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Phosphorus deficiency enhances plasma membrane H^+-ATPase activity and citrate exudation in greater purple lupin (*Lupinus pilosus*)

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Abstract. The response of greater purple lupin (*Lupinus pilosus* L.) to a combination of phosphorus (P) deficiency and aluminium (Al) toxicity is unknown, and the mechanisms involved in the exudation of organic anions from greater purple lupin have not been reported. Therefore, plants grown with (+P) or without (–P) 250 μM P were exposed to 0 or 50 μM $AlCl_3$ and the amount of organic anions exuded and the activities of plasma membrane H^+-ATPase (E.C 3.6.3.6) and H^+-pumps were investigated. Twenty days of P deficiency resulted in significantly reduced shoot growth and increased proteoid root formation. Exposure to 50 μM $AlCl_3$ did not induce citrate exudation but did induce some malate exudation in –P plants. In contrast, P deficiency did induce exudation of citrate. Enhanced citrate exudation was attributed to the large increase in the activity of plasma membrane H^+-ATPase and associated H^+ transport. This was shown by the inhibitory effect of vanadate on plasma membrane H^+-ATPase activity *in vitro* and on citrate exudation *in vivo*. However, vanadate did not suppress the exudation of malate. During 9 h of Al exposure, exudation of citrate showed a continuing increase for both –P and +P plants, while malate exudation increased only during the first 3 h, after which it rapidly declined. The total amount of organic anion exudation was significantly higher for –P plants. In the presence of 50 μM anion channel blockers [anthracene-9-carboxylic acid (A-9-C), niflumic acid (NIF) and phenylglyoxal (PG)], the exudation of citrate and malate was reduced by 25–40%. It was concluded that P deficiency induces citrate exudation by enhancing the activity of plasma membrane H^+-ATPase and H^+ export. In *L. pilosus*, exudation of organic anions occurs primarily in response to P deficiency but not Al toxicity. This contrasts with previous results obtained in *Brassica napus* L.

Keywords: Al toxicity, H^+-ATPase, H^+-pump activity, *Lupinus pilosus*, organic anions, P deficiency.

Introduction

Phosphorus is one of the most important plant nutrients as it significantly affects growth and metabolism. Phosphorus deficiency is a major limiting factor for crop production in both acidic soils, where P is fixed to oxides and hydroxides of Al and Fe, and in calcareous soils where P is precipitated as calcium phosphate (Marschner 1995). Higher plants have developed various physiological and morphological strategies to use unavailable P. The release of organic anions is one of these mechanisms that has received considerable attention. In acidic mineral soils where P deficiency and Al toxicity are commonly reported, these organic anions are involved in the chelation of toxic metals such as Al (Matsumoto 2002). In such soils, organic anions play a dual role: first, they alleviate the direct toxic effects of Al and second, they make the fixed P available for plants. The release of organic anions in response to P deficiency has been reported for rape (Hoffland *et al*. 1989), white lupin (Gardner *et al*. 1983) and alfalfa (Lipton *et al*. 1987). Similarly, enhanced exudation of citrate, malate and oxalate in response to Al toxicity has been reported in snap bean, maize, *Cassia tora* L., wheat and buckwheat (Miyasaka *et al*. 1991; Delhaize *et al*. 1993; Pellet *et al*. 1995; Ma *et al*. 1997*a*, *b*). However, both P deficiency and Al toxicity can fail to induce the exudation of organic anions. Phosphorus deficiency did not induce the exudation of malate, oxalate

Abbreviations used: A-9-C, anthracene-9-carboxylic acid; NIF, niflumic acid; PG, phenylglyoxal; P_i, inorganic phosphate; RFW, root fresh weight.

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and citrate from wheat, buckwheat, taro, soybean or rape (Delhaize *et al*. 1993; Ma *et al*. 1997*a*; Ma and Miyasaka 1998; Yang *et al*. 2000; Ligaba *et al*. 2004). Similarly, Al did not induce exudation in oat (Zheng *et al*. 1998), Al-sensitive cultivars of wheat (Ryan *et al*. 1995) or maize (Pellet *et al*. 1995). This indicates that plant species, and cultivars of the same species, may respond differently to Al toxicity and P deficiency.

In addition to physiological changes, the modification of root architecture is a common response to P deficiency in a range of plant species. Some plant species form proteoid roots, including the majority of species belonging to the family Proteaceae (Lamont 1972). The formation of proteoid roots under P deficiency is also documented for some plant species other than the Proteaceae, including *L. albus* L. (Gardner *et al*. 1981; Johnson *et al*. 1996; Neumann *et al*. 1999), *L. consentinii* Guss. (Trinick 1977), and *Viminaria juncea* (Schrad. & Wendl.) Hoffmanns (Walker and Pate 1986). The same authors indicated that proteoid root formation is influenced predominantly by the P status of the plant.

Proteoid roots have a strong capacity to acidify the rhizosphere. According to Dinkelaker *et al*. (1989), proteoid roots of white lupin can acidify the rhizosphere, changing the pH from 7.5 to 4.8. This dramatic decrease in pH can mobilise sparingly available P from calcium phosphate. Rhizosphere acidification is attributed to the release of citric and malic acid (Gardner *et al*. 1982; Dinkelaker *et al*. 1989; Keerthisinghe *et al*. 1998; Neumann *et al*. 1999). The release of organic acid across the plasma membrane must involve two separate transport processes because of high cytosolic pH (> 7.0) Yan *et al*. (2002). Firstly, citrate transport is mediated by the anion channel. This is supported by findings of Neumann *et al*. (1999) in which the release of citrate from proteoid roots of white lupin is mediated by an anion channels. Similarly, Ryan *et al*. (1995) reported that the Al-induced malate efflux was mediated by an anion channel. Secondly, the export of H^+ from the plant cells is mediated by the plasma membrane H^+-ATPase (Serrano 1989; Yan *et al*. 2002). In most plant species, including greater purple lupin, the effect of P deficiency and associated responses have not been investigated, and the mechanism of organic anion exudation from the roots is unknown. However, the effect of Al on plasma membrane H^+-ATPase activity and H^+-pump activity has been investigated. In Al-treated roots of barley seedlings, the plasma membrane bound H^+-ATPase activity and associated H^+ extrusion from the roots was significantly inhibited (Matsumoto 1988). Similarly, in squash and wheat, Al inhibited plasma membrane H^+-ATPase activity by depolarising the surface potential (Ahn *et al.* 2001, 2002, 2004). As with P deficiency, the effect of Al on H^+-ATPase activity and associated H^+ transport in greater purple lupin has not been reported.

In the current study, the effect of P deficiency on the growth of greater purple lupin, the combined effect of P and Al stress on the exudation of organic anions, and the mechanisms involved in exudation of organic anions were investigated. We report that P deficiency induces the exudation of the organic anions by enhancing the activity of plasma membrane H^+-ATPase and associated H^+-transport. Furthermore, our results indicate that Al has no significant effect on the response.

Materials and methods

Plant growth conditions

Greater purple lupin (*Lupinus pilosus* L.) seeds were purchased from Takii Seed Co. (Kyoto, Japan). The seeds were surface-sterilised in 5% (v / v) sodium hypochlorite for 5 min and thoroughly washed with deionised water. Seeds were then scarified in concentrated H_2SO_4 with continuous agitation for 4 h. The scarified seeds were washed thoroughly, wrapped in filter paper and soaked in 0.5 mM $CaCl_2$ solution for germination. After 3 d, uniformly sized seedlings were transferred to a complete nutrient solution similar to that by Neumann *et al*. (1999). The composition of the nutrient solution was (in mM): $Ca(NO_3)_2$ (5), K_2SO_4 (1.75), $MgSO_4$ (1.5), KCl (0.25), KH_2PO_4 (0.25) and (in µM): Fe(III)-EDTA (20), H_3BO_3 (25), $MnSO_4$ (1.5), $ZnSO_4$ (1.5), $CuSO_4$ (0.5), $(NH_4)_6Mo_7O_{24}$ (0.025). The pH of the nutrient solution was adjusted to 5.0. The nutrient solution was renewed every 3 d. Seedlings were grown in a cultivation chamber (CFH-405, Tomy Co., Tokyo, Japan) with a cycle of 14 h / 25°C day and 10 h / 20°C night and a light intensity of 40 µmol s^{-1} m^{-2}. The relative humidity of the chamber was adjusted to 65%. After 7 d in the complete nutrient solution, seedlings were transferred to 3.5-L pots with an aerated nutrient solution containing either 0.25 mM P (+P) or no P (–P). Four plants were grown in each pot. The plants were grown for an additional 20 d in a naturally-illuminated phytotron at a day temperature of 25°C and night temperature of 20°C.

For the Al treatment, plants grown with or without P for 3 weeks were transferred to 1-L pots, filled with 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 (–Al) or 50 µM (+Al) $AlCl_3$. All treatments were replicated at least three times and each set of experiments was repeated at least twice. SE values were used to show mean differences.

Determination of dry weight and P concentration

Shoots and roots were dried separately in an oven at 70°C for 24 h to determine dry weight (DW). The phosphorus concentration in roots and shoots was determined by the vanado-molybdate-yellow method of Gericke and Kurmis (1952). Briefly, 200 mg of milled DW was dry-ashed at a temperature of 480°C for 12 h. The resulting ash was dissolved in 1 : 3 diluted HNO_3. After the colour reaction, P concentration was determined spectrophotometrically at 450 nm (UV-1600, Shimadzu Co., Kyoto, Japan).

The effect of short-term and prolonged Al exposure on organic anions exudation

To study the effect of Al on the exudation of organic anions, 30-d-old +P and –P plants were exposed to 0.5 mM $CaCl_2$ solution (pH 4.5) containing +Al or –Al for 3 h. Subsequently, solutions containing root exudates was collected for analysis of organic anions. A more prolonged effect of Al was studied over a period of 9 h, during which root exudates were collected every 3 h for analysis of organic anions.

The effect of vanadate and anion channel blockers (A-9-C, NIF and PG) on P-deficiency-induced exudation of organic anions

To determine the effect of vanadate on the exudation of organic anions, 30-d-old –P plants were exposed to 0.5 mM $CaCl_2$ solution (pH 5.0) containing 0 or 1 mM Na_3VO_4 for 3 h. To study the role of anion channels in organic anion exudation, 30-d-old –P plants were exposed to 0.5 mM $CaCl_2$ solution (pH 5.0) containing various anion channel blockers (A-9-C, NIF or PG) at a final concentration of 50 µM for 3 h. The solution containing root exudates was collected for analysis of organic anions.

Analysis of organic anions

The $CaCl_2$ solution containing root exudates was first passed through a cation exchange column (16 mm × 14 cm) filled with 5 g Dowex 50W × 8 (50–100 mesh, H^+ form) resin (Muromachi Technos Co. Ltd, Tokyo, Japan) and then through an anion exchange column filled with 2 g Dowex 1 × 8 resin (50–100 mesh, Cl^- form) without adjusting the pH. This procedure was conducted in a cold room (4°C) under non-sterile conditions and the organic anions retained in the anion exchange resin were eluted with 8 N formic acid. Organic anion residues were obtained and quantified by HPLC according to Ma *et al*. (1997*a*).

Plasma membrane isolation

Plasma membranes were isolated following the method described by Palmgren *et al*. (1990). The 30-d-old +P and –P plants previously exposed for 3 h to the +Al or –Al treatments were used. Apical root zones (2–3 cm) were excised and washed twice in an ice-cold homogenisation buffer. The homogenisation buffer contained 25 mM Tris (pH 7.5), 5 mM EGTA, 250 mM sucrose, 1 mM DTT, 0.1% (w / v) BSA (Sigma, Tokyo, Japan), 2.5 mM $K_2S_2O_5$, 2 mM PMSF (phenylmethysulphonyl fluoride), 2 mM salicylhydroxamic acid (SHAM, ICN Biomedicals Inc., OH), 1.5% (w / v) polyvinyl-polypyrrolidone (PVP, Nacalai Tesque Inc., Kyoto, Japan) and 0.2% (w / v) casein. The samples were homogenised with a mortar and pestle. The homogenate was transferred to a glass tube and further homogenised with a mechanical homogeniser. The homogenate was centrifuged at 12 000 *g* for 10 min at 0°C. The supernatant was ultra-centrifuged at 100 000 *g* for 50 min (45 Ti Rotor, Beckman, CA). The resulting pellet was washed in a microsome suspension buffer containing 5 mM K_2PO_4 (pH 7.8), 250 mM sucrose, 0.1 mM EDTA and 1 mM DTT, at 100 000 *g* for 40 min. The pellet was then suspended in a fresh microsome suspension buffer with a glass homogeniser to yield 12 g. This was loaded on a 36-g phase system containing 6.5% (w / w) Dextran T500, 6.5% (w / w) polyethylene glycol 3350, 330 mM sucrose, 4 mM K_2PO_4 (pH 7.8) and 4 mM KCl. Three to five batch procedures were conducted as described by Larsson (1985). The final upper phases that were rich in plasma membrane were diluted several-fold with a suspension buffer containing 40 mM Tris–acetate (pH 7.4), 2 mM EDTA, 250 mM sucrose and 4 mM DTT and centrifuged at 156 000 *g* for 40 min. The pellet was further washed with a fresh suspension buffer at 156 000 *g* for 20 min. The plasma membrane pellet was then suspended in a final suspension buffer containing 40 mM Tris (pH 7.4), 2 mM EDTA, 20% (v / v) glycerol and 4 mM DTT and immediately stored at –80°C until assayed. The plasma membrane protein was quantified following the method by Bradford (1976), and compared against BSA as a standard.

Plasma membrane H^+-ATPase assay

Plasma membrane H^+-ATPase activity was measured following a modified Faraday and Spanswick (1992) method. The assay was conducted in 0.5 mL reaction volume containing 30 mM BTP (Bis–Tris propane) / MES (pH 6.5), 5 mM $MgSO_4$, 50 mM KCl, 4 mM Na_2-ATP and 0.02% (w / v) Brij 58 (polyoxyethelene 20-cetyl-ether, Sigma, Tokyo, Japan) to create inside-out plasma membrane vesicles. Reactions were initiated by addition of 1–5 µg plasma membrane protein. The reactions were proceeded for 30 min at 30°C and were stopped with 1 mL of stopping reagent containing 2% (v / v) concentrated H_2SO_4, 5% (w / v) SDS and 0.7% (w / v) sodium molybdate followed immediately by 50 µL of 10% (v / v) ascorbic acid. Colour development of the phosphomolybdate complex proceeded for 30 min. Absorbance at 820 nm was measured with a spectrophotometer (Shimadzu, Kyoto, Japan). H^+-ATPase activity was calculated from the increase in absorbance at 820 nm due to liberation of inorganic phosphate (P_i).

Determination of plasma membrane H^+ pump activity

H^+-pump activity was monitored as the decrease in absorbance at 495 nm of acridine orange, following the method of Palmgren *et al*. (1990) with a slight modification. The change of the quenching was continuously monitored by a spectrophotometer. The assay media contained 20 µM acridine orange, 2 mM ATP–BTP, 4 mM $MgCl_2$, 10 mM MOPS–BTP (pH 7.0), 140 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mg mL^{-1} BSA (fatty acid free), 0.05% (w / v) Brij 58 and 20 µg membrane protein in a total volume of 1 mL. After 5 min pre-incubation at 20°C, the reaction was initiated by adding $MgCl_2$. The rate of H^+-accumulation was estimated from the initial slope of absorbance quenching (Δ 495 nm) of acridine orange. Gramicidine (50 µM) was added to abolish the established pH gradient.

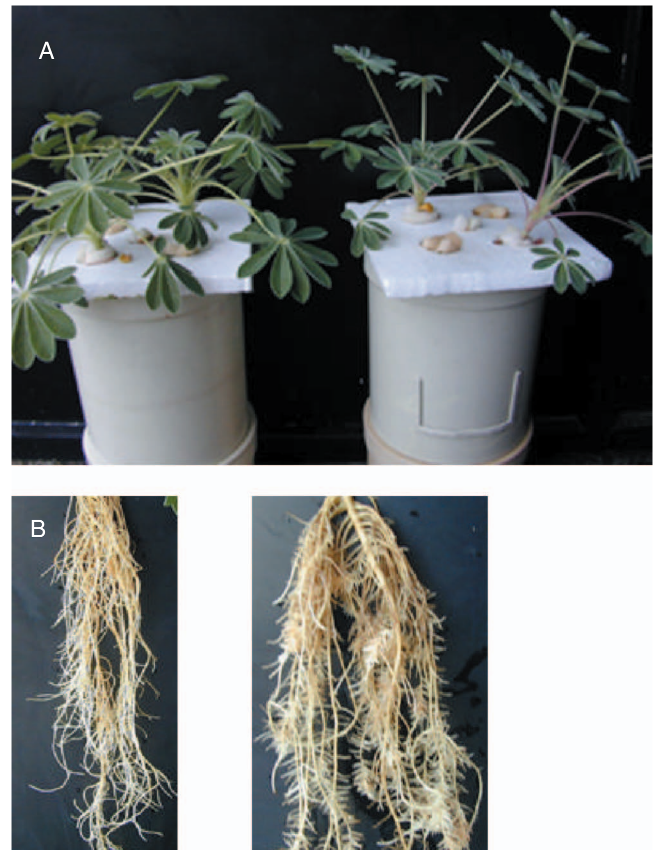

Fig. 1. The effects of P deficiency on shoot and root growth. Ten-day-old plants were transferred to +P or –P nutrient solution for an additional 20 d. (*A*) Shoot growth of +P (*left*) and –P (*right*). (*B*) –P roots with numerous clusters (*right*) and +P roots without clusters (*left*) of proteoid roots.

Results

The effect of P supply on biomass and tissue P concentration

After 3 weeks of plant growth, shoot and root growth parameters of +P and –P plants were compared, and conspicuous differences were observed (Fig. 1). The –P plants exhibited obvious P-deficiency symptoms including general growth reduction, purple coloration of leaves and development of numerous proteoid roots. P deficiency resulted in approximately a 40% reduction in shoot biomass compared with +P plants, but did not significantly affect root biomass (Fig. 2). Total P concentration of both roots and shoots was significantly lower for –P plants than +P plants (Fig. 3). Root P concentration of +P plants was 8-fold that of –P plants. Similarly, shoot P concentration of +P plants was 4-fold that of –P plants. This indicates that P-deficient plants experienced severe P deficiency.

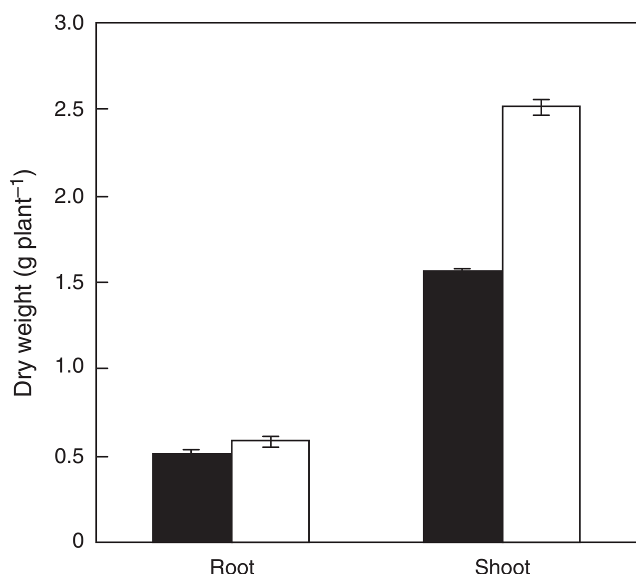

Fig. 2. The effect of P supply on root and shoot DW. Black bars, –P; white bars, +P. Bars represent means ± SE of three replicates and three independent experiments.

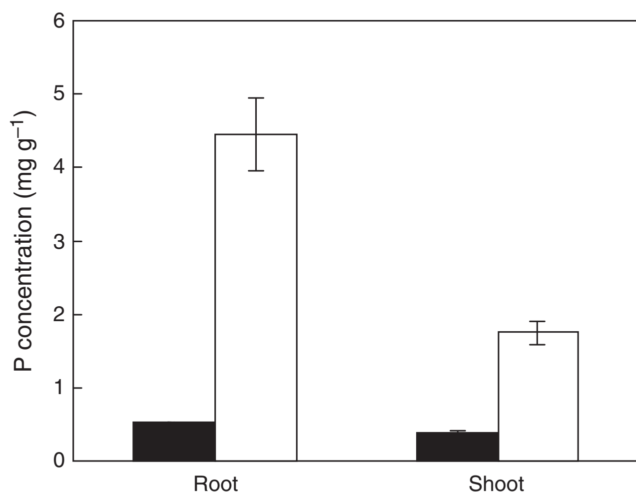

Fig. 3. The effect of P supply on root and shoot P concentration. Black bars, –P; white bars, +P. Bars represent means ± SE of three replicates and three independent experiments.

The combined effect of P and Al stresses

To study the combined effects of P and Al stresses, –P plants and +P plants were exposed to +Al or –Al and the amount of organic anions (citrate and malate) exuded was quantified. As shown in Fig. 4*A*, +Al did not affect the exudation of citrate significantly in either –P or +P plants. However, P deficiency significantly enhanced exudation of citrate. The exudation of citrate was approximately 3–4 times higher in –P plants than +P plants. By comparison, +Al slightly induced exudation of malate (Fig. 4*B*) in –P plants. P-deficient plants exuded 2–3 times as much malate as +P plants. In general, P deficiency enhanced exudation of citrate and malate significantly and Al seems to have no significant role in the response.

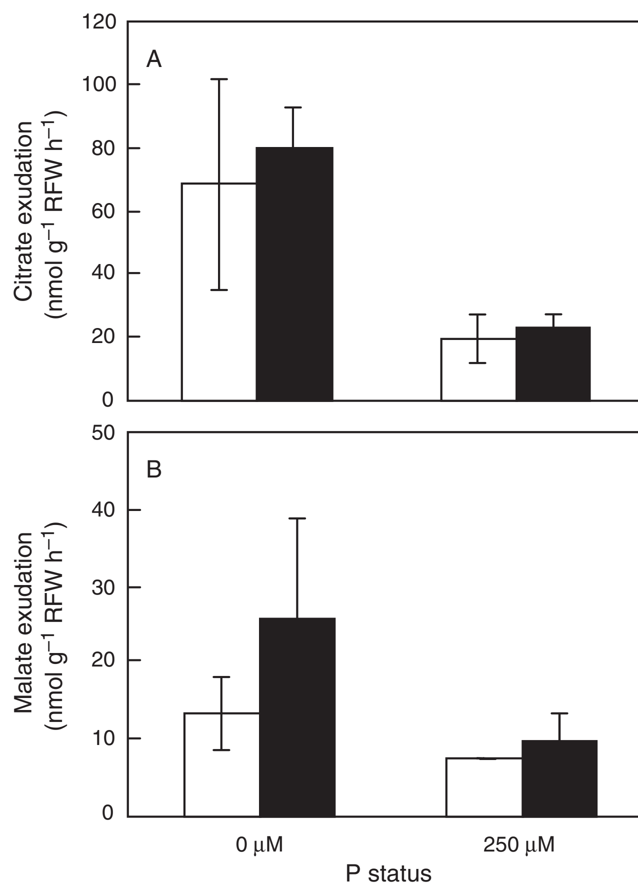

Fig. 4. The effect of P supply and Al treatment on citrate (*A*) malate (*B*) exudation. Black bars, –Al; white bars, +Al. Bars represent means ± SE of three replicates and three independent experiments.

The effect of prolonged Al exposure on citrate and malate exudation

The prolonged effect of +Al was studied during 9 h of exposure. For both –P and +P, a slight increase in citrate exudation was observed (Fig. 5*A*). Citrate exudation increased continuously and was higher in –P plants than +P plants. Malate exudation increased in both +P and –P plants for only the first 3 h, after which it decreased sharply (Fig. 5*B*). However, malate exudation was significantly higher in –P plants than +P plants. This finding, together with the above results, indicates that P deficiency alone can induce the exudation of organic anions from greater purple lupin.

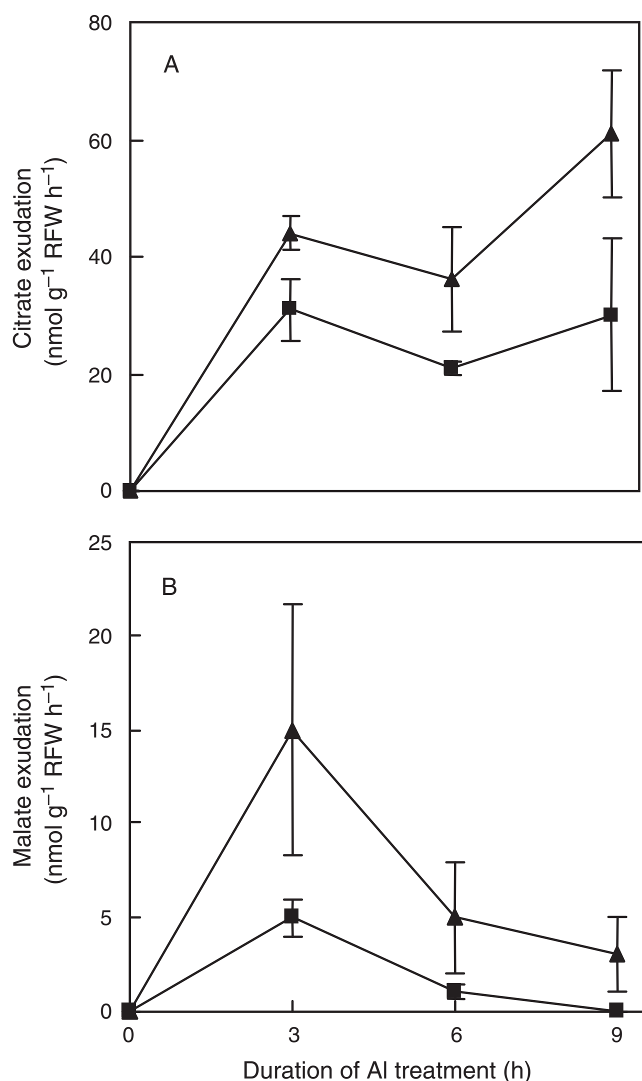

Fig. 5. The time course of effect of Al on citrate (*A*) and malate (*B*) exudation from –P and +P plants. ▲, 0 µM P; (–P); ■, 250 µM P (+P). Curves indicate means ± SE of three replicates and two independent experiments.

The effect of vanadate and anion channel blockers on organic anion exudation

The effect of vanadate on the exudation of organic anions was studied *in vivo*. The presence of 1 mM vanadate resulted in a 60% reduction in citrate exudation (Fig. 6*A*), but had no significant effect on the exudation of malate (Fig. 6*B*). The involvement of anion channels in organic anion exudation was confirmed by the effect of anion channel blockers (A-9-C, NIF and PG). Citrate exudation was significantly inhibited by these anion channel blockers in the order of PG (40%) > A-9-C (30%) > NIF (25%) (Fig. 6*A*). Similarly, malate exudation was significantly inhibited by A-9-C (30%), NIF (25%) and PG (25%) (Fig. 6*B*).

Phosphorus deficiency enhances H^+-ATPase activity

To understand the mechanisms involved in the enhanced exudation of organic anions from –P plants, plasma membrane vesicles were isolated from both +P and –P roots

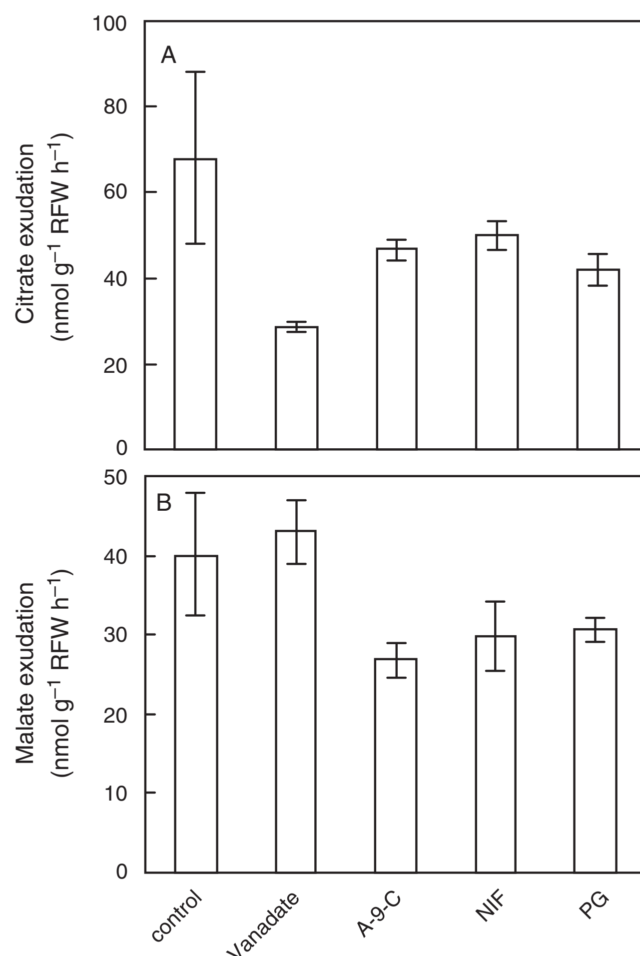

Fig. 6. The effect of 1 mM vanadate and 50 µM anion channel blockers (A-9-C, NIF and PG) on citrate (*A*) and malate (*B*) exudation. Bars indicate means ± SE of three replicates and two independent experiments.

exposed to +Al or –Al and H^+-ATPase activity was assayed. There was no significant effect of +Al on H^+-ATPase activity, while –P resulted in a 3-fold increase (Fig. 7). To confirm the purity of plasma membrane vesicles, H^+-ATPase activity was assayed in the presence of the inhibitors vanadate, nitrate, azide, and molybdate. Vanadate, which specifically inhibits plasma membrane H^+-ATPase activity, resulted in 80% inhibition. Nitrate and azide (inhibitors of tonoplast and mitochondrial H^+-ATPase, respectively) resulted in 2% inhibition indicating a very low level of contamination by tonoplast and mitochondria. However, H^+-ATPase activity showed a significant sensitivity to molybdate (25%, data not shown) indicating the presence of unspecific acid phosphatase activity (Yan *et al.* 2002).

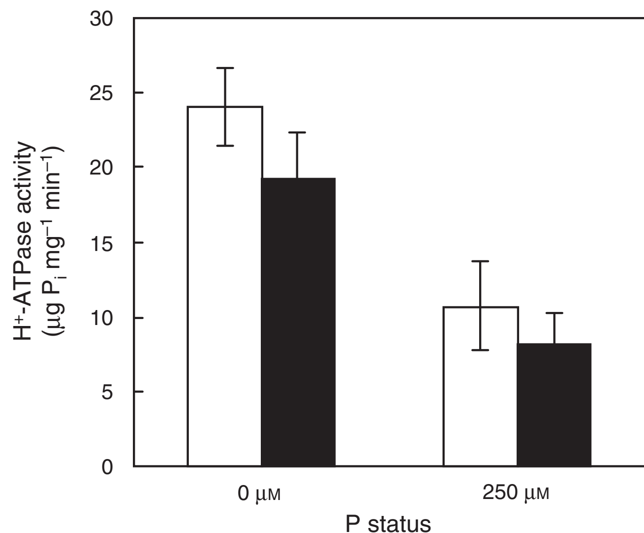

Fig. 7. The effect of P supply and Al treatment on plasma membrane H^+-ATPase activity. P_i, inorganic phosphate. Black bars, +Al; white bars, –Al. Bars represent means ± SE of three replicates and four independent experiments.

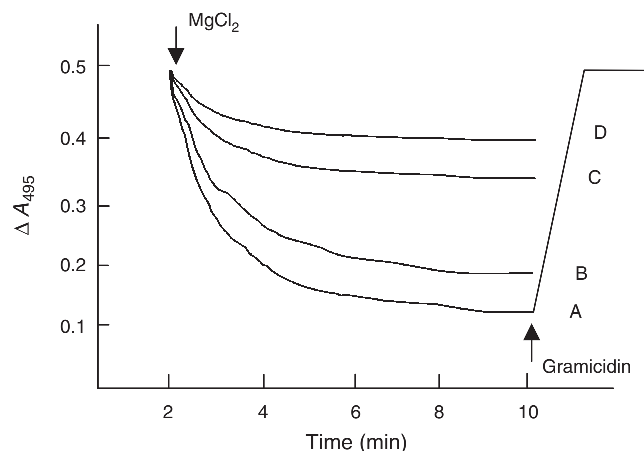

Fig. 8. The effects of P supply and Al treatment on plasma membrane H^+-pump activity. Plasma membrane vesicles were isolated and H^+-pump activity was determined as ΔA_{495} of acridine orange. The treatments were *A* –P, –Al; *B* –P, +Al; *C* +P, –Al; and *D* +P, +Al. The maximum quenching in treatments *A* and *B* is proportional to their higher H^+-efflux compared with treatments *C* and *D*.

Phosphorus deficiency enhances plasma membrane H^+-pump activity

Plasma membrane H^+-pump activity was determined by the absorbance quenching of acridine orange. Quenching occurred after addition of $MgCl_2$ in the presence of Brij 58, indicating that the plasma membrane vesicles were originally right-side-out. The increase in H^+-ATPase activity in –P plants was coupled with an increase in H^+-pump activity (Fig. 8). The level of H^+-pump activity in –P plants was twice as high as that in +P plants. In both –P and +P plants, Al slightly inhibited H^+-pump activity. The established pH gradient was abolished when gramicidin was added.

Discussion

Root and shoot growth parameters of phosphorus-sufficient and -deficient plants were compared. Although P deficiency significantly reduced the shoot growth (40%), it did not affect the total root DW (Figs 1, 2) and it resulted in the formation of numerous proteoid roots (Fig. 1*B*). The formation of proteoid roots is regulated by the P status of the plant. Decreased formation of proteoid roots with increased P was reported for *L. consentinii* (Trinick 1977), *V. juncea* (Walker and Pate 1986) and *L. albus* (Gardner *et al.* 1982; Keerthisinghe *et al.* 1998). We observed abundant proteoid roots in *L. pilosus* when the root and shoot P concentrations were very low ($0.54\,mg\,g^{-1}$ and $0.39\,mg\,g^{-1}$, respectively; Fig. 3), but proteoid roots were not observed in +P plants, indicating that proteoid roots form in response to P deficiency.

The combined effect of P and Al stress on organic anion exudation has been investigated. Although exudation of organic anions has been reported as an Al-tolerance mechanism in snapbean, wheat, maize, tora, soybean and rape (Miyasaka *et al.* 1991; Delhaize *et al.* 1993; Pellet *et al.* 1995; Ma *et al.* 1997*a*; Yang *et al.* 2000; Ligaba *et al.* 2004), our results showed that +Al did not induce citrate exudation from either –P plants or +P plants. Unlike citrate, +Al slightly induced malate exudation from –P plants (Fig. 4*A*, *B*). However, the effects of P deficiency on both citrate and malate exudation was significant. Exudation from –P plants with numerous proteoid roots was significantly higher than from +P plants devoid of proteoid roots. Similar effects of P deficiency have been reported for proteoid roots of white lupin (Gardner *et al.* 1983; Neumann *et al.* 1999), alfalfa (Lipton *et al.* 1987) and rape (Hoffland *et al.* 1989).

However, P deficiency did not induce exudation of organic anions from wheat (Delhaize *et al*. 1993), buckwheat and taro (Ma *et al.* 1997*a*, 1998), soybean (Yang *et al*. 2000) or rape (Ligaba *et al*. 2004). In soybean, Al-induced citrate exudation was significantly higher in the presence of 10 µM P than in the absence of P (Nian *et al*. 2003). Similarly, a previous study indicated that P-sufficient plants of rape exuded twice as much malate as P-deficient plants (Ligaba *et al*. 2004). These findings indicate that the type and amount of organic anions released depends on the plant species and external factors including P and Al stresses. When plants were exposed to +Al for 9 h, citrate exudation continued to increase in both –P and +P plants, whereas malate exudation increased significantly during the first 3 h, then decreased sharply. In an Al-tolerant wheat cultivar, Al-induced malate efflux from the root apex was detected within 15 min (Delhaize *et al*. 1993) and 5 min (Osawa and Matsumoto 2001) of Al exposure. The exudation of both citrate and malate was significantly higher in –P plants than +P plants. Hence, enhanced exudation of citrate and malate from greater purple lupin is a specific response to P deficiency, and Al seems to have no significant role in the response.

In the present study, exudation of organic anions from the proteoid roots of –P plants was significantly higher than that of the normal roots of +P plants. Previous reports have indicated that proteoid roots have a strong capacity under P-deficient conditions to acidify the rhizosphere by releasing citrate and malate (Gardner *et al*. 1983; Dinkelaker *et al*. 1989; Neumann *et al*. 1999). Measurement of plasma membrane H^+-ATPase activity and H^+-pump activity showed that the enhanced exudation of mainly citrate from –P plants could be attributed to the increase in the H^+-ATPase activity and the H^+-pump activity across the plasma membrane (Figs 7, 8). Previous work on proteoid roots of white lupin indicated that the release of organic anions involve an active H^+ efflux driven by the plasma membrane H^+-ATPase, and a passive efflux of organic anions mediated by the anion channels (Johnson *et al*. 1996; Neumann *et al*. 1999; Yan *et al*. 2002). Similarly, Ohne *et al*. (2003) reported that citrate release in carrot mutant cells (IPG, insoluble phosphate growers) was concomitant with H^+-efflux. In active proteoid roots of white lupin, acidification was not observed in the presence of 1 mM vanadate (Yan *et al*. 2002). In carrot mutant cells, 0.5–1 mM vanadate inhibited citrate transport by 50% (Ohne *et al*. 2003). In the current study, we also found that the presence of 1 mM vanadate (a specific inhibitor of plasma membrane H^+-ATPase) suppressed the activity of H^+-ATPase of greater purple lupin by 80% *in vitro* and inhibited *in vivo* citrate exudation by 60%. However, the exudation of malate was not sensitive to vanadate. Previous reports indicated that Al-induced malate efflux from wheat was accompanied by the release of K^+ (Ryan *et al*. 1995; Osawa and Matsumoto 2002). These results indicate that the transport of citrate and malate might involve separate pathways. In greater purple lupin, the efflux of citrate, but not malate, could be attributed to the efflux of H^+ upon the increase in H^+-ATPase activity as well as H^+-pump activity. However, in tomato and wheat the release of H^+ did not coincide with increased exudation of carboxylic acids from roots (Neumann and Römheld 1999).

The current study shows that +Al did not markedly affect the activity of H^+-ATPase in either –P or +P plants (Fig. 7). In contrast, Matsumoto (1988) reported that in roots of barley seedlings treated with 1 mM Al for 1 d, proton extrusion was significantly inhibited. He also observed that the K_m (Michaelis constant)for ATP in *in vitro* H^+-transport activity of microsomal fractions of roots was 0.25 mM and 0.45 mM in the presence or absence of 100 µM Al, respectively. Similarly, Ahn *et al.* (2001, 2002, 2004) reported that treatment of wheat *in vivo* with 2.26 µM Al for 4 h inhibited plasma membrane H^+-ATPase activity by depolarising the surface potential in squashed roots of an Al-sensitive cultivar (ES8), but not an Al-tolerant one (ET8). These results indicate that the effect of Al on H^+-ATPase activity might vary with plant species, age and Al concentration.

The effect of anion channels on organic anion exudation was investigated. In the presence of anion channel blockers, the exudation of citrate and malate was inhibited by 25–40%. Studies of wheat (Ryan *et al*. 1995) and white lupin (Neumann *et al*. 1999) have also found that anion channel blockers inhibit the exudation of organic anions. Ligaba *et al*. (2004) reported that in rape, 10 µM PG could inhibit the exudation of citrate and malate by 50%. This suggests that anion channels are involved in the process of organic anion exudation along the plasma membrane.

In conclusion, enhanced exudation of citrate and malate from greater purple lupin is a specific response to P deficiency, and Al did not significantly alter the response. Significantly higher H^+-ATPase levels and associated H^+ pump activity could be a possible reason for the elevated exudation of citrate but not malate from –P plants compared with +P plants. However, further work on the property of H^+-ATPase at a molecular level is required to understand the relationship between ATPase activity and citrate exudation under P deficiency in the presence or absence of Al.

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