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Phytophthora nicotianae PnPMA1 encodes an atypical plasma membrane H^+-ATPase that is functional in yeast and developmentally regulated

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Abstract

PnPMA1, a gene encoding a putative P-type plasma membrane H^+-ATPase, has been isolated by differential screening of a *Phytophthora nicotianae* germinated cyst cDNA library. *PnPMA1* is differentially expressed during pathogen asexual development with a more than 10-fold increase in expression in germinated cysts, the stage at which plant infection is initiated, compared to vegetative or sporulating hyphae or motile zoospores. PnPMA1 proteins are encoded by two closely linked genes that have no introns and encode identical proteins having 1068 amino acid residues and a molecular mass of 116.3 kDa. PnPMA1 shows moderate identity (30–50%) to plant and fungal plasma membrane H^+-ATPases and weak identity to other P-type cation-transporting ATPases. PnPMA1 contains all the catalytic domains characteristic of H^+-ATPases but also has a distinct domain of ~155 amino acids that forms a putative cytoplasmic loop between transmembrane domains 8 and 9, a feature that is not present in PMA1 proteins from other organisms. Polyclonal antibodies raised against the 155 residue domain were shown by immunogold labelling to react with a protein in the plasma membrane of *P. nicotianae* germinated cysts but not with the plasma membrane of motile zoospores. Genetic complementation experiments demonstrated that the *P. nicotianae* PnPMA1 is functional in yeast, *Saccharomyces cerevisiae*.

Keywords: H^+-ATPase; Oomycetes; *Phytophthora nicotianae*; Plasma membrane; Yeast complementation

1. Introduction

Phytophthora species belong to a group of micro-organisms classified as Oomycetes. Oomycetes are fungus-like in appearance but are phylogenetically distant from true fungi, being more closely related to the heterokont algae (Förster et al., 1990; Gunderson et al., 1987; Sachay et al., 1993). The Oomycetes, heterokont algae and several other categories of protists have been grouped together as the Stramenopiles (Van de Peer and De Wachter, 1997). The Oomycetes include many obligate and facultative pathogens that cause disease on a wide range of plants worldwide and some are also important animal pathogens. Among the more notorious species is *Phytophthora infestans*, the organism which caused the potato famine in Europe in the 1840s (Ristaino et al., 2001) and which is still a worldwide problem in potato-growing regions (Birch and Whisson, 2001). Other highly destructive species include *Phytophthora cinnamomi* which infects important crops and causes severe damage to forests and heathlands in Australia (Hardham, 2005), *Phytophthora ramorum* the cause of Sudden Oak Death (Rizzo et al., 2002) and *Phytophthora sojae* which causes major problems for soybean production in North America (Tyler, 2001). Oomycetes are fundamentally different from true fungi in terms of cell wall composition, reproductive biology, and genetics (Judelson, 1997); most fungicides are ineffective in controlling Oomycete diseases

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(Erwin and Ribeiro, 1996). These features make it likely that distinct biochemical and genetic mechanisms may underlie plant infection and colonization.

Our studies aim to elucidate the genetic and biochemical mechanisms governing *Phytophthora* development and interaction with host plants using *Phytophthora nicotianae* as a model species. *P. nicotianae* is well-known as the causal agent of tobacco black shank but also infects a diverse range of plant species including many vegetable and fruit crops (Erwin and Ribeiro, 1996). Deployment of resistance genes has been the most effective way for controlling tobacco black shank but the level of resistance is limited. Development of alternative disease control measures will be greatly aided by the understanding of how the pathogen infects plants and completes its life cycle.

During our analysis of genes differentially expressed during asexual development, we identified a *P. nicotianae* cDNA clone displaying significant similarity to plasma membrane H^+-ATPase (*PMA*) genes from algae, plants and true fungi (Shan et al., 2004). PMA proteins couple ATP hydrolysis to proton transport across the plasma membrane of cells. The resultant proton motive force drives the transport of nutrients, metabolites and other solutes across the plasma membrane via various carriers and channels. PMAs are integral membrane proteins of ~100 kDa that are P-type ATPases. In higher plants, they belong to multigene families often containing about 10 members (Arango et al., 2003; Baxter et al., 2003; Robertson et al., 2004). Different genes are expressed in different tissues (Ewing and Bennett, 1994; Frias et al., 1996; Robertson et al., 2004), providing underlying energy for a variety of fundamental plant processes such as cellular homeostasis, mineral and metabolite transport, plant morphogenesis, and responses to environment stimuli.

Numerous *PMA* genes have been cloned from yeasts and filamentous fungi and their encoded proteins subjected to extensive genetic and biochemical characterization. These studies have led to the conclusion that PMA proteins play essential roles in fungal cell physiology and pathogenicity (Balhadère and Talbot, 2001; Wirsel et al., 2001). There are usually only one or two *PMA* genes in fungal genomes (Struck et al., 1998). These properties, together with the availability of numerous in vivo and in vitro systems for high-throughput screening for antagonistic compounds, make PMA proteins a desirable target for therapeutic drugs (Monk and Perlin, 1994; Perlin et al., 1997; Seto-Young et al., 1997).

In the present study, we describe the cloning and sequencing of two closely linked *P. nicotianae* genes that encode proteins with homology to plant and fungal plasma membrane H^+-ATPases. The *P. nicotianae PMA1* genes are differentially expressed during the pathogen's life cycle, with the highest levels of expression in germinated cysts. The *P. nicotianae* H^+-ATPase differs from other PMA1 proteins due to the presence of a 155-amino acid cytoplasmic loop near the C-terminus. Despite this unusual structure, immunocytochemical studies showed that the *P. nicotianae* H^+-ATPase was localized in the plasma membrane of *P. nicotianae* germinated cysts and its functionality confirmed by genetic complementation of a *Saccharomyces cerevisiae* mutant conditionally deficient in its endogenous PMA1 activity.

2. Results

2.1. Sequencing and genomic organization of *PnPMA1* genes in *P. nicotianae*

WS66 (GenBank Accession No. CF891682) was one of six cDNA clones identified during differential screening of a cDNA library made from *P. nicotianae* germinated cysts (Shan et al., 2004). WS66 is 870 bp in length, is highly expressed in germinated cysts and has homology to P-type ATPases from various organisms.

In the present study, the WS66 cDNA was used to determine the copy number of this gene in the *P. nicotianae* genome and to isolate genomic clones containing the WS66 sequence by screening a *P. nicotianae* genomic BAC library constructed with DNA fragments partially digested with *Hin*dIII (Shan and Hardham, 2004). Under stringent conditions, two *Bam*HI bands and four *Hin*dIII bands (the two smaller bands co-migrate) were labelled in Southern blots (Figs. 1A and B). There are no restriction sites for these enzymes within the probe sequence. Taking into account the fact that *Phytophthora* is diploid, these results indicate that the gene corresponding to the WS66 cDNA, designated *PnPMA1b*, is likely to be a member of a small gene family comprising two to four members. Three BACs that hybridized with the WS66 probe were selected for sequencing and characterization.

Physical mapping indicated that BAC1 and BAC2 span the whole gene locus and represent two different alleles in the diploid *P. nicotianae* genome. BAC3 is the same allele as BAC2 but covers only part of the *PnPMA1* locus. The combined restriction enzyme cleavage patterns of BAC1 and BAC2 were identical to the pattern revealed by Southern blot analysis of *P. nicotianae* genomic DNA (compare Figs. 1A and 1B).

All WS66-hybridizing *Eco*RI and *Hin*dIII fragments from BAC1 and BAC2 were purified and subcloned. Sequencing and restriction analysis revealed that both *Hin*dIII fragments lie within the single 27 kb *Eco*RI fragment (Fig. 1C). Partial sequence alignment indicated that the PnPMA1-coding regions in the two *Hin*dIII fragments from BAC1 were almost identical (99% identity) but they showed a few nucleotide differences to their corresponding alleles in BAC2. Gene-specific primers were used, in combination with sequencing and restriction analysis, to determine the genomic organization of the *PnPMA1* genes. This led to the identification of two physically linked, nearly identical *PnPMA1* genes, designated *PnPMA1a* and *PnPMA1b* (GenBank Accession Nos DQ533871 and DQ533872), arrayed in a head-to-tail orientation in the genome of *P. nicotianae* (Fig. 1C).

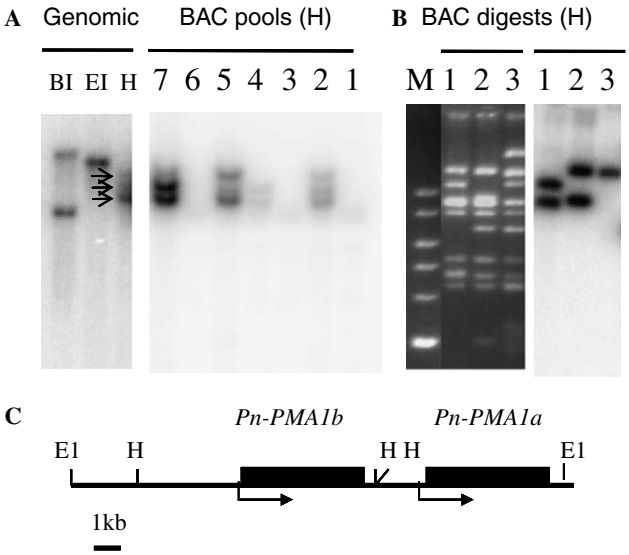


Fig. 1. The *PnPMA1* locus of *P. nicotianae*. (A) Southern blot analyses of *P. nicotianae* genomic and BAC DNA probed with WS66 cDNA identified BAC pools that contained *Hin*dIII fragments that correspond to the *Hin*dIII fragments in the genomic DNA. Lanes marked BI, EI, and HIII on left show genomic DNA cut with *Bam*HI, *Eco*RI, and *Hin*dIII, respectively. Lanes marked 1–7 show DNA from pooled BAC clones, cut with *Hin*dIII. Arrowheads show the four *Hin*dIII fragments that hybridize with the probe. The smallest band is a doublet composed of two co-migrating fragments. (B) Restriction analyses of three BAC clones that span the *PnPMA1* locus. The left hand panel is an ethidium bromide stained gel showing BAC DNA digested by *Hin*dIII. The right hand panel shows a blot from the gel on the left labelled with the WS66 cDNA. Digestion with *Hin*dIII reveals that BAC1 and BAC2 differ in their restriction pattern and that they represent the two alleles at this locus. BAC3 only partially spans the *PnPMA1* locus. (C) The *PnPMA1* locus contains two nearly identical members located in a single *Eco*RI fragment in the *P. nicotianae* genome. Physical organization of the *PnPMA1* genes in the *Eco*R1 fragment is derived from BAC2. E1, *Eco*RI restriction site; H, *Hin*dIII restriction site; M, 1 kb ladder (showing sizes from 3 to 8 kb).

The full length coding sequence of the *PnPMA1b* cDNA and further upstream non-translated sequences were obtained by PCR amplification using the germinated cyst cDNA library as a template. Comparison of the cDNA sequence with that of the genomic sequence indicated that the *PnPMA1b* gene has no introns. The cDNA sequences obtained include 131 bp upstream of the putative ATG start codon.

Initial sequence alignment indicated that all six cDNA clones are derived from the *PnPMA1b* gene contained in the 9.5 kb *Hin*dIII fragment from BAC2. This restriction fragment spans the complete predicted *PnPMA1b* open reading frame. The genomic sequence has two stop codons 72 and 93 bp upstream of the putative translation start site. The putative translation start site has a clear Kozak consensus sequence [(GCC)RCCATGG] (Kozak, 1987) and no additional ATG codons were found upstream of the putative initiation codon. These features strongly suggest that the proposed translation initiation codon actually represents the start of the PnPMA1b protein. The 7.5 kb *Hin*dIII subclone spanned the open reading frame of the second gene, *PnPMA1a*, and shared nearly identical promoter sequences over 755 bp upstream of the start codon. Although they share only 192 bp of sequence downstream of the translation stop codon in the terminator region, *PnPMA1a* contains clear AT-rich polyadenylation sites.

2.2. *PnPMA1 encodes an atypical plasma membrane H$^+$-ATPase*

The *PnPMA1a* and *PnPMA1b* genes differ by only three nucleotides and encode a predicted identical protein of 1068 residues with a molecular mass of 116.3 kDa. This size is larger than that reported for homologous P-type ATPases in plants and true fungi (~100 kDa). A Blast search against protein databases showed that, over its full length, the PnPMA1 protein has 30–40% sequence identity to various fungal and plant plasma membrane H$^+$-ATPases and in general is more similar to plant than fungal H$^+$-ATPases. The protein contains 10 predicted transmembrane domains and all the highly conserved signature sequences characteristic of the non-heavy metal-transporting P-type ATPase family including CSDKTGT, TGES, ML/VTGD, and GDGVN motifs that are involved in ATP binding, phosphorylation and dephosphorylation. Hydropathy analysis also revealed that the structure of PnPMA1 was more similar to that of homologous proteins in plants and algae than in true fungi (Supplementary Fig. 1).

Sequence comparisons revealed that the PnPMA1 protein has a distinct domain of ~155 residues that is, with one possible exception, not present in homologous H$^+$-ATPases in other organisms. This domain occurs between transmembrane domains 8 and 9 and is predicted to be cytoplasmic (Fig. 2). Sequence comparisons of selected regions of the PnPMA1 protein showed that it is 30–40% and 40–50% identical to plant or fungal H$^+$-ATPases in the regions on either side of the 155-residue cytoplasmic domain. Currently, there is only one non-*Phytophthora* ATPase sequence in the NCBI database that is predicted to have a similar cytoplasmic loop between transmembrane domains 8 and 9 and this is an H$^+$-ATPase from *Dunaliella*

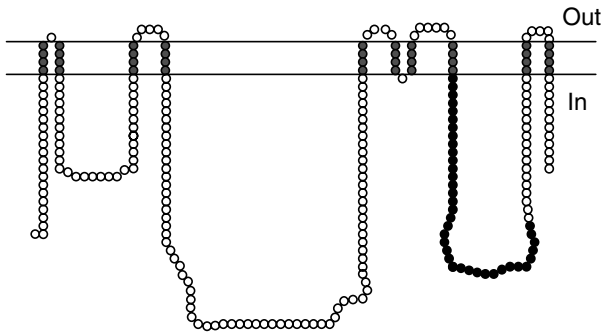


Fig. 2. Predicted structure of PnPMA1 protein showing putative transmembrane domains, cytoplasmic N- and C-termini and three cytoplasmic loops. The loop closest to the C-terminus, between transmembrane domains 8 and 9, contains ~155 amino acids (black circles) that are specific to *Phytophthora* plasma membrane H$^+$-ATPase proteins. Each circle represents about four amino acids.

acidophila (GenBank Accession No. U54690) (Weiss and Pick, 1996) (see Supplementary Fig. 1). The *D. acidophila* protein shows 41% overall sequence identity with PnPMA1 but sequence comparisons restricted to the 155-amino acid domain show only limited homology.

PnPMA1b has 95% sequence identity at the nucleotide level and 98% identity at the amino acid level over the protein-coding region to *PiPMA1*, a putative plasma membrane H^+-ATPase gene in *P. infestans* (GenBank Accession No. AAQ23136). The *P. infestans* H^+-ATPase also possesses the cytoplasmic loop between transmembrane domains 8 and 9. Database searches against the *P. sojae* genome (http://genome.jgi-psf.org/sojae1/sojae1.home.html) identified two physically linked homologues in scaffold 1 sharing sequence identities of 96.4% and 93.0% to the PnPMA1 protein. In contrast to the situation in *P. nicotianae*, these two genes are organized in a head-to-head orientation. A third putatively unlinked homologue in scaffold 136 shares 96.63% sequence identity to the PnPMA1 protein. In the *P. ramorum* genome, there were two homologous sequences showing over 90% identity to the PnPMA1 protein (http://genome.jgi-psf.org/ramorum1/ramorum1.home.html). They had a tail-to-tail orientation and thus differed with the arrangements in *P. nicotianae* and *P. sojae*.

The database search of the *P. sojae* genome using the PnPMA1 protein also identified a fourth homologue with a moderate sequence identity. Blast searches of GenBank using the protein sequence of this fourth PMA homologue showed that the predicted protein has significant sequence identity over its full length to algal (~50%) and plant (~40%) plasma membrane H^+-ATPases. The protein contains 10 predicted transmembrane domains and all the highly conserved signature sequences of plasma membrane P-type H^+-ATPases. It putatively encodes a protein of 964 residues that does not contain the 155-residue cytoplasmic loop seen in PnPMA1. The predicted protein is thus more similar to plasma membrane P-type H^+-ATPase proteins present in other organisms than is PnPMA1. At the nucleotide level, the fourth PMA homologue in *P. sojae* has no significant sequence identity with *PnPMA1* or with any of the other three *P. sojae* homologues. Further database searches (Phytophthora Functional Genomics Database, http://www.pfgd.org/) identified ESTs encoding homologues lacking the 155-residue domain in both *P. sojae* and *P. infestans*.

2.3. Genetic complementation of PMA1 mutation in S. cerevisiae

Database searches against *S. cerevisiae* genome sequences identified two genes encoding PMA1 (Serrano et al., 1986) and PMA2 (Schlesser et al., 1988) proteins which were highly similar to PnPMA1. The yeast PMA1 and PMA2 proteins had 30.2% and 28.6% overall sequence identity with PnPMA1, respectively. When the 155-residue putative *Phytophthora*-specific domain was excluded, the yeast and *P. nicotianae* proteins showed 34.6% and 32.7% identity, respectively.

To confirm the functionality of PnPMA1 as a plasma membrane H^+-ATPase, yeast genetic complementation experiments were carried out to test if the *PnPMA1* gene is functional in yeast. A yeast *pma1* conditional mutant was generated in this study by a PCR-based gene knockout approach and used as a recipient strain to test if the mutation could be complemented by the expression of wild-type *P. nicotianae PnPMA1*. The *PnPMA1* coding region was cloned into the *Ura*3-based yeast expression vector and was under the control of the *ADH*1 promoter. PCR analysis confirmed integration of the *PnPMA1* gene into the yeast genome and reverse-transcriptase PCR showed its expression. Genetic complementation experiments indicated that transformants carrying the *P. nicotianae PnPMA1* gene construct were able to complement the *pma1* mutant phenotype in the absence of galactose, confirming its functional identity as a plasma membrane H^+-ATPase gene (Fig. 3).

2.4. Expression of PnPMA1 during asexual development and plant infection

Northern blot analysis was used to investigate *PnPMA1* gene expression during asexual development and plant infection in *P. nicotianae*. *PnPMA1* expression is significantly up-regulated in germinated cysts compared to that in vegetative hyphae, sporulating hyphae and zoospores, consistent with previous results which indicated that the *PnPMA1* transcription was increased by over 12-fold in germinated cysts relative to vegetative hyphae (Shan et al., 2004). During infection of tobacco by *P. nicotianae*, levels of *PnPMA1* transcripts in early (water-soaked tissues) and late (necrotic tissues) stages of plant infection were at least as low as that in vegetative hyphae (data not shown).

2.5. Immunocytochemical localization of PnPMA1

Two regions of the PnPMA1 protein were chosen for the production of polyclonal antibodies. The first was a region that is highly conserved in plant and fungal PMA proteins and encompassed amino acids 492–718 of the *P. nicotianae*

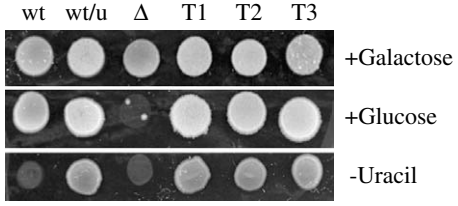


Fig. 3. Functional complementation of the PMA1-deficient yeast cells by *PnPMA1*. Wild-type (wt) and wild-type cells transformed with the empty plasmid containing the uracil selection marker (wt/u) can grow on galactose- and glucose-containing medium. PMA1-deficient mutant (Δ) can grow only in the presence of galactose, which induces expression of *PMA1*. The PMA1-deficient mutants transformed with *PnPMA1* (T1–T3) can grow in the presence or absence of galactose. The −uracil control shows the successful introduction of the PnPMA1 construct that is carried on the Ura3 vector.

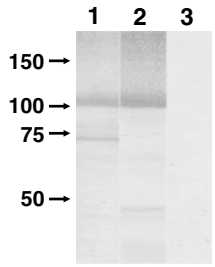


Fig. 4. Immunoblot of *P. nicotianae* proteins isolated from 2 h germinated cysts and labelled with antisera raised against the 155-amino acid *Phytophthora*-specific region of the C-terminal predicted cytoplasmic loop in PnPMA1. Antisera from rabbit 1 (lane 1) and rabbit 2 (lane 2) were diluted 1:2000. Lane 3 shows a control membrane strip incubated in dilution buffer and then alkaline phosphatase-conjugated secondary antibody. Positions of molecular weight standards are shown on left.

protein. Attempts to express a histidine-tagged version of this polypeptide in *Escherichia coli*, were, however, not successful. The second region consisted of the distinct 155-residue domain (amino acids 740–894) in the third cytoplasmic loop in PnPMA1 (Fig. 2). A histidine-tagged version of this polypeptide was successfully expressed in *E. coli*, purified and used to raise polyclonal antibodies in two rabbits. On Western blots, both antisera reacted with a polypeptide of approximately 115 kDa (Fig. 4). The sera from the two rabbits also reacted weakly with lower molecular weight bands that differed between the two antisera. Affinity purification of the sera against the fusion protein immobilised on nitrocellulose membranes did not eliminate the weak reaction of the lower molecular weight bands (data not shown).

In immunofluorescence assays, both PnPMA1 antisera labelled the surface of *P. nicotianae* cysts (Fig. 5A) and germinated cysts (Fig. 5B) but not the surface of zoospores (Fig. 5A). Immunolabelling of sectioned material at the ultrastructural level gave similar results. The plasma membrane of zoospores was not labelled by the antisera (Fig. 5C). Low levels of immunogold labelling were associated with the plasma membrane and cell wall of cysts 5 min after the induction of encystment (Fig. 5D). The density of labelling of the cyst plasma membrane and cell wall increased as the cysts aged (Figs. 5E–H). Dense labelling of the plasma membrane of the cyst body was often observed, with little or no labelling of the cyst cell wall (Fig. 5G). On germ tubes the antisera labelled the cell wall and the plasma membrane (Figs. 5F and H). Affinity purification of the antisera against the PnPMA1b fusion protein resulted in a low-titre antiserum that labelled the plasma membrane of the cyst body and germ tubes in some cells, although the majority were unlabelled (Fig. 5I).

3. Discussion

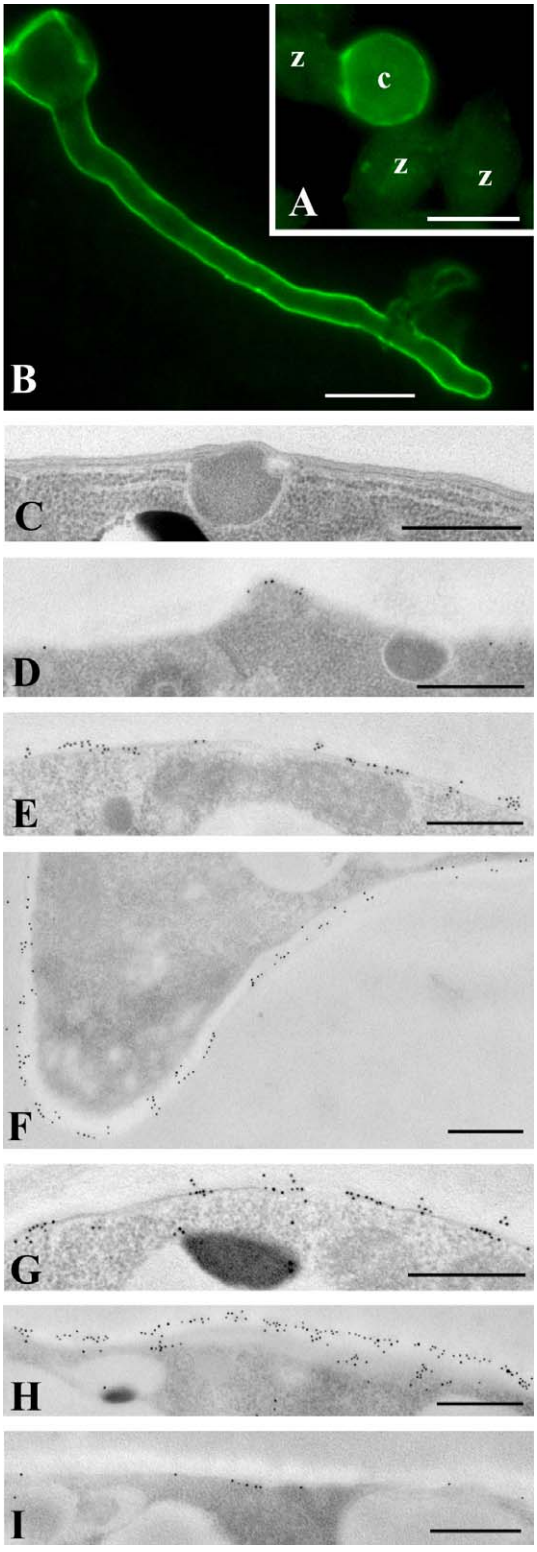
In this study, we describe the cloning and characterization of a gene family consisting of two genes, *PnPMA1a* and *PnPMA1b*, that are closely linked in the *P. nicotianae* genome. The genes encode an identical protein with high homology to plasma membrane H^+-ATPases. The PnPMA1 H^+-ATPase is, however, unusual in that it has a 155-amino acid cytoplasmic loop between transmembrane domains 8 and 9, a domain not found in H^+-ATPases from other organisms. Nevertheless, *PnPMA1* successfully complemented a yeast strain conditionally deficient in *PMA1* activity, providing evidence that *PnPMA1* encodes a functional plasma membrane proton pump.

The two *PnPMA1* genes are located in a single genetic locus as determined by genomic Southern blot analysis and BAC characterization. The genes are nearly identical in both the protein-coding region and in putative regulatory sequences. We also identified the second allele corresponding to each of the two *PnPMA1* genes in this diploid organism. Genomic sequencing indicated that there is more variation between alleles of the same gene than between the two members of the gene family on the same chromosome. Analysis of six *PnPMA1* ESTs indicated that all of them originated from the same allele of the *PnPMA1b* gene, giving evidence of allele-specific gene expression in *P. nicotianae* and of possible preferential expression of *PnPMA1b* over *PnPMA1a*.

PnPMA1b was identified by differential hybridization, showing enhanced expression in germinated cysts as compared to mycelium (Shan et al., 2004). Northern blots verified that the *PnPMA1* gene(s) are under transcriptional control during *Phytophthora* development, with the highest transcriptional levels occurring in germinated cysts and the lowest levels during asexual sporulation. Immunolabelling of PnPMA1 indicated that the H^+-ATPase protein was also abundant in germinated cysts. The 115 kDa polypeptide, corresponding to the predicted size of PnPMA1, was strongly labelled in Western blots even though total protein extracts from germinated cysts rather than the more usual microsomal or plasma membrane protein extracts were loaded on the gels. In ultrathin sections of embedded cells, the plasma membrane of germinated cysts was strongly labelled. These results suggest that regulation of PnPMA1 plasma membrane H^+-ATPase synthesis in *P. nicotianae* is not under post-translational control as appears to be the case in several fungal and plant systems, including *Uromyces fabae* (*Uf-PMA1*) (Struck et al., 1998), *S. cerevisiae* (*PMA1*) (Capieaux et al., 1989), and *Nicotiana plumbaginifolia* (Michelet et al., 1994; Perez et al., 1992).

The zoospore plasma membrane is assembled during cleavage of sporangia formed during asexual sporulation and thus the absence of the PnPMA1 protein in the zoospore plasma membrane is consistent with the absence or low levels of *PnPMA1* transcription during asexual sporulation. Nevertheless, zoospores would be expected to have a plasma membrane H^+-ATPase and indeed immunofluorescence labelling with an antibody raised against the plasma membrane H^+-ATPase from *N. plumbaginifolia* (Morsomme et al., 1996) has demonstrated the presence of a P-type ATPase in the plasma membrane of *P. nicotianae* zoospores (Mitchell and Hardham, 1999). In addition, bioinformatic analysis confirms the presence

of PMA genes that lack the 155-residue domain in *P. sojae*, *P. ramorum*, and *P. infestans*. A *P. nicotianae* homologue of these ATPases would not be detected by the polyclonal antibodies used in this study, as they were generated against the 155-residue domain. Differences in nucleotide sequence would also lead to lack of detection in DNA and RNA blots.



Labelling of the cell wall, in particular that surrounding the germ tube of germinated cysts, by both polyclonal antibodies is difficult to explain and is likely to be non-specific. Extracellular ATPases (ecto- or E-type ATPases) do occur in fungi, plants and animals but they have a different structure to typical P-type ATPases (Smith and Kirley, 1998) and to PnPMA1. It seems unlikely that the cell wall labelling is due to cross-reaction of the antibodies with the lower molecular weight polypeptides seen on the Western blots because the weakly-labelled bands differed between the two antibodies. Although only a low proportion of sectioned cells were labelled by the affinity purified antibody, in those that were labelled, the gold particles were mainly associated with the plasma membrane, even in germ tubes, supporting the interpretation that the cell wall labelling is a non-specific reaction.

Reasons for the enhanced expression of *PnPMA1* and the precise role of the PnPMA1 plasma membrane H^+-ATPase in germinated cysts of *P. nicotianae* remain to be determined, however, an increase in expression of *PMA* genes following spore germination has been observed in other plant pathogens. In the pathogen causing powdery mildew in barley, *Blumeria graminis*, there is an 8-fold increase in mRNA encoding a plasma membrane H^+-ATPase during the first 8 hours after conidial germination (Both et al., 2005). Similarly in the bean rust pathogen, *U. fabae*, *PMA1* mRNA levels increase after conidium germination and are maintained during ensuing germling growth (Struck et al., 1998). In *B. graminis*, after a transient decrease, H^+-ATPase mRNA levels are also high during the next stage in plant infection, namely appressorium development (Both et al., 2005). In *U. fabae*, although *PMA1* transcription is low in haustoria, the presence of H^+-ATPase proteins in the plasma membrane and high levels of H^+-ATPase activity have been demonstrated in

Fig. 5. Immunocytochemical labelling of *P. nicotianae* cells with polyclonal antisera raised against the 155-residue cytoplasmic loop of PnPMA1. (A and B) Immunofluoresence labelling with sera from rabbit 2 (A) and rabbit 1 (B) diluted 1:100. (C–H) Immunogold labelling with serum from rabbit 2. (I) Immunogold labelling with rabbit 2 serum after affinity purification against the PnPMA1 fusion protein. (A) The anti-PnPMA1 antibody labels the surface of a cyst (c) but not the surface of zoospores (z). (B) The anti-PnPMA1 antibody labels the surface of a germinated cyst. (C) There was no labelling of the plasma membrane of zoospores. Rabbit 2 serum diluted 1:500. (D) In cysts, 5 min after the induction of encystment, there is light labelling of the plasma membrane. Rabbit 2 antiserum diluted 1:800. (E) In cysts, 30 min after the induction of encystment, the antibodies react with the plasma membrane and the cell wall. The cell wall is generally electron-lucent in the non-osmicated material used for immunogold labelling but is about 10 nm in thickness. Rabbit 2 antiserum diluted 1:500. (F) In germinated cysts, fixed 45 min after the induction of encystment, the antibodies label the plasma membrane and germ tube wall. Rabbit 2 antiserum diluted 1:600. (G) The antibodies show dense labelling of the plasma membrane of the cyst body in 3 h germinated cysts. Rabbit 2 antiserum diluted 1:1000. (H) The antibodies label the surface of the germ tube of 3 h germinated cysts. Rabbit 2 antiserum diluted 1:600. (I) A germ tube of a 3 h germinated cyst labelled with rabbit 2 antiserum after affinity purification against PnPMA1 fusion protein. The purified antiserum labels the plasma membrane of the germ tube.

haustoria, the specialized feeding structures that form during biotrophic host-pathogen interactions (Struck et al., 1996). The haustorial plasma membrane H^+-ATPase is believed to be responsible for the generation of an electrochemical gradient that enables the haustorium to take up nutrients from the surrounding plant cell (Schulze-Lefert and Panstruga, 2003; Struck et al., 1996; Struck et al., 1998). *B. graminis* germlings 4–8 h after conidium germination do not absorb nutrients from the environment so the reason for the increase in plasma membrane H^+-ATPase in these cells is unclear (Both et al., 2005). However, in *Phytophthora*, continued germling development after cyst germination is dependent on supply of exogenous nutrients (A.R. Hardham, unpublished observations), and thus the presence and activity of H^+-ATPases in the plasma membrane of the germinated cysts may be required to support nutrient uptake. The differential expression of *PnPMA1* genes and the unusual structure of the PnPMA1 ATPase may be indicative of a specialised role of this protein in *Phytophthora* development.

4. Materials and methods

4.1. Microbial strains and culture conditions

Phytophthora nicotianae H1111 (ATCC MYA 141), kindly supplied by Dr. David Guest (University of Sydney), was cultured as described previously (Shan and Hardham, 2004). *E. coli* strain DH10B (Gibcol, USA) was used for BAC library construction (Shan and Hardham, 2004) and sub-cloning, strain TunerBlue (Stratagene, USA) for production of recombinant proteins and strain XL1-blue for cDNA library construction. Standard culturing conditions were used for cloning and protein expression following Sambrook et al. (Sambrook et al., 1989) or the manufacturer's recommended procedures.

Methods for preparation of different asexual developmental stages of *P. nicotianae* were as described previously (Mitchell and Hardham, 1999; Shan et al., 2004). To prepare *P. nicotianae* infected tissues, detached susceptible tobacco leaves (6-week old) were placed in a tray, inoculated with small pieces of strain H1111 mycelia and the trays covered with clear, plastic wrap. At 25 °C with light, water-soaked lesions (early stage of infection) formed in 1–2 days and brown, necrotic lesions (late stage of infection) developed in 3–5 days.

Saccharomyces cerevisiae strain CS27-48C (*MAT*α, *leu2*, *ura3*, and *trp1*) and its derivatives were grown at 30 °C in a rich medium containing 2% tryptone, 1% yeast extract (Difco) and 2% glucose (YPD medium) or 2% galactose (YPG medium), or in a synthetic medium containing minimum SD base and -Ura supplements (BD Biosciences, USA). Solid media were supplemented with 2% agar.

4.2. Subcloning and plasmid construction

To clone genomic sequences hybridizing to the WS66 probe, all three *Hin*dIII and one *Eco*RI fragment were released from 0.5 μg of BAC DNA and cloned into the corresponding restriction sites in pBluescript KS (Stratagene, USA) following standard methods.

To make the construct for PCR-based gene replacement in *S. cerevisiae*, the yeast *Gal10* promoter was derived from pCXJ28 as a 0.95 kb *Sal*I/*Eco*RI fragment and fused to the dominant NPTII gene flanked by the *Ashbya gossypii TEF* (translation elongation factor) regulatory sequences which were derived from pUG6 (Güldener et al., 1996) as a 2.2 kb *Sal*I/*Spe*I fragment. These two fragments were cloned into the *Spe*I and *Eco*RI sites of pBluescript KS.

To clone the *PnPMA1b* gene from *P. nicotianae* into the yeast expression vector pCXJ9, which contains the *Ura3* selection marker under the *ADH1* promoter, *PnPMA1b* gene-specific primers were designed to amplify an ~3.3 kb fragment starting 21 bp upstream of the translation initiation ATG of the open reading frame and extending 31 bp beyond the stop codon. To facilitate cloning, *Eco*RI sites were adapted to the primers. Recombinant plasmids with the correct orientation were identified by restriction analysis and were sequenced to exclude possible introduction of mutations.

4.3. Yeast transformation and mutant generation

Saccharomyces cerevisiae strain CS27-48C (*MAT*α, *leu2*, *ura3*, and *trp1*) was used to generate conditional mutants deficient in the *PMA1* gene. Competent yeast cells were prepared following a standard lithium acetate/PEG–mediated procedure. A PCR-based homologous recombination approach (Reid et al., 2002) was used to generate conditional endogenous plasma membrane H^+-ATPase genes by replacing the native *PMA1* gene promoter with the *Gal10* promoter. Under control of the galactose-inducible promoter, growth of the yeast mutants was repressed in media not supplemented with galactose as the carbon source. The *NPTII* gene driven by the *TEF* promoter of *A. gossypii* was fused with the *Gal10* promoter and used as a marker for geneticin selection. Primers (TCCATCATGAAAAATC TCTCGAGACCGTTTATCCATTGCTACTAGTGGAT CTGATATCACC and AGGATGATGTATCAGTCAT ATTGATATTGTTTGATAATTACCTTGAATTTTCA AAAATTC TTAC), which included 40-bp sequences derived from the yeast *PMA1* promoter region (underlined bases) as extracted from the yeast genome database (http://db.yeastgenome.org/), were designed to amplify the fusion of the *Gal10* promoter and *NPTII* expression cassette. The resultant PCR product was cleaned using PCR Purification columns (Qiagen, Germany) and was used to directly transform yeast strain CS27-48C. Two rounds of selection were carried out to identify successful conditional mutants. First round selection was determined by geneticin resistance on YPG media, with sequential streaking on YPG agar to obtain stable geneticin-resistant colonies. Second round selection was then carried out by replicating cultures on YPD agar plates. As the *PMA1* gene is essential for yeast cell function (Serrano et al., 1986), transformants that grew

on YPG but were suppressed on YPD were successful disruptants. The resulting *PMA1*-deficient mutants were used for genetic complementation experiments using *URA3* plasmids carrying *PnPMA1b* constructs.

4.4. Library screening

An 870 bp EST clone, WS66 (GenBank Accession No. CF891682), was used to screen a bacterial artificial chromosome (BAC) library constructed with nuclear genomic DNA of *P. nicotianae* H1111. The library contains 10,752 clones with an average insert size of 90 kb and is equivalent to 10.1× *P. nicotianae* genome (Shan and Hardham, 2004). The 10,752 clones are arrayed in 28 374-well plates. For library screening, the whole library was replicated and clones from groups of four plates (a total of 1536 clones) combined into seven pools. Clones from the same groups of four plates were also replicated onto nylon filters. DNA was extracted from each of the seven pools and digested to completion with *Hin*dIII, the restriction enzyme used for library construction. The digested DNA was transferred to Hybond N+ membranes (Amersham) and hybridised with [^{32}P]-dCTP labelled WS66. Genomic *P. nicotianae* DNA was also digested to completion with *Eco*RI, *Bam*HI, and *Hin*dIII and included on the library blots for comparative analysis of positive BAC clones. This screening led to the identification of four positive BAC pools. BAC filters corresponding to two of the positive BAC pools were hybridised with WS66 to identify individual positive BAC clones. Fifteen BAC clones hybridized with the probe and three were chosen for detailed characterization.

4.5. Nucleic acid methods and gel blot analysis

For the isolation of *Phytophthora* total genomic DNA, *P. nicotianae* H1111 mycelia were ground into a fine powder in liquid nitrogen in a mortar before dispersing into DNA extraction buffer (Dudler, 1990). BAC pool DNA was isolated using a PlasmidMidi kit (Qiagen, Germany) as described (Shan and Hardham, 2004). For Southern blot analysis, 2 μg of total *P. nicotianae* genomic DNA or 3 μg of BAC pool DNA were digested with appropriate restriction enzyme(s), resolved in 0.8% agarose gels and transferred to Hybond N+ nylon membranes (Amersham–Pharmacia) using 0.4 N NaOH as the transfer agent following a standard procedure (Sambrook et al., 1989).

Total RNA was isolated using a method of Chirgwin et al. (1979). For Northern hybridizations, 10 μg of total RNA were mixed in 20 μl of 1× RNA-treating buffer (15 μl of DMSO, 3 μl of glyoxal, and 2 μl of 10× Hepes buffer) and heat-treated at 70 °C for 5 min before being separated in 1% agarose gels in 1× Hepes buffer (20 mM Hepes, 1 mM EDTA, pH 7) and transferred to Hybond N nylon membranes (Amersham–Pharmacia) with 10× SSC (1× SSC contains 0.15 M NaCl and 15 mM sodium citrate) following a standard procedure (Sambrook et al., 1989). The membranes were baked at 80 °C for 2 h with vacuum to fix RNA samples and were stained with 0.05% methylene blue (w/v, in 0.3 M sodium acetate) to check for RNA integrity and equal loading of RNA samples. The probes were released from cDNA clones by *Eco*RI and *Xho*I double digestion. Random priming using a Megaprime kit was used to prepare ^{32}P labeled radioactive probes.

Membrane hybridizations followed the same conditions used for both Southern and Northern blots and were done at 65 °C for 16 h using either 6× SSPE or 6× SSC with 5× Denhardt's solution (1× Denhardt's solution contains 0.02% Ficoll 400, 0.02% PVP, 0.02% BSA) and 0.1% SDS as the hybridization buffer as described (Sambrook et al., 1989). Following hybridization, the membranes were washed at 65 °C twice in 2× SSC and 0.1% SDS each 15 min, and twice in 0.2× SSC and 0.1% SDS each 15 min. Signals were detected by exposing the filters to a PhosphoImager screen for 2 h to 3 days and were quantified by using ImageQuant software (Version 5.0) (Molecular Dynamics, CA, USA).

4.6. PCR amplification

For PCR amplification, standard (VentR DNA polymerase, New England Biolabs, USA) or hotstart (JumpStart Taq DNA polymerase, Sigma–Aldrich, Germany) conditions were used following the manufacturer's recommendation with minor modifications in which 5 ng BAC DNA or 0.5 ng plasmid DNA was used, $MgCl_2$ was supplemented to 2 mM final concentration, and dNTP mix was used at 200 μM final concentration. PCR amplification was done in a PTC-200 Peltier Thermal Cycler (MJ Research, Finland) using the following conditions: initial denaturation at 94 °C for 2 min followed by 30 cycles of 94 °C for 30 s, 56–60 °C for 40 s, and 72 °C for 1–3.5 min, and a final 5 min extension at 72 °C. For colony PCR, initial denaturation at 94 °C was extended to 5 min.

4.7. Recombinant protein expression in E. coli

For prokaryotic expression of recombinant protein, the pET-Blue system was used (Novagen, USA). A 0.46 kb PCR fragment encoding a 155-amino acid cytoplasmic domain which appeared to be unique to *Phytophthora* PMA1 proteins was cloned into the *Eco*RV site of pET-Blue-2 vector, resulting in a construct that encoded the protein fused to a 6-histidine tag in the C-terminus. The expression construct was confirmed by DNA sequencing and delivered into Tuner cells for IPTG-inducible protein expression under the control of the T7 promoter following the manufacturer's recommendations. The recombinant protein is mainly expressed as inclusion bodies which were isolated using BugBuster reagent and purified by passage through a resin column and released into 1× Elute Buffer containing 6 M urea. The recombinant protein extract was cleaned using ultrafiltration with a Centricon column and suspended in 1× PBS containing 3 M urea before purification of the recombinant protein on a nickel-agarose column.

4.8. Antibody production and immunolabelling

Two rabbits were immunized with the purified 155-amino acid fusion protein from PnPMA1b with six boost immunizations at 3-week intervals. Serum was collected before initial injection (preimmune serum) and 7–10 days after each boost.

Phytophthora nicotianae proteins were extracted from 2 h germinated cysts in a buffer containing 8 M urea, 5% SDS, 40 mM Tris–HCl, pH 6.8, 0.1 mM EDTA, and 0.4 mg/ml bromphenol blue. Ten microlitres of β-mercaptoethanol, 10 μl 10× protease inhibitor cocktail solution, and 50 μl 100× PMSF solution were freshly added to 1 ml extraction buffer, and the samples boiled for 5 min. Proteins were separated in 10% SDS–polyacrylamide gels and transferred to Hybond C Extra (Amersham, USA) membranes. The membranes were blocked for 1 h in 1% BSA and 2% skimmed milk powder in PBS and rinsed in Tris-buffered saline containing 0.05% Tween 20 (TBST). Membrane strips were incubated in anti-PnPMA1 antisera diluted in TBST containing 1% BSA for 45 min. Strips were rinsed in TBST and incubated in sheep anti-rabbit secondary antibody conjugated to alkaline phosphatase (Chemicon, Australia) for 45 min before incubation in alkaline phosphatase substrate.

For electron microscopy, *P. nicotianae* cells were fixed in suspension for 30 min in 1% (v/v) glutaraldehyde in 100 mM Pipes buffer (pH7). After washing in 100 mM Pipes buffer, cells were embedded in 2% low-melting point agarose, dehydrated in a graded series of ethanol and embedded in Lowicryl K4M resin under N_2 and UV light (Hardham, 2001). Sections were cut on a Reichert Ultracut microtome and collected on gold grids coated with formvar. Sections were blocked for 1 h in PBS containing 1% BSA and 0.1% gelatin and then incubated sequentially for 1 h each in anti-PnPMA1 antiserum and goat anti-rabbit secondary antibody conjugated to 10 nm gold (Amersham). Sections were stained with uranyl acetate for 4 min and lead citrate for 1 min and examined on a Hitachi 7100 transmission electron microscope. For some experiments, the anti-PnPMA1 antiserum was affinity purified against PnPMA1 fusion protein immobilised on nitrocellulose membrane according to the general procedure described in Smith and Fisher (Smith and Fisher, 1984).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2006.03.002.

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