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Phosphite induces expression of a putative proteophosphoglycan gene in *Phytophthora cinnamomi*

Mee-Hua Wong[A,B], *Jennifer McComb*[B], *Giles E. St J. Hardy*[B] *and Philip A. O'Brien*[B,C]

[A]Agricultural Research Centre, Semongok, PO Box 977, 93720 Kuching, Sarawak, Malaysia.
[B]Centre for Phytophthora Science and Management, School of Biological Sciences and Biotechnology, Murdoch University, Murdoch, WA 6150, Australia.
[C]Corresponding author. Email: P.Obrien@murdoch.edu.au

Abstract. The phosphate analogue phosphite is widely used to control diseases of plants caused by oomycete pathogens such as those within the genus *Phytophthora*. Phosphite inhibits zoospore production and growth of *P. cinnamomi*. However, very little is known about the underlying mechanism of action. In the present study, we grew *P. cinnamomi* in Ribero's liquid medium with 0.1 mM phosphate, with and without 5 μg phosphite/mL, and used differential display reverse transcriptase–PCR (DDRT–PCR) to identify *P. cinnamomi* genes that are transcriptionally repressed or induced by phosphite. By using this technique, four differentially expressed bands were identified. However, quantitative measurement of the amount of mRNA transcript by RT–PCR revealed that only one gene was actually phosphite inducible. On the basis of the homology of the deduced amino acid sequence, this gene encodes a proteophosphoglycan. The remaining three bands did not show differential expression.

Additional keywords: DDRT–PCR, phosphite, *Phytophthora.*

Introduction

Phytophthora cinnamomi occurs worldwide on a broad range of plant species, causing significant losses in the horticulture, agriculture and forestry industries, and disrupting the ecological balance in natural communities (Shearer *et al*. 2007). *P. cinnamomi* is established in more than several hundred thousand hectares of native forests across the southern part of Australia where it presents a major threat to native ecosystems, resulting in disruption of plant-community structure, a decline in species richness and plant abundance, degradation of faunal habitat and increased soil erosion. Given the resources required, complete eradication of *P. cinnamomi* from native ecosystems is not a practical goal. Disease management is achieved through the use of hygiene, quarantine and the use of chemicals. In Australia the principal chemical used for control is phosphite, an inexpensive, non-toxic analogue of phosphate (Shearer *et al*. 2004). Phosphite is used as either the potassium salt of phosphite, or in the form of ethyl phosphonate (marketed as Aliette™ or Fosetyl™) in which three ethyl-phosphonate groups are ionically bonded to a single Al ion (McDonald *et al*. 2001*a*). It is phosphite, released in the plant by hydrolysis of ethyl-phosphonate, that is responsible for protection of plants against *P. cinnamomi* and other oomycete pathogens.

Phosphite is highly effective in reducing the impact of *P. cinnamomi* on susceptible species from a range of native plant communities. Studies on native plant communities in Western Australia (Hardy *et al*. 2001; Shearer *et al*. 2004) have shown that phosphite reduces the rate at which disease fronts move through a plant community from 6 months to more than 5 years, whereas Aberton *et al*. (1999) showed that application of Fosetyl to *Xanthorrhoea australis* plants in Decline stage 2 delayed further development of symptoms for more than 2 years. The mechanism by which exposure to phosphite makes plants resistant to subsequent infection by *P. cinnamomi* is unknown. However, it appears to be mediated by induction of the defence responses of the plant. Application of Fosetyl to *Arabidopsis thaliana* resulted in induction of PR1 transcription, and resistance to *Peronospora parasitica* (Molina *et al*. 1998). These effects were not observed in *Nah*G plants deficient in salicyclic acid-induced resistance, suggesting that Fosetyl-induced resistance is mediated through induction of salicyclic-acid signalling. More recently, Daniel and Guest (2006) showed that phosphite treatment of *A. thaliana* resulted in resistance to *Phytophthora palmivora*. On infection, phosphite-treated plants showed enhanced development of cytoplasmic aggregates, increased superoxide production and increases in localised cell death and accumulation of phenolics at infected cells. Pathogen development and sporangial production were severely restricted in the phosphite-treated plants and the hyphae were distorted. Induction of defence responses by phosphite has also been observed in species of native Australian plants. In *Banksia brownii*, phosphite treatment resulted in more rapid and extensive lignin formation around infection sites caused by *P. cinnamomi* (Smith *et al*. 1997*a*).

Phosphite can also act directly on *Phytophthora. In vitro* experiments have shown that exposure of *P. cinnamomi* to phosphite causes inhibition of growth (Wilkinson *et al*. 2001*b*; Wong 2006) and zoospore formation (Wilkinson *et al*. 2001*a*).

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Direct exposure may contribute to the resistance of phosphite-treated plants as phosphite is not metabolised by plants (McDonald *et al*. 2001*a*) but accumulates in tissues such as fruit, leaves, stems and roots where it may persist for some time (Pilbeam *et al*. 2000; Barrett *et al*. 2004; Malusa and Tosi 2005). Pathogens infecting phosphite-treated plants will be directly exposed to phosphite. Experiments have shown that in lupin phosphite induces resistance to *Phytophthora nicotiana* only when it accumulates in sufficient concentration to inhibit growth *in vitro* (Smillie *et al*. 1989). In their study on infection of avocado by *P. cinnamomi*, van der Merwe and Kotze (1994) observed a close correlation between the concentration of phosphite at the site of infection and the degree of protection. Work with *Eucalyptus marginata* also suggests a direct effect of phosphite on the invading pathogen at high phosphite concentrations (Jackson *et al*. 2000). As an approach to understanding the mechanism of the direct action of phosphite on *P. cinnamomi*, we used DDRT–PCR (Liang *et al*. 1995) to identify *P. cinnamomi* genes that are induced or repressed by phosphite.

Materials and methods

Isolates of P. cinnamomi

The isolates used in the present study were as follows: MP9448, MP62 and MP9411, isolated from *Eucalyptus marginata*; MP9418, isolated from *Corymbia callophyla*; and MU33, isolated from soil. The isolates were obtained from the Centre for Phytophthora Science and Management (CPSM) culture collection at Murdoch University.

Phosphite solution

The phosphite used was phosphorous acid (H3PO3, 99%, Aldrich, St Louis, MO, US). A stock solution was prepared with distilled water. The pH of the solution was adjusted to 6.5 with 6 M potassium hydroxide. The solution was sterilised by filtering through a 0.2-μm millipore filter (Schleicher and Schuell, Keene, NH, US).

Growth-inhibition experiments

Growth-inhibition experiments were carried out in 9-cm Petri plates containing 20 mL of liquid modified Ribeiro's medium (Ribeiro 1978). Phosphate or phosphite was added to the sterilised medium by addition of an aliquot of a concentrated stock solution to the autoclaved (10.5 kg/m^2 for 15 min) medium. The medium was inoculated with a 6-mm-diameter plug of agar from a Ribeiro's agar plate that had been inoculated from the stock cornmeal agar plate and incubated in the dark for 7 days at 26°C. The mycelium was harvested by filtration and the dry weight measured. The results were expressed as percentage growth inhibition of mycelium. Results were calculated as.

$$(y1 - y2)/y1 \times 100,$$

where $y1$ = mean dry weight of phosphite-free mycelium and $y2$ = mean dry weight of phosphite-treated mycelium. There were three replicate Petri plates per concentration and the experiments were carried out twice.

Mycelial growth and extraction of RNA

Three 6-mm-diameter mycelium plugs from a 5-day-old agar culture were placed in a 9-cm Petri plate containing 20 mL of modified (0.1 mM phosphate) Rebeiro's liquid medium. Where required phosphite was added to the medium at a concentration of 5 μg/mL. The cultures were incubated at 26°C for 3 days, at which time the mycelium was harvested, rinsed twice with sterilised distilled water, blotted with filter paper and snap-frozen in liquid nitrogen before storing at −80°C. Total RNA was extracted from phosphite-treated and untreated mycelium, according to Logemann *et al*. (1987).

DDRT–PCR

DDRT–PCR was carried out essentially as described by Jorgensen *et al*. (1997), with two-base anchor primers $ET_{12}VA$, $ET_{12}VC$ and $ET_{12}VG$ and $ET_{12}VT$ (E is an *Eco*R1 site, 5′-GAATTC-3′ and V is a degenerate base) and equal amounts of RNA from phosphite-treated and untreated mycelium. First-strand cDNA was synthesised using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, US), according to the manufacturers instructions. The first-strand cDNA was used for PCR amplification with the 3′ anchor primer and an arbitrary primer. Amplification products were analysed by electrophoresis in a 2% high-resolution agarose (Progen, Redwood City, CA, US) gel at 5 V/cm in TBE buffer (Sambrook *et al*. 1989) for 3.5 h.

Differentially expressed cDNA bands were excised from gels and the DNA purified with the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI, US) as described by the manufacturer. Purified cDNA (2 μL) was re-amplified with the same anchor and arbitrary primers that were used in the DDRT–PCR to generate the band under the same amplification conditions except that the annealing temperature was increased to 60°C to provide extra stringency in the amplification process. The products were purified with the Wizard PCR cleanup system (Promega) as recommended and cloned into pTOPO (Invitrogen) as instructed by the supplier.

DNA sequencing

For sequencing of cloned bands, plasmid minipreps were prepared from overnight cultures of *Escherichia coli* by using the Wizard SV Plus Minipreps DNA purification system (Promega). Sequencing reactions were performed with an ABI PRISM Big Dye Terminator kit (Applied Biosystems) and sequence reads generated on an ABI 3730 automated DNA sequencer (Applied Biosystems). Both strands of each clone were sequenced.

Sequence data were analysed with the software program Sequence Editor V 1.03 (Applied Biosystems, Carlsbad, CA, US). Sequences were edited manually to remove vector sequences and to amend ambiguous bases by comparison with the chromatograms from the program. Basic local aligment search tool (BLAST) program (Altschul *et al*. 1997) was used to search the GenBank database of the National Centre for Biotechnology Information database (http://www.ncbi.nlm.nih.gov) for possible sequence homology. Each nucleotide sequence was subjected to BLASTX analysis which translates the query nucleotide

sequence in six reading frames and compares the translational products to protein database.

qRT–PCR

The SuperScript™ III Platinum Two-Step qRT–PCR kit with SYBR Green (Invitrogen) was used for quantitative reverse transcriptase PCR (qRT–PCR) on mRNA extracted from untreated and phosphite-treated mycelium as described by the supplier. Amplification was carried out by using the ABI PRISM 7700 sequence detector (Applied Biosystems). For qRT–PCR, forward (f) and reverse (r) primers were designed for each of the isolated differential bands. The sequences of the primers for each gene are CP1f: 5′-AATCGCTACGAGCTTCCGCC, CP1r: 5′-GGGCAGCGCAAG TTGTCTGA; CP6f: 5′-TTGA TCTCGTCGCTGCTGGG, CP6r: 5′-GCGGTGGTCG AATC GTCGTA; CP22f: 5′-CGCTCCCAATCACGATGTT, CP22r: 5′-AGCACCC sCGATGAGATATGG; and CP29f: 5′-GTG CATCACTTCGCGTCGCT, CP29r: 5′-TTCACCCCCAGTT TGCGTCC. The thermal cycling conditions consisted of one cycle at 50°C with a 2-min hold, one cycle at 95°C with a 2-min hold for denaturation and 'hot-start', followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s.

Amplification plots (ΔR_n *v.* cycle number) were generated with the software program Sequence Detector V 1.7 which is part of the ABI PRISM 7700 Sequence Detection System. The standard curves were generated with Sequence Detector V 1.7 and Microsoft Excel 2004. The difference in transcript level between the phosphite-treated and untreated samples was then calculated on the basis of the formula $\Delta C_T = C_T (+P) - C_T (-P)$.

Reference genes for qRT–PCR

For the development of endogenous reference genes used for normalisation of the amounts of RNA (Livak and Schmittgen 2001; Ponchel *et al*. 2003), forward (f) and reverse (r) primers were designed for the following candidate genes: *P. cinnamomi* tubulin (Accession no. AY766221) (HK1f, TCGTGCTGCT TTTGGATGCG: HK1r, CATACA CGCTCCGCGGATCA), *P. infestans* cyclophilin (Accession no. AF424658) (CYC1f, TAGATGCCGAACCCGCAGGT: CYC1r,CTGTGCAAAGA GCGCGGAAG) and *P. palmivora* actin (Accession no. AY 729846) (ACT1f, TTTGACTGAAGCGCCGCTCA: ACT1r, AATCGCGTCCAGCCAGGTTC). Amplification conditions used with these primers were as described above.

The efficiency of amplification of the endogenous reference and the target gene was tested by amplification of a series of 10-fold dilutions of the cDNA from the untreated mycelium. For each dilution, the change in fluorescence (ΔRn) was measured and plotted against the cycle number (C_T) in the amplification plot. A standard curve for both reference and target genes was then constructed by plotting the C_T values against the logarithm of the 10-fold serial dilutions of cDNA. From the slope of each standard curve, the PCR amplification efficiency was calculated by using the following formula: Efficiency (E) = $(10^{(-1/\text{slope})} - 1) \times 100\%$ (Guide to performing relative quantification of gene expression using real-time quantitative PCR, Applied Biosystems).

The identity of the gene that was differentially expressed was determined by using the DNA sequence to query the GenBank database using the BLASTN algorithm (Altschul *et al*. 1997).

Results

Growth-inhibition experiments

To identify genes that are transcriptionally responsive to phosphite, we need to use a phosphite concentration that at least partially inhibits growth yet still allows production of sufficient mycelium for RNA extraction. Since growth inhibition by phosphite has been observed only at concentrations of phosphate that are limiting for growth (Barchietto *et al*. 1988; Griffith *et al*. 1993; McDonald *et al*. 2001*b*), we determined the growth response of *P. cinnamomi* at different concentrations of phosphite and phosphate. Across the range of 0–0.5 mM phosphate, *P. cinnamomi* showed a strong positive growth response to phosphate concentration (Fig. 1), with a slower rate of increase across the range 0.5–10 mM phosphate. These results showed that a phosphate concentration of 0.1 mM, although limiting for growth, would still allow production of sufficient mycelium for RNA extraction. This level of phosphate was used in media when testing the effects of phosphite on growth of *P. cinnamomi*.

The five isolates of *P. cinnamomi* were incubated in medium with 0.1 mM phosphate and different concentrations of phosphite to assess the effects of phosphite on growth (Fig. 2). The mycelial growth of all five isolates of *P. cinnamomi* was significantly ($P < 0.001$) inhibited by phosphite from 5 µg/mL through to 100 µg/mL, with the percentage inhibition increasing as phosphite concentration increased. These results showed that a concentration of phosphite of 5 µg/mL is appropriate for the growth of mycelium for DDRT–PCR, as growth was 30–60% reduced yet a sufficient mass of mycelium was produced for RNA extraction.

DDRT–PCR

RNA was extracted from untreated and phosphite-treated mycelium and used for DDRT–PCR analysis with 76 primer-pair combinations. The products were electrophoresed on a high-definition agarose gel. To reduce the effects of natural variation in band pattern that occurs between PCR-amplified samples, each sample was analysed in duplicate, and only those differential

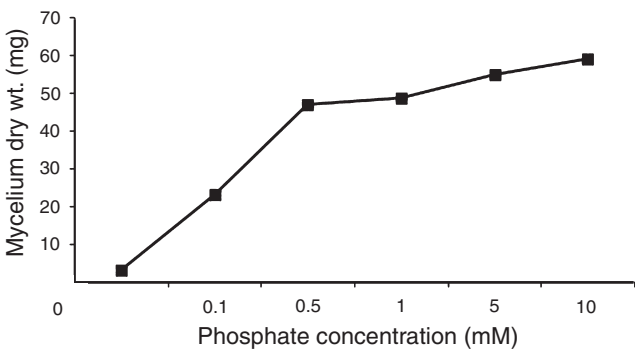

Fig. 1. Effect of phosphate concentration on the growth of *Phytophthora cinnamomi.* The experiment was carried out twice, with three replicates in each experiment. Each point represents the mean of six replicates.

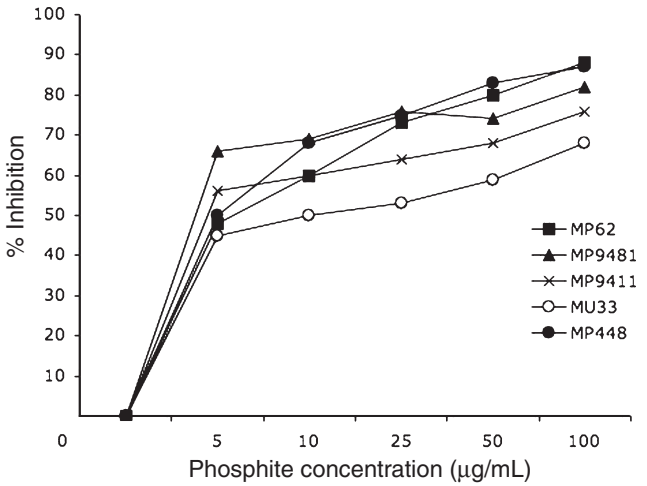

Fig. 2. Effect of phosphite on the growth of *Phytophthora cinnamomi*. Five isolates of *P. cinnamomi* were tested for their sensitivity to growth inhibition by phosphite. The experiment was carried out twice, with three replicates in each experiment. Each point represents the mean of six replicates.

bands that were present in both of the duplicates were chosen for further analysis (Fig. 3).

Four differential bands were identified, excised from the gel and cloned in pTOPO. Transformant *E. coli* colonies were screened for the presence of the correct-sized band by colony PCR. Plasmids were extracted from the colonies and sequenced. Screening the nucleotide sequences against the GenBank database by using the BLASTN algorithm revealed that three of the sequences showed high-level homology (78–98%) to *Phytophthora sojae* clones. The identity of these clones was not available from the *P. sojae* genome database. No result was obtained for the fourth clone, CP22 (Table 1). To identify potential proteins encoded by these sequences, the deduced amino-acid sequences were screened against the SwissProt database by using BLASTX. From this, potential protein products were identified for all four sequences (Table 1).

Selection of endogenous reference genes for qRT–PCR

In order to further confirm differential expression, the amount of the mRNA in treated and untreated mycelium was measured by qRT–PCR using SYBR Green binding. However, accurate measurement of mRNA transcripts by qRT–PCR requires that

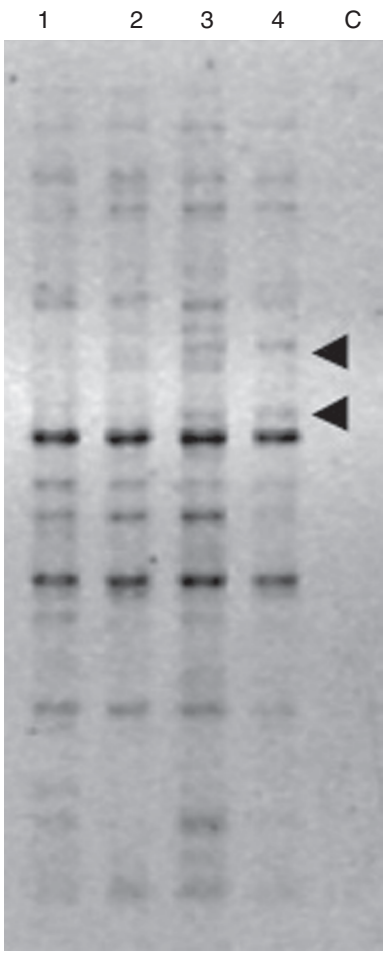

Fig. 3. Electrophoresis of DDRT–PCR products. Differential bands are indicated. Lanes 1 and 2, RNA from untreated mycelium; Lanes 3 and 4, RNA from phosphite treated mycelium. Lane 5, no cDNA in the DDRT–PCR reaction.

the signals from the target gene are normalised to an endogenous reference gene whose expression does not vary between the treatments, and which amplifies with an efficiency that is within 10% of that of the target gene (Livak and Schmittgen 2001). Initially, we evaluated genes for three proteins, tubulin from *P. cinnamomi*, cyclophilin from *P. infestans* and actin from *P. palmivora*, as potential reference genes as these genes are

Table 1. Identification of the protein products of the differential display sequences

SEQ	GenBank accession #	Type of BLAST search	Putative protein product	Percentage identity	Length of homologous region (bp)	*E*-value	Fold change in expression
CP1	EU170011	BLASTN	*Phytophthora sojae* clone expressed during infection	98	710 bp	0	1.6
		BLASTX	Mitochondrial protein from *Kluyveromyces lactis*	64	112 aa	6e–19	
CP6	EU170010	BLASTN	*Phytophthora sojae* clone expressed during infection	73	281 bp	0	3.1
		BLASTX	*Leishmania brazielensis* proteophosphoglycan	41	214 aa	0.002	
CP22	EU170012	BLASTN	No result				1.2
		BLASTX	Signal transduction kinase	50	146 aa	3e–13	
CP29	EU170013	BLASTN	*Phytophthora sojae* clone expressed during infection	78	237 bp	2e–41	1.4
		BLASTX	Angiotonin-activated protein	43	246 aa	6e–11	

commonly used for this purpose (Nicot *et al*. 2005). Analysis of amplification of a DNA dilution series with the tubulin primers showed that the efficiency of amplification of the tubulin gene is within 5% of the target gene amplification (Fig. 4), and showed little variation in expression between the untreated and phosphite-treated mycelium, with a ΔC_T value of 0.22 or less (Table 2) (a fold change of two or greater is considered to be significant (Livak and Schmittgen 2001)). Although the efficiency of amplification of the other two candidate reference genes was also within 5% of the target genes (data not shown), tubulin was selected as the reference gene as it is the most highly expressed of the three candidate genes.

qRT–PCR

Quantitative RT–PCR was used to confirm differential expression of the four bands identified by DDRT–PCR. RNA was extracted from untreated and phosphite-treated mycelium and used for qRT–PCR. The fold difference in the expression level of the four genes is shown in Table 1. Three genes showed less than a 2-fold change in expression. Only one gene, CP6 showed a greater than 2-fold change in mRNA levels, which is considered indicative of induction (Rajeevan *et al*. 2001). One of the translation products of the CP6 gene showed a high level of homology to a phosphoproteoglycan from *Leishmania brazielensis.*

Discussion

In the present study, we used DDRT–PCR to identify *P. cinnamomi* genes that are induced or repressed by treatment of mycelium with phosphite. Four differentially expressed bands were identified, cloned and sequenced. By using qRT–PCR, we were able to confirm that one of the bands shows induction of expression in phosphite-treated mycelium. This gene shows 41% homology at the amino-acid level to a phosphoproteoglycan from *Leishmania*. Genome-sequencing analysis has revealed that the Alveolates, of which *Leishmania* is a member, are grouped together with the Stramenophiles, which include *Phytophthora* to form the Chromoalveolates (Tyler *et al*. 2006), hence indicating a relatively close relationship between these organisms.

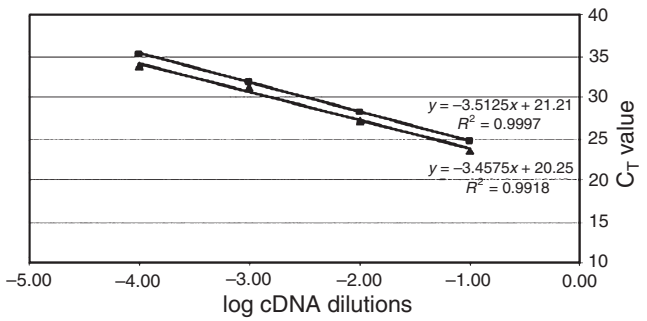

Fig. 4. Comparison of the amplification efficiency of tubulin and a target gene. The two standard curves shown are tubulin (bottom) and CP22 (top). Each point represents the mean of duplicate C_T values.

Table 2. Effect of phosphite on expression of candidate normalising genes

	C_T (+Phi)	C_T (−Phi)	ΔC_T
Tubulin	24.35	24.13	0.22
Cyclophilin	26.05	25.9	0.15
Actin	34.2	34.02	0.18

The fold change in expression is given by ΔC_T. A ΔC_T of 2 indicates induction.

The differentially expressed bands identified in the present study were cloned, and bands corresponding in size to the band on the DDRT–PCR gel selected for development of qRT–PCR primers. Subsequent qRT–PCR with primers based on the sequence of the selected clones showed that only three of the four bands were differentially expressed. An explanation is provided from the work of Smith *et al*. (1997*b*) who showed that single DDRT–PCR bands may consist of several products and that selection of clones on the basis of size may result in selection of the wrong clone. They recommend selection of clones on the basis of frequency, i.e. selecting the most frequent product among the cloned products.

The differentially expressed cDNA clone, CP6 (1564 bp), was found to share 41% sequence identity with the proteophosphoglycan gene from *Leishmania brazielensis* across a region of 214 amino acids (25.6% of the phosphoproteoglycan sequence). Proteophosphoglycan molecules are made up of phosphoglycan chains linked to the polypeptide backbone via phosphodiester linkages to serine, a protein modification termed phosphoglycosylation.

Phosphoproteoglycans have been identified in several species of *Phytophthora* (Robold and Hardham 2005) as well as in other oomycetes such as *Pythium*, *Albugo* and *Plasmopara* (Hardham 2005). They appear to enable infective propagules to adhere to the substratum. Homologues of the *Phytophthora* proteins are found in the green alga *Ulva linza* (Stanley *et al*. 2005) and in the apicomplexan parasite *Cryptosporidium parvum* (Hardham 2005). In *Leishmania*, phosphoproteoglycans play important roles in survival, development and virulence (Gopfert *et al*. 1999; Klein *et al*. 1999).

Adhesive proteins also play a role in activation of the host-plant defence responses. The CBEL protein of *P. parasitica* var. *nicotiana* functions both to adhere oospores and hyphae to the substratum (Mateos *et al*. 1997; Gaulin *et al*. 2002) and to induce lipoxygenase activity and the accumulation of hydroxyproline-rich cell-wall proteins in host plants (Khatib *et al*. 2004). Lipoxygenase activation is a trigger for activation of the host defence response (Thatcher *et al*. 2005). Consistent with this is the finding that application of CBEL to tobacco plants protected the plants against subsequent infection by a virulent strain of *P. parasitica* var. *nicotiana* (Mateos *et al*. 1997).

The outcome of host infection by *Phytophthora* is determined by the defence systems of the host and by protein factors produced by the infecting pathogen. *Phytophthora* var proteins interact with cognate host R proteins to activate the host defence systems and limit infection (Tyler 2001). In addition to the avr proteins, *Phytophthora* also secretes other proteins as well as non-protein elicitors that serve to activate the host defence systems. Protein elicitors include the elicitins and glycoproteins such as the CBEL protein of *P. parasitica* (Mateos *et al*. 1997). Several studies have shown that phosphite activates host defence systems (Smith *et al*. 1997*a*; Molina *et al*. 1998; Daniel and Guest 2006). In treated plants phosphite accumulates in various

tissues where it can persist for some considerable time (Pilbeam *et al*. 2000; Malusa and Tosi 2005). Pathogens such as *P. cinnamomi* infecting the root tissue would be exposed to this phosphite and this may serve to limit infection of the host as phosphite has been shown to inhibit growth and zoospore production in *P. cinnamomi* (Wilkinson *et al*. 2001*a*, 2001*b*) and to induce a variety of metabolic changes in other species of *Phytophthora* (Griffith *et al*. 1990; Niere *et al*. 1994, 2001; Perez *et al*. 1995; Martin *et al*. 1998). The contribution this direct exposure to accumulated phosphite makes to host resistance will be the focus of future studies.

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