14 days after inoculation, indicating that pathogen growth was limited to the root tissue.

Leaf inoculation of the mutant/over-expressor lines resulted in germination of zoospores and formation of appressorium-like swellings across all mutant lines to the same extent that was

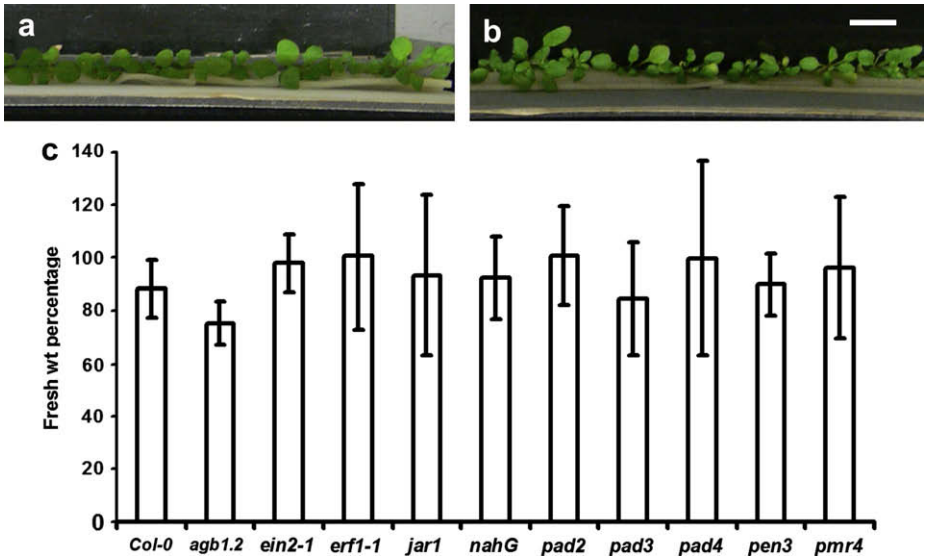

Fig. 3. Root observation tray-grown Arabidopsis Col-0 and gene mutant/over-expressor lines inoculated with *P. cinnamomi*. Aerial photos of root-inoculated Col-0 plants grown in root observation trays 14 days after mock inoculation (a) or inoculation with *P. cinnamomi* zoospores (b), scale bar = 15 mm. Inoculation of roots with zoospores did not cause any visible disease symptoms in either root or aerial tissue. (c) Effect of zoospore-root inoculations on plant growth in Col-0 and various mutants/over-expressor lines. Fresh weight measurements were recorded 14 days p.i. with zoospores (inoculated with 5×10^4 zoospores ml^{-1}, 5 μl per plant). Bars represent the fresh weight percentage of plants inoculated with *P. cinnamomi* compared to mock-inoculated plants of the same line. No significant differences ($P > 0.05$, Student's *t*-test) in fresh weight were found between Col-0 and any of the mutants/over-expressor lines. Data are the average of three independent experiments (±SE), with a minimum of 12 plants per experimental group.

observed in Col-0. Similarly, staining inoculated leaves with LPTB displayed no breakout of inter- or intracellular *P. cinnamomi* hyphae, no development of asexual reproductive structures and restricted HR-like cell death in all the mutant/over-expressor lines tested (data not shown). When leaves from mutant/over-expressor lines were stained for the presence of callose following inoculation, the two mutants *pmr4* and *pen3* showed differences in callose production at pathogen challenge sites when compared to Col-0. Not surprisingly, *pmr4* (a stress-induced callose biosynthesis mutant) did not produce callose in response to *P. cinnamomi* challenge, while *pen3* (a putative ATP binding cassette transporter mutant) displayed more callose producing sites per leaf than Col-0 following inoculation (Fig. 4a–c). While more callose accumulation was evident in *pen3*, production was still limited to single cells or small clumps of cells as was observed in Col-0. Neither Col-0 nor *pen3* displayed callose production in response to mock inoculation.

3.3. Northern analysis of Arabidopsis defence-related genes following infection with P. cinnamomi

The expression of several defence-related genes was examined to assess the involvement of key components of Arabidopsis defence; namely the SA pathway (*PR1*), ET/JA pathways (*PDF1.2*) and accumulation of ROS (*GST1*). In time-course experiments of RNA extracted from Arabidopsis Col-0 leaf tissue inoculated with *P. cinnamomi* zoospores it was apparent that the pathogen caused the induction of defence-related genes (Fig. 5a). Following inoculation, *PDF1.2* showed elevated expression 12–72 h p.i. compared to mock-inoculated leaves, with a maximum level at 24 h p.i. *PR1* displayed low level but detectable induction at 12–48 h p.i. *GST1* also showed increased expression, particularly at the earlier time points of 12 h and 24 h p.i.

The three tested defence-related genes showed a different pattern of expression in inoculated roots compared with that of leaves (Fig. 5b). Low level *PR1* gene expression was found in control roots and no induction was apparent following inoculation. Similarly, expression of *PDF1.2* was not induced and could not be detected (data not shown). *GST1* expression was elevated directly following the mock-inoculation procedure (0 h time point) but quickly dropped to a low basal level at later time points. In contrast, *P. cinnamomi*-inoculated samples displayed some elevation of *GST1* expression between 12 and 72 h p.i. when compared to those which were mock-inoculated.

3.4. Induction of green fluorescence in Arabidopsis PALGFP plants inoculated with P. cinnamomi

PAL1 encodes phenylalanine ammonia-lyase, a key enzyme of the phenylpropanoid pathway that is responsible for the production of secondary metabolites implicated plant defence (such as lignin). *PALGFP* plants containing a GFP reporter gene under the control of the *PAL1* promoter were inoculated with *P. cinnamomi* zoospores to assess whether the phenylpropanoid pathway was activated during defence. Mock inoculations of leaf tissue showed that the basal level of *PAL1* promoter-controlled GFP expression was low (Fig. 6a). Following the introduction of *P. cinnamomi* zoospores to leaf surfaces, GFP was expressed (Fig. 6b). Infection-associated GFP expression was initially observed at approximately 24 h p.i., exhibited its highest intensity by 48 h and dissipated within 120 h. Intense GFP accumulation was predominantly localised to cells in direct contact with the pathogen, although expression was often also present in small clusters of surrounding cells (typically ranging between two and six cells) (Fig. 6c,d). *PAL1* promoter-controlled GFP expression was observed in both epidermal and mesophyll cells, with expression more obvious in mesophyll cells. While induction of GFP was consistently observed across leaves and experimental replicates, the incidence of GFP-expressing cells was low, with only a small number of individual sites (10.0 ± 1.8 SE, following inoculation with ~100 zoospores, $n = 12$) displaying green fluorescence per inoculated leaf at 48 h p.i. Some cells expressing GFP adjoined cells that had likely undergone HR-like cell death. These cells were assumed to have undergone HR-like cell death due to a lack of green fluorescence while under direct challenge by *P. cinnamomi*, when the surrounding cells exhibited GFP (Fig. 6d). Another indicator that HR-like cell death had occurred was the production of cellular autofluorescence under

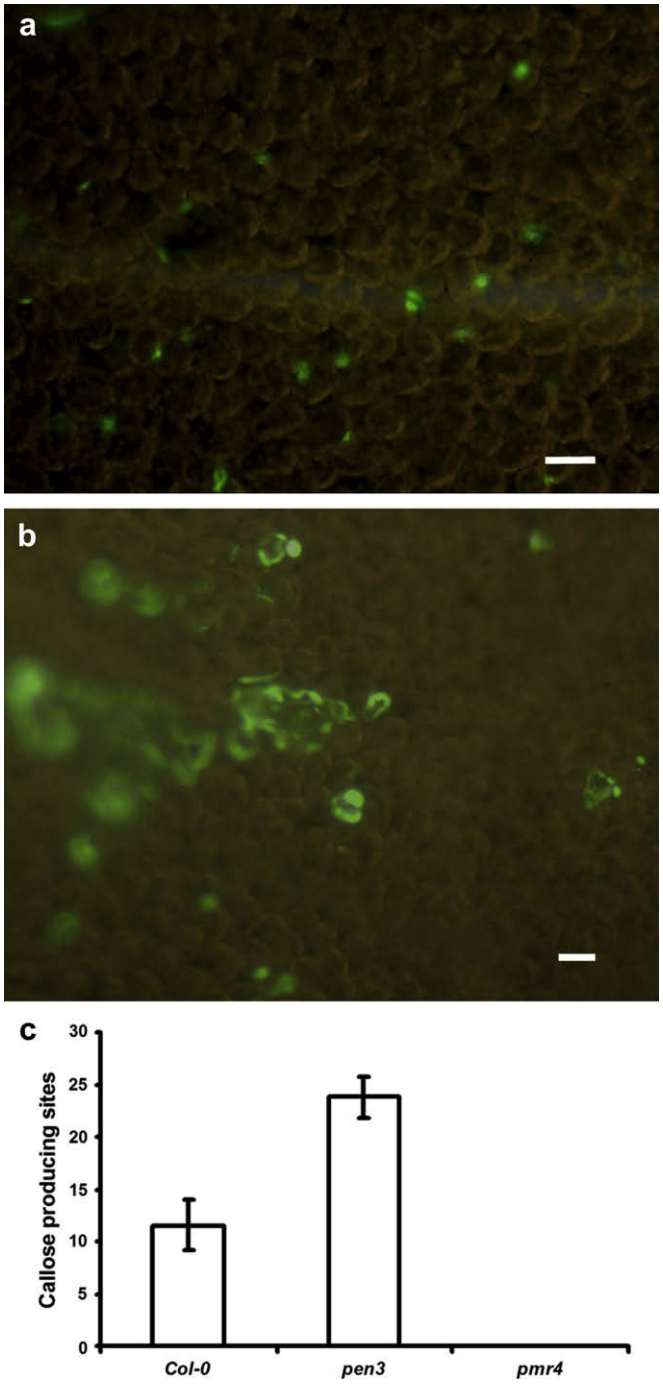

Fig. 4. Induction of callose in Arabidopsis leaf tissue at *P. cinnamomi*-challenged sites. Callose induction in leaf tissue of Col-0 (a) and the *pen3* mutant (b), 48 h p.i. with *P. cinnamomi* zoospores, scale bars = 50 μm. (c) Number of callose producing sites (associated with *P. cinnamomi*) per leaf at 48 h p.i. Leaves were inoculated with 2 μl droplets (of 5×10^4 zoospores ml^{-1}), data are the average of three independent experiments (±SE) with leaves from nine plants per experimental group counted. A significant difference ($P < 0.05$, Student's *t*-test) in the number of callose producing sites was found between Col-0 and *pen3*.

UV light in some *P. cinnamomi*-challenged non-GFP-expressing cells (data not shown). This parameter has been used as a marker of Arabidopsis cell death in other studies [8,37].

Roots of *PALGFP* plants were also inoculated with *P. cinnamomi* zoospores to examine *PAL1* induction. GFP expression was observed in the stele of mock and *P. cinnamomi*-inoculated *PALGFP* roots (Fig. 6e,f) but was not found in epidermal or cortical cells that were

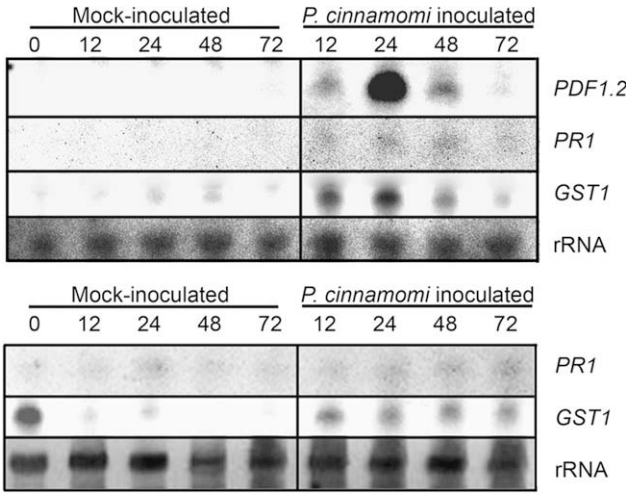

Fig. 5. Expression of defence-related Arabidopsis genes following mock inoculation or inoculation with *P. cinnamomi*. (a) Arabidopsis Col-0 leaf tissue, (b) Arabidopsis Col-0 root tissue. Tissue was collected during time-course experiments at 0, 12, 24, 48 and 72 h p.i. RNA blots were hybridised with the probes labelled to the right of figure. Ribosomal RNA (rRNA) was used to show even loading. Time-course experiments were performed twice with similar results.

in contact with the pathogen (Fig. 6g,h) up to 7 days after inoculation.

4. Discussion

Inoculation of Arabidopsis Col-0 plants with the pathogen *P. cinnamomi* resulted in pathogen encystment, germination and hyphal growth. In leaf tissue, penetration of cells was rare and when it did occur, further growth was contained and associated with typical Arabidopsis defence responses which included the induction of ROS, HR-like cell death, callose production and lignin biosynthesis. The induction of these defence responses was very specific to challenged cells and was tightly controlled, which is typical of the responses displayed in non-host resistance [14,15,18]. As *P. cinnamomi* is predominantly a root pathogen, its inability to colonise and cause disease in Arabidopsis Col-0 leaf tissue was somewhat expected. A previous study [12] showed a similar response of Arabidopsis Col-0 leaf tissue to *P. cinnamomi*, although low level chlamydospore production was observed, which may have resulted from different experimental procedures and conditions. In contrast, inoculation of Arabidopsis Col-0 root tissue with *P. cinnamomi* did result in penetration of root tissue and the subsequent emergence of asexual reproductive structures in the form of chlamydospores and sporangia. This infection did not, however, lead to disease symptoms and there was no reduction in growth or impediment of plant development. Therefore, in the Arabidopsis Col-0–*P. cinnamomi* interaction, Col-0 is probably best described as tolerant to the pathogen, a term commonly used to for plants that are infected by *P. cinnamomi* but are able to survive infection [38,39]. Tolerance to *P. cinnamomi* is displayed by many species and relatively few plants have been shown to be truly resistant to infection by *P. cinnamomi* [3]. Following root inoculation, the pathogen could be re-isolated from root but not aerial tissue, indicating that the infection was limited to root tissue. The development of new lateral root growth with no sign of *P. cinnamomi* colonisation adjacent to infected root tissue provides further evidence that although infection clearly occurs, there is some degree of pathogen restriction. This 'tolerant' response of *P. cinnamomi*-inoculated Arabidopsis Col-0 is very different to that of highly susceptible species, such as *L. angustifolius* which displays

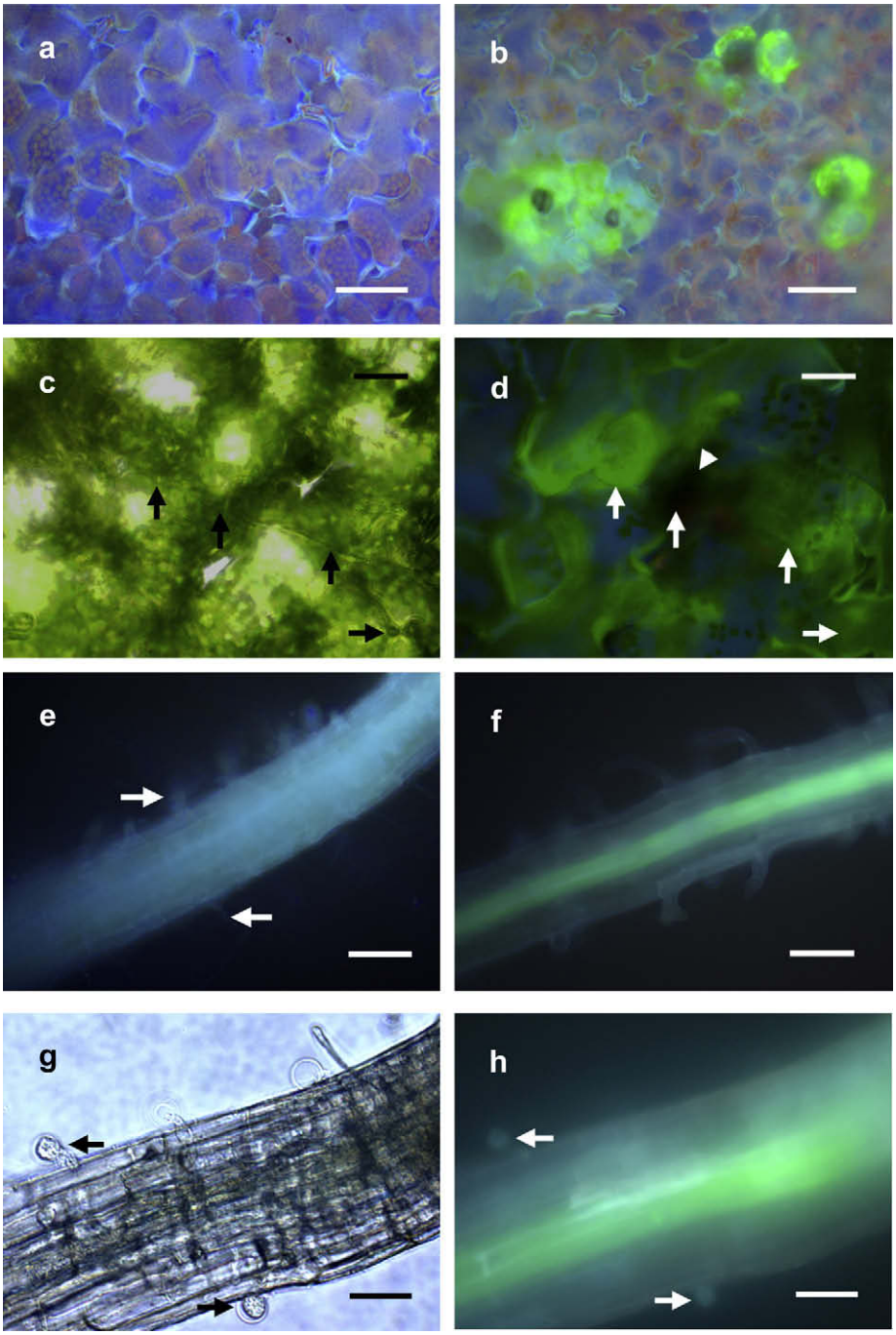

Fig. 6. *PAL1* promoter-controlled GFP expression following inoculation with *P. cinnamomi*. (a) Arabidopsis *PALGFP* leaf tissue 48 h after mock inoculation, scale bar = 50 μm. (b) Arabidopsis *PALGFP* leaf tissue at 48 h p.i. with zoospores, scale bar = 100 μm. Expression of GFP is associated with *PAL1* promoter induction which is restricted to individual or small groups of cells directly under pathogen challenge. (c) *PALGFP* leaf tissue under attack from germinated cysts and hyphae (arrows) on the leaf surface, 48 h p.i., scale bar = 20 μm. (d) Same section as in image (c), displaying GFP expression in cells in contact with *P. cinnamomi* structures. The presence of a cell that appears to have undergone HR-like cell death due to the proximity of the pathogen and a lack of GFP expression is depicted by an arrowhead. (e) Col-0 root tissue under attack from germinated cysts and hyphae (arrows), 48 h p.i., scale bar = 50 μm. No green fluorescence is present. (f) Arabidopsis *PALGFP* root tissue 48 h after mock inoculation, scale bar = 50 μm. GFP is expressed in the root stele. (g) *PALGFP* root tissue under attack from germinated cysts (arrows) and hyphae, 48 h p.i., scale bar = 20 μm. (h) Same root as in image (g), displaying GFP within root stele but GFP expression is not evident in cells under immediate *P. cinnamomi* challenge. All images were photographed under ultraviolet light, except for (c) and (g) which were photographed under white light. Experiments were independently repeated a minimum of three times.

necrosis of roots, chlorosis of leaves and subsequent plant death when grown and inoculated in the same manner.

Hypersensitive response-like cell death was observed in leaf cells following challenge with *P. cinnamomi* and when it was triggered the pathogen was tightly contained. This was not found to occur in root tissue and may be a contributing factor to the different infection levels displayed by inoculated root and leaf tissue. Similarly, HR-cell death in root tissue is in the most part absent in other root–pathogen interactions studied to date, presumably because the occurrence of HR-cell death in root tissue would have stark physiological consequences on the whole plant and the myriad of necrotrophic microorganisms found in soil could take advantage of such a response [40]. Research conducted by Takemoto et al. [25] found that treatment of Arabidopsis leaves with *P.*

cinnamomi-derived elicitins did not induce HR-like cell death, while it was observed in other plant species tested. This suggests that for *P. cinnamomi* to induce the HR-like cell death in Arabidopsis leaf cells, direct challenge from the pathogen is required. This agrees with our findings while many cells were exposed to cysts and/or hyphae across the leaf surface, HR-like cell death was limited and was usually correlated with a germ tube displaying an appressorium-like swelling, indicating penetration or attempted penetration.

One of the great advantages of using Arabidopsis in the study of plant–pathogen interactions is the availability of a wide range of gene mutants and gene over-expressing lines. In this study a variety of the defence-related gene mutant/over-expressing lines were screened for their responses to challenge with *P. cinnamomi*, but none caused a significant increase in the development of disease or pathogen infection. The only clear difference following inoculation was found in the levels of callose production in inoculated leaf tissue in the mutant lines *pmr4* and *pen3*. *pmr4* is incapable of producing callose (in response to wounding or pathogen challenge) but this did not result in the development of disease symptoms or pathogen proliferation, which indicates that although callose production is induced by *P. cinnamomi* challenge, it is not responsible for controlling the invasion. Callose induction has been implicated in Arabidopsis resistance against the pathogens *Alternaria brassicicola* [41] and *Plectosphaerella cucumerina* [42] but its absence has been shown to enhance resistance to pathogens such as *Pseudomonas syringae* [41] and *Hyaloperonospora parasitica* [31]. It has been demonstrated that *pmr4* displays elevated levels of SA-related defence [41,43], adding complexity to the relationship between callose induction and plant defence. In contrast, the *pen3* mutant showed increased callose accumulation in leaf tissue within cells under challenge by *P. cinnamomi*. Increased levels of callose production can be associated with increased levels of pathogen penetration or attempted penetration [6,36]. Arabidopsis *pen3* mutants have previously been shown to display elevated disease symptoms and increased pathogen penetration following inoculation with several other pathogens including *P. infestans*, which produces a non-host interaction with Arabidopsis [36,44]. *PEN3* has been characterised as encoding an ATP binding cassette transporter that may be involved in exporting toxic materials to attempted pathogen invasion sites and is thought to be an important component of non-host resistance [36]. Other than the increased callose level in inoculated leaf tissue there was no macro or microscopic evidence that the *pen3* mutation caused a marked increase in *P. cinnamomi* infection levels following either leaf or root inoculations.

In all the other mutant lines tested there were no obvious differences in the responses against *P. cinnamomi* when compared to the Arabidopsis Col-0 wild-type. The gene mutant/over-expressing lines used in this study included a variety of signalling pathways and responses that have been shown to be important in defence against other pathogens. Of particular interest was the response of mutants defective in the plant defence pathways ET, JA and SA, which have been shown to be highly influential in the outcomes of many plant–pathogen interactions. The inability of these mutations to impact on Arabidopsis defence against *P. cinnamomi* is not unique, however, as resistance in Arabidopsis against other pathogens such as *P. brassicae* [9] and *Leptosphaeria maculans* [4] does not require the involvement of these pathways. In a study by Roetschi et al. [9], it was found that the *pad2* mutant (recently identified as a γ-glutamylcysteine synthetase [21]) exhibited increased susceptibility to *P. brassicae*. This was not the case in the interaction of *pad2* with *P. cinnamomi*, but as Arabidopsis leaf tissue is susceptible to *P. brassicae* (isolate dependent) it is likely that the requirements for defence against these two oomycetes may vary considerably. Likewise the *pad2* mutant did not affect Arabidopsis resistance against *P. infestans* [9].

Analysis of gene expression levels showed that inoculation of Arabidopsis Col-0 leaf tissue with *P. cinnamomi* caused the induction of defence-related genes. The most apparent induction was observed in the expression of *PDF1.2*, indicating that ET/JA signalling pathways were activated. Induction of these pathways has been shown in Arabidopsis in response to other *Phytophthora* spp. such as *P. infestans* [8] and *P. brassicae* [9]. It has been reported that the ET/JA pathways, rather than SA pathways are preferentially activated in non-host resistance interactions [36,45] and the Arabidopsis Col-0 leaf tissue–*P. cinnamomi* interaction shows a similar pattern of gene expression. Although *PDF1.2* was activated, neither the JA pathway mutants *jar1* and *coi1* or the ET pathway mutant *ein2* was compromised in their resistance to *P. cinnamomi*, implying that ET/JA pathways are involved but other components of Arabidopsis defence also provide protection against *P. cinnamomi*. In the interaction between Arabidopsis and the non-host pathogen *Blumeria graminis* f. sp. *hordei* a similar result was found with increased expression of ET/JA responsive defensin genes, while *coi1* and *ein2* mutants produced resistant interactions to the same degree as wild-type plants [45]. The nominal induction of *PR1* (a SA-responsive gene) suggests that the SA defence pathway has only minor involvement in the response. As *P. cinnamomi* is predominantly a necrotroph this is not surprising, however, some necrotrophic pathogens such as *Fusarium oxysporum* have also been shown to up-regulate SA pathways [46]. It was interesting to find that *PDF1.2* induction could be detected in leaf but not root tissue. A similar pattern of *PDF1.2* expression in root and leaf tissue has been observed following root inoculations of Arabidopsis with *F. oxysporum* [47]. Organ specificity of plant defence responses has been described previously [40,48] and the limited amount of active defence responses triggered in Arabidopsis Col-0 root tissue inoculated with *P. cinnamomi* is a possible explanation for the differences in *P. cinnamomi* infection exhibited between root and leaf tissue. In comparison to foliar plant pathogens, we still know little about the mechanisms plants use to combat root pathogens and indeed only a small proportion of defence responses described in foliar pathogen interactions have been reported to occur in roots [49]. Further understanding of root–pathogen interactions is required to provide us with the tools to tackle these pathogens both in agricultural situations and native ecosystems.

GST1, a marker gene for the oxidative burst [50] was up-regulated in response to *P. cinnamomi* in leaf and to a lesser degree, in root tissue. In leaf tissue this expression was evident at the earlier time points measured (12–24 h) which correlates with ROS production being one of the early active defence responses triggered by pathogens [51]. Leaf hydrogen peroxide staining provides a corresponding line of evidence for early activation of the oxidative burst in this interaction. The expression of *GST1* in root tissue was observed in the initial mock-inoculated sample, indicating that the preparation and inoculation of plants may have caused some induction of ROS (this was consistently displayed across several experiments). Although initial tissue sampling was conducted within 15 min of mock inoculations, the root observation trays containing the plants were moved and prepared for inoculation approximately 1 h prior. It is most likely that these processes caused some wounding and/or stress to the plants to cause this *GST1* expression at the initial time point. *GST1* has been reported to be quickly up-regulated in response to wounding or environmental stresses [52] and indeed its early induction during mock inoculations in other studies has been shown [50,52,53]. The initial induction quickly dropped in the mock-inoculation time course, however, while *GST1* remained moderately elevated in the 12–72 h *P. cinnamomi*-inoculated samples. It is possible that this was due to continued *P. cinnamomi* growth causing a repeated stimulus of ROS. DAB-hydrogen peroxide staining did not show *P. cinnamomi*-related hydrogen peroxide accumulation in roots, although a low

level of sparse ROS production may not be detectable using this staining technique. Alternatively, *GST1* expression may have been caused by other factors than hydrogen peroxide *per se*.

Expression of *PAL1* promoter-controlled GFP provided a visual approach to examining induction of a defence-related gene in the interaction. *PAL1* encodes for the enzyme phenylalanine ammonia-lyase, a key enzyme of the phenylpropanoid pathway which is responsible for the production of many secondary metabolites implicated in plant defence and its expression has been shown to be up-regulated in response to a variety of abiotic factors and various pathogens [5]. Localised *PAL1* promoter-controlled GFP expression was observed in leaf cells directly under challenge or in close proximity to *P. cinnamomi* and maximal GFP expression was observed at 48 h p.i. This further suggests that the pathogen was effectively contained, otherwise GFP expression would have most likely spread to neighbouring cells if infection progressed. While the induction of the phenylpropanoid pathway was demonstrated in *P. cinnamomi*-challenged Col-0 leaf tissue (through *PAL1* promoter analysis), its induction has also been previously shown in response to leaf pathogens such as *H. parasitica* and *P. syringae* pv. *tomato* [5], although the magnitude of GFP expression was much higher in these host–pathogen interactions. This supports the proposition that the Arabidopsis Col-0 leaf tissue–*P. cinnamomi* interaction resembles non-host resistance. Adding further weight to this finding was the presence of dead cells often adjoining GFP-expressing cells, suggesting that tightly defined HR-like cell death had occurred.

This study demonstrates the difficulty in developing strategies to protect plants from pathogens with wide host ranges such as *P. cinnamomi*. Arabidopsis Col-0 does not succumb to the pathogen, but as asexual reproduction of the pathogen occurred in root tissue, within a natural ecosystem containing a variety of plant species this would likely result in propagation of the pathogen and lead to infection of other species. The ability to asexually reproduce on the roots of a wide variety of plant species is a major reason why *P. cinnamomi* poses such a threat to natural ecosystems [3]. The activation of particular defence responses in root tissue may be an avenue to provide resistance to this pathogen, although constitutive expression of the ethylene response factor 1 (ERF1), transcription factor which can enhance resistance against other pathogens such as *F. oxysporum* [46], *B. cinerea* and *P. cucumerina* [54] did not prevent *P. cinnamomi* colonisation or asexual reproduction on root tissue in this study. In accordance, the ET pathway mutant *ein2* was not compromised in resistance against *P. cinnamomi* which further suggests that the ET pathway is not a key element in Arabidopsis defence against this pathogen. It can, however, be concluded that the resistance/tolerance of Arabidopsis Col-0 to *P. cinnamomi* involves the induction of various defence responses and signalling pathways, but does not appear to singly rely upon any of the defence responses or pathways that were represented by the various mutant/gene over-expressing lines examined. The resistance/tolerance displayed is, therefore, likely to be multi-faceted. As there was considerable variation in the defence responses exhibited by leaf and root tissue, this could be a major factor in the differences in *P. cinnamomi* infection between the two tissue types. Further research is required to understand the mechanisms that allow some plants to resist or tolerate this pathogen while others remain vulnerable.

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