Title	Induction of high affinity phosphate transporter in the duckweed Spirodela oligorrhiza
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Citation	Physiologia Plantarum, 120(2), 271-279 https://doi.org/10.1111/j.0031-9317.2004.0231.x
Issue Date	2004-02
Doc URL	https://hdl.handle.net/2115/15840
Rights	The definitive version is available at www.blackwell-synergy.com.
Type	journal article
File Information	PP120-2.pdf

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Induction of high affinity phosphate transporter in the duckweed *Spirodela oligorrhiza*

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Abstract

Duckweed plants (*Spirodela oligorrhiza*) grown under phosphate (Pi)-deficient conditions (–P plants) exhibited more than 50-fold higher Pi uptake activity than plants grown under Pi-sufficient conditions (+P plants). The Pi uptake activity of –P plants measured using ^{32}Pi was significantly inhibited by carbonylcyanide *m*-chlorophenylhydrazone, indicating that Pi uptake is energized by the electrochemical proton gradient across the plasma membrane (PM). When Pi uptake was examined at various concentrations of Pi, more active uptake of Pi was observed in –P plants than in +P plants, irrespective of the Pi concentrations. An immunoblot analysis of the PM proteins using antiserum against the conserved sequence of the high-affinity Pi transporter recognized the occurrence and large accumulation of a novel protein band at 48 kDa in –P plants. The protein was almost completely extracted with chloroform-methanol (2:1, v/v), but only a trace amount of the protein was detected in +P plants. Immunohistochemical studies of plant roots using the same antiserum demonstrated a large accumulation of high-affinity Pi transporters at the outermost cortical cells of –P plants, but not of +P plants. When an immunoblot analysis of PM proteins was performed using antiserum against the PM H^+-ATPase, a positive band of about 96 kDa was detected in both plants with a similar signal intensity. Furthermore, ATP-hydrolytic and ATP-dependent H^+-transporting activities of PM H^+-ATPase in –P plants were not higher than those in +P plants. However, kinetic analyses showed that the PM H^+-ATPase in –P plants had a lower K_m value and a higher coupling efficiency between ATP hydrolysis and H^+ pumping than the corresponding values in +P plants. These results suggest that the significant stimulation of Pi uptake in –P plants may be due mainly to the induction and accumulation of the high-affinity Pi transporter in the PM, and that the electrochemical proton gradient across the PM may be generated by the high-ATP-affinity and energy-efficient H^+ pump in –P plants. This would facilitate the acquisition of Pi in *S. oligorrhiza* under Pi-depleted conditions.

Abbreviations: BTP, bis-tris propane; CCCP, carbonylcyanide *m*-chlorophenylhydrazone; CM, chloroform-methanol; GPI, glycosylphosphatidylinositol; KLH, keyhole limpet hemocyanin; PAP, purple acid phosphatase; PM, plasma membrane.

Introduction

Duckweed (*Spirodela oligorrhiza*) is a plant that responds to phosphate (Pi)-depletion by various modifications. These include slowing the growth rate, significant elongation of the roots, changes in color from green to yellow in aged fronds, heavy development of anthocyanin in newly formed fronds, and a huge accumulation of phosphatases (Bieleski 1968a, b, Reid and Bieleski 1970a, b). These kinds of modifications found in plants grown under Pi-depleted conditions are well summarized by Raghothama (1999).

Morita et al. (1996) provided evidence that one of the Pi-depletion-induced phosphatases of *S. oligorrhiza* is a glycosylphosphatidylinositol (GPI)-anchored protein. Interestingly, this type of phosphatase is recovered mainly as a soluble protein in *S. oligorrhiza*, suggesting that the GPI-anchored phosphatase is cleaved from the plasma membrane (PM) by one or more GPI-specific phospholipases (Morita et al. 1996, Nishikoori et al. 2001). Nakazato et al. (1998) showed that the GPI-anchored phosphatase is a purple acid phosphatase (PAP). Immunohistochemical results using Pi-starved plant roots demonstrate that PAP is preferentially distributed in the outermost cortical cells of roots, but not in the epidermis (Nishikoori et al. 2001). These results suggest that the PAP of this plant would play a role in acquiring inorganic phosphate under Pi-deficient conditions.

Phosphate is taken up by plant roots in an energy-dependent cotransport process, which is driven by the proton gradient generated by PM H^+-ATPases (Ullrich-Eberius et al. 1981, Sakano 1990, Raghothama 1999). It is well known that Pi-starved plants (Ullrich-Eberius et al. 1984, Shimogawara and Usuda 1995), as well as many microorganisms (Harrison and van Buuren 1995), exhibit an increased capability for Pi uptake. The kinetic characterization of the Pi-uptake activity of plant cells indicates the presence of a high- and low-affinity Pi-transporting system (Furihata et al. 1992, Sakano et al. 1995, Shimogawara and Usuda 1995). The low-affinity system operates under normal circumstances, and the high-affinity system can be induced under Pi-depleted conditions (Furihata et al. 1992). Thus, it is considered that plants subjected to Pi-depletion would develop a Pi-acquisition system that consisted of a high-affinity Pi transporter, a PM H^+-ATPase, and probably a phosphatase as a Pi-recruiting enzyme to overcome Pi deficiency. Muchhal and Raghothama (1999) provided evidence that Pi-depletion in the roots of tomato plants resulted in accumulated levels of not only high-affinity Pi transporter mRNA but also its protein. However, little has been reported on the effects of Pi-depletion on the whole system, including the PM H^+-ATPase and other related enzymes for Pi-acquisition in plants (see Raghothama 1999). In this study, we describe the enhancement of the Pi uptake activity of Pi-starved plants of *S. oligorrhiza*, accompanied by an increased accumulation of the Pi transporter in the PM, and its preferential distribution at the outermost cortical cells of roots, and with a qualitative alteration of the PM H^+-ATPase. A possible involvement of GPI-anchored PAP in Pi-acquisition in this plant is also discussed.

Materials and methods

Plant material and growth conditions

S. oligorrhiza plants were grown in modified Hoagland's medium (Posner 1967) containing either 1.5 or 0 m*M* KH_2PO_4 for 2 weeks at 25ºC under 16-h day length with illumination from fluorescent lamps (80 μE m^{-2} s^{-1}) (Nakazato et al. 1997). Plants grown in the presence of Pi and in the absence of Pi were designated +P plants and –P plants, respectively. Fresh plants immediately after harvest were used for Pi-transporting experiments and for membrane preparations.

Pi transport

Harvested *S. oligorrhiza* plants were washed once with 50 m*M* Tris-maleic acid buffer, pH 8.5. Five plants (10 to 20 mg) were incubated in 2 ml of Tris-maleic acid buffer containing 0.1 m*M* non-radioactive Pi (as KH_2PO_4) and 1 μCi ^{32}Pi (as $H_3{}^{32}PO_4$, 500 mCi ml^{-1}; ICN Biomedicals, Inc., Irvine, CA, USA). After incubation plants were transferred to 2 ml water and then washed two times with another 2 ml of water. Finally plants were transferred to 2 ml of 1.5 m*M* phosphate buffer and incubated for 30 sec. Inhibition of the Pi uptake activity was investigated by adding carbonylcyanide *m*-chlorophenylhydrazone (CCCP) dissolved in ethanol to the incubation mixture of plants. Plant samples were then blotted on Kimwipe paper (type S-200; Crecia, Tokyo, Japan) and immersed in 5 ml of scintillation liquid (Scintizol EX-H; Dojindo Lab., Kumamoto, Japan). Radioactivity was measured with a liquid scintillation counter (type LC-1000; Aloka, Tokyo). The specific Pi-transport activity was expressed as nmol of Pi taken-up per mg of plants per 10 min. For the kinetic analysis of Pi uptake, the activity of +P and –P plants was examined at various concentrations of Pi from 0 to 4000 μ*M*.

Preparation of PM vesicles and chloroform-methanol treatment

Right-side out PM vesicles of +P and –P plants cultured for 2 weeks or designated days were prepared as described by Yoshida et al. (1983) with some modifications (Hase, 1993). Briefly, whole plants (about 10 g) were homogenized with a Polytron homogenizer in a homogenizing medium (50 ml) at 0°C. The homogenizing medium consisted of 0.25 *M* sucrose, 75 m*M* MOPS-KOH (pH 7.6), 1.5% (w/v) soluble PVP (mol wt 24,500), 0.5% (w/v) defatted BSA, 5 m*M* EGTA, 5 m*M* EDTA, 1 m*M* phenylmethylsulfonyl fluoride, 4 m*M* salicylhydroxamic acid, 10 m*M* KF, 10 mg ml^{-1} butylated hydroxytoluene, and 5 m*M* potassium metabisulfite. Five grams of washed Polyclar SB-100 were then added, the homogenate was filtered through two layers of cheesecloth and microsomal fractions were obtained by differential centrifugation. PM vesicles were isolated from the microsomal fraction by aqueous two-polymer phase partitioning using a phase system composed of 5.6% (w/v) PEG P3640, 5.6% (w/v) dextran T500, 30 m*M* NaCl, 10 m*M* K-phosphate (pH 7.8) and 0.25 *M* sucrose, as described by Yoshida et al. (1983).

An aliquot of PM vesicles was subjected to chloroform-methanol (CM, 2:1, v/v) treatment to extract intrinsic membrane proteins according to the method of Seigneurin-Berny et al. (1999), and CM-insoluble and soluble fractions were recovered.

Assay of ATP-hydrolytic and H^+-transport activities

ATPase activity in the PM vesicles was measured by determining the amount of released Pi at 25°C for 10 min as described by Ames (1966). The assay mixture (125 µl) consisted of the indicated concentration of ATP, 3 m*M* $MgSO_4$, 50 m*M* KCl, 1 m*M* Na_2MoO_4, 5 m*M* NaN_3, 0.05% (w/v) Brij 58, 25 m*M* HEPES/bis-tris propane (BTP) adjusted to the specified pH, and 10 to 20 µg PM protein. Under the conditions used in the present assays, the reaction progressed linearly at least for 15 min. The activity measured in the presence of 0.1 m*M* vanadate was subtracted from that measured in its absence and vanadate-sensitive ATPase activity was determined.

For the measurement of H^+-transporting activity, the right-side out PM vesicles prepared by the aqueous two-polymer phase partitioning were converted to inside-out oriented vesicles by treatment with 0.05% (w/v) Brij 58 as described by Johansson et al. (1995), and activity of ATP-dependent H^+ transport was measured as the initial rate of fluorescence-quenching of the permeant dye acridine orange and expressed as $\%\Delta F$ mg^{-1} min^{-1} (Yoshida et al., 1989). The assay mixture (1 ml) contained 0.25 *M* sorbitol, the indicated concentration of ATP, 50 m*M* KCl, 3 µ*M* acridine orange, 0.05% (w/v) Brij 58, 25 m*M* HEPES/BTP adjusted to the specified pH, and 20 µg PM protein. The reaction was initiated by addition of $MgSO_4$ to a final concentration of 3 m*M* to the above mixture. Changes in fluorescence were monitored with a Hitachi 650-10S fluorescence spectrophotometer set at 493 nm for excitation and 520 nm for emission at 25°C.

Electrophoresis and immunoblot analysis

PM proteins were solubilized with a sample buffer containing 2% (w/v) SDS, 100 m*M* DTT and 0.125 *M* Tris-HCl (pH 6.8) and separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS as described by Laemmli (1970). Separated proteins were visualized by silver stain as described previously (Hase, 1993) or subjected to immunoblotting.

For raising specific antisera against the Pi transporter, PM H^+-ATPase and 14-3-3 protein, oligopeptides with a highly conserved sequence (SATIMSEYANKKTRG) corresponding to amino acids 148-162 of *Arabidopsis* AtPT1 and AtPT2 (Muchhal et al., 1996) in the predicted second cytoplasmic loop of the high-affinity Pi transporter, a conserved sequence (MTGDGVNDAPALKKAD) corresponding to amino acids 500-515 of tobacco PMA1 (Boutry at al., 1989) at an ATP-binding site in the large cytoplasmic loop of the PM H^+-ATPase and a conserved sequence (EAIAELDTLGEESYKD) corresponding to amino acids 204-219 of *Arabidopsis* GF14 (Lu et al., 1992), respectively, were synthesized, conjugated to keyhole limpet hemocyanin (KLH), and then injected in rabbits as described by Liu et al. (1979). PM proteins were electroblotted from the SDS-PAGE gels to nitrocellulose filters using a semidry blotting apparatus. Immuno-reactive proteins were visualized by an enhanced peroxidase-chemiluminescence system (ImmunoStar Reagent, Wako Pure Chemicals, Tokyo, Japan). The abundance of antigens was densitometrically quantified on the image-developed X-ray films using a densitometer (DM-303, Advantec-Toyo, Tokyo, Japan).

Immunohistochemical analysis of Pi transporter

For immunohistochemical analysis of Pi transporter whole +P and –P plants were fixed and embedded in LR White resin (type medium; London Resin, Berkshire, UK) as described previously (Nishikoori et al. 2001). 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 Zymed Histostain-SP kit (Zymed Laboratories, S. San Francisco, CA) according to the manufacturer’s protocol. The primary antiserum against the Pi transporter was diluted to 1:100 and treated for 1 h at 25°C. Resulting peroxidase-labeled complexes were visualized with TrueBlue Peroxidase Substrate (KPL, Gaithersburg, MD).

Protein determination

Protein content was measured by the methods of Lowry et al. (1951), after precipitation with TCA, or by Bradford (1976), with BSA as a standard.

Results and discussion

Pi uptake activity of +P and –P *S. oligorrhiza* plants

The Pi uptake activity of *S. oligorrhiza* +P and –P plants cultured for two weeks was measured using KH_2PO_4 containing $H_3{}^{32}PO_4$ as a substrate. The time-dependent linear uptake of Pi in a concentration of Pi at 0.1 m*M* was observed for 30 min for +P and –P plants (Fig. 1). The specific Pi uptake activity was calculated as 0.0025 ± 0.0010 and 0.129 ± 0.008 nmol Pi mg^{-1} plant (10 min)$^{-1}$ for +P and –P plants, respectively. A similar significant enhancement of Pi uptake in plant cells grown under Pi depletion has commonly been observed. Namely, the Pi uptake activity of Pi-starved suspension-cultured cells of *Nicotiana tobacum* (Shimogawara and Usuda 1995) and *Catharanthus roseus* (Schmidt et al. 1992) was approximately five times greater than that of non-starved cells. The V_{max} for Pi uptake increased over 10-fold in Pi starved cells of *Chlamydomonas reinhardtii* cells (Shimogawara et al. 1999) and *Brassica nigra* suspension-cultured cells (Lefebvre et al. 1990). To our knowledge, however, a 50-fold enhancement in the specific Pi uptake activity of *S. oligorrhiza* is the maximum value ever reported for plant sources. This increase in Pi uptake could mainly be explained by the remarkable induction of high-affinity Pi transporter(s) in –P plants of *S. oligorrhiza*, as described below.

To understand the transporting mechanism of Pi in *S. oligorrhiza*, the uptake of Pi was examined using –P plants in the presence of CCCP, a proton ionophore. The uptake of Pi for 10 min was 64% and 89% when inhibited by CCCP at 1×10^{-5} and 1×10^{-4} *M*, respectively (data not shown). This indicated that the Pi transport of the plant is energized by the proton gradient across the PM, as is the case with other plants and microorganisms (Ullrich-Eberius et al. 1984, Harrison and van Buuren 1995). No inhibition by CCCP was observed in Pi uptake in +P and –P plants for the first minute (data not shown). This could be explained by low incorporation of CCCP in the roots during this short time.

The Pi uptake activity of +P and –P plants was examined at various concentrations of Pi from 0 to 4000 μ*M*. More active uptake of Pi was observed in –P plants than in +P plants, irrespective of Pi concentrations (Fig. 2), but in particular, at lower concentrations of Pi (up to 300 μ*M*) the Pi-transporting activity in –P plants was significantly enhanced compared to that in +P plants. In terms of K_m for the Pi of high-affinity Pi transporters, values of 2 to 10 μ*M* have been reported in *Lemna gibba* plants (6 to 8 μ*M*; Ullrich-Eberius et al. 1984), *Catharanthus roseus* protoplasts (3 μ*M*; Furihata et al. 1992), and tobacco cultured cells (2.5 μ*M*; Shimogawara and Usuda 1995). A relatively high K_m value of 12.3 μ*M* has been reported for the high affinity Pi transporter of *Arabidopsis thaliana* (Dunlop et al. 1997). On the other hand, K_m values of low-affinity plant Pi transporters ranged from approximately 50 to 100 μ*M* in the plants tested (see Shimogawara and Usuda 1995). Although apparent K_m values of Pi uptake have not been determined in *S. oligorrhiza* because of their large fluctuation, enhancement of Pi uptake in –P plants at Pi concentrations higher than 1 μ*M* (Fig. 2) suggests that both high- and low-affinity Pi transport would be involved in Pi uptake in –P plants. This is because a low-affinity Pi transporter(s) is generally operative even in plants grown under low Pi conditions (Furihata et al. 1992; Shimogawara and Usuda 1995). In general, Pi transporters are preferentially distributed in roots under Pi starvation. However, some Pi transporters in plants such as the tomato (Liu et al. 1998), potato (Leggewie et al. 1997) and Arabidopsis

(Karthikeyan et al. 2002) are also expressed in other organs. Therefore, it is considered that Pi transporter(s) would be expressed not only in the roots (see below), but also in the leaves of *S. oligorrhiza* –P plants. Ultimately, it is suggested that one or several Pi transporters having high affinity for Pi are induced and accumulated in *S. oligorrhiza* under low Pi conditions. Thus, the very high specific Pi uptake activity in –P plants would be affected by both low-affinity Pi transport and the induced high-affinity Pi transport. However, we have no evidence that low-affinity Pi uptake increases in –P plants.

Occurrence and accumulation of the Pi transporter in the PM of *S. oligorrhiza*

PM vesicles were purified from +P plants (day 0) and –P plants cultured for up to 14 days (Fig. 3). Solubilized PM proteins were subjected to SDS-PAGE and immunoblotting with several antisera against PM proteins. As shown in Fig. 3A (Total), significant changes in protein profiles were not found during Pi starvation in the total PM protein fractions, although a few polypeptides decreased. The antiserum against the conserved sequence of high-affinity Pi transporters reacted with a polypeptide of 48 kDa of the total PM proteins from –P plants (Fig. 3B) and the intensity of its immunodetectable signal increased drastically with the time of Pi starvation (Fig. 3B, C). A trace signal was only observed in the blot of the +P plants (day 0). These findings are comparable with the results of the Pi uptake experiments (Figs. 1 and 2).

The 48 kDa size was 10 kDa smaller than the predicted molecular mass (58 kDa) of the high-affinity Pi transporter of higher plants (Muchhal et al. 1996). Therefore, to clarify the specificity of the antiserum against the Pi transporter, several experiments were conducted, and the following results were obtained. First, preimmune serum and other antisera against KLH-conjugated oligopeptides, after dilution to the same concentration of the antiserum against the Pi transporter, did not react with the 48 kDa polypeptide (data not shown). Second, immunoblotting of the crude microsomal fractions from –P plants showed only a trace of the signal at 48 kDa (data not shown); the signal was enriched in PM fractions of –P plants (Fig. 3B, Total). Third, when CM-insoluble fractions obtained by treating PM vesicles with chloroform-methanol (CM) (2:1, v/v) were subjected to SDS-PAGE and immunoblotting, only the signal of 48 kDa diminished to a large extent (Fig. 3B, CM-Insoluble).

The CM-insoluble fractions showed electrophoretic profiles with a lower background than total PM fractions, while the polypeptide patterns of both fractions were similar (Fig. 3A). This indicated that the high background of the total PM fractions would be due to the involvement of CM-soluble, highly hydrophobic membrane proteins. Unfortunately, no hydrophobic proteins could be re-extracted from the CM-soluble fractions, probably because of the formation of hard aggregates.

The immunodetectable signal of 48 kDa was scarcely found in the CM-insoluble PM fractions from –P plants. However, signals of the PM H^+-ATPase, which is an intrinsic membrane protein with a large hydrophilic cytoplasmic loop and a long hydrophilic C-terminal region and the 14-3-3 protein, a soluble PM H^+-ATPase-binding protein, were clearly observed in the CM-insoluble fractions of +P and –P plants (Fig. 3B). The signal intensity of the PM H^+-ATPase in the CM-insoluble fractions was 50 to 60% of that of the total PM fractions, and the signal intensity of the 14-3-3 was almost identical in both fractions. These results strongly suggest that the 48 kDa polypeptide is so highly hydrophobic as to be almost completely extracted with CM. An increase of about 10 kDa in the mobility of very hydrophobic proteins in SDS-PAGE gels has been also observed in tonoplast H^+-pyrophosphatase, a highly hydrophobic tonoplast protein, as described by Maeshima (2001). Consequently, we concluded that the immunoreacted 48 kDa polypeptide is a high-affinity Pi transporter of duckweed PM.

Localization of the Pi transporter in *S. oligorrhiza* roots

S. oligorrhiza +P and –P plants show various types of morphological difference in their roots (Reid and Bieleski 1970b, Nishikoori et al. 2001); namely, +P plants have short roots covered with a root sheath, and in –P plants the root sheath is lost and the roots are fine and long. The size of the cortex cells of –P plant roots extended two to three times that of +P plants (Nishikoori et al. 2001, see Fig. 4A, C). In this study, the distribution of the Pi transporter in *S. oligorrhiza* roots was examined by immunostaining, using the same antiserum as described above. As shown in Fig. 5B, accumulation of the Pi transporter signal was scarcely observed in +P plant roots. However, the outward surface of flattened cells situated just outside enlarged cortical cells was heavily stained in –P plants (Fig. 4D). Although the possibility cannot be excluded that the inward surface of epidermal cells was stained, it is reasonable to consider that only the outward surface of flattened cortical cells was stained. As described in our previous article (Nishikoori et al. 2001), the root epidermis of –P plants of *S. oligorrhiza* could be converted into a protection tissue having a suberinized cell wall and lose its uptake activity, and the outermost cortex might take the place of the epidermis for this function. This type of localization of the Pi transporter would drastically affect Pi fluxes and the local concentrations of Pi near the Pi transporters, as discussed by Raghothama (1999). The finding that the high-affinity Pi transporter is preferentially distributed only at the outermost cortical cells of *S. oligorrhiza* roots is very different from the finding that two high-affinity Pi transporters are dominantly expressed in the root epidermis and root hairs of Pi-starved tomato roots (Daram et al. 1998). However, the role of Pi transporters in both cases can be regarded as analogous in acquiring Pi from the rhizosphere. On the other hand, the dominant distribution of the high-affinity Pi transporter at the outer surface of the outermost cortical cells in –P plants indicates that its specific localization is at the epidermis-facing PM of the same cells. This was very different from the distribution of PAP localized in the surrounding cell wall areas of the same cells (Nishikoori et al. 2001).

ATP-hydrolytic and H^+-transport activities in PM vesicles from *S. oligorrhiza*

When ATP-hydrolytic and ATP-dependent H^+-transport activities in the PM vesicles from the duckweed were assayed at ATP concentrations higher than 2 m*M*, inhibitions of the activities occurred (data not shown). Therefore, all the comparative studies between the +P and –P plants were performed using assay mixtures containing 2 m*M* or lower concentrations of ATP in a HEPES/BTP buffer at pH 6.5 (the optimum pH of the enzyme) or pH 7.0 (near the physiological pH of the cytoplasm). As shown in Table 1, ATP-hydrolytic and H^+-transport activities at pH 6.5 were about 1.5-fold higher than those at pH 7.0 in both plants. The relative amount of the PM H^+-ATPase in –P plants was 94% that of +P plants. This was consistent with the result that the H^+-transport activities of the PM vesicles in –P plants were 91% and 94% those of +P plants at pH 6.5 and 7.0, respectively. Nonetheless, the vanadate-sensitive ATP-hydrolytic activity in –P plants was only 50 to 60% that of +P plants (Table 1). Kinetic parameters (K_m and V_{max}) of the vanadate-sensitive ATPase activity and the ATP-dependent H^+-transport activity, which were calculated from the activities assayed with 3 m*M* $MgSO_4$ and ATP levels from 0 to 2 m*M* at pH 6.5 or pH 7.0, are listed in Table 2. At the optimum pH (pH 6.5), the apparent K_m values for the ATP of both activities in –P plants were significantly lower than those in +P plants, and they increased at pH 7.0 (Table 2). On the contrary, the V_{max} values of both activities in +P plants at pH 6.5 were much higher than those in –P plants (Table 2). Thus, in –P plants, a high-affinity and lower-reaction-rate type of H^+ pumping activity might be induced by Pi starvation. Alternatively, as reviewed by Morsomme and Boutry (2000), it is also possible that PM H^+-ATPase of –P plants would be modulated by its phosphorylation or by the binding of regulatory 14-3-3 proteins, which would modify the H^+-ATPase activity. As shown in Fig. 3B, however, the content of 14-3-3 proteins in the PM fractions did not change during Pi starvation. Therefore, their involvement in the modification of H^+-ATPase activity in –P plants is obscure at present.

Despite the large activation of Pi uptake (Figs. 1 and 2) and the accumulation of a Pi transporter in –P plants (Fig. 3B, C), the relative amount and the ATP-hydrolytic and ATP-dependent H^+-transport activities of PM H^+-ATPase in –P plants were not higher than those in +P plants (Table 1, Fig. 3B). The PM H^+-ATPase is a housekeeping enzyme that generates an electrochemical H^+ gradient and energizes the active transport of nutrients and ions. Therefore, metabolically active cells and tissues such as guard cells, elongating root hairs and expanding leaf border parenchyma cells have a relatively large accumulation of the PM H^+-ATPase (Michelet and Boutry 1995). Since +P plants of duckweed grow more rapidly than –P plants and have very short roots, the relatively high activity of the PM H^+-ATPase in +P plants may be mainly due to the expanding leaves. On the other hand, the PM H^+-ATPase of –P plants had a lower K_m value and a higher coupling efficiency between ATP hydrolysis and H^+ pumping than +P plants, especially when assayed at pH 6.5 (see Tables 1 and 2). Since –P plants have very long surface-undulant roots, the kinetic properties of the root PM H^+-ATPase in –P plants may at least partly reflect the increased proportion of root tissue. To verify the occurrence of the new-type PM H^+-ATPase in –P plant roots, PM vesicles must be isolated from the roots of *S. oligorrhiza* and characterized for the enzyme. However, as the duckweed is a very small plant and the roots of –P plants are very fine (about 100 µm in diameter), PM isolation from –P plant roots in a sufficient amount for assay is difficult to achieve.

In conclusion, the large stimulation of Pi uptake in –P plants of *S. oligorrhiza* may be mainly due to the induction and accumulation of one or several high-affinity Pi transporter(s) in –P plants by Pi starvation (Figs. 1 and 3). The high ATP-affinity and energy-efficient H^+ pump in –P plants may facilitate the Pi uptake by increasing the H^+ gradient across the PM under the ATP-deficient conditions enforced by Pi removal. This suggests that a new PM H^+-ATPase isoform having a high affinity against ATP and improved coupling efficiency is induced by Pi starvation and accumulated in –P plants. Immunohistochemical studies clearly demonstrated large accumulations of high-affinity Pi transporters (Fig. 4D) and PAP (Nishikoori et al. 2001) at the PM and cell wall of the outermost cortical cells, respectively. Our preliminary immunohistochemical observations of *S. oligorrhiza* –P plant roots shows that PM H^+-ATPase is localized at the outermost cortical cells, as was the case with the high-affinity Pi transporter (M. Nishikoori, A. Hase and H. Okuyama, unpublished data). Thus, the accumulation of the high-affinity Pi transporter(s) at a place close to the root surface and the large stimulation of Pi uptake could be regarded as a response coordinated with the induction of PAP and the probable occurrence of the high-affinity and energy-efficient H^+ pump in the –P plants. As a result, *S. oligorrhiza* creates a multifunctional system for Pi acquisition.

Acknowledgments. We are grateful to Emeritus Professor G. A. Thompson, Jr of the University of Texas at Austin for his critical review of the manuscript. This work was partially supported by Grants-in-Aid for Scientific Research (C) (No. 10640623) from the Ministry of Education, Science and Culture of Japan and by The Suhara Memorial Foundation.

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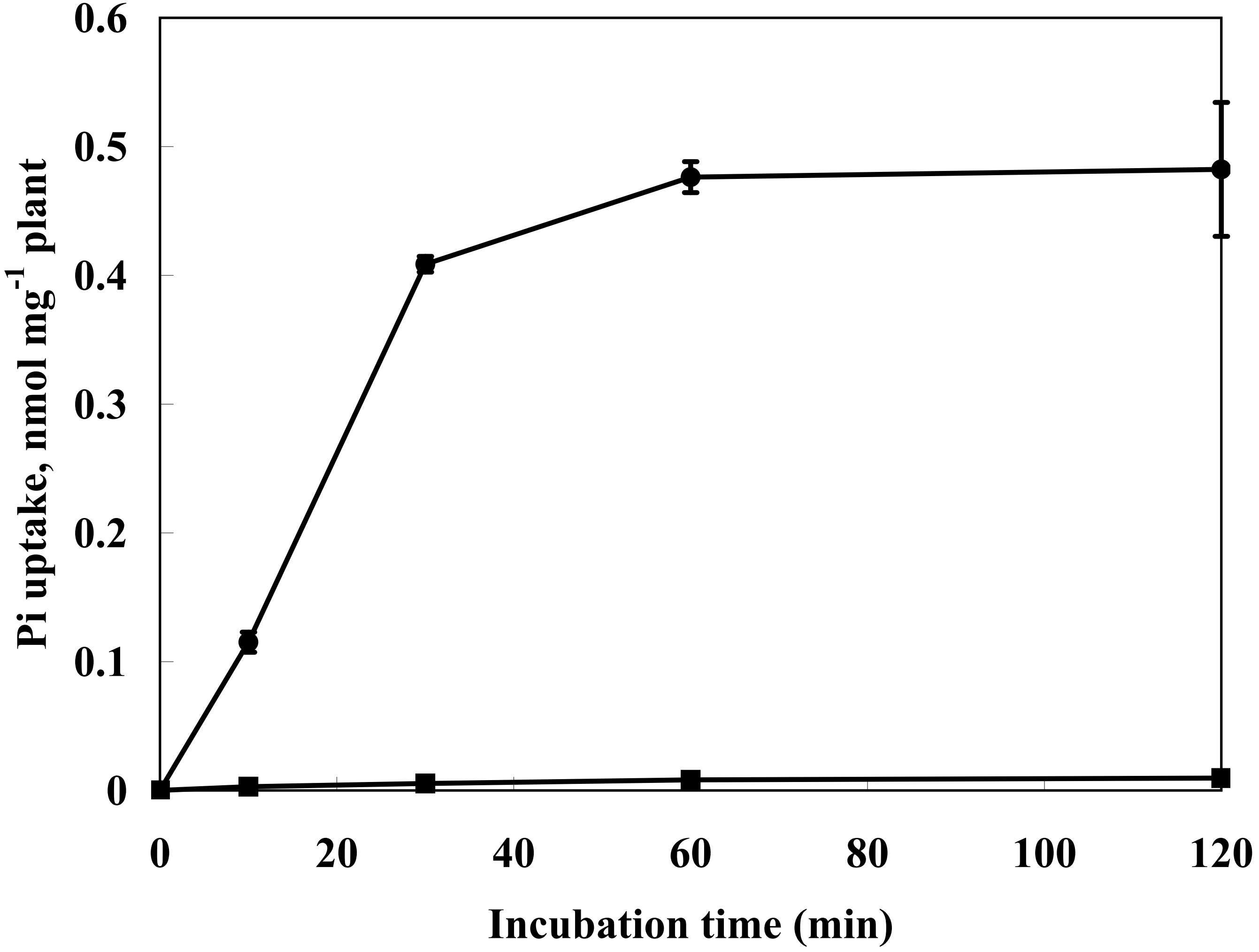
Figure legends

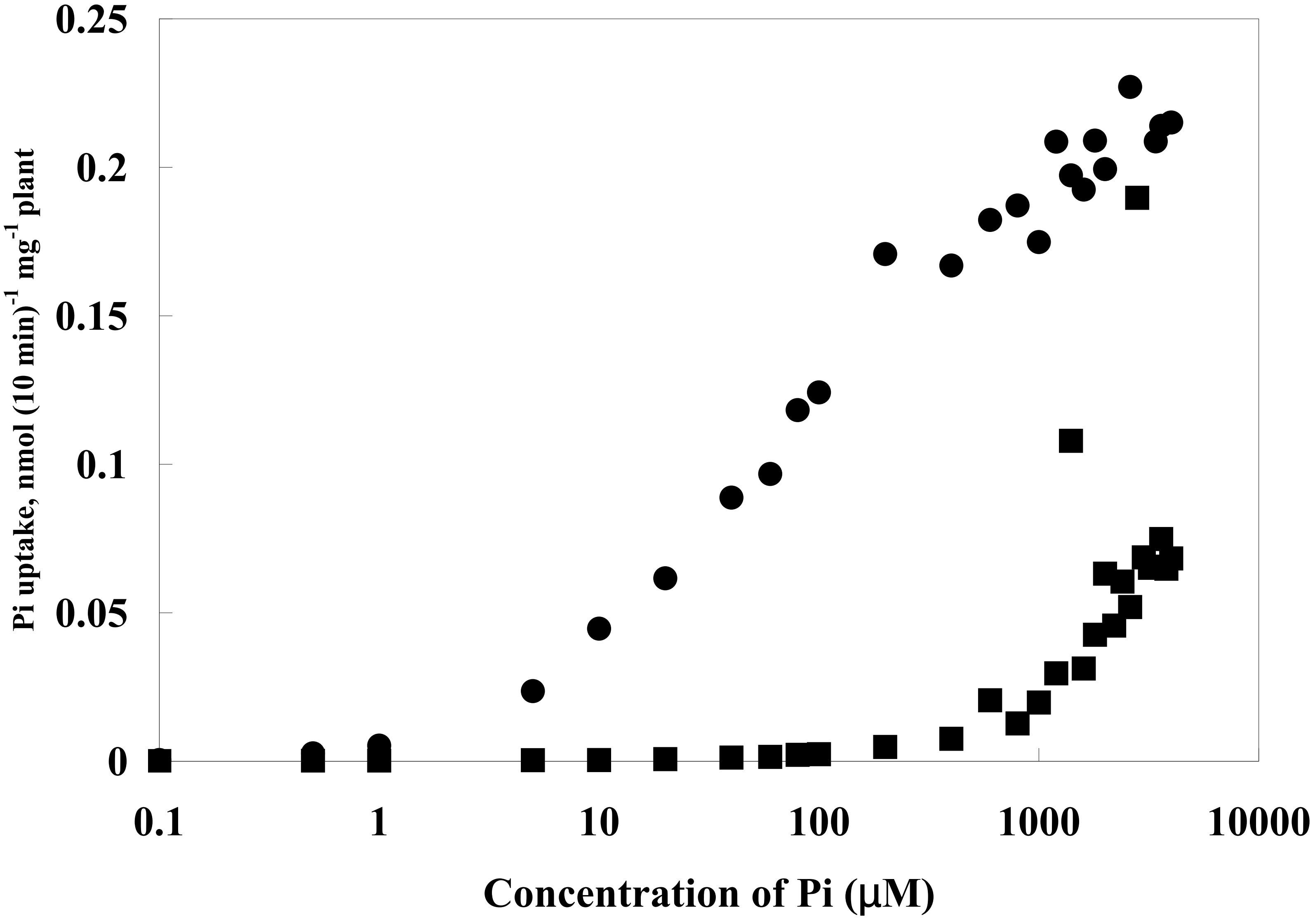
Fig. 1. Time dependence of the Pi uptake activity of *S. oligorrhiza*. Five each of +P (■) and –P (●) *S. oligorrhiza* plants (10 to 20 mg) were floated in 2 ml of Tris-maleic acid buffer containing 0.1 m*M* non-radioactive Pi (as KH_2PO_4) and 1 µCi ^{32}Pi (as $H_3{}^{32}PO_4$) for the indicated times. The radioactivity of plants was measured as described in the **Materials and methods** section. Each value is the mean of three independent assays with SE.

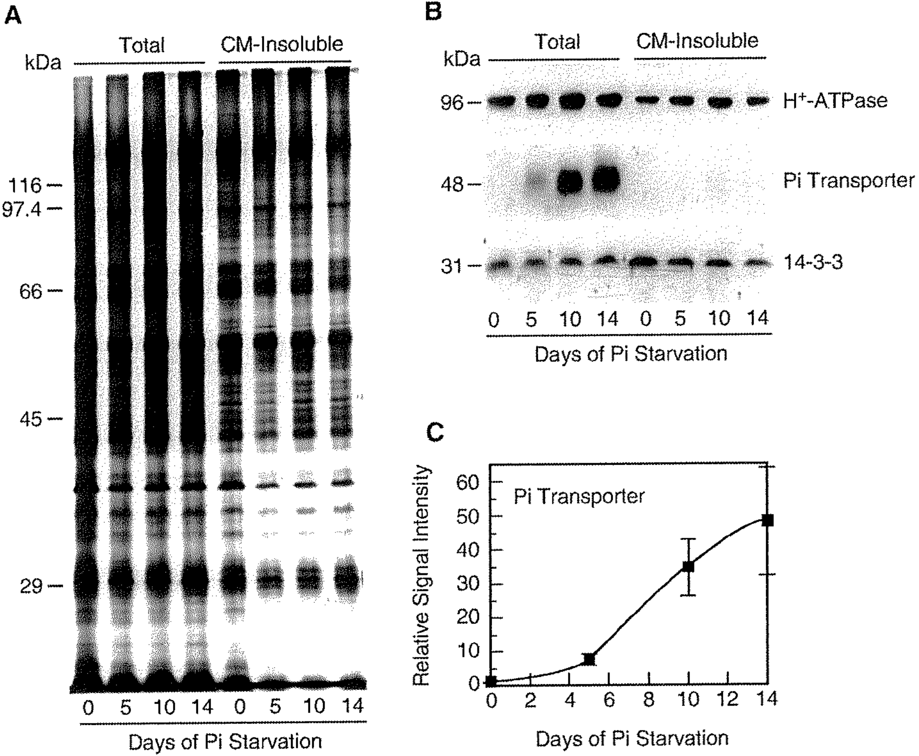
Fig. 2. Effects of the concentration of Pi on the Pi uptake activity of *S. oligorrhiza*. +P (■) and –P plants (●), which were incubated at various concentrations of Pi for 10 min. The radioactivity of the plants was measured as described in the **Materials and methods**. This is one of three independent measurements with similar tendencies.

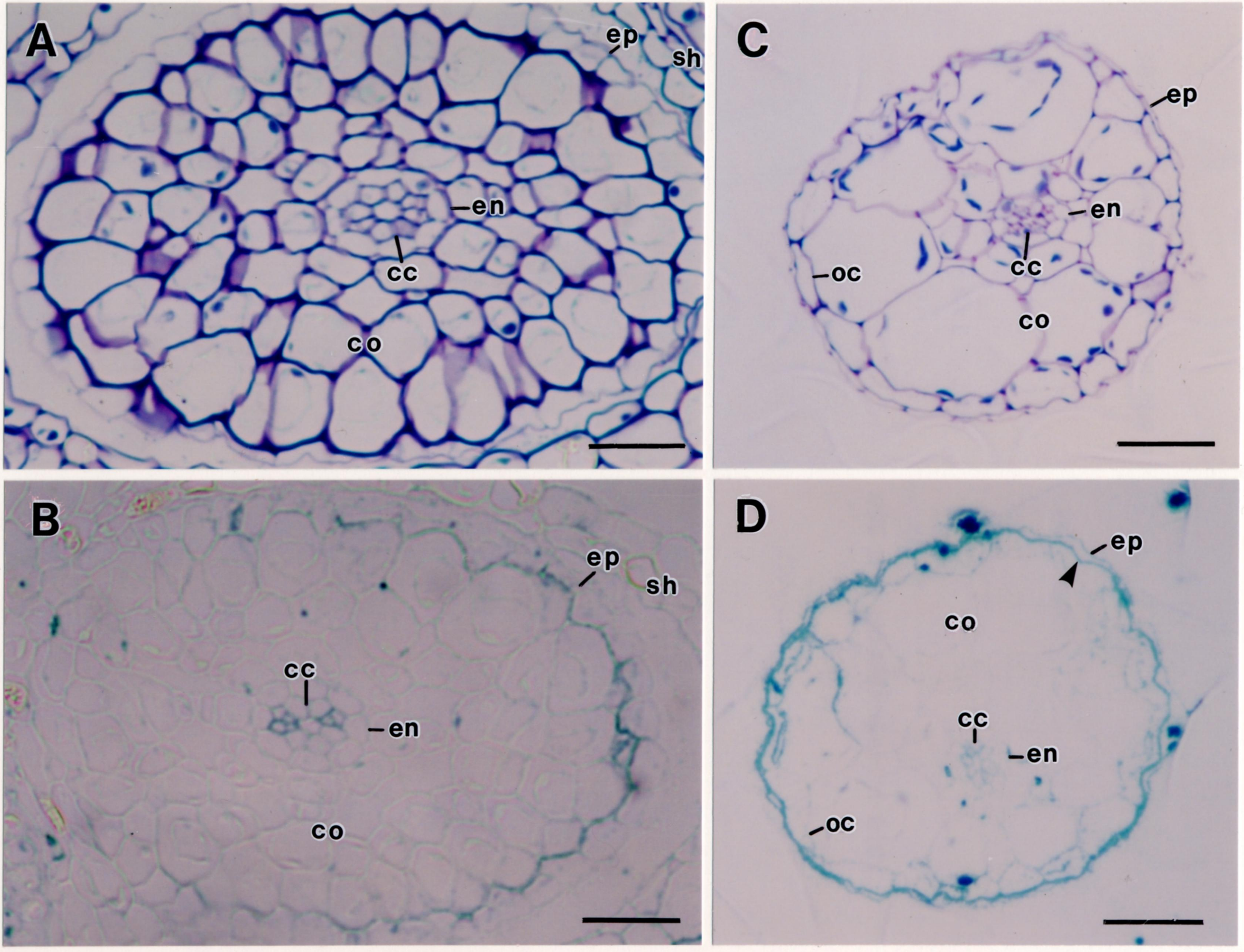
Fig. 3. SDS-PAGE and immunoblot analyses of PM proteins from *S. oligorrhiza.* (A) Silver-staining profiles of PM proteins from +P plants (day 0) and –P plants cultured for the indicated days. (B) Immuno-detection of PM H^+-ATPase, the high-affinity Pi transporter and 14-3-3 protein. (C) Changes in the immunodetected signal intensity of the Pi transporter in the total PM proteins. Data in C are relative intensities against day 0 (+P plants) and represent the means with SE of three independent measurements. Total, total PM proteins; CM-Insoluble, CM-insoluble PM proteins.

Fig. 4. Immunolocalization of Pi transporters in roots of *S. oligorrhiza*. Transverse sections (2 µm) of roots were prepared from LR-White embedded +P (A, B) and –P (C, D) plants of *S. oligorrhiza* and stained with 0.1% toluidine blue (A, C) or immunolabeled for the Pi transporter (B, D). Intense immunolabeling (arrowhead) is found at the outer surface of the outermost cortical cells of the –P plant roots (D). Vascular tissues in the central cylinder of –P plant root are also immunolabeled. cc, central cylinder; co, cortex; en, endodermis; ep, epidermis; oc, outermost cortex; sh, root sheath; and scale bars, 20 µm.


0.6
0.5
0.4
0.3
0.2
0.1
0
Pi uptake, nmol mg^{-1} plant
0
20
40
60
80
100
120
Incubation time (min)


0.25
0.2
0.15
0.1
0.05
0
Pi uptake, nmol (10 min)-1 mg-1 plant
0.1
1
10
100
1000
10000
Concentration of Pi (µM)


A
Total
CM-Insoluble
kDa
116
97.4
66
45
29
0
5
10
14
0
5
10
14
Days of Pi Starvation
B
Total
CM-Insoluble
kDa
96
48
31
H+-ATPase
Pi Transporter
14-3-3
0
5
10
14
0
5
10
14
Days of Pi Starvation
C
Pi Transporter
Relative Signal Intensity
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
Days of Pi Starvation


A
ep
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B
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C
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D
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oc

Table 1. Vanadate-sensitive ATPase and ATP-dependent H^+-transport activities and relative amounts of H^+-ATPase in PM vesicles from *Spirodela oligorrhiza*. PM vesicles were prepared from two-week cultured +P and –P plants and assayed for vanadate-sensitive ATPase and ATP-dependent H^+-transport activities with 2 m*M* ATP and 3 m*M* $MgSO_4$ at pH 6.5 or pH 7. Relative amounts of the proteins were measured by densitometry on the western blots. Each value is the mean of at least three independent assays with SE.

Culture conditions	Relative amount	Vanadate-sensitive ATPase activity		ATP-dependent H^+-transport activity	
		pH 6.5	pH 7	pH 6.5	pH 7
		μmol Pi h^{-1} mg^{-1}		%Δ*F* min^{-1} mg^{-1}	
+P	1	20.0±2.0	14.6±0.3	1112±139	756±203
–P	0.94±0.19	12.0±1.9	7.4±1.3	1012±47	708±32

Table 2. Kinetic parameters of vanadate-sensitive ATPase and ATP-dependent H^+-transport activities in PM vesicles from *Spirodela oligorrhiza*. PM vesicles were prepared from two-week cultured +P and –P plants and assayed for vanadate-sensitive ATPase and ATP-dependent H^+-transport activities with 3 m*M* $MgSO_4$ and ATP levels from 0 to 2 m*M* at pH 6.5 or pH 7. The K_m and V_{max} values were calculated from Eadie-Hofstee plots. Each value is the mean of three independent assays with SE.

Culture conditions	Vanadate-sensitive ATPase activity				ATP-dependent H^+-transport activity			
	K_m		V_{max}		K_m		V_{max}	
	pH 6.5	pH 7	pH 6.5	pH 7	pH 6.5	pH 7	pH 6.5	pH 7
	m*M*		μmol Pi h^{-1} mg^{-1}		m*M*		%Δ*F* min^{-1} mg^{-1}	
+P	0.8±0.2	0.9±0.1	28.1±3.3	18.3±4.9	0.7±0.2	0.5±0.1	2301±412	1012±319
–P	0.3±0.1	0.6±0.1	14.3±2.5	10.5±1.9	0.3±0.1	0.8±0.1	1420±29	1052±43