Chapter 6

Clarification of lipid composition for Al tolerance in other crop species

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6.1 Introduction

As in rice, PM lipid composition has been clarified as one of the main Al tolerance mechanism, I further tried to clarify whether that mechanism is unique for rice or not. I selected sorghum, wheat, triticale, maize and soybean crops. Among the cultivars of the selected crop species, OA exudation mechanism has already clarified for some cultivars. For example, wheat OA exudation as Al tolerance mechanism has well reported (Sasaki et al. 2004). In wheat and triticale, PM intact ness has been reported as main Al tolerance mechanism (Wagatsuma et al. 2005a, b). Therefore, it was my intension to know whether lipid composition confers Al tolerance to these crops as clarified in the previous chapter for rice. Tolerance mechanism other than OA release need to be clarified yet.

6.2 Materials and methods

6.2.1 Selection of cultivars of the crop species

I chose tolerant and sensitive cultivars in these crop species. Al-tolerant and -sensitive cultivars of sorghum (Kaneko as sensitive and Super Sugar as tolerant), soybean (Enrei and sensitive and Ryokuheki as tolerant) and maize (Golddent KD850 as sensitive and Golddent KD520 as tolerant) were selected based on Afrin et al. (2009) and those of wheat, triticale and maize were selected based on Wagatsuma et al. (2005a, b). Al tolerance of sorghum was based on the screening in 2.5μM $AlCl_3$ in 0.2mM $CaCl_2$ for

24h (pH 5.0). For all other crop species, Al tolerance was based on screening in 20μM AlCl$_3$ in 0.2mM CaCl$_2$ for 24h (pH 4.9).

6.2.2 Study on OA exudation

Seeds of soorghum, maize and soybean were germinated and precultured as described previously. Five-day-old seedlings with similar root length (5cm) were pretreated in 0.2mM CaCl$_2$ (pH 4.9) for 5h (10 seedlings 300 mL^{-1} solution). Thereafter, roots were treated with or without 20μM AlCl$_3$ in 0.2mM CaCl$_2$ (pH 4.9) for 5h (300 mL^{-1} solution). Both pretreatment and treatment was conducted under 25°C temperature, aeration and constant light as described in Chapter 2. Exuded organic acids in the solution were then measured by the enzyme cycling method (Kihara et al. 2003). Shortly, citrate and malate were converted to lyase/citrate dehydrogenase and malate dehydrogenase/glutamate oxaloacetate transaminase (Roche, Basel, Switzerland), respectively. The NAD$^+$ and NADH were then measured according to the method described by Kato et al. (1973). This experiment and measurement was replicated three times.

6.2.3 Study on PM permeability and Al accumulation

PM permeability of sorghum, soybean and maize has been studied after 24h treatment in control and Al at pH 4.9. After washing the roots with deionized water, roots were stained with FDA-PI for PM permeabilization study or with hematoxylin for Al accumulation study. These study procedures has been described in Chapter 1.

6.2.3 Root-tip collection, extraction and analysis

After control and Al treatment ($20\mu M$ $AlCl_3$ in $0.2mM$ $CaCl_2$ for 24h at pH 4.9), root tips (0-10mm) were collected. After washing with deionized water and removing excess water, roots were stored in freezer at $-18^{\circ}C$ before analysis. Extraction, purification and measurement of phospholipids and Δ^5-sterols were performed as described in the previous chapter (Chapter- 5).

6.3 Results

Al tolerance of the representative cultivars of sorghum, wheat, triticale, rice, maize and soybean has been presented in Fig. 6.1 based on the already reported data (shown in caption). Al tolerance of the crop species was in order of maize>rice, soybean>triticale, wheat>sorghum (Al tolerance of sorghum was expressed in $2.5\mu M$ $AlCl_3$ at pH 5.0). There was wide variation of Al tolerances among the cultivars of each crop species.

Citrate and malate exudation of sorghum, maize and soybean has been presented in Fig. 6.2. Citrate was main organic acid exuded by the cultivars of these crop species. For sorghum, citrate or malate exudation did not show any difference irrespective of Al-tolerant and Al-sensitive cultivars in control or in Al treatment. For maize, greater citrate exudation was observed for Al-tolerant Golddent KD520 in Al treatment indicating involvement of citrate exudation for Al tolerance. Although malate exudation did not correspond similar tendency to citrate but considering the greater exudation of citrate, this malate exudation may have less impact. On the other hand, soybean showed almost reverse tendency of OA exudation to Al-toelrance. Both citrate and malate exudation in soybean followed almost siliar irrelevance to Al-tolerance.
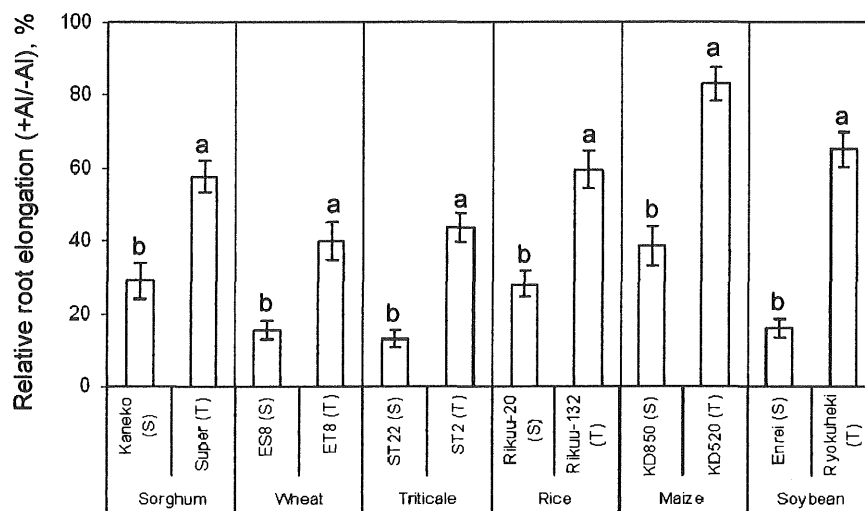
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Fig. 6.1 Al tolerance of the representative cultivars of sorghum, wheat, triticale, rice maize and soybean. Al treatment for sorghum was 2.5μM AlCl₃, in all other cases Al tolerance was in 20μM AlCl₃. Al tolerance of sorghum, maize and soybean based on Afrin et al. (2009). Al tolerance of wheat, triticale were based on Wagatsuma et al. 2005 and Al tolerance of rice was based on Khan et al. (2009).
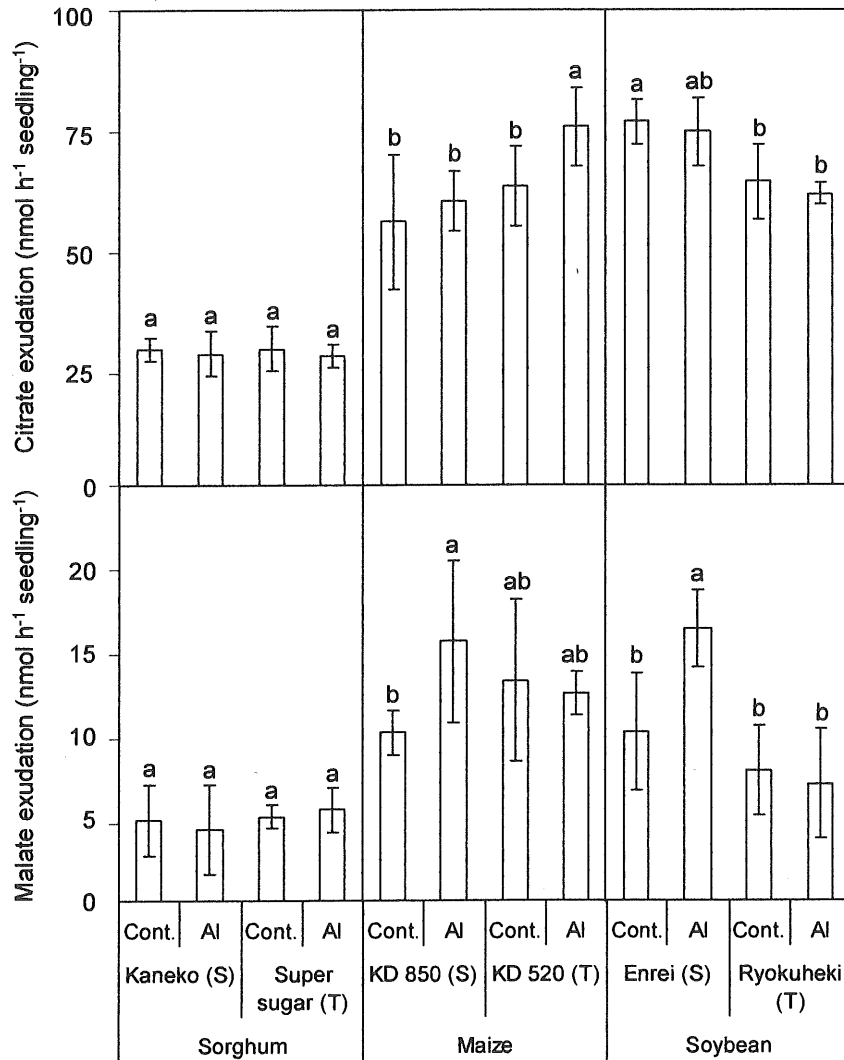
Fig. 6.2. Citrate (A) and malate (B) exuded from sorghum, maize and soybean. Five-day-old seedlings were treated for 5 h with or without Al in 0.2 mM $CaCl_2$ (pH 4.9) following 5 h of pretreatment with 0.2 mM $CaCl_2$ (pH 4.9). Exudates were collected during the 5 h treatment. Values are means ± SE (n = 3). Bars having different letters are significant at 5% level of significance within same crop species.

PM permeabilization of sorghum, maize and soybean cultivars showed same intact PM in control irrespective of their tolerance to Al (Fig. 6.2). On the other hand, in Al treatment, only Al-sensitive cultivars or lines showed greater permeabilization which is ascribed as the red fluorescence. Al accumulation in these cultivars were greater than tolerant cultivars ascribed as brownish purple color (Fig. 6.3).

This tendency of greater PM permeabilization and Al accumulation in the sensitive cultivars were similar to that in rice (Khan et al. 2008), wheat (Wagatsuma et al. 2005a) and tricale (wagatsuma et al. 2005b) (Fig. 6.4).

Total phospholipid content was greater in the control of sensitive cultivars than that of tolerant cultivars (Fig. 6.5). Except for Al-tolerant soybean cultivar, all the cultivars and lines showed a general increase of phospholipids following Al treatment irrespective of their Al tolerance. A slight decrease in phospholipids was observed for Al tolerant Ryokoheki cultivar though this decrease was non-significant. Following the control, Al treatment also showed a less phospholipids content in Al-tolerant cultivars or lines for all the crop speciesexcept for soybean. However, tolerant soybean cultivar (Ryokuheki) showed greater phospholipid content than sensitive and further showed decreasing tendency while treated with Al (Fig. 6.5).

On the other hand, Δ^5-sterol content showed wide variation among the crop species, i.e., least was in Al treatment of Kaneko (sorghum) (0.8µmol) and highest in control of Rikuu-132 (rice) (2.86µmol) (Fig. 6.6). Within the same crop species, Δ^5-sterol was greater in control of Al-tolerant cultivars or lines except for soybean (Fig. 6.6). In Al treatment, Δ^5-sterol content decreased in all cultivars or lines except for sensitive maize

cultivar (KD850) and soybean cultivars (both cultivars). In fact, tolerant soybean cultivars showed an increasing tendency for Δ^5-sterol in Al treatment than that in control. As negatively changed phospholipids makes less tolerant PM and neutral Δ^5-sterol makes strong PM, to consider the influence on PM permeabilization of these two lipids, lipid ratio (phospholipids/Δ^5-sterol) was considered to be more influential. Though lipid ratio within the crop species did not show any specific trend, but within the same crop species, lipid ratio was greater for Al sensitive crop species except for soybean. Tolerant wheat showed least lipid ratio whereas tolerant soybean showed highest lipid ratio (Fig. 6.7). In Al treatment, lipid ratio showed increasing tendency except for soybean. In soybean lipid ratio was greater in tolerant cultivar (Ryokuheki) than sensitive cultivar which further showed decreasing tendency in Al treatment.
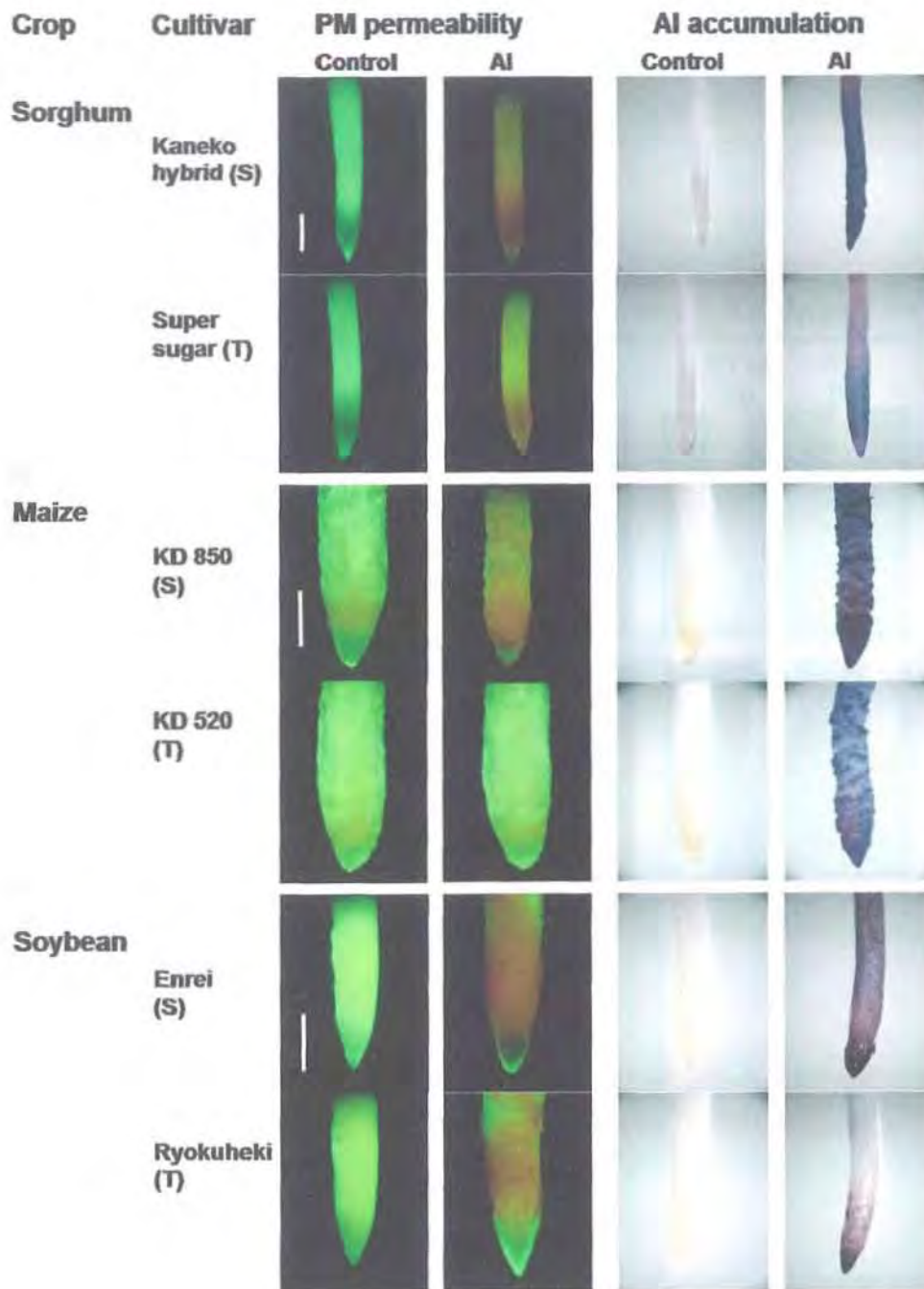
Fig. 6.3: Plasma membrane (PM) permeability by FDA-PI fluorescence microscopy and Al accumulation by hematoxylin staining light microscopy. Al treatment conditions were same as in Fig. 6.1. Green fluorescence in FDA-PI staining indicates intact PM, red fluorescence indicates permeabilized PM. Dark purple color in hematoxylin staining indicates heavy accumulation of Al. Fluorescence excitation filter 450-490nm; barrier filter 520nm. Photograph are representative of at least three independent observ ations. Bar = 1mm.
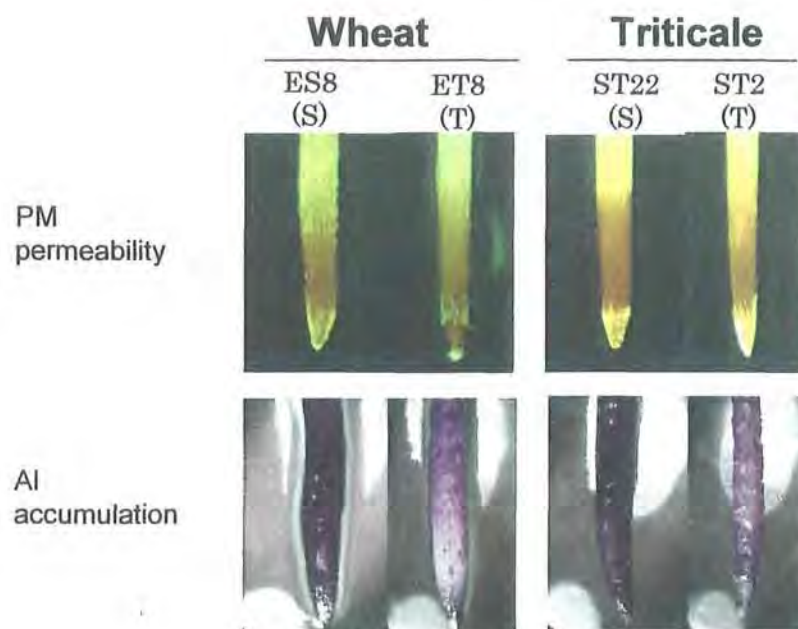
Fig. 6.4: PM permeability and Al accumulation of wheat (Wagatsuma et al. 2005) and triticale (Wagatsuma et al. 2005). Treatment conditions were same as in Fig. 6.1. Photograph are representative of at least three independent observations.
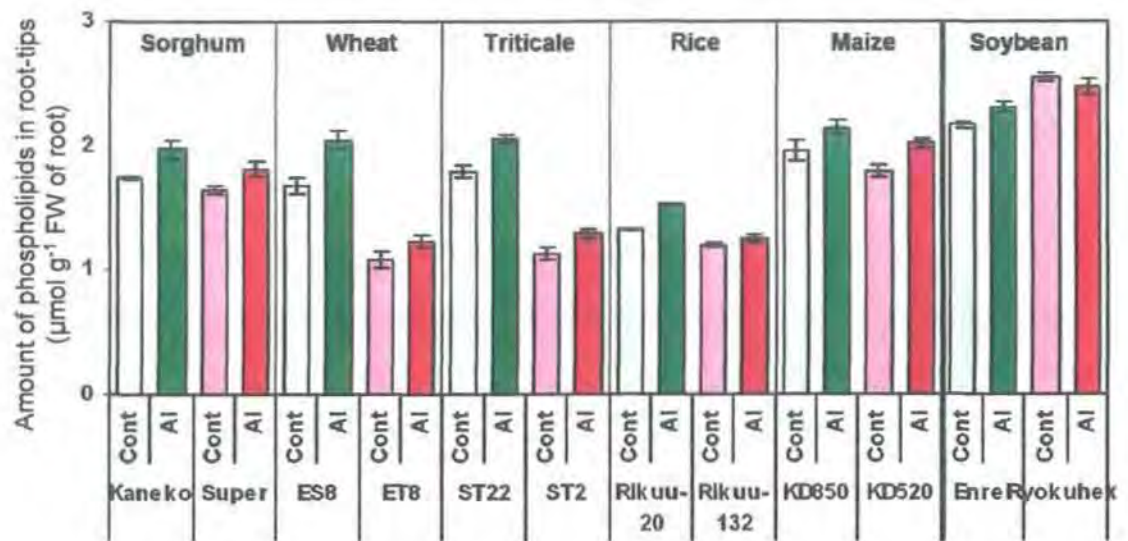
Fig. 6.5: Amount of phospholipids in the root tips for control and Al treatment of the selected cultivars. Treatment conditions were same as described in Fig. 6.1. Data are average ±SE (n = 2).
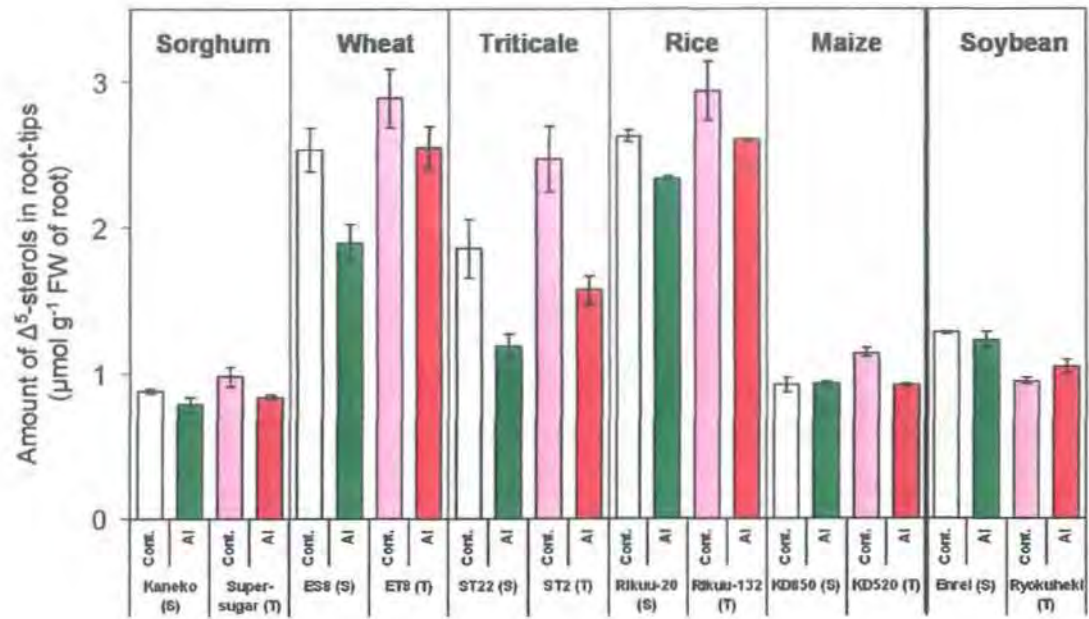
Fig. 6.6: Amount of Δ^5-sterols in the root-tips (0-10mm) for control and Al treatment. Treatment conditions were same as described in Fig. 6.1. Data are mean±SE (n = 2).
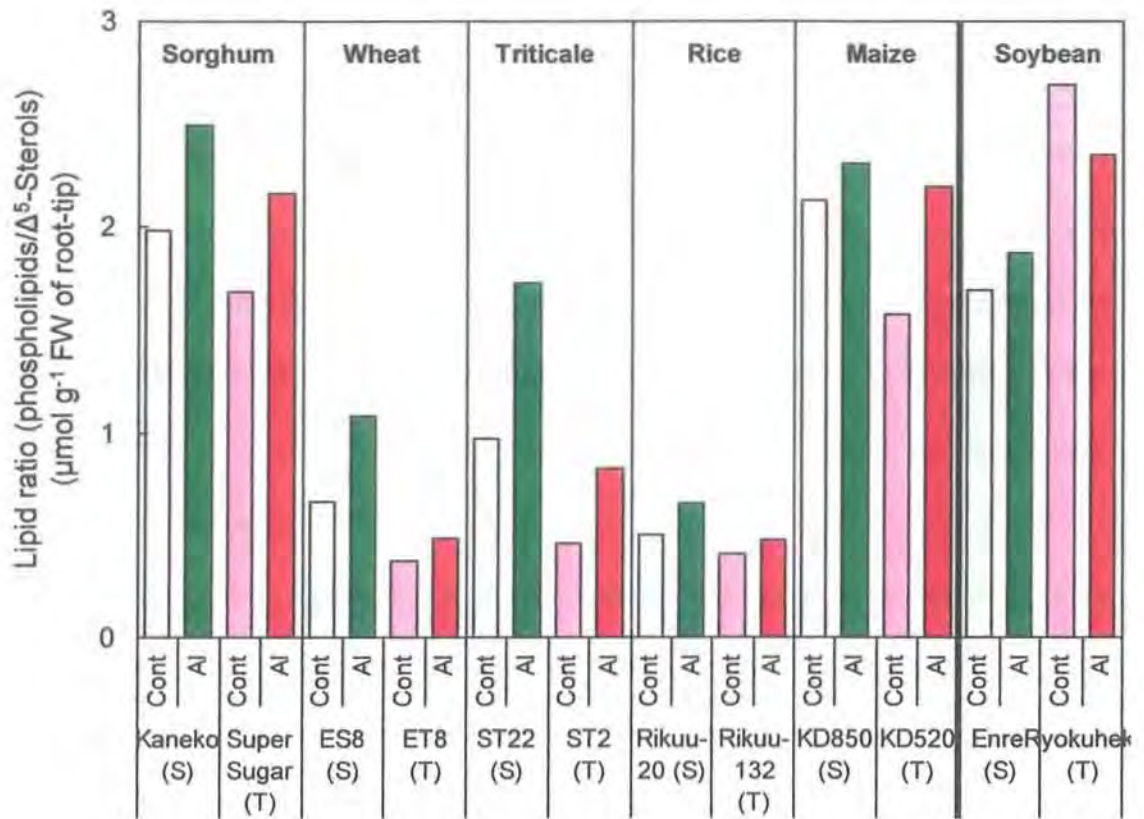
Fig. 6.7: Lipid ratio (phospholipids/Δ^5-sterol) in root tip portion. Data are ratio of average values obtained in Fig. 6.4 and 6.5.

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6.4 Discussion

Among the crops studied in this experiment, greater permeabilization and Al accumulation was observed in the sensitive cultivars or lines for all crop species (Fig. 6.2-6.3) which is ascribed to higher phospholipids (Fig. 6.4) content in sensitive cultivars or lines. On the other hand, a neutral lipid, Δ^5-sterol contribute greater intactness in the PM which was greater in the tolerant cultivars (Fig. 6.5) and finally lipid ratio (phospholipids/Δ^5-sterol) was greater in control and in Al treatment for sensitive cultivars than that of tolerant cultivars. This tendency follows the results shown in the previous Chapter 5 where lipid ratio showed a significant correlation with Al tolerance in rice. In Figs. 6.4-6.6., it could be observed that phospholipids, Δ^5-sterol and lipid ratio are independent among the crop plant species. On the other hand, results showed clear tendency within the cultivars of same crop species. Suggested Al tolerance mechanisms by the researchers among these crop species has been summarized in Table 6.1.

Table 6.1: Major Al tolerance mechanisms in crop species used in this study

Crop	Al tolerance mechanism(s)
Sorghum	: Less PL/Δ^5-sterol (present study), citrate exudation (Magalhaes et al. 2007)
Wheat	: Less PL/Δ^5-sterol (present study), malate exudation (Sasaki et al. 2004)
Triticale	: Less PL/Δ^5-sterol (present study)
Rice	: Less PL/Δ^5-sterol (present study), less pectin methyl esterase (Yang et al. 2008)
Maize	: Less PL/Δ^5-sterol (present study), citrate exudation (Piñeros et al. 2002)
Soybean	: Other than OA exudation (present study)

Although OA exudation has been reported as one of the Al tolerance mechanism in wheat, the present study clearly shows the significance of PM lipid composition for Al tolerance. It could be possible that both Al tolerance mechanism (less PL/Δ^5-sterol ratio and greater OA exudation) is function simultaneously for wheat Al tolerance. In sorghum, citrate or malate exudation did not show any relevance with Al tolerance among the cultivars in this study (Fig. 6.2) rather it posses a mechanism of less lipid ratio for Al tolerance. Although citrate exudation has been reported as Al tolerance mechanism (Magalhaes et al. 2007), cultivars studied by Magalhaes et al. (2007) were different from the present study. Moreover, sorghum cultivars in the present study were selected by screening which were widely used in Japan and were highly susceptible to Al whereas cultivars used by Magalhaes et al. (2007) were so strong against Al stress.

In triticale, till now, Al tolerance mechanism other than less phoshpolipid/ Δ^5-sterol ratio has not yet been proposed. In maize, greater citrate exudation (Fig. 6.2) as well as less lipid ratio (PL/Δ^5-sterol) may simultaneously acting for greater Al resistance. In some other study, citrate exudation has been suggested as one Al tolerance mechanism in maize, but Piñeros et al. (2005) reported a non relevance of OA exudation with Al tolerance. They also studied some other possible Al tolerance mechanism in North American and Colombian maize cultivars but could not draw any positive results for Al tolerance. Considering all together, less lipid ratio would be primary mechanism for Al tolerance and this would be first clear report to show Al tolerance mechanism in maize.

Soybean, however, showed different tendency to other crop species which were used in the present study. Moreover it did not snow any correlation of Al tolerance with citrate or malate exudation. Yang et al. (2001) showed that citrate exudation would be the Al

tolerance mechanism in soybean though cultivars studied in the present study were different from those used by them. In my study, I could not confirm the Al tolerance mechanism for soybean. In another study with other dicot leguminuous plant (pea) we could find relationship of Al tolerance with less lipid ratio (PL/Δ^5-sterol). Based on this point, I can suggest the Al tolerance mechanism for soybean would be different even from another leguminous plant (data not shown).

Chapter 7

Long-term tolerance of rice in high Al and low ionic strength conditions

Chapter 7

Long-term tolerance of rice in high Al and low ionic strength conditions

7.1 Introduction

7.1.1 Extent, occurrences and characters of Acid soils

Soil acidity has long been considered as one of the major constraint for agricultural production. About 40% of world arable land consists of low pH condition (Kochian et al. 2005) and this area is increasing day by day. Causes of soil acidification are intensive weathering (Oxisols, Ultisols, Andisols and Alfisols), acid sulfate soil (Inceptisols and Entisols), parent materials poor in basic cations (Spodosol, Histosol and Entisol), acid deposition, intensively managed row crop agriculture and pasture system (Sumner and Noble 2003). Intensive weathering of parent materials is the major acid soil occurring factor in the humid tropics, leaving higher amount of iron and aluminum oxides instead of other nutrients (Sumner and Noble, 2003) e.g., Ferralosols, Acrisols, Andosols (FAO-UNESCO soil classification). Therefore, these acid soils consist of high Al and low nutrient as accompanying predicament. Beside this, other kinds of acid soils are occurred due to parent materials poor in basic cations, acid sulfate in delta areas of the great rivers, acid deposition through acid precipitation owing to fossil fuel combustion, acidified as a consequence of agricultural practice especially due to ammoniacal N fertilization. Though there are many acid soils but the extent of acidity and nutrient content or ionic strength widely varied. Low nutrient is one of the major accompanying predicament in naturally occurred acid soils with high concentration of Al.

Rice is especially grown in the areas where Al toxicity and low nutrient stress hampers rice production simultaneously. On the other hand, population increasing rate in that rice growing region is very high. Rice is rather Al tolerant crop species. Till now we do not know the primary stress factor reducing the rice yield. While simulating Al and low nutrient stress syndrome in nutrient solutions, Wenzl et al. (2003) found that less-adapted *B. ruziziensis* became more sensitive to Al toxicity as the level of nutrients in the growth medium was reduced. Watanabe and Okada (2005) studied interactive effect of Al and other cations in Indica and Japonica rice cultivars and suggested that the primary mechanism of Al toxicity in rice changes depending on the ionic strength hence the nutrient content where it is grown. Pintro and Taylor (2004) also pointed out that the nutrient concentration should be considered carefully to simulate natural soil solutions when screening for Al tolerance.

7.1.2 Interaction of Al ion with other ions in the growth media

High concentration of Al in high nutrient solution might be alleviated due to physicochemical interactions between Al and other ions and formation of nontoxic complexes with anions (e.g. OH^-, SO_4^{2-}) and silicate ions (Blamey et al. 1991). Al can inhibit uptake of particular nutrient element (e.g. P) by forming complex with nutrient making unavailable form or by competing with cationic nutrient elements with higher potentials or by blocking the cation channels. Not only the binding of Al with other anions at low pH conditions but other metal ions like Cu, Cd showed interaction in the medium and also promoted or inhibited Al accumulation depending on barley genotypes (Guo et al. 2007). Moreover, Al ion activity is also regulated depending on the ionic

strength of the medium. Given that addition of similar amount of Al to the different ionic strength medium makes the different ionic activity of $\{Al\}^{3+}$.

Given that among the monomeric Al ions $\{Al\}^{3+}$ is the most toxic to plants which is followed by other ions. Phosphorus deficiency has also been reported as a major yield limiting factor in acid alfisols, oxisols, ultisols, and andepts (Clark 1984).

7.1.3 Primary growth limiting factor in tropical acid soil

Soil mineralogy is one of the major factors regulating the relationships between pH, exchangeable and soluble Al, and for a given pH the amount of soluble Al may increase three times with increase in clay content (Sierra et al. 2005). In spite of mimicking true acid soil conditions in tropics, Al research typically carried out in nutrient conditions higher than those typically found in acid soil solutions (Gillman and Bell 1978, Blamey et al. 1991, Edmeades et al. 1985, Wenzl et al. 2003). Unfertilized soil solution of Colombian Savannas acid soils are extremely poor in nutrients (<1.7mM) (Parker and Norvell 1999) though experiments designed for low ionic strength nutrient solutions are 5.4-13.4mM (Gillman and Bell 1978, Edmeades et al. 1985). Actual Al toxicity in such high nutrient hydroponic culture alters by building better rooting environment (Pintro and Taylor 2004) and finally reduces the activity of metal ions in solution (Pintro et al. 1999). Wenzl et al. (2003) reported that Al tolerance in low nutrient condition can only be mimicked to actual acid soils for two tropical grasses, *Brachiaria decumbens* and *B. ruziziensis*. Okada et al. (2003) reported that the relative yield of Al-sensitive varieties of upland rice was correlated with the exchangeable Ca in highly weathered soils with low cation exchange capacity suggesting that Ca has an important role for Al tolerance in acid

soils. Many solution culture studies have used nutrient and Al concentrations which are far from those found in the soil solutions of acid soils. Most studies have focused on the effect of Al to plant adaptation (Foy 1992, Kinraide 1997). To my knowledge, determination of primary factor for better crop production in acid soil not yet been carried out except for Wenzl et al. (2003) for two *Brachiaria* spp. Therefore, clarification of each stress condition is needed to differentiate Al toxicity with other stress factors occurring in true acid soil.

7.1.4 New aspect of Al tolerance in tropical acid soils

Ionic strength of Savanas acid soil has been reported as low as <1.7mM (1.3-1.7mM in general) which upon fertilization increased 5.4-13.4μM (Edmeades et al. 1985). This ionic strength was far lower than the acid soils in Australia and New Zealand (Gillman and Bell, 1978). It was imperative to know the actual situation of crop production in actual acid soil. There are so many works on Al tolerance or Al toxicity which was mainly carried out in nutrient medium. On the contrary, till now, very few researchers focus on this aspect of Al tolerance in low nutrient medium.

7.1.5 Objectives

Tropical acid soil contains not only the toxic concentration of Al but low nutrient availability is also a major factor to be considered. We do not know what is the primary factor for these low nutrient acid soils and the regulating factor to tolerate both stress simultaneously. Objectives of the present study were to know the primary stress factor

among both stress conditions and to know the role of minerals to regulate combined stress conditions.

7.2 Materials and Methods

7.2.1 Sources of seeds and reagents

The seeds of Indica type Bangladesh rice cultivars were collected from the Bangladesh Rice Research Institute, Gazipur, Bangladesh. Seeds of Japonica cultivars were collected from the Faculty of Agriculture, Yamagata University, Japan. Seeds of Sasanishiki pedigree cultivars were collected from the Faculty of Agriculture, Yamagata University, Japan; Shonai Branch Station, Yamagata Prefectural Agriculture Experiment Station, Yamagata, Japan or National Institute of Agro-Environmental Science, Tsukuba, Japan. All the chemicals were purchased from Wako Pure Chemical Industries Ltd., Japan unless otherwise stated.

7.2.2 Long-term tolerances for Al, low nutrient and combined stresses:

Seed germination and preculturing was carried out following Ofei-Manu et al. (2001). Briefly, seeds preliminarily soaked 1 d were spread on nylon screen that was put on a polypropylene container filled with 9 L of tap water under aeration at 27°C in cultivation root and germination. Just after sprouting, seedlings were transferred to the glasshouse for preculturing.

Seedlings were precultured for 5 days in a 40 L container filled with tap water under aeration. The tap water was renewed every 2 days to prevent the nutrient deficiency. Then the seedlings were transplanted in a piece of PVC tube supported by saffron. After

transplanting, seedlings were cultured in $1/5^{th}$ full nutrient solution (composition of full nutrient solution has been given hereafter) in the 40 L container. Full nutrient solution composed of 2.86mM NH_4NO_3, 1.43mM $NaNO_3$, 0.26mM $NaH_2PO_4 \cdot 2H_2O$ (Kanto Chemical Co., Inc., Japan), 0.77mM K_2SO_4, 2mM $CaCl_2 \cdot 2H_2O$, 1.7mM $MgSO_4 \cdot 7H_2O$ (Kanto Chemical Co., Inc., Japan), 36μM $FeSO_4 \cdot 7H_2O$, 18μM $MnSO_4 \cdot 5H_2O$, 37μM H_3BO_3 (Kanto Chemical Co., Inc., Japan), 3μM $ZnCl_2$, 0.16μM $CuSO_4 \cdot 5H_2O$, 0.05μM $(NH_4)Mo_7O_{24} \cdot 4H_2O$. Four treatments were applied as follows: 1) Control (adequate nutrient, AN; pH 5.2); 2) Al in AN (after filtration with a membrane filter having pore size of 0.45μm soluble ionic Al ranged from 48μM to 37μM and soluble P ranged from 6.1 to 5.2; pH 4.3); 3) low nutrient (LN) ($1/5^{th}$ concentration of nutrients used for AN; pH 5.2) and 4) Al in LN (after filtration with a membrane filter having pore size of 0.2μm soluble ionic Al ranged from 45μM to 37μM and soluble P ranged from 6.1 to 4.0; pH 4.3).

In the Al treatments, the mean soluble ionic Al concentration of 42.6μM Al was obtained by mixing 370μM Al and 230μM P and allowing standing for 1 d. The soluble ionic concentrations of Al and P were measured every day by inductively coupled plasma atomic absorption spectrophotometer, ICP-AES (Liberty 220, Varian Australia Ptv. Ltd., Australia) and have been presented in Fig. 1A and B. If the soluble P concentration goes below the asking level (0.2ppm) required amount of P was supplemented to the solution. Ionic activity of Al in the solutions were calculated by computer program by Wada and Seki (1984). The seedlings with 4 replications were treated for 5 wk under aeration with daily pH maintenance and weekly renewal of the culture solutions. Seedlings were

separated into shoots and roots after harvest, thoroughly washed, dried for 3 d at 70°C in draft oven, and weighed.

7.2.3 Calculation of tolerances

The stress tolerances of the respective crops were calculated as % relative growth with respect to the plant dry weight, i.e.

$$\% \text{ Al tolerance in AN} = \frac{\text{Dry weight in AN + AL}}{\text{Dry weight in AN}} \times 100$$

$$\%\text{Al tolerance in LN} = \frac{\text{Dry weight in LN + Al}}{\text{Dry weight in LN}} \times 100$$

$$\% \text{ low nutrient tolerance} = \frac{\text{Dry weightin LN}}{\text{Dry weightin AN}} \times 100$$

$$\% \text{ Combined tolerance} = \frac{\text{Dry weight in LN + Al}}{\text{Dry weight in AN}} \times 100$$

7.2.4 Analysis of minerals in the plant samples

Dry root and shoot samples were homogenized and 0.1 g of each samples were taken for analysis. Wet ashing of samples were done by adding 4 ml of acid mixture (HNO_3:60%$HClO_4$ = 5:3, v/v) to the sample and heating. The ash was resolubilized with 1 M HCl followed by deionized water with repetitions and filtered. Measurement of P, K, Ca, Mg, Fe, Mn and Al concentration in the sample was carried out by inductively coupled plasma atomic absorption spectrophotometer (ICP-AES, Liberty 220, Varian Australia Pvt. Ltd., Australia).

Calcium translocation capability was calculated as below-

$$\text{Ca translocation capability} = \frac{\text{Ca conc. in shoot}}{\text{Ca conc. in root}}$$

7.3 Results

Average Al tolerance in short-term single nutrient solution of all the cultivars was 43.3% (Fig. 7.3). Al tolerance was in the order of Rikuu-132, Kamenou>Sasanishiki, BR41>>>Aikokuu, Rikuu-20, Domannaka, BR34. In long-term experiment the whole plant average Al tolerance was 83% and 72% in AN and LN condition respectively (Fig 7.4 A, B). Al stress in both nutritional conditions decreased the plant growth, but tolerant and sensitive cultivars maintained identical tendency i.e. whole plant Al tolerance in AN was in the order of BR41, Sasanishiki>Rikuu-132, Kamenou>>> Domannaka> Aikoku>Rikuu-20, BR34 and whole plant Al tolerance in LN was in the order of BR41 > Rikuu-132, Sasanishiki> Domannaka > Kamenou >>> BR34, Rikuu-20>Aikoku (Fig. 7.4A, B).

Low nutrient tolerance was almost reverse condition to Al tolerances except for Rikuu-132 (Fig 7.4C) and Al sensitive plants showed rather higher low nutrient tolerances. In combined stress condition, i.e. combined tolerance did not followed any trend and are in random order when compared with Al tolerance and low nutrient tolerance. Further, the combined tolerance did not show any correlation with Al tolerance or low nutrient conditions (Table 7.1). Short-term Al tolerance showed significant positive relationship with Al tolerances in both nutrition (AN and LN) conditions (Table 7.1). Significant negative correlation (R^2 = -0.884**) was observed between root Al

concentration and Al tolerance in LN condition (Fig. 7.5A). On the other hand, root Al concentration did not showed any relationship with combined tolerance (Fig. 7.5B).

Ca concentration in the shoot showed significant positive correlation with combined tolerance of shoot (R^2 = 0.507*) indicating the Ca in the shoot playing the most important role to ameliorate or minimize the combined stress condition (Fig. 7.6). However, without combined stress condition this Ca did play his enthusiastic role (Fig. 7.6).

Typical cultivars were isolated from the results obtained in this experiment.. It could be found that Rikuu-132 is tolerant to high Al and combined stress condition. BR34 was most sensitive to Al but tolerant to low nutrient condition finally this greater tolerance to low nutrient makes it high combined tolerant. On the other hand, Sasanishiki is tolerant to high Al but sensitive to low nutrient stress which finally shows its lower tolerance to combined stress condition. Considering low nutrient as the main regulating factor to determine combined tolerance, Ca translocation capability was highest in Rikuu-132 for low nutrient stress condition which was followed by BR34 (Fig. 7.7). Least Ca translocation from root to shoot was observed in Sasanishiki.
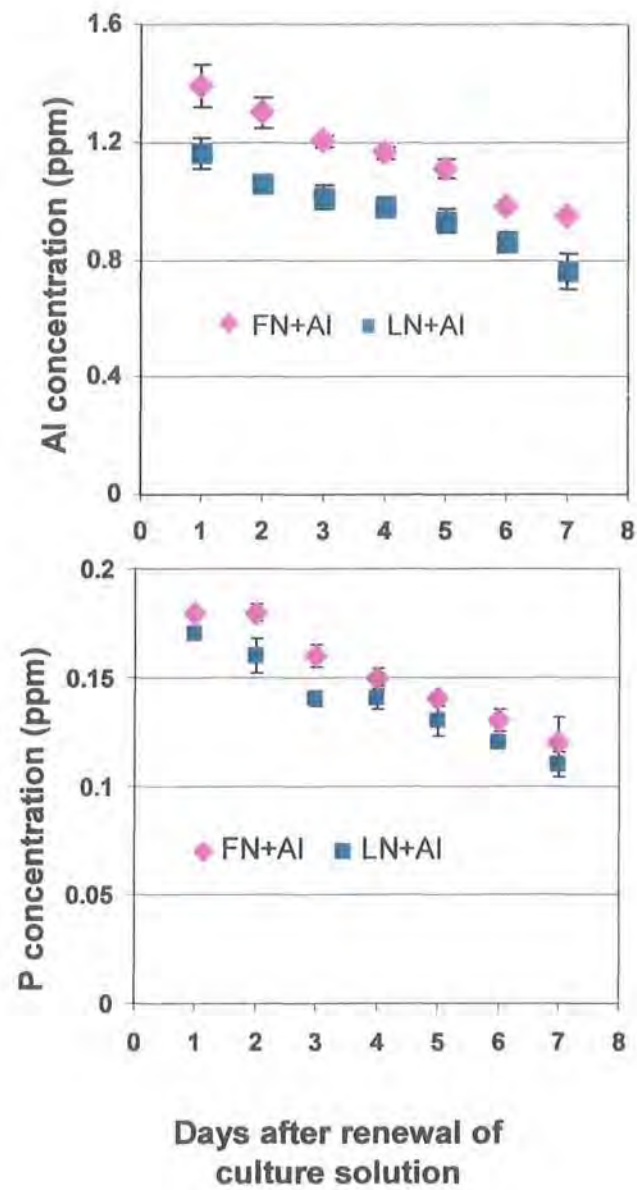
Fig. 7.1: Soluble Al and P concentration in the treatment media in adequate nutrient and low nutrient condition
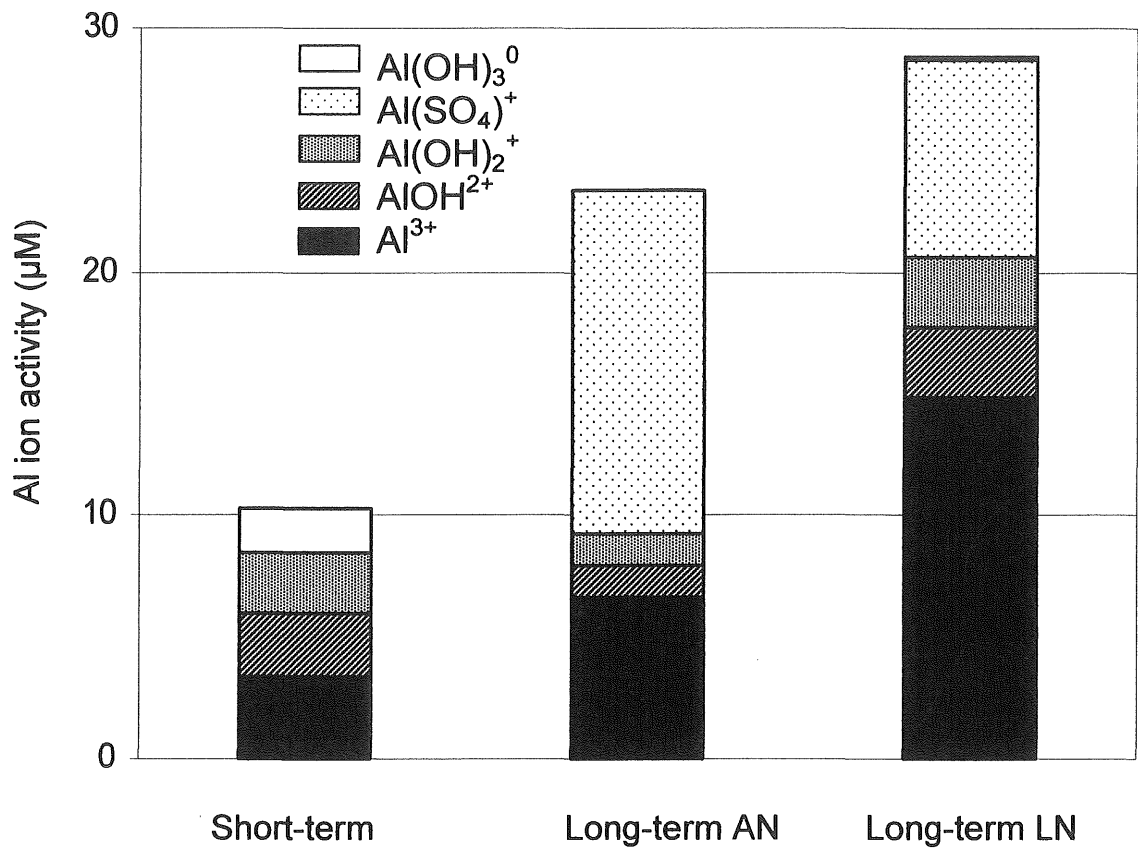
Fig. 7.2: Al ion activity in the culture solution. In short-term (24 h) added Al concentration was 20µM. In long-term experiments added Al and P concentration was 370µM and 230µM respectively. AN, adequate nutrient; LN, low nutrient (added nutrient concentration is $1/5^{th}$ of AN).
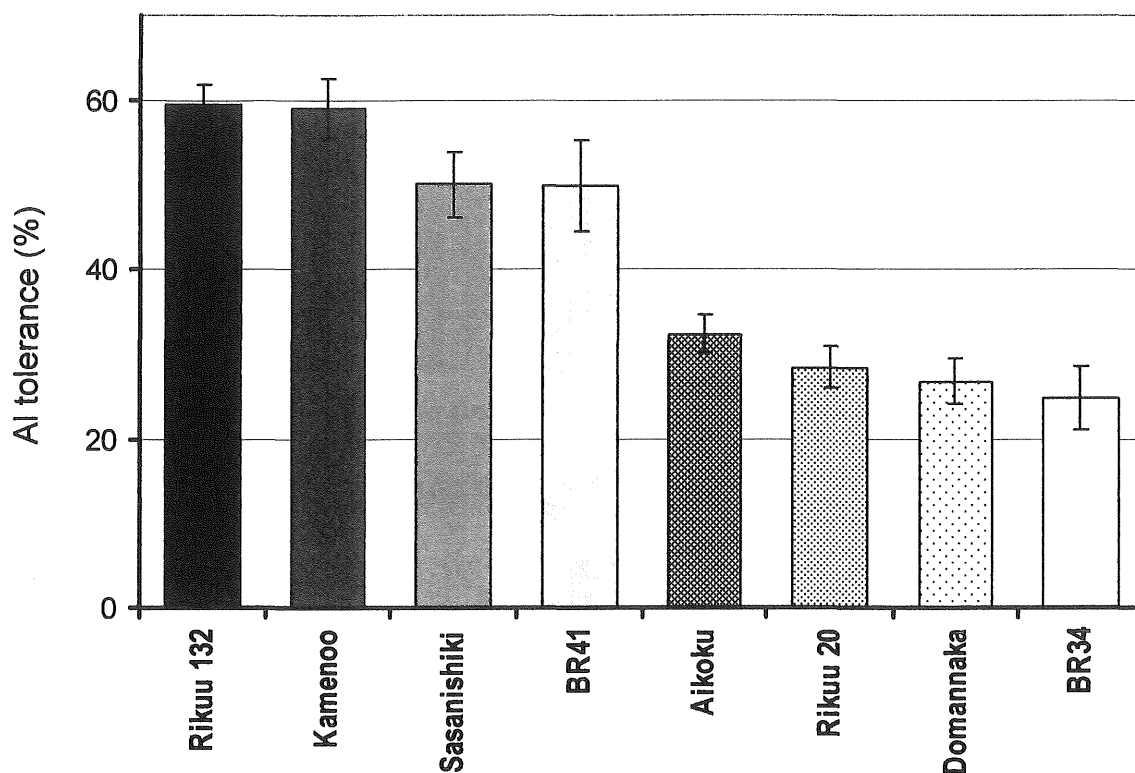
Fig. 7.3: Short-term Al tolerance of the rice cultivars. Control treatment: 0.2mM $CaCl_2$ at pH 4.9 for 24h. Al treatment: 20µM $AlCl_3$ in 0.2mM $CaCl_2$ at pH 4.9 for 24h. Relative net root elongation was used as Al tolerance. Dotted line indicates average Al tolerance in all cultivars. Denser color indicate higher Al tolerance. Bar indicate ±SE, n = 12.
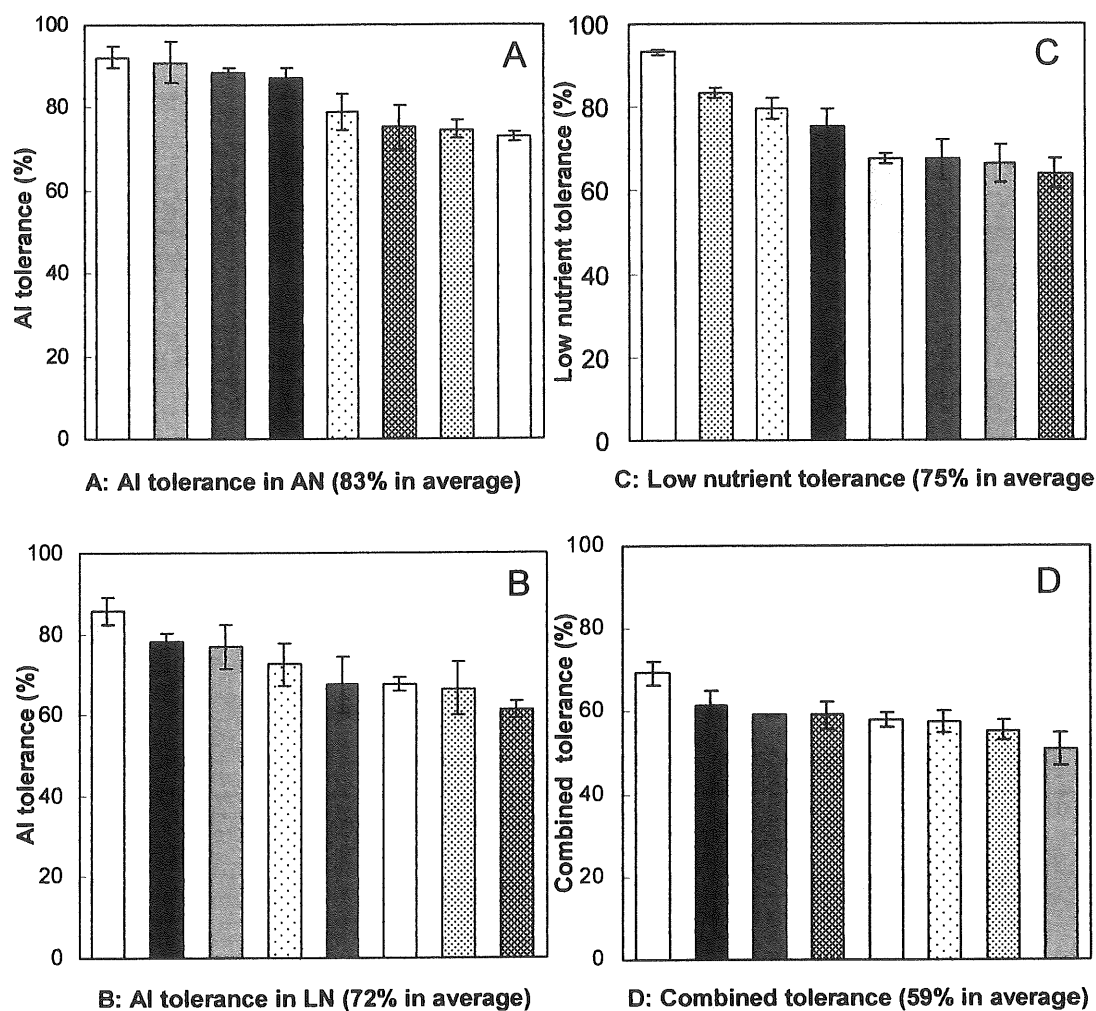
Fig. 7.4: Relative tolerances in long-term (35 d) hydroponics culturing experiment. A. Al tolerance in AN conditions, B. Al tolerance in LN conditions, C. Low nutrient tolerance and D. Combined tolerance. Definitions of the tolerances were described in the chapter Materials and Methods. Color of each column represents same cultivar as shown in Fig. 2. Bar indicate ±SE, n=4

Table 7.1: Correlations (r value) among each tolerance (whole plant)

	Al tolerance in AN	Al tolerance in LN	Short-term Al tolerance	Low nutrient tolerance	Combined tolerance
Al tolerance in AN	1				
Al tolerance in LN	0.711*	1			
Short-term Al tolerance	0.884**	0.808*	1		
Low nutrient tolerance	-0.638	-0.389	-0.609	1	
Combined tolerance	-0.449	-0.187	-0.300	0.605	1

Table 7.2: Isolation of typical cultivars and it's cause

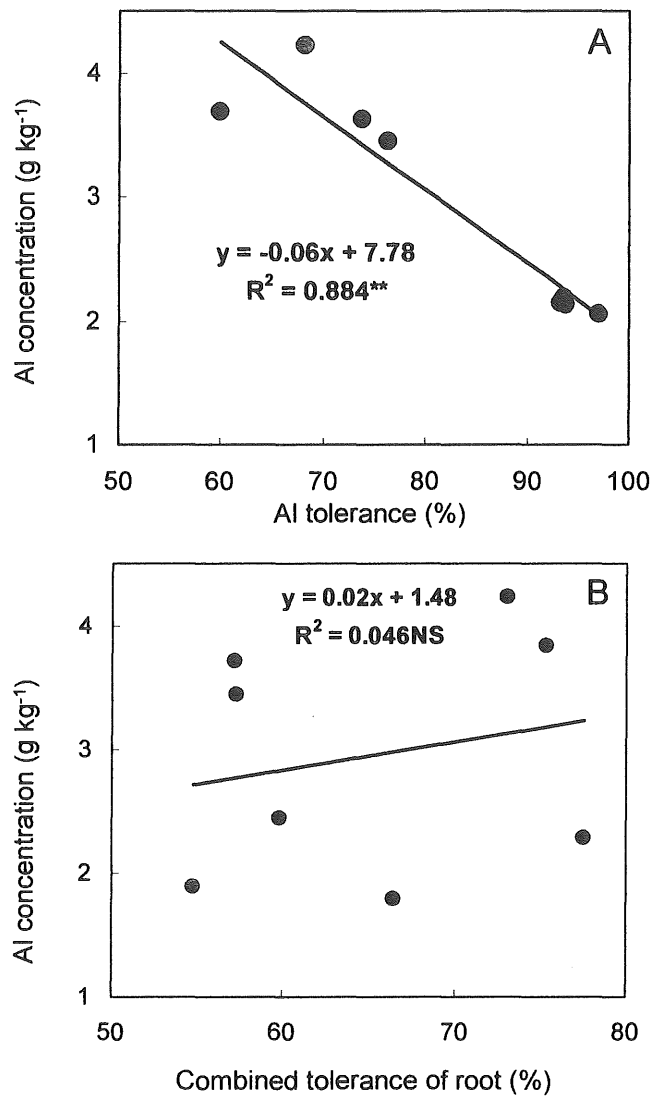|	
Rikuu-132	: Al tolerant and combined tolerant
Sasanishiki	: Al tolerant but combined sensitive due to high sensitivity to low nutrient stress
BR34	: Al sensitive but combined tolerance due to high tolerance to low nutrient stress

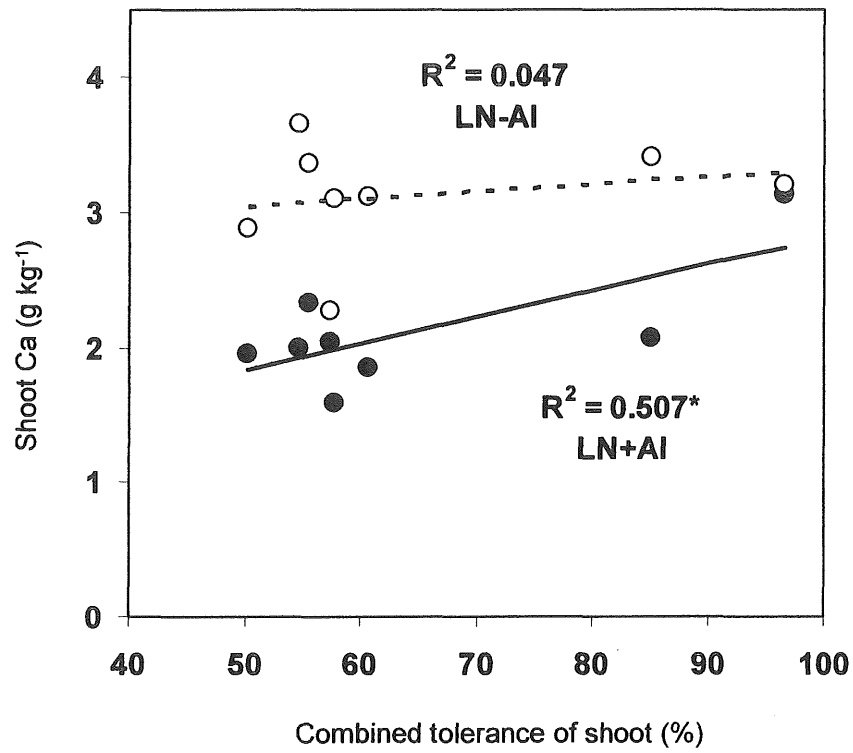Fig. 7.5: Relationship of Al concentration with Al tolerance (A) and combined tolerance (B)

Fig. 7.6: Relationship of shoot Ca concentration with combined tolerance. Open circles are in LN condition, closed circles in LN+Al conditions
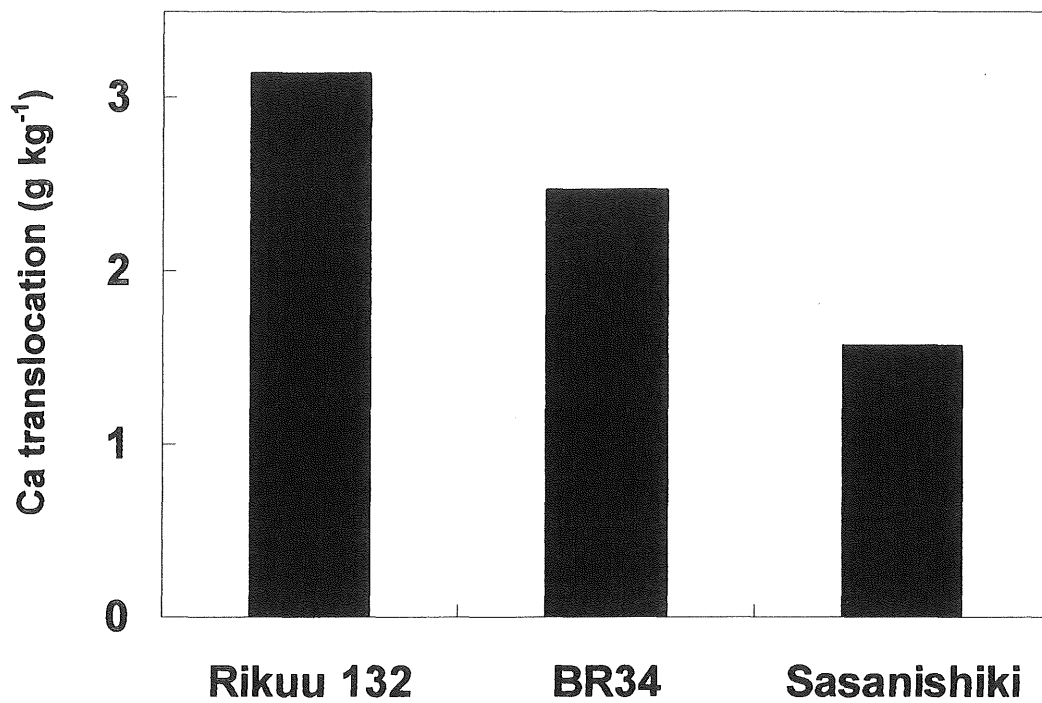
Fig. 7.7: Ca translocation from root to shoot of typical cultivars. Values are mean of 3 replicated samples

7.4 Discussion

Ionic strength of Savanas acid soil has been reported as low as <1.7mM (1.3-1.7mM in general) which upon fertilization increased 5.4-13.4μM (Edmeades et al. 1985). This ionic strength was far lower than the acid soils in Australia and New Zealand (Gillman and Bell, 1978). Watanabe and Okada (2005) also found less Al ion activity while increased Ca concentration in the medium. In the present experiment, Al tolerance in LN is lower than that was found in AN conditions. In this experiment, I used a solution having ionic strength 4.5mM which mimicks Australian and New Zealand acid soil.

Al concentration in the root is significantly related with Al concentration in the roots. Pineros et al. (2005) also found negative correlation Al tolerance with root Al concentration in maize cultivars. Al tolerance did not show any relationship with combined tolerance (Table 7.1). The mechanism for Al tolerance would be different from the mechanism of tolerance in combined stress conditions. Plant physiological function might differently response to these stress conditions. Combined tolerance did not show any significant correlation with Al tolerance in LN ($R^2 = 0.187$) or low-nutrient tolerance ($R^2 = 0.605$) (Table 7.1). Although these Al tolerance and low-nutrient tolerance does effect independently to combined tolerance but effect of low-nutrient tolerance is greater than Al tolerance ($0.605 >> 0.187$). This result suggests that both factor, Al tolerance and low-nutrient tolerance simultaneously interact with combined tolerance and contribution of low-nutrient tolerance is fur greater than that of Al tolerance for rice. Akhter et al. (2008) also found similar greater contribution of low nutrient tolerance than Al tolerance to combined tolerance while using

In the present study, root Al content showed significant positive correlation with Al tolerance ($R^2 = 0.884**$) (Figure 7.5A). Clear differential Al content was also found in tolerant and sensitive group of cultivars. In AN nutrient condition Al tolerance increases with the decrease in root Al connect which was also reported by several researchers (Wagatsuma et al. 1991, Ofei-Manu et al. 2001, Pineros et al. 2005).

Although, Al tolerance is related mainly with the Al status in the plant in any nutrient condition, in the present experiment while using only rice crop for Al tolerance study, Al toxicity was not found as a main factor in combined condition (Figure 7.5B. For combined tolerance, Ca status can be ascribed as the main factor for better rice growing (Figure 7.7). Other coexisting factors may also have important relations. Ca also showed significant relation positively with Mg and Fe in the shoot (data not shown).

As more important factor for plant growth in acid soils, shoot Ca content was found to be ascribed for combined tolerance (Figure 7.6). In the present study, it was observed that Ca translocation from root to shoot was greater in reasonably in combined tolerant cultivar (Rikuu-132) (Fig. 7.7). On the other hand, least Ca translocation was observed in Al tolerant but combined sensitive Sasanishiki. Among the selected typical cultivars, BR34 was Al sensitive but combined tolerant which shows intermediate type of Ca translocation. The order of Ca translocation follows the same order of combined tolerance but not the Al tolerance (Figs. 7.4 and 7.7). Further Ca showed significant positive correlation with Mg and Fe content (data not shown) which indicate that uptake of these two nutrients also regulated by uptake of Ca. Wenzl et al. (2003) found that in the tropical sub humid savannas having highly weathered acid soil, the susceptible crop plant growth is reduced not only for Al concentration, soil pH but also exchangeable Ca.

Babourina et al. (2005) investigated potassium transport in the root elongation zone of *Arabidopsis* seedlings and suggested that elevated external Ca activities can sustain K influx in the root elongation zone during Al exposure either by maintaining $[Ca^{2+}]_{cyt}$ or by affecting Al uptake across the plasma membrane. Nutrient uptake from the medium is not the only mechanism for efficient growing in low fertile soil but utilization efficiency of the uptaken nutrients which implies specific physiological mechanism is also important (Rengel 2003, Sattelmacher et al. 1994).

7.5 Conclusion

Considering the complex stress condition of tropical acid soils, high Al and low-nutrient would be the major growth limiting factors. For combined tolerance (tolerance to high Al along with tolerance to low-nutrient), although both factor is contribution simultaneously, low-nutrient tolerance is the primary regulating factor. This low-nutrient tolerance is controlled by the Ca uptake and translocation from root to shoot. Further consideration is needed to know the role of other nutrients for combined tolerance.
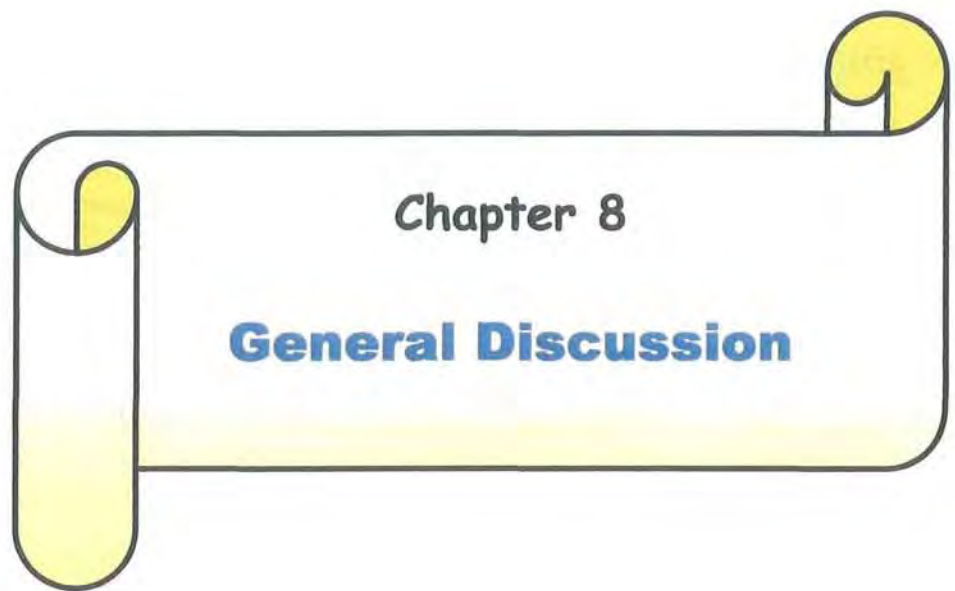
Chapter 8

General Discussion

Chapter 8

General Discussion

Al tolerance of Indica rice (from Bangladesh) cultivars were more widely distributed than Japonica rice cultivars though Indica rice were more sensitive to Al than that of Japonica rice cultivars (Fig. 2.1). Ma et al. (2005) also reported similar higher sensitivity to Al for Indica rice cv. Kasalath than Japonica cv. Kushihikari. The sensitive cultivars also showed greater Al accumulation (Fig. 2.2) and PM permeabilization (Fig. 2.3) indicating higher negative site in the PM (greater Al accumulation) and finally PM showed greater permeabilization. After higher accumulation of Al in the root-tip cells, PM of the cells became permeabilized which is shown in permeability experiment by FDA-PI staining (Figure 5). Higher zeta potential of the root-tip cells PM of sensitive cultivars is liable to bind more Al on its surface (Wagatsuma et al. 1991) which intern makes the PM permeable after making partial rigidification in the PM (Ishikawa and Wagatsuma, 1998). Yermiyahu et al. (1997) and Ahn et al. (2004) also stated that the differences in the magnitude of negative charges on the surface of the PM differentially attract the positively charged Al ion and alters phospholipids profile.

In general, Al remains as hexahydrated form in aqueous medium. On the other hand, phospholipids of the PM remain dispersed in the liquid crystal state and embedded proteins which manifest maximal biological activity moves freely in this fluid phase (Leshem et al. 1992). When negatively charged phosphate group of phospholipids comes in contact of $[Al(H_2O)_6]^{3+}$, they bind together covalently (Caldwell 1989) all the water molecules of hexahydrated Al and maximum water molecules of phosphate group (7-8

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molecules of water, Cevc 1982) dehydrated by Eigen mechanism (Ishikawa and Wagatsuma 1998). This type of binding characteristics of phosphates and protein groups of the PM have been reported by several researchers (Ahn et al. 2004, Chen et al. 1991, Ishikawa et al. 1996, Ishikawa and Wagatsuma, 1998, Jones and Kochian 1997, Wagatsuma et al. 1991). By dehydrating, dispersed phospholipid molecules in the normal PM, loose it's hydrated form as a result membrane in a liquid crystal state becomes rigid and gel-like (Hauser and Phillips 1979, Chen et al. 1991, Leshem 1992). The packing area becomes more hydrophobic after this dehydration (Ishikawa and Wagatsuma 1998). This type of decrease in membrane fluidity was also reported for *Thermoplasma acidophilum* for isolated and intact cell membranes (Vierstra and Haug 1978).

Among the cultivars presented in Fig. 2.1, Sasanishiki showed outstanding Al tolerance. In the next stage, characterization of the 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 were studied (Fig. 3.1B). This rice cv. Sasanishiki was bred at Furukawa Agricultural Experiment Station, Japan in 1963, cultivated vastly as one of the most famous and popular rice cultivar especially in Tohoku district, north east area in Japan, in 1980's–1990's. Ancestor cultivars showed a wide range of Al tolerance (from 23 to 60%) (Fig. 3.1A), and originated from Al-tolerant and -sensitive ancestors. The family tree suggests that greater Al tolerance of Sasanishiki is considered to be originated basically from the most Al-tolerant Kamenoo (17) which was bred by a farming breeder in 1893. Based on the results in Fig. 3.1B, I selected Kamenoo (17) and Rikuu-132 (18) as the most Al-tolerant

cultivars and Rikuu-20 (2) and Aikoku (7) as the Al-sensitive cultivars with intimate genetic connections for the later stage of more detailed experiments.

Differential PM permeabilities were found after 24h of Al treatment, i.e., less permeabilization of PM in Al-tolerant cultivars Kamenoo (17) and Rikuu-132 (18) (Fig. 3.4). Differential Al accumulation were also found after 24h of Al treatment, i.e., less Al accumulation in the former two Al- tolerant cultivars. Consequently, PM permeabilities after 24h of Al treatment was consistent with those after 1h of Al treatment (Fig. 3.4). In a study, Ofei-Manu et al. (2001) reported similar tendencies: less Al accumulation and less PM permeabilization in Al-tolerant woody plant species also after short-term of Al treatment. Ishikawa et al. (2001) reported less PM permeabilization in Al-tolerant cultivars than that of Al-sensitive cultivars among five crops. Finally, it is obvious that Al-tolerant plants and cultivars accumulate less Al and showed less PM permeabilization.

There are several expected mechanisms for Al tolerance. Among already reported mechanisms for Al tolerance, OA exudation is most frequently reported (Kochian et al. 2004). Although OA excretion is considered as a major and widely applicable mechanism for Al tolerance in many plant species, cultivars and lines, this did not explain the variation in Al tolerance between Rikuu-20 and Rikuu-132. In this case, the sensitive cultivar Rikuu-20 excreted more citrate than Rikuu-132, while no difference was observed in malate excretion (Fig. 4.2). This suggests that other Al-tolerance mechanisms may account for the difference in 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). While studying OA exudation in sorghum, maize and soybean, I identified that only maize citrate exudation may partially

be explained for greater Al tolerance (Fig. 6.2). Although citrate exudation for maize also been reported by some other researchers, Pineros et al. (2005) could not find any correspondence of Al tolerance with citrate or malate exudation though they could not suggest the actual mechanism.

To identify the main causes to induce less Al accumulation and less PM permeabilization in Al-tolerant rice cultivars (Fig. 5.4 and 5.5), I investigated the relationship between Al tolerance, PM permeabilization, Al accumulation and lipid composition of root-tip portion as we have already noticed the significance of PM and related characteristics in Al tolerance (Ishikawa and Wagatsuma 1998, Ofei-Manu et al. 2001, Ishikawa et al. 2001, Wagatsuma et al. 2005a, b).

While treatments with sterol metabolism inhibitors especially with uniconazole-P, decreased Al tolerance predominantly of Al-tolerant Rikuu-132, but on the contrary, there were no significant inhibitory effect for Al-sensitive Rikuu-20 (Fig. 5.10A). Although we investigated using the selected two cultivars with both extreme Al tolerances, similar results can be expected based on the similar responses of other two cultivars with both extreme Al tolerances to sterol metabolism inhibitors (Fig. 5.10B). All sterol metabolism inhibitors were found to induce also greater PM permeabilization and Al accumulation only for Al-tolerant Rikuu-132 (Fig. 5.4, 5.5). Except for phospholipids in Al treatment for Rikuu-132, Δ^5-sterols were decreased and conversely phospholipids were increased by all the treatments with Al, inhibitors and Al with inhibitors (Fig. 5.8). However, the causes for the increase in phospholipids after inhibitor treatment was not clear. To my knowledge, this is the first report to show the increase in phospholipids after inhibitor treatment.

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 (negative correlation between Al tolerance and lipid ratio, i.e., $R^2=0.667^*$, Fig. 5.9). Although I 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, and the parent cultivars Kamenoo and Kyoku, which suggested that membrane lipid make-up contributed to higher Al tolerance in Rikuu-132 (Fig. 5.8). After inhibiting Δ^5-sterols synthesis in the tolerant Rikuu-132, the relative ratio of phospholipids in root tip membranes was increased (Fig. 5.9). 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 (DLVO) 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.

The differential response to inhibitors of Δ^5-sterols synthesis in Rikuu-132 suggests that an alternative model may explain differential Al tolerance. Both fenpropimorph and

uniconazole-P enhanced Al sensitivity in Rikuu-132. However, these inhibitors inhibit different enzymes in the Δ^5-sterols synthesis pathway (Fig 5.2). Fenpropimorph inhibits cycloeucalenol obtusifoliol isomerase (COI) as the primary target (Burden et al. 1987; Grandmougin et al. 1989), while uniconazole-P inhibits obtusifoliol-14α-demethylase (OBT 14DM) (Haughan et al. 1988; Rademacher 2000). As a result, each inhibitor produces a different type of abnormal sterols. Fenpropimorph treatment produces 24-methylpollinastanol, 24-dihydrocycloeucalenol, and cycloeucalenol, and uniconazole-P treatment produces obtusifoliol, dihydroobtusifoliol, and 14α-methyl-Δ^8-ergostenol. Because these abnormal sterols have larger van der Waals volumes (Milon et al. 1989), they may increase permeability of the PM (Dahl et al. 1980). Based on computer modeling, abnormal sterols resulting from uniconazole-P have larger van der Waals volume than those resulting from fenpropimorph (see Appendix S1 in Supplementary material). This may account for greater negative impact of uniconazole-P on Al tolerance of Rikuu-132.

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. Our 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.

Fenpropimorph considerably decreases phytosterols with least van der Waals volume (Milon et al. 1989), instead considerably increases in cycloeucalenol, 24-dihydrocycloeucalenol and 24-methylpollinastanol with intermediate van der Waals volume (Grandmougin et al. 1989). On the other hand, (2RS,3RS)-paclobutrazol and uniconazole-P considerably decreases in phytosterols, instead considerably increases in obtusifoliol, dihydroobtusifoliol and 14α-methyl-Δ^8-ergostenol with greatest van der Waals volume (Figs. 5.2 and 5.11). At least a part of the decrease in the summarized amounts of Δ^5-sterols and phospholipids as compared with those in control is considered as the increase in abnormal sterols in PM after Al with inhibitor treatments (Fig. 5.4). Abnormal sterols having 14α-methyl group such as obtusifoliol induces the greatest van der Waals volume (Milon et al. 1989); this induces the greatest flexibility or permeability (Dahl et al. 1980). Abnormal sterols having 14α-methyl group accompanied with a 9β,19-cyclopropane ring such as cycloeucalenol induces the intermediate van der Waals volume; this induces the intermediate flexibility or permeability (Schuler et al. 1991, Cerdon et al. 1996). Phytosterols without 14α-methyl group has the least van der Waals volume; this induces the least flexibility or permeability of the membrane (Dahl et al.

1980, Milon et al. 1989). Differences in Al tolerances after the treatments with paclobutrazol and uniconazole-P in spite of same inhibitory target will be ascribed to the complexity of their stereochemical structures which induce complicated side reactions related to plant hormone (GA), sterols and other plant constituents (Burden et al. 1987, Rahier and Taton 1997, Rademacher 2000).

Significant exponential negative correlation was observed between Al tolerance and the molar ratio of phospholipids/Δ^5-sterols (Y = $81.1e^{-0.88x}$, R^2 = 0.668*) (Fig. 5.9). However, no significant relationship could be found between Al tolerance and phospholipids (R^2 = 0.604[NS]) or Δ^5-sterols (R^2 = 0.548[NS]) (data not shown). From these relationships, it is suggested that the simultaneous status of lipid composition with less phospholipids together with greater Δ^5-sterols is more effective for Al tolerance; the former status will contribute to the less Al binding sites with lipid layers, and the latter status will contribute to the less permeabilization of lipid layers. Even in control treatment phospholipids/Δ^5-sterols (0.41±0.003) was less in Al-tolerant cultivar than that of Al-sensitive cultivar (0.51±0.007). This tendency agreed with Zhang et al. (1996) where phospholipids/Δ^5-sterols of microsomes from root-tips of Al-tolerant wheat was slightly less than that of Al-sensitive one. This indigenous lipid composition will also beneficial for the less permeabilized membrane from the start of Al treatment. Higher glucocerebrosides in Al-tolerant cultivar (Rikuu-132) and considerable decrease in glucocerebrosides after treatment with Al+inhibitor also suggests the significance of the glucocerebrosides in Al tolerance by HPTLC (Fig. 5.5). Although glucocerebrosides with normal fatty acyl chain is reported to be able to contribute to the less permeabilization and higher Al tolerance in *Arabidopsis* (Ryan et al. 2007), greater

contribution of Δ^5-sterols as compared with glucocerebrosides to the less permeabilization is expected because of the greater occupation of Δ^5-sterols within PM relative to glucocerebrosides. Finally, we speculated as follows: after binding Al ions, dispersed phospholipids molecules in the normal PM will be dehydrated and form a partial packing area as a result of salting-out effect based on DLVO theory (Wagatsuma et al. 1995). Less formation of packing prea after dehydration and greater Δ^5-sterols will induce less permeabilization which is more beneficial for greater Al tolerance. The greatest adverse effects of uniconazole-P on PM permeability, Al accumulation and Al tolerance (Figs. 5.4, 5.5 5.10) conclusively suggest OBT 14DM as a promising target for future research on Al tolerance at least in rice. These findings was also supported by the later stage of experiments where several crop species were analyzed for PM permeabilization, Al accumulation and lipid analysis (Figs. 6.2-6.6). These results shows that except for soybean, tolerant and sensitive cultivars or lines within the same crop species showed similar tendency to tolerant and sensitive cultivars of rice, respectively.

The negative site of plasma membrane (PM) from root-tip portion binds aluminum (Al) covalently. This negative charge originated from the phosphate groups of phospholipids and carboxyl groups of the protein in the PM (Nagata and Melchers 1978). Oka et al. (1988) estimated surface negativity of roots of *Vigna mungo* by using a basic fluorescent dye, 9-amino acridine. By using Tb^{3+} phosphorescence Caldwell (1989) demonstrated that PM of Al sensitive what cultivar (Anza) binds Al with a higher affinity than an Al tolerant cultivar (BH 1146). Wagatsuma and Akiba (1989) suggested that Al tolerance increases with the increase of average zeta potential of root protoplast. Wagatsuma et al. (1995) proposed a new technique (PCSM- positively charged silica

microbed) to isolate Al-tolerant protoplast based on DLVO theory and suggested that the areas of PM rich in negatively charged sites are specifically and preferentially susceptible to Al-toxicity. Ishikawa et al (1996) studied comparative response to other trivalent metal ions (e.g. Yb^{3+}, La^{3+}) to the root-tip cells differing in Al tolerance and suggested that Al binds to the negative sites of PM with highest ionic potential and thereafter dehydrated. Wagatsuma et al. (1991) demonstrated that Al tolerance of root-tip protoplasts can be measured by using methylene blue which is a basic dye and can bind with the negative sites of PM and exhibit a blue color and suggested that with the increase of Al-tolerance blue color intensity increases indicating a low surface negativity in these protoplasts.

Phospholipids of PM is the primary site for Al toxicity (Takabatake and Shimmen 1997, Jones and Kochian, 1997). Yermiyahu et al. (1997) suggested that PM surface negativity and Al sorptive capacity probably responsible for some of the sensitivity to Al^{3+}. After binding with Al^{3+} ions, dispersed phospholipid molecules in the normal PM, loose it's hydrated form as a result membrane in a liquid crystal state becomes rigid and gel-like (Hauser and Phillips 1979, Chen et al. 1991, Leshem 1992). The packing area becomes more hydrophobic after this dehydration (Ishikawa and Wagatsuma 1998). This type of decrease in membrane fluidity was also reported for *Thermoplasma acidophilum* for isolated and intact cell membranes using electron paramagnetic resonance spectroscopy (Vierstra and Haug 1978).

Based on the above discussion, a schematic representation of Al-tolerant and Al-sensitive PM of wheat and maize has been presented in Fig. 8.1. As wheat malate exudation on same line has already clarified (Sasaki et al. 2004) and maize citrate exudation was partly connected with Al tolerance, in this figure, PM lipid bilayer with

less PM negativity, less permeability (due to greater amount of Δ^5-sterols) as well as OA exudation has been ascribed for higher Al-tolerance. Schematic representation for Al-tolerance in sorghum, triticale and rice has been presented in Fig. 8.2. For these three crop species, till now, only suggested Al tolerance mechanism is less PM negativity (ascribed as less phospholipids) and less permeability (ascribed as greater amount of Δ^5-sterols) could be suggested as the mechanism of Al tolerance. Future model lipid layer with greater Al tolerance has been represented schematically in Fig. 8.3. It can be suggested that Al-tolerant PM contains greater OA transporter, less phospholipids/Δ^5-sterol ratio and greater sphingolipids (Z-isomer). These PM features may be regulated by the genes which finally determines these special features. Even though I could not suggest specific mechanism of Al tolerance for soybean, but it could be suggested that Al tolerance mechanism in soybean is neither OA exudation nor less lipid ratio. Molecular and genetic analysis is further needed to make crop plant with these special features which confers Al-tolerance.
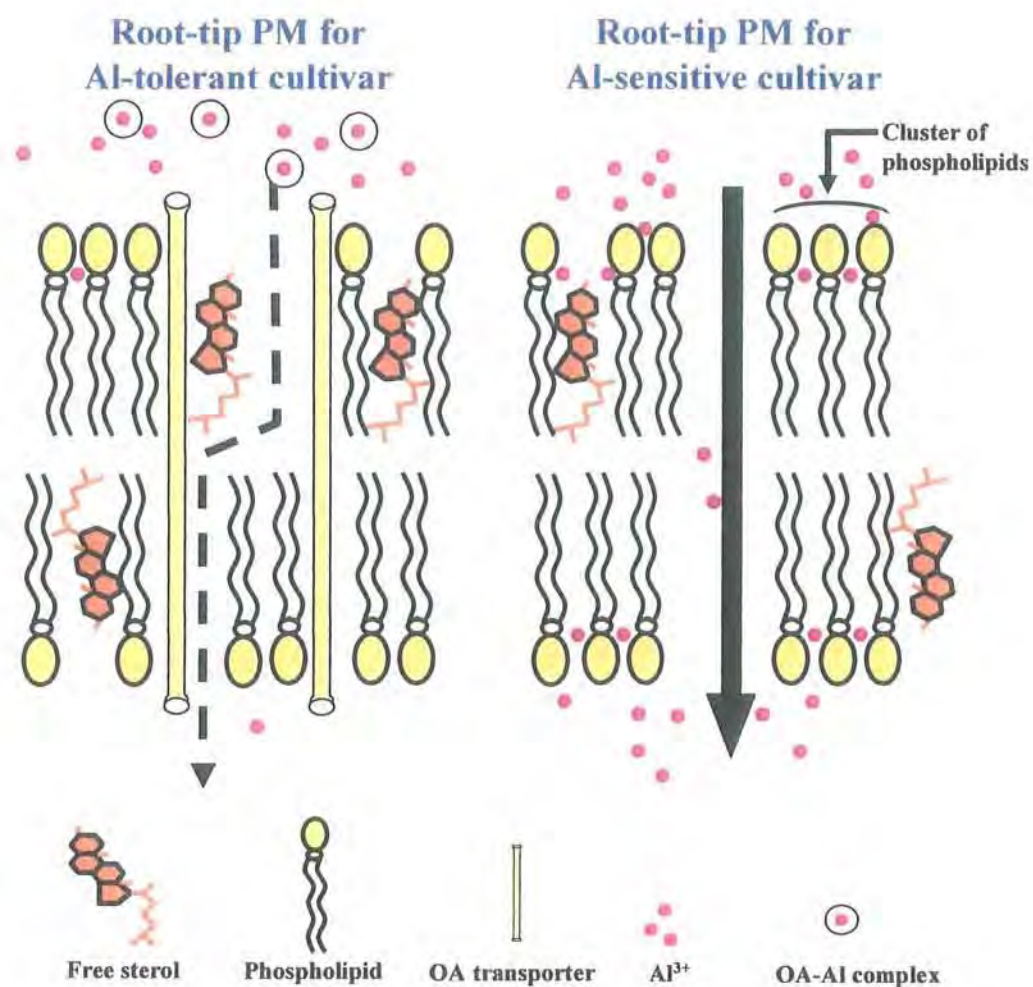
Fig. 8.1. Schematic representation of Al-tolerance strategy by PM lipid bilayer with less PM negativity, less permeability and OA exudation in wheat and maize.

Root-tip PM for Al-tolerant cultivar

Root-tip PM for Al-sensitive cultivar

Cluster of phospholipids

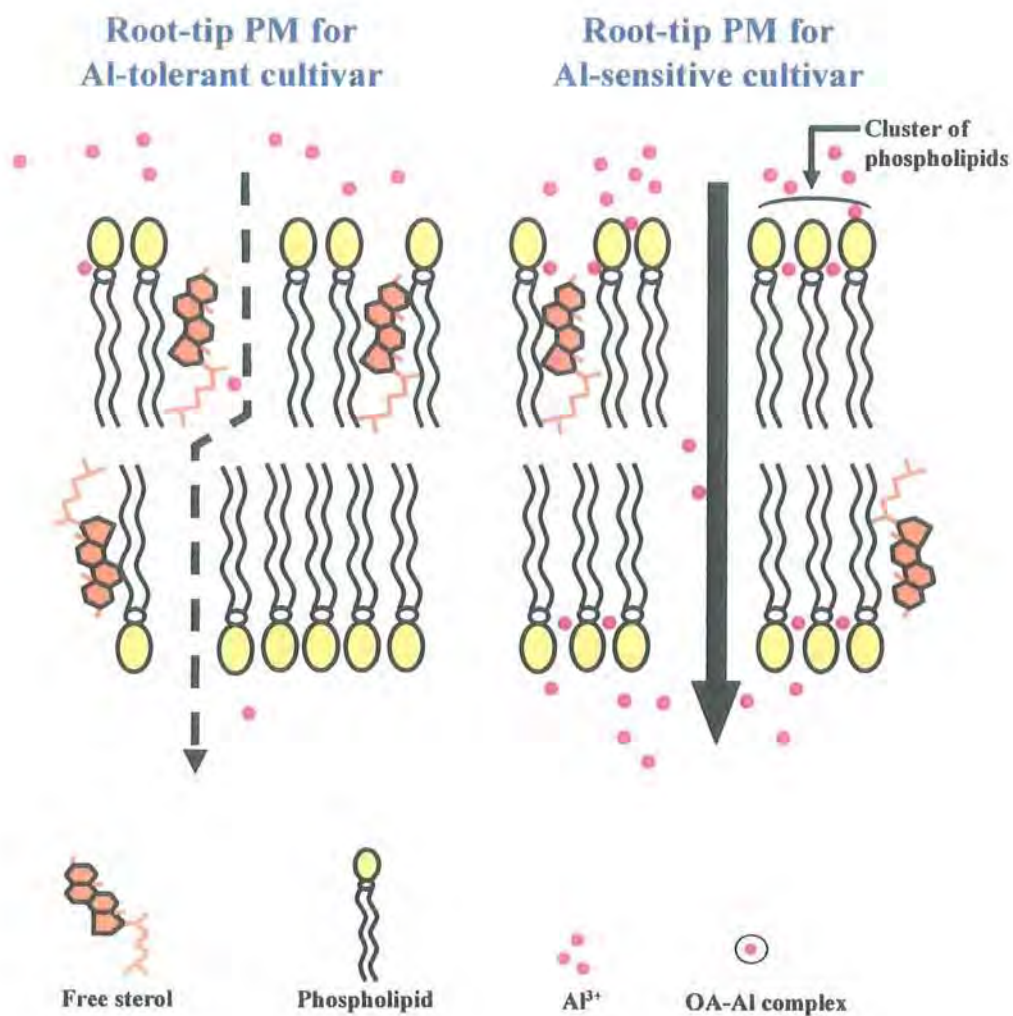Free sterol   Phospholipid   Al³⁺   OA-Al complex

Fig. 8.2. Schematic representation of Al-tolerance strategy by PM lipid bilayer with less PM negativity and less permeability in sorghum, triticale and rice.
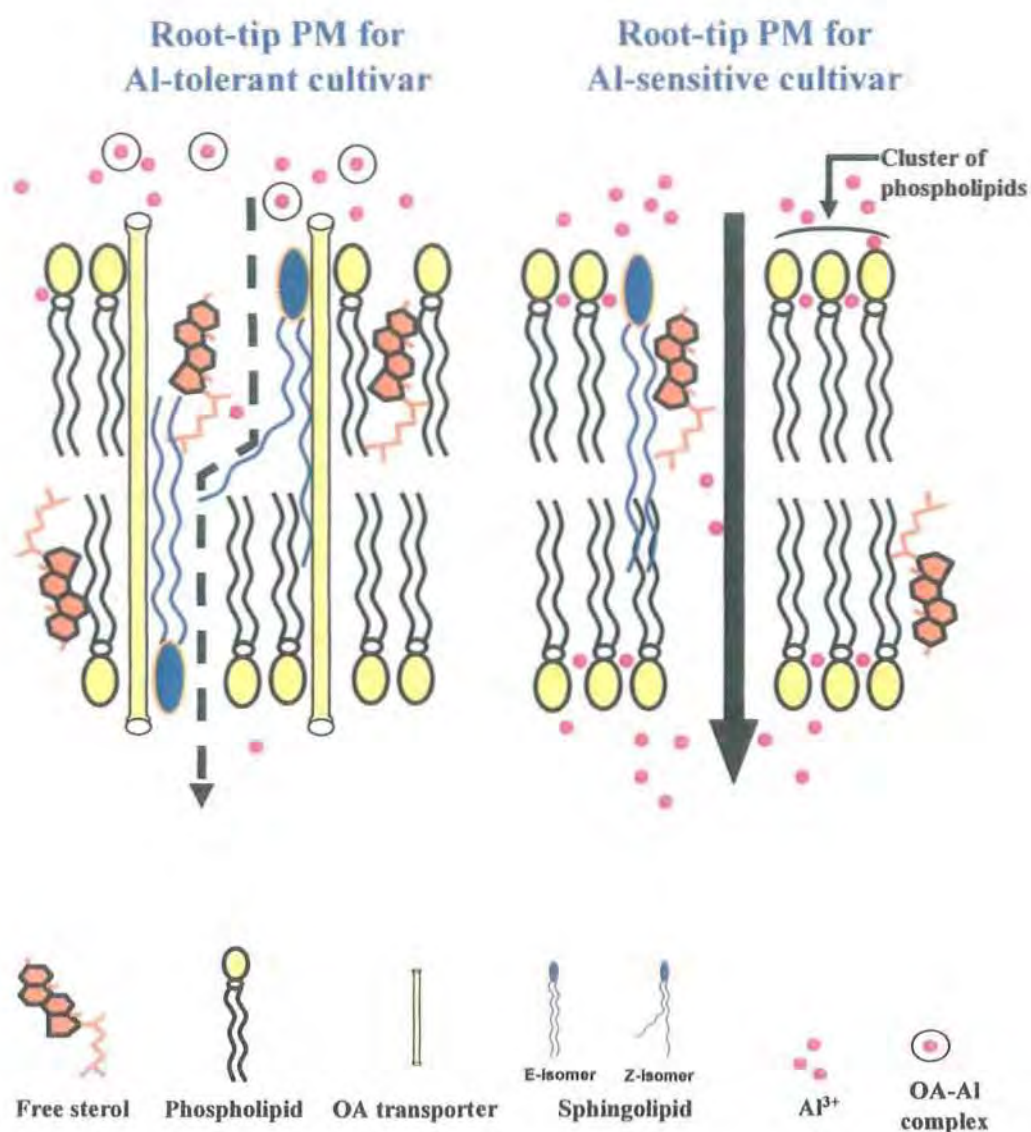
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Fig. 8.3. Schematic representation of Al-tolerant PM to be prepare in the future with less PL, less permeability, greater Z-isomer of sphingolipids and greater OA transporter.

In practical sense, tropical acid soils consists not only the toxic level of Al but there are other major growth limiting factors like low nutrient. After investigation in a combination of high Al and low-nutrient condition for long-term, it was also found that in combined stress conditions (high Al and low-nutrient), both factors simultaneously affect on the growth (Table 1).

In the present study, root Al content showed significant positive correlation with Al tolerance (Figure 7.5A). Clear differential Al content was also found in tolerant and sensitive group of cultivars. In AN nutrient condition Al tolerance increases with the decrease in root Al connect which was also reported by several researchers (Wagatsuma et al. 1991, Ofei-Manu et al. 2001, Pineros et al. 2005).

Although, Al tolerance is related mainly with the Al status in the plant in any nutrient condition, in the present experiment while using only rice crop for Al tolerance study, Al toxicity was not found as a main factor in LN condition (Figure 7.5B, Table 7.1). In other words, contribution of low-nutrient tolerance is fur greater than that of Al tolerance. After finding this truth, I searched the nutritional reasons for low-nutrient tolerance. I could find that, low-nutrient tolerance is mainly controlled by Ca status in plant and can be ascribed as the main factor for better rice growing (Figure 7.6). Other coexisting factors may also have important relations. Further, Ca showed significant relation positively with Mg and Fe in the shoot (data not shown) indicating greater influence of Ca for other nutrient also.

As more important factor for plant growth in acid soils, shoot Ca content was found to be ascribed for combined tolerance (Figure 7.6). Further Ca showed significant positive correlation with Mg and Fe content (data not shown) which indicate that uptake

of these two nutrients also regulated by uptake of Ca. Wenzl et al. (2003) found that in the tropical sub humid savannas having highly weathered acid soil, the susceptible crop plant growth is reduced not only for Al concentration, soil pH but also exchangeable Ca. In the present study, it was observed that Ca translocation from root to shoot was greater in reasonably in combined tolerant cultivar (Rikuu-132) (Fig. 7.7). On the other hand, least Ca translocation was observed in Al tolerant but combined sensitive Sasanishiki. Among the selected typical cultivars, BR34 was Al sensitive but combined tolerant which shows intermediate type of Ca translocation. The order of Ca translocation follows the same order of combined tolerance but not the Al tolerance (Figs. 7.4 and 7.7).

Based on the results, it was concluded that both factor, Al-tolerance and low-nutrient tolerance should simultaneously be considered to solve acid soil problems in the tropics but greater emphasis should be given to low-nutrient tolerance.

Summary

Summary

Summary in English

Aluminum (Al) tolerance mechanisms have been reported by many researchers for different crop plant species. Organic acid (OA) anion exudation has been clarified as main Al-tolerance mechanism in wheat, maize, buckwheat, soybean and some other crop plant species. On the other hand, it was also suggested that only one mechanism is not enough to explain Al tolerance even in one crop plant species. Rice, one of the primary crop plant species in the world, is famous for Al-tolerant crop plant species, however its tolerance mechanism is almost unknown. In the first stage, Al tolerance mechanism was investigated with special interest in plasma membrane (PM) lipid layer using rice root-tips (1 cm). Thereafter, Al tolerance mechanism for other crop plant species was investigated whether the same tolerance mechanism is working or not.

In the first stage of experiments, investigations were carried out to determine the Al tolerance mechanism in rice (*Oryza sativa* L.) using 23 Bangladeshi (*Indica*) and 6 Japanese (*Japonica*) cultivars. Al tolerance was screened with 20 μM $AlCl_3$ in 0.2 mM $CaCl_2$ solution (pH 4.9) for 24h. Of the Bangladeshi cultivars, BRRIdhan41 and Rahmat were found to be most Al-tolerant, and Moyna and BRRIdhan34 to be most Al-sensitive cultivars. Of the Japanese cultivars, Sasanishiki and Domannaka were found to be both extremes of tolerant and sensitive cultivars, respectively. Hematoxylin staining of root-tip portions and root-tip sections from Al-tolerant cv. Sasanishiki and cv. BRRIdhan41, and Al-sensitive cv. Domannaka and cv. BRRIdhan34 indicated that rice possesses an Al exclusion mechanism. Staining with fluorescein diacetate-propidium iodide (FDA-PI)

indicated that PM permeability of Al-tolerant rice cultivars remains almost intact, whereas even after 1-h Al treatment, the PM of sensitive rice cultivars becomes permeable. These results suggest that PM permeability is the key factor in early stage of Al tolerance of rice.

Among many rice cultivars ever screened, Sasanishiki was found to be one of the most tolerant cultivar to high concentration of Al in medium. However, wide variation of Al tolerance was found in almost all pedigree cultivars of Sasanishiki. I selected and investigated further using mainly two cultivars with both extreme tolerances to high Al. The cultivar Rikuu-20 was Al sensitive, whereas a closely related cultivar that is a descendant of Rikuu-20, Rikuu-132, was Al tolerant. The sensitive cultivar Rikuu-20 showed increased permeability of PM within 1 h of Al treatment. Furthermore, greater Al accumulation was observed in the root-tip portion of sensitive Rikuu-20. Differential Al tolerance and Al uptake could not be explained by the difference in the release capability of malate and citrate as sensitive cultivar secreted more malate and citrate than tolerant cultivar even after Al treatment. Lipid composition of the PM differed between these cultivars. Sensitive cultivar contains more phospholipids and less Δ^5-sterols than tolerant cultivars in control and Al treatment. In general, phospholipids content increased and Δ^5-sterols decreased after Al treatment compared to that in control irrespective of their Al tolerance. After inclusion of sterol metabolism inhibitors with Al, greatest increase of phospholipids was observed in the Al+uniconazole treatment for sensitive cultivar's root-tip compared to control and Al treatment. Conversely, greater decrease of Δ^5-sterols was observed in Al+uniconazole treatment for tolerant Rikuu-132. Also, the tolerant cultivar Rikuu-132 had a lower ratio of phospholipids to Δ^5-sterols than the sensitive cultivar

Rikuu-20, suggesting that the PM of Rikuu-132 is less negatively charged and less permeabilized than that of Rikuu-20. I used inhibitors of Δ^5-sterol synthesis (uniconazole-P, an inhibitor of obtusifoliol-14α-demethylase [OBT 14DM], and fenpropimorph, an inhibitor of cycloeucalenol obtusifoliol isomerase) to lower the content of Δ^5-sterols in both cultivar. Rikuu-132 showed a similar level of Al sensitivity when the ratio of phospholipids to Δ^5-sterols was increased to match that of Rikuu-20 after treatment with uniconazole-P. This inhibitor reduced Al tolerance in Rikuu-132 and its Al-tolerant ancestor cultivars Kamenoo and Kyoku to the same level of Al tolerance for Al-sensitive Rikuu-20 and Aikoku. Al tolerance was negatively correlated with the ratio of phospholipids to Δ^5-sterols in root-tip portions of both cultivars in the existence of Al and inhibitors. This indicates that greater contribution of this lipid ratio as phospholipids makes more sensitive (negative impact) and Δ^5-sterols makes intact (positive effect) on PM permeabilization which offers tolerance to Al. Differentially induced permeabilizations could be discussed based on van der Waals conformational differences in phytosterols (stigmasterol) and abnormal sterols (cycloeucalenol and obtusifoliol) synthesized greater after the treatment with inhibitors. This is the first investigation which suggests the significant roles of relative abundance of Δ^5-sterols within PM and of OBT 14DM in Al tolerance of rice.

In the second stage of experiments, further investigations were carried out using other crop plant species to clarify whether the same Al tolerance mechanism is working or not. I used the Al-tolerant and Al-sensitive cultivars or lines of sorghum (Super sugar and Kaneko, respectively), wheat (ET8 and ES8, respectively), triticale (ST22 and ST2, respectively), maize (Golddent KD520 and Golddent KD500, respectively) and soybean

(Enrei and Ryokuheki, respectively) for these experiments. Among the crop species studied, only maize showed greater citrate exudation for Al-tolerant cultivar in Al treatment. For wheat, malate exudation has been reported as one of the Al-tolerance mechanism using same lines. As a matter, I could conclude that greater OA exudation is partially connected with Al-tolerance for wheat and maize only. On the other hand, greater Al accumulation and PM permeabilization were also observed in the Al-sensitive cultivars of all the crop plant species studied. Greater Δ^5-sterol and less phospholipids content were also found in tolerant cultivars or lines for all the crop species except for soybean, although this tendency did not show any trend among different crop plant species. Additionally, it was observed that Al treatment increased phospholipids and decreased Δ^5-sterol for all the crop plant species. This result surely shows the existence of the different mechanism for Al tolerance additionally to the mechanism on OA release for maize and wheat. No exceptions have been observed yet in monocot plants on the greater lipid ratio for Al-tolerance.

In practical sense, major growth limiting factors in tropical acid soils are not only the toxic level of Al but also low nutrients. After investigation under the combination of high Al and low-nutrient conditions in long-term, it was found that Al tolerance in full nutrient condition is greater than that of low-nutrient conditions indicating simultaneous effect of toxic Al and low-nutrient. Though Al concentration in roots can explain Al tolerance, however, it can not explain combined tolerance (high Al and low nutrient tolerance) or low nutrient tolerance. From the above results, it was suggested that contribution of low-nutrient tolerance is greater than that of Al tolerance for rice. Transport capability of Ca to shoots was suggested as an important component for low-nutrient tolerance in rice.

In conclusion, I demonstrated for the first time the significant role of plasma membrane lipid layer (especially, sterol molecule) in Al tolerance and suggested also the significant role of transport capability for Ca to rice shoot in better growth on tropical acid soils.

Summary in Japanese

要　約

アルミニウム（Al）耐性機構は種々の植物で多数報告されている。有機酸放出はコムギ、トウモロコシ、ソバ、ダイズなどの植物での主要なAl耐性機構とされている。しかしながら、Al耐性機構は単一でないことも解っている。イネは重要作物であり、Al耐性作物としても有名であるが、その耐性機構は不明である。そこで、まず最初にイネを用い、根端細胞膜（PM）脂質層に注目してAl耐性機構を、ついで、イネ以外の植物での同様の機構の関与を調査した。

　バングラデシュのイネ２３品種と日本のイネ６品種のAl耐性を調査した。その結果、Al耐性は BRRIdhan41、Rhamat が最強で、Moyna、BRRIdhan34 が最弱であり、日本のイネではササニシキが最強で、どまんなかは最弱であった。根端のヘマトキシリン染色と FDA-PI 蛍光染色結果から、Al耐性品種はAl排除機構が共通して認められ、また、PM透過性はAl存在下でも増大しにくいことが明らかとなった。

　Al耐性の強いササニシキのほぼ全部の系統品種に該当する１８品種に関してAl耐性を調査した結果、広範な耐性差を認めた。それらのなかで、陸羽２０号は著しく感受性なのに対し、その直系の子孫である陸羽１３２号は著しく耐性であった。これら両品種間にも上記のAl耐性機構の違いが認められたが、根端からのリンゴ酸やクエン酸放出能には差が認められず、有機酸放出機構でAl耐性を説明できなかった。

　根端のリン脂質/Δ^5-ステロール（PL／S）比はAl耐性品種の陸羽１３２号で小さかった。ウニコナゾールPは、obutusifoliol-14α-demethylase（OBT 14DM）の阻害剤であり、通常は極く微量しか含まれていない obutusifoliol などの含量を増大させ、他方最終生成物であるSの含量を低下させることが既に解っている。また、フェンプロピモーフは、

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cycloeucalenol obutusifoliol isomerase (COI) の阻害剤であり、同様に cycloeucalenol などを増やし、他方Sを減らす。これら二つの阻害剤処理で、陸羽１３２号根端部のS含量は低下し、他方ＰＬ含量は増加し、その結果 PL/S 比、Ａ１含有率、膜透過性のいずれもが増大し、Ａ１耐性は低下した。ステロール合成阻害剤は根端細胞膜脂質層中のステロール含量を低下させることによって、膜の負荷電性を高め、その結果Ａ１の膜脂質層への結合能を高め、同時に abnormal ステロール量の増大により膜の透過性を高めるため、Ａ１耐性を低下させると解釈された。ステロール合成阻害剤とＡ１の同時処理のデータを総合した結果、PL/S 比とＡ１耐性の間に負の相関が認められた。また、ウニコナゾールＰ処理でＡ１感受性品種である陸羽２０号、愛国のＡ１耐性値は変わらないのに対し、Ａ１耐性品種である亀の尾、旭、ササニシキのＡ１耐性値は、感受性品種の値にまで低下した。以上の結果、イネのＡ１耐性におけるＰＭ中のΔ^5-ステロールと、OBT 14DM の重要な役割が示唆された。本研究は、Ａ１耐性における根端細胞膜脂質の重要な意義を示した最初のものである。

つぎに、このイネのＡ１耐性機構が他の植物でも関与しているのかを調査した。まず、Ａ１耐性の最強（T）、最弱（S）をあらかじめ選抜した。すなわち、ソルゴーではカネコ・ハイブリッド（T）とスーパーシュガー（S）、コムギでは ET8 (T) と ES (8)、ライコムギではＳＴ（２）とＳＴ（２２）、トウモロコシではＫＤ５２０(T)とＫＤ８５０(S)を用いた。その結果、Ａ１耐性品種間差と根端のＡ１集積性、膜透過性、ＰＬ／Ｓ比の品種間差の間に、イネと同様の傾向を認めた。しかしながら、ダイズ品種間では、この機構の関与が認められなかった。

最後に、典型的酸性土壌である熱帯酸性土壌での作物生育支配要因を検討した。実際のこれら酸性土壌に似せたＡ１と養分の濃度に各種組み合わせ、多くのイネ品種を長期間水耕栽培し、生育量と体内養分組成を調査した。その結果、Ａ１耐性植物であるイネでは、低養分耐性が生育をより大きく支配し、カルシウム（Ｃａ）の茎葉部への輸送能力が品種間生育差の大きな要因であることを示唆する結果を得た。

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