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, digalactosylgiacylglycerol; HC, hemicellulose; MDA, malondialdehyde; MGDG, monogalactosylgiacylglycerol; 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

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

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

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

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

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

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

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

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

MATERIALS AND METHODS

P pretreatment

Seeds of rice (*Oryza sativa* L. cv. Koshihikari) were surface sterilized with sodium 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 (K_2SO_4:KCl = 1:1), 1.2 mM Ca ($CaCl_2 \cdot 2H_2O$), 0.82 mM Mg ($MgSO_4 \cdot 7H_2O$), 35 μM Fe ($FeSO_4 \cdot 7H_2O$), 9.1 μM Mn ($MnSO_4 \cdot 5H_2O$), 46.3 μM B (H_3BO_4), 3.1 μM Zn ($ZnSO_4 \cdot 7H_2O$), 0.016 μM Cu ($CuSO_4 \cdot 5H_2O$), and 0.05 μM Mo ($(NH_4)_6Mo7O_{24} \cdot 4H_2O$), with (+P) or without (−P) 0.32 mM P ($NaH_2PO_4 \cdot 4H_2O$). 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 ($AlCl_3 \cdot 6H_2O$) 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 ×*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 Na_2SO_4, 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 F_{254}, 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).

1 Sterols

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

9

10 *Cell wall isolation and fractionation*

11 Crude cell walls were prepared as described by Zhong and Lauchli (1993) with
12 minor modifications. In brief, approximately 10 mg of a lyophilized root powder sample
13 was homogenized with a mortar and pestle in 75% ethanol on ice. The homogenates
14 were centrifuged at 10,000 $\times g$ for 10 min, and the supernatant was discarded. The
15 pellets were washed three times each with an ice-cold acetone, methanol:chloroform
16 mixture (1:1, v:v), and then with methanol. The supernatant of each wash was discarded,
17 and the final pellet was dried under vacuum. The dried cell wall material was treated
18 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% H_2SO_4 (containing 12.5 mM $Na_2B_4O_7 \cdot 10H_2O$) 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% H_2SO_4 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 Al 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 $CaCl_2$ solution with or without 100 μM $AlCl_3$ (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

A lyophilized shoot or root sample (50 mg) was digested in HNO_3−H_2O_2, and the mineral concentrations were determined using inductively coupled plasma mass spectrophotometry (ELAN DRC−e, Perkin Elmer, Waltham, MA, USA).

Hematoxylin staining

After Al treatment, the roots were washed with deionized water and stained in 0.2% hematoxylin solution with 0.02% $NaIO_3$ for 15 min (Polle et al., 1978). Then, the roots were washed with deionized water for 20 min and photographed.

RESULTS

P pretreatment

No significant difference was observed in the dry weight of the roots between the +P and −P pretreatments, whereas the dry weight of the shoots receiving the +P pretreatment was significantly higher than that of the shoots receiving the −P pretreatment (Fig. 1). The P concentration in the rice shoots and roots receiving +P pretreatment was three and eight times higher, than that of the shoots and roots 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 b

een elucidated (Zhu et al., 2012). The binding of Al to pectins is one of the most

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

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

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

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

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

Andersson MX, Stridh MH, Larsson KE, Lijenberg C, Sandelius AS. Phosphate-deficient oat replaces a major portion of the plasma membrane phospholipids with the galactolipid digalactosyldiacylglycerol. Febs Lett 2003;537:128–32.

Andersson MX, Larsson KE, Tjellström H, Liljenberg C, Sandelius AS. The plasma membrane and the tonoplast as major targets for phospholipid-to-glycolipid replacement

and stimulation of phospholipases in the plasma membrane. J Biol Chem 2005;280:27578–86.

Barceló J, Poschenrieder C. Fast root growth responses, root exudates, and internal detoxification as clues to the mechanisms of aluminium toxicity and resistance: a review. Environ Exp Bot 2002;48:75–92.

Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–7.

Blumenkrantz N, Asboe-Hansen G. New method for quantitative determination of uronic acids. Anal Biochem 1973;54:484–9.

Chen J, Sucoff EI, Stadelmann EJ. Aluminum and temperature alteration of cell membrane permeability of *Quercus rubra*. Plant Physiol 1991;96:644–9.

Deleers M, Servais JP, Wulfert E. Neurotoxic cations induce membrane rigidification and membrane fusion at micromolar concentrations. Biochim Biophys Acta 1986;855:271–6.

Delhaize E, Ma JF, Ryan PR. Transcriptional regulation of aluminium tolerance genes. Trends Plant Sci 2012;17:341–8.

DuBois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6.

Fernandes JC, García-Angulo P, Goulao LF, Acebes JL, Amâncio S. Mineral stress affects the cell wall composition of grapevine (*Vitis vinifera* L.) callus. Plant Sci 2013;205–206:111–20.

Härtel H, Dormann P, Benning C. DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in *Arabidopsis*. Proc Natl Acad Sci U S A 2000;97:10649–54.

Hartmann MA, Benveniste P. Plant membrane sterols: Isolation, identification, and biosynthesis. Methods Enzymol 1987;148:635–50.

Horst WJ. The role of the apoplast in aluminium toxicity and resistance of higher plants: A review. J Plant Nutr Soil Sci 1995;158:419–28.

Jouhet J, Marechal E, Baldan B, Bligny R, Joyard J, Block MA. Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria. J Cell Biol 2004;167:863–74.

Khan MSH, Wagatsuma T, Tawaraya K, Ishikawa S. Plasma membrane lipids as an early device for detecting aluminum tolerance in rice. In: Li CJ, Zhang FS, Dobermann A, Hinsinger P, Lambers H, Li XL, Marschner P, Maene L, McGrath S, Oenema O, Peng SB, Rengel Z, Shen QR,Welch R, von Wirén N, Yan XL, Zhu YG, editors. Plant Nutrition for Food Security, Human Health and Environmental Protection Beijing,

China, 2005. p740–1.

Khan MSH, Tawaraya K, Sekimoto H, Koyama H, Kobayashi Y, Murayama T, et al. Relative abundance of Delta(5)-sterols in plasma membrane lipids of root-tip cells correlates with aluminum tolerance of rice. Physiol Plant 2009;135:73–83.

Kobayashi Y, Kobayashi Y, Watanabe T, Shaff JE, Ohta H, Kochian LV, et al. Molecular and physiological analysis of $Al3^{+}$ and H^{+} rhizotoxicities at moderately acidic conditions. Plant Physiol 2013;163:180–92.

Kochian LV, Hoekenga OA, Pineros MA. How do crop plants tolerate acid soils? Mechanisms of aluminum tolerance and phosphorous efficiency. Annu Rev Plant Biol 2004;55:459–93.

Legge RL, Thompson JE, Baker JE, Lieberman M. The effect of calcium on the fluidity and phase properties of microsomal membranes isolated from postclimacteric golden delicious apples. Plant Cell Physiol 1982;23:161–9.

Ma JF, Shen RF, Zhao ZQ, Wissuwa M, Takeuchi Y, Ebitani T, et al. Response of rice to Al stress and identification of quantitative trait loci for Al tolerance. Plant Cell Physiol 2002;43:652–9.

MacKinnon N, Ridgway J, Crowell KJ, Macdonald PM. Aluminum binding to phosphatidylcholine lipid bilayer membranes: aluminum exchange lifetimes from P−31

NMR spectroscopy. Chem Phys Lipids 2006;139:85–95.

Marschner H, Handley R, Overstreet R. Potassium loss and changes in the fine structure of corn root tips induced by H−ion. Plant Physiol 1966;41:1725–35.

Matoh T, Kobayashi M. Boron and calcium, essential inorganic constituents of pectic polysaccharides in higher plant cell walls J Plant Res 1998;111:179–90.

Munné-Bosch S, Peñuelas J, Asensio D, Llusià J. Airborne ethylene may alter antioxidant protection and reduce tolerance of holm oak to heat and drought stress. Plant Physiol 2004;136:2937–47.

Nakamura Y, Koizumi R, Shui G, Shimojima M, Wenk M, Ito T, et al. *Arabidopsis* lipins mediate eukaryotic pathway of lipid metabolism and cope critically with phosphate starvation. Proc Natl Acad Sci U S A 2009;106:20978–83.

Nian H, Ahn SJ, Yang ZM, Matsumoto H. Effect of phosphorus deficiency on aluminium-induced citrate exudation in soybean (*Glycine max*). Physiol Plant 2003;117:229–36.

Nishitani K, Masuda Y. Auxin-induced changes in the cell wall xyloglucans: effects of auxin on the two different subtractions of xyloglucans in the epicotyl cell wall of *Vigna angularis*. Plant Cell Physiol 1983;24:345–55.

Polle E, Konzak CF, Kittrick JA. Visual detection of aluminum tolerance levels in

wheat by hematoxylin staining of seedling roots. Crop Sci 1978;18:823–7.

Poschenrieder C, Gunse B, Corrales I, Barcelo J. A glance into aluminum toxicity and resistance in plants. Sci Total Environ 2008;400: 356–68.

Russo MA, Quartacci MF, Izzo R, Belligno A, Navari-Izzo F. Long- and short-term phosphate deprivation in bean roots: Plasma membrane lipid alterations and transient stimulation of phospholipases. Phytochemistry 2007;68:1564–71.

Sawaki Y, Iuchi S, Kobayashi Y, Kobayashi Y, Ikka T, Sakurai N, et al. STOP1 regulates multiple genes that protect *Arabidopsis* from proton and aluminum toxicities. Plant Physiol 2009;150:281–94.

Shoemaker SD, Vanderlick TK. Calcium modulates the mechanical properties of anionic phospholipid membranes. J Colloid Interface Sci 2003;266:314–21.

Tjellström H, Hellgren LI, Wieslander A, Sandelius AS. Lipid asymmetry in plant plasma membranes: phosphate deficiency-induced phospholipid replacement is restricted to the cytosolic leaflet. Faseb J 2010;24:1128–38.

Uemura M, Yoshida S. Involvement of plasma membrane alterations in cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol 1984;75:818–26.

Vanveldhoven PP, Mannaerts GP. Inorganic and organic phosphate measurements in

the nanomolar range. Anal Biochem 1987;161:45–8.

Virk S, Cleland R. The role of wall calcium in the extension of cell walls of soybean hypocotyls. Planta 1990;182:559–64.

Wagatsuma T, Akiba R. Low surface negativity of root protoplasts from aluminum−tolerant plant species. Soil Sci Plant Nutr 1989;35:443–52.

Wagatsuma T, Khan MSH, Rao IM, Wenzl P, Tawaraya K, Yamamoto T, et al. Methylene blue stainability of root-tip protoplasts as an indicator of aluminum tolerance in a wide range of plant species, cultivars and lines. Soil Sci Plant Nutr 2005;51:991–8.

Watanabe T, Osaki M, Yoshihara T, Tadano T. Distribution and chemical speciation of aluminum in the Al accumulator plant, *Melastoma malabathricum* L. Plant Soil 1998;201:165–73.

Watanabe T, Okada K. Interactive effects of Al, Ca and other cations on root elongation of rice cultivars under low pH. Ann Bot-London 2005;95:379–85.

Yamaji N, Huang CF, Nagao S, Yano M, Sato Y, Nagamura Y, et al. A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. Plant Cell 2009;21:3339–49.

Yamamoto Y, Masamoto K, Rikiishi S, Hachiya A, Yamaguchi Y, Matsumoto H. Aluminum tolerance acquired during phosphate starvation in cultured tobacco cells.

1 Plant Physiol 1996;112:217–27.

2 Yang JL, Li YY, Zhang YJ, Zhang SS, Wu YR, Wu P, et al. Cell wall polysaccharides

3 are specifically involved in the exclusion of aluminum from the rice root apex. Plant

4 Physiol 2008;146:602–11.

5 Yang JL, Zhu XF, Zheng C, Zhang YJ, Zheng SJ. Genotypic differences in Al

6 resistance and the role of cell-wall pectin in Al exclusion from the root apex in

7 *Fagopyrum tataricum*. Ann Bot-London 2011;107: 371–8.

8 Zhong H, Lauchli A. Changes of cell wall composition and polymer size in primary

9 roots of cotton seedlings under high salinity. J Exp Bot 1993;44:773–8.

10 Zhu XF, Lei GJ, Jiang T, Liu Y, Li GX, Zheng SJ. Cell wall polysaccharides are

11 involved in P-deficiency-induced Cd exclusion in *Arabidopsis thaliana*. Planta

12 2012;236:989–97.

13 Zlatkis A, Zak B. Study of a new cholesterol reagent. Anal Biochem 1969;29:143–8.

14

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 ($P < 0.05$).

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		TBARS (μmol g^{-1} FW)	Citrate (fmol g^{-1}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

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

	+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 ±standard errors. Asterisk indicates a significant difference (**$P < 0.01$).

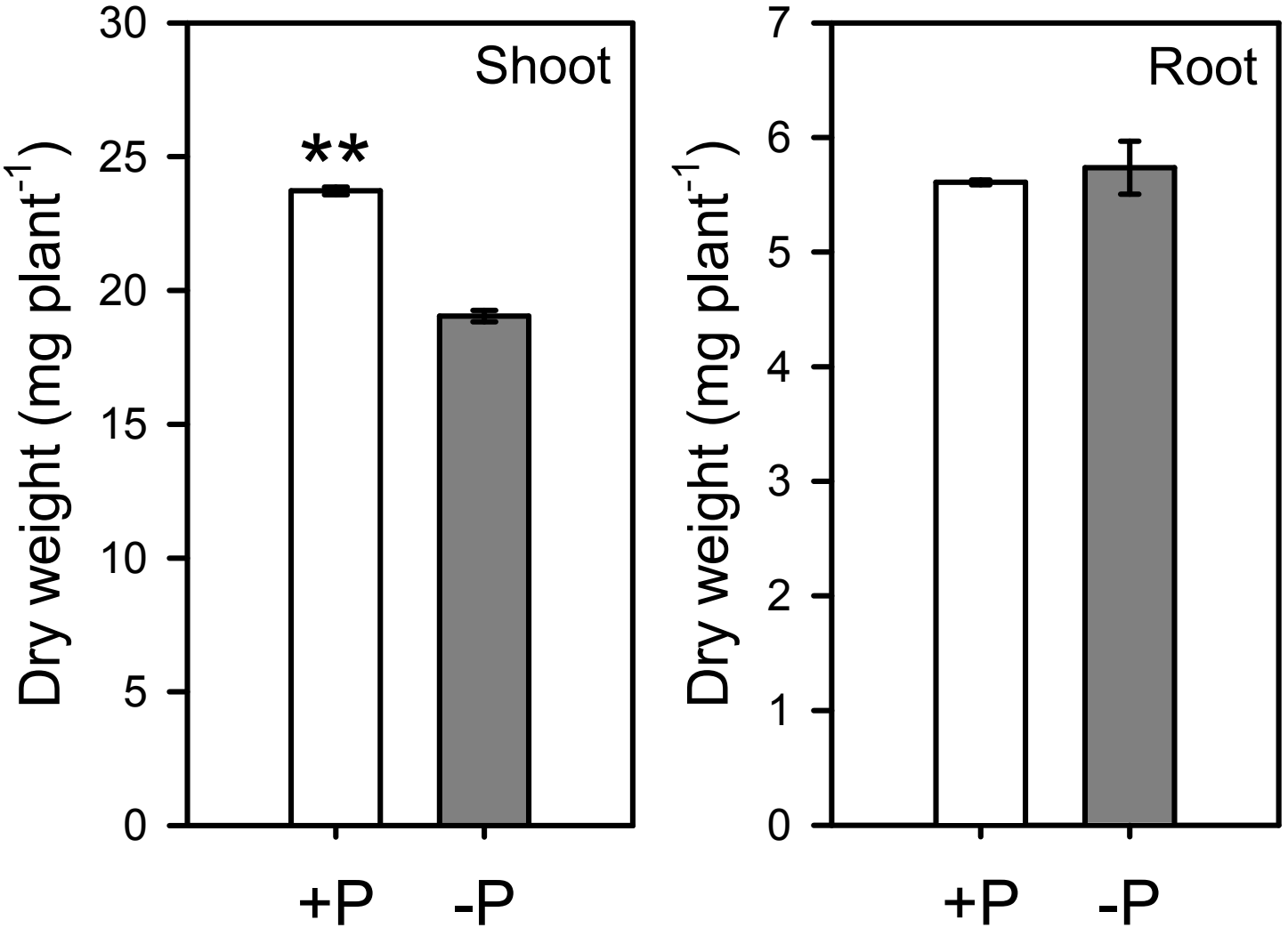
Fig. 2. P concentration in the shoots and roots after the P pretreatments: +P pretreatment (0.32 mM P) and −P pretreatment (0 mM P). Values are means (n = 3), and bars indicate ±standard errors. Asterisk indicates a significant difference (*$P < 0.05$, **$P < 0.01$).

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 ±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 ±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 ±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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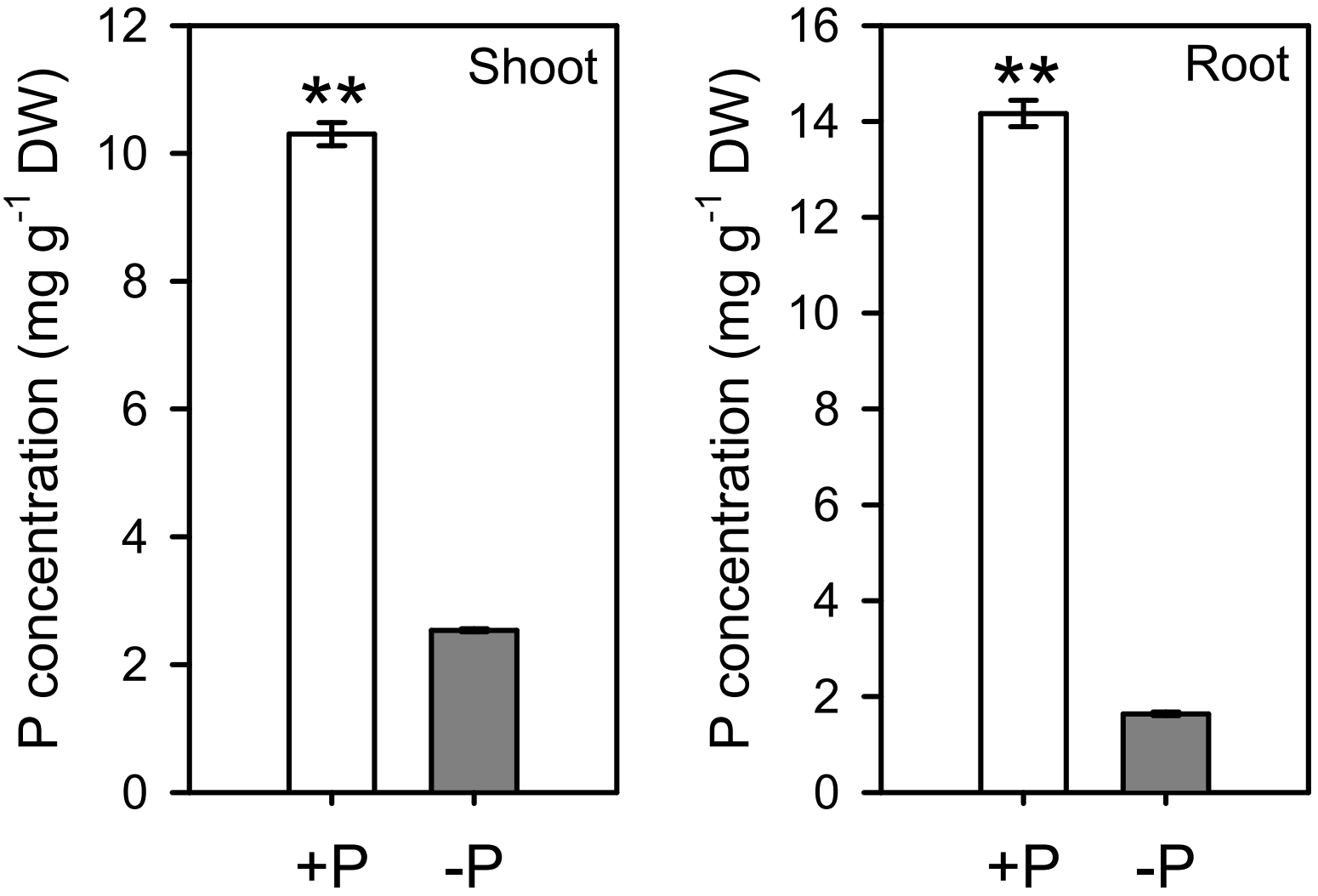

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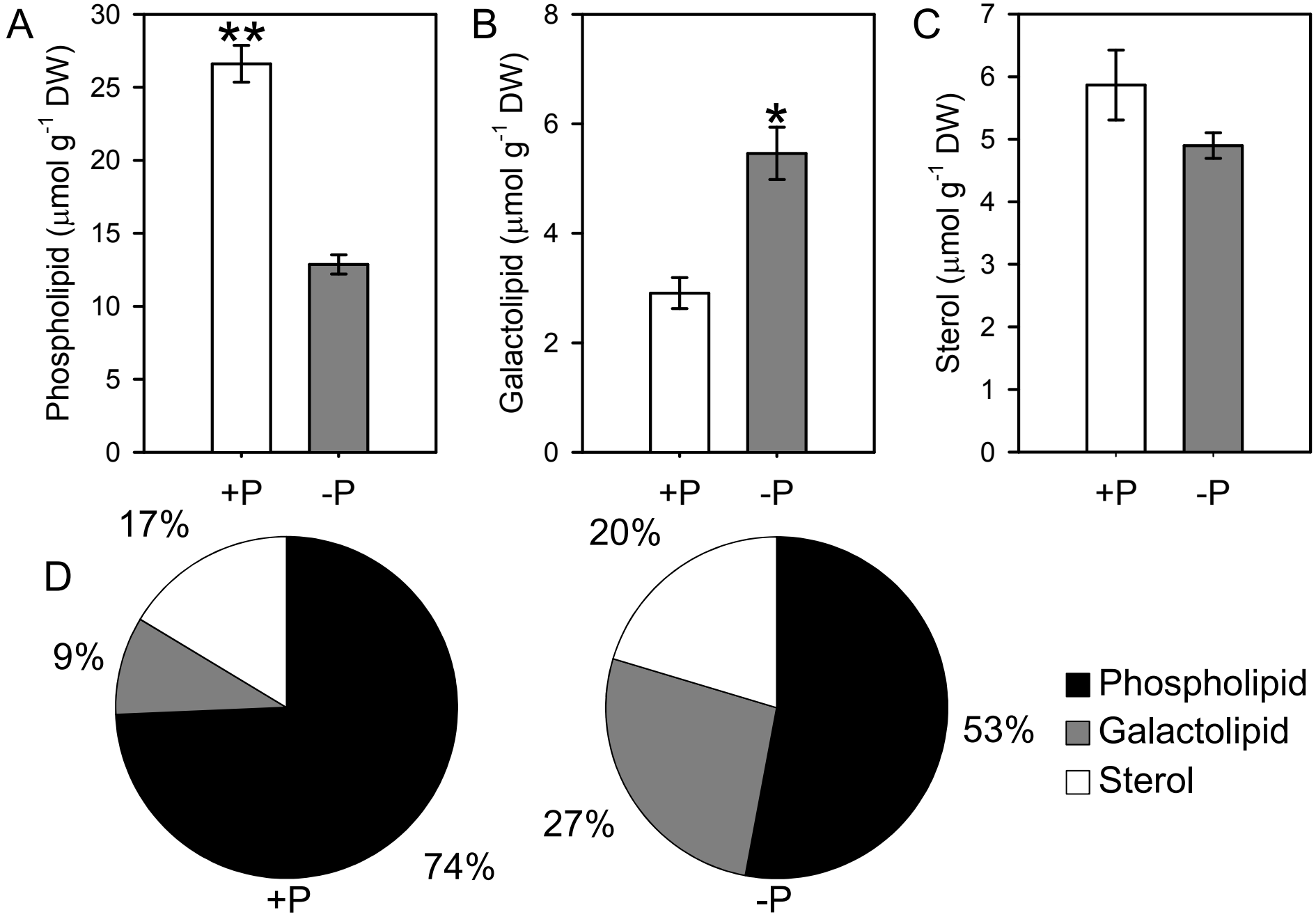

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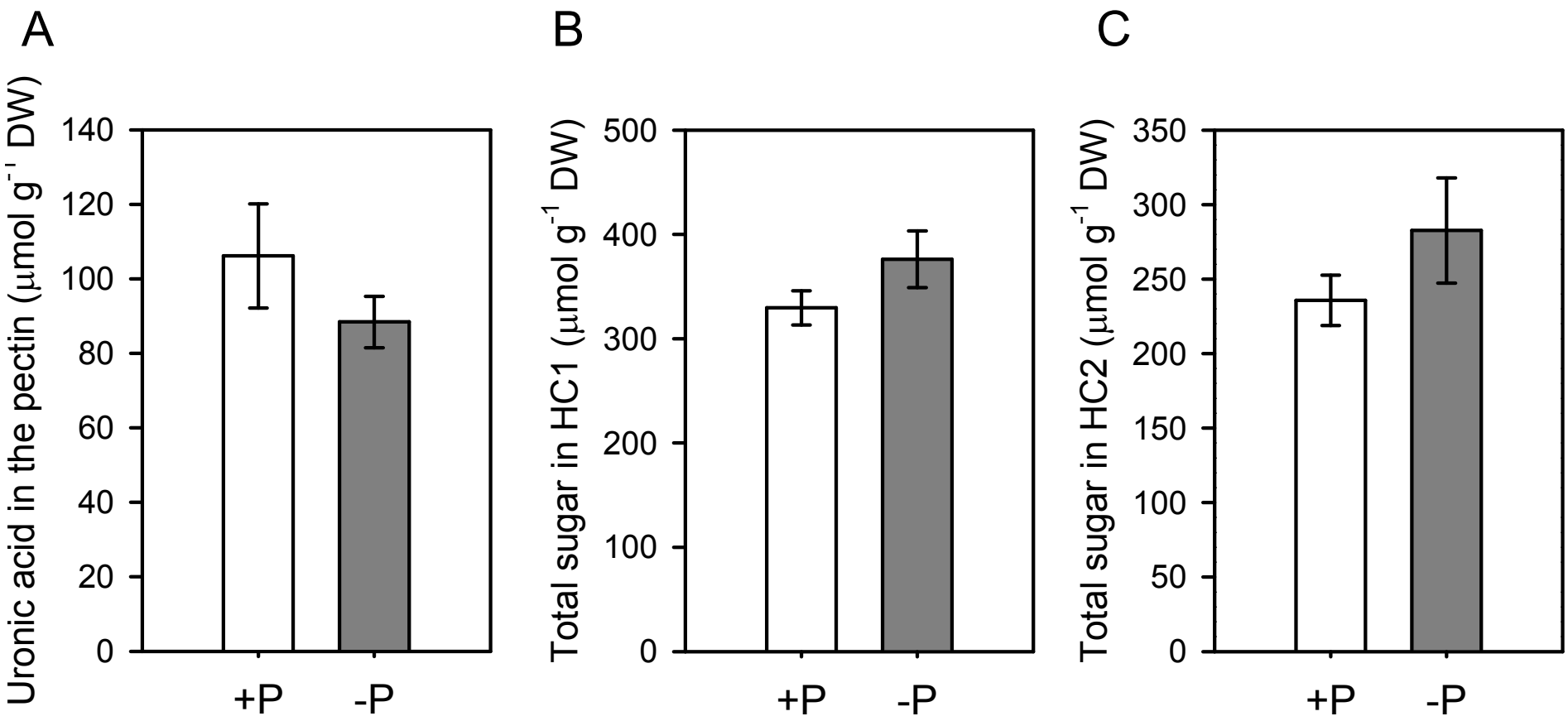

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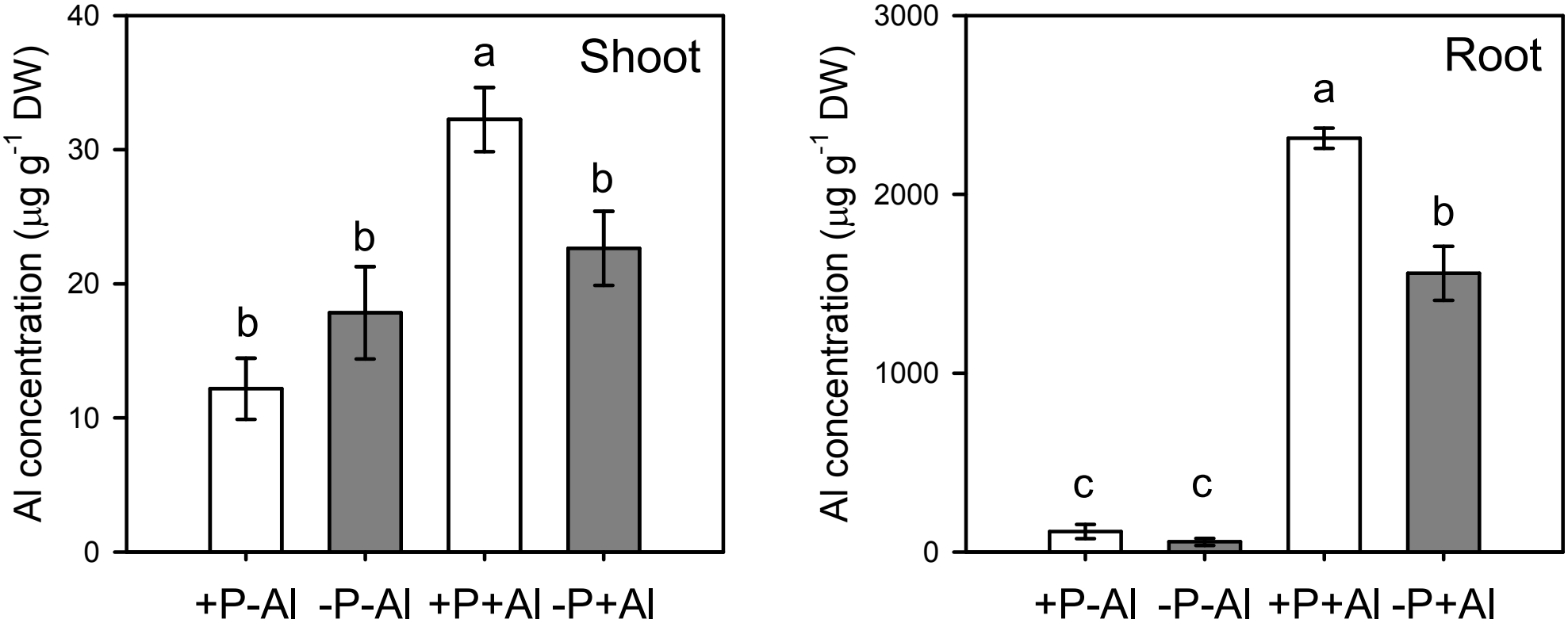

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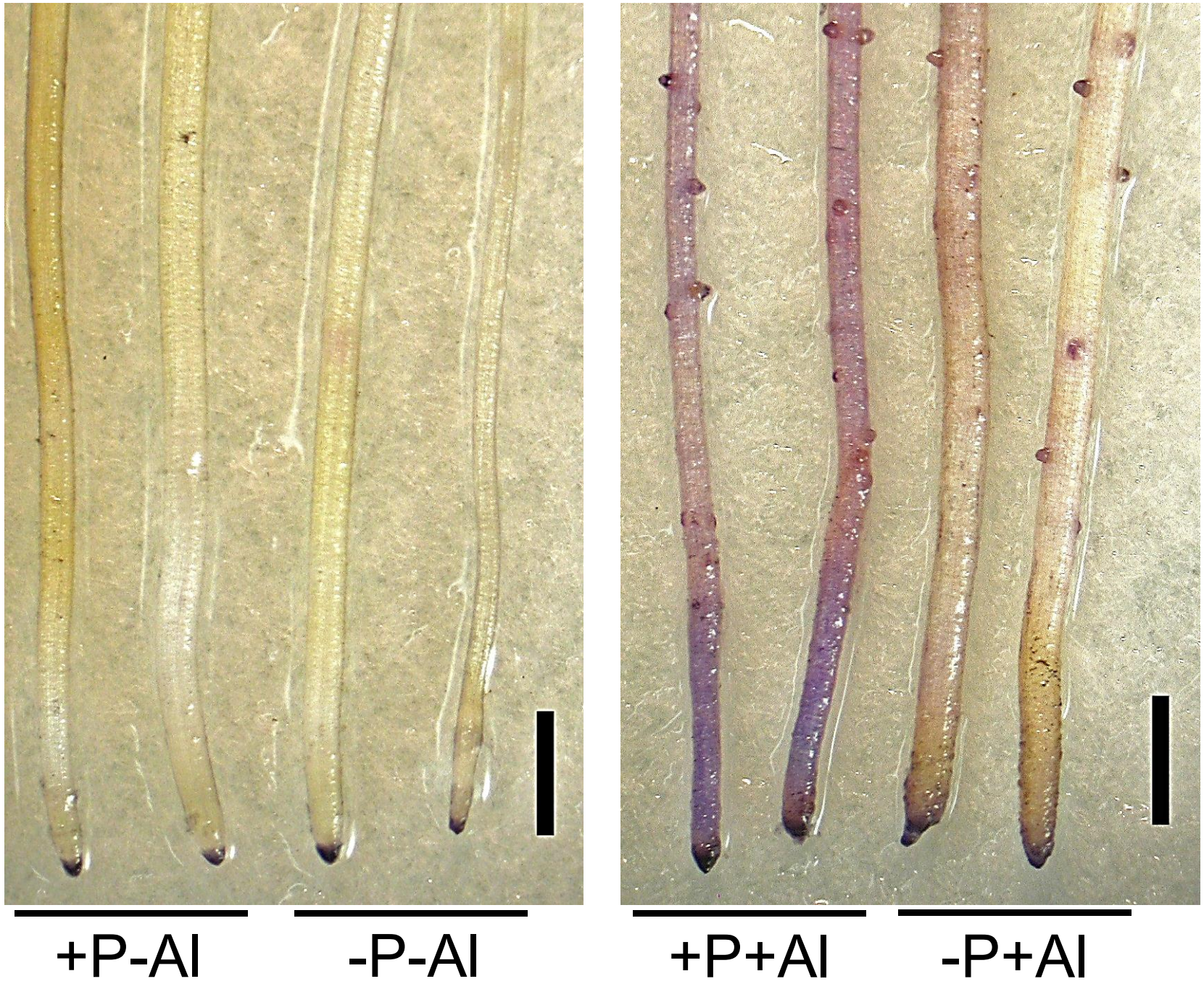

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