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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.