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ORIGINAL ARTICLE

Localization of acid phosphatase activities in the roots of white lupin plants grown under phosphorus-deficient conditions

Jun WASAKI[1], Soichi KOJIMA[2†], Hayato MARUYAMA[3], Susan HAASE[4], Mitsuru OSAKI[3] and Ellen KANDELER[4]

[1]*Creative Research Initiative "Sousei", Hokkaido University, Sapporo 001-0021,* [3]*Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan;* [2]*Institute of Plant Nutrition, University of Hohenheim, Fruwirthstrasse 20, 70593 Stuttgart, and* [4]*Institute of Soil Science, University of Hohenheim, 70599 Stuttgart, Germany*

Abstract

Acid phosphatase (APase) produced by the cluster roots of white lupin (*Lupinus albus* L.) plays an important role in inorganic phosphate (Pi) acquisition. Although the importance of cluster roots in Pi acquisition is well known, information on the distribution of APase within tissues of normal and cluster roots is lacking. Isoelectric focusing of APase isoforms as well as histochemical localization and visualization of APase were used to clarify the importance of secretory APase for P nutrition of white lupin grown under P deficiency. Isoelectric focusing revealed that both the secretory type and other major APase isoforms probably involved in P translocation were inducible. The major activity in the rhizosphere soil of cluster roots and roots grown under hydroponic conditions corresponded to LASAP2, a previously purified APase secreted from white lupin roots. Histochemical localization using enzyme-labeled fluorescence (ELF)-97 phosphate as a substrate was applied to rhizosphere samples. This substrate provides fluorescent precipitates after hydrolysis by phosphatase. Strong APase activity in the epidermal tissues of normal roots and cluster rootlets and in root hairs of cluster rootlets under P deficiency was detected. These results support the hypothesis that APase activities in the rhizosphere liberate Pi and supply it to white lupin plants grown under P-deficient conditions.

Key words: acid phosphatase, cluster roots, ELF-97 phosphate, phosphorus deficiency, *Lupinus albus*.

INTRODUCTION

The white lupin plant has developed several mechanisms to obtain phosphorus from the soil. Abundant exudates, such as acid phosphatase (APase), and organic acids from the roots cause inorganic phosphate (Pi) to be released from organic and sparingly soluble P compounds (Dinkelaker *et al*. 1989; Gardner *et al*. 1983; Tadano and Sakai 1991). White lupin forms unique root clusters resembling bottle brushes, designated as "cluster roots" (or proteoid roots), under P and Fe deficiency (Neumann and Martinoia 2002). Cluster roots yield P not only by increasing the root surface area, but also by the upregulation of Pi transporters and APases (Liu *et al*. 2001; Miller *et al*. 2001; Wasaki *et al*. 2003).

Ozawa *et al*. (1995) used isoelectric focusing to demonstrate that secreted APase was one of the APase isoforms in the roots. They also purified and characterized the secreted APase protein: it is a homodimer with an estimated molecular weight of 140 kDa, 72 kDa for each subunit, it has wide substrate specificity, and is stable at pH 4.0–9.0. Like many other secreted proteins, the APase is glycosylated, which protects it against proteolytic enzymes and contributes to its stability over a wide pH range (Ozawa *et al*. 1995). The half-life of the enzyme in soil solution at 25°C was estimated to be 2 weeks, suggesting high stability (Tadano *et al*. 1993). These properties are consistent with the role suggested for APase in the hydrolysis of organic P in soil.

The APase secreted from lupin roots is important for P acquisition from organic P sources in soils. Injecting

Correspondence: J. WASAKI, Creative Research Initiative "Sousei" (CRIS), Hokkaido University, N21W10, Kita-ku, Sapporo 001-0021, Japan. Email: junw@chem.agr.hokudai.ac.jp

Present address: †Plant Science Center, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan.

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crude APase collected from lupin exudates into the rhizosphere of tomato and sugar beet plants increased plant growth and P absorption (Tadano and Komatsu 1994). This result suggests that APase contributes to phosphorus acquisition from organic phosphate compounds. *LASAP2* cDNA for the secreted APase has been isolated from phosphorus-deficient white lupin (Wasaki *et al*. 2000). The mRNA was not constitutive, but inducible in phosphorus-deficient roots, especially in cluster roots (Wasaki *et al*. 2000, 2003). The age of the root cluster and the cultivation methods influenced the activity of the secreted APase. Mature and senescent cluster roots showed the highest APase activity in hydroponic and soil cultures, respectively (Neumann *et al*. 2000; Wasaki *et al*. 2005).

Although the importance of cluster roots for the local release of Pi is well known, no information is available on the small-scale distribution of APase within roots and cluster roots. APase was localized histochemically using a new type of fluorogenic compound, enzyme-labeled fluorescence (ELF)-97 phosphate (Nedoma and Vrva 2006; Nedoma *et al*. 2003). This substrate is converted to a water-insoluble, crystalline, fluorescent product at the site of enzymatic hydrolysis, thus localizing active enzymes when viewed by fluorescence microscopy (Kandeler 2007).

The present study evaluated the physiological importance of APase activities in cluster roots formed under phosphorus-deficient conditions in hydroponic and in soil culture. The approach used involved isoelectric focusing of APase isoforms and histochemical localization and visualization of APase.

MATERIALS AND METHODS

Plant culture

Hydroponic culture

White lupin (*Lupinus albus* L. cv. Amiga) seeds were sterilized by immersion in 30% H_2O_2 for 15 min with subsequent washing in deionized water. Sterilized seeds were incubated for 4 h in 10 mmol L^{-1} $CaSO_4$ and germinated in the dark for 4 days on filter paper soaked with 2.5 mmol L^{-1} $CaSO_4$. After 3 days in the light, the seedlings were transferred to a nutrient solution (10 plants per 2.5-L pot). Plants were cultivated under controlled environmental conditions in closed growth chambers with a 16/8 h day/night cycle, a light intensity of 300 µmol m^{-2} s^{-1}, and a 25/20°C day/night temperature regime with a relative humidity of 60%.

The nutrient solution was composed of 2 mmol L^{-1} $Ca(NO_3)_2$, 0.7 mmol L^{-1} K_2SO_4, 0.5 mmol L^{-1} $MgSO_4$, 0.1 mmol L^{-1} KCl, 1 µmol L^{-1} H_3BO_4, 0.5 µmol L^{-1} $MnSO_4$, 0.5 µmol L^{-1} $ZnSO_4$, 0.2 µmol L^{-1} $CuSO_4$, 0.01 µmol L^{-1} $(NH_4)Mo_7O_{24}$, and 20 µmol L^{-1} Fe-ethylenediaminetetraacetic acid (Fe-EDTA) with (+P) or without (–P) 0.25 mmol L^{-1} KH_2PO_4 application in continuously aerated 2.5-L pots. The solution was adjusted to pH 5.6 and renewed every 2 days.

Soil culture

Rhizoboxes (26 cm × 11.5 cm × 1.9 cm) developed by Dinkelaker and Marschner (1992) were used for soil culture. White lupin seedlings were grown as described for hydroponic culture. The seedlings were transferred to rhizoboxes (two seedlings per box) that contained 250 g dry matter of a P-deficient calcareous loess sub-soil ($CaCO_3$: 21.5%; pH 7.5; C_{org}: 0.1%; P (CAL): 3 mg kg^{-1} soil (Schüller 1969); P (Bray1): 2 mg kg^{-1} soil (Bray and Kurtz 1945); P (Olsen): 1 mg kg^{-1} soil (Olsen *et al*. 1954) (representing easily plant-available P fractions); total acid-soluble P (H_2SO_4): 332 mg kg^{-1} soil (Walker and Adams 1958) and total organic P: 2 mg kg^{-1} soil (Walker and Adams 1958); N_{total}: 0.02%; K: 40 mg kg^{-1} soil), mixed with 10% (w/w) of fresh field soil for microbial inoculation, 50% (w/w) fine quartz sand and fertilizers (described below) and sieved through a 2-mm mesh. Fertilization occurred prior to transplanting the seedlings by applying 100 mg N kg^{-1} as $Ca(NO_3)_2$; 150 mg K kg^{-1} as K_2SO_4; 50 mg Mg kg^{-1} as $MgSO_4$ and 20 µmol Fe kg^{-1} as Fe-EDTA to the soil, without applying P. Soil moisture was adjusted to 20% (w/v) and appropriate volumes of water were added daily by gravimetric determination. The rhizoboxes were fixed at a horizontal angle of 50° to stimulate root growth along the transparent root observation window of the boxes.

Isoelectric focusing and activity staining of acid phosphatase on a polyacrylamide gel

White lupin plants were hydroponically grown with or without P for 14 days after transfer. Roots were collected, the moisture was removed using paper towels, and the roots were then frozen immediately in liquid nitrogen. Crude protein from the roots was extracted according to Wasaki *et al*. (1997). Five micrograms of crude protein was loaded onto each lane. An additional two white lupin plants cultured in hydroponics with or without P were transferred to a bottle containing 150 mL of 1 mmol L^{-1} $CaCl_2$ solution and incubated for 24 h in a greenhouse. The solution was collected and filtrated after incubation, then transferred to a clean dialysis tube. The secreted protein was concentrated with polyethyleneglycol up to 1 mL. The rhizosphere soil suspension of cluster roots was prepared according to Wasaki *et al*. (2005). These solutions containing crude protein were used directly for electrophoretic separation, which was carried out using a mini gel system (Mini-Protean III; Bio-Rad Laboratories, Hercules,

CA, USA) according to Ozawa *et al.* (1995). In brief, the crude protein was applied on a 5% polyacrylamide gel containing 2% BioLyte (pH range 3–10; Bio-Rad Laboratories) and 10% glycerol and separated for 2 h at 200 V and then for 2 h at 400 V using 20 mmol L^{-1} AcOH and 25 mmol L^{-1} NaOH as the cathode and anode solutions, respectively. After electrophoresis, the gel was transferred into a container with staining solution (400 mmol L^{-1} Citrate-NaOH at pH 5.0 containing 20 mmol L^{-1} 4-methylumbelliferyl phosphate and 20 mmol L^{-1} EDTA). The fluorescence of methylumbelliferone liberated by phosphatase activity was visualized under ultraviolet light (260 nm). The pI was estimated with a pI marker (Serva Electrophoresis, Heidelberg, Germany).

Activity staining using fluorescent substrate

At 35 days after transplanting (DAT), normal and cluster roots were harvested from the plants grown in hydroponics and in soil. Cluster roots collected from soil were transferred to a microcentrifuge tube containing 1 mL of 75 mmol L^{-1} Tris-malate buffer (pH 4.3) and washed by gentle agitation several times using forceps and paper towel to remove soil particles and water around the cluster roots. The roots were frozen in optimum cutting temperature (OCT) compound embedding medium at –20°C and sliced at 50 µm thickness using cryostats (Cicrom HM500M; Microm International, Waldorf, Germany or CM3050S; Leica Microsystems, Wetzler, Germany). Sliced root sections were stretched onto a slide glass (Histobond; Marienfeld, Lauda-Königshofen, Germany or Superfrost; Matsunami Glass, Osaka, Japan).

ELF97 phosphate (Invitrogen, Carlsbad, CA, USA) was used as a substrate for APase (Nedoma *et al.* 2003; Nedoma and Vrva 2006) in the histochemical activity staining analysis. The buffer for substrate dilution and washing was adjusted to pH 4.3, which was the optimal condition for purified APase secreted from white lupin roots (Ozawa *et al.* 1995). ELF97 phosphate was diluted to 25 µmol L^{-1} with 75 mmol L^{-1} Tris-malate buffer. Thirty to fifty microliters of diluted substrate solution was dropped onto the stretched root section and incubated in a dark box at room temperature for 10–20 min. As a negative control, the same volume of Tris-malate buffer without substrate was dropped onto a root section. After the incubation, the substrate solution was removed with a micropipette and washed with the same volume of Tris-malate buffer three times to remove the remaining substrate.

Fifty microliters of Tris-malate buffer was dropped onto the washed root section and covered with a cover glass. ELF97, the hydrolyzed product of APase activity, was observed using fluorescence microscopes (Axiovert 200M; Carl Zeiss, Oberkochen, Germany or Leica FW4000; Leica Microsystems) with 360 and 450 nm for excitation and emission, respectively. Fluorescent images were recorded using high-resolution CCD cameras (AxioCam HRm; Carl Zeiss or Leica DFC300FX; Leica Microsystems). Exposure time was set as short as possible to avoid the influence of autofluorescence. Unstained sections, chosen from the closest part of the stained section, were also captured as a negative control using the same method. Sections captured by the same exposure time were compared to each other. A fluorescent image was merged with the transmitted image.

RESULTS

Profile of APase in white lupin roots and secreted APase

At 14 DAT, the dry matter production of white lupin was similar in –P and +P treated plants. The inorganic phosphate concentration differed between treatments (1.2 and 0.7 mg P per gram dry weight (DW) in +P and –P roots, respectively). The profile of APase isoforms in the roots of white lupin grown in nutrient solution is shown in Fig. 1A. These isoforms were detected as 10 independent bands by isoelectric focusing. High activity was detected at pI 4.7, which corresponded to the pI of purified secretory APase (Ozawa *et al.* 1995). Image analysis also revealed that the APase activities of all isoforms were higher in –P than in +P (data not shown).

The APase activity in secreted proteins was also detected with isoelectric focusing (Fig. 1B). Activity was low in secreted protein from +P plants grown under hydroponic conditions, but strong at pI 4.7 in –P plants. In contrast, a strong signal at pI 4.7 and a broad and weak signal at a higher pI were detected in the lane of soil suspension prepared from the rhizosphere soil of mature cluster roots (Fig. 1B).

Distribution of APase activity in normal roots of white lupin

Histochemical activity staining of APase using a fluorescent substrate was carried out for transverse sections of normal roots of white lupin grown under hydroponic conditions (Fig. 2). As shown in the right panels in Fig. 2, autofluorescence was negligible. The intensity of fluorescence, indicating APase activity, was stronger in –P roots than +P roots. Fluorescence was distributed over the whole section of –P-treated roots (Fig. 2). Specific enrichment of APase was found in the epidermis of –P roots.

Distribution of APase activity in rootlets of white lupin cluster roots

Histochemical activity staining of APase using a fluorescent substrate was carried out for a vertical section of rootlet

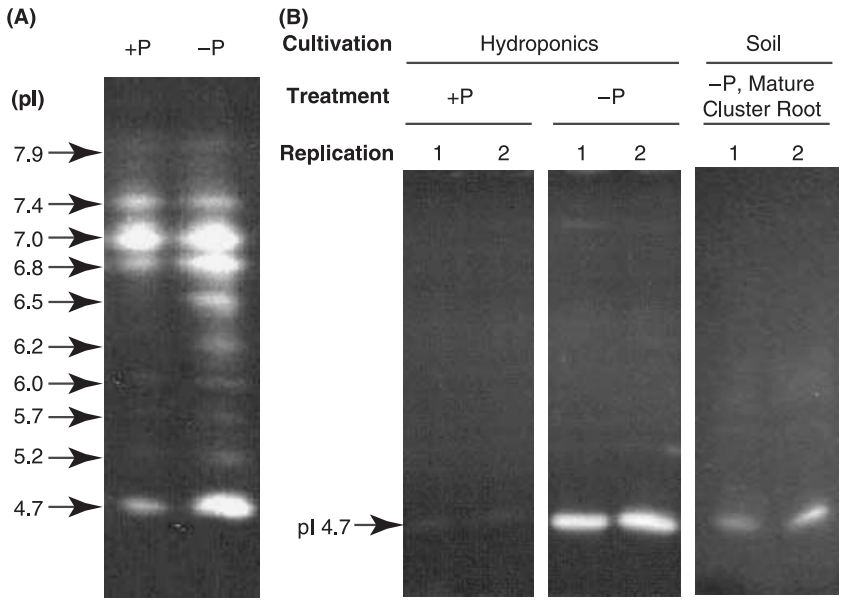

Figure 1 Activity staining of APase separated by isoelectric focusing. (A) Profile of APase isoforms in crude protein extracted from roots of hydroponically cultured white lupin. (B) Profile of secreted crude protein from roots of hydroponically cultured white lupin and extracted protein of rhizosphere cluster roots cultured in soil. pI, xxxx.

of cluster roots formed under hydroponic conditions (Fig. 3). The distribution of APase activity differed considerably between +P and –P rootlets. In the former, activity was weak in vascular tissue, whereas in the latter it was strong both in vascular tissue and in the epidermis (Fig. 3). Fluorescence was most intense in the root tip of rootlets (Fig. 3). Fluorescence in epidermal tissue was higher in the rootlet than in the secondary root, which was the basal root of the cluster rootlets (Fig. 3).

Distribution of APase activity in cluster rootlets of P-deficient white lupin grown under hydroponic and soil conditions

Activity staining of APase for cluster rootlets of P-deficient plants grown under hydroponic and soil conditions were conducted using the method described in the previous section (Fig. 4). In hydroponic plants, activity was strong not only in vascular tissues but also in the epidermis and root hairs, both in vertical and transverse sections (Fig. 4A,C). The APase distribution in vertical sections of cluster rootlets was similar to that in normal roots (Figs 2,4C). The activity of cluster rootlets was distributed similarly in hydroponic and soil-cultured plants (Fig. 4C,E). Autofluorescence was negligible in both plant types (Fig. 4B,D,F), indicating that the fluorescence from APase of root tissue is far stronger than the influence of autofluorescence of soil particles.

DISCUSSION

In a previous study, secretion of APase from lupin roots was induced by decreasing external P; the function essentially depended on inducing *LASAP2* expression (Wasaki *et al.* 2003). Therefore, *LASAP2* mRNA is accumulated by internal P decrease followed by active secretion of the enzyme. The present study induced APase activities at pI 4.7 and other isoforms (Fig. 1A), suggesting that APase isoforms involved in internal P recycling were also induced after internal P concentrations dropped. The response of each APase isoform to P nutrition has been described: Bosse and Köck (1998) examined the pattern of these isoforms in tomato roots and showed that the activity of one root-specific isoform increased and a new isoform appeared. Using *Arabidopsis* as a model plant, only one of five isoforms was induced under low P conditions (Trull *et al.* 1997). Our results revealed that low P availability induced many isoforms of APase.

A strong signal at pI 4.7 was detected both in secreted proteins and in a suspension of the rhizosphere soil of mature cluster roots (Fig. 1B). This indicates that the major activity of APase secreted from cluster roots is LASAP2 at pI 4.7. Only a broad and weak signal at higher pI was detected in the lane of soil suspension (Fig. 1B). APase activity from other isoforms of plants and microbes contributed very little to the phosphorus mineralization in the rhizosphere of cluster roots.

APase activity at pH 4.3 was high in the epidermis of normal roots and cluster rootlets (Figs 2,3,4). This APase fraction was probably responsible for Pi release from the organic substrate in the rhizosphere. A previous study of Wasaki *et al.* (1997) using histochemical immunostaining provides evidence that this APase fraction at pH 4.3 is accumulated in epidermal cells. Therefore, LASAP2 occupies a large proportion of

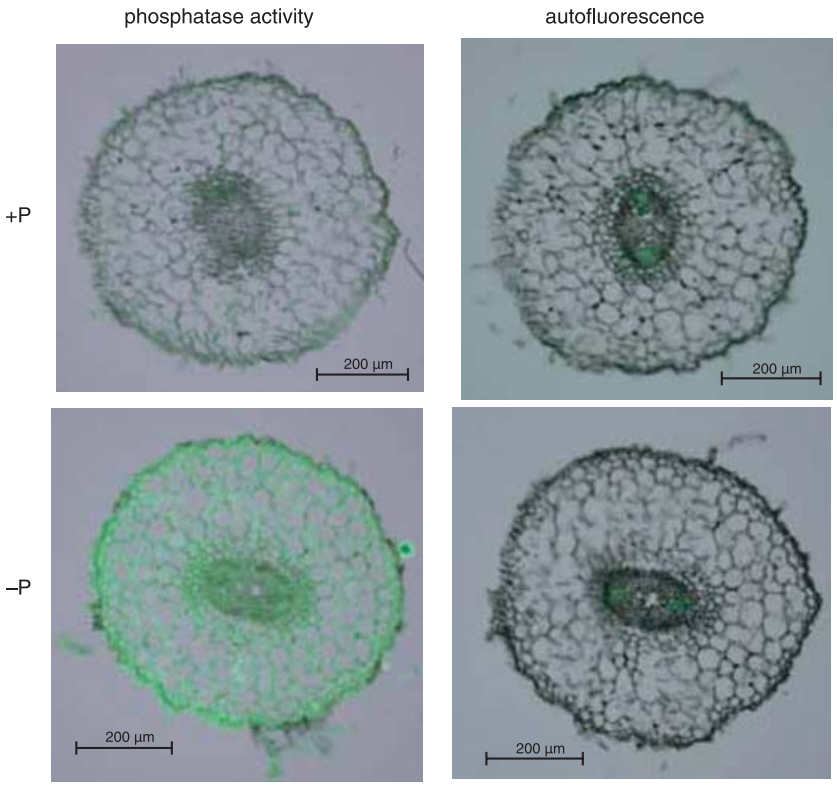

Figure 2 Visualization of the histochemical activity of APase for normal roots using ELF97 phosphate as a substrate. Hydroponically cultured white lupin roots were used for this experiment. Fluorescent precipitates of ELF97 (green color), the product of phosphatase activities, were observed using a fluorescence microscope. The results of the activity staining and autofluorescence are shown in the left and right panels, respectively. Upper and lower panels indicate the transverse sections of normal roots under +P and –P conditions, respectively.

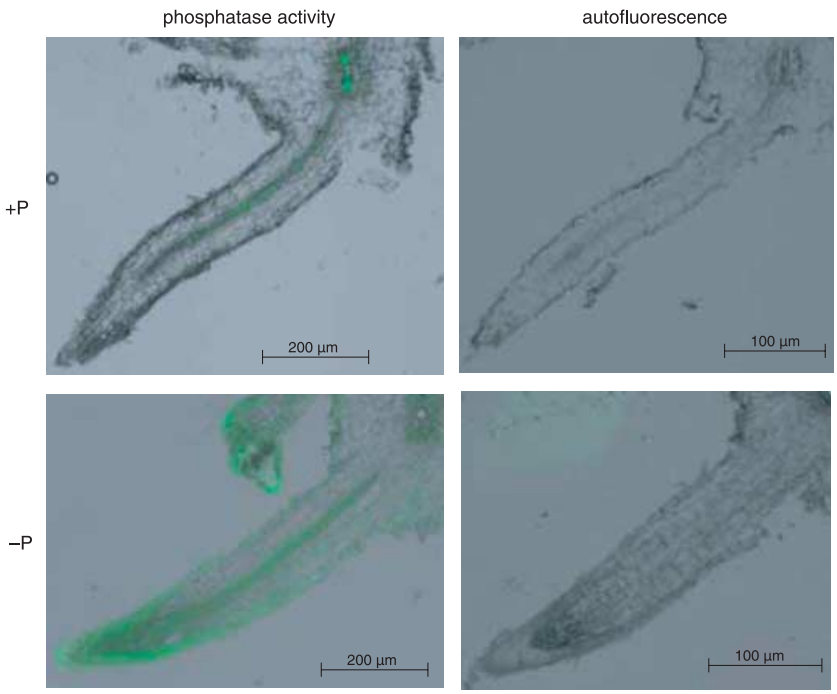

Figure 3 Visualization of the histochemical activity of APase for cluster rootlets using ELF97 phosphate as a substrate. Hydroponically cultured white lupin roots were used for this experiment. The results of the activity staining and autofluorescence are shown in the left and right panels, respectively. Upper and lower panels indicate the cluster rootlets formed under +P and –P conditions, respectively.

APase acting in the rhizosphere of white lupin. In addition, cluster root formation is significantly induced under low P conditions (Gardner *et al.* 1983; Neumann and Martinoia 2002; Shane and Lambers 2005; Wasaki *et al.* 2005). APase activity in the rhizosphere and the accumulation of mRNA for secretory APase were high in cluster roots (Wasaki *et al.* 2003, 2005). A higher activity in the epidermis of cluster rootlets

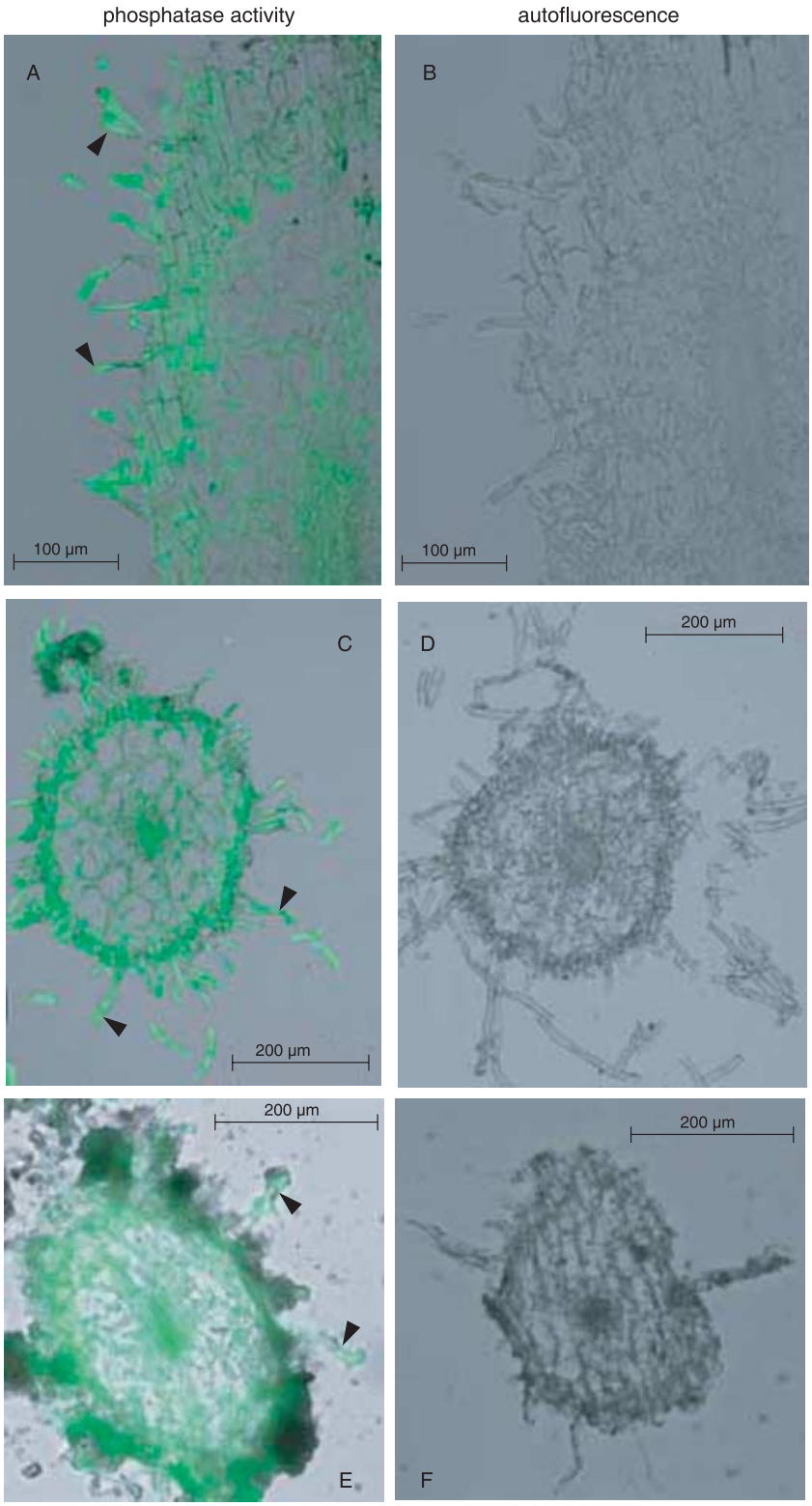

Figure 4 Visualization of the histochemical activity of APase for cluster rootlets formed under –P conditions using ELF97 phosphate as a substrate. Hydroponically and soil-cultured white lupin roots were used for this experiment. The results of the activity staining and autofluorescence are shown in the left (A,C,E) and right (B,D,F) panels, respectively. (A,B) Vertical sections of a cluster rootlet in a hydroponic plant; (C,D) transverse sections of a cluster rootlet in a hydroponic plant; (E,F) transverse sections of a cluster rootlet in a soil culture plant. Triangles indicate typical root hairs.

supports these results (Fig. 3). In white lupin, increased cluster root formation and APase secretion could contribute to Pi release from organic P compounds and Pi uptake.

Clear APase activity took place at pI 4.7 in +P crude root extracts, whereas it was very low in +P crude secreted protein (Fig. 1A). Wasaki *et al*. (2003) have shown that *LASAP2* mRNA accumulated only in –P roots. A more constitutive APase activity representing an isoform differing from LASAP2 probably exists at pI 4.7. This isoform might correspond to LASAP1, which has been isolated and characterized as a probable cell wall or plasma membrane binding protein in a previous study (Wasaki *et al*. 1999).

The promoter region of secretory APase has been isolated and characterized from *Arabidopsis thaliana* (Haran *et al*. 2000). Their experiment showed the strongest expression in the meristematic regions of lateral roots, corresponding to our result for cluster rootlets (Fig. 3). Those authors also showed that P starvation induced expression of the reporter gene in vascular tissue soon after germination; within 10 days this extended throughout most of the root tissue. This finding corresponds to our results: high activity in the central cylinder of the cluster rootlet under P deficiency and distribution of the activity in the whole rootlet (Fig. 3). High APase activities in lateral roots of *Arabidopsis* also correspond to the strategy of white lupin for P acquisition by increasing tertiary lateral rootlets, that is, cluster root formation under low P conditions.

APase activity was very strong in the aquatic plant duckweed *Spirodela oligorrhiza*, mainly because of the production of a glycosylphosphatidylinositol (GPI)-anchored APase (Morita *et al*. 1996). Immunostaining using polyclonal antibody to purify GPI-anchored APase revealed that this enzyme is located in the outermost cortical cells of the roots (Nishikoori *et al*. 2001). Such APase is apparently present in root surfaces and contributes to the P acquisition of *S. oligorrhiza*. In this plant, the main phosphatase activity was localized near the root surface and was enriched in the cell wall (Bieleski and Johnson 1972), minimizing enzyme loss to the aquatic environment. In contrast, white lupin produces cell wall-bound APase and other APases, which are released into the rhizosphere. An APase isoform secreted from roots of white lupin grown in soil was also high (Fig. 1). Therefore, the activities of inducible secreted APase as well as the relatively constitutive epidermal APase might help white lupin acquire P.

In conclusion, APase secreted by cluster roots plays an important role in P uptake under P-deficient conditions. Moreover, other isoforms of APase induced under P deficiency might be responsible for P translocation within roots and cluster roots.

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