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PnPMA1, an atypical plasma membrane H^+-ATPase, is required for zoospore development in *Phytophthora parasitica*

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ABSTRACT

Biflagellate zoospores are the major infective agents that initiate plant infection for most *Phytophthora* species. Once released from sporangia, zoospores swim and use a number of tactic responses to actively target host tissues. However, the molecular mechanisms controlling zoospore development and behaviour are largely unknown. Previous studies have shown that the *PnPMA1* gene is highly expressed in zoospores and germinated cysts of *Phytophthora parasitica* and encodes an atypical plasma membrane H^+-ATPase containing an insertion of ~155 amino acid residues at the C terminus. Using topology determination we now show that the C-terminal insertion loop in the PnPMA1 protein is located in the extracellular space. To elucidate the biological function of *PnPMA1*, *PnPMA1*-deficient transformants were generated by homology-dependent gene silencing and were confirmed by quantitative PCR of *PnPMA1* transcripts and detection of associated small interfering RNAs (siRNAs). High levels of *PnPMA1* silencing in *P. parasitica* resulted in production of nonflagellate and large aberrant zoospores, rapid transition from zoospores to cysts, and a decreased germination rate of cysts. These results indicate that PnPMA1 plays important roles in zoospore development.

Introduction

Species in the genus *Phytophthora*, which belongs to a group of fungus-like organisms in the Oomycetes, include many destructive plant pathogens. The late blight disease caused by *Phytophthora infestans* led to the Irish Potato Famine in the 1840s, and has been a serious threat to sustainable potato production (Ristaino *et al.* 2001). *Phytophthora sojae* is one of the most devastating pathogens of soybean, causing substantial yield losses worldwide (Tyler 2001). Other highly destructive species include *Phytophthora ramorum*, the causal agent of Sudden Oak Death (Rizzo *et al.* 2002), *Phytophthora cinnamomi* that causes dieback in Australia (Hardham 2005), and *Phytophthora capsici* which causes fruit rot of peppers (Lamour *et al.* 2012). Oomycetes are similar to true fungi in morphology and growth habits, but are different in cell wall composition, reproductive biology and genetics (Judelson 1997). Phylogenetic analyses reveal that Oomycetes are phylogenetically distant from true

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fungi (Harper et al. 2005). As a consequence, most fungicides are ineffective in controlling diseases caused by oomycete pathogens (Madoui et al. 2009). In order to provide insights that will help development of novel control strategies, we aim to elucidate molecular mechanisms that control *Phytophthora* asexual development and interaction with host plants using *Phytophthora parasitica* (synonymous with *Phytophthora nicotianae*), a species that is emerging as a model for the studies of oomycete biology and pathology (Attard et al. 2008, 2010; Wang et al. 2011).

Oomycetes reproduce asexually by forming multinucleate sporangia. Sporangia can germinate directly but usually undergo cytoplasmic cleavage to produce uninucleate zoospores. The motile zoospores are considered to be the major infective agents that initiate plant diseases for most *Phytophthora* species. Zoospores are wall-less cells having two flagella that allow them to swim, and they are able to target host tissues by various tactic responses, such as chemotaxis, autotaxis, and electrotaxis (Walker & van West 2007). Once the zoospores reach the host, they encyst and subsequently germinate. The germ tubes emerging from cysts often develop appressorium-like structures that facilitate plant penetration (Hardham 2007).

Only a few genes involved in *Phytophthora* zoospore development have been characterized and little is known about the molecular mechanisms that control this process. The important roles of zoospore motility for successful infection have been shown through silencing of genes encoding a G protein α subunit and a bZip transcription factor (Latijnhouwers et al. 2004; Blanco & Judelson 2005; Hua et al. 2008). Phospholipase D (PLD) has also been shown to control zoospore behaviour (Latijnhouwers et al. 2002). PLD generates the signalling molecule phosphatidic acid (PA) by hydrolysis of structural phospholipids and PA induces zoospore encystment. A putative DEAD-box RNA-helicase gene, which was highly expressed in zoospores, is required for normal zoospore development in *P. infestans* since silencing of this gene results in production of large aberrant zoospores as a result of abnormal sporangial cleavage (Walker et al. 2008). Silencing of the *NIFC* genes in *P. infestans* impaired cyst germination but did not affect other aspects of the asexual lifecycle (Judelson & Tani 2007), indicating that these transcriptional regulators are required for cyst germination. More recently, a stress activated MAP kinase, PsSAK1, which represents a novel group of MAPKs containing a pleckstrin domain, was shown to control zoospore viability and virulence in *P. sojae* (Li et al. 2010). In *P. parasitica*, disruption of dynein light chain 1 inhibited development of flagella in zoospores, and demonstrated that zoospore motility is not essential for zoospore release from sporangia (Narayan et al. 2010).

Analysis of gene expression during *Phytophthora* development led to the identification and characterization of two tandemly arrayed duplicated genes that differ by only three nucleotides and that encode an atypical plasma membrane H^+-ATPase (PnPMA1) (Shan et al. 2004, 2006). The *PnPMA1* gene/genes is/are highly expressed in zoospores and germinated cysts of *P. parasitica*. Plasma membrane H^+-ATPases (PMAs) are 100-kDa integral membrane proteins in fungi and plants that belong to the P-type ATPase family because of the formation of a phosphorylated intermediate during the catalytic cycle (Palmgren 2001). PMAs couple ATP hydrolysis to proton transport out of the cell. The resultant proton motive force is then used by secondary transporters to move ions and metabolites across the plasma membrane.

In plants, PMAs are involved in a variety of biological processes such as pH homeostasis, nutrient uptake, plant morphogenesis, and responses to biotic and abiotic stresses (Morsomme & Boutry 2000; Palmgren 2001; Elmore & Coaker 2011). PMAs have also been extensively studied in yeast and filamentous fungi and shown to play crucial roles in fungal cell physiology and pathogenicity (Portillo 2000; Remy et al. 2008). PnPMA1 in *Phytophthora* is different from other PMA proteins because it contains an insertion loop of ~155 amino acid residues near its C terminus. Immunocytochemical studies showed that PnPMA1 was localized in the plasma membrane of germinated cysts and functional analysis showed that it could complement a yeast mutant deficient in endogenous PMA activity (Shan et al. 2006).

The present study was aimed at understanding the unusual structure of PnPMA1. We show that the insertion loop is located in the extracellular space by mapping the membrane topology of PnPMA1 using *lacZ* gene fusion approach. To investigate the roles of PnPMA1 in *P. parasitica* development and pathogenicity, we generated PnPMA1-deficient transformants by homology-dependent gene silencing. Phenotypic characterization of the transformants revealed roles of PnPMA1 in zoospore flagella formation, zoospore encystment, and cyst germination.

Materials and methods

Phytophthora parasitica *strains and culture conditions*

Phytophthora parasitica strain Pp016 (ATCC MYA-141; H1111) was grown on 5 % (v/v) carrot juice agar (CA) supplemented with 0.002 % (w/v) β-sitosterol and 0.01 % (w/v) $CaCO_3$ at 25 °C. Asexually sporulating mycelium was prepared as previously described (Wang et al. 2011). Briefly, after growing cultures on 5 % (v/v) carrot broth for 2–3 d, they were washed twice with sterile water before transfer to mineral salts solution for 3–5 d at 25 °C. To release zoospores, the cultures were washed twice with cold sterilized distilled water and released in ~15 mL of cold sterile distilled water per plate by placing at 4 °C for 0.5 h followed by incubation at 25 °C for about 1 h.

Topology characterization

The *lacZ* gene was amplified from genomic DNA of *Escherichia coli* strain BL21(DE3)pLysS (Promega, USA) using the primers (5′-TTA TTC CCA AGC TTA TGG TCG TTT TAC AAC GTC GTG A-3′ and 5′-TTA TTC CCA AGC TTT TAT TAT TTT TGA CAC CAG ACC AAC TGG-3′), and cloned into pGEM-T-easy vector (Promega, USA). The resulting plasmid was verified by DNA sequencing (GenScipt, China), and subcloned into expression vector pGADT7 (Clontech, USA) as a positive control. The *PnPMA1::lacZ* fusion was generated by fusion PCR (primers for truncated *PnPMA1*: 5′-TTA TTC CCA AGC TTA TGG CTG GTG CCG CAG GTA A-3′ and 5′-TCA CGA CGT TGT AAA ACG

ACC ATG AAA GCA CCC ACG TCC GAC AC-3′; primers for *lacZ*: 5′-ATG GTC GTT TTA CAA CGT CGT GA-3′ and 5′-TTA TTC CCA AGC TTT TAT TAT TTT TGA CAC CAG ACC AAC TGG-3′), and inserted into pGADT7 vector (Clontech, USA). The artificial transmembrane domain (Fire *et al.* 1990) (TM domain: 5′-CCT CGT GAA AGT TGG CAA AGA GCT CTT GTC CTG CTA ATC GTA CTA CTA TTC ATC GTC ATC TTC GTT ATT ACT GTT TTG TTC GTC ATA AGA TCT AAC AAG GTA CCA GTG GGT GAA GAC CAG AAA CAG CAT CTA GAA CTG AGT CGT GAT ATT GCC CAG CGT TTC AAC GCT CTG TAT GGT GAG ATC-3′) was synthesized by GenScript Corporation and ligated to pUC57 plasmid (GenScipt, China). The *PnPMA1::TM::lacZ* fusion was prepared by ligating *lacZ* (primers: 5′-GCT ATA GGA CTA GTG TCG TTT TAC AAC GTC GTG A-3′ and 5′-GCA TAC CCA AGC TTT TAT TAT TTT TGA CAC CAG ACC AAC TGG-3′) and truncated *PnPMA1* (primers: 5′-TCA TCG CGG ATC CAA GCT TAT GGC TGG TGC CGC AGG TAA-3′ and 5′-TCA TCG CGG ATC CGA AAG CAC CCA CGT CCG ACA C-3′) into the pUC57 plasmid containing the TM domain separately, and then transferring the resultant fusion into pGADT7.

Modelling of the three-dimensional structure of PnPMA1 was performed by Swiss-Model homology modelling programs (Arnold *et al.* 2006).

β-Galactosidase (β-Gal) assay

A colony-lift filter assay for measuring β-Gal activity in yeast transformants was performed as described in the Yeast Protocols Handbook (Clontech, USA). The constructs for topology characterization were introduced into yeast strain cdc25H (Merck, USA) by PEG/LiAc method (Gietz & Schiestl 1995). Transformants were grown on SD/-Leu agar plates at 25 °C for 5 d, and the colonies on the plates were lifted onto Whatman No. 5 filter. The cells on the filter were then frozen in liquid nitrogen for 10 s and placed on another sterile Whatman No. 5 filter presoaked with Z buffer/X-gal solution [60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$ (pH 7.0)] and 0.02 % 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. The filter was incubated at 25 °C for 3 h for colour development.

Vector construction and PnPMA1 *silencing*

To generate the hairpin construct, a 262 bp fragment within the insertion loop of *PnPMA1* coding region was amplified using forward primer F: 5′-GGA CTA GTA CGT GCA CCT CAA CTG GCT G-3′ and reverse primer R: 5′-CCA TCG ATA TCA TCT CAT TGT CGC GAG TCC-3′, digested with *Spe*I and *Cla*I, and ligated to a *Cla*I-digested kanamycin resistance gene linker to generate a hairpin structure, and the hairpin structure was then inserted into *Spe*I-linearized pBluescript II KS. The PMA1-kanamycin-PMA1 fragment was released from pBluescript II KS using *Spe*I and blunt-ended by *Pfu* DNA Polymerase (Fermentas, USA), then ligated into *Sma*I-linearized expression plasmid pTH210 (Judelson *et al.* 1991). To generate the antisense construct, the ORF of *PnPMA1* was amplified and inserted in the reverse orientation into *Sma*I-linearized pTH210.

Phytophthora parasitica transformation was carried out as described previously (Bottin *et al.* 1999). The *PnPMA1*-silencing constructs were cotransformed with pTH210 by using the polyethylene glycol–$CaCl_2$ method. Transformants were recovered 3–7 d after regeneration on 5 % CA supplemented with 80 μg mL^{-1} hygromycin. The primary transformants were transferred to 5 % CA with 100 μg mL^{-1} hygromycin and maintained for following analyses.

Analyses of PnPMA1*-silencing transformants*

Primary transformants were subcultured onto 5 % CA plates supplemented with 100 μg mL^{-1} hygromycin for 5 d, then a piece of agar culture was transferred to 5 % CA medium without hygromycin and incubated at 25 °C for 6 d before induction of sporangia formation and zoospore release as described previously (Wang *et al.* 2011).

To observe zoospore behaviour, *Phytophthora parasitica* sporangia were monitored under a light microscope during zoospore release. To analyze zoospore encystment, 40 μL aliquots of zoospore suspension were transferred onto glass plates placed on distilled water-saturated filter paper in a 150 mm Petri dish, and incubated at 25 °C. The number of encysted zoospores was counted after 0, 0.5, and 1 h. To measure cyst germination, 200 μL of zoospore suspension in a 2 mL tube was vortexed at 2200 rpm for 60 s to induce encystment, and then 4 μL of carrot juice was added to the encysted zoospore suspension. Cyst germination was measured by transferring 50 μL of cyst suspension onto glass plates placed on distilled water-saturated filter paper in a 150 mm Petri dish and incubated at 25 °C for 0, 1, 2, and 3 h.

Scanning electron microscopy (SEM)

Thirty minutes after zoospore induction, the zoospore suspensions were filtered through sterile Miracloth to remove mycelia and sporangia, fixed in 0.1 mol L^{-1} phosphate buffer (pH 6.8) containing 2 % (w/v) glutaraldehyde overnight at 4 °C, rinsed with the same buffer for 1 h, dehydrated in a series of aqueous solutions of increasing ethanol concentration (30, 50, 70, 80, 90, and 100 %) for 20 min each, critical point dried, and finally mounted on stubs and sputter-coated with gold–palladium. Specimens were observed with a JSM-6360LV (JEOL) scanning electron microscope at 15 kV.

Quantitative real-time PCR analyses

Total RNA was isolated from *Phytophthora parasitica* using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol and three biological replicates were performed. Total RNA was treated with gDNA Eraser and reverse transcribed using PrimeScriptRT reagent Kit with gDNA Eraser (TaKaRa, China) according to the manufacturer's instructions. To generate first-strand cDNA, 0.5 μg of total RNA was reverse transcribed in 10 μL volume. Real-time PCR experiments were carried out using 5 μL of a 1:20 dilution of the first-strand cDNA, using SYBR Premix Ex Taq™ II (TaKaRa, China) according to the manufacturer's instructions. Relative levels of *PnPMA1* transcripts in *P. parasitica* were quantified using the iQ5 real-time PCR detection system (BioRad, USA). The PCR program ran as follows: an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. *PnPMA1* transcripts were detected with the primer pair that amplifies both copies of *PnPMA1*: 5′-ATG AGT GCC ACG

ACT TCT TCC-3′ and 5′-GCA CGC TAC CCG TCA TCT C-3′. *WS041* (GenBank accession number: CF891677), a gene shown to be constitutively expressed throughout the *P. parasitica* life-cycle (Shan *et al.* 2004) was selected as a normalizing reference gene (*WS041* primer pair: 5′-CAC GTA CAC ATG CCC GAG AC-3′ and 5′-TTC CCA TGT AGG CCG AGT ATT C-3′). A melt curve was generated after each qPCR run to validate specificity.

Northern blot

For analyses of small RNAs in *PnPMA1*-silenced lines, total RNA was isolated from *Phytophthora parasitica* using TRIzol reagent (Invitrogen, USA) as described above according to the manufacturer's instructions. RNA was separated in a 15 % polyacrylamide-8 M urea gel, and transferred to Hybond-N^+ by electroblotting at constant current (3 mA cm^{-2}) for 20 min, then the filter was UV cross-linked at 1200× 100 μJ cm^{-2} energy. The 262 bp PCR products, the same region as that in the hairpin construct, were gel purified and labelled with alpha-^{32}P-dCTP using the Random Primer DNA labeling Kit (TaKaRa, China). The filters were hybridized overnight at 42 °C in hybridization solution (0.2 M Na_2HPO_4, pH 7.2, 200 μg mL^{-1} denatured herring sperm DNA, 7 % SDS). Membranes were washed twice for 15 min at room temperature with 2× SSC and 0.5 % SDS before being exposed to the intensifying screens and scanned using an FLA-7000 Phosphorimager (Fuji Photo Film, Japan). U6 small nuclear RNA (snRNA) was used as the loading control. RNA oligomers of 21 and 24-nt RNAs were used as size markers.

Results

Membrane topology analysis of PnPMA1 protein

In the initial study, bioinformatic prediction of transmembrane sequences and comparison of conserved PMA domains suggested that the C-terminal insertion loop of the PnPMA1 protein occurred on the cytoplasmic side of the membrane (Shan *et al.* 2006). However, in the current study, further analysis using a homology modelling method indicates that the C-terminal insertion loop is located in the extracellular space. The crystal structure of the plasma membrane proton pump from *Arabidopsis*, AHA2, has been determined (Pedersen *et al.* 2007) and was used as a template for homology modelling of the three-dimensional structure of PnPMA1. As shown in Fig 1A, the overall structure of PnPMA1 is similar to that of AHA2. As in AHA2, PnPMA1 contains ten transmembrane helices and three cytoplasmic domains including the nucleotide-binding domain (N), the phosphorylation domain (P), and the actuator domain (A). The insertion loop (I) is predicted to be located in the extracellular space (Fig 1A).

We employed the *lacZ* gene fusion-based biochemical approach to experimentally determine the localization of the large C-terminal insertion loop in PnPMA1. The *lacZ* gene fusion approach has been widely used to characterize the topology structure of membrane proteins (Schulein *et al.* 1997; van Geest & Lolkema 2000). Topological information is gained by the fusion of lacZ to putative cytoplasmic and extracellular loops of membrane proteins. Fusion proteins in which lacZ is located in the cytosol exhibit β-Gal activity, whereas fusion proteins in which lacZ is located in the extracellular space do not exhibit β-Gal activity (Li & Greenwald 1996). We generated *PnPMA1::lacZ* chimeric constructs consisting of *PnPMA1* truncated after the predicted transmembrane domain 8 (TM8) (the middle of the insertion loop) and fused to *lacZ* or to a synthetic transmembrane domain followed by *lacZ* (Fire *et al.* 1990; Li & Greenwald 1996). The *PnPMA1::TM::lacZ* yeast transformants exhibited β-Gal activity, whereas the *PnPMA1::lacZ* transformants did not (Fig 1B). Accordingly, we confirmed in yeast that the C-terminal insertion loop in PnPMA1 is localized in the extracellular space.

Variable small interfering RNAs (siRNAs) accumulation in the PnPMA1-silencing transformants

Previous work has demonstrated that gene silencing can be achieved by transforming *Phytophthora parasitica* with sense,

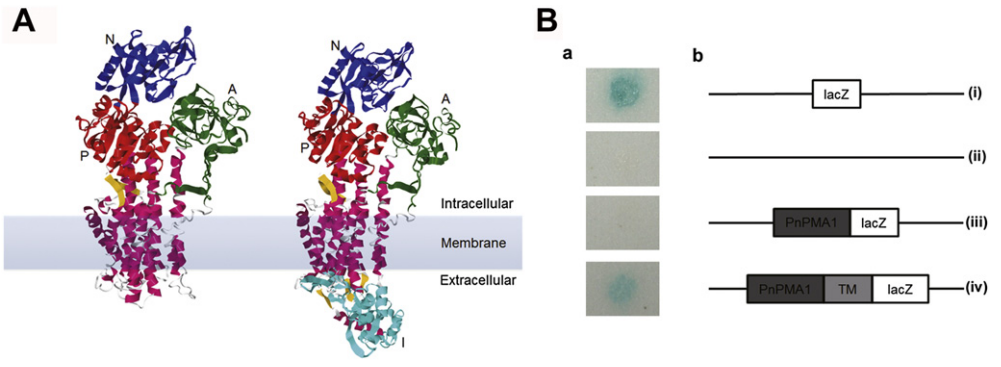

Fig 1 – Three-dimensional structure and topology of *P. parasitica* PnPMA1. (A) The *Arabidopsis* AHA2 structure (left) was used as a template for homology modelling of PnPMA1 (right). The structures represent active forms of the proton pumps without their auto-inhibitory C-terminal domains. Indicated are: ten transmembrane α-helics, purple; the nucleotide-binding domain (N), blue; the phosphorylation domain (P), red; the actuator domain (A), green; and the C-terminal insertion loop (I), cyan. The light blue shaded area represents the plasma membrane. (B) Topological characterization of PnPMA1. (a) β-Gal staining of the transformed yeast colonies; (b) constructs used in the transformations. (i) the *lacZ* construct, the positive control; (ii) the pGADT7 vector, the negative control; (iii) the *PnPMA1::lacZ* fusion construct; (iv) the *PnPMA1::TM::lacZ* construct.

antisense, and hairpin constructs (Gaulin *et al.* 2002; Narayan *et al.* 2010). In a previous study, we showed the accumulation of *PnPMA1* siRNAs in *P. parasitica* cells transformed with *PnPMA1* hairpin constructs, indicating that production of siRNAs is part of the RNA-silencing mechanism in *P. parasitica* (Zhang *et al.* 2011), as it is in *Phytophthora infestans* (Ah-Fong *et al.* 2008). In the previous *P. parasitica* study, the transformants with the greatest *PnPMA1* silencing had *PnPMA1* transcript levels 30–35 % of that in wild-type cells. No phenotypic differences to wild-type cells were observed. To further investigate the biological function of the PnPMA1 protein, in the present study we generated numerous new *PnPMA1*-silenced transformants by transforming *P. parasitica* with antisense and hairpin constructs (Fig 2). In eight cotransformation experiments, a total of 263 primary transformants, including 121 hairpin-derived transformants and 142 antisense-derived transformants, were generated.

In wild-type *P. parasitica*, *PnPMA1* expression is upregulated in zoospores and germinated cysts compared to levels in vegetative hyphae (Shan *et al.* 2004), suggesting that PnPMA1 is likely to play a role in zoospores and germinated cysts. We therefore examined the primary transformants for possible phenotypic changes in zoospores and cysts. Sporulating hyphae were prepared and zoospore release was monitored for each transformant. In parallel, cyst germination was measured for each transformant. The results showed that transformants T77 and T129 were the only lines that showed significant phenotypic changes, as described in the next section.

Quantitative RT-PCR was conducted to measure transcript levels of *PnPMA1* in transformants T77 and T129. The results showed that the accumulation of *PnPMA1* transcripts in the transformants T77 and T129 was 8 % and 20 % of that in the wild-type *P. parasitica*, respectively (Fig 3). In all the other transformants examined, the expression levels of *PnPMA1* were not less than 30 % of that in the wild-type, suggesting that levels of *PnPMA1* expression of 30 % or more relative to wild-type were sufficient to confer a normal phenotype.

We examined levels of siRNAs homologous to the transgene in 120 *P. parasitica* hairpin-derived transformants by Northern blot. This led to the identification of 13 transformants (11 %) that accumulated siRNAs. Of these, the T77 transformant accumulated the highest level of siRNAs (Fig 4). The remaining 12 transformants in which siRNAs accumulation were detected had *PnPMA1* expression levels that were 30–50 % of those in wild-type *P. parasitica*. Moderate level of siRNA accumulation was detected in T15 (Fig 4) corresponded with moderate level of *PnPMA1* downregulation (Fig 3). Taken together, these results show a negative correlation between levels of *PnPMA1* transcripts and siRNA accumulation, giving further evidence that siRNAs are associated with hairpin-mediated gene silencing in *P. parasitica*.

PnPMA1-silencing transformants showed normal colony morphology but produced nonmotile zoospores

The silenced *Phytophthora parasitica* transformants showed normal colony morphology and growth rate when grown on 5 % CA plates compared to the wild-type and control transformants.

Zoospore release experiments showed that the silenced lines released zoospores from sporangia but the zoospores were not motile (Supplementary Video 2), whereas zoospores released in the wild-type strain quickly swam away (Supplementary Video 1) (Fig 5A). All of the zoospores released from T77 were nonmotile. In the T129 transformant line, about 3 % of the zoospores released from sporangia could swim for several minutes, a much shorter time than wild-type zoospores which maintain motility for over 1 h. The remaining 97 % of zoospores from the T129 line were nonmotile. This indicates that even an 80 % reduction in *PnPMA1* transcript levels is not sufficient to completely block PnPMA1 function in generating motile zoospores in *P. parasitica*. Single-zoospore lines from the T77 and T129 transformants displayed the same phenotypic changes, suggesting that the transformants were mitotically stable.

Pathogenicity assays on *Nicotiana benthamiana* leaves by inoculation with 50 μL zoospores (~100 spores/μL) showed that spores of the silenced transformant T77 were unable to initiate infection of this normally-susceptible plant, both when the inoculum was applied to wounded or nonwound tissue. To test whether *PnPMA1* silencing affected plant colonization by *P. parasitica* hyphae, detached *Arabidopsis thaliana* (ecotype Landserg erecta, Ler) leaves were inoculated with mycelial

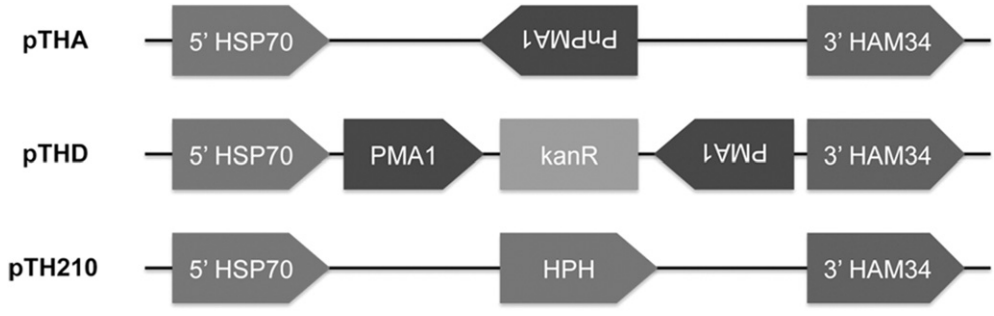

Fig 2 – Constructs used for silencing of *P. parasitica PnPMA1*. Expression cassettes are shown. In all constructs, transgenes are driven by the constitutive *Bremia lactuca* HSP70 promotor and terminator HAM34. pTHA contains full-length *PnPMA1* in the antisense orientation. pTHD contains 262 bp of *PnPMA1* in the sense and antisense orientations separated by the kanamycin resistant gene (kanR). Plasmid TH210 contains the hygromycin-resistance gene (HPH) and was used as the selection marker in the cotransformation of *P. parasitica*.

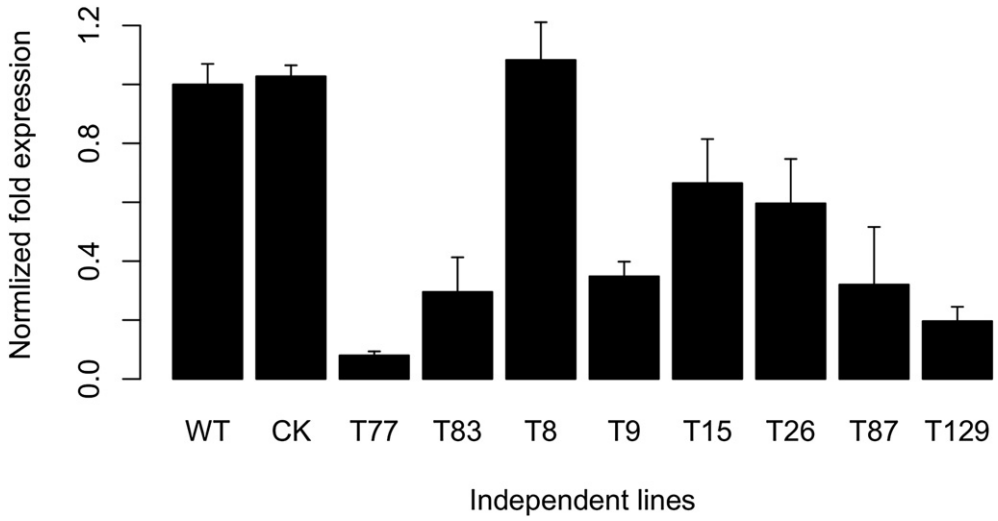

Fig 3 – Quantitative PCR analysis of *PnPMA1* expression in wild-type *P. parasitica* and silencing transformants. WT, wild-type *P. parasitica* strain Pp016; CK, Pp016 transformed with hygromycin-resistance gene; T83, T8, T9, T15, T26, and T87, Pp016 transformants that did not show changes in the zoospore motility phenotype; T77 and T129, Pp016 transformants that showed the nonmotile zoospore phenotype. *PnPMA1* expression levels are relative to that of the constitutively expressed *P. parasitica* gene *WS041*. Bars represent the standard errors of three biological replicates.

plugs. The results showed the *PnPMA1*-silenced transformants were, similar to the wild-type strain, pathogenic and resulted in water-soaked lesions leaves 3 d postinoculation. Microscopic observation showed development of multiple sporangia on the diseased leaves (Fig 5B). Zoospore release by cold treatment showed that, similar to the results in *in vitro* experiments, zoospores released from *in planta* sporangia of the *PnPMA1*-silenced lines (T77 and T129) were not motile and aggregated on the bottom of the Petri dishes (Fig 5B).

PnPMA1-*deficient transformants produced large aberrant zoospores*

Both the *PnPMA1*-silenced and control *Phytophthora parasitica* transformants produced similar numbers of sporangia that were capable of releasing zoospores. However, in addition to zoospores having a normal size and shape, sporangia of the *PnPMA1*-silenced lines released larger than normal, aberrantly-shaped zoospores (Fig 6A). These large zoospores appeared to consist of two, three or four adjoined zoospores and to have arisen through incomplete cleavage of the sporangia (Supplementary Video 3). They contained multiple water expulsion vacuoles (WEVs) which continued to pulse (Supplementary Video 3), indicating that PnPMA1 is not required for the function of the WEV. Up to 30 % of zoospores released from sporangia in the T77 transformant were aberrant and about 6 % in T129. Similar large aberrant zoospores were rarely found in the wild-type and control *P. parasitica* lines (<0.2 %).

To examine possible mechanisms that account for the loss of zoospore motility, zoospore suspensions were fixed and subjected to microscopic characterization. SEM showed that the *PnPMA1*-silenced transformants produced nonflagellate zoospores (Fig 7). Two flagella emerged from the ventral groove in wild-type zoospores, whereas zoospores of the silenced lines did not develop flagella although the ventral grooves were still formed (Fig 7). About 3 % of zoospores produced by *PnPMA1*-silenced transformant T129 developed two flagella, which was consistent with the results described above that about 3 % of zoospores of this transformant were

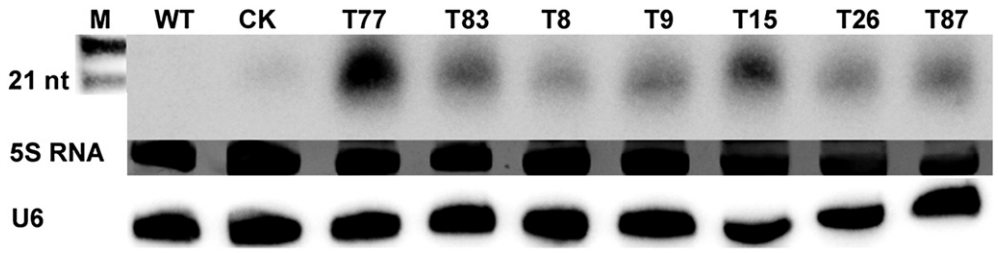

Fig 4 – Detection of siRNAs in *P. parasitica* transformants. The top panel shows hybridization of the *PnPMA1* probe to the hairpin-derived *P. parasitica* transformants. Each lane contains 15 μg of total RNA isolated from mycelia. The middle panel displays ethidium bromide-stained 5S RNA as a loading control. The bottom panel shows hybridization of a probe complementary to U6 snRNA as the loading control. The same blot was used by stripping and rehybridization.

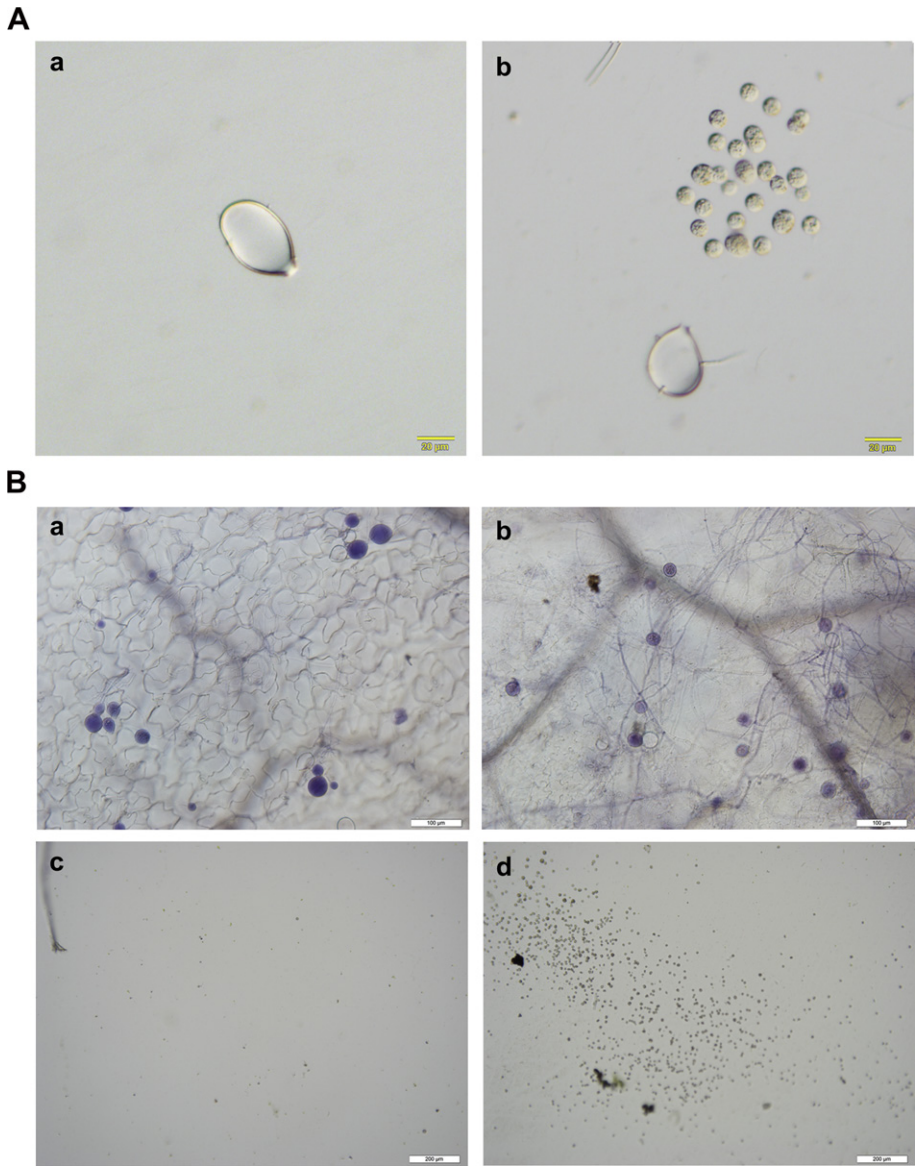

Fig 5 – *PnPMA1*-silenced *P. parasitica* transformants produced nonmotile zoospores. (A) Zoospore release from *P. parasitica* sporangia grown *in vitro*. (a) Empty sporangium of the wild-type strain Pp016 after zoospore release. The zoospores quickly swam away. (b) Zoospores of the *PnPMA1*-silenced transformants remained near the sporangium after their release because they were nonmotile (Scale bar: 20 μm). (B) Zoospores released from sporangia that have formed on the surface of infected plant tissues. (a) Abundant sporangia developed on the surface of the *Arabidopsis* (ecotype Landserg erecta) leaf inoculated with the wild-type strain Pp016. (b) Sporangia developed on the plant leaf inoculated with a *PnPMA1*-silenced transformant. (c) Zoospores released from the Pp016-infected plant tissue (a) quickly swam away and few zoospores could be observed on the bottom of the Petri dish. (d) Zoospores released from the silenced transformant-infected plant tissue (b) were nonmotile and could not swim away. The zoospores aggregated on the bottom of Petri dish (Scale bar: 200 μm).

able to swim for a short period. The results indicated that the loss of zoospore motility in the *PnPMA1*-silenced transformants resulted from the inhibition of flagella development.

Zoospores released from sporangia in the *PnPMA1*-silenced transformants T77 and T129 quickly encysted (Fig 6B), whereas zoospores from the wild-type strain were able to swim for several hours. The large aberrant zoospores were also able to encyst (Fig 6B). However, about 20 % of cysts ruptured when zoospores were encysted by vigorous shaking, which was rarely found in the wild-type (data not shown). In addition, the germination rate of cysts from the T77 *PnPMA1*-silenced line was dramatically reduced, from 97 % in the wild-type strain to about 15 % in T77 (Fig 6C), indicating that PnPMA1 was involved in cyst germination. About 85 % of the encysted zoospores were unable to germinate when they were incubated in 2 % carrot juice for 3 h. However, most encysted zoospores were able to germinate after extended incubation of 16 h in 2 % carrot juice.

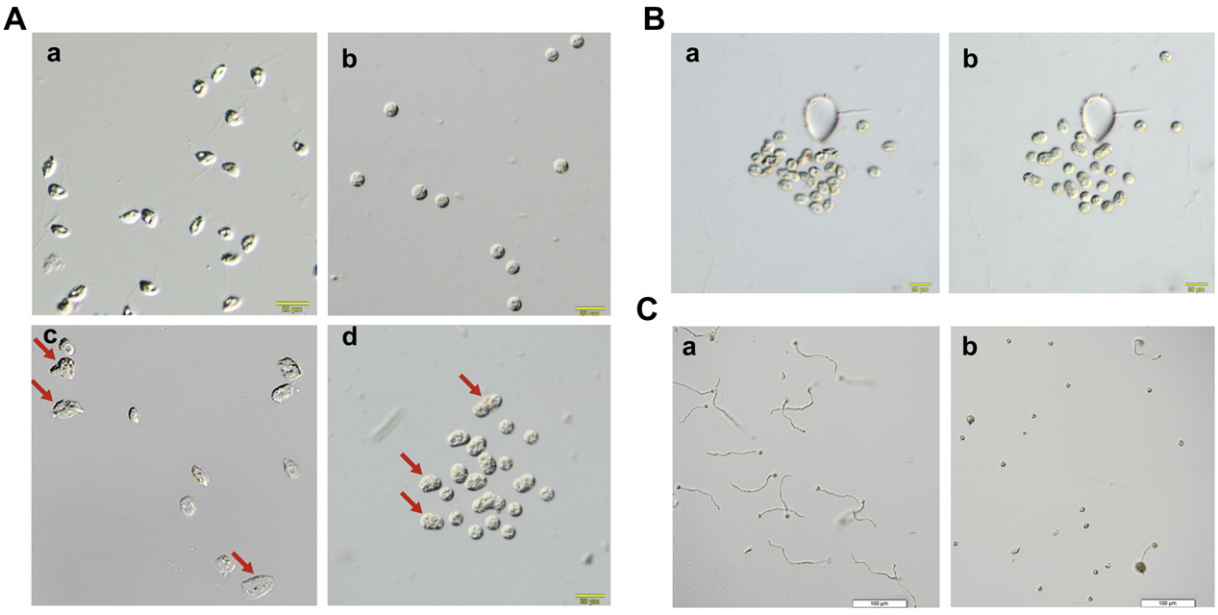

Fig 6 – Phenotypic characterization of the *PnPMA1*-silenced *P. parasitica* transformants. (A) The *PnPMA1*-silenced transformants produced large aberrant zoospores. (a) Zoospores of the wild-type strain Pp016 showed normal zoospore morphology. (b) Cysts of Pp016. (c) Large aberrant zoospores produced by the *PnPMA1*-silenced transformant T77. (d) Cysts of the *PnPMA1*-silenced transformant T77. Scale bars: 20 μm. The red arrows indicate the aberrant zoospores. (B) Rapid zoospore encystment in the *PnPMA1*-silenced transformants. Zoospores released from a *PnPMA1*-silenced transformant (a) and quickly (less than 30 min) developed into cysts (b). Scale bars represent 20 μm. (C) Cyst germination was affected in the *PnPMA1*-silenced transformants. Zoospores were encysted and the resulting cysts were incubated in 2 % carrot juice for 3 h. (a) Germinated cysts of Pp016. (b) Germinated cysts of *PnPMA1*-silenced transformant. Scale bars: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

Discussion

We report here that PnPMA1, an atypical plasma membrane H^+-ATPase, is involved in zoospore development in *Phytophthora parasitica*. Our results demonstrate that the C-terminal insertion domain is located in the extracellular space. PMAs are conserved plasma membrane ATPases in fungi and plants. The typical PMA has ten transmembrane domains. The cytoplasmic regions of the protein consist of three well-characterized conserved domains designated as P, N, and A that are critical to phosphorylation, ATP binding, and dephosphorylation, respectively. Crystal structures show that the overall structure of P-type ATPases is conserved among different subfamilies, although they share low sequence similarity (Pedersen *et al.* 2007). Bioinformatic analysis indicates that PnPMA1 contains ten transmembrane spanning helices with both N- and C-termini located in the cytoplasm. Three-dimensional structure modelling and experimental topology analysis of PnPMA1 demonstrates that the C-terminal insertion domain of PnPMA1 is a large extracellular loop which is absent from typical PMAs. The role of the large extracellular domain in the PnPMA1 protein remains to be elucidated. It might function in the perception of extracellular signals or in the connection between the cell wall and plasma membrane.

Reduction of *PnPMA1* transcripts to 20 % or less of their normal levels in *P. parasitica* led to the production of zoospores that lacked flagella and encysted more rapidly, and to inhibition of cyst germination. Zoospore motility is important for *Phytophthora* pathogens to target suitable infection sites and is thus important for pathogenicity (Latijnhouwers *et al.* 2004; Hua *et al.* 2008). In *P. parasitica*, production of zoospores that do not have flagella also occurs when expression of the dynein light chain 1 gene is silenced (Narayan *et al.* 2010). In both cases, zoospores were still released from sporangia, indicating that this process does not depend on active zoospore motility.

Plasma membrane H^+-ATPases transport protons out of the cell, and the resultant proton motive force plays important roles in the regulation of diverse biological processes (Palmgren 2001; Elmore & Coaker 2011). PMAs play an important role in cell pH homeostasis in plants and fungi (Portillo 2000). In *Phytophthora cinnamomi*, initiation of cytoplasmic cleavage in zoosporangia requires a rise in pH and zoosporogenesis is blocked when the intracellular pH of the sporangium is held constant at pH 7.0 (Suzaki *et al.* 1996). Intracellular pH has also been shown to participate in the initiation of cytokinesis in other organisms. In *Urechis* eggs, a rise in intracellular pH is required after fertilization for germinal vesicle breakdown (Gould & Stephano 1993). Production of the large aberrant zoospores in the *PnPMA1*-silenced *P. parasitica* transformants may be due to lack of pH fluctuation, and a consequent interference in cytoplasmic cleavage.

Silencing of *PnPMA1* in *P. parasitica* resulted in production of nonflagellate zoospores but at this stage the molecular mechanisms underlying this process are unclear. It has been

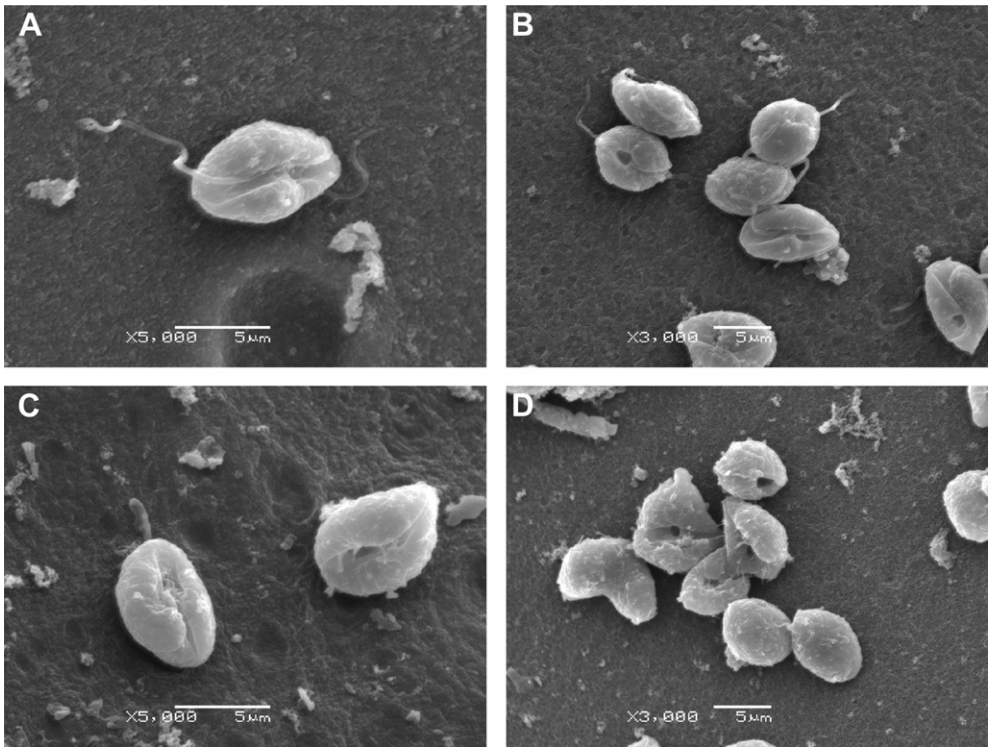

Fig 7 – *PnPMA1*-silenced *P. parasitica* transformants produced nonflagellate zoospores. (A and B) Zoospores of the wild-type strain Pp016 developed two flagella from the ventral groove. (C and D) Zoospores of the *PnPMA1*-silenced transformants lacked flagella although the ventral groove was still formed. Scale bars: 5 μm.

shown in *Chlamydomonas* that deflagellation is initiated by pH shock (Piao *et al.* 2009). High levels of expression of *PnPMA1* in *P. parasitica* zoospores may indicate a need for high levels of proton export from the zoospores. Disruption of *PnPMA1* might block this process and as a consequence may lead to a pH shock similar to that which causes deflagellation in *Chlamydomonas*.

Zoospores of the *PnPMA1*-silenced *P. parasitica* transformants encysted rapidly after release from sporangia, and this may be associated with disruption of ion homeostasis. It was shown that potassium homeostasis affects zoospore behaviour and encystment in Oomycetes (Appiah *et al.* 2005). High external concentrations of potassium salts reduced swimming speeds, and pharmacological inhibition of K^+ translocation resulted in reduced swimming speeds and changes in the swimming patterns of *Phytophthora* species, indicating that potassium ions play an important role in regulating zoospore behaviour (Appiah *et al.* 2005). Inhibition of the plasma membrane H^+-ATPase by DCCD caused a reversal of K^+ flux in zoospores of *Phytophthora* (Holker *et al.* 1993), and the effect of DCCD on zoospores is similar to that of agitation regarding K^+/H^+ exchange. The rapid encystment of zoospores may be related to transient inhibition of the plasma membrane H^+-ATPase (Holker *et al.* 1993). Changes in intracellular Ca^{2+} concentration are often linked to changes in intracellular pH (Nishiguchi *et al.* 1997). Ca^{2+}, a secondary messenger molecule, plays important roles in many physiological processes. It was reported that transmembrane Ca^{2+} fluxes are associated with cyst germination in *P. parasitica* (Warburton & Deacon 1998).

Finally, silencing of *PnPMA1* in *P. parasitica* may lead to abnormal zoospore and cyst development by interrupting cell wall–plasma membrane interaction potentially mediated by the extracellular C-terminal loop. Proteins mediating interactions between plant cell walls and plasma membranes are considered to participate in a monitoring system required for the perception and transduction of environmental and developmental signals (Gouget *et al.* 2006). Most of the zoospores of the *PnPMA1*-silenced lines can develop into cysts naturally after release, but when zoospores were encysted by vigorous shaking, a proportion of cysts ruptured and this was rarely found in the wild-type. It is possible that silencing of the *PnPMA1* gene in *P. parasitica* affected the rapid response of zoospores to environmental stimuli or disrupted the intimate interaction between cell wall and plasma membrane in cysts.

In conclusion, we show that the *PnPMA1* gene of *P. parasitica* is involved in zoospore development, and we postulate that *PnPMA1* controls biological processes by regulation of ion homeostasis and cell wall–plasma membrane interaction. However, further studies are needed to confirm this hypothesis.

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