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Title page

Phosphorus deficiency enhances aluminum tolerance of rice (*Oryza sativa*) by changing the physicochemical characteristics of root plasma membranes and cell walls

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SUMMARY

The negative charge at the root surface is mainly derived from the phosphate group of phospholipids in plasma membranes (PMs) and the carboxyl group of pectins in cell walls, which are usually neutralized by calcium (Ca) ions contributing to maintain the root integrity. The major toxic effect of aluminum (Al) in plants is the inhibition of root elongation due to Al binding tightly to these negative sites in exchange for Ca. Because phospholipid and pectin concentrations decrease in roots of some plant species under phosphorus (P)-limiting conditions, we hypothesized that rice (*Oryza sativa* L.) seedlings grown under P-limiting conditions would demonstrate enhanced Al tolerance because of their fewer sites on their roots. For pretreatment, rice seedlings were grown in a culture solution with (+P) or without (–P) P. Thereafter, the seedlings were transferred to a solution with or without Al, and the lipid, pectin, hemicellulose, and mineral concentrations as well as Al tolerance were then determined. Furthermore, the low-Ca tolerance of P-pretreated seedlings was investigated under different pH conditions. The concentrations of phospholipids and pectins in the roots of rice receiving –P pretreatment were lower than those receiving +P pretreatment. As expected, seedlings receiving the –P pretreatment showed enhanced Al tolerance, accompanied by the decrease in Al accumulation in their roots and shoots. This low P-induced enhanced

Al tolerance was not explained by enhanced antioxidant activities or organic acid secretion from roots but by the decrease in phospholipid and pectin concentrations in the roots. In addition, low-Ca tolerance of the roots was enhanced by the –P pretreatment under low pH conditions. This low P-induced enhancement of low-Ca tolerance may be related to the lower Ca requirement to maintain PM and cell wall structures in roots of rice with fewer phospholipids and pectins.

Key words

aluminum tolerance; galactolipid; low calcium tolerance; *Oryza sativa*; phosphorus deficiency; phospholipid; pectin; plasma membrane

Abbreviations

BHT, butylated hydroxytoluene; DGDG, digalactosylglycerol; HC, hemicellulose; MDA, malondialdehyde; MGDG, monogalactosylglycerol; PM, plasma membrane; TBARS, thiobarbituric acid reactive substance

INTRODUCTION

High aluminum (Al) concentrations in soil solution are the most important factor restricting plant growth in acidic soils. Various studies on Al tolerance mechanisms in crop plants have been conducted to improve crop productivity in acidic soils. Till date, various Al tolerance mechanisms have been reported in plants, such as Al exclusion and internal Al tolerance (Kochian et al., 2004; Poschenrieder et al., 2008). Organic acid anion release from roots is a major Al tolerance factor in various plant species (Barceló and Poschenrieder, 2002). Rice (*Oryza sativa* L.) is relatively more tolerant to Al than other crops, but organic acid anion release alone cannot explain its Al tolerance (Ma et al., 2002), suggesting that other critical mechanism(s) are operational in rice.

Exposure to Al ions induces the coordinated expression of a range of Al tolerance genes. In *Arabidopsis thaliana*, the zinc finger protein sensitive to proton rhizotoxicity 1 (STOP1) regulates transcription of multiple genes critical for Al tolerance and low pH (Sawaki et al., 2009). Similarly, the ADP-ribosyltransferase 1 (ART1) protein is found in rice and shares significant sequence similarity with STOP1 (Yamaji et al., 2009). Al ions interact with a receptor on the plasma membrane (PM) to initiate an unknown signal transduction pathway that activates ART1 (Delhaize et al., 2012). ART1 regulates the expression of at least 31 genes, some of which have been implicated in both internal

1 and external detoxification mechanisms during Al tolerance (Yamaji et al., 2009).
2 However, the structural characteristics of roots have also been suggested to be involved
3 in Al tolerance in rice (Watanabe and Okada, 2005).

4 The negative charge on the PM surface of the root cell caused by the dissociation of
5 protons (H^+) from the anionic ligand, mainly phospholipids, and is a major factor
6 altering Al accumulation at the PM surface and may affect Al tolerance (Wagatsuma and
7 Akiba, 1989; Kobayashi et al., 2013). PM is usually stabilized by calcium (Ca) binding
8 to the phosphate group of phospholipids (Shoemaker and Vanderlick, 2003). Al replaces
9 membrane-bound Ca and tightly binds to the negative sites of the phosphate groups of
10 phospholipids, making the membrane rigid and gel-like, finally resulting in increase in
11 the PM permeability (Deleers et al., 1986). PMs of root cells of Al-tolerant plant species,
12 including rice, are less negatively charged than those of Al-sensitive species
13 (Wagatsuma et al., 2005).

14 A previous study demonstrated a contrasting difference in the lipid composition of
15 PM between Al-tolerant and Al-sensitive rice cultivars (Khan et al., 2005). The ratio of
16 phospholipids/sterols in root-tip membranes is lower in Al tolerant rice cultivars than in
17 Al sensitive cultivars (Khan et al., 2009). These findings indicate that the phospholipid
18 concentration (proportion) in PM of root cell is an important factor in Al tolerance in

1 rice. Thus, it may be possible to enhance Al tolerance in rice by decreasing the
2 proportion of the phospholipids in root cells.

3 Some reports indicate that the negativity of carboxyl groups in the root cell wall is
4 also involved in Al tolerance. Cell wall structure is maintained with gelled galacturonic
5 acid, which is a major component of pectin, by Ca binding to the non-esterified
6 carboxyl groups of galacturonic acid. Al binds to root cell wall components, particularly
7 the carboxyl groups of pectins, replacing Ca and making the cell wall rigid, thus leading
8 to inhibition of root elongation. Therefore, the different cell wall composition may alter
9 the amount of Al accumulating in the cell wall and the tolerance to Al. In fact, a greater
10 amount of pectin in the cell wall leads to greater accumulation of Al in roots of maize
11 (Horst et al., 1999) and buckwheat (Yang et al., 2011). Moreover, lower uronic acid
12 content in the cell wall with a higher degree of methylesterification results in fewer
13 carboxyl groups, which serve as Al-binding sites, inducing greater Al exclusion, as
14 observed in Al-resistant rice (Yang et al., 2008). Thus, cell wall composition is related to
15 Al tolerance.

16 Phosphorus (P) is one of the major essential elements for plant growth; however, its
17 availability is often low in soil. Thus, plants have developed various mechanisms to
18 adapt to P-deficient conditions. One of the mechanisms is to improve P-use efficiency. P

1 starvation triggers membrane lipid remodeling in root cells, a process that replaces a
2 significant portion of membrane phospholipids with non-P-containing galactolipids,
3 presumably to use the phospholipids as an internal P reserve (Andersson et al., 2003;
4 2005; Nakamura et al., 2009; Tjellström et al., 2010). Not only membrane lipids but
5 also cell wall components could change under P-deficient conditions (Fernandes et al.,
6 2013), but their role in low P tolerance is unclear. P deficiency significantly decreases
7 the pectin concentration in the root cell wall, which leads to decrease in cadmium (Cd)
8 bound to the root cell wall and alleviation of Cd toxicity in *A. thaliana* (Zhu et al.,
9 2012).

10 Therefore, we hypothesized that rice plants grown under low-P conditions will be
11 more tolerant to Al stress in acidic soil than those grown under moderate P conditions
12 because of the fewer Al-binding sites available on the PM surface and/or cell wall.

15 MATERIALS AND METHODS

16 *P pretreatment*

17 Seeds of rice (*Oryza sativa* L. cv. Koshihikari) were surface sterilized with sodium
18 hypochlorite (0.5–1% available chlorine) for 10 min, washed with water, and soaked in

running water for 3 days. The seeds were germinated on a nylon screen placed in a polypropylene container filled with 36 L of nutrient solution, including 2.14 mM N (NH_4NO_3), 0.77 mM K ($\text{K}_2\text{SO}_4\text{:KCl} = 1\text{:}1$), 1.2 mM Ca ($\text{CaCl}_2\cdot 2\text{H}_2\text{O}$), 0.82 mM Mg ($\text{MgSO}_4\cdot 7\text{H}_2\text{O}$), 35 μM Fe ($\text{FeSO}_4\cdot 7\text{H}_2\text{O}$), 9.1 μM Mn ($\text{MnSO}_4\cdot 5\text{H}_2\text{O}$), 46.3 μM B (H_3BO_4), 3.1 μM Zn ($\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$), 0.016 μM Cu ($\text{CuSO}_4\cdot 5\text{H}_2\text{O}$), and 0.05 μM Mo ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$), with (+P) or without (–P) 0.32 mM P ($\text{NaH}_2\text{PO}_4\cdot 4\text{H}_2\text{O}$). The seedlings were grown in a greenhouse at Hokkaido University for 2 weeks (14 h photoperiod and day/night temperature of 25–28 °C /18–22 °C, respectively). The pH of the solution was adjusted daily to pH 4.9. After P pretreatment, the seedlings were sampled, washed with deionized water, and cut to separate the roots from the shoots. The longest roots and the fresh weight of the shoots and roots were determined. The fresh samples were rapidly frozen in liquid nitrogen, lyophilized, weighed, and ground. Concentrations of each element in the shoots and roots and the concentrations of phospholipids, galactolipids and sterols in the roots were determined as described later.

Al treatment

After P pretreatment, the seedlings were immersed in tap water (pH 4.9) overnight to remove P from the root-free space, transferred to a 0.5 mM CaCl_2 solution (pH 4.5) with

(+Al) or without (–Al) 0.1 mM Al ($\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$) and cultivated for 1 week. Some seedlings were used to estimate organic acid exudation from the roots, as described later. After Al treatment, the seedlings were sampled as described above. No nutrient deficiency symptoms were observed during the Al treatment.

Ca treatment

After P pretreatment, the seedlings were immersed in tap water (pH 4.9) overnight to remove P from the root-free space, transferred to a solution (pH 4.9 or 4.2) with 0.5 mM CaCl_2 (high-Ca) or 0.1 mM CaCl_2 (low-Ca), and cultured for 1 week. Thereafter the roots were determined.

Analysis of phospholipids, galactolipids, and sterols in roots

Phospholipids and galactolipids

Phospholipids and galactolipids were extracted using the method described by Bligh and Dyer (1959), and modified by Uemura and Yoshida (1984). The lyophilized root sample (50 mg) was homogenized three times in a mixture that included 2.5 mL 2-propanol, 2.5 mL chloroform, and 1.25 mL H_2O , using a mortar and pestle. The homogenized sample was centrifuged at $1000 \times g$ for 5 min, and the chloroform layer

was separated. Subsequently, 5 mL of chloroform was added to the residue, shaken for 5 min, and centrifuged, and the chloroform layer was removed. This layer was then filtered through a filter paper (No.6, Advantec, Tokyo, Japan) and shaken with the same volume of 0.1 M KCl several times to remove protein and water soluble molecules (e.g., ATP). Then, the chloroform layer was dehydrated with Na₂SO₄, evaporated at 40 °C, and resolubilized in 1 mL chloroform:methanol (2:1, v:v). Phospholipids were quantified by measuring the P concentration in the lipid extract using the malachite green spectrophotometric method (Vanveldhoven and Mannaerts, 1987) after wet digestion with sulfuric acid. The lipid extracts were separated on silicagel thin-layer chromatography plates (silica gel 60 F₂₅₄, Merck, Japan) with chloroform:methanol:water (65:15:2, v:v:v) to determine the galactolipid concentration. Monogalactosyldiacylglycerol (MGDG) (Funakoshi, Tokyo, Japan) and digalactosyldiacylglycerol (DGDG) (Funakoshi) were used as standards. To visualize the lipid bands, the plates were sprayed with 2% anthrone–sulfuric acid and heated at 90 °C for 5 min. The plates were scanned with a scanner (GT–9300UF, EPSON, Tokyo, Japan) and analyzed using ImageJ ver. 1.46 (Wayne Raband, National Institutes, of Health, Bethesda, MD USA; <http://rsb.info.nih.gov/ij>).

Sterols

Sterols were extracted as described by Hartmann and Benveniste (1987) with slight modifications. The lyophilized root sample (50 mg) was homogenized three times in 5 mL dichloromethane:methanol (2:1, v:v) using a mortar and pestle. The homogenates were filtered through a filter paper (No.6, Advantec) and washed several times with the same solution. The crude lipid extract was purified, dehydrated and resolubilized as described above. The Δ^5 -sterol concentration was determined using the method of Zlatkis and Zak (1969). Stigmasterol (Funakoshi) was used as a standard.

Cell wall isolation and fractionation

Crude cell walls were prepared as described by Zhong and Lauchli (1993) with minor modifications. In brief, approximately 10 mg of a lyophilized root powder sample was homogenized with a mortar and pestle in 75% ethanol on ice. The homogenates were centrifuged at 10,000 $\times g$ for 10 min, and the supernatant was discarded. The pellets were washed three times each with an ice-cold acetone, methanol:chloroform mixture (1:1, v:v), and then with methanol. The supernatant of each wash was discarded, and the final pellet was dried under vacuum. The dried cell wall material was treated with 20 units of α -amylase.

Crude cell walls were fractionated according to the procedure described by Nishitani and Masuda (1983), with minor modifications according to Zhu et al. (2012). Pectins were extracted from the crude cell walls by incubating three times with 1 mL of 20 mM ammonium oxalate at 70 °C for 1 h each. The supernatants containing the oxalate-soluble pectins were collected after centrifugation at 10,000 ×g for 10 min. The pellet was subjected to triple extraction with 1 mL of 4% KOH solution at room temperature for a 24 h, followed by a similar extraction with 24% KOH. The pooled supernatants from the 4% and 24% KOH extractions yielded the hemicellulose 1 (HC1) and hemicellulose 2 (HC2) fractions, respectively. The HC2 fraction was neutralized with acetic acid before analysis.

Uronic acid and total sugar analyses

The uronic acid concentration in the pectin fraction was assayed using galacturonic acid as the standard, according to the method of Blumenkrantz and Asboe-Hansen (1973). In brief, 200 µL of pectin extract was incubated with 1.2 mL of 98% H₂SO₄ (containing 12.5 mM Na₂B₄O₇·10H₂O) at 100 °C for 5 min. After cooling, 20 µL of 0.15% m-hydroxy-diphenyl in 0.5% NaOH was added to the solution. The sample was allowed to stand at room temperature for 20 min, and the absorbance was

spectrophotometrically measured at 492 nm. Because carbohydrates produce a pinkish chromogen with sulfuric acid/tetraborate at 100 °C, the absorbance of a blank sample was measured without m-hydroxy-diphenyl, which was replaced with 20 µL 0.5% NaOH. The absorbance of the blank sample was subtracted from the total absorbance.

The total sugar concentration in the HC1 and HC2 fractions was analyzed by the phenol-sulfuric acid method, with glucose as the standard (DuBois et al., 1956). In brief, 200 µL of the HC1 or HC2 extract was incubated with 1 mL of 98% H₂SO₄ and 10 µL of 80% phenol at room temperature for 15 min and then at 100 °C for 15 min. After cooling, the absorbance was spectrophotometrically measured at 492 nm.

Determination of lipid peroxidation

After the P pretreatments and AI treatments, lipid peroxidation was estimated in the roots by measuring the malondialdehyde (MDA) content, as described by Munne-Bosch et al. (2004). Fresh root tips (<50 mm) were repeatedly extracted (three times) in ethanol:water (80:20, v:v) with 1 µg mL⁻¹ butylated hydroxytoluene (BHT) using a homogenizer and ultrasonicator. After centrifugation, the supernatants were pooled, and 1 mL of sample was added to a test tube with 1 mL of either A = -thiobarbituric acid (TBA) solution with 20% (w/v) trichloroacetic acid and 0.01%

(w/v) BHT or a +TBA solution with the above plus 0.65% (w/v) TBA. The samples were heated at 95 °C for 25 min and then centrifuged at 10,000 ×g for 15 min after cooling. The absorbance of the supernatant was read at 440, 532, and 600 nm. MDA equivalents (nmol mL⁻¹) were calculated using the formula:

$$10^6 \times [(A - B)/157000],$$

where $A = [(Abs_{532} + TBA - Abs_{600} + TBA) - (Abs_{532} - TBA - Abs_{600} - TBA)]$ and

$$B = [(Abs_{440} + TBA - Abs_{600} + TBA) \times 0.0571].$$

Analysis of organic acids exuded from roots

After P pretreatment, the seedlings were transplanted to a tube containing 45 mL of 500 µM CaCl₂ solution with or without 100 µM AlCl₃ (pH 4.5) and cultured for 6 h. The solution was filtered through a membrane filter (pore size = 0.45 µm, Advantec), frozen, and lyophilized. The residue was dissolved in 0.02 M HCl, and the organic acid concentration was determined by capillary electrophoresis (Quanta 4000 CE, Waters, Milford, MA, USA), as described by Watanabe et al. (1998).

Mineral analysis

1 A lyophilized shoot or root sample (50 mg) was digested in HNO₃–H₂O₂, and the
2 mineral concentrations were determined using inductively coupled plasma mass
3 spectrophotometry (ELAN DRC–e, Perkin Elmer, Waltham, MA, USA).

4 5 *Hematoxylin staining*

6 After Al treatment, the roots were washed with deionized water and stained in 0.2%
7 hematoxylin solution with 0.02% NaIO₃ for 15 min (Polle et al., 1978). Then, the roots
8 were washed with deionized water for 20 min and photographed.

9 10 11 RESULTS

12 *P pretreatment*

13 No significant difference was observed in the dry weight of the roots between the +P
14 and –P pretreatments, whereas the dry weight of the shoots receiving the +P
15 pretreatment was significantly higher than that of the shoots receiving the –P
16 pretreatment (Fig. 1). The P concentration in the rice shoots and roots receiving +P
17 pretreatment was three and eight times higher, than that of the shoots and roots
18 receiving the –P pretreatment, respectively (Fig. 2), demonstrating that rice plants

receiving the $-P$ pretreatment had low-P nutrient status. The concentrations of phospholipids and galactolipids in roots with $-P$ pretreatment were lower and higher than those in plants receiving $+P$ pretreatment, respectively (Fig. 3A, B). Because the concentrations of MGDG were below the quantification limit in all the samples, the concentration of galactolipids was the same as the concentration of DGDG. No significant difference was observed in sterol concentration between the $+P$ and $-P$ pretreatments (Fig. 3C). Figure 3D depicts the proportion of phospholipids, galactolipids, and sterols in the roots. The ratio of phospholipids:galactolipids:sterols was 74:9:17 in the roots receiving the $+P$ pretreatment and 53:27:20 in those receiving $-P$ pretreatment, indicating that the proportion of galactolipids was higher in the roots receiving the $-P$ pretreatment than in those receiving the $+P$ pretreatment. The proportion of sterols did not change considerably by either of the pretreatments. Although no significant difference was observed in the concentration of pectin, HC1, and HC2 in the roots receiving the $+P$ and $-P$ pretreatments (Fig. 4), a tendency for decrease and increase in the pectin and HC concentrations, respectively, was observed in the $-P$ pretreatment.

Al treatment

The P pretreatment affected Al tolerance in the rice plants. The longest roots after +Al treatment relative to -Al treatment was calculated for each of the P pretreatments. Relative root length was longer in the roots receiving +Al treatment after the -P pretreatment (-P+Al) than in those receiving the +Al treatment after the +P pretreatment (+P+Al) (Table 2). The Al concentration in the shoots and roots receiving the -P+Al treatment was lower than that in the shoots and roots receiving the +P+Al treatment (Fig. 5). Furthermore, the roots receiving the -P+Al treatment showed lower Al accumulation in their tips when stained with hematoxylin than those receiving the +P+Al treatment (Fig. 6). Irrespective of the P pretreatment, the plants grown without Al demonstrated no Al accumulation in their root tips (Fig. 6). P pretreatment did not affect the level of lipid peroxidation in the +Al treated roots (Table 1). Capillary electrophoresis quantitatively detected that only citrate was released from the roots, and P pretreatment and Al treatment did not affect the levels of citrate released (Table 1).

Ca treatment

The largest relative root length of plants receiving low-Ca treatment relative to that in plants receiving high-Ca treatment (low-Ca/high-Ca) was calculated at each pH and for

each P pretreatment to evaluate low-Ca tolerance. The extent of inhibition was lesser in the roots receiving the -P pretreatment than in those receiving the +P pretreatment, but only at pH 4.2 (Table 2).

DISCUSSION

As expected, in the present study, Al tolerance in rice was enhanced by P starvation (Table 2). Some plant species under P-deficient conditions show enhanced antioxidant activity (e.g., tobacco cultured cells; Yamamoto et al., 1996) and/or organic acid secretion from their roots (e.g., soybean; Nian et al., 2003), which enhances Al tolerance. However, these mechanisms were not involved in enhanced Al tolerance in the rice plants in our study (Table 1). Thus, what mechanism(s) is related to enhanced Al tolerance under the P-deficient condition in rice?

Replacement of phospholipids with non-phosphate-containing membrane lipids is one strategy to adapt to P deficiency. We here demonstrated that galactolipids replaced a portion of the phospholipids in the rice roots under P-limiting conditions (Figs. 2, and 3A, B). Low-P-induced replacement of phospholipids with galactolipids has been reported in various plant species, such as in the leaves of *A. thaliana* and oat, and the roots of *A. thaliana*, oat and bean (Härtel et al., 2000; Andersson et al., 2003; Russo et

al., 2007). This replacement of phospholipids with galactolipids under P-deficient conditions has been demonstrated in various membrane fractions, including PM (Andersson et al., 2003), the mitochondrial membrane (Jouhet et al., 2004), the tonoplast membrane (Andersson et al., 2005), and the chloroplast membrane (Härtel et al., 2000). Thus, P-deficiency-induced lipid replacement in whole roots reflect that in PM. Al binds to phospholipids in PM and induces reduction in membrane fluidity and/or increase in membrane permeability (Chen et al., 1991; MacKinnon et al., 2006). Rice plants receiving the -P pretreatment, which contained a lower proportion of phospholipids and higher proportion of galactolipids than the rice plants receiving the +P pretreatment, were thus expected to maintain membrane fluidity and membrane permeability in root cells under Al stress because of reduction in the amount of Al binding to PM, resulting in higher Al tolerance.

In addition to alterations in the lipid composition, P deficiency may affect the cell wall composition (Fernandes et al., 2013). Although it was not statistically significant, the pectin concentration in the root cells tended to decrease under the P deficient condition (Fig. 4). Similarly, P deficiency decreases pectin and hemicellulose concentrations in the cell wall of *A. thaliana*, although the mechanism has not been elucidated (Zhu et al., 2012). The binding of Al to pectins is one of the most

1 important factors in the expression of Al toxicity, and a negative correlation between the
2 amount of pectin in roots and Al tolerance has been suggested. Therefore, decrease in
3 the pectin concentration may also be involved in the mechanism of the enhanced Al
4 tolerance in rice under P-deficient conditions.

5 The enhanced Al tolerance in rice under P-deficient conditions was accompanied by
6 decrease in the Al accumulation in the shoots and roots (Fig. 5). furthermore,
7 hematoxylin staining of the root tips receiving the -P+Al treatment showed less Al
8 accumulation compared with that of root tips receiving the +P+Al (Fig. 6). This
9 low-P-induced decrease in Al accumulation in roots may have been caused by decrease
10 in the amount of Al binding to PM and the cell wall of the roots and/or by decrease in
11 the entry of Al into the root cells.

12 In addition to excess Al stress, Ca deficiency and low pH stress are major factors
13 restricting plant growth in acidic soils. The -P pretreatment significantly enhanced
14 low-Ca tolerance as well as Al tolerance of the rice plants under low pH conditions
15 (Table 2). This can also be explained by replacement of phospholipids with galactolipids
16 in PM and decrease in cell wall pectin. In general, Ca binds to the phosphate residues of
17 PM phospholipids (Legge et al., 1982; Shoemaker and Vanderlick, 2003) and to pectins
18 in the cell wall (Matoh and Kobayashi, 1998) to maintain structures. Similar to Al, H⁺

1 replaces Ca by binding to phospholipids, increasing membrane permeability under low
2 pH conditions (Marschner et al., 1966), and by binding to the cell wall, which weakens
3 its structure (Virk and Cleland, 1990). Therefore, low-Ca stress in roots is more
4 remarkable under low-pH conditions (Table 2). Because rice grown under low-P
5 conditions, contains a lower proportion of phospholipids in PM and a lower
6 concentration of pectin in the cell wall, it requires less Ca to maintain the PM and cell
7 wall structures, and could acquire tolerance to Ca deficiency under low pH conditions.

8 In conclusion, we report that the -P pretreatment enhanced tolerance to excess Al and
9 Ca deficiency under low pH conditions by decreasing the phospholipid content and
10 increasing the galactolipid content in PM and decreasing pectins in the cell wall.
11 Because P deficiency often occurs in acidic soils, we speculate that such alterations
12 have already occurred in plants growing under such conditions. However, the PAH gene
13 encodes phosphatidate phosphatase, a key enzyme involved in the replacing
14 phospholipids with galactolipids (Nakamura et al., 2009). Kobayashi et al. (2013)
15 reported that *pah1pah2* double mutants, which are expected to have greater amounts of
16 phospholipids in cell membranes under P-deficient conditions, are more sensitive to Al
17 than wild-type plants when grown in P-deficient medium. If replacement of
18 phospholipids with galactolipids is promoted by *PAH* over expression, it may be

possible to generate transgenic plants more tolerant to acidic soils. Although many studies have reported the mechanisms of Al tolerance in plants, this is the first study to demonstrate the possibility of enhancing tolerance of plants to multiple stressors in acidic soils by changing structural components (e.g., decrease phospholipids and/or pectins) in root cells of rice.

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1 TABLES

2 Table 1. Effects of P pretreatments on the level of lipid peroxidation and the amount of
 3 organic acid released from roots. The level of lipid peroxidation was estimated by the
 4 level of thiobarbituric acid reactive substances (TBARS). Values are means (n = 3).
 5 Different letters within the same column indicate a significant difference ($P < 0.05$).

6

7

8

		TBARS ($\mu\text{mol g}^{-1}$ FW)	Citrate ($\text{fmol g}^{-1}\text{DW h}^{-1}$)
+P pretreatment	-Al	12.7 ± 1.88 ab	77.7 ± 15.4 a
	+Al	12.2 ± 0.86 ab	53.1 ± 6.77 a
-P pretreatment	-Al	7.64 ± 1.73 b	66.7 ± 11.4 a
	+Al	13.1 ± 1.29 a	73.2 ± 8.08 a

1 Table 2. Relative maximum length of roots in each treatment. +Al/-Al = maximum
2 length of roots receiving the +Al treatment/maximum length of roots receiving the -Al
3 treatment; low-Ca/high-Ca = maximum length of roots receiving the low-Ca treatment/
4 maximum length of roots receiving the high-Ca treatment. Values are means (n = 3). An
5 asterisk within the same column indicates a significant difference ($P < 0.05$).

6

	+Al/-Al	low-Ca/high-Ca	
		pH 4.2	pH 4.9
+P pretreatment	0.859	0.861	0.951
-P pretreatment	0.919*	0.922*	0.955

LEGENDS OF FIGURES

Fig. 1. Dry weight of the shoots and roots after the P pretreatments: +P pretreatment (0.32 mM P) and -P pretreatment (0 mM P). Values are means ($n = 3$), and bars indicate $\pm$ standard errors. Asterisk indicates a significant difference ($**P < 0.01$).

Fig. 2. P concentration in the shoots and roots after the P pretreatments: +P pretreatment (0.32 mM P) and -P pretreatment (0 mM P). Values are means ($n = 3$), and bars indicate $\pm$ standard errors. Asterisk indicates a significant difference ($*P < 0.05$, $**P < 0.01$).

Fig. 3. Concentration of each lipid type [phospholipids (A), galactolipids (B), and sterols (C)] and their proportion (D) after the P pretreatments: +P pretreatment (0.32 mM P) and -P pretreatment (0 mM P). Values are means ($n = 3$), and bars indicate $\pm$ standard errors. Asterisk indicates a significant difference ($*P < 0.05$, $**P < 0.01$).

Fig. 4. Concentrations of pectin (A), hemicellulose 1 (B), and hemicellulose 2 (C) after the P pretreatments: +P pretreatment (0.32 mM P) and -P pretreatment (0 mM P). Values are means ($n = 3$), and bars indicate $\pm$ standard errors.

Fig. 5. Al concentration in the shoots and roots after the Al treatments: +P–Al = –Al treatment (0 mM Al) after the +P pretreatment; –P–Al = –Al treatment (0 mM Al) after the –P pretreatment; +P+Al = +Al treatment (0.1 mM Al) after the +P pretreatment; –P+Al = +Al treatment (0.1 mM Al) after the –P pretreatment. Values are means ($n = 3$), and bars indicate $\pm$ standard errors. Different letters indicate a significant difference ($P < 0.05$).

Fig. 6. Roots visualized by hematoxylin staining. Roots were obtained from seedlings receiving the Al treatments at pH 4.5 for 7 days: +P–Al = –Al treatment (0 mM Al) after the +P pretreatment; –P–Al = –Al treatment (0 mM Al) after the –P pretreatment; +P+Al = +Al treatment (0.1 mM Al) after the +P pretreatment; –P+Al = +Al treatment (0.1 mM Al) after the –P pretreatment. Bar = 1 mm.

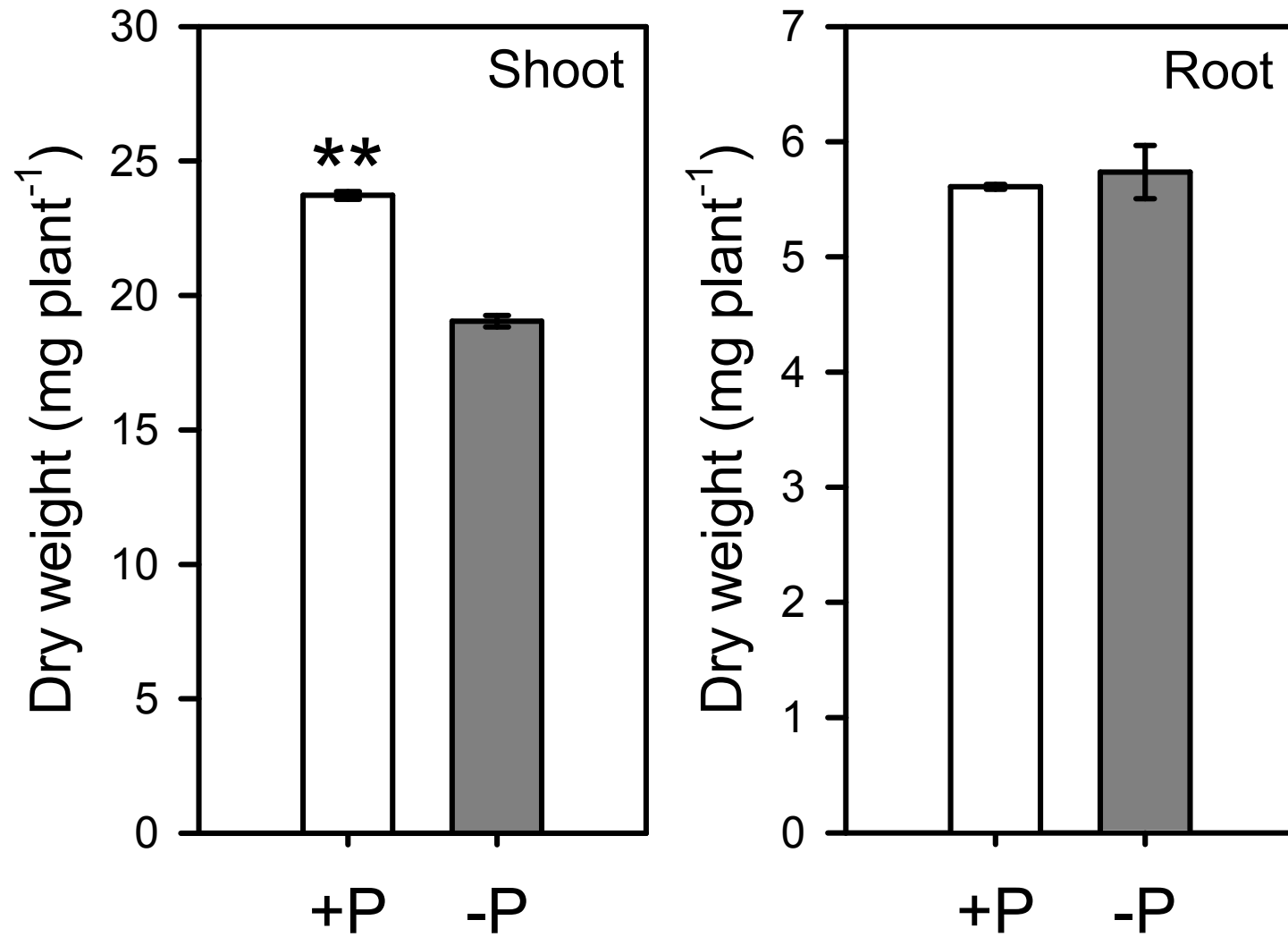


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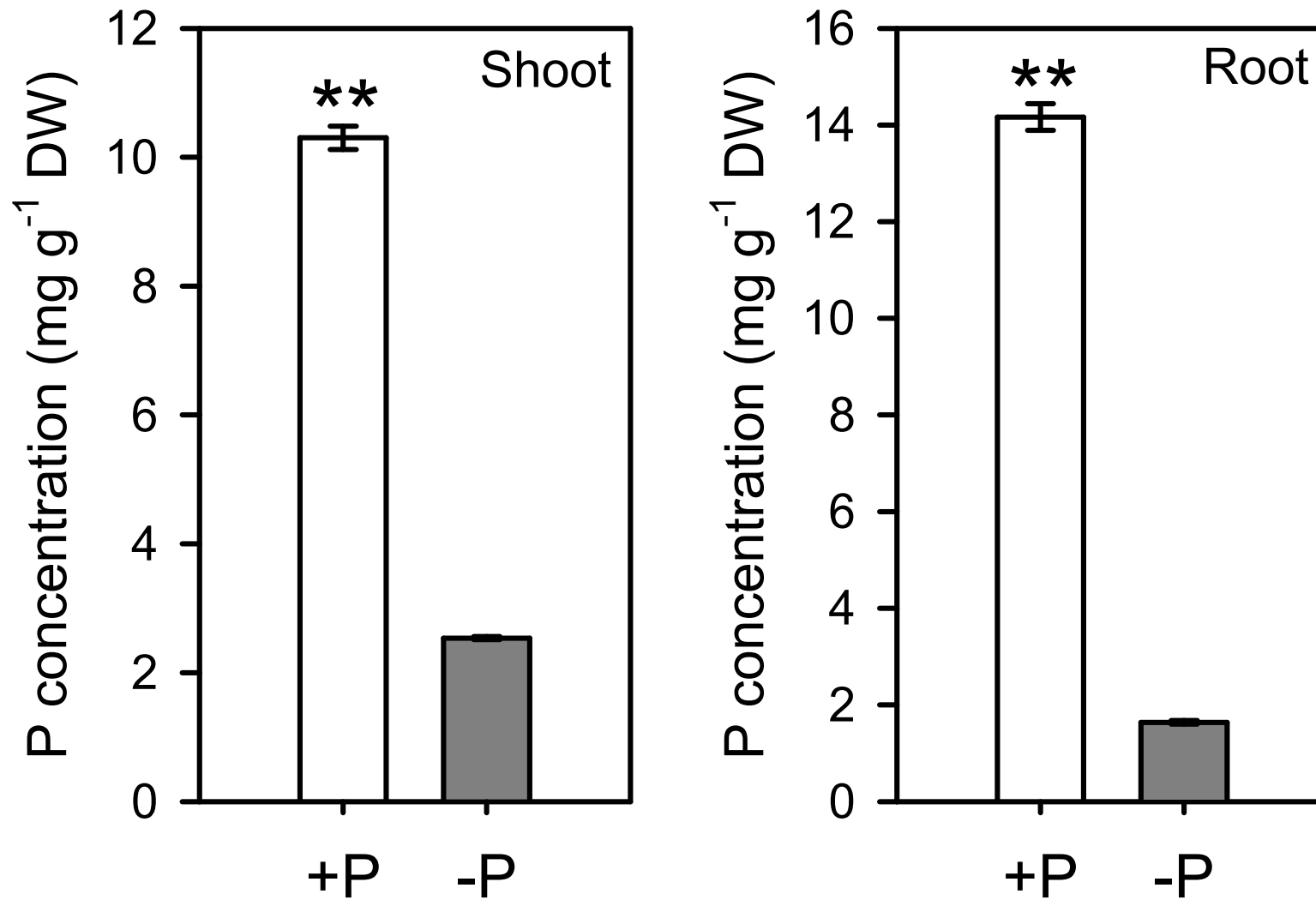


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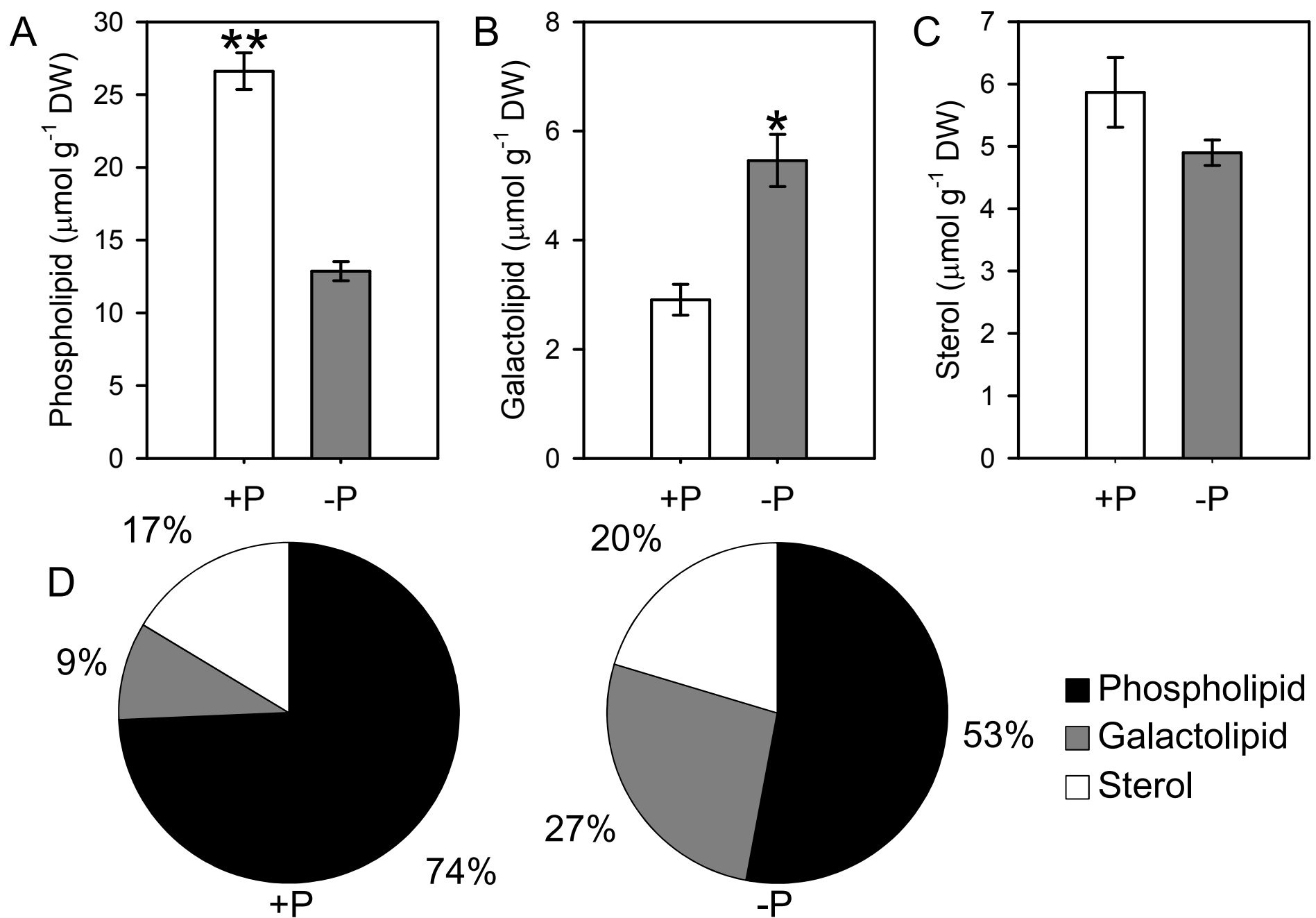


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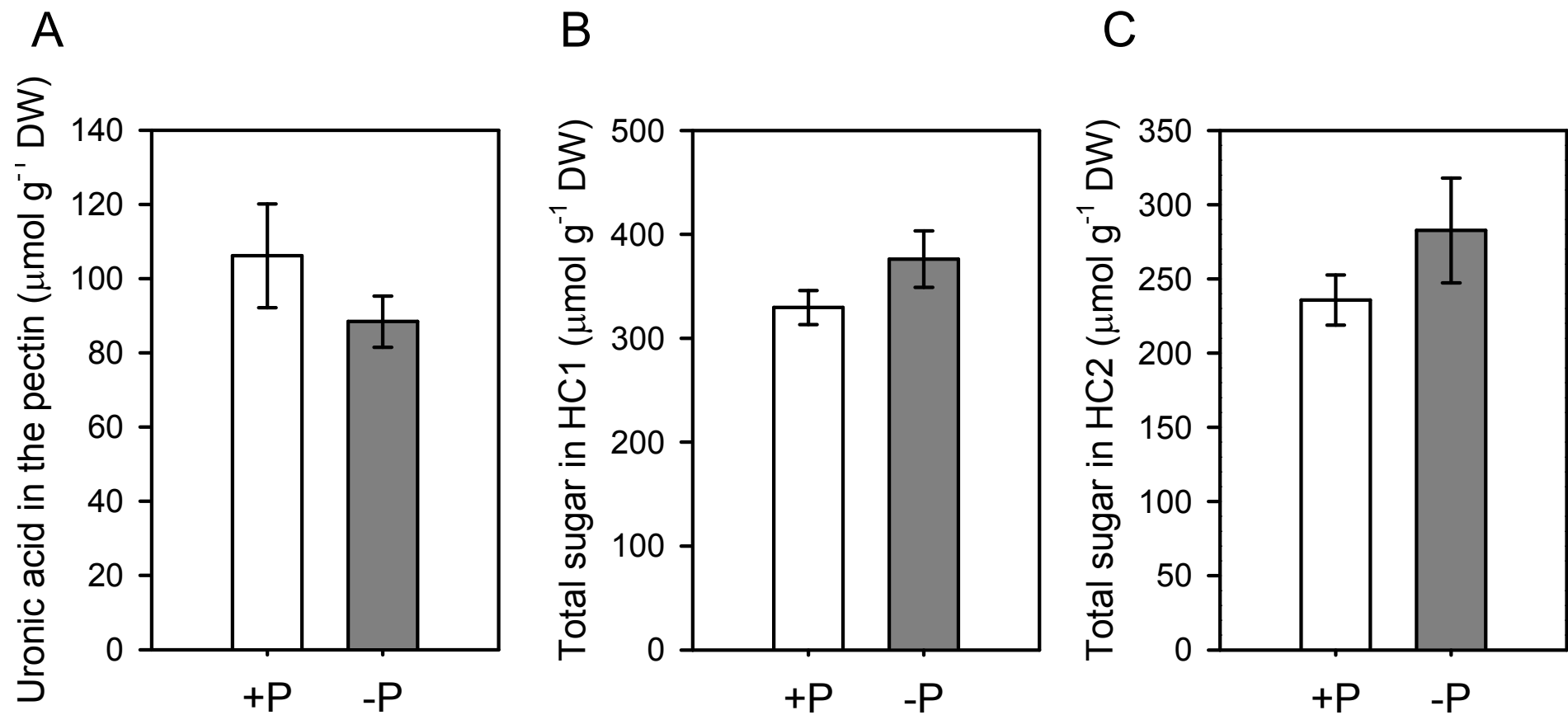


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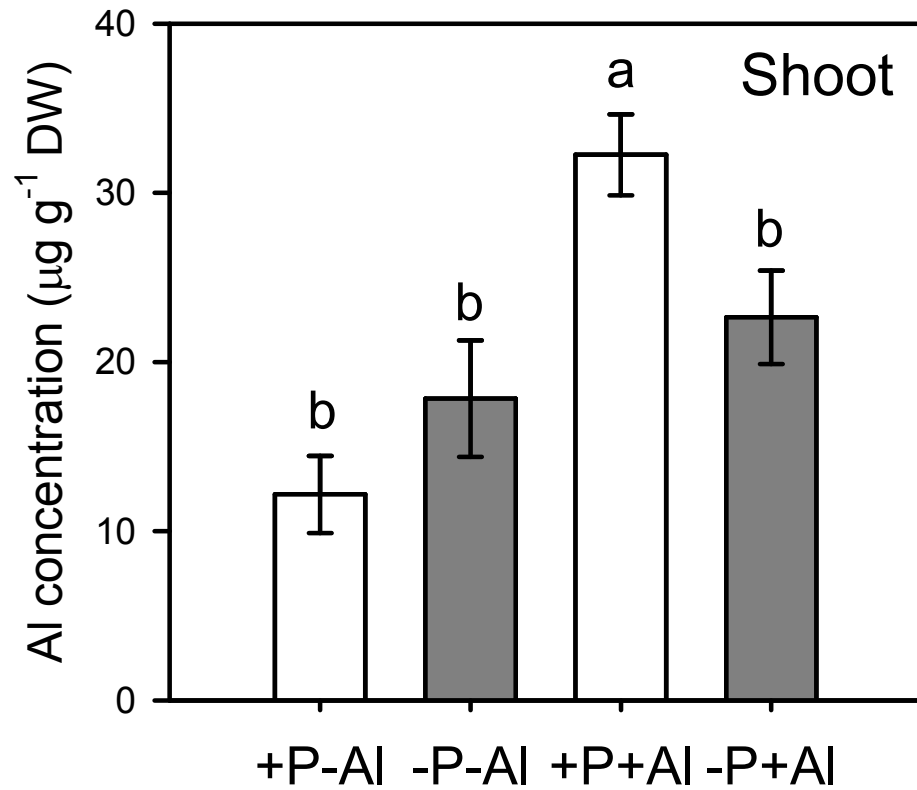
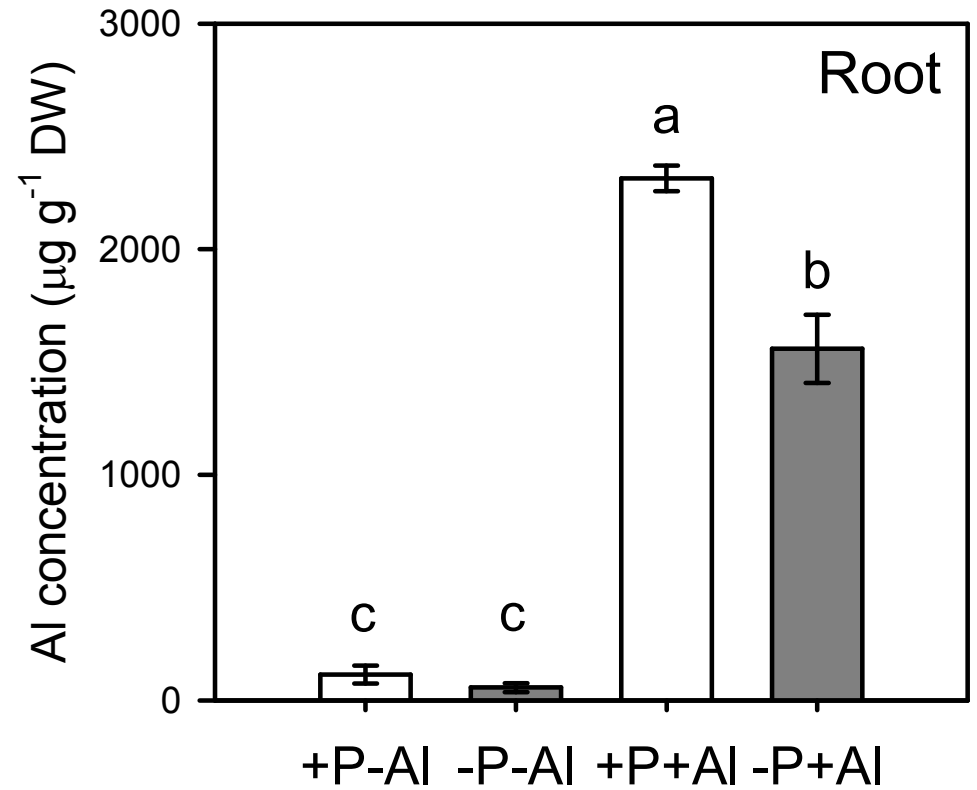


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+P-Al

-P-Al



+P+Al

-P+Al

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