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Spatial distribution and expression of intracellular and extracellular acid phosphatases of cluster roots at different developmental stages in white lupin

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ABSTRACT

Acid phosphatases (APases) play a key role in phosphorus (P) acquisition and recycling in plants. White lupin (*Lupinus albus* L.) forms cluster roots (CRs) and produces large amounts of APases under P deficiency. However, the relationships between the activity of intracellular and extracellular APases (EC 3.1.3.2) and CR development are not fully understood. Here, comparative studies were conducted to examine the spatial variation pattern of APase activity during CR development using the enzyme-labelled fluorescence-97 (ELF-97) and the *p*-nitrophenyl phosphate methods. The activity of intracellular and extracellular APases was significantly enhanced under P deficiency in the non-CRs and CRs at different developmental stages. These two APases exhibited different spatial distribution patterns during CR development, and these distribution patterns were highly modified by P deficiency. The activity of extracellular APase increased steadily with CR development from meristematic, juvenile, mature to senescent stages under P deficiency. In comparison, P deficiency-induced increase in the activity of intracellular APase remained relatively constant during CR development. Increased activity of intracellular and extracellular APases was associated with enhanced expression of *LaSAP1* encoding intracellular APase and *LaSAP2* encoding extracellular APase. The expression levels of these two genes were significantly higher at transcriptional level in both mature and senescent CRs. Taken together, these findings demonstrate that both activity and gene expression of intracellular or extracellular APases exhibit a differential response pattern during CR development, depending on root types, CR developmental stages and P supply. Simultaneous *in situ* determination of intracellular and extracellular APase activity has proved to be an effective approach for studying spatial variation of APases during CR development.

Introduction

Phosphorus (P) is frequently a limiting factor for plant growth. Most P is immobilized by inorganic minerals or organic compounds in soil, and thus becomes poorly available to most plants (Schachtman et al., 1998). Rhizosphere acidification and exudation of organic acids contribute to the mobilization of sparingly soluble soil P (Ryan et al., 2001). In soil, at least 30% and up to 80% of total P is found in organic forms that mainly comprise phosphate monoesters. Inositol hexaphosphate is the main component of phosphate monoesters and accounts for 20–50% of total organic P in soil (Richardson et al., 2009). To be available to plants, soil organic P must first be hydrolyzed by acid phosphatase (APase, EC 3.1.3.2) to release orthophosphate (Pi). Most intracellular APases exist in the vacuole as soluble proteins, while extracellular APases are localized in the cell wall or secreted by roots into the rhizosphere (Duff et al., 1994). These two types of APase play a dominant role in the recycling of internal organic P as well as the acquisition of external organic P from the rhizosphere.

White lupin forms cluster roots (CRs) when grown under P-deficient conditions (Neumann and Martinoia, 2002). This special root structure increases the availability of soil P by expanding the root surface area, acidifying the rhizosphere and increasing the exudation of citrate (Dinkelaker et al., 1995; Neumann et al., 2000). The acquisition of soil P by white lupin is also associated with the secretion of APase, which hydrolyzes soil organic P, and hence

Abbreviations: APase, acid phosphatase; CRs, cluster roots; ELF-97, enzyme-labelled fluorescence-97; P, phosphorus; Pi, orthophosphate; *p*-NPP, *p*-nitrophenyl phosphate; non-CRs, non-cluster roots; 4-MU, 4-methylumbelliferone; 4-MUP, 4-methylumbelliferone phosphate.

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increases the availability of soil P (Gilbert et al., 1999). Previous reports have shown that expression and secretion of APase are highly induced under P deficiency (Tadano et al., 1993; Miller et al., 2001; Wasaki et al., 2003). A two-fold increase in APase activity was observed in root exudates of the CRs as compared with non-cluster roots (non-CRs) under P deficiency (Gilbert et al., 1999). When crude APase excreted from CRs of white lupin was added to the rhizosphere of tomato and sugar beet, a positive effect on plant growth and P uptake were observed in both species (Tadano et al., 1993). Ozawa et al. (1995) purified and characterized the root secretory APase, designated as *LaSAP2*, from roots of P-deficient white lupin. Subsequent work has confirmed this result (Miller et al., 2001; Wasaki et al., 2003). Interestingly, another APase cDNA, designated as *LaSAP1* that significantly differs from *LaSAP2* reported previously, was also isolated and sequenced from P-deficient white lupin roots (Wasaki et al., 1999). The results from PSORT analysis indicated that *LaSAP1* was located in plasmalemma, while *LaSAP2* was secreted from root cells in association with external organic P mobilization (Miller et al., 2001; Wasaki et al., 2003). Over-expression of *LaSAP2* for secretory APase in white lupin significantly enhanced P uptake and growth of transgenic tobacco plants when grown on media containing soluble phytate (Wasaki et al., 2009). These findings suggest that APase secreted by the CRs of white lupin may play an important role in organic P acquisition.

In white lupin, CRs can be separated into four developmental stages: meristematic, juvenile, mature, and senescent stages (Massonneau et al., 2001). These CR stages showed significant difference in rhizosphere pH and organic acid excretion (Neumann and Römheld, 1999), sucrose metabolism (Massonneau et al., 2001), isoflavonoid exudation (Weisskopf et al., 2006), and even distribution of endogenous nitric oxide (Wang et al., 2010). It is possible that production of intracellular and extracellular APases might also exhibit distinctive spatial distribution patterns at different developmental stages of CRs.

Phosphatase activity excreted into the growth medium is commonly quantified spectrophotometrically with the *p*-nitrophenyl phosphate (*p*-NPP) method (Lee, 1988; Gilbert et al., 1999; Ciereszko et al., 2011). Root-secreted APase has also been visualized by staining with fast blue RR salt in the present of 1-naphthyl phosphate (Gilbert et al., 1999), or by using 4-methylumbelliferone phosphate (4-MUP) as a substrate, which can be hydrolyzed to release a fluorescent product, 4-methylumbelliferone (4-MU) measured by UV illumination at 260 nm (Wasaki et al., 2003). However, these methods cannot indicate where APase is localized in the plant tissue. The development of the substrate enzyme-labelled fluorescence-97 (ELF-97) phosphate allows localization of intracellular APase activity (Huang et al., 1993). The substrate is normally slightly fluorescent in the blue range, but after the phosphate group is enzymatically removed, it forms a bright green fluorescent precipitate at the site of APase activity (Huang et al., 1993). The exact visualization and localization of intracellular and extracellular APases under contrasting P conditions contribute to understanding the role of these enzymes in mineralization or recycling of organic P for the model system of white lupin.

The objectives of the present study were to investigate the spatial patterns of intracellular and extracellular APases, and expression of corresponding genes *LaSAP1* and *LaSAP2* from CRs of white lupin at different developmental stages in response to different P supply. We hypothesized that spatial distribution and expression of intracellular and extracellular APases are strongly related to the efficiency of internal and external organic P mobilization, and that the variation in distribution patterns and activity of APases is strongly dependent on P supply and CR development.

Materials and methods

Plant growth

White lupin (*Lupinus albus* L. cv. Kiev Mutant) seeds were surface-sterilized in 10% (v/v) H_2O_2 for 15 min and soaked in saturated $CaSO_4$ solution for germination at 25 °C in darkness. After 4 d, similar size seedlings were transferred to porcelain pots containing 2 L of a continuously aerated nutrient solution with the following nutrient composition (μM): $Ca(NO_3)_2$ (2000), K_2SO_4 (700), $MgSO_4$ (500), KCl (100), H_3BO_3 (10), $ZnSO_4$ (0.5), $MnSO_4$ (0.5), $CuSO_4$ (0.2), $(NH4)_6Mo_7O_{24}$ (0.01) and Fe-ethylene diamine tetraacetic acid (EDTA) (20). Phosphorus was supplied as KH_2PO_4 at 0 μM P (P deficient) and 75 μM P (P sufficient). The nutrient solution was daily adjusted to pH 5.8 with 0.1 M NaOH and renewed every 3 days. Seedlings were grown in a growth chamber with a day/night cycle of 22/18 °C and 16/8 h, light at 250 μmol m^{-2} s^{-1} and relative humidity of 75–85%. Three seedlings were grown in each pot. The pots were completely randomized and re-positioned weekly to minimize environmental effects.

Biomass and P concentration determination

After harvest, roots and shoots of white lupin were dried at 70 °C for 3 days and weighed. Shoots were digested in concentrated H_2O_2–H_2SO_4, and the P concentration was measured using the vanado-molybdate colorimetric reaction (Westerman, 1990).

Sampling of CRs at different developmental stages

Before CRs sampling, a functional test, using bromocresol purple as an indicator, was performed to differentiate the different developmental stages of CRs according to the reported method (Massonneau et al., 2001). The entire root systems of white lupin were divided into the non-CRs and CRs 14 days after emergence, and excised CR segments were further separated into four clearly distinguishable developmental stages: meristematic (non-emerging), juvenile, mature and senescent CRs (Fig. 1). A subsample was kept at −80 °C for subsequent RNA extraction.

Protein concentration determination in tissues

Leaf, stem and root tissue samples were ground in liquid nitrogen, extracted with an extraction buffer (50 mM sodium acetate with 1 mM dithiothreitol, pH 5.2) and centrifuged at 12,000 × *g* for 10 min at 4 °C (Ciereszko et al., 2011). Protein content in tissue extracts was determined by the Bradford method (Bradford, 1976). Protein concentration was expressed in mg g^{-1} root FW.

In situ fluorescence staining and activity assay of intracellular APase

Intracellular APase in root tissues was visualized using the ELF-97 endogenous phosphatase detection kit (Invitrogen, Carlsbad, CA, USA) as previously described by van Aarle (2009). Excised CR segments at different developmental stages were washed thoroughly 3–5 times with acetate buffer (0.2 M, pH 5.5) and incubated in the 40-fold dilution of the ELF-97 substrate solutions at 25 °C for 60–90 s. After incubation, CRs segments were repeatedly rinsed with Tris-wash buffer (30 mM Tris, 1.5 M NaCl, 0.05% (w/v) Triton X-100, pH 8.0) and mounted onto a glass slide. Intracellular APase fluorescence from the CRs segments was visualized with a fluorescence microscope (Leica FW4000, Leica Microsystems, Mannheim, Germany). The fluorescence was excited at 345 nm, and emission fluorescence signals were measured at 530 nm. The intensity of