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Sterol Biosynthesis Inhibition by Paclobutrazol Induces Greater Aluminum (Al) Sensitivity in Al-Tolerant Rice

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Abstract: Al tolerance is one of the main growth and yield limiting factor in world and mechanism for Al-tolerance or Al-sensitivity yet to be clarified. We previously reported Japonica rice (*Oryza sariva* L.) cultivar Rikuu-20 as Al-sensitive, whereas a closely related cultivar that is a descendant of Rikuu-20, Rikuu-132, was Al tolerant. The objective of the present study was to clarify the role of plasma membrane lipid layer for Al tolerance in rice. The previously stated two cultivars were compared to determine mechanisms underlying variations in Al tolerance. The sensitive cultivar Rikuu-20 showed increased permeability of the Plasma Membrane (PM) and greater Al uptake within 24 h of Al treatment. Lipid composition of the PM differed between these cultivars was considered to be the primary account for the difference in Al tolerance. The tolerant cultivar Rikuu-132 showed a less PM permeabilization and Al accumulation which was drastically decreased in presence of paclobutrazol, a sterol metabolism inhibitor which reduces Δ^5-sterols and accumulates abnormal sterols by inhibiting obtusifoliol-14α-demethylase. The tolerant cultivar Rikuu-132 had lower phospholipids than that of sensitive cultivar Rikuu-20, suggesting that the PM of Rikuu-132 is less negatively charged and less permeabilized than that of Rikuu-20. We used inhibitor of Δ^5-sterol synthesis to alter the ratio of phospholipids to Δ^5-sterols in both cultivars. These inhibitors reduced Al tolerance in Rikuu-132 whereas Al tolerance of Rikuu-20 was unchanged suggesting that PM lipid composition greatly regulating Al tolerance in rice.

Key words: Aluminum, paclobutrazol, rice, tolerance

INTRODUCTION

Al tolerance is one of the main growth and yield limiting factor in the world. There are various aluminum (Al) tolerance mechanisms in plants, such as Al exclusion and internal Al tolerance mechanisms (Kochian *et al*., 2004; Sasaki *et al*., 2004; Poschenrieder *et al*., 2008). Although, Organic Acid (OA) release has been identified as the major Al tolerance mechanism in various plant species (e.g., wheat and *Arabidopsis*), this does not explain Al tolerance in some other plant species (Ishikawa *et al*., 2000; Wagatsuma *et al*., 2005a; Yang *et al*., 2008). Rice is the most important crop in southern Asian countries and different types of Al-sensitive mutants have been isolated (Ma *et al*., 2005). In rice, OA release was less significant in Al tolerance (Ma *et al*., 2002; Yang *et al*., 2008; Khan *et al*., 2009),

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suggesting that other mechanisms underlie differences in Al tolerance between cultivars. The complex nature of Al tolerance is still poorly understood.

Modeling studies of Al^{3+} toxicity in a solution culture system showed that $\{Al^{3+}\}_{PM}$ (activity at the plasma membrane surface) is a more reliable index than $\{Al^{3+}\}_{bulk}$ (activity in the solution) to explain Al-rhizotoxicity (Kinraide and Sweeney, 2001). Using this model, surface negativity caused by dissociation of H^+ from the anionic ligand (e.g., phospholipids) would be a major factor in altering Al accumulation at the PM surface and could possibly affect Al tolerance (Kinraide, 1999; Wagatsuma *et al*., 2005a, b; Wagatsuma and Akiba, 1989; Yermiyahu *et al*., 1997). As previously reported, Al-tolerant plant species show less membrane surface negativity than sensitive ones, as indicated by staining with the non-phytotoxic cationic dye, methylene blue (Wagatsuma *et al*., 2005a). This factor, namely PM negativity, is one mechanism that may underlie variations in Al tolerance within species, including rice. In the methylene blue method, a sensitive plant shows a more dense blue stain than a tolerant one. Membrane lipid composition has not yet been compared, but the difference in methylene blue staining among a wide range of plant species, cultivars and lines indicates that more research should be carried out to clarify the role of membrane lipids in Al tolerance.

An *Arabidopsis* mutant carrying a dysfunctional CYP51G1, the obtusifoliol-14α-demethylase, showed defects in membrane integrity (Kim *et al*., 2005), but the effects of this on Al tolerance are unknown. These results suggest that lipid composition of the PM is a potentially important factor controlling Al tolerance in plants, especially for plants in which the mechanisms underlying variations in Al tolerance are still unknown. Using an ectopic expression system in yeast and *Arabidopsis*, a Δ^8-sphingolipid desaturase was identified as one of the genes useful for enhancing Al tolerance via molecular breeding (Ryan *et al*., 2007). In this case, over expression of the enzyme might modify the structure of sphingolipids and stabilize the PM structure (i.e., preventing membrane leakiness). In this study, we studied PM lipids and pharmaceutical experiments using inhibitors of sterol biosynthesis which was widely used for gibberellin biosynthesis (Rademacher, 2000) and fungicidal function (Benveniste, 2004) indicated that PM lipid composition plays an important role in Al tolerance in rice.

MATERIALS AND METHODS

Growth Conditions

Seeds of rice were soaked with tap water for 24 h under aeration and then spread on a nylon mesh over 9 L of tap water for germination with an average light intensity of 0.6 cd m^2 m^{-4} (klux) at 25°C following the procedure of Khan *et al*. (2009). This tap water contains (mg L^{-1}) 8.0 Ca, 2.92 Mg, 1.95 K and minor quantity of other elements. All treatment experiments were carried out at Yamagata University Japan during 2006-2009.

Al Treatment

Twelve seedlings having almost same root length (ca. 4 cm) were selected for treatments in all screening experiments. Roots were pretreated with 0.2 mM $CaCl_2$ at pH 4.9 for 6 h and the root length of each seedling was measured by a ruler. Afterwards, seedlings were treated continuously with (20 μM $AlCl_3$) or without (control) Al containing 0.2 mM $CaCl_2$ for 24 h at pH 4.9. Just after 24 h root lengths were measured again and root elongation in control and Al treatments was calculated.

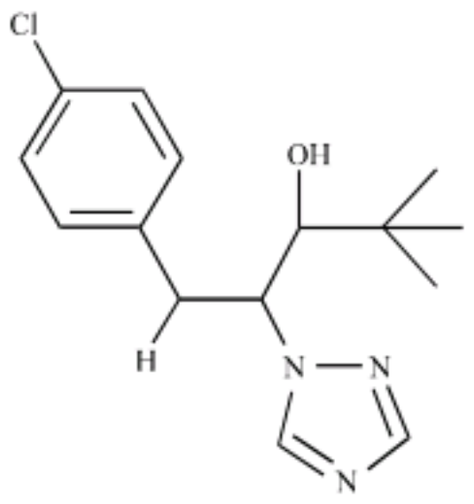

Fig. 1: Chemical structure of 2RS, 3RS-paclobutrazol

Paclobutrazol Treatment

Tween 20 was used for preparation of 1.7 µM paclobutrazol ((2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol; Wako Pure Chemical Industries Ltd., Japan) (Fig. 1). Four-days-old seedlings were treated for 24 h with graded concentrations up to 1.7 µM of paclobutrazol in the existence of 0.2 mM $CaCl_2$ at pH 5.2. Thereafter, root elongation was measured and PM permeability was checked. Inhibition of root elongation was calculated as the relative percentage to that in control medium without inhibitor.

$$\text{Al tolerance (\%)} = \frac{\text{Root elongation in Al treatment (cm)}}{\text{Root elongation in control without Al (cm)}} \times 100$$

More than 12 seedlings were used for each screening experiment and highest and lowest values were abandoned to get more authentic result.

Histochemical Analysis

Histochemical analysis was conducted following the procedure of Khan *et al*. (2009). Shortly, after growing 4 days on the nylon screen in tap water, rice cultivars were pretreated with 0.2 mM Ca (pH 4.9, 6 h) followed by 20 µM Al in 0.2 mM Ca (pH 4.9, 24 h) or in Al + paclobutrazol (0.68 µM paclobutrazol) (pH 4.9, 24 h). After washing the roots with deionized water, roots were immersed in hematoxylin solution for 15 min. Hematoxylin solution was made using 0.2% hematoxylin (w/v) (Wako, Japan), 0.02% sodium iodated (w/v) (Junsei Chemial Co., Japan), pH 4.8. After staining, roots were washed several times with deionized water to remove the extra dye. Water on the surface of the roots was removed by Kimwipes and roots were observed under light microscope (Nikon, Japan) and photographed by a digital camera (Coolpix 4000, Nikon, Japan). This experiment was replicated 3-4 times.

PM Permeability Study

Roots of 4-days-old seedlings were treated with control, or without 20 µM $AlCl_3$ in 0.2 mM $CaCl_2$ at pH 4.9 for 24 h. the roots were stained for 5 min with fluorescein diacetate-propidium iodide (FDA-PI) (12.5 mg L^{-1} FDA, 5 mg L^{-1} PI) following Ishikawa *et al*. (2001). After removing extra-dyes with deionized water, the root-tips were observed under a fluorescent microscope (SMZ-10, Nikon, Japan) equipped with a UV light (Nikon, Japan) (ex. 390 nm, ba. 520 nm) and photographed with a digital camera. This experiment was replicated 3-4 times.

Preparation of Root Samples for Lipid Analysis

Preparation of root samples for lipid analysis was conducted following the procedure of Khan *et al.* (2009). Briefly, roots of 4-days-old seedlings were treated with control (0.2 mM Ca), Al (20 μM Al in 0.2 mM Ca), (2RS,3RS)-paclobutrazol (0.68 μM paclobutrazol in 0.2 mM Ca) and Al+paclobutrazol (20 μM Al and 0.68 μM paclobutrazol in 0.2 mM Ca) for 24 h at pH 4.9. for 24 h at pH 4.9. Treatment solution was changed every 8 h to equalize the treatment conditions. Just after the treatment, tip 1 cm roots were collected. Adhered water was removed by Kimwipes after washing the samples several times with deionized water under vacuum pressure. Two gram fresh weight of root sample was preserved in freezer (-18°C) before extraction. This experiment was replicated 2 times.

Extraction and Measurement of Phospholipids and Glucocerebrosides

Phospholipids and glucocerebosides were extracted basically by Bligh and Dyer (1959) method modified partially by Uemura and Yoshida (1984). Each portion of 2.5 mL n-propanol, 2.5 mL chloroform and 1.25 mL H_2O was added to the root sample and the mixture was homogenized. Homogenization was repeated additionally twice. After filtering, the filtrate was shaken with similar volume of 0.1M KCl to remove proteins and water soluble molecules (e.g., ATP). Recovered chloroform layer was dehydrated with Na_2SO_4 (10 g per 100 mL solution), concentrated at 40°C and finally purged with N_2 gas. This concentrate was resolubilized with 200 mL per gram of fresh root weight with chloroform.

Extracted phospholipids and glucocerebrosides were developed on HPTLC (Silica gel 60 F_{254}, Merck Ltd., Japan) using a development solvent mixture of chloroform:methanol:acetic acid = 65:25:8 following the procedure of Uemura *et al*. (2003). Amount of each lipid species were quantified qualitatively by developing color with 20% H_2SO_4 in methanol and heating. This experiment was replicated 2 times.

Extraction and Measurement of Δ^5-Sterol

Extraction of free sterols fraction of the root-tip plasma membrane was carried out following Hartmann and Benveniste (1987) with slight modification. Free sterols and sterol conjugates of membrane fractions were extracted from 2 g of frozen root-tip with 12 mL of dichloromethane-methanol (2:1, v/v). Extraction was repeated 3 times and was filtered. The combined solvent extracts were vigorously shaken with mixing same volume of 0.1 M KCl to remove protein. Adhered water molecules were dried over anhydrous sodium sulfate. The extract was concentrated with rotary evaporator, transferred to vial and evaporated to dryness with N_2 gas. Finally it was re-solubilized with 200 μL of chloroform.

Sterols were developed on HPTLC (Silica gel 60 F_{254}, Merck Ltd., Japan) and using a development solvent mixture of dichloromethane: methanol = 85:15 following the procedure of Hartmann and Benvenisie (1987) with some modifications. Amount of each lipid species were quantified with standard ones qualitatively by developing color with 20% H_2SO_4 in methanol and heating on sand bath.

Al Tolerance of the Selected Cultivars in Presence of Inhibitors

Four-days-old seedlings were treated with 0.2 mM $CaCl_2$ (Cont) or 0.68 μM paclobutrazol in 0.2 mM $CaCl_2$ which induced the greatest difference in the inhibition of root elongation based on the former experiment using graded concentrations of each inhibitor. All treatment solutions were renewed at every 8 h. No less than ten seedlings were used for each experiment. Al tolerance in the presence of inhibitor was calculated as follows:

$$\frac{\text{Root elongation in Al treatment with inhibitor (cm)}}{\text{Root elongation in control treatment with inhibitor (cm)}} \times 100$$

Statistical analysis of Fisher's LSD was carried out using Kaleida Graph 4.0.

RESULTS

Root Elongation and PM Permeability in Paclobutrazol

As paclobutrazol was solubilized in Tween 20, previous checking was done whether it makes any remarkable effect for 24 h treatment period on root growth. The concentration of 0.0005% of Tween 20 exhibited similar greater relative root elongation for both cultivar (90.4-95.4%) indicating almost no harmful effects on root elongation (data not shown). Consequently, PM permeability and following to this, root elongation inhibition had been studied. Greater root elongation inhibition was observed in the tolerant Rikuu-132 cultivar than that of sensitive Rikuu-20 cultivar (Fig. 2). Consequent studies on PM permeability showed similar red fluorescence indicating almost similar effect on both cultivars which is similar to PM permeabilization without inhibitor (Fig. 2).

Effect of Paclobutrazol on PM Permeabilization and Al Accumulation

All rice cultivars showed an increase in PM permeabilization after Al-treatment in presence of lipid metabolism inhibitors (Fig. 3). In fact, Al-treatment with lipid metabolism inhibitors showed almost similar greater PM permeabilization irrespective of the cultivar having differential Al tolerance. Increase in PM permeabilization was greater in Al-tolerant cultivar (Rikuu-132). On the other hand, all rice cultivars showed an increase of Al

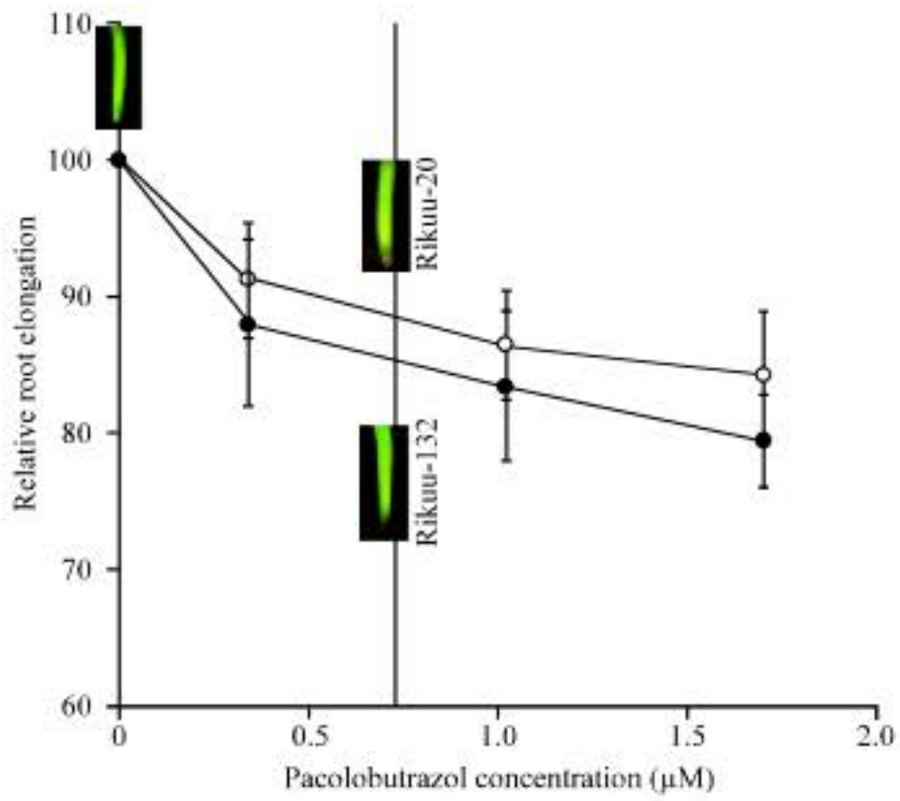

Fig. 2: Relative root elongation in different concentration of paclobutrazol Open circles, Rikuu-20; closed circles, Rikuu-132. Vertical line indicates the selected concentration of paclobutrazol for next stage of experiments. Roots having FDA- PI treatment showing PM permeabilization with that concentration of paclobutrazol. Photographs are the representative ones at least from three independent observations. Values are Mean±SE, n = 10

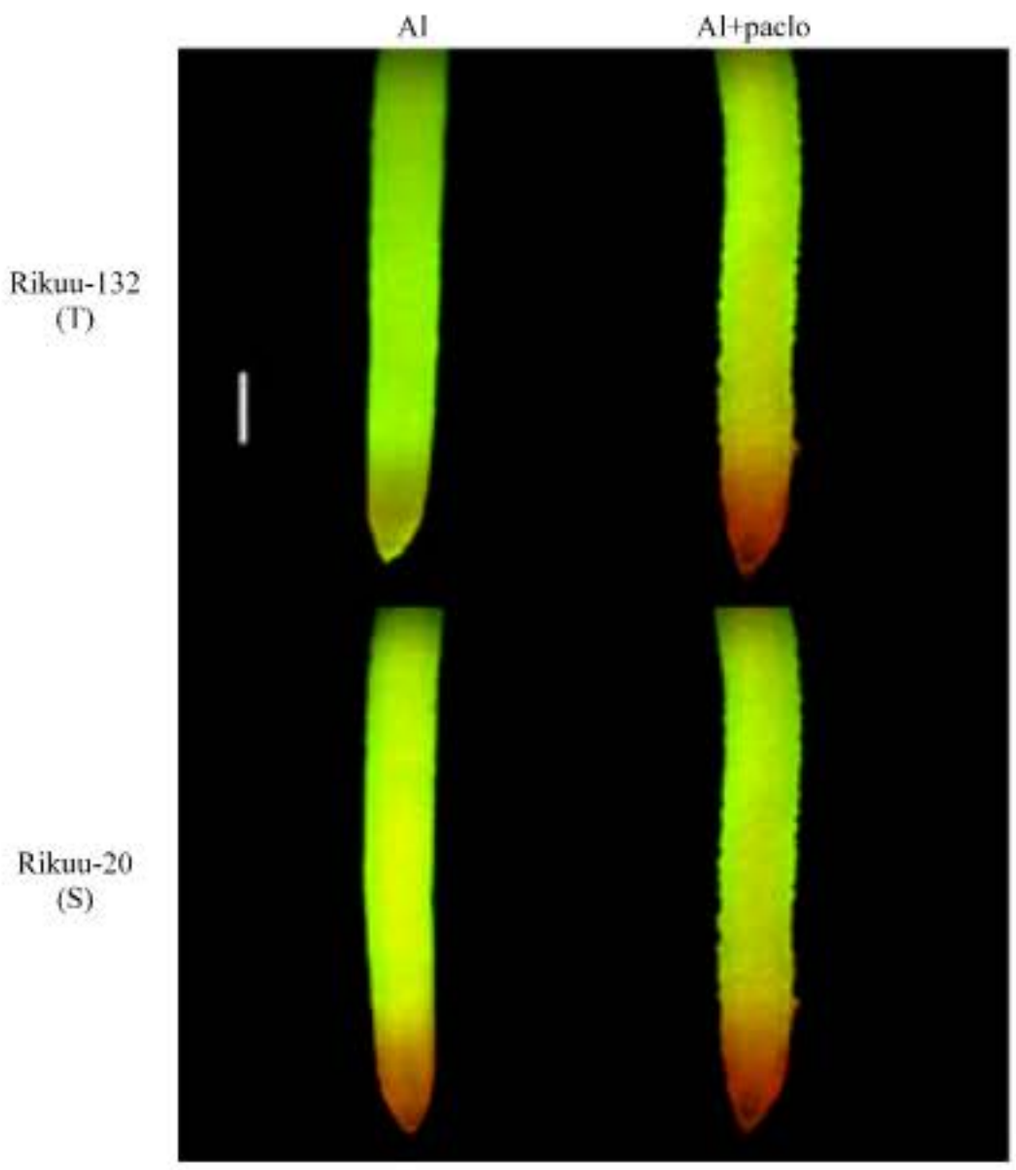

Fig. 3: Plasma membrane permeability of root- tip portion of two rice cultivars Rikuu-132 and Rikuu-20 observed with an FDA- PI flourescence microscope after 24 h Al or Al+pacobutrazol treatment. Photographs are the representative ones at least from three inde pendent observations

accumulation after Al-treatment in presence of lipid metabolism inhibitor (Fig. 4). These results show almost similar greater Al accumulation in Al treatment with lipid metabolism inhibitor irrespective of the Al tolerance among the cultivars. It also can be observed that increase in Al accumulation was greater in Al tolerant cultivars.

Paclobutrazol Effect on PM Lipid Composition

Among the phospholipids, PC, PE content was greater in control of Rikuu-20 (Al-sensitive) than that of Rikuu-132 (Al-tolerant) (Fig. 5). Among the lipid species, PE content was greater than PC for in Rikuu-20 and was reverse in Rikuu-132. However, total phospholipids contents were greater in Rikuu-20 than that of Rikuu-132. On the other hand, total Δ^5-Sterol content was greater in control of Rikuu-132 than that of sensitive Rikuu-20.

Effect of Paclobutrazol on Root Elongation

Relative root elongation in presence of Al or Al+paclobutrazol has been presented in Fig. 7. In Al solution, root elongation of Al sensitive Rikuu-20 was severely inhibited. When Al was accompanied with sterol metabolism inhibitor, paclobutrazol, only Al-tolerant Rikuu-132 showed a drastic significant decline of relative root elongation. On the other hand, Al-sensitive Rikuu-20 did not respond in root elongation behavior (Fig. 7).

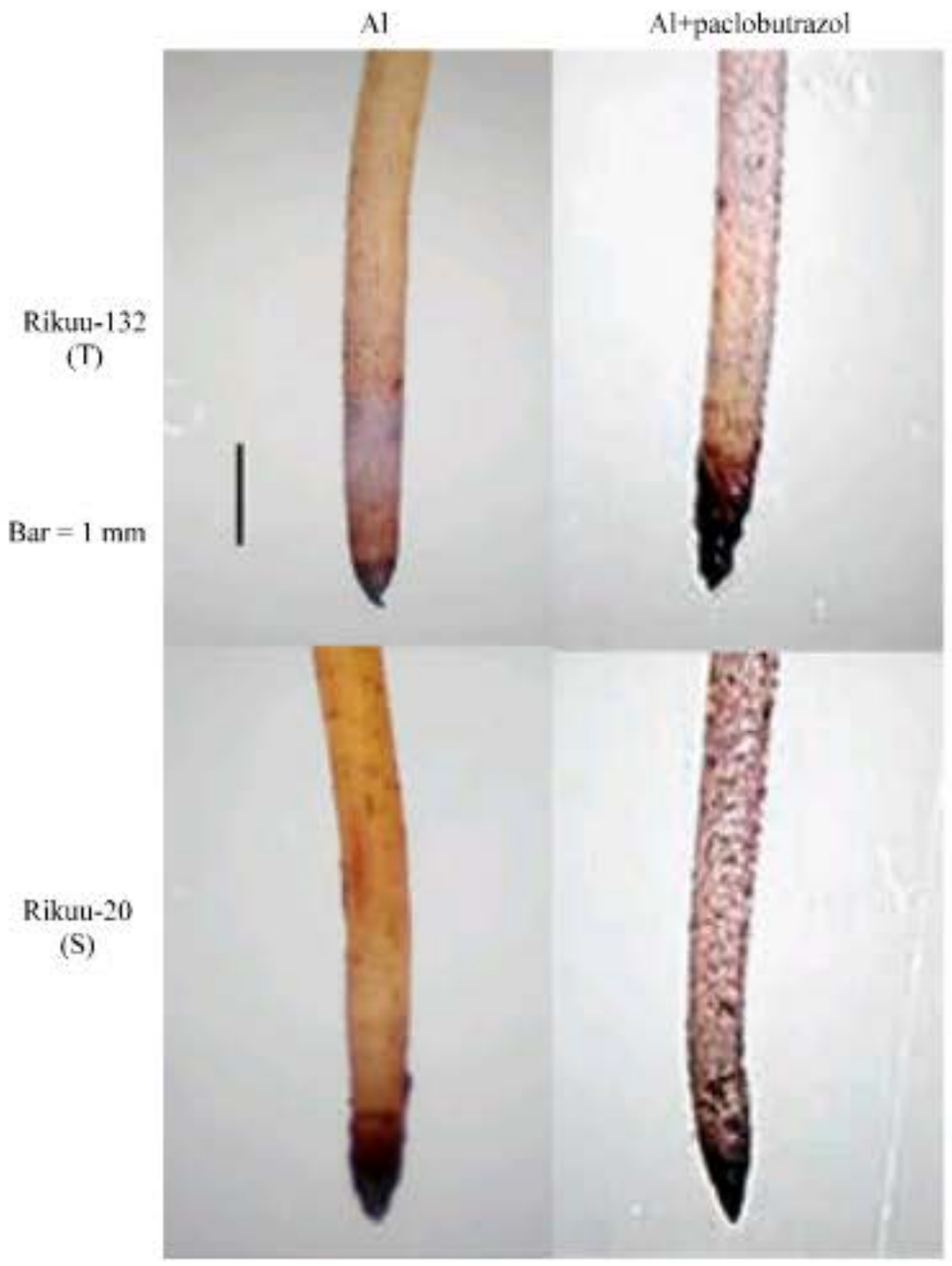

Fig. 4: Al accumulation in root- tip portion of two rice cultivars Rikuu-132 and Rikuu-20. Hematoxylin staining after 24 h Al or Al+paclobutrazol treatment. Photographs are the representative ones at least from three independent observations

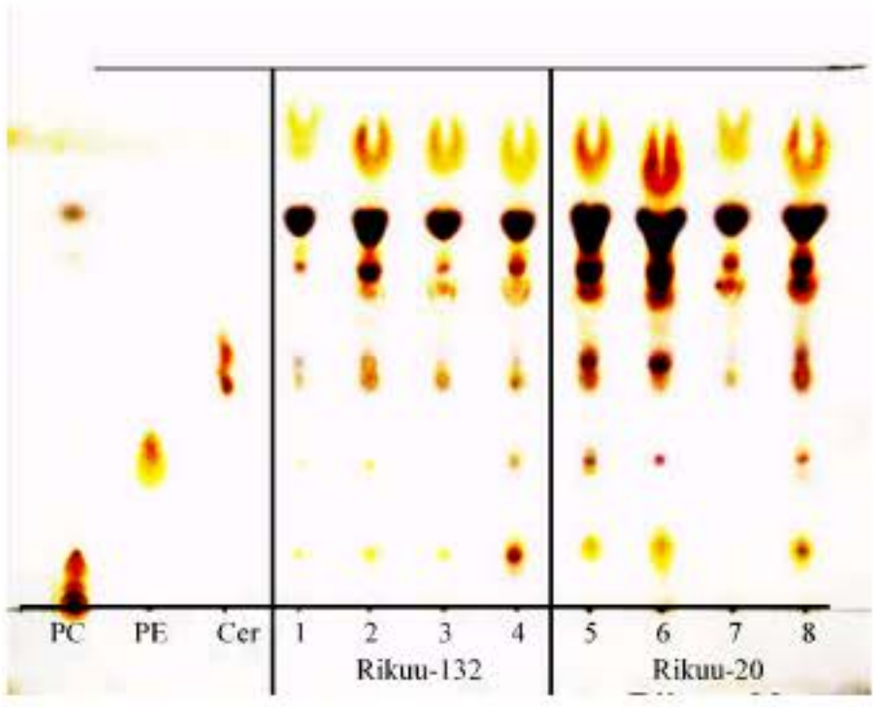

Fig. 5: Phosphotidyl choline (PC), phosphotidyl ethanol amine (PE) and cerebroside (Cer) after control (1 and 5), Al (2 and 6), paclobutrazol (3 and 7) and Al+paclobutrazol (4 and 8) treatment of Rikuu-132 and Rikuu-20. Photograph are representative of two individual experiments

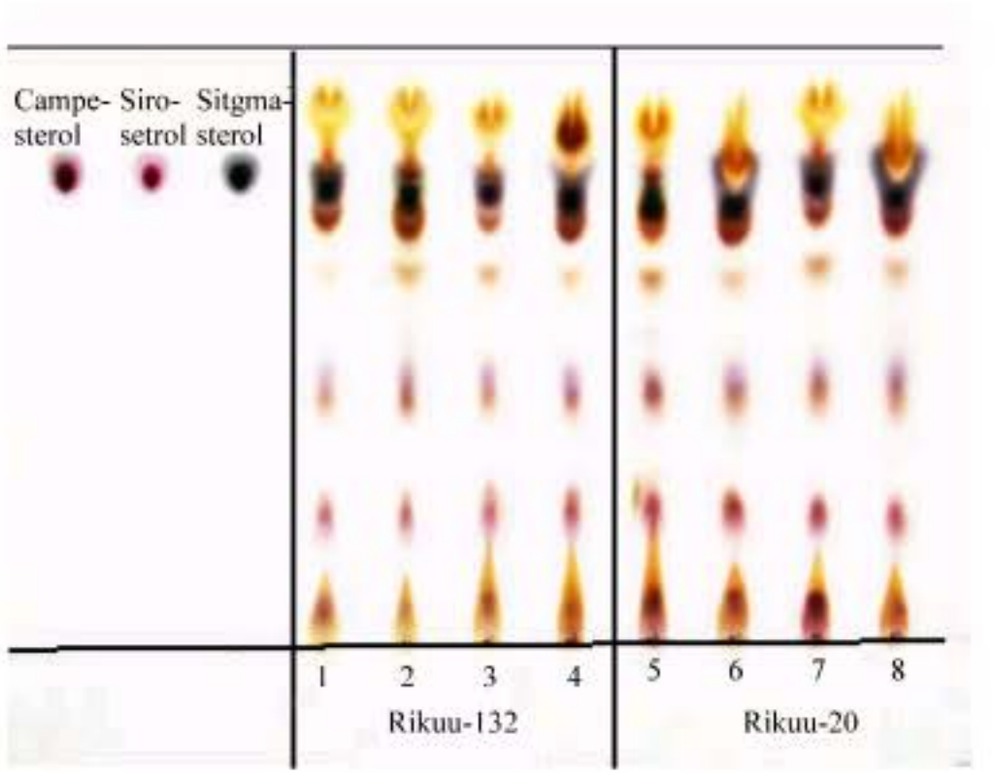

Fig. 6: Δ^5-sterols (Campesterol, sitosterol and stigmasterol) after control (1 and 5), Al (2 and 6), paclobutrazol (3 and 7) and Al+paclobutrazol (4 and 8) treatment of Rikuu-132 and Rikuu-20. Photograph are representative of two individual experiments

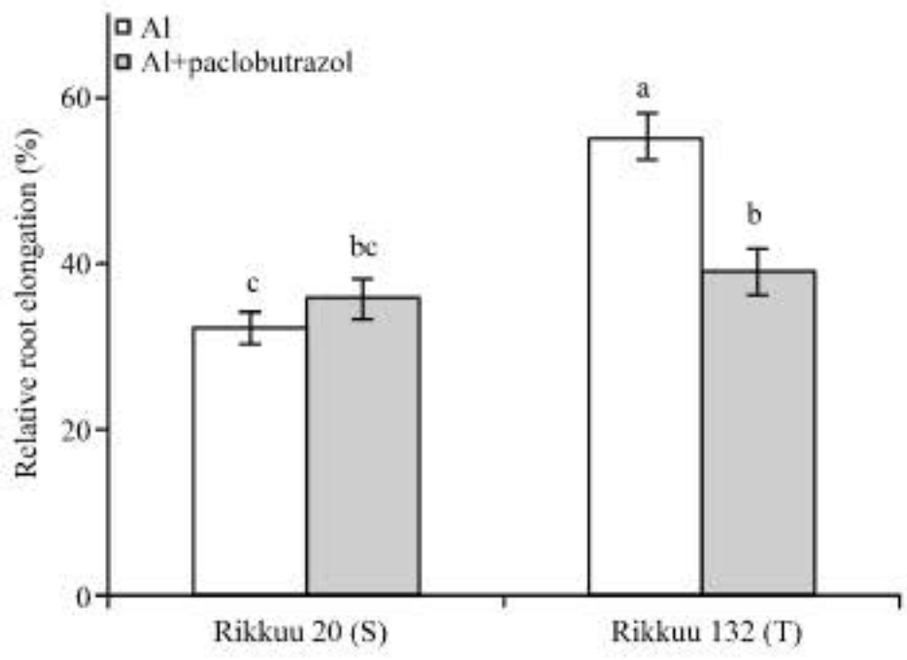

Fig. 7: Relative root elongation in Al or Al+paclobutrazol treatment. Values are Mean±SE (n =10). Values having same letters are not significantly different at 5% level of significance

DISCUSSION

2RS, 3RS-paclobutrazol is a triazole type fungicide with high plant growth regulatory activity on a wide variety of crops (Sugavanam, 1984). Although, there are several stereoisomers of paclobutrazol but (2RS, 3RS)-diasterioisomer is most effective for plant growth regulatory activity (Sugavanam, 1984) and further inhibition of obtusifoliol isomerase in Δ^5-sterol synthesis pathway (Burden *et al*., 1987). On the other hand, (2RS, 3RS)-paclobutrazol accumulates obtusifoliol, dihydroobtusifoliol and 14α-methyl-Δ^8-ergostenol and these effect on PM permeabilization are severe (Dahl *et al*., 1980). Moreover, 2RS-3RS-paclobutrazol also has an inhibitory effect on *ent*-kaurene (CYP51A2).

In a earlier study, we found that some Japonica cultivars, such as Sasanishiki, are highly Al tolerant (Khan *et al*., 2005). In the present study, we characterized mechanisms underlying variations in Al tolerance between the tolerant cultivar Rikuu-132 and the sensitive cultivar Rikuu-20, both of which are ancestor cultivars of the same Sasanishiki family line (Khan *et al*., 2009). Although, organic acid excretion is a major Al tolerance mechanism in some plant species, this did not explain the variation in Al tolerance between Rikuu-20 and Rikuu-132 (Khan *et al*., 2009) suggesting greater importance of mechanism (s) other than organic acid exudation for differential expression of Al tolerance between these cultivars. Previous research in rice has indicated that variations in Al tolerance are not associated with OA release (Ishikawa *et al*., 2000; Ma *et al*., 2002; Yang *et al*., 2008; Khan *et al*., 2009).

Differential Al accumulation was found after 24 h of Al treatment, i.e., less Al accumulated in the Al-tolerant Rikuu-132 cultivars (Fig. 4). Al accumulation was associated with permeabilization of the PM at the root tip, which was greater in the sensitive cultivar Rikuu-20 (Fig. 3). The sensitive cultivar Rikuu-20 had a greater proportion of phospholipids than the tolerant cultivar Rikuu-132. This is one possible explanation for the difference in Al tolerance. Although, we did not determine lipid composition in the isolated PMs, increased permeability and Al accumulation in the root tip of Rikuu-132 suggested that PM lipids might be modified as to increase the ratio of phospholipids. This possibility was further supported by pharmaceutical characterization of Al tolerance in Rikuu-20, Rikuu-132, which suggested that membrane lipid make-up contributed to higher Al tolerance in Rikuu-132 (Fig. 3, 5-7). The greater proportion of phospholipids in the sensitive cultivar Rikuu-20 may enhance Al accumulation and PM permeability via a complex mechanism. According to the Gouy-Chapmann-Stern model of Al rhizotoxicity, a greater amount of phospholipids in Rikuu-20 could lead to increased Al concentration at the PM surface than in Rikuu-132, due to the greater negative charge of the PM surface created by phospholipids (Kinraide, 1999). On the other hand, the Deljaguin-Landau-Verway-Overbeek theory would predict that a greater amount of phospholipids increases membrane leakiness in Rikuu-20, because the greater amount of packed Al-phospholipids increases permeability of the membrane (Wagatsuma *et al*., 1995). This could be the mechanism by which the sensitive cultivar Rikuu-20 accumulated more Al than the tolerant cultivar Rikuu-132.

Among the phospholipids, PC, PE content was greater in control of Rikuu-20 (Al-sensitive) than that of Rikuu-132 (Al-tolerant) (Fig. 5). Among the lipid species, PE content was greater than PC for in Rikuu-20 and was reverse in Rikuu-132. However, total phospholipids contents were greater in Rikuu-20 than that of Rikuu-132. Although, I did not measure other lipid species like phosphatidyl serine, phosphatidyl glycerol or phosphotidyl inositol by HPTLC, these may constitute fewer fractions within the PM considering the total content of PC and PE. On the other hand, however, cerebrosides content was greater in the PM of Rikuu-20 (Fig. 5). Although cerebroside was considering to contribute for Al tolerance in triticale (Wagatsuma *et al*., 2005a), results of the present study suggests a less contribution of cerebrosides for Al tolerance in rice at least for the cultivars under study.

Inhibition of root elongation was great for Rikuu-132 than that of Rikuu-20 in Al+paclobutrazol medium. A slight increase of root elongation was observed for Rikuu-20 which differed from the results obtained for another sterol metabolism inhibitor uniconazole-P. This might be due to milder effect of paclobutrazol than that of uniconazole-P on inhibition of sterol metabolism.

In the present study, we identified the difference in membrane lipid compositions between contrasting Al-tolerant and -sensitive rice cultivars. The sensitive cultivar's PM had a greater proportion of phospholipids compared to the tolerant cultivar, which may account for Al tolerance in the tolerant cultivar. Present results suggest that the relative amount of Δ^5-sterols is an important factor in Al tolerance in some rice cultivars. Although, the difference between tolerant and sensitive cultivars was small, similar data has been reported previously for wheat cultivars. That is, a lower phospholipids/Δ^5-sterols ratio in control root-tips was observed in the Al-tolerant cultivar (Zhang *et al*., 1996). In addition, Ryan *et al*. (2007) recently reported that genetically modified *Arabidopsis thaliana* with altered membrane lipids showed greater Al tolerance. In this case, over expression of the Δ^8-sphingolipid desaturase altered the glucocerebroside side chain, which may have reduced permeation of Al into the cytosol by stabilizing PM during Al treatment. These results also suggest that PM lipid composition plays a significant role in Al tolerance. Further research, such as comparison of PM lipid composition among different plant species, may lead to greater understanding of the significance of PM lipids in plant Al tolerance.

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