Cloning and characterization of cDNA of the GPI-anchored purple acid phosphatase and its root tissue distribution in *Spirodela oligorrhiza*

Miwa Nishikoori[a], Kenji Washio[a], Akira Hase[b], Naoki Morita[c] and Hidetoshi Okuyama[a,*]

[a]*Laboratory of Environmental Molecular Biology, Graduate School of Environmental Earth Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan*
[b]*Biological Laboratory, Hakodate College, Hokkaido University of Education, Hachiman-cho, Hakodate 040-8567, Japan*
[c]*Molecular Adaptation Research Group, Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-higashi, Toyohira-ku, Sapporo 062-8517, Japan*
Corresponding author, e-mail: hoku@ees.hokudai.ac.jp

A cDNA clone of the glycosylphosphatidylinositol (GPI)-anchored purple acid phosphatase (PAP) has been obtained by a combination of cDNA library screening and 5′ rapid amplification of cDNA ends from *Spirodela oligorrhiza* plants grown under phosphate-deficient (− P) conditions. The open reading frame of the *S. oligorrhiza* PAP cDNA consists of 1 365 bp encoding a 455 amino acid protein. Its deduced amino acid sequence shows 82 and 80% similarity to *Arabidopsis thaliana* and *Phaseolus vulgaris* PAP, respectively. The amino acid residue, Ala439, followed by two more small amino acid residues, Asp and Ser, is predicted to be the GPI-anchoring (ω) site. The absence of a dibasic motif upstream of the putative ω site suggests that the PAP is a cell wall protein.

This presumption is supported by the finding that PAP was released by digestion of the cell wall fraction with cellulase. The GPI anchor is speculated to be a signal for transporting PAP to the cell wall. Immunohistochemical results using − P plant roots demonstrate that PAP is preferentially distributed in the outermost cortical cells of roots but not in the epidermis, suggesting its role in acquiring inorganic phosphate under phosphate-deficient conditions. Northern blot analysis using the *S. oligorrhiza* PAP cDNA as a probe demonstrates that expression of the PAP gene increased during growth of − P plants and this time-dependent occurrence in mRNA levels of the PAP in − P plants was also observed in their protein and activity levels.

Introduction

The *Spirodela oligorrhiza* phosphatase, inducibly synthesized under phosphate-deficient conditions, was proved to be a glycosylphosphatidylinositol (GPI)-anchored protein found in higher plants (Morita et al. 1996). Recently, Nakazato et al. (1998) presented evidence showing that this *S. oligorrhiza* GPI-anchored phosphatase is a purple acid phosphatase (PAP). The phosphatase was found to be a purple-colored metalloenzyme containing Fe and Mn atoms. Western blot experiments showed that anti-*Arabidopsis thaliana* PAP antibodies cross-reacted with the *S. oligorrhiza* phosphatase. The finding that the N-terminal amino acid sequence of this phosphatase was almost identical to that of PAPs of *A. thaliana* (accession no. U48448), *Phaseolus vulgaris* (Klabunde et al. 1994) and *Glycine max* (LeBansky et al. 1992), confirmed that the GPI-anchored phosphatase of *S. oligorrhiza* is a PAP (Nakazato et al. 1998).

Among these plant PAPs, only the *S. oligorrhiza* PAP has been shown to be GPI-anchored. Although the *S. oligorrhiza* PAP has been purified (Nakazato et al. 1997a) and its biochemical properties and kinetic parameters presented (Nakazato et al. 1997a, 1998), the primary structure of the PAP and the fine structure of the GPI anchor itself have yet to be elucidated.

As reported by Englund (1993), animal and yeast GPI-anchored proteins have several characteristic amino acid sequences in their C-terminal region. A comparison of amino acid sequences reveals the consistent presence of 15–20 hydrophobic residues at the extreme C-terminus, although primary structures of these proteins vary greatly. Upstream of the hydrophobic sequence is a spacer region (usually about 5–10 residues) that contains hydrophilic amino acids. The spacer region is preceded by one of the small amino

---

acids, either Gly, Asp, Asn, Ala, Ser or Cys. It is this residue that becomes joined to the GPI anchor by an amide bond following enzymatic cleavage of the downstream peptide. Two small amino acid residues downstream of the anchoring site complete the domain, specifying cleavage and GPI anchorage (Englund 1993).

In this study, cDNA of the *S. oligorrhiza* GPI-anchored PAP has been cloned and its deduced amino acid sequence was compared to that of genes and cDNAs of the GPI-anchored proteins known so far, with a special emphasis on the presence of the C-terminal signal sequences. Cellular and subcellular localization of PAP and effects of phosphate starvation on expression of the PAP gene and accumulation of the PAP in *S. oligorrhiza* have also been investigated.

Materials and methods

Plant material and extraction of total RNA and mRNA

Duckweed *Spirodela oligorrhiza* plants were grown in Hoagland's medium (Posner 1967) for 2–3 weeks at 25°C as described previously (Nakazato et al. 1997a). Plants grown in the presence and in the absence of 1.5 mM sodium phosphate were termed $+ P$ and $- P$ plants, respectively. Harvested plants were stored at $- 80$°C until use.

About 0.2 g (wet weight) of $- P$ plants in a 1.5 ml microfuge tube was frozen in liquid nitrogen and then ground to a fine powder with a hand-type homogenizer (type S-203; Ikedarika, Tokyo, Japan). The powdered plant material was subjected to total RNA extraction by the SDS-phenol method (Kingston et al. 1999). Poly(A)$^+$ RNA was isolated from the total RNA using an oligo(dT) cellulose column (CLONTECH, Palo Alto, CA, USA) according to the published method (Reddy and Gilman 1999).

Cloning of PAP cDNA

The cDNA library of $- P$ plants was made using a cDNA synthesis module kit (Amersham, Buckinghamshire, UK), a cDNA rapid cloning module-lambda gt11 (Amersham), a cDNA rapid adaptor ligation module (Amersham) and Gigapack III Gold phage extracts (Stratagene, La Jolla, CA, USA), according to the manufacturer's instructions. A cDNA of PAP from *Arabidopsis thaliana* in the Bluescript SK plasmid (provided by T. McKnight, Texas A&M University) was used as a probe. The 1 400 bp fragment thus obtained was inserted into the pBluscript SK($+$) vector at the *Eco*RI site (pHIP1). As this cDNA lacked the 5′ region of the PAP cDNA, 3 gene-specific primers (5′-TTGATT-GAGTACCAAAAT-3′ (5′-GSP1), 5′-CTCTGGAGCATA-ATCAATTTCGTGGTTC-3′ (5′-GSP2) and 5′-ACGTGTC-CCATCTTACATTATCATGATC-3′ (5′-nested GSP)) were synthesized to obtain the full-length PAP cDNA by the 5′ rapid amplification of cDNA ends (5′RACE) method. Total RNA (1 µg) prepared from $- P$ plants grown for 7 days was used for 5′RACE, version 2.0 (GIBCO BRL) with the 5′-GSP1, 5′-GSP2 and 5′-nested GSP primers. The 1 100 bp fragment obtained was subcloned into pT7-blue (Novagen, Madison, WI, USA) and designated pHIP2. The pHIP2

fragment digested with *Bam*HI and the pHIP1 fragment digested with *Bam*HI were ligated with Ligation kit Ver. 2 (TaKaRa, Tokyo, Japan). The resulting PAP cDNA clone was designated pHIP and was used for preparation of a DNA probe.

DNA sequencing and computer analysis

The sequences of the cDNA insert were determined by a Thermo Sequence fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham). Homology searches were performed through NCBI using the BLASTX program against the GenBank database.

Northern blotting

Total RNA (10 µg/lane) was electrophoretically separated on 1.3% formaldehyde denaturing agarose gels and blotted onto nylon membrane (Hybond N$^+$; Amersham) with a vacuum blotter (ATTO, Tokyo, Japan). The membrane was hybridized overnight at 42°C with a ^{32}P-labeled probe (10^6 cpm ml^{-1}) in a solution containing 50% (w/v) deionized formamide, 5% (w/v) Irish cream liqueur, 0.5% (w/v) SDS, $6 \times$ SSPE (0.9 M NaCl, 52 mM Na$_2$HPO$_4$, 7.5 mM EDTA, pH 7.4) and 20 µg ml^{-1} denatured salmon sperm DNA. Filters were washed twice in $2 \times$ SSPE at room temperature for 15 min, and twice in $2 \times$ SSPE and 0.1% SDS at 65°C for 45 min each before being placed on Imaging Plate BAS 2000 film (Fuji Photo Film, Tokyo, Japan).

SDS-PAGE and activity stain of PAP

Frozen plants were ground with a homogenizer and extracted with 200 µl of 10 mM Tris-HCl, pH 8.5. The mixture was centrifuged for 10 min and supernatants were used as cell-free extracts. The cell-free extracts were analyzed by SDS-PAGE by the method of Laemmli (1970), using gels with a linear gradient of 5–20% polyacrylamide (type NPG-520 L; ATTO). Protein samples dissolved in the SDS sample buffer not containing 2-mercaptoethanol were not heated, thereby maintaining the incompletely denatured proteins in a solubilized but enzymatically active state (Nakazato et al. 1997a). Gels were treated with phosphatase activity stain buffer (1 mM 5-bromo-4-chrolo-3-indolyl phosphate in 100 mM tricine-NaOH, pH 8.0) (Nakazato et al. 1997a). Protein concentrations of the supernatants were determined by the method of Bradford (1976).

Immunoblot and immunohistochemical analysis of PAP

The cell-free extracts (10 µg of protein/lane), which were denatured in the SDS sample buffer containing 2% (v/v) 2-mercaptoethanol by heating at 100°C for 3 min, were resolved by SDS-PAGE and then were electrotransferred to poly(vinylidene difluoride) (PVDF) membrane (Millipore, Milford, MA, USA) with wet transblotting apparatus (Mini-trans blot; Bio-Rad, Hercules, CA, USA), as described by Nakazato et al. (1997a).

For immunohistochemical analysis of PAP, whole $+P$ and $-P$ plants were deaerated and fixed in 4% (w/v) paraformaldehyde, 1% (v/v) glutaraldehyde, 0.06 M sucrose and 0.05 M Na-cacodylate-HCl (pH 7.4) for 3 h at room temperature. Fixed plants were rinsed twice in 0.06 M sucrose and 0.05 M Na-cacodylate-HCl (pH 7.4) and then dehydrated through an ascending ethanol series to 100% and embedded in LR White resin (type medium; London Resin, Berkshire, UK). Root transverse sections (2 μm) were cut from the embedded plants and stained with 0.1% (w/v) toluidine blue or immunodecorated by the streptavidin-biotin-peroxidase complex method using the Zimed Histostain-SP kit (Zymed Laboratories, S. San Francisco, CA, USA) according to the manufacturer's instructions. The primary antiserum against *S. oligorrhiza* PAP was diluted 1:2 000 and treated for 1 h at 25°C. Resulting peroxidase-labeled complexes were visualized with TrueBlue Peroxidase Substrate (KPL, Gaithersburg, MD, USA).

Purification of the phosphatase from *S. oligorrhiza*

Purification of *S. oligorrhiza* phosphatase was carried out as described previously (Nakazato et al. 1997a). The electrophoretically purified phosphatase was used as experimental material.

Preparation of cell wall from *S. oligorrhiza*

Plant materials were freeze-dried, ground and extracted with 50 mM Tris-maleic buffer 5 times and then extracted with 50 mM Tris-maleic buffer containing 1.0 M NaCl twice. The washed cell walls were freeze-dried. Dried cell walls (2.5 mg) were digested either with 1 mg of cellulase from *Tricodelma viride* (Wako, Tokyo, Japan) or with 1 mg of pectinase (macerozyme R-200; Yakult, Tokyo, Japan) in 200 μl of 100 mM sodium acetate (pH 4.5) for 16 h at 37°C.

Results and discussion

Cloning of PAP cDNA

For isolation of the cDNA clone of the *S. oligorrhiza* PAP, the full-length of cDNA of the *A. thaliana* PAP was used as a probe. Among 70 000 plaques, a few positive plaques were obtained in the screening of the cDNA library. One of the resulting cDNA clones, pHIP1 (1 400 bp), included the predicted sequence of Thr-Arg-Thr-His-Ala-His-Tyr-Ser, which was identical to the partial sequence of a peptide derived from the lysyl endopeptidase-digested purified PAP (data not shown), but it did not include the whole sequence of the *S. oligorrhiza* PAP cDNA.

In order to get the remaining half of the *S. oligorrhiza* PAP cDNA clone, 5′RACE was carried out and only one positive clone, pHIP2 (1 100 bp), was obtained. pHIP2 included the sequence of Ala-Val-Asp-Met-Pro-Leu-His-Ala-Asp-Val-Phe-Arg-Met-Pro-Pro-Gly-Tyr-Asn-Ala, which was identical to the N-terminal amino acid sequence of the purified *S. oligorrhiza* PAP (Nakazato et al. 1998).

The full-length cDNA of the *S. oligorrhiza* GPI-anchored PAP was obtained by ligating the two cDNA fragments, pHIP1 and pHIP2, at the *Bam*HI site. The combined DNA product was sequenced and shown to be properly ligated. The ligated clone (pHIP; accession no. AB039746) had one open reading frame consisting of 1 365 bp encoding 455 amino acids (Fig. 1). Since the residue of Ala33 is the N-terminus of the mature protein, the domain consisting of 32 amino acid residues from Met1 to Ser32 was regarded as a signal sequence. In the case of the *P. vulgaris* PAP, Phe28 is the N-terminal amino acid (see Fig. 1), as determined by matrix-assisted laser desorption/ionization mass spectrometry of the purified enzyme protein (Klabunde et al. 1994). Interestingly, according to Klabunde et al. (1994), the N-terminal amino acid of the mature *P. vulgaris* PAP was heterogeneous, suggesting that the size of the signal sequence of the plant PAP might depend on its source plant and on the enzyme protein itself. Since the N-terminus of the mature *A. thaliana* PAP has never been determined, the length of its signal peptide is unknown.

The signal for GPI anchor addition to the immature protein (so-called preproprotein) usually consists of a 3-small amino acid domain including the attachment (ω) site of the anchor and a C-terminal hydrophobic signal sequence (Englund 1993). The ω site (the C-terminal end of the mature protein) is a small amino acid, generally either Gly, Asp, Asn, Ala, Ser or Cys that is followed by two small amino acids ($\omega + 1$ and $\omega + 2$). In some cases, the second amino acid ($\omega + 1$) of this cleavage domain is not a small amino acid (Udenfriend and Kodukula 1995). In the deduced amino acid sequence of the *S. oligorrhiza* PAP cDNA, two domains consisting of 3 small amino acids were identified as candidates for the cleavage domain giving rise to the mature protein's C-terminus. These are Asn420-Arg421-Thr422 and Ala439-Asp440-Ser441 (see Figs 1 and 2). However, the former can be excluded from the candidates because the peptide fragment including this sequence has been obtained as a product of lysyl endopeptidase digests of the purified protein (Nakazato et al. unpublished result). Thus, Ala439 is predicted to be the GPI-anchoring site of the *S. oligorrhiza* GPI-anchored PAP, although no biochemical evidence has been obtained to support this assignment.

In animal and yeast GPI-anchored proteins, a proprotein has 15–20 hydrophobic residues at the extreme C-terminus, and just upstream of the hydrophobic sequence is a 5–10 amino acid spacer (Englund 1993). Recently, it was shown that classical arabinogalactan proteins of *A. thaliana* and other plant sources have a 4–8 amino acid spacer which is followed by a 14–18 residue hydrophobic signal sequence (Schultz et al. 1998, Youl et al. 1998, Thompson and Okuyama 2000). However, compared to the typical structure of these GPI-anchored proteins, the appropriate C-terminal domain of the *S. oligorrhiza* PAP is less hydrophobic and too short (Fig. 2).

The deduced amino acid sequence of the 3 PAP cDNAs shown in Fig. 1 was analyzed with the PSORT program (http://psort.nibb.ac.jp:8800/), a computer program for prediction of protein localization sites in cells. This program recognizes proteins having N- and C-terminal hydrophobic

```
                                              * * * * * * * * * *
S.oligorrhiza              MARLVLAVMLLLNAAILCSGGITSEFVRLQESAVDMPLHADV     42
A.thaliana           MSSRSDLKIKRVSLIIFLLSVLVEFCYGGFTSEYVRGSDLPDDMPLDSDV
P.vulgaris             MGVVKGLLALALVLNVVVVSNGGKSSNFVRKTNKNRDMPLDSDV
                                                                ^

               * * * * * * * *
S.oligorrhiza  FRMPPGYNAPQQVHITQGDHEGRSIIVSWITPSEKGSSTVFYGTSENKLD     92
A.thaliana     FEVPPGPNSPQQVHVTQGNHEGNGVIISWVTPVKPGSKTVRYW-CENKKS
P.vulgaris     FRVPPGYNAPQQVHITQGDLVGRAMIISWVTMDEPGSSAVRYW-SEKNGR

S.oligorrhiza  QH-AEGTVTMYKFYTYTSGYIHHCVLTDLKYDRKYFYKVGEGSAARLFWF    141
A.thaliana     RKQAEATVNTYRFFNYTSGYIHHCLIDDLEFDTKYYYEIGSGKWSRRFWF
P.vulgaris     KRIAKGKMSTYRFFNYSSGFIHHTTIRKLKYNTKYYYEVGLRNTTRRFSF

S.oligorrhiza  KTPPEVGPDVPYTFGLIGDLGQTFDSNVTLTHYESNPG-GQAVLYVGDLS    190
A.thaliana     FTPPKSGPDVPYTFGLIGDLGQTVDSNSTLSHYEMNPGKGQAVLFVGDLS
P.vulgaris     ITPPQTGLDVPYTFGLIGDLGQSFDSNTTLSHYELSPKKGQTVLFVGDLS

S.oligorrhiza  YADVYPDHDNVRWDTWGRFVERSTAYQPWIWTTGNHEIDYAPEIGEYVPF    240
A.thaliana     YADRYPNHDNNRWDTWGRFVERSVAYQPWIWTAGNHEIDFVPDIGEIEPF
P.vulgaris     YADRYPNHDNVRWDTWGRFTERSVAYQPWIWTAGNHEIEFAPEINETEPF

S.oligorrhiza  KPFTHRYHVPHKSSGSGSPFWYSIKRASAYIIVLASYSAFGKYTPQSEWL    290
A.thaliana     KPFMNRYHTPHKASGSISPLWYSIKRASAYIIVMSCYSSYGIYTPQYKWL
P.vulgaris     KPFSYRYHVPYEASQSTSPFWYSIKRASAHIIVLSSHIAYGRGTPQYTWL

S.oligorrhiza  EQEFPKVNRSETPWLIVLMHSPLYNSYNYHYMEGETMRVMYEPLFVTYKV    340
A.thaliana     EKELQGVNRTETPWLIVLVHSPFYSSYVHHYMEGETLRVMYEQWFVKYKV
P.vulgaris     KKELRKVKRSETPWLIVLMHSPLYNSYNHHFMEGEAMRTKFEAWFVKYKV

S.oligorrhiza  DVIFAGHVHAYERSYRISNVAYNITDGKCTPTSDLSAPVYITVGDGGNQE    390
A.thaliana     DVVFAGHVHAYERSERVSNIAYNIVNGLCEPISDESAPIYITIGDGGNSE
P.vulgaris     DVVFAGHVHAYERSERVSNIAYKITDGLCTPVKDQSAPVYITIGDAGNYG

                                            0 1
S.oligorrhiza  GLASSMTEPQPNYSAYREASFGHAIFGIKNRTHAYYNWYRNQDGNAVEAD    440
A.thaliana     GLLTDMMQPQPKYSAFREASFGHGLLEIKNRTHAYFSWNRNQDGNAVAAD
P.vulgaris     VIDSNMIQPQPEYSAFREASFGHGMFDIKNRTHAHFSWNRNQDGVAVEAD

               2
S.oligorrhiza  SLWFFNRVWNPREES    455
A.thaliana     SVWLLNRFWRAQKKTWLDAF
P.vulgaris     SVWFFNRHWYPVDDST
```
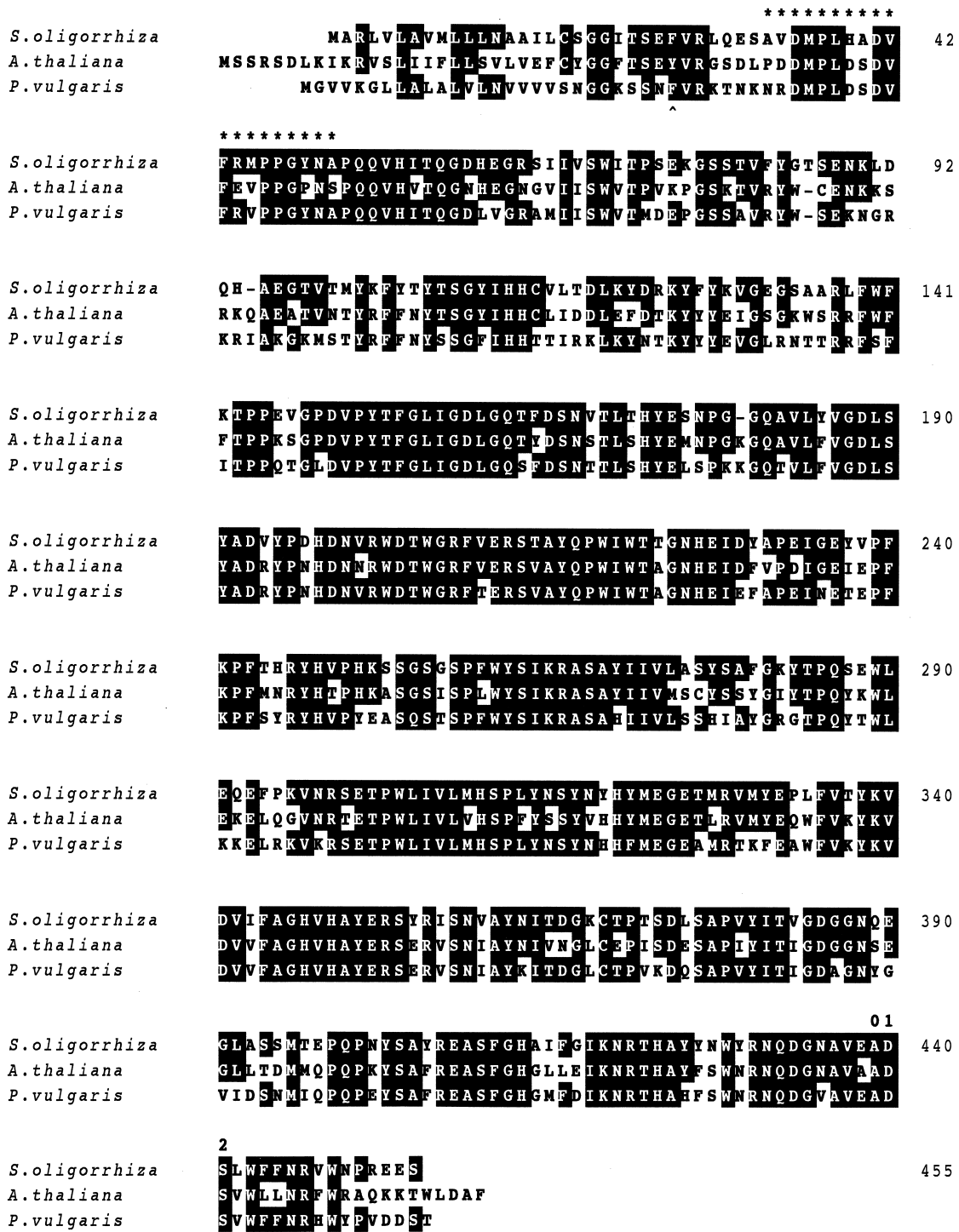
Fig. 1. Comparison of the deduced amino acid sequence of pHIP with those of the PAPs of *A. thaliana* and *P. vulgaris*. The conserved amino acids among sequences of *S. oligorrhiza*, *A. thaliana* and *P. vulgaris* PAPs are shown in white on black. Amino acids determined by sequencing the purified protein are indicated by asterisks. The phenylalanine residue marked by ^ is the N-terminal amino acid of the *P. vulgaris* PAP (Klabunde et al. 1994). The ω, $\omega + 1$ and $\omega + 2$ sites are indicated by 0, 1 and 2, respectively. Partial nucleotide and deduced amino acid sequences of pHIP were presented at the XIII International Plant Nutrition Colloquium, September 13–19, 1997, in Tokyo, Japan (Nakazato et al. 1997b).

Plasma membrane-localized GPI proteins

```
                                                 ***  *    ****  *  *  *  * *  ****  **** * *
  Exg2 (Yeast)        VLSSTTTSRKSKN    ↓         AAISNKLTTSQLLPIKNMSLTWKASVCALAITIAALCASL

                                                 *   *** *** ** ** ******
  Yap3 (Yeast)        STASATSTSSKRN    ↓         VGDHIVPSLPLTLISLLFAFI

                                                   * ***** ** *** *
  Mkc7 (Yeast)        LSPTSSSSPRKEN    ↓         GGHNLNPPFFARFITAIFHHI
```

Cell wall proteins

```
                                                 ***** * ** ******
  Sed1 (Yeast)        SSASSHSVVINSN    ↓         GAVVVPGLGLAGVAMLFL

                                                 * ** * **** ******
  Tip1 (Yeast)        TVETASNAGQRVN    ↓         AGAASFGAVVAGAAALLL

                                                   *** ********  ***
  Aga1 (Yeast)        TSSMVTISQYMGG    ↓         SGSQTRLPLGKLVFAIMAVACNVIFS

                                                 ** *  ****  ******** ***
  NaAGP1 (Nicotiana)  GSAPAGSPTSSPN    ↓         AASLNRVAVAGSAVVAIFAASLMF

                                   439             **** ** *
  PAP (Spirodela)     NWYRNQDGNAVEA    ↓         DSLWFFNRVWNPREES
```

signal sequences and no transmembrane domain in the mature protein as GPI-anchored proteins. Using PSORT, none of the PAPs were predicted to be GPI-anchored proteins. The PSORT result was probably based on the absence of a C-terminal transmembrane domain. However, the criteria utilized by PSORT in making its prediction may only select very typical GPI-anchored proteins. Indeed, proteins such as the animal Sm23 protein (Köster and Strand 1994) and the csA protein of slime mold (Stadler et al. 1989), which have been biochemically identified as GPI-anchored proteins, are not predicted to be GPI-anchored by PSORT.

Expression of the PAP gene and accumulation of PAP

Expression of the PAP gene in $+P$ and $-P$ plants of *S. oligorrhiza* was examined by northern blot analysis using pHIP1 as a probe. As shown in Fig. 3A, expression of the PAP gene depended on the duration of growth under phosphate-deficient conditions; that is, levels of the message of PAP that were negligible from day 0 ($+P$ plant) to day 2, significantly increased after day 3 and reached a maximum at day 8. Unfortunately, however, no positive signals have been obtained in plant samples after day 9 (data not shown), because sufficient amounts of RNA could not be recovered from these plant samples. According to Bieleski (1968b), phosphate depletion from *S. oligorrhiza* medium brings about remarkable enhancement of the degradation activity of endogenous phosphorus compounds; thus, it is considered that RNAs including mRNA would be almost completely degraded by hydrolytic enzymes in the preparation process of cell-free extracts. However, it can be presumed that the time-dependent enhancement of transcripts and

accumulation of PAP mRNA should be occurring in $-P$ plants after day 9 from results of western blot analysis of PAP (see below). The tendency of the time-dependent accumulation of PAP in $-P$ plants was found also in levels of
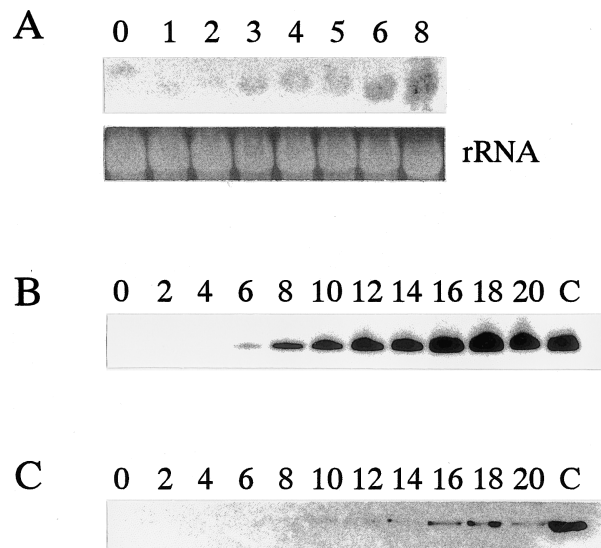

Fig. 3. Effects of phosphate depletion on induction of *S. oligorrhiza* PAP. (A) Northern blot analysis of PAP. $+P$ plants were transferred to phosphate-deficient medium and then grown for a number of days. Total RNA, 10 µg in each lane, was hybridized to the pHIP probe. Ethidium bromide staining of the gel is shown as loading controls. (B) Analysis of phosphatase activity. Cell-free extracts, 10 µg of protein in each lane and pure PAP in lane C, were electrophoresed under incompletely denaturing conditions (see Materials and methods). After electrophoresis, gels were stained for phosphatase activity. (C) Western blot analysis of PAP using the anti-*S. oligorrhiza* PAP antibody.
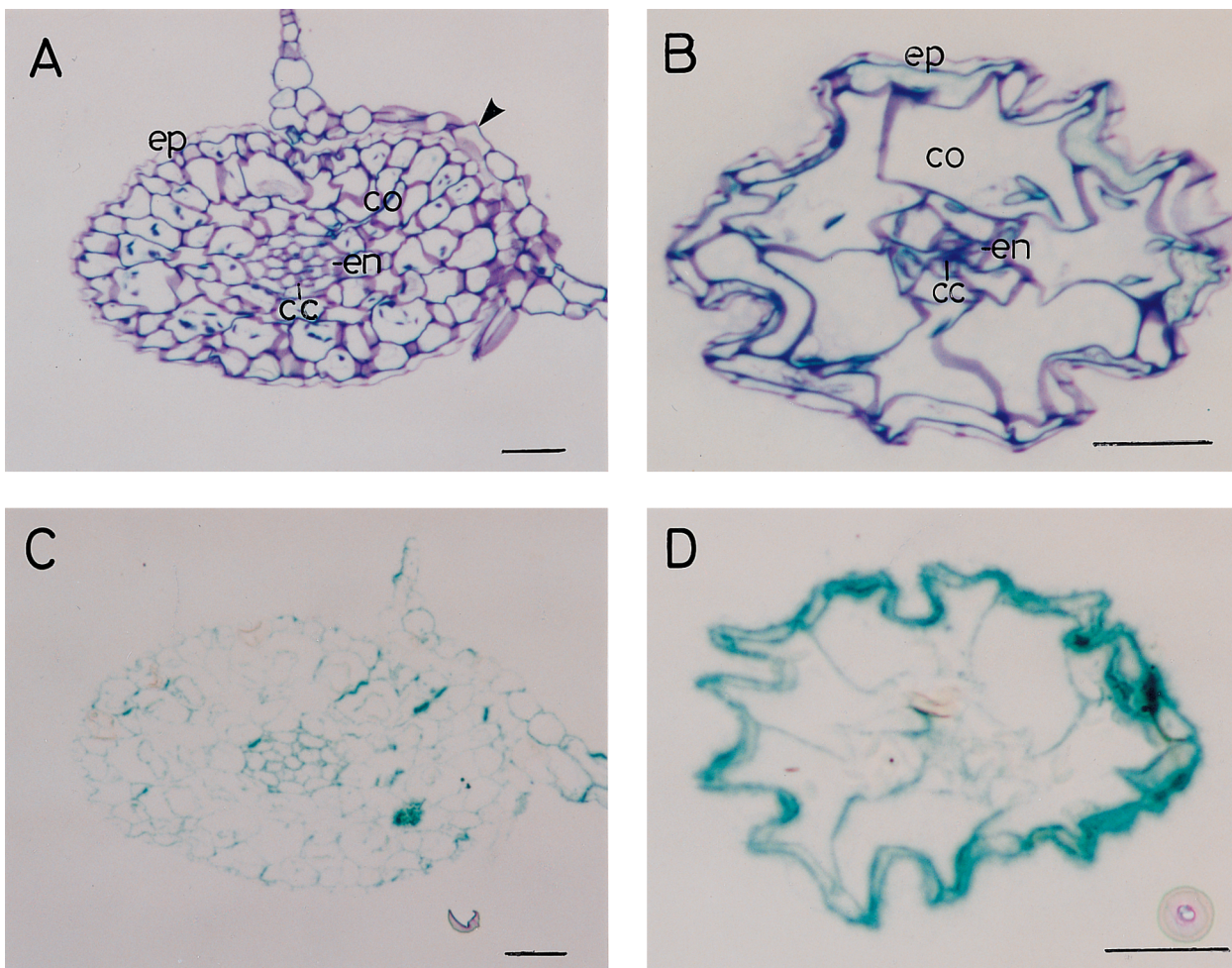
Fig. 4. Immunolocalization of PAP in roots of *S. oligorrhiza*. Transverse sections (2 μm) of roots were prepared from LR-White embedded + P (A,C) and − P (B,D) plants of *S. oligorrhiza* and stained in 0.1% toluidine blue (A,B) or immunolabeled for PAP (C,D). Intense PAP labeling is found around the outermost cortical cells of − P plant root (D). Arrowhead, root sheath; cc, central cylinder; co, cortex; en, endodermis; ep, epidermis; scale bars, 50 μm.

its protein and enzymatic activity. As shown in Fig. 3B, the increase in the phosphatase activity was visualized by activity staining of the phosphatase after SDS-PAGE of crude extracts under incomplete denaturing conditions. The phosphatase-active band appeared at day 6 and became increasingly intense with time. This increase in the PAP activity level coincided with the increase in its protein levels (Fig. 3C). These results suggest that the accumulation of PAP mRNA proceeds the accumulation of its protein by a few days.

Localization of PAP in *S. oligorrhiza*

More than 30 years ago Bieleski and coworkers presented the symptoms observed in *S. oligorrhiza* grown under phosphate-deficient conditions (Bieleski 1968a,b, Reid and Bieleski 1970a,b). These symptoms included retardation of the growth rate, changes in color from green to yellow in aged fronds, heavy development of anthocyanin in newly formed fronds, and so on, in addition to which induction of phosphatases and significant root elongation are listed as

the most significant symptoms. In + P plants each frond has 3 short (ca 5 mm) green roots, whereas in − P plants, fronds have roots which elongate up to 5 cm. Bieleski and Johnson (1972) showed induction and accumulation of phosphatase on the surface of roots and leaves of − P plants, which were visualized by in situ active staining of phosphatase.

In this study, the distribution of *S. oligorrhiza* PAP in roots was examined by immunostaining. Root tissue structures were first compared between + P and − P plants that had been stained with toluidine blue. As shown in Fig. 4A,B, + P plants had roots covered with root sheath, whereas in − P plants, root sheath was lost and roots had an undulated surface. The size of cortex cells of − P plants extended 2–3 times of that of + P plants and the epidermal cells of − P plants became longer than those of + P plants.

In addition to these morphological differences, a large accumulation of PAP was observed in − P plants. As shown in Fig. 4D, flattened cells situated just outside enlarged cortical cells were heavily immunolabeled in − P plants. Almost all the epidermal cells of roots in + P and − P

plants lost nuclei and their water-cell interface was weakly stained with toluidine blue (Fig. 4A, 4B). However, the epidermal cells in $-P$ plants did not show any immunolabeling of PAP (Fig. 4D). These results indicate that PAP is located in the outermost cortical cells, but not in the epidermal cells in $-P$ plants. By contrast, the accumulation of PAP was scarcely observed in $+P$ plant roots (Fig. 4C). Furthermore, anti-plasma membrane H^+-ATPase antiserum intensely reacted with the outermost cortical cells but not with the epidermal cells in $-P$ plants (M. Nishikoori, A. Hase and H. Okuyama, unpublished data), suggesting that the epidermis could be converted into a protection tissue having a suberinized cell wall and lose its uptake activity and that the outermost cortex might take the place of the epidermis for its functions. Thus, it is likely that adaptive morphological and biochemical changes would occur in the duckweed roots under starvation of phosphate and that a large amount of PAP would be accumulated in the cell wall of outermost cortical cells of the roots for the acquisition of phosphate.

In order to establish the distribution of PAP in the cell wall, total phosphatase activity was first measured by using ground dry material of $-P$ plants. The phosphatase activity at pH 8 was 301.6 μmol mg^{-1} [10 min]$^{-1}$ of dry mass (plant). This included the activity of at least two types of phosphatase, PAP and a low molecular mass phosphatase (Morita et al. 1996). Proteins were repeatedly extracted from powdered materials with 50 mM Tris-HCl. The cell wall fraction retained an activity of 35.1 μmol mg^{-1} [10 min]$^{-1}$ of dry mass (cell wall) after being washed with 50 mM Tris-HCl containing 1.0 M NaCl, indicating that approximately 12% of the total phosphatase activity was resistant to salt extraction. After the 1.0 M NaCl wash, cell walls were further treated with cellulase, supernatants of the digests were centrifuged at 10 000 g for 20 min and subjected to SDS-PAGE, and subsequently to activity staining and immunoblot analysis using the anti-PAP antibody. As shown in Fig. 5, only a phosphatase corresponding to PAP was liberated by treating the cell wall with cellulase, suggesting that some part (more than 12%) of the total S. oligorrhiza PAP binds to the cell wall by hydrophobic and/or by

covalent associations. Since the treatment of the cell wall with pectinase brought about almost the same results (data not shown), some PAP molecules would nonspecifically associate with the cell wall and other molecules might specifically link to cellulose or pectin of the cell wall. Although we do not yet have any direct evidence, we believe that cell wall unbound PAP is located outside of the cell, namely in the apoplastic space. Cashikar et al. (1997) have provided immunomicroscopic evidence for localization of the P. vulgaris PAP at the cell wall. Taken together, it is speculated that the final destination of PAP in S. oligorrhiza as well as P. vulgaris is the cell wall.

According to Vossen et al. (1997), GPI-anchored proteins of yeast have two destinations from the ER, one is the plasma membrane and the other is the cell wall. Interestingly, GPI-anchored proteins targeted to plasma membranes have a dibasic motif, 1–5 amino acids upstream of the ω site of the protein, while no such motif is present in GPI-anchored proteins targeted to the cell walls. As shown in Fig. 2, there is no sequence corresponding to the dibasic motif in the S. oligorrhiza PAP, suggesting that the final target of this protein is the cell wall. NaAGP1, a secreted alabinogalactan protein of $Nicotiana alata$, also has no dibasic motif (see Fig. 2). In yeast, signals for cell wall localization of GPI-anchored proteins are proposed (Hamada et al. 1999), that is, the specific amino acid residues Val, Ile or Leu at the side 4 or 5 amino acids upstream of the ω site and Tyr or Asn at the site 2 amino acid residues upstream of the ω site. However, such signals were not found in either S. oligorrhiza PAP or N. alata NaAGP1 (see Fig. 2), suggesting that cell wall-localized GPI proteins of plants would have a specific signal(s) for their localization to the cell wall.

The deduced amino acid sequence of the S. oligorrhiza PAP showed 82 and 80% similarity to that of PAP from A. thaliana and P. vulgaris, respectively, implying that the latter two PAPs are also originally synthesized as GPI-anchored proteins. We speculate that the GPI anchor could be used as a signal that leads the GPI-anchored protein to the cell wall in plants and yeast.
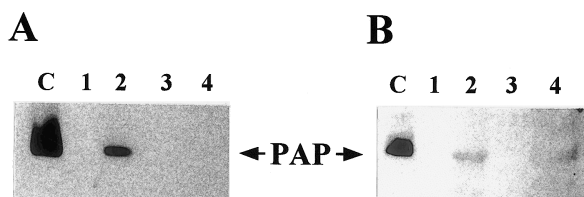
Fig. 5. Release of PAP from the cell wall fraction by digestion with cellulase. The cell wall fraction was prepared from $-P$ plants of S. oligorrhiza. Plant materials were freeze-dried, ground and extracted with 50 mM Tris-maleic buffer 5 times and then extracted with 50 mM Tris-maleic buffer containing 1.0 M NaCl twice. The cell wall fraction, which did not include soluble phosphatase, was treated with cellulase. The digests were centrifuged and the resulting supernatants were subjected to SDS-PAGE. Activity stains (A) and western blots (B) of PAP from supernatants of cellulase-treated digests. Lane 1, cellulase; lane 2, supernatants of digested cell walls with cellulase; lane 3, supernatants from cell walls incubated in water; lane 4, supernatants from cell walls incubated in 1 M of NaCl; lane C, pure PAP from S. oligorrhiza.

References

Bieleski RL (1968a) Levels of phosphate esters in Spirodela. Plant Physiol 43: 1297–1308

Bieleski RL (1968b) Effect of phosphorus deficiency on levels of phosphorus compounds in Spirodela. Plant Physiol 43: 1309–1316

Bieleski RL, Johnson PN (1972) The external location of phosphatase activity in phosphorus-deficient Spirodela oligorrhiza. Aust J Biol Sci 25: 707–720

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Cashikar AG, Kumaresan R, Rao NM (1997) Biochemical characterization and subcellular localization of the red kindney bean purple acid phosphatase. Plant Physiol 114: 907–915

Englund PT (1993) The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. Annu Rev Biochem 62: 121–138

Hamada K, Terashima H, Arisawa M, Yabuki N, Kitada K (1999) Amino acid residues in the ω-minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. J Bacteriol 181: 3886–3889

Kingston RE, Chomczynski P, Sacchi N (1999) Phenol/SDS method for plant RNA preparation. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl KS (eds) Short Protocols in Molecular Biology. John Wiley & Sons, New York, NY, pp 7–8

Klabunde T, Stahl B, Suerbaum H, Hahner S, Karas M, Hillenkamp F, Krebs B, Witzel H (1994) The amino acid sequence of the red kidney bean Fe(III)-Zn(II) purple acid phosphatase. Eur J Biochem 226: 369–375

Köster B, Strand M (1994) *Schistosoma mansoni*: Sm23 is a transmembrane protein that also contains a glycosylphosphatidylinositol anchor. Arch Biochem Biophys 310: 108–117

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

LeBansky BR, McKnight TD, Griffing LR (1992) Purification and characterization of a secreted purple phosphatase from soybean suspension cultures. Plant Physiol 99: 391–395

Morita N, Nakazato H, Okuyama H, Kim Y, Thompson GA Jr (1996) Evidence for a glycosylinositolphospholipid-anchored alkaline phosphatase in the aquatic plant *Spirodela oligorrhiza*. Biochim Biophys Acta 1290: 53–62

Nakazato H, Okamoto T, Ishikawa K, Okuyama H (1997a) Purification and characterization of phosphatase inducibly synthesized in *Spirodela oligorrhiza* grown under phosphate-deficient conditions. Plant Physiol Biochem 35: 437–446

Nakazato H, Okamoto T, Nishikoori M, Washio K, Morita N, Haraguchi K, Thompson GA Jr, Okuyama H (1997b) Characterization and cDNA cloning of the GPI-anchored phosphatase from *Spirodela oligorrhiza*. In: Ando T, Fujita K, Mae T, Matsumoto H, Mori S, Skiya J (eds) Plant Nutrition – For Sustainable Food Production and Environment. Kluwer Academic Publishers, Dordrecht, pp 229–230

Nakazato H, Okamoto T, Nishikoori M, Washio K, Morita N, Haraguchi K, Thompson GA Jr, Okuyama H (1998) The glycosylphosphatidylinositol-anchored phosphatase from *Spirodela oligorrhiza* is a purple acid phosphatase. Plant Physiol 118: 1015–1020

Posner HB (1967) Aquatic vascular plants. In: Witt FA, Wessels NK (eds) Methods in Developmental Biology. Crowell, New York, NY, pp 301–317

Reddy KJ, Gilman M (1999) Preparation of Poly(A) $^+$ RNA. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl KS (eds) Short Protocols in Molecular Biology. John Wiley & Sons, New York, NY, pp 11–12

Reid MS, Bieleski RL (1970a) Changes in phosphatase activity in phosphorus-deficient *Spirodela*. Planta 94: 273–281

Reid MS, Bieleski RL (1970b) Response of *Spirodela oligorrhiza* to phosphorus deficiency. Plant Physiol 46: 609–613

Schultz C, Gilson P, Oxley D, Youl J, Bacic A (1998) GPI-anchors on arabinogalactan-proteins: Implications for signalling in plants. Trends Plant Sci 3: 426–431

Stadler J, Keenan TW, Bauer G, Gerisch G (1989) The contact site a glycoprotein of *Dictyostelium discoideum* carries a phospholipid anchor of a novel type. EMBO J 8: 371–377

Thompson GA Jr, Okuyama H (2000) Lipid-linked proteins of plants. Prog Lipid Res 39: 19–39

Udenfriend S, Kodukula K (1995) Prediction of ω site in nascent precursor of glycosylphosphatidylinositol protein. Methods Enzymol 250: 571–582

Vossen J, Muller HW, Lipke NP, Klis MF (1997) Restrictive glycosylphosphatidylinositol anchor synthesis in cwh6/gpi3 yeast cells causes aberrant biogenesis of cell wall proteins. J Bacteriol 179: 2202–2209

Youl JJ, Bacic A, Oxley D (1998) Arabinogalactan proteins from *Nicotiana alata* and *Pyrus communis* contain glycosylphosphatidylinositol membrane anchor. Proc Natl Acad Sci USA 95: 7921–7926

Edited by L. McIntosh