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Identification and characterization of PiORP1, a *Petunia* oxysterol-binding-protein related protein involved in receptor-kinase mediated signaling in pollen, and analysis of the *ORP* gene family in *Arabidopsis*

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Abstract Oxysterol-binding proteins (OSBPs) and oxysterol-binding-protein related proteins (ORPs) are encoded by most eukaryotic genomes examined to date; however, they have not yet been characterized in plants. Here we report the identification and characterization of PiORP1, an ORP of *Petunia inflata* that interacts with the cytoplasmic kinase domain of a receptor-like kinase, named PRK1, of *P. inflata*. PiORP1 is phosphorylated by PRK1 *in vitro* and therefore may be involved in PRK1 signaling during pollen development and growth. RNA gel blot analysis showed that *PiORP1* and *PRK1* had very similar expression patterns in developing pollen, mature pollen and pollen tubes. GFP fusion proteins of PiORP1 localized in the plasma membrane of pollen tubes at distinct foci and its PH domain alone was sufficient to mediate this localization. The sequence for the oxysterol-binding domain of PiORP1 was used to search the genome of *Arabidopsis*; 12 *ORPs* were identified and phylogenetic analysis revealed that they fell into two distinct clades, consistent with the ORPs of other eukaryotes. RT-PCR analysis showed that all 12 Arabidopsis *ORPs* were expressed; 10 were expressed in most of the tissues examined under normal growth conditions, but only three were expressed in pollen.

Keywords Oxysterol-binding-protein related proteins · Pollen · Pollen tube · Receptor-like kinase

Abbreviations

ORP	oxysterol-binding-protein related protein
OSBP	oxysterol-binding protein
RT-PCR	reverse transcriptase polymerase chain reaction

GenBank accession number for PiORP1: DQ241801

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Introduction

Signal transduction pathways mediated by receptor kinases are essential for many aspects of growth and development in higher eukaryotes (Morris and Walker, 2003). Plants possess a large number of receptor-like kinases (e.g., ~417 predicted in *Arabidopsis*; see Shiu and Bleecker, 2001), but the functions of only a small number have been characterized. Among them, CLAVATA1 of *Arabidopsis* controls shoot and floral meristem size (Clark et al. 1997); BRI1 of *Arabidopsis* mediates a brassinosteroid signal transduction pathway (Li and Chory, 1997); LePRK1 and LePRK2 of tomato are involved in polarized pollen tube growth (Kaothien et al. 2005). We previously identified a predominantly pollen-expressed receptor-like kinase of *Petunia inflata*,

named PRK1 (Mu et al. 1994), essential for pollen (Lee et al. 1996) and embryo sac development (Lee et al. 1997). PRK1 is likely to have an additional role in pollen tube growth in the style, because its message is abundant in both mature pollen and in *in vitro* germinated pollen tubes. PRK1 was previously reported to interact with and phosphorylate KIP1, a cytoplasmic protein with sequence homology to myosin-like and microtubule-binding proteins (Skirpan et al. 2001). To further understand the role of PRK1 in pollen development and pollen tube growth, the yeast two-hybrid system was used to identify additional PRK1 interacting proteins. A protein named P*etunia* i*nflata* Oxysterol-binding-protein-Related Protein 1 (PiORP1), was identified in a yeast two-hybrid library screen as a PRK1-interacting protein.

OSBPs and their related proteins, ORPs, have been identified and studied in a variety of organisms, including humans (Levanon et al. 1990), *Drosophila* (Alphey et al. 1998) and fungi (Jiang et al. 1994; Schmalix and Babdlow 1994; Fang et al. 1996; Daum et al. 1999; Hull and Johnson, 1999). Genes encoding OSBPs and ORPs have also been found in practically all eukaryotic organisms whose genomes have been extensively sequenced. For example, 7 *ORP* genes (named *OSH-1* through *OSH-7*) have been identified in yeast (Beh et al. 2001), and 12 *OSBP/ORP* genes have been identified in both human (Jaworski et al. 2001; Lehto et al. 2001) and mouse (Anniss et al. 2002). Although no plant *ORP* genes have been characterized to date, sequence analysis of the *Arabidopsis* genome has revealed 12 putative *ORP* genes (Jaworski et al. 2001). OSBPs/ORPs are believed to be involved in membrane trafficking (Fang et al. 1996), tumor metastasis (Fournier et al. 1999) and cell cycle progression (Alphey et al. 1998), whereas the oxysterols themselves have been implicated in regulating processes as diverse as Ca^{2+} uptake and apoptosis, to transcriptional activity and cellular differentiation (Jaworski et al. 2001).

Here, we report the isolation and characterization of PiORP1 of *P. inflata*. PiORP1 was phosphorylated by PRK1 *in vitro*, was found to localize to distinct foci or islands on pollen tube plasma membrane, and its PH domain alone was found to be sufficient to mediate this localization. In addition, we report the phylogenetic and expression analyses of the *ORP* gene family in *Arabidopsis*.

Materials and methods

Yeast two-hybrid and cDNA library screens

The yeast two-hybrid library and the cDNA library were constructed using poly(A)$^+$ RNA of mature pollen collected from open flowers as previously reported (Skirpan et al. 2001). *pGBT9/PRK1*$^{351–720}$ was generated by PCR using the forward primer PRK1051RI-F (5′-CGGAATTCCG TCGCCGTAGCCATAGCA-3′) and the reverse primer PRK2163SAL-R (5′-AAGTCGACTCAAACTCCAGCAT CATGCATTTG-3′). The product was cloned into pGBT9 at the *Eco*RI and *Sal*I sites. The yeast two-hybrid library was screened in yeast strain PJ69-4a (James et al. 1996) according to Clontech's protocol for the Matchmaker Two-Hybrid System. Colonies positive for growth on SD/leu$^-$/trp$^-$/his$^-$ plates were assayed for growth on SD/leu$^-$/trp$^-$/ade$^-$ plates. The cDNA insert of the clone KDI-47 was labeled with ^{32}P using the RTS RadPrime DNA Labeling Kit (Life Technologies); screening of the pollen cDNA library followed the procedure described by Skirpan et al. (2001).

DNA sequencing and sequence analysis

Cycle sequencing reactions were performed as described in Skirpan et al. (2001). Database searches were conducted with the BLAST program at the National Center for Biotechnology Information website (http://www.ncbi.n-lm.nih.gov). The *Arabidopsis* ORPs were identified by searching TAIR (http://www.arabidopsis.org) with the OSBP domain of PiORP1. Sequences were aligned using Clustal W and Boxshade (http://www2.igh.cnrs.fr/bin/clustalw-guess.cgi) and analyzed using SMART (http://www.smart.embl-heidelberg.de). Phylogenetic analysis was performed with MEGA2 (Kumar et al. 2001) using the neighbor-joining algorithm and 500 bootstrap replications.

RNA gel blot analysis

RNA blot analysis was performed as described by Skirpan et al. (2001), using the *PiORP1* cDNA from clone KDI-47 as a probe.

Recombinant protein expression

The N-terminal 6× His-tagged PiORP1$^{2–290}$ construct was generated by digesting cDNA clone KDI-47 with *Sal*I and ligating the resulting cDNA insert at the *Sal*I site of pET28a. Protein expression in *E. coli* strain BL21 DS3 (plysS) was induced at OD_{600}=0.6 with 0.5 mM IPTG overnight at 22°C. The pellet was lysed with 5 ml of B-PER reagent (Pierce) plus 1 mM PMSF, and then sonicated. The volume of the lysate was brought to 12 ml with Talon sonication buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10% [v/v] glycerol) and incubated with 0.5 ml Talon resin (Clontech) for 2 h at 4°C. The resin was washed twice with 10 ml Talon sonication buffer. The bound proteins were eluted with 200 mM imidazole, 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10% (v/v) glycerol, and stored at −20°C.

The GST fusion constructs, *GST:PRK1-K* and *GST:PRK1-K462R*, and their expression in yeast were described in Skirpan et al. (2001), except that the yeast pellets were washed in 1.2 M sorbitol and digested with zymolase (1.2 M sorbitol, 50 mM KH_2PO_4, pH 7.4, 14 mM β-mercaptoethanol, 2 mg/ml Zymolase 20T) for 1 h at 37°C with gentle shaking. To purify the fusion proteins, yeast protoplasts were washed with 1.2 M sorbitol and then lysed in glass bead disruption buffer (20 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM EDTA, 5% [v/v] glycerol, 1 mM DTT, 0.3 mM ammonium sulfate, 1 mM PMSF) by vortexing with 0.5 g acid washed glass beads. The supernatant was cleared by centrifugation, and incubated with Glutathione Sepharose 4 Fast Flow resin (GE Healthcare). The resin was washed three times with GST wash buffer (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 10 mM EDTA, 1 mM DTT, 0.4% [w/v] Triton X-100) and the bound proteins were eluted with 50 mM Tris–HCl (pH 8.0) and 20 mM glutathione.

In vitro binding assay

GST, GST:PRK1-K, and GST:PRK1-K462R were bound to Glutathione Sepharose 4 Fast Flow resin and washed to remove contaminating yeast proteins. Three hundred microliters of binding buffer (25 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.01% [v/v] NP40) were added to each tube containing the resin along with 40 μl purified 6× His-tagged $PiORP1^{2-290}$. The mixture was incubated with rotation for 1 h at room temperature. The resin was then washed three times with 1 ml binding buffer and the bound proteins were eluted with 1× SDS-PAGE buffer (50 mM Tris–HCl, pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 1% [v/v] β-mercaptoethanol, 0.1% [w/v] bromophenol blue) at 100°C for 5 min. The proteins were separated on a 10% (w/v) SDS-polyacrylamide gel and transferred to a PVDF membrane (Millipore). Protein blotting was performed as described in McCubbin et al. (1997). Primary antibody, anti-His (Novagen), was added at 1:5,000 dilution for 1.5 h. Secondary antibody, AP conjugated goat anti-mouse (Life Technologies), was added at 1:5,000 dilution for 1.5 h. The signal was detected using a Bio-Rad AP conjugate kit.

β-galactosidase and phosphorylation assays

The β-galactosidase and phosphorylation assays were performed as described by Skirpan et al. (2001).

Construction of GFP fusion constructs of PiORP1 and biolistic bombardment of pollen

For the *PiORP1:GFP* construct, the complete coding sequence for PiORP1 was amplified from the full-length cDNA clone by standard PCR using the forward primer 5′-CCATGGCGGCGGCACCAAAGCCG-3′ and the reverse primer 5′-GGCGGCCGCAGTCTCTTCAGATATTTCAC C-3′. The resulting product was cloned into the *Nco*I and *Not*I sites of a previously constructed cassette plasmid containing the *LAT52* promoter of tomato (Twell et al. 1990) and a *NOS* terminator in pGBT9. A *Not*I GFP fragment containing an $(Ala)_3(Gly)_{10}$ linker sequence was ligated to the 3′ end of *PiORP1* at the *Not*I site. $GFP:PiORP1^{1-201}$ was amplified by using the forward primer 5′-CCATGGCGGCGGCACCAAAGCCG-3′ and the reverse primer 5′-GCGGCCGCTCAAAACATATTTT TTGCCGCAAT-3′; $GFP:PiORP1^{363-775}$ was amplified by using the forward primer 5′-CCATGGAGTTCCATCCTT CTGAAGATGGT-3′ and the reverse primer 5′-GCGGCC GCTCAAGTCTCTTCAGATATTTCACC-3′. Each of the PCR products was cloned into the *Nco*I and *Not*I sites of the same cassette plasmid described above, followed by the cloning of a *Nco*I GFP fragment containing a $(Gly)_{10}$ linker sequence.

Pollen grains collected from 10 flowers of greenhouse grown *P. inflata* plants were suspended in 200 μl of germination media (0.07% [w/v] $Ca(NO_3)_2$, 0.02% [w/v] $Mg(SO_4)_2{\cdot}7\ H_2O$, 0.01% [w/v] KNO, 0.01% [w/v] H_3BO_3, 0.2% [w/v] sucrose, 15% [w/v] PEG 4000, 20 mM MES (pH 6.0)), and bombarded with DNA-coated gold particles (1.0 μm in diameter; 2 μg of plasmid DNA coated per 0.5 mg of gold particles) according to Fu et al. (2001). The bombarded pollen grains were incubated at room temperature for 30 min, washed into culture tubes with 1 ml germination media and shaken at 200 rpm at 30°C for 5 h before observation. GFP fluorescence was observed using a Zeiss LSM 510 Meta, equipped with 488 nm Argon laser and 510–560 nm emission filter. 3D projections were compiled from images taken every 0.5 μm through the depth of the pollen tube using the LSM 510 3D reconstruction software.

RT-PCR

Total RNA was extracted from various tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out using 5 μg of RNA isolated from each tissue and Superscript II (Stratagene) according to the manufacture's protocol. The resulting cDNA was diluted 20-fold, and 0.5 μl was used in PCR. The primers used in the amplification of the 12 *Arabidopsis ORP* genes were: At2g31030-193-F (5′-TACGAAGGCAGACTTAGCGAA-3′), At2g31030-923-R (5′-ATCAGCATAGTCCCATAATGA-3′), At2g31020-884-F (5′-GGCAACGGAGACTCAGAAGAAAC-3′), At2g31020-1724-R (5′-ATCTTCATTGTCCCGTAGTGA-3′),

At4g08180-2090-F (5′-GCGTATTGAGGGTAGTGCAG-3′), At4g08180-2667-R (5′-ATCGGAATCGATGTGGCC GA-3′), At1g13170-2239-F (5′-GACAACAGAACCGGG GAGAA-3′), At1g13170-2729-R (5′-GATGAGAGCGAG TTACGCTGTTG-3′), At4g22540-2007-F (5′-GTCGAAA GTCAGTGATCCTGCATC-3′), At4g22540-2428-R (5′-AGCTTTCTAAGCGGCGTCCGCT-3′), At4g12460-1669-F (5′-GTTGCTGGTGATGGAATCAA-3′), At4g12460-2081-R (5′-GCACAATCTGCAACCTCTTCG-3′), At5g59420-811-F (5′-GGATTTGGTGCCGCCTCTCACTA-3′), At5g59420-1470-R (5′-TCCGTTTCGTTGGAGGCAC-TAC-3′), At5g02100-941-F (5′-GGACGGTTACGTGT ATAATTCAGC-3′), At5g02100-1494-R (5′-TGGCACC AAAGGATTAAGCAGAG-3′), At3g09300-1028-F (5′-GGATGGATATGTCTATAACGCCTC-3′), At3g09300-1675-R (5′-TGGGAAAGCTAGCAGATGTAGAA-3′), At5g57240-773-F (5′-AGGACTCGGAGCCGAATTG-CAG-3′), At5g57240-1326-R (5′-A CACTTCTCATGAG TTCTTACTTCT-3′), At4g25860-862-F (5′-GTGGGCT CGAGGTTATCTA-3′), At4g25860-1263-R (5′-TTGGTTTATGTTACATGGAATTG-3′), At4g25850-1041-F (5′-CTGGTGAGCTCGAGGTCATTTAC-3′), At4 g25850-1386-R (5′-CGGTGAATGATTCGTCCTTGC-3′). The primers used for the controls were: PGA4 F (5′-GCCCCAACACCGATGGTAT-3′), PGA4 R (5′-TGCCT ACATAGTCCAAGTTCA-3′) RBCS-1B F (5′-CTCTGC CGCTGTGGTTACCT-3′), RBCS-1B R (5′-GAC-TTGACGGGTGTTGTCGAAT-3′), actin 2/11 F (5′-GATTTGGCATCACACTTTCTACAATGA-3′), actin 2/11 R (5′-CCTTCCTGATATCCACATCACA-3′). The PCRs were carried out with initial annealing at 94°C for 1 min, followed by 30 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 55°C or 61°C for 30 s, and extension at 72°C for 2 min. After the final cycle, the extension continued at 72°C for an additional 10 min. The annealing temperature for the primers for At2g31030, At2g31020, At4g08180, At5g57240 and At4g25860 was 55°C, and the annealing temperature for all the other primers was 61°C.

Results

PiORP1 interacts with the kinase domain of PRK1

To identify downstream components of the PRK1-mediated signaling pathways in pollen, PRK1$^{351–720}$, encompassing the entire cytoplasmic domain of PRK1, was used as bait in a yeast two-hybrid screen of mature pollen cDNAs. Approximately 5×10^6 yeast transformants were screened, and five different classes of cDNA clones encoding potential PRK1-interacting proteins were isolated, including the previously reported KIP1 (Skirpan et al. 2001). One class contained a single clone, named KDI-47, with a cDNA insert of 864 bp. The longest reading frame of this cDNA (encoding 288 amino acids) was completely in phase with the coding sequence for the activation domain of GAL4. *pGBT9/PRK1*$^{351–720}$ and *pGAD424/KDI-47* were re-transformed into yeast strains PJ69-4A and SFY526, and the transformants were assayed for interaction, as well as tested against control plasmids. In both yeast strains, PJ69-4A and SFY526, the activation of the reporter genes was specific to the *pGBT9/PRK1*$^{351-720}$ and *pGAD424/KDI-47* constructs. All control interactions were negative suggesting that the gene encoded a *bona fide* interacting partner of PRK1.

The *KDI-47* clone was used to screen a cDNA library to obtain a full-length clone. Sequence analysis using BLAST revealed that the full-length clone showed homology to oxysterol-binding proteins and was therefore named P. *inflata* Oxysterol-binding-protein Related Protein 1 (PiORP1). The full-length PiORP1 cDNA encoded 775 amino acids and contained a predicted pleckstrin homology (PH) domain at amino acid residues 73–198, a predicted coiled-coil region at amino acid residues 253–286, and a predicted oxysterol-binding domain at amino acid residues 365–758. The region of PiORP1 identified in the yeast two-hybrid library screen as an interacting protein of PRK1 (KDI-47) spanned amino acids 2–290 of the full-length protein, including the entire PH domain. BLAST searches of the non-redundant protein database showed that PiORP1 was most similar to the putative proteins encoded by At4g08180 and At2g31020 of *Arabidopsis*. At the amino acid sequence level, PiORP1 is 61.3% identical to At4g08180 and 61% identical to At2g31020 over the entire length of the protein, 73.1% identical to At4g08180 and 73.6% identical to At2g31020 in the PH domain, and 73.9% identical to At4g08180 and 68.9% identical to At2g31020 in the oxysterol-binding domain.

PiORP1 interacts with the kinase domain of PRK1 *in vitro*

To further substantiate the results of the yeast two-hybrid screens and to rule out the possibility that the observed interaction was mediated by yeast protein(s), an *in vitro* binding assay was used to ascertain whether PRK1 and PiORP1 can interact directly. GST, GST:PRK1-K and GST:PRK1-K462R were recombinantly expressed and bound to GST resin. The resin beads containing bound GST or the GST fusion proteins were then incubated with the 6× His:PiORP1$^{2–290}$ protein, and the bound proteins were eluted and separated by SDS-PAGE (Fig. 1A). As shown in Fig. 1B, an anti-His antibody was used to detect

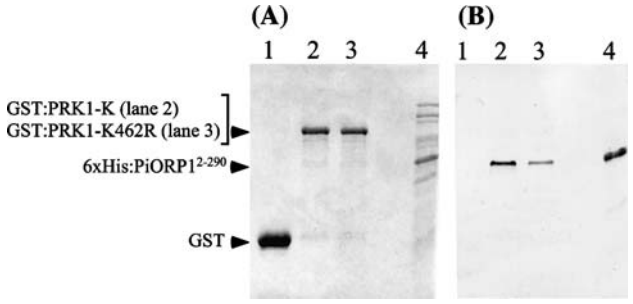


Fig. 1 *In vitro* binding assay of the interaction between PRK1 and PiORP1. 6× His:PiORP1$^{2–290}$ was incubated with purified GST (lane 1), GST:PRK1-K (lane 2) and GST:PRK1-K462R (lane 3) bound to GST resin. Lane 4 contains ~25% of the input 6× His:PiORP1$^{2–290}$ protein. (**A**) Coomassie blue staining of a duplicate gel shown in (**B**). (**B**) Protein gel-blot to detect 6× His:PiORP1$^{2–290}$ using an anti-His tag antibody

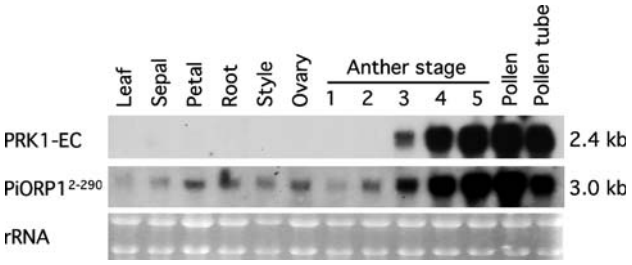


Fig. 2 RNA gel-blot analysis of *PiORP1* and *PRK1* expression. Each lane contains 20 μg of total RNA isolated from the indicated tissue. The blot was first probed with *KDI-47* cDNA, encoding PiORP1$^{2–290}$. After autoradiography, the blot was stripped of the probe and re-hybridized with cDNA encoding the extracellular domain of PRK1. Anther stages are as described in Skirpan et al. (2001). The sizes of the transcripts are indicated. The rRNAs stained with ethidium bromide are shown as controls for RNA loading

the presence of 6× His:PiORP1$^{2–290}$ in the lanes containing GST:PRK1-K and GST:PRK1-K462R (lanes 2 and 3) but not GST alone (lane 1). Due to a conserved substitution, PRK1-K462R has reduced, but not abolished, kinase activity (Skirpan et al. 2001). The intensity of the 6×His:PiORP1$^{2–290}$ band detected in the GST:PRK1-K lane was approximately two-fold that in the GST:PRK1-K462R lane, suggesting a preference for GST:PRK1-K binding. However, when the interactions of PiORP1$^{2–290}$ with PRK1-K and PRK1-K462R were evaluated quantitatively in the yeast two-hybrid assay using ONPG as substrate, there were no significant differences in the strength of the interactions as determined by galactosidase activity (in Miller units) (results not shown). Thus, PiORP1$^{2–290}$ interacts directly with the kinase domain of PRK1 *in vitro*.

PiORP1 is predominantly expressed in pollen and shows a similar expression profile as *PRK1*

A gel blot containing RNA isolated from various tissues, anthers at different developmental stages, as well as pollen and *in vitro* germinated pollen tubes was probed with KDI-47 cDNA (encoding PiORP1$^{2–290}$). To increase the specificity of hybridization, the coding region for the oxysterol-binding domain was not included in the probe as, by analogy to other organisms, we would predict a family of ORPs with this domain in *Petunia*. RNA transcript of the expected size, ~3 kb, was detected predominantly in anthers that contained developing pollen, and in mature pollen and pollen tubes (Fig. 2). The transcript reached the maximum level in mature dehydrated pollen collected from dehisced anthers and then declined slightly in the *in vitro* germinated pollen tubes. To compare the expression patterns of *PiORP1* and *PRK1*, the RNA gel blot was re-hybridized with cDNA for PRK1-EC, the extracellular domain of PRK1 (Fig. 2). *PiORP1* and *PRK1* showed very similar expression patterns in developing pollen, mature pollen and pollen tubes. The *PiORP1* transcript was present at low levels in leaf, sepal, petal, root, style and ovary. In contrast, the *PRK1* transcript was not detectable in any of these other tissues. However, we cannot rule out the possibility that the weak signals detected with the *PiORP1*$^{2–290}$ probe were due to cross hybridization with transcripts of genes encoding other PH-domain containing proteins.

PRK1 phosphorylates PiORP1 *in vitro*

Since PiORP1$^{2–290}$ interacted with the kinase domain of PRKI in the yeast two-hybrid and *in vitro* binding assays, we wished to determine whether PRK1 was capable of phosphorylating PiORP1$^{2–290}$. Purified GST:PRK1-K, GST:PRK1-K462R, and 6× His:PiORP1$^{2–290}$ were used in the phosphorylation assay shown in Fig. 3. Consistent with our previous findings (Mu et al. 1994; Skirpan et al. 2001), GST:PRK1-K auto-phosphorylated and GST:PRK1-K462R also auto-phosphorylated, but to a lesser degree. 6× His:PiORP1$^{2–290}$ was phosphorylated in the presence of GST:PRK1-K, and, to a lesser degree, in the presence of GST:PRK1-K462R. No phosphorylation of 6× His:-PiORP1$^{2–290}$ was detected when the assay was performed with 6× His:PiORP1$^{2–290}$ alone, or with GST and 6× His:PiORP1$^{2–290}$. Moreover, the GST control was completely free of background phosphorylation, demonstrating that the phosphorylation of 6× His:PiORP1$^{2–290}$ was not due to any potential contamination by yeast or bacterial kinases.

PiORP1 is membrane localized in growing pollen tubes

OSBPs and their homologues that contain a PH domain in the N-terminal region have been shown to localize predominantly to the cytosol and membranes of the Golgi

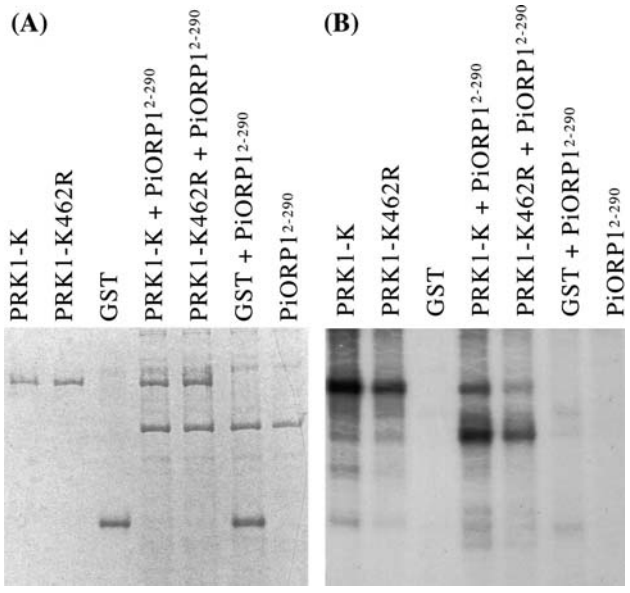


Fig. 3 Phosphorylation of PiORP1$^{2–290}$ by PRK1-K *in vitro*. GST and GST fusion proteins of PRK1-K and PRK1-K462R along with 6× His:PiORP1$^{2–290}$ were combined in phosphorylation assays in the presence of [^{32}P]ATP. Different reaction mixtures, as indicated, were electrophoresed on a 12% (w/v) SDS-polyacrylamide gel that was stained with Coomassie blue (**A**) and dried for autoradiography (**B**)

apparatus (Levine and Munro, 1998). To determine the subcellular localization of PiORP1, the coding sequences of PiORP1, PiORP1$^{1–201}$, encoding the N-terminal PH domain, and PiORP1$^{363–775}$, encoding the C-terminal oxysterol-binding domain, were separately fused to the coding sequence of GFP, and the fusion genes were placed under the control of the pollen-specific *LAT52* promoter of tomato (Twell et al. 1990).

The control construct, *pLAT52/GFP*, encoding soluble GFP, yielded fluorescence throughout the entire cytoplasm of the pollen tube (Fig. 4A). However, when PiORP1:GFP was visualized, islands of fluorescence dispersed within the plasma membrane of the entire pollen tube were observed, as well as some cytoplasmic signal (Fig. 4B, C, E). In non-growing pollen tubes, the foci extended to the very apex of the tube (Fig. 4B) and time-lapse visualization revealed these were non-dynamic in their placement (results not shown). However, in growing tubes, these islands were stationary along the shanks of the tube but were excluded from the very apex of the tip (Fig. 4C and supplementary movie). A higher magnified image of PiORP1:GFP in a growing tube is shown in Fig. 4E. Just behind the apex of the pollen tube, these foci appear to be inserted into the still expanding plasma membrane (supplemental movie). In pollen tubes that showed very high levels of PiORP1:GFP, the localization was no longer apparent and the fluorescence appeared completely cytoplasmic; these pollen tubes ceased to elongate (data not shown). When GFP:PiORP1$^{1–201}$ was visualized in pollen tubes, a similar punctate plasma membrane localization was observed (Fig. 4D). However, these pollen tubes had very little cytoplasmic signal and the fluorescent islands were visible even in pollen tubes that showed very high expression levels. As was the case with PiORP1:GFP, at the time when expression of GFP:PiORP1$^{1–201}$ was clearly visible, pollen tubes generally stopped growing. For GFP:PiORP1$^{363–775}$, the fluorescent signals resembled soluble GFP, and the pollen tubes appeared normal (results not shown).

PiORP1 shares sequence similarity with a family of *Arabidopsis* ORPs

The sequence of the oxysterol-binding domain of PiORP1 was used to search the *Arabidopsis* genome to identify the *ORP* gene family. Consistent with the unpublished results mentioned in Jaworski et al. (2001), 12 *ORP* genes were identified. The proteins encoded by these genes are schematically shown in Fig. 5A, along with PiORP1 for comparison. These predicted *Arabidopsis* proteins are similar in structural organization to PiORP1 and members of the human and yeast OSBP/ORP families (Jaworski et al. 2001). The *Arabidopsis* ORP family contains long variants, which have an N-terminal PH domain, and short variants, which do not have this domain. Like PiORP1, each of the five long variants of *Arabidopsis* ORPs contains a predicted coiled-coiled region. This region may be involved in dimerization, as noted for the coiled-coiled region of a rabbit OSBP by Ridgeway et al. (1992). Coiled-coil regions are predicted in some other ORPs, but it is not known whether they can homo- or heterodimerize. The ankyrin repeats and GOLD protein-protein interaction domains found in human and yeast OSBPs/ORPs are not present in any of the plant ORPs.

The PH domains of the five *Arabidopsis* long variants and PiORP1 are highly conserved, as are the oxysterol-binding domains of all 12 *Arabidopsis* ORPs and PiORP1. In contrast, other regions of these ORPs are not very similar. Thus, sequence comparisons were made only in the PH domain and the oxysterol-binding domain. An alignment of the PH domains of PiORP1 and the five *Arabidopsis* long variants is shown in Supplemental Fig. A, and an alignment of the oxysterol-binding domains of PiORP1 and all 12 *Arabidopsis* ORPs is shown in Supplemental Fig. B. The oxysterol-binding domains of 2 of these 13 ORPs contain the perfect mammalian signature sequence, EQVSHHPP, and those of the other ORPs differ from the signature sequence by 1 or 2 residues. Moreover, the oxysterol-binding domains of all 12 *Arabidopsis* ORPs and PiORP1 contain the 4 invariant amino acids (marked with an asterisk in Supplemental Fig. B) present in all the reported ORPs to date, including those from metazoans, plants and fungi (Beh et al. 2001).

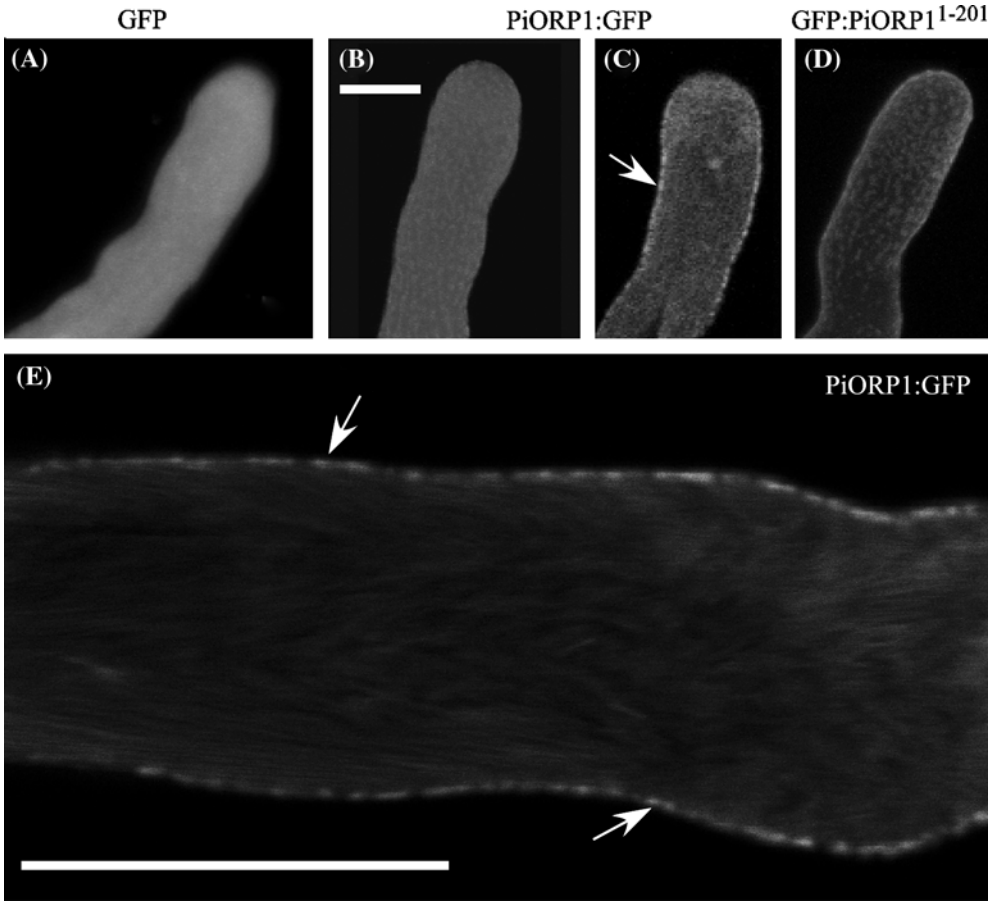


Fig. 4 Membrane localization of PiORP1 and PiORP1$^{1–201}$ in pollen tubes. Soluble GFP was transiently expressed as a control (**A**). PiORP1:GFP was expressed in a non-growing pollen tube (**B**) and a growing pollen tube (**C**). (**D**) GFP:PiORP1$^{1–201}$ expression in a non-growing tube (3D reconstruction). (**E**) Higher magnification image of GFP:PiORP1$^{1–201}$ in the subapical shank a growing tube. The image shown in (**B**) represents a 3D reconstruction, whereas the image shown in (**C**) is a mid-plane individual optical section (as the time necessary to collect the optical sections required for 3D reconstruction makes it impossible to perform on the moving tip of a growing tube). Note that the punctate staining over the plasma membrane (arrows) of the pollen tube shown in (**C**) is more clearly visible in the image shown in (**E**). All constructs were under the control of the pollen-specific promoter *LAT52* and transformed into hydrated pollen by particle bombardment according to Fu et al. 2001. Scale bar is equal to 10 μm

A neighbor-joining tree was constructed from the sequence distance data of the aligned oxysterol-binding domains of PiORP1 and the 12 *Arabidopsis* ORPs (Fig. 5B). The topology of the tree shows that the 12 *Arabidopsis* ORPs fall into 2 main clusters: 9 of them, along with PiORP1, are in 1 cluster, and the remaining 3 are in the other cluster (comprising group IV). The former cluster is further divided into three groups (designated I, II, and III).

The oxysterol-binding domains of PiORP1 and 12 *Arabidopsis* ORPs were compared with the corresponding domains of 12 human OSBPs/ORPs (OSBP-1, OSBP-2, OSBPL-1 to OSBPL-3, and OSBPL-5 to OSBPL-11), seven yeast ORPs (OSH-1 to OSH-7) and four *Drosophila* ORPs (FLY-455, -762, -764, -784). A cladogram of these OSBPs/ORPs is shown in Fig. 6. The two major divisions observed in the human OSBPs/OPRs (Jaworski et al. 2001) hold true for the ORPs of all the other organisms examined here, including *Arabidopsis*. However, it is interesting to note that, whereas some yeast ORPs are clustered with some human and/or *Drosophila* OSBPs/ORPs, the *Arabidopsis* ORPs and PiORP1 are clustered separately within these two major divisions. For example, two plant clusters (one including PiORP1) fall within one major division, and one plant cluster falls within the other. The significance of this topology is unknown, but it may be due to differentiation of ORPs for the purpose of binding plant-specific sterols/lipids, or due to differentiation of ORP functions in plants. The conservation of the overall gene family structures is consistent with the belief that a divergent family of specialized ORPs was present at early stages of evolutionary history (Jaworski et al. 2001). The independent clustering in the cladogram suggests that the plant genes evolved separately from those in other organisms.

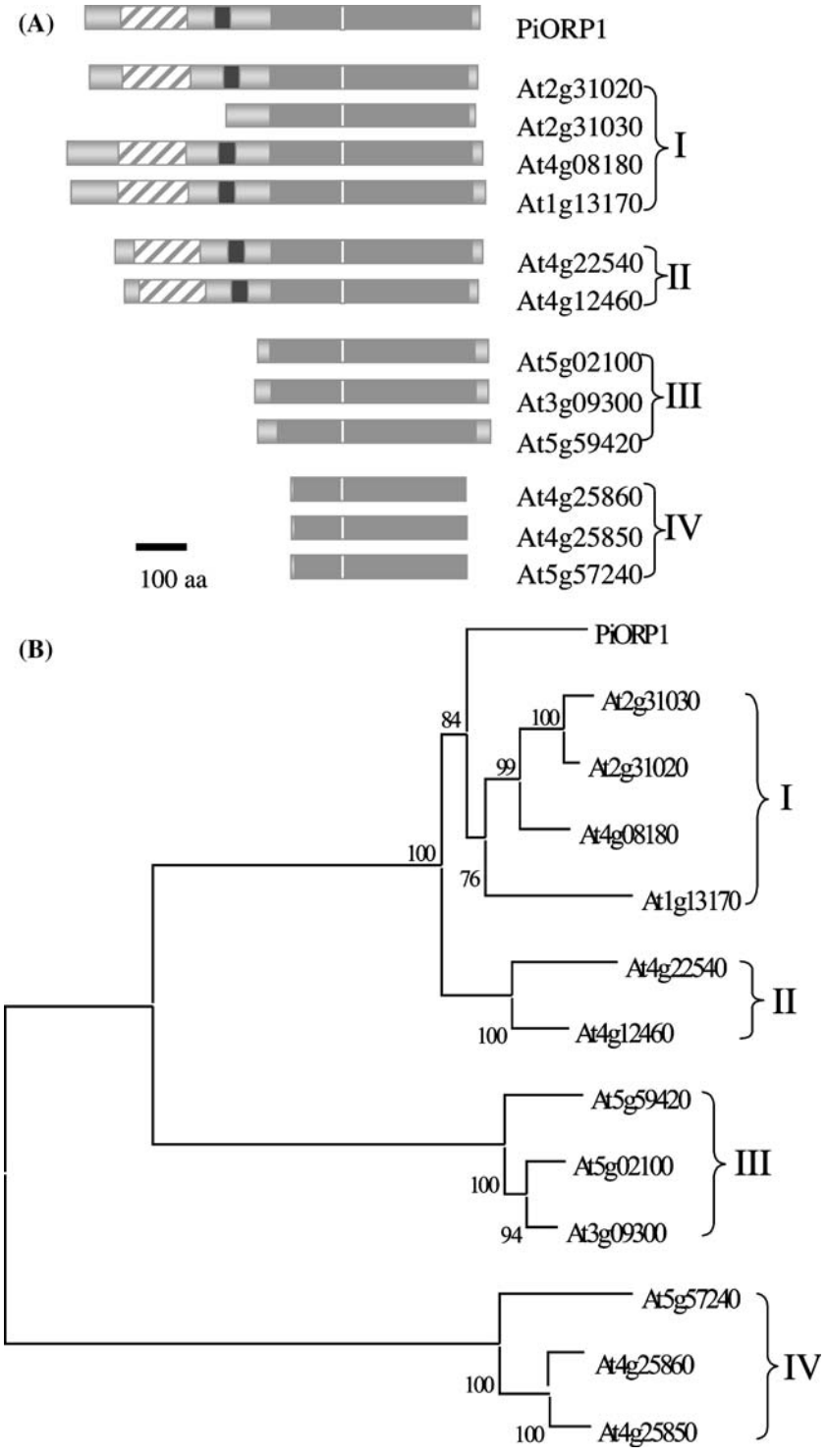


Fig. 5 Comparison of the 12 *Arabidopsis* ORPs and PiORP1. (**A**) Schematic representation of the domain structures of PiORP1 and the 12 ORPs of *Arabidopsis*. The diagonally striped boxes indicate the PH domains, the dark gray shaded boxes indicate the oxysterol-binding domains and the black boxes represent coiled coil regions. Proteins are aligned by a highly conserved protein sequence (EQVSHHPP) indicated by the white box in the oxysterol-binding domain. The *Arabidpsis* ORPs fall into four distinct subgroups, designated I, II, III, and IV (see (**B**) below), and PiORP1 is most similar to members of Subgroup I. (**B**) Cladogram of the 12 *Arabidopsis* ORPs and PiORP1. A neighbor-joining tree was produced from the sequence distance data of the aligned oxysterol-binding domains. The tree branching represents the relationships among the 13 ORPs based on sequence similarities. The *Arabidopsis* genes fall into three distinct clusters and four subgroups. The numbers represent results of 500 bootstrap replications. The GenBank accession number for PiORP1 is DQ241801

The *Arabidopsis ORPs* show diverse expression patterns but lack pollen specificity

To determine the tissue specificity of the *Arabidopsis ORP* gene family, the expression profiles were analyzed using RT-PCR. Since isolating pollen that is free of contaminating tissues and isolating tissues that are free of pollen contamination can be difficult for *Arabidopsis*, we included *RBCS-1B*, encoding the small subunit 1B of ribulose bisphosphate carboxylase, and *PGA4*, a pollen-specific gene (Honys and Twell, 2003), as controls to ensure the purity of all the tissues used for RNA isolation. As expected, the

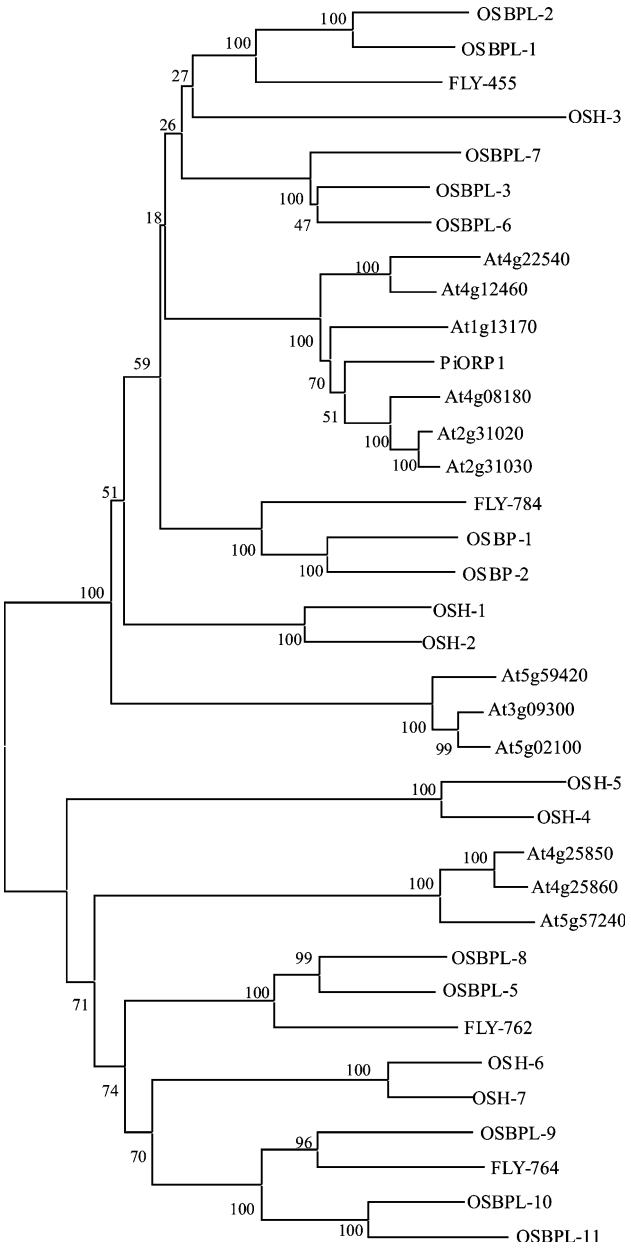
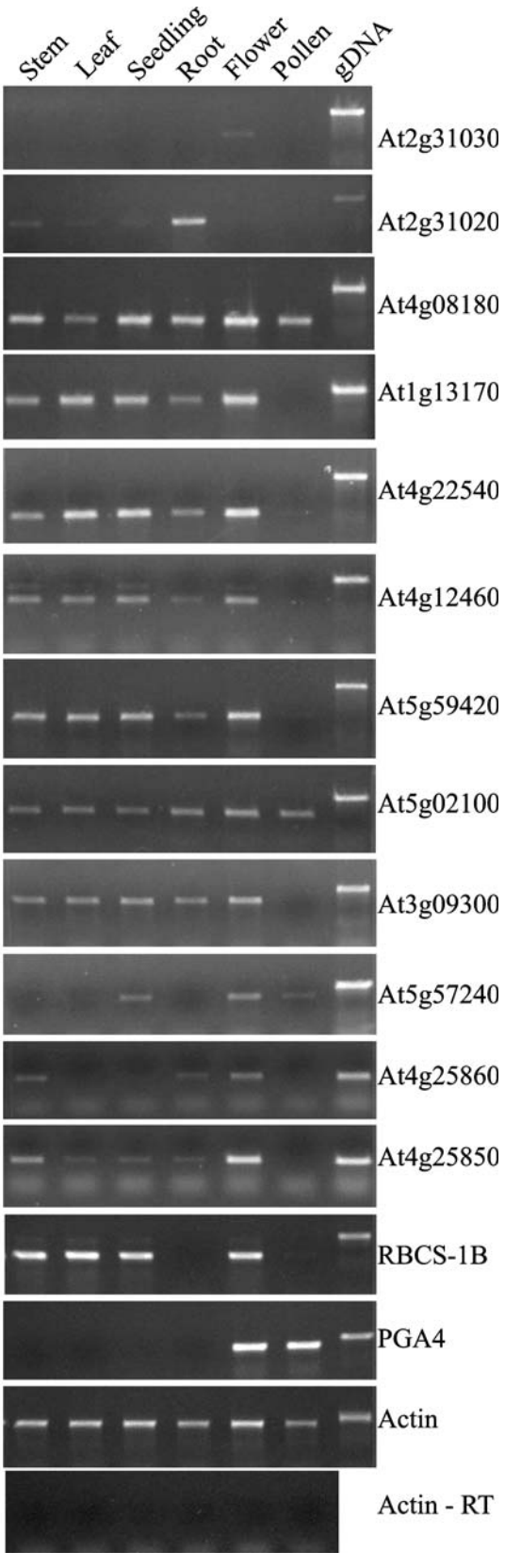


Fig. 6 Cladogram of OSBPs/ORPs of human, yeast, fruit fly and plants. A neighbor-joining tree was produced from the sequence distance data of the aligned oxysterol-binding domains. The tree branching represents the relationships among the proteins based on sequence similarities. The plant genes separate with the genes of the other organisms into the two major divisions, but always remain clustered separately within these divisions. The numbers represent results of 500 bootstrap replications. The human proteins are designated OSBP-1, OSBP-2, OSBPL-1 to OSBPL-3, and OSBPL-5 through OSBPL-11; the yeast proteins are designated OSH-1 through OSH-7, the fruit fly proteins are designated FLY-(number of amino acid residues) and the *Arabidopsis* proteins are designated as those shown in Fig. 5**A**. The GeneBank accession numbers are: fly-784, CAA74289; fly-455, AAF47130; fly-762, AAG22160; fly-764, AAF58878; OSH-1, NP009421; OSH-2, NP010265; OSH-3, NP011940; OSH-4, NP015180; OSH-5, NP014880; OSH-6, NP012928; OSH-7, NP011863; OSBP-1, AAG28373; OSBP-2, AAG53406; OSBPL-1, BAA91496.1; OSBPL-2, BAA34492; OSBPL-3, BAA31679; OSBPL-5, BAA96058; OSBPL-6, AAG53409; OSBPL-7, AAG53410; OSBPL-8, BAA95975; OSBPL-9, BAA14096; OSBPL-10, BAA91118; OSBPL-11, BAA14391

PCR product for *RBCS-1B* was obtained in all but root and pollen (lacking chloroplasts), and the PCR product for *PGA4* was only obtained in flower (containing mature anthers) and pollen (Fig. 7).

As shown in Fig. 7, all the 12 predicted *ORP* genes of *Arabidopsis* were expressed, but their expression profiles varied. At2g31030 had the lowest level of expression and it was specific to flowers; At2g31020 was predominantly



expressed in roots. Most of the other 10 *ORPs* were expressed in all the tissues examined, but only three were expressed in pollen. At4g08180 and At5g02100 had the strongest pollen expression, and At5g57240 was weakly expressed in pollen. The pollen enriched expression patterns of At4g08180, At5g02100 and At5g57240 determined by our PCR analysis are consistent with those defined by microarray analysis in Pina et al. (2005). Among the four *ORP*s that are in the same subgroup as *PiORP1* (Fig. 5B), only At4g08180 is expressed in pollen.

Discussion

The receptor-like kinase PRK1 has been shown to be essential for pollen development, as suppression of *PRK1* expression by antisense RNA resulted in the arrest of developing microspores at the unicellular stage (Lee et al. 1996). However, PRK1 is also likely to have an additional role in pollen tube growth in the style, because the transcript of *PRK1* is most abundant in mature pollen and *in vitro* germinated pollen tubes. To address this role by an antisense RNA or RNAi approach requires a pollen-tube specific promoter for suppressing the expression of *PRK1*. Such a promoter has not been identified, so we chose to search for PRK1 interacting partners in order to elucidate the role of PRK1 in pollen growth and development. Using yeast two-hybrid and *in vitro* binding assays, we have identified an oxysterol-binding-protein related protein of *P. inflata*, PiORP1, which interacts with the cytoplasmic kinase domain of PRK1. PiORP1 is phosphorylated by PRK1 *in vitro*, suggesting a potential regulatory role for PRK1 in PiORP1 action in the pollen tube.

Oxysterol-binding proteins and their homologues in animals and fungi are characterized by a highly conserved oxysterol-binding domain that is also present in the *Petunia* and *Arabidopsis* proteins. This domain is responsible for the sterol interaction exhibited by this class of proteins. The most characterized sterol in animals is cholesterol, which, in addition to being an essential component of plasma membranes, is involved in signaling related to cell

◀ **Fig. 7** Expression analysis of *Arabidopsis ORPs*. Total RNA (1 μg) isolated from stem, leaf, seedling, root, flower and pollen was used for RT-PCR. Primers specific to each of the 12 *ORP* genes, as well as primers specific to *Arabidopsis RBCS-1B* gene (encoding the small subunit of ribulose bisphosphate carboxylase), *PGA4* (a pollen-specific gene), and the actin genes are described in the Materials and methods. *RBCS-1B* and *PGA4* were included as controls for purity of tissue, and the actin genes were included as a control for equal amounts of RNA used in each RT-PCR. ''Actin – RT'' indicates PCR of the actin genes without reverse transcriptase. Genomic DNA (gDNA) was also included to demonstrate the size of the genomic DNA band

division, cell growth and various developmental processes (Edwards and Ericsson, 1999). Mammalian OSBPs have been implicated in the sensor system that regulates cholesterol levels (Im et al. 2005; Wang et al. 2005). In contrast, the functional role and substrate for PiORP1 and its *Arabidopsis* homologues remains unknown. One report shows that an *ORP* is up-regulated in barley as a result of infection with powdery mildew (*Blumeria graminis* f. sp. *hordei*); in this case it may play a role in cell death (Hein et al. 2004) similar to that proposed for oxysterols in apoptosis in animal cells (Bakos et al. 1993; Thompson and Ayala-Torres, 1999; Rusinol et al. 2000). However, there are few other reports of OSBP action in plant responses. As well as binding oxysterols, OSBPs have also been reported to bind phosphoinositides and phosphatidic acid (Xu et al. 2001), both of which have been proposed to regulate pollen tube growth through control of the apical Ca^{2+} gradient required to drive tip growth (Monteiro et al. 2005 and references therein). Thus, it is possible that PiORP1 is acting to control the levels and/or distribution of lipids that are key facilitators of pollen tube elongation. Indeed, oxysterols have been implicated in regulating calcium uptake in animal cells (Kolsch et al. 1999), raising the possibility that PiORP1 may play a role in controlling the growth-related Ca^{2+} gradient at the pollen tube apex. Interestingly, a disruption in tip growth is observed when both PiORP1:GFP and GFP:PiORP1$^{1–201}$ fusions are expressed at high levels in the pollen tube.

Localization by GFP fusion revealed that the PiORP1 PH domain alone is capable of generating the plasma membrane foci observed with the full-length PiORP1 protein. PH domains are classically thought of as phosphoinositide binding domains (Lemmon, 2004). Therefore, the punctate localization may reflect binding to lipid microdomains in the pollen tube plasma membrane. The only lipid to be localized in the pollen tube plasma membrane to date is phosphatidyl inositol (4,5) bisphosphate (Kost et al. 1999). This lipid does bind to PH domains; however, it is uniformly distributed around the apical plasma membrane and does not form foci. Therefore, an intriguing possibility is that the interaction with PRK1 is responsible for PiORP1 localization. We have shown that PRK1 interacts with PiORP1$^{2–290}$, which lacks the oxysterol-binding motif but does contain the coiled-coil and PH domains. LePRK1, LePRK2 and LePRK3, which are homologues of PRK1 in tomato, are integral membrane proteins known to be localized to the plasma membrane, providing one potential localization site for the ORPs. When Kim et al. (2002) localized these receptor-like kinases to the plasma membrane/cell wall of the pollen tube, each displayed distinct patterns of distribution, yet each produced images similar to the foci observed for PiORP1:GFP. In particular, the pattern of distribution displayed by LePRK3 is similar to that of PiORP1:GFP. The phosphorylation of PiORP1$^{2–290}$ by PRK1 could therefore act not only to regulate OSBP function but also to potentially alter its lipid association, allowing shuttling on and off the plasma membrane in response to signals from, for example, the transmitting tract of the style. Indeed, in animals, OSBP is proposed to be regulated in part by phosphorylation (Storey et al. 1998; Mohammadi et al. 2001). Although it is tempting to speculate that the phosphorylation of PiORP1 by PRK1serves a similar role, the physiological function of the phosphorylation is still being investigated.

PiORP1 possesses the conserved structural motifs and phosphorylation seen in OSBPs across kingdoms, suggesting that understanding PiORP1 action may help explain OSBP functions in general. In addition, the novel plasma membrane localization and role in a tip growing system suggested by our results raises the possibility of specific plant-related functions for this class of proteins. Our future work is aimed at defining these conserved and potentially novel activities.

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