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Cloning and characterization of a theta class glutathione transferase from the potato pathogen *Phytophthora infestans*

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Abstract

A glutathione transferase (GST) related to the theta (T) class of enzymes found in plants and animals has been cloned from the potato pathogen *Phytophthora infestans*. The cDNA encoded a 25 kDa polypeptide termed *Pi*GSTT1 which was expressed in *E. coli* as the native protein. The purified recombinant enzyme behaved as a dimer (*Pi*GSTT1-1) and while being unable to catalyse the glutathione conjugation of 1-chloro-2,4-dintrobenzene, was highly active as a glutathione peroxidase with organic hydroperoxide substrates. In addition to reducing the synthetic substrate cumene hydroperoxide, *Pi*GSTT1-1 was shown to be highly active toward 9(*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid = 9(*S*)-HPOT, which is formed in potato plants during infection by *P. infestans* as a precursor of the antifungal oxylipin colnelenic acid. An antiserum was raised to *Pi*GSTT1-1 and used to demonstrate that the respective enzyme was abundantly expressed in *P. infestans* both cultured on pea agar and during the infection of potato plants.

Keywords: Detoxification; Glutathione peroxidase; Oxylipin; Phytoalexin; Phytopathogenic fungi, *Phytophthora infestans*; Potato, *Solanum tuberosum*

1. Introduction

The oomycete *Phytophthora infestans*, the causative agent of late blight, is a phytopathogen of considerable agronomic importance in both tomato and potato (Carlile et al., 2001). As part of the defence response to *P. infestans*, potato (*Solanum tuberosum* L.) plants produce a specific group of antimicrobial divinyl ether oxylipins, derived from fatty acid hydroperoxides (Grechkin, 1998). The antimicrobial oxylipins colneleic acid (9-[1′(*E*),3′(*Z*)-nonadienyloxy]-8(*E*)-nonenoic acid; **1**) and colnelenic acid (9-[1′(*E*),3′(*Z*),6′(Z)-nonatrienyloxy]-8(*E*)-nonenoic acid; **2**) have been linked to resistance to *P. infestans*. Thus, potato cultivars showing an incompatible response to infection accumulated significantly more of these compounds than susceptible individuals during infection (Weber et al., 1999). The antifungal activity of oxylipins is broad-ranging and includes the inhibition of mycelial growth and the prevention of cytospore germination (Kato et al., 1992).

The precursors of colneleic acid (**1**) and colnelenic acid (**2**) are the 9(*S*)-hydroperoxides of linoleic acid (9(*S*)-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid = 9(*S*)-HPOD; **3**) and linolenic acid (9(*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid = 9(*S*)-HPOT; **4**) respectively (Grechkin, 1998). These hydroperoxides are formed by the action of a potato 9-lipoxygenase (9-LOX, EC 1.13.11.12), which catalyses a relatively unusual reaction compared with the more common formation of the respective 13(*S*)-hydroperoxides (Howe and Schmiller, 2002). In potato tubers, 9(*S*)-HPOD (**3**) and 9(*S*)-HPOT (**4**) are subsequently converted to the antifungal oxylipins by the enzyme divinyl ether synthase. The corresponding pathway leading from 9(*S*)-HPOT (**4**) to colnelenic acid (**2**) is shown in Fig. 1. The importance of the 9-hydroperoxidation of polyenoic fatty acids in disease resistance in plants has been demonstrated

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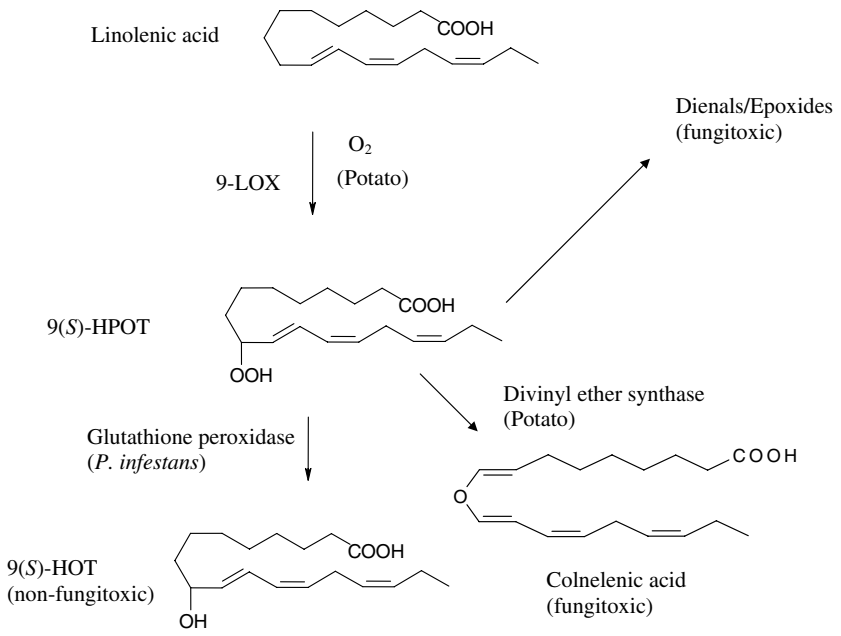

Fig. 1. Potential routes of metabolism of oxylipins derived from linolenic acid in potato infected with *P. infestans*. Linolenic acid is converted to the central precursor 9(*S*)-HPOT through the action of 9-LOX. If absorbed by the invading pathogen, the 9(*S*)-HPOT (**4**) would be reduced to the non-toxic monohydroxy derivative 9(*S*)-HOT. Alternatively, the 9(*S*)-HPOT will be metabolized in potato to fungitoxic dienal or epoxide derivatives, or through the action of divinyl ether synthase transformed to the oxylipin colnelenic acid (**2**).

in transgenic tobacco, with antisense suppression of 9-LOX leading to a loss in endogenous resistance to *Phytophthora parasitica* var. *nicotianae* and *Rhizoctonia solani* (Rancé et al., 1998).

To sustain virulence, phytopathogens have evolved protective mechanisms to overcome plant-derived inducible antimicrobial toxins, also known as phytoalexins (Hammerschmidt, 1999). Classic protective responses by the fungus include the exclusion of phytoalexins through the use of ATP-binding cassette transporter proteins (Zwiers et al., 2003), or their detoxification by cytochrome P450 mixed function oxidases (George and VanEtten, 2001). Another set of detoxifying proteins in phytopathogens which might be expected to be involved in counteracting phytoalexins are the glutathione transferases (GSTs). GSTs are known to be involved in the tolerance to synthetic and natural chemical control agents in animals (Lien et al., 2002), bacteria (Allocati et al., 1999), plants (Cummins et al., 1999) and insects (Enayati et al., 2005). Although GSTs have been described in many fungal species (McGoldrick et al., 2005), there are relatively few reports of these proteins in phytopathogenic species. GST activities toward the xenobiotic 1-chloro-2,4-dinitrobenzene (CDNB) were determined in extracts from *Fusarium oxysporum* and *R. solani* (Cohen et al., 1986) and in a range of *Phytophthora* species (Oros and Kömives, 1991). More recently, genes encoding three GSTs have been determined in the pathogenic grey mold *Botrytis cinerea*, though the activities of the respective proteins were not determined (Prins et al., 2000; Schulze Gronover et al., 2004, 2005).

With this background, we have been interested in further characterizing GSTs in *P. infestans* which may be involved in counteracting phytoalexins. We now report on the cloning of a GST from *P. infestans* which is most closely related to the plant theta class of these proteins and have determined its functional activities as a recombinant protein and monitored its expression during plant infection.

2. Results

2.1. Cloning, expression and characterization of a theta class GST from P. infestans

The *Phytophthora* genome consortium database (http://www.pfgd.org/pfgd/filter.html) was interrogated with GST sequences derived from the plant-specific tau and phi classes, as well as the zeta and theta class sequences which are found in both animals and plants (Dixon et al., 2002b). One partial sequence (Accession piMY025aC05r) showing similarity to the theta class of GSTs from *Arabidopsis thaliana* was identified and termed *P. infestans* GST theta 1 (*Pi*GSTT1). The respective 800 bp cDNA was amplified from cultured *P. infestans* using RT-PCR and sequenced (EMBL Accession AM261440), showing that the full-length sequence of *Pi*GSTT1 encoded a 25 kDa polypeptide that showed significant identity with theta class GSTs from soybean, rice and *Arabidopsis* (Fig. 2). The coding sequence was then ligated into the pET24a plasmid for expression of the untagged protein in *E. coli*. The transformed bacteria were treated with IPTG for 2.5 h and the lysate analysed by SDS–PAGE. A 25 kDa polypeptide, which was absent from the empty vector controls, was

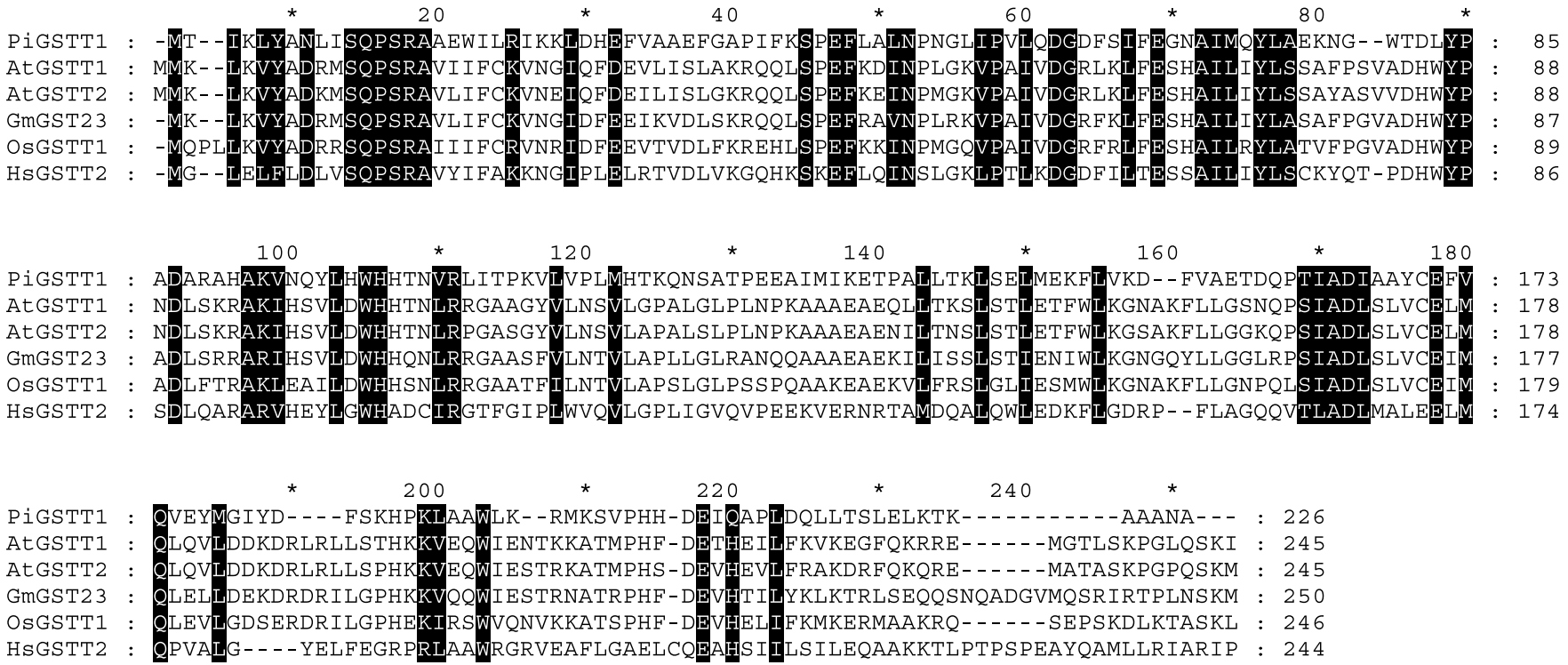

Fig. 2. Alignment of *Pi*GSTT1 with theta class GST sequences from *Arabidopsis thaliana* (*At*GSTT1; Uniprot Q9ZRT5, AtGSTT2; UniProt Q9ZRR6), soybean (GmGST23; UniProt Q9FQD5), rice (*Os*GSTT1; UniProt Q945X2), and *Homo sapiens* (*Hs*GSTT2; UniProt P30712) with conserved amino acid residues highlighted.

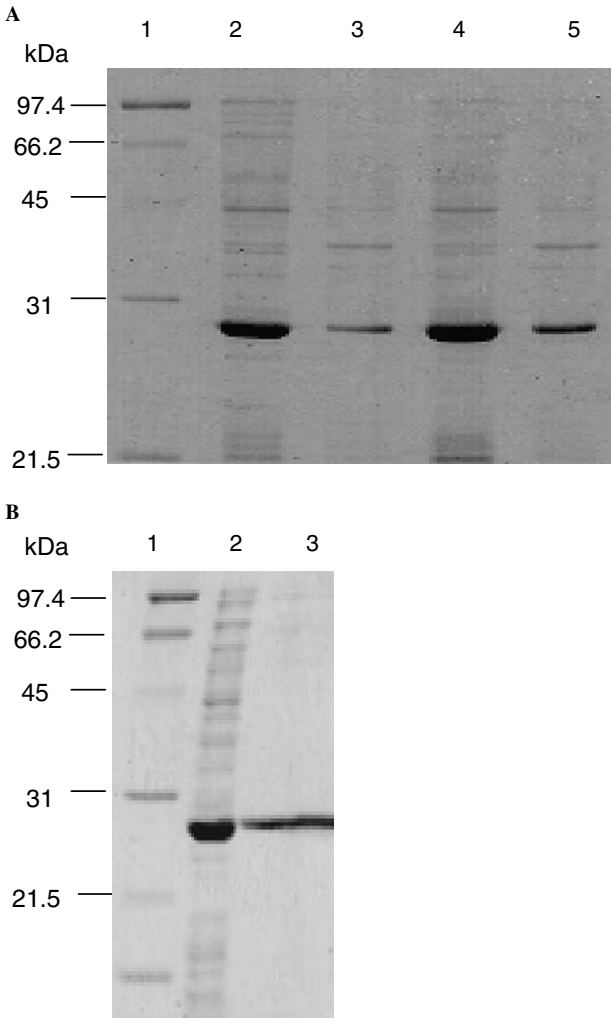

Fig. 3. SDS–PAGE analysis of recombinant *Pi*GSTT1 in crude lysates from *E. coli* following expression at 30 and 37 °C (A) and following purification by hydrophobic interaction chromatography (B). A. Lane 1, molecular mass markers; lane 2, total soluble (30 °C); lane 3, insoluble (30 °C); lane 4, soluble (37 °C); lane 5, insoluble (37 °C). B. Lane 1, molecular mass markers; lane 2, total soluble (30 °C); lane 3, purified protein.

determined in the soluble and insoluble fraction (Fig. 3A). The yield of soluble vs. insoluble recombinant protein was found to be improved by inducing the bacteria at 30 °C rather than 37 °C (Fig. 3A). Lysates from the pET24-*Pi*GSTT1 transformants showed no detectable GST activity with CDNB as substrate, after correcting for the minor rates of conjugation determined in extracts from the empty vector controls. However, the extracts from the *Pi*GSTT1 transformants were found to contain appreciable glutathione peroxidase (GPOX) activity (9.8 nkat mg^{-1} protein) when assayed using the synthetic cumene hydroperoxide. In contrast GPOX activity in the empty vector controls was negligible (<0.1 nkat mg^{-1}). The untagged recombinant protein was purified from the lysate by hydrophobic interaction chromatography using phenyl Sepharose. As determined by SDS–PAGE, the resulting protein was determined to be over 95% pure (Fig. 3B), having a final specific GPOX activity of 15.8 nkat mg^{-1} protein when assayed with cumene hydroperoxide. When the purified *Pi*GSTT1 polypeptide was analysed by time-of-flight mass spectrometry following electrospray ionization (ESI-ToF-MS), a dominant mass ion of 25,450 Da was determined on deconvolution, consistent with a predicted mass of 25449.5 Da (after loss of the N-terminal methionine). When analysed on a pre-calibrated gel filtration column, the recombinant *Pi*GSTT1 and GPOX activity eluted as a 46 kDa protein, consistent with the protein being a homodimer. Thereafter, the native recombinant protein was described as being *Pi*GSTT1-1.

The pure recombinant enzyme had no detectable activity as a GST when assayed with CDNB as substrate. When assayed for GPOX activity with cumene hydroperoxide, the reduction of the hydroperoxide was found to be strictly dependent on protein content within the range of 0 to 48 micrograms of protein per assay. The pH optimum of the GPOX activity was between pH 7.2 and 7.4 and the enzyme was found to be reasonably thermotolerant, showing no loss of activity after being heated at 57 °C for 10 min. However, a similar treatment at 67 °C abolished all activity. The apparent K_M of the enzyme toward cumene hydroperoxide was 14.2 mM and 1 mM toward glutathione. To determine GPOX activity with organic hydroperoxides derived from host potato plants, 9(*S*)-HPOT (**4**) was generated from linolenic acid by the action of a 9-LOX preparation from potato tubers (Grechkin et al., 1991). Prior to its use, the identity of the 9(*S*)-HPOT (**4**) was confirmed by HPLC-MS (m/z for $M-H^+ = 309.276$). When 9(*S*)-HPOT was used in the coupled assay in place of cumene hydroperoxide, the *Pi*GSTT1 was shown to have a specific activity of 1051 ± 42 nkat mg^{-1} pure protein. Due to the limited solubility of the 9(*S*)-HPOT detailed kinetic analysis with this substrate was not undertaken.

2.2. Expression of PiGSTT1 in P. infestans

In order to study the expression of the *Pi*GSTT1 polypeptide in *P. infestans*, a polyclonal antiserum to the pure recombinant GST was raised in rabbits and used in Western blotting studies. The antiserum selectively recognized the recombinant *Pi*GSTT1 and when used to probe mycelial extracts from *P. infestans* grown for 12 days on pea medium recognized a corresponding 25 kDa polypeptide (Fig. 4B). By using known amounts of recombinant *Pi*GSTT1, it was possible to calculate that the *Pi*GSTT1 polypeptide was a highly expressed protein in the cultured mycelia, representing 0.6% of the extracted protein present. When mycelial extracts were resolved by 2D-gel electrophoresis and then Western-blotted, a single immunoreactive polypeptide was observed with a molecular mass of 25 kDa, and p*I* 6.8 This suggested that *Pi*GSTT1-1 was expressed as a single isoenzyme, though since the predicted p*I* of the GSTT was pH 6.13 it is possible that the protein had undergone post-translational processing to alter its final ionic charge.

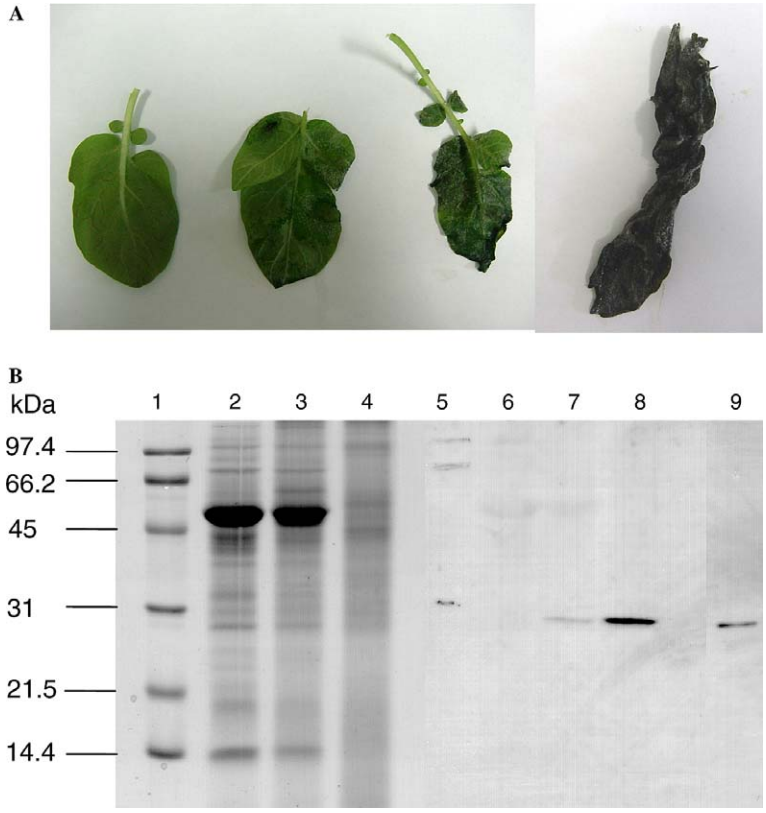

Fig. 4. Expression of *Pi*GSTT1 in potato leaves infected with a compatible strain of *P. infestans*. (A) Progression of blight symptoms in leaves following infection. From left to right, uninfected control (day 7); infected (day 0); infected (day 5) and infected (day 7). (B) Composite of total protein from the infected leaves resolved by SDS–PAGE and visualized by staining (lanes 2–3) and after Western blotting using the anti-*Pi*GSTT1 serum (lanes 6–8). Lanes 1 and 4 show pre-stained reference molecular mass markers. For each time point, total protein and Western blots, respectively, are shown for day 0 samples in lanes 2 and 5, for day 5 in lanes 3 and 6 and for day 7 in lanes 4 and 8. Lane 9 shows the Western blot analysis, of *Pi*GSTT1 detected in total protein extracts from the mycelia of *P. infestans* grown on pea agar.

The expression of *Pi*GSTT1 during the infection of potato plants with *P. infestans* was then monitored. The plants, which showed a compatible response to the fungus were visually assessed, 5 days and 7 days after infection (Fig. 4A) and the presence of *Pi*GSTT1 determined in the infected tissue using the antiserum (Fig. 4B). No immunoreactive polypeptides were observed in uninfected potato plants, demonstrating that the antiserum was not cross-reacting with plant proteins. An immunoreactive 25 kDa polypeptide was observed 5 days following infection, when visible signs of disease were evident and continued to accumulate up to 7 days, at a time when the potato leaves were badly blighted and undergoing extensive proteolysis (Fig. 4).

3. Discussion

Phylogenetic analysis of the relationship between *Pi*GSTT1 and other GSTs was performed as described (Dixon et al., 2002a), with half-jacknife bootstapping being used to assess the reliability of the resulting phylogenetic tree. From this analysis, *Pi*GSTT1-1 could be shown to be a 'true' theta class GST with clearly related homologues in plants and animals (Dixon et al., 2002b). When compared with families of GSTs which are specific to the fungi (McGoldrick et al., 2005), it could be shown that *Pi*GSTT1 was more closely related to plant rather than other fungal GSTs (Fig. 5). Based on sequence similarity searches, related theta class GSTs could also be readily identified in the databases in a diverse range of fungal genera including *Coccidioides, Aspergillus, Paxillus, Gibberella* and *Trichoderma* sp. Confusingly, unrelated GSTs, also described as being of the theta class, have previously been identified in *B. cinerea* (Prins et al., 2000; Schulze Gronover et al., 2004, 2005) and *Aspergillus nidulans* (Fraser et al., 2002). The classification of these GSTs as theta enzymes was based on a default nomenclature which termed all non-mammalian GSTs which could not be classified into an existing clade to be theta class (McGoldrick et al., 2005). In fact, the previously identified GSTs from *B. cinerea* and *A. nidulans* appear to be part of a fungal-specific class also identified in *Schizosaccharomyces pombe* (Veal et al., 2002), which are distinct from the yeast GSTs previously identified in *Saccharomyces cereviseae* (Fraser et al., 2002). For reference, based on the existing nomenclature (McGoldrick et al., 2005), the relatedness of these fungal specific classes of GSTs (clusters I and II and URE2) to one another and toward the theta GSTs are shown (Fig. 5).

*Pi*GSTT1-1 showed similar characteristics to the dimeric theta GSTs characterized from plants (Dixon et al., 2002b);

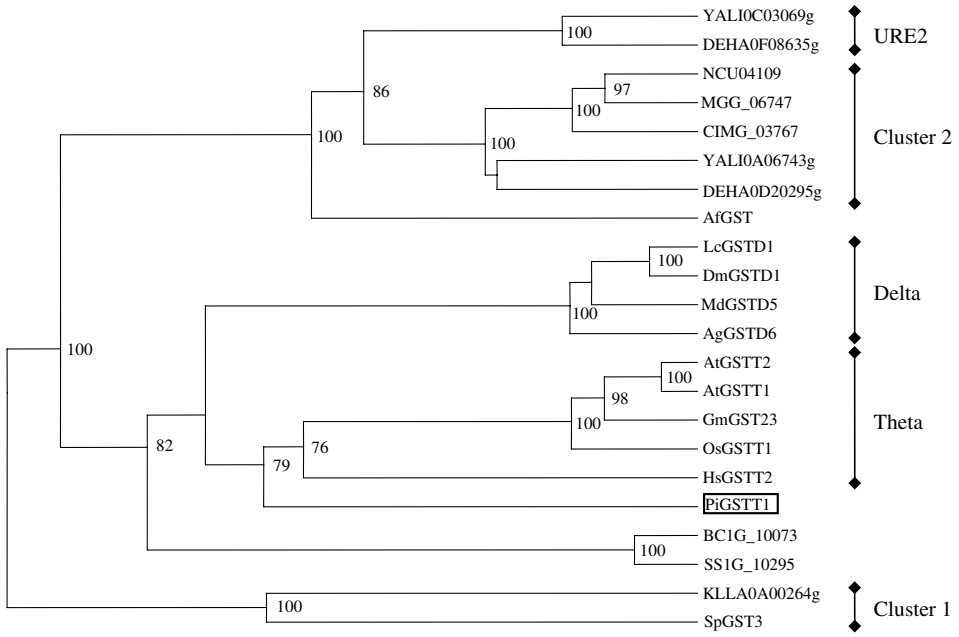

Fig. 5. Dendrogram illustrating relationship of *Pi*GSTT1 (boxed) with related fungal and other GST sequences, showing clustering with theta-class enzymes. Clade nomenclature is taken from McGoldrick et al. (2005). Bootstrap values at nodes present in at least 75% of 100 replicates are shown. Sequences and sources are as follows. Representative members from the fungal-specific clusters 1 and 2 and URE2 clades are from Genolevures (http://cbi.labri.u-bordeaux.fr/Genolevures/) and have Accession Nos. as shown (YALI0C03069g and YALI0A06743g; *Yarrowia lipolytica*, DEHA0F08635g and DEHA0D20295g; *Debaryomyces hansenii*, KLLA0A00264g; *Kluyveromyces lactis*), except for *Sp*GST3 (*Schizosaccharomyces pombe*; UniProt Q9P6M1). Also shown are the most closely related fungal GSTs to *Pi*GSTT1 found through the Fungal Genome Initiative (http://www.broad.mit.edu/annotation/fgi/); Accession Nos. are shown (NCU04109; *Neurospora crassa*, MGG_06747; *Magneporthe grisea*, CIMG_03767; *Coccidioides immitis*, BC1G_10073; *Botrytis cinerea*, SS1G_10295; *Sclerotinia sclerotiorum*). Finally, additional related GSTs in the UniProt database are shown – *Af*GST (*Aspergillus fumigatus*; Q4W9G5), *Lc*GSTD1 (*Lucila cuprina*; P42860), *Dm*GSTD1 (*Drosophila melanogaster*; Q4JFI6), *Md*GSTD5 (*Musca domestica*; Q254450), *Ag*GSTD6 (*Anopheles gambia*; Q93113), *At*GSTT1 (*Arabidopsis thaliana*; Q9ZRT5), *At*GSTT2 (*Arabidopsis thaliana*; Q9ZRR6), *Gm*GST23 (*Glycine max*; Q9FQD5), *Os*GSTT1 (*Oryza sativa*; Q945X2) and *Hs*GSTT2 (*Homo sapiens*; P30712).

being highly active as a GPOX in reducing organic hydroperoxides, while having limited glutathione conjugating activity with classic GST substrates such as CDNB. While the functions of theta GSTs have yet to be unequivocally determined, their conservation in eukaryotes is suggestive of essential house-keeping functions, probably linked to their ability to counteract fatty acid hydroperoxide formation (Dixon et al., 1999, 2002b). Studies with the anti-*Pi*GSTT1 serum showed that this theta GST was highly expressed during both vegetative growth and pathogenesis. In *P. infestans* the 5-(*S*)-hydroperoxide of arachidonic acid, would be the most abundant endogenous substrate of *Pi*GSTT1-1 present (Griffiths et al., 2003). However, attempts to prepare this substrate for assay proved unsuccessful. Instead, we tested the possibility that *Pi*GSTT1-1 could be involved in metabolizing lipid hydroperoxides formed in host potato plants during infection (Grechkin, 1998). *Pi*GSTT1-1 had high GPOX activity toward the potato hydroperoxide 9(*S*)-HPOT (**4**), effectively detoxifying it by catalyzing the reduction to the respective monohydroxyalcohol (Fig. 1). While it is known that phytopathogenic fungi can absorb hydroperoxides (Prins et al., 2000), the functional importance of such a GPOX activity in detoxifying potato oxylipin precursors and assisting the pathogenesis of *P. infestans* is beyond the scope of this report.

It has been suggested that three recently described GSTs in *B. cinerea* are important in promoting plant infection, though the activities of the respective proteins were not tested and the respective gene knockouts had no obvious effect on pathogenesis (Prins et al., 2000; Schulze Gronover et al., 2004, 2005). Interestingly, a related GST in *A. nidulans* was shown to contribute to tolerance to heavy metals and toxic xenobiotics (Fraser et al., 2002). Similarly, a further three related GSTs in *S. pombe* were shown to be involved in counteracting peroxidatively imposed stress and in promoting drug resistance, with one of these enzymes shown to have GPOX activity (Veal et al., 2002). In view of the known importance of GSTs in conferring tolerance to chemicals in bacteria (Allocati et al., 1999), plants (Cummins et al., 1999), insects (Enayati et al., 2005) and mammals (Lien et al., 2002) the roles of these enzymes in counteracting the action of fungicides of natural and synthetic origin in phytopathogenic fungi is deserving of further study.

4. Experimental

4.1. Growth and analysis of fungal cultures

Phytophthora infestans host compatible strain 9.5.1.1 was obtained from Bayer Crop Science, Lyon and mycelial

cultures maintained on pea agar (Griffiths et al., 2003). Mycelial agar plugs were used to inoculate liquid pea media, with cultures grown in the dark at 23 °C for 12 days on an orbital shaker (120 rpm). Fresh mycelia were harvested by vacuum filtration onto hardened filter paper (Whatman 54), and washed with 50 mM K.Pi buffer pH 7 containing 1 mM EDTA and 0.4 M glucose. RNA was prepared from the mycelia after extracting with 8 M guanidine hydrochloride (Gurr and McPherson, 1992). For protein extraction, the mycelia were re-suspended in 1 v/w ice cold 0.1 M K.Pi buffer pH 7.0 containing 1 mM EDTA, 1 mM PMSF and 2 mM DTT and then homogenized at 4 °C using a glass-bead beater (Bio-Spec, Bartlesville, OK 74005, USA) operating with 3×30 s bursts. Homogenates were filtered and centrifuged (20 min, 16,000*g*, 4 °C) and the supernatant adjusted to 80% saturation with $(NH_4)_2SO_4$ prior to re-centrifugation to recover the protein precipitate. After desalting into 2 mM K.Pi buffer, pH 7.0, protein concentrations were determined using the Bio-Rad protein assay reagent and referenced to γ-globulin. The protein content of each sample was then normalized, prior to analysis by SDS–PAGE and Western blotting, which was carried out using the procedures described previously (Cummins et al., 1997).

4.2. Potato infection studies

Sporocysts of *P. infestans* from infected potato (*Solanum tuberosum* L. var. Bintje), were rinsed from the plants with water and adjusted to 40,000 sporocysts ml^{-1} as determined using a haemocytometer. The fresh suspension was then administered to the underside of the foliage of 20 healthy potato plants (var. Bintje) using a Fischer spray gun. Plants were maintained in growth cabinets at 22 °C, with 100% humidity, with 20 uninfected plants placed in a control chamber under identical conditions. Plants were assessed for visible signs of infection and harvested ($n = 5$) at timed intervals for analysis. The leaves were ground to a fine powder in liquid nitrogen and the protein extracted as described for the mycelia and used to prepare $(NH_4)_2SO_4$ precipitates for Western blotting studies.

4.3. Enzyme assays

Enzyme preparations were assayed for GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) and for glutathione peroxidase (GPOX) activity with cumene hydroperoxide after quantifying the protein present (Cummins et al., 1999). 9(*S*)-HPOT (**4**) was prepared from linolenic acid using a crude 9-LOX preparation from potato tubers (var Desiree) using a published procedure (Grechkin et al., 1991). The resulting 9-hydroperoxide was purified and quantified using the procedure described for the preparation of 13-hydroperoxylinoleic acid (Cummins et al., 1997). The identity of the 9(*S*)-HPOT (**4**) was confirmed by injecting the reaction product onto an HPLC column (C4; 2 mm i.d. × 50 mm) and washing at 0.2 ml min^{-1} with MeOH:water (5:95 v/v). After 0.5 min, the bound material was eluted with a linearly increasing proportion of MeOH (5–100%) over 4.5 min. The eluate was fed directly into a Micromass LCT time-of-flight mass spectrometer (TOF-MS) after ionisation in negative mode using electrospray ionisation (ESI). The MS was tuned with raffinose and calibrated with NaI. 9(*S*)-HPOT (0.25 mM) was then used as a GPOX substrate in the standard coupled assay (Cummins et al., 1999).

4.4. cDNA cloning, expression and purification of the recombinant GST

cDNA was prepared from total RNA isolated from *P. infestans* mycelia using reverse transcriptase with the oligonucleotide primer og2 ($GAGAGAGGATCCTCGAG[T]_{17}$) and used to PCR amplify a GST sequence using the primers og9 (CGCACTGAGAGAGGATCCTCGAG) and *Pi*23(2)-NDE1 (GTCATATGACCATCAAGCTCTACGCCAAC), essentially as described previously (Dixon et al., 2002a). Primer *Pi*23(2)NDE1 was designed from the N-terminal sequence of a GST-like sequence (EST Accession PiMY025aC05r) identified in the *Phytophthora* genome consortium database (http://www.pfgd.org/pfgd/filter.html). The PCR amplification product (800 bp) was purified by electrophoresis and cloned into the pGEM-T easy vector (Promega, Southampton, UK). The *Pi*GSTT1 coding sequence was subsequently excised, ligated into the pET-24a vector (Novagen, Nottingham, UK) such that the resulting expressed protein was untagged, and then re-sequenced prior to expression in *E. coli* strain BL21(DE3) as described previously (Dixon et al., 2002a). On harvest, pelleted bacteria were re-suspended in 5 ml 20 mM Tris–HCl buffer, pH 7.4, containing 2 mM DTT and sonicated (30 s, 4 °C) prior to centrifuging (12,000*g*, 10 min). $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 1 M and the clarified sample applied to a phenyl Sepharose column (25 ml). After washing with 20 mM K-Pi buffer, pH 7.4, containing 2 mM DTT and 1 M $(NH_4)_2SO_4$, bound protein was eluted at 1 ml min^{-1} with a linearly decreasing concentration of $(NH_4)_2SO_4$ (1–0 M, total vol. 75 ml). Fractions (5 ml) were monitored for GPOX activity and polypeptide composition by SDS–PAGE using 12.5% gels, with the molecular mass of the native protein determined by gel permeation chromatography (Cummins et al., 1997).

4.5. Preparation and use of antisera

Purified recombinant *Pi*GSTT1-1 was used to raise polyclonal antiserum in two female New Zealand white rabbits. Immunization consisted of an initial dosage of 0.2 mg of pure protein formulated with an equal volume of Titermax Gold adjuvant (Stratech Scientific, Cambs, UK) followed 6 weeks later by a booster of 0.2 mg. The antisera were used in Western blotting experiments at a 1:10,000 dilution following resolution of polypeptides by SDS–PAGE or 2D-gel electrophoresis (Cummins et al., 1997).

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