CLARIFICATION OF ALUMINUM TOLERANCE MECHANISMS WITH SPECIAL INTEREST IN THE PLASMA MEMBRANE LIPID LAYER OF ROOT-TIP PORTION MAINLY OF RICE

（主にイネの根端細胞膜脂質層に注目したアルミニウム耐性機構の解明）

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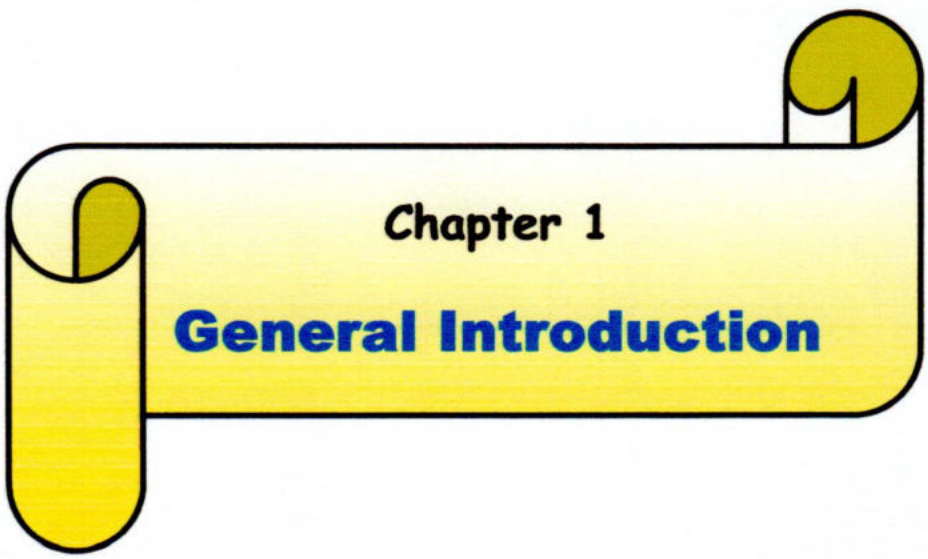

Chapter 1
General Introduction

Chapter 1

General Introduction

1.1 Plant growth retardation in acid soil

Crop productivity on acid soils is restricted by multiple abiotic stress factors. Among those aluminum (Al) would be the most important growth-limiting factors for most of the acid soils. Several wild and crop plant species can exhibit a higher tolerance to this toxic element though this tolerance varies widely between the crop or plant species even within the cultivars and lines in one species (Wagatsuma et al. 2005a).

Roots injured by high aluminum are become stubby and thick, dark colored, brittle, poorly branched and rubberized with a reduced root length and volume (Nguyen et al. 2001). Shoot is also inhibited due to limiting supply of water and nutrients. Al toxicity caused Ca deficiency or reduced Ca transport within the plant by curling or rolling of young leaves, inhibited growth of lateral branches or a collapse of growing points or petioles. Young seedlings are affected more than older plants (Thaworuwong and van Diest, 1975). But plant growth retardation in acid soil occurs not only by toxic elements but also by low pH. Moreover, low availability of nutrients such as Ca, Mg, K and Mo are reported by several researchers in the naturally occurring acid soils.

1.2 Mechanisms of Al tolerances

There are various aluminum (Al) tolerance mechanisms in plants, such as Al exclusion and internal Al tolerance mechanisms (Kochian et al. 2004, Poschenrieder et al. 2008). Taylor (1991) also categorized the proposed Al tolerance mechanisms into two

groups as A) exclusion of Al from the root apex (secretion of Al-chelating ligands, binding of Al with the cell wall and mucilage, plant-induced pH barrier in the rhizosphere or root apoplasm, selective permeability of the plasma membrane and Al efflux) and B) internal tolerance when Al enters the plant symplasm (Al-chelating in the cytosol, compartmentation in the vacuole, Al-binding by protein, and elevated enzyme activity).

1.2.1 Organic acid (OA) anion exudation mechanism

Organic acid (OA) (e.g. citrate, malate and oxalate) anion exudation has widely been accepted by several related researchers as key Al tolerance mechanism. The Al-dependent stimulation of organic acid efflux from roots has now been reported in more than ten species, and this response has been associated with an increase in Al resistance (Yang et al. 2005). However, plant or crop species are existing which organic acid exudation does not correlate with Al tolerance. Irrelavance of organic acid exudation also has been reported for signalgrass (*Brachiaria decumbens*) (Wenzl et al. 2001), maize (Piñeros et al. 2005), triticale (Wagatsuma et al. 2005b) and oat (*Avena sativa*) (Zheng et al. 1998a) and also for rice (Khan et al. 2009, Ma et al. 2002). Although several Al tolerance mechanisms have been reported, detailed information on Al tolerance mechanisms are limited except for organic acid anion (OA) exudation mechanism (Ma et al. 2001, Kochian et al. 2004, Hoekenga et al. 2006). Sasaki et al. (2004) identified aluminum induced malate transporter (ALMT) in the root cells of tolerant wheat (ET8) which was less abundant in Al sensitive ES8. On the other hand, Delhaize et al. (2004) successfully made Al tolerant plant by transferring ALMT from *Arabidopsis* to barley plant by genetically engineering.

1.2.2 Al tolerance mechanisms other than OA exudation

There are also other reports that do not support the hypotheis that organic acids efflux enhances Al resistance of plants (Ishikawa et al. 2000, Parker and Pedler 1998, Wenzl et al. 2001). There are also evidences to the existence of Al tolerance mechanisms other than OA anion exudation in many crops e.g. *Cassia tora* (Ishikawa et al. 2000), *Brachiaria decumbens* (Wenzl et al. 2001), Pea (Kobayashi et al. 2004) and buckwheat (Zheng et al. 2005). Zheng et al. (2005) also found irrelevance between Al tolerance and oxalate efflux in seven cultivars of buckwheat and suggested that oxalate efflux plays only a minor role in high Al tolerance of that species. Piñeros et al. (2005) also found no correlation between differential Al resistance and root citrate exudation in maize and suggested that root organic acid release may play a role in maize Al resistance in some extent, but it is clearly not the only or the main resistance mechanism operating in maize root system. They further tested a number of other potential Al-resistance mechanisms including release of other Al-chelating ligands, Al-induced alkalinization of rhizosphere pH, changes in internal levels of Al-chelating compounds in the root, and Al translocation to the shoot and found no correlation of Al tolerance with these mechanisms. Therefore, the role of organic acid secretion in Al resistance should not be overemphasized, as alternative mechanisms may play an equal or even more important role in some plants (Yang et al. 2005).

1.2.3 Molecular or genetic studies regarding Al tolerance

Zhang et al. (2007) identified 37 genes using two constricting (differential response to Al) in rice cultivars by gene transcriptional responses to Al. Among these genes, five

have been previously known as Al regulated previously while the others are novel genes. Among the up-regulated genes, four encode ion transporters, two are involved in signal transduction, and five in the synthesis of cysteine and metallothionein. They suggested that these could be members that are potentially involved in Al adaptation or resistance. Furthermore, they studied transcription of 17 genes and found strong inhibition under Al stress. These genes are associated with cytoskeletal dynamics and metabolism, and could be possible targets associated with Al toxicity. In *Arabidopsis*, a homologue of the wheat malate transporter (TaALMT1; Sasaki et al. 2004) AtALMT1 (Hoekenga et al. 2006) and a possible Al translocator, ALS3 (Larsen et al. 2005) were identifies as critical Al tolerance genes. Recent mutant and QTL (quantitative trait loci) studies indicate that multiple factors can regulate Al tolerance within a plant species (Kobayashi et al. 2005).

1.2.4 Al tolerance mechanism in connection with plasma membrane lipid composition

Wagatsuma et al. (1991) reported lower zeta potential of protoplasts from Al tolerant plant species. Plasma membrane (PM) negativity is the main reason of Al tolerance and in addition to this, PM intactness is also an important factor to regulate the entrance of Al in to the cytoplasm. Further, Wagatsuma et al. (2005a) reported that PM lipid is more powerful strategy than OA anion release for initial stage of Al tolerance in triticale suggesting that sterols and glucocerebroside play vital role to make stronger PM.

PM lipids are the primary site for Al-toxicity due to activity of several kinds of soluble and membrane-bound enzymes in this region (Jones and Kochian 1997). Ishikawa et al. (2001) investigated Al tolerance mechanisms in the cultivars of five plant species

and suggested that PM is the primary factor to influence the Al-tolerance and it can be regulated by maintaining PM flexibility. Al rhizotoxicity may be related to a disruption of membrane function, probably due to changes in the structure and function of the root-cell plasmalemma (Zhao et al. 1987). PM of the root-apex cells seems to be a major target of Al toxicity (Mossor-Pietraszewska 2001). It (Al) also can bind to membrane proteins and lipids (Campbell et al. 1994; Ishikawa and Wagatsuma 1998) and finally reduces membrane integrity (Foy and Fleming 1982). PM lipid layer regulates not only the influx and efflux of nutrients but also influx of toxic cations like Al.

Phospholipids, glycolipids, and sterols generally make up the biological membranes. Other lipids (relatively small quantities) play crucial roles in electron carriers, hydrophobic anchors, intracellular messengers etc. (Lehninger et al. 1993). Al binds to the negative sites of the phosphate group of phospholipids, makes the membrane rigid and gel like and finally PM becomes permeable (Leshem 1992). Fatty acid compositions are independent among the plant species, cultivars or lines and that composition controls the fluidity of membranes. Increase in short fatty acid chains and unsaturated fatty acids causes increase in PM fluidity and decreased by saturated long fatty acid chains. This loss of water molecule alters the PM fluidity (Ishikawa and Wagatsuma 1998) thus makes the permeable PM. Higher phospholipids content in the PM resulted the binding much Al^{3+} and finally makes greater permeabilized area.

Role of sterols in the PM is of particular interest as these are essential component of biological membrane and play an integral role in PM organization, dynamics and function as well as in the structural integrity of the lipid bilayer. In the plant PM there are at least 4 major sterols, cholesterol, campesterol, sitostero and stigmasterol (Larsson

1992). Although sterols are non-polar and do not bind with Al but small difference in sterol structure can markedly differ in membrane properties especially on membrane integrity (Henriksen et al. 2004). Free sterols in the PM contribute to fluidity and permeability and also participated in the control of membrane-associated processes (Umebayashi and Nakano 2003). Change in sterol composition have been reported to alter the sensitivity to certain drugs in yeast cells (Zweytick et al. 2000). Sterol/phospholipids molar ratio was considered to regulate the membrane fluidity in yeast (Sharma and Dietz 2006).

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.

The make-up of PM lipids would also affect physical and structural properties of the PM, which would affect fluidity and integrity (Shinitzky, 1984). 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, overexpression of the enzyme might modify the structure of sphingolipids and stabilize the PM structure (i.e. preventing membrane leakiness). 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.

1.2.5 Al tolerance mechanisms for rice

Rice is the most important crop in South Asian countries where population density is so high and shortage of food occurs so often. In rice, OA release for Al tolerance was less significant (Ma et al. 2005, Yang et al. 2008, Khan et al. 2009), suggesting that other mechanisms underlie differences in Al tolerance between cultivars. Yang et al. (2008) recently suggested that the formation of cell was methylesterified pectins would provide the exclusion of Al from the root apex. Ma et al. (2005) identified Al sensitive mutant for wild type rice.

In previous screening studies, we found several Al-tolerant rice cultivars among the Japonica and Indica cultivars, mainly from Bangladesh (Khan et al. 2005). This result suggested that Japonica germplasms would be a useful genetic source for breeding of Al-

tolerant rice cultivars. However, we also found several Al-sensitive cultivars among Japonica germplasms (Khan et al. 2005). Many cultivars among the same family line are available for some Japanese major rice cultivars. These may be useful for molecular genetics studies to identify key genes regulating Al tolerance, if Al tolerance is segregated among the same family line. Another approach is the use of various pharmaceuticals that alter sterol content. Such methods are well developed, and have been used widely e.g., in studies on gibberellin biosynthesis (Rademacher 2000) and fungicidal function (Benveniste 2004). In this study, I screened Al tolerance among well characterized cultivars in the family line of Japonica rice, and investigated Al tolerance with respect to PM lipid composition. Both analyses of the PM lipids and pharmaceutical experiments using inhibitors of sterol biosynthesis indicated that PM lipid composition plays an important role in Al tolerance in rice.

Rice is the main food as carbohydrate source especially of Asian people and is known as Al-tolerant crop species among small grain cereals (Foy 1988). Also, there are wide variation of Al tolerances among many Japonica and Indica rice cultivars (Khan et al. 2005). Although several Al tolerance mechanisms have been reported, detailed information on Al tolerance mechanisms are limited except for organic acid anion (OA) exudation mechanism (Ma et al. 2001, Kochian et al. 2004, Hoekenga et al. 2006). Rice has been reported to secrete citrate and malate with Al induction, however the secreted OA possessed less significance for Al tolerance (Ishikawa et al. 2000, Ma et al. 2002, Yang et al. 2008). As the alternative mechanism for Al tolerance of rice, Yang et al. (2008) reported the important role of cell wall pectins through excluding Al from the root apex.

Rice is known as an Al-tolerant crop (Ishikawa et al. 2000) although its tolerance is widely different among cultivars and the mechanism of Al tolerance in rice is still to be clarified. However, organic acid secretion from roots is not a primary mechanism for Al tolerance in rice (Ishikawa et al. 2000, Ma et al. 2002). In this study, rice was selected to study Al tolerance mechanism in detail. Further, to know whether the newly found mechanism is specific to rice or not, selected cultivars or lines of sorghum, wheat, triticale, maize, and soybean were used.

1.3 Mechanism of the tolerance to high Al under low fertility

In addition to Al toxicity in acid-soils, low nutrient concentration is also a major accompanying predicament. Al can inhibit uptake the 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. Blockage of K (Gassmann and Schroeder 1994) and Ca (Huang et al. 1993) channels in wheat root cells reportedly affected by Al. 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 in Al tolerance of rice in acid soils. Phosphorus deficiency is a major yield limiting factor in acid alfisols, oxisols, ultisols, and andepts (Clark 1984). In spite of considering true acid soil conditions in tropics, (i.e., high Al with low nutrient stress) Al research popularly carried out in high nutrient conditions. Wenzl et al. (2003) reported using *Brachiaria* spp. (*B. decumbens* and *B. ruziziensis*) that Al tolerance in low nutrient condition can only be mimicked to actual acid soils. Therefore, clarification of each stress

condition is needed to differentiate Al toxicity with other stress factors occurring in true acid soil.

1.4 New aspect of Al tolerance for tropical acid soils

Methylene blue stainability of root-tip protoplasts was negatively correlated with Al tolerance among 18 different plant samples (species, cultivars and lines), suggesting the common importance of permeation characteristics of plasma membrane (PM) in addition to PM negativity for Al tolerance (Wagatsuma et al. 2005a). We proposed as an important topic for future studies the negativity and permeation of PM for clarification of Al tolerance mechanism. In the present paper, we investigated the differential composition of phospholipids and Δ^5-sterols in connection with differential Al tolerances between rice cultivars using sterol metabolism inhibitors. This study conclusively suggested for the first time the significant role of obtusifoliol-14α-demethylase in Al tolerance of rice. Further, phospholipids and Δ^5-sterols composition in several crop species were studied and recognized similar tendency for those crops.

1.5 Objectives

1. To know the role of plasma membrane (PM) lipid in Al tolerance mechanism in rice.
2. To know the PM lipid composition conferring Al tolerance in rice by changing the PM lipid status pharmaceutically.
3. To know whether PM lipid compositional mechanism for Al tolerance is underlie within several crop species or not.

4. Most acid soil in the tropics and subtropics are lack of availability of essential nutrients. Therefore, it was my intention to know the tolerance mechanism in high Al and low nutrient condition as in nature, acid soil generally lack of adequate nutrient for crop production.
5. To know the mineral absorption characteristics of rice cultivars in high Al and low fertility condition.
6. To know the determining factor or mineral for Al and/or low nutrient tolerance.

Chapter 2

Selection of representative rice cultivars by short-term Al tolerance screening

Chapter 2

Selection of representative rice cultivars by short-term Al tolerance screening

2.1 Introduction

The major symptom of Al toxicity is a rapid inhibition of root growth (Zhang et al. 2007). Al inhibits root cell expansion and elongation and, if over the long term, cell division as well. Al can inhibit cytoskeletal dynamics, and interacts with both microtubules and actin filaments (Sivaguru et al. 1999, 2003). This growth inhibition of root further cause reduced plant vigor and yield (Rengel 1992, Kochian et al. 2005). Toxicity symptoms of Al are similar to nutrient deficiencies (Bennet et al. 1986, Taylor 1988) though these general symptoms appear to be the consequence of inhibition of root development caused by targeted action of Al at root tips (Ryan et al. 1993). Visible symptoms of Al toxicity include inhibition of root growth (Delhaize and Ryan 1995), swelling of the root tip, and/or sloughing off the epidermis, plasma membrane depolarization, alteration of Ca^{2+} fluxes at the root-tip, stimulation of callose deposition (Schreiner et al. 1994, Zhang et al. 1994), and induction of rigor in the actin cytoskeleton (Grabski and Schindler 1995).

There are complexities to definite identification of Al toxicity, however, the initial and most dramatic symptom of Al toxicity would be the inhibition of root elongation as a consequence of toxicity to the root apex (Kochian 1995). Delhaize and Ryan (1995) also revealed that a typical symptom of Al toxicity in plants is the inhibition of root elongation, and this has become a widely accepted measures of Al sensitivity. In general,

sensitive plants exhibit inhibition of root elongation after approximately 0.5 to 2h of exposure to 1–10mM of Al (Barceló and Poschenrieder 2002, Wenzl et al. 2001).

Al exclusion mechanism has already been reported in several crop species. Ma et al. (2005) reported Al exclusion mechanism in Al-tolerant rice cv. Koshihiari comparing to Al-sensitive rice Kasalath. Ishikawa and Wagatsuma (1998) also reported exclusion in rice, maize and pea.

To know the intactness of the PM followed by Al treatment, FDA-PI technique is widely been used by researchers. Ishikawa and Wagatsuma (1998) also reported greater permeabilization in the Al-sensitive cultivars of rice, maize and pea. Wagatsuma et al. (2005a) showed that Al-tolerant triticale line ST22 posses more intact PM after Al treatment whereas Al-sensitive line ST2 became permeabilized ascribed as the more red fluorescence from the roots.

To study the mechanism(s) of Al tolerance in rice, selection of extreme tolerant and sensitive cultivars would be contributive. In this context, study on Al tolerance screening of Bangladeshi and Japanese rice cultivars has been conducted to select extreme tolerant and sensitive rice cultivars.

2.2 Materials and Methods

2.2.1 Source of seeds

Seeds of *Indica* type Bangladesh rice (*Oryza sativa* L.) cultivars (Chandina, Mala, Biplob, Dulabhog, Brribalam, Asha, Shufola, Mukta, Moyna, Gazi, Shahjalal, Niamot, Kiron, Rahmat, Noya Pajam, BRRIdhan27, BRRIdhan28, BRRIdhan29, BRRIdhan34, BRRIdhan36, BRRIdhan37, BRRIdhan39, BRRIdhan41) were collected from the

Bangladesh Rice Research Institute, Gazipur, Bangladesh. Seeds of *Japonica* type Japanese rice cultivars (Akitakumachi, Domannaka, Haenuki, Koshihikari, Hitomebore, Sasanishiki) were collected from Kanto Seed Co. Ltd., Japan.

2.2.2 Growth conditions

Seeds of rice were soaked with tap water for 24h 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 $cd \cdot m^2 \cdot m^{-4}$ (klux). 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 25°C under aeration.

2.2.3 Al treatment

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

To search early effect of Al, 1h Al treatment was carried in the following way. Seedlings were primarily conditioned with 0.2mM $CaCl_2$ at pH 4.9 for 5h. Seedlings were then subjected to pretreatments with or without Al (20μM $AlCl_3$) containing 0.2mM $CaCl_2$ at pH 4.9 for 1h. After rinsing of roots with deionized water, roots of seedlings

were re-elongated in Al free medium (0.2mM $CaCl_2$) for 12h. Al tolerance was calculated as follows:

Al tolerance in 24h of Al treatment (%) =

$$\frac{\text{Root elongation in Al treatment during 24h (cm)}}{\text{Root elongation in control treatment during 24h (cm)}} \times 100$$

Al tolerance in 1h of Al treatment from the start of 1h Al treatment until the finishing of 12h of re-elongation period = $\frac{\text{Root elongation in Al treatment (cm)}}{\text{Root elongation in control without Al (cm)}} \times 100$

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

2.2.4 Histochemical analysis

2.2.4.1 Al accumulation in root tips

After growing 4 days on the nylon screen in tap water, selected rice cultivars were pretreated with 0.2 mM Ca (pH 4.9, 6h) followed by 20 μM Al in 0.2 mM Ca (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 were 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.

2.2.4.2 Al accumulation in root-tip sections

Free hand root-tip (1-3mm) sections were made by razor blade, stained with hematoxylin and observed under light microscope after covering with a coverslip like in the case of intact root. This experiment was replicated 3-4 times.

2.2.4.3 PM permeability study

Roots were treated with or without 20μM $AlCl_3$ in 0.2mM $CaCl_2$ at pH 4.9 for 1h followed by reelongation in Al free $CaCl_2$ medium at pH 5.2 for 12 h. After 1-h Al treatment and after 12-h re-cultivation, the roots were stained for 5 min with fluorescein diacetate-propidium iodide (FDA-PI) (12.5 mg l^{-1} FDA, 5mg 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. 390nm, ba. 520nm) and photographed with a digital camera.

2.3 Results

2.3.1 Al tolerance screening for 24 h

Al tolerance of Bangladesh rice cultivars varied widely (Figure 2.1). Among 23 Bangladesh rice cultivars, Rahmat (BR24) (51.3%) and BRRIdhan41 (49.8%) were tolerant, Gazi (BR14) (36.6%) and BRRIdhan29 (35.0%) were intermediate and Moyna (BR12) (24.8%) and BRRIdhan34 (24.8%) were found sensitive whereas among Japanese rice Sasanishiki (50.0%) found tolerant and Domannaka (26.5%) was found sensitive to Al. Another rice species from Africa, *Oryza glaberrima* was found sensitive

to Al. Among the tested rice cultivars, Sasanishiki was found highly tolerant to Al though its tolerance was almost similar to Bangladeshi tolerant cultivars. Wide variation of Al tolerance of Bangladeshi rice cultivars were remarkable. This kind of wide variation of Al tolerance among rice cultivars also have been reported by Jan and Pettersson, (1993).

2.3.2 Al tolerance screening for 1-h

As inhibition of root elongation is the primary target for Al toxicity which is followed by several other toxic syndromes, Al tolerance study using shorter time would be crucial. Therefore, selected rice cultivars were screened for 1-h Al tolerance to isolate early expression of Al tolerance and found that tolerant and sensitive cultivars expressed similar tendency of tolerance. Results presented in Figure 2.2 indicate Al tolerance screening with 1-h of Al treatment followed by re-elongation in Al free medium suggesting that even 1-h of Al treatment is enough to make differential Al tolerance in rice. Ishikawa et al. (2000) also found differential Al tolerance having 1 h of Al treatment among tolerant and sensitive cultivars.

2.3.3 Al accumulation

After staining by hematoxylin it was found that roots of Al-tolerant cultivars (Sasanishiki and BR41) accumulated less amount of Al which is indicated by light purple color in intact root and root tip sections (Figure 2.3). On the other hand, intact roots and root-tip sections of Al-sensitive cultivars (Domannaka and BR34) accumulated Al more densely indicated by denser purple color. This result indicate that rice primarily posses Al exclusion mechanism. This kind exclusion mechanism has already been reported in some

other crops including rice. Ma et al. (2002) found Al exclusion mechanism in tolerant Kushihikari (Japonica type) rice cultivar when comparing sensitive Kasalath (Indica type) cultivar. In my study, I used two Japanese and two Indica type cultivars of same *Oryza sativa* and found similar trend of exclusion.

2.3.4 PM permeabilization

Immediately after 1h Al treatment, only Domannaka showed slight PM permeabilization, as shown by weak red fluorescence (Fig. 2.4). When 1h Al treatment was followed by 12h re-elongation in Al-free medium, the PM permeability of Al-tolerant cultivars (Sasanishiki and BR41) was almost unchanged, but Al-sensitive cultivars (Domannaka and BR34), on the other hand, exhibited strong red fluorescence. When stained with FDA-PI, FDA entered intact cells and exhibited green fluorescence under UV light. On the other hand, PI was absorbed only by permeabilized cells that exhibited red fluorescence when excited with the same UV light. This red fluorescence corresponds to PM permeability. The weak red fluorescence exhibited by Sasanishiki suggests higher PM strength compared with Domannaka.

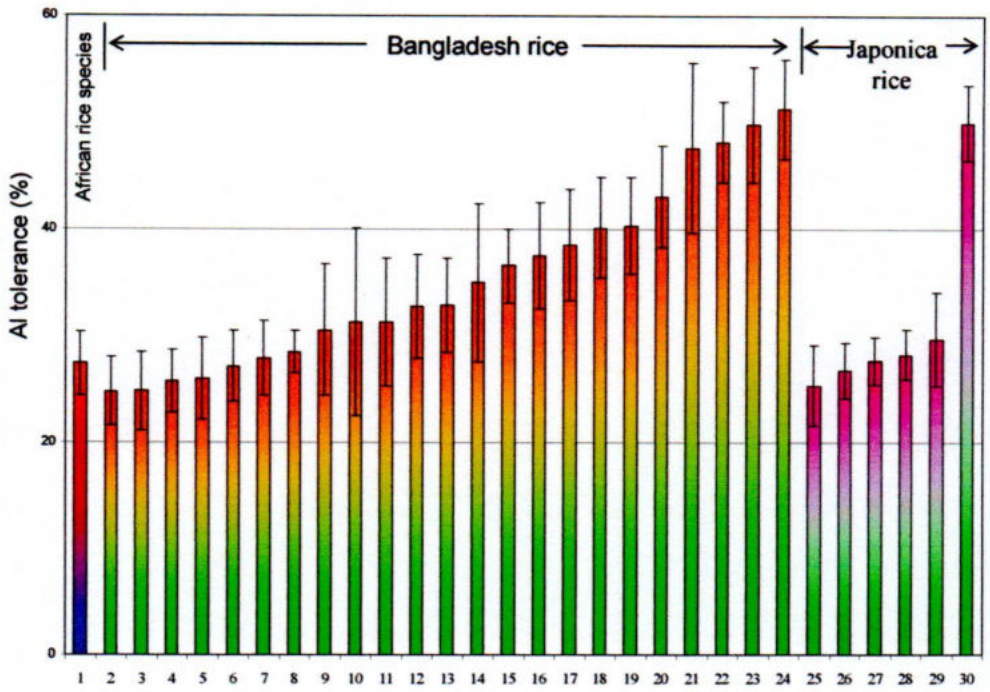


Figure 2.1: Aluminum tolerance screening with 20μM $AlCl_3$ in 0.2 mM $CaCl_2$ (pH 4.9, 24h). 1, *Oryza glaberrima sp.*; 2, cv. Moyna; 3, cv. BRRIdhan34 4, cv. Asha; 5, cv. Dulabhog; 6, cv. BRRIdhan27; 7, cv. Chandina; 8, cv. Niamat; 9, cv. BRRIdhan37; 10, cv. Kiron; 11, cv. Naya Pajam; 12, cv. Asha; 13, cv. Mala; 14, cv. BRRIdhan29; 15, cv. Gazi; 16, cv. Sufala; 17, cv. BRRIdhan36; 18, cv. BRRIdhan39; 19, cv. Biplab; 20, cv. Brribalam; 21, cv. BRRIdhan28; 22, cv. Mukta; 23, cv. BRRIdhan41; 24, cv. BRRIdhan24; 25, cv. Akitakomachi; 26, cv. Domannaka; 27, cv. Haenuki; 28, cv. Koshihikari; 29, cv. Hitomebore; 30, cv. Sasanishiki.

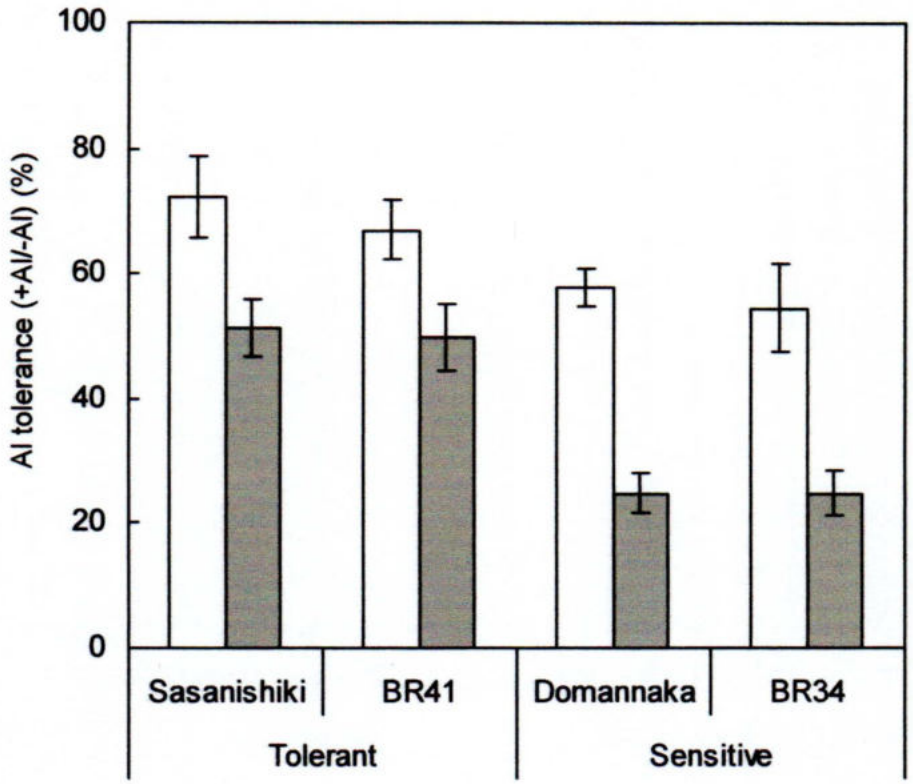


Figure 2.2: Al tolerance tolerances for 1h (open bar) and 24h Al treatments. 1h Al treatment, 1h treatment in ±20μM $AlCl_3$ in 0.2mM $CaCl_2$ (pH 4.9) and reculturing in Al free medium (0.2mM $CaCl_2$ at pH 5.2); 24h Al treatment, continuous treatment with ±20μM $AlCl_3$ in 0.2mM $CaCl_2$ (pH 4.9). Al tolerance was calculated as the ratio of root elongation in Al to that in control. Data are mean ±SE ($n \geq 10$).

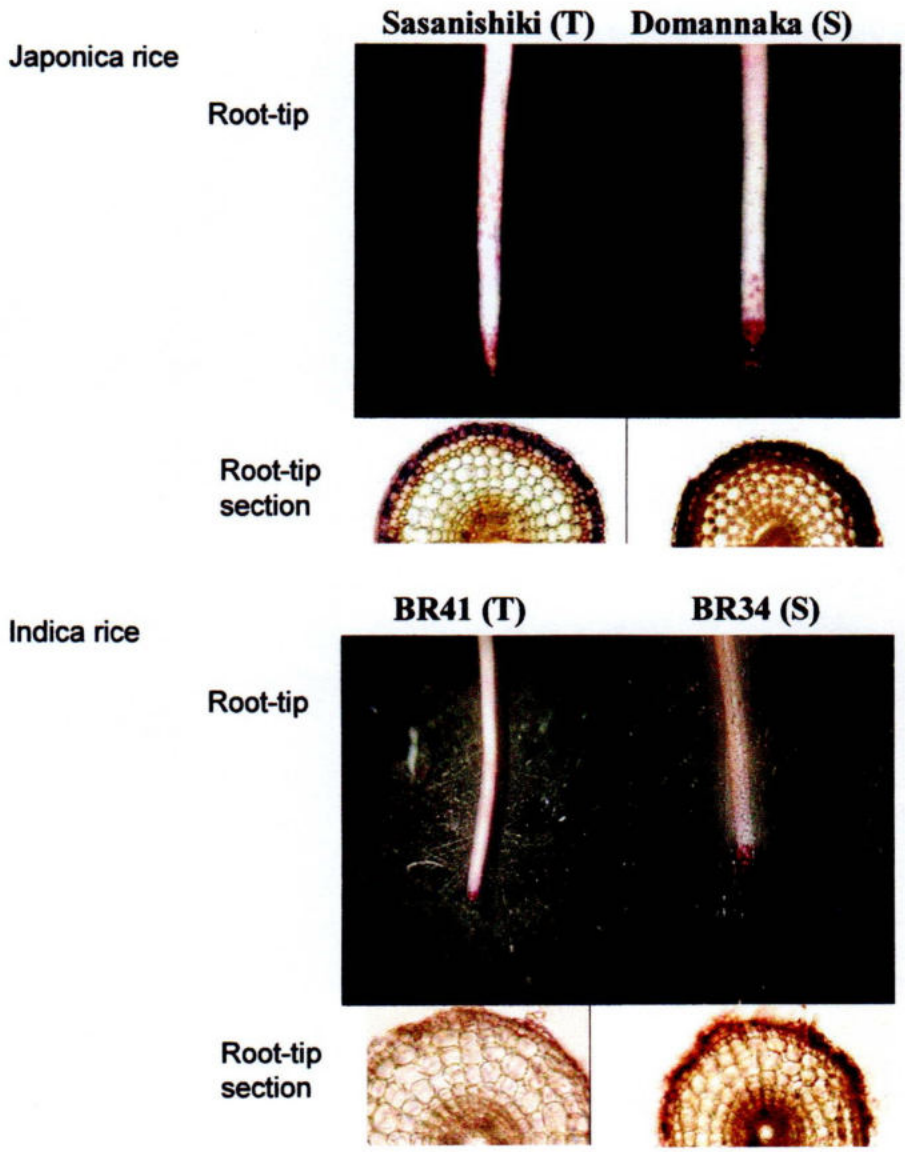


Figure 2.3: Localization of Al in 1-cm root-tip and 2-3 mm sections from apex detected by hematoxylin staining method. Deeper brownish purple color indicates higher Al accumulation

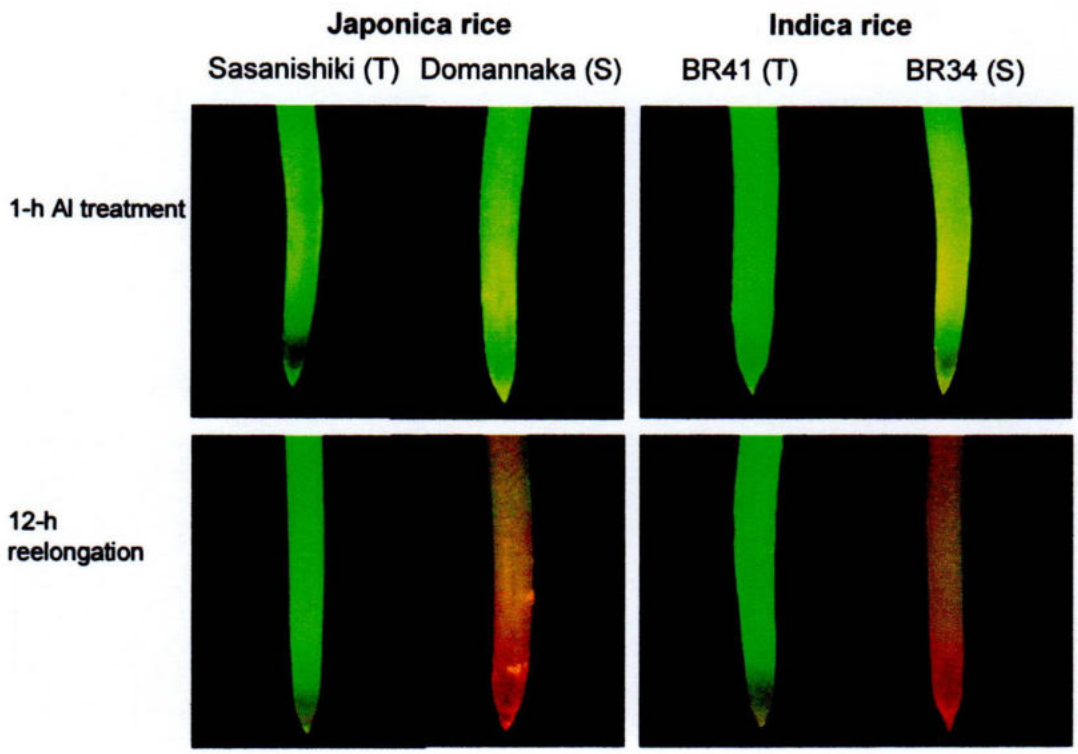


Figure 2.4: Permeability of root-tip cells after 1h Al treatment (50μM $AlCl_3$ in 0.5mM $CaCl_2$, pH 4.5) and after reelongation in Al free medium was observed by FDA-PI staining

2.4 Discussion

Though, relative root elongation (Al tolerance) varied widely among the cultivars, wider variation was observed among the Indica rice (Bangladeshi rice) cultivars. Among the Japanese rice cultivars, Sasanishiki showed a distinct greater Al tolerance (Fig. 2.1). To know the primary mechanism of Al tolerance, Al accumulation was study was conduced by hematoxylin staining light microscopy. This technique is widely been used by several researchers to detect Al in the roots. Primary Al tolerance mechanism was found to be exclusion.

Immediately after 1h Al treatment, the PM of root-tip cells was barely permeabilized irrespective of Al tolerance. Thereafter, when re-elongated in Al-free medium, root-tip cells of sensitive cultivars irrespective of their types (Indica or Japonica) became permeabilized, indicating that an irreversible arrangement of PM lipid molecules occurred in sensitive plants. Al binds to PM lipids during 1h Al treatment, altering PM fluidity and making it permeable to Al (Ishikawa and Wagatsuma, 1998). We previously reported that PM strength plays a potential role in Al tolerance in triticales (Wagatsuma et al., 2005a). The results of this study suggest that PM intactness/strength is the key factor of Al tolerance in Sasanishiki.

The results on hematoxylin staining of root tip sections shows a large numbers of cortex cells especially in the cytoplasm of sensitive cultivars were heavily stained with hematoxylin-Al complex of purple color. Yang et al. (1988) found that the surface of root cells of *G. triacanthos* and *P. taeda* bound larger amounts of Al immediately after exposure to Al ions. Cronan (1991) reported that the accumulation of Al in root cortex cells walls of *Picea rubens* was pH dependent. Steinen and Bauch (1988) found out

highest amount of Al in the cortex of *P. abies* whereas small quantities in the xylem. In the cortex cell wall of *P. rubens* (Schroeder 1988, Schlegel et al. (1992) and *P. albies* (Godbold 1988) was found to be the major accumulation site. Ofei-Manu (2001) also found same accumulation pattern in woody plants.

When stained with FDA-PI, cells with normal permeability can exclude PI from their PM's lipid layer; in such cells, FDA passes through the PM and is hydrolyzed by intracelluloar esterases to produce fluorescein, and exhibits green fluorescence when excited by UV light. On the other hand, the permeabilized cells exhibit a bright red fluorescence due to the passage of PI through their PM's and intercalation with DNA and RNA. Al tolerant cultivars showed light red color indicating intact PM after 1-h Al treatment and even after reelongation (Figure 2.4). Even though, after 1-h Al treatment, sensitive cultivars exhibited little increase in PM lipid permeability but after reelongation, it showed wide variation of permeability in the root-tip cells.

To know the early response to Al, 1h Al tolerance screening was conducted. This result showed increasing tendency of Al tolerance for 1h Al treatment than 24h though similar tendency was observed between these two tolerances for tolerant and sensitive cultivars. Even though rice is relatively tolerant crop species, but root tip of sensitive rice became permeabilized even after 1 h of Al treatment and subsequent reelongation in Al free medium. During short-term Al treatment, PM of the cells transformed from crystal liquid phase to rigid phase. This kind of transformation occurs during Al treatment. Rigidified PM can not be observed by FDA-PI staining just after Al treatment as these are not permeabilized. Once this kind of rigidification occurs due to Al, it can not retransformed into liquid phase due to permanence of alteration. During reelongation

period, cells try to elongate but PM further can not be elongated due to its rigidness; rather rupture occurs on the PM and finally it become sit becomes permeabilized. This permeabilized PM can permit PI to enter into the cytoplasm and finally emit red fluorescence. After 24 h Al treatment, permeabilization pattern was also changed. In 24 h, Al treatment makes permeabilized not only the limited root tip but also proximal part of the root. This result indicates that even short term Al treatment makes irreversible alteration of PM by decreasing the fluidity. Al treatment and reelongation has little effect on the PM of tolerant cultivars which is emitting green fluorescence.

Less permeability after 1-h Al tolerance may be ascribed as the rigidification of the liquid PM layer. During short time treatment, Al binds with the PM lipids and PM lipid losses its crystal liquid form by dehydration (Leshem et al. 1992, Ishikawa and Wagatsuma 1998). This time, though PM become leaky but the space was not enough to enter PI. During reelongation, leaky space on gel like rigid PM enlarged and enough space created to enter PI.

This result suggests that even after 1-h of Al treatment in sensitive cultivars, Al makes some irreversible change in the PM lipid structure which makes it permeable to Al. Ishikawa et al. (2001) showed differential permeability among tolerant and sensitive cultivars of rice, maize, pea, wheat and sorghum crop species by FDA-PI staining technique and found no induction of change in permeability of the PM just after short-term Al treatment. But, when short-term exposure to Al was followed by re-elongation in Al free medium, the PM of root-tip cells was clearly permeabilized depending on the Al tolerance. Ishikawa and Wagatsuma (1998) suggested that PM of the Al sensitive cultivars became rigid and reduces its extension but it becomes permeabilized during the

re-elongation time and inhibits elongation.

When root of the plants coexist with Al in the medium, 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). 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. Figure showing the lesser permeability in intact roots indicate that Al tolerant rice posses higher strength in PM lipid layer (Figure 2.4). 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.

But in membranes of Al-tolerant plant species, the other long chain materials of PM may also be involved which makes the membrane higher tolerance against Al-stress and prevent the membranes to make easy entry points even after binding with Al. Very long chains of glucocerebroside in the phospholipids bilayer overlaps each other and also overlaps with other phospholipids. Less permeable PM also can be attributed by increasing the content of free sterols in the PM layer. As sterols has a complex plate like structure, higher amount of sterols makes the PM more rigid by decreasing fluidity. And

finally it makes less permeable PM.

Although similar Al tolerance, Al accumulation and PM permeabilization was observed among Al-tolerant cultivars of Japonica and Indica and Al-sensitive cultivars of both type, Al tolerance of Sasanishiki was extraordinary. Therefore, I considered that there might be some gene underlie within the Sasanishiki and that tolerant gene is originating from ancestor cultivars. To know the basis of this high tolerance of Sasanishiki, I decided to study Sasanishiki pedigree cultivars as these will confer Al tolerance mechanism with genetic clarification.

Chapter 3

Clarification of Al tolerance in Sasanishiki pedigree cultivars

Chapter 3

Clarification of Al tolerance in Sasanishiki pedigree cultivars

3.1 Introduction

In my primary study with several rice cultivars of Indica and Japonica types, I could conclude that there might be some cultivars consisting Al tolerant gene and transfering to the Sasanihsiki. Clarification of background source of Al tolerance in Sasanishiki would clarify further the genetic connection of Al tolerance. It was also considered that Al tolerance in closely related cultivars would be more contributive to make Al tolerant crop species. Considering this point I decide to check the Al tolerance mechanism in these pedigree cultivars and collect pedigree cultivars of Sasanishiki. Further it was my intention to know whether exclusion mechanism is also underlie in the cultivars of same family line.

3.2 Materials and Methods

3.2.1 Source of seeds

Seeds of Sasanishiki pedigree cultivars i.e., Sen-ichi, Rikuu-20, Nourin-8, Tougou, Joushu, Nourin-1, Aikoku, Asahi, Moritawase, Kamenoo-4, Ginbouzu, Nourin-22, Sasanishiki, Asahi (Kyoku), Hatsunishiki, Sasashigure, Kamenoo and Rikuu-132 were collected from the Faculty of Agriculture, Yamagata University, Japan; Shonai Branch Yamagata Regional Prefectural Agricultural Station, Japan; and National Institute for Agricultural and Environmental Science, Japan. The seeds of Touhoku-24 and Nourin-6 could not be found from all related institutes within Japan.

3.2.2 Al tolerance screening

Al tolerance of the pedigree cultivars of Sasanishiki were conduced following the same procedure as described in the previous chapter (Chapter 1).

3.2.3 Al accumulation and PM permeability

Al accumulation and PM permeability was studied following same procedure as described in the previous chapter.

3.3 Results

3.3.1 Al tolerance of Sasanishiki pedigree cultivars

Wide variation of Al tolerances were found among Sasanishiki pedigree cultivars in the range from 23 to 60%: Rikuu-132 (18), Kamenoo (17), Sasashigure (16), Hatsunishiki (15) > Asahi (Kyoku) (14), Sasanishiki (13) > Nourin-22 (12), Ginbouzu (11), Kamenoo-4 (10), Moritawase (9), Asahi (8) > Aikoku (7), Nourin-1 (6), Joushu (5), Tougou (4), Nourin-8 (3), Rikuu-20 (2) > Sen-ichi (1) (Fig. 3.1A). To my knowledge, there are no seeds of Nourin-6 and Touhoku-24 in Japan. Based on the differences in Al tolerance, all the cultivars were grouped for convenience sake into 5 categories which were shown as the depth of black color density, i.e., the denser the color the higher the tolerance. Sasanishiki (13) had been bred from Sasashigure (16) and Hatsunishiki (15) as parents; both were most tolerant to Al (Fig. 3.1B). Additionally to these two cultivars, Kamenoo (17) and Rikuu-132 (18) were found to be most Al-tolerant cultivars. Rikuu-132 (18) had been bred from Al-sensitive Rikuu-20 (2) and intermediate Al-tolerant Kamenoo-4 (10) as parents. Although the former had been bred from Al-sensitive Aikoku

(7), the latter had been bred from most Al-tolerant Kamenoo (17). On the other hand, there were 7 sensitive cultivars, i.e., Sen-ichi (1), Rikuu-20 (2), Nourin-8 (3), Tougou (4), Joushu (5), Nourin-1 (6) and Aikoku (7) (Al tolerance ranged from 23-32%). Al-tolerance of some cultivars were considered to be intermediate, i.e., Asahi, Mouritawase, Kamenoo-4, Ginbousu and Nourin-22.

Kamenoo and Rikuu-132 were significantly more Al-tolerant than Aikoku and Rikuu-20 after 24h of continuous Al treatment (Fig. 3.2). To know the timing to induce the differential Al tolerance between these two groups of cultivars, we further screened cultivars for 1h with 20μM $AlCl_3$, followed by 12h in 0.2mM $CaCl_2$. Although the difference in Al tolerance was less than that in continuous 24h Al treatment, the former two cultivars were also found to be more Al tolerant than the latter cultivars: Al tolerance was confirmed to be expressed within 1h of Al treatment (Fig. 3.2).

3.3.2 PM permeability and Al accumulation

Greater Al accumulation and PM permeabilization was observed in the sensitive Rikuu-20 and Aikoku cultivars than tolerant Rikuu-132 and Kamenoo (Fig. 3.3). This result follows the similar tendency to the results in the previous experiment where different types of rice (Indica and Japonica) had been used. From this result it can be concluded that Al exclusion (as primary mechanism) underlie in these pedigree cultivars which is ascribed as the intactness of the PM (Fig. 3.4).

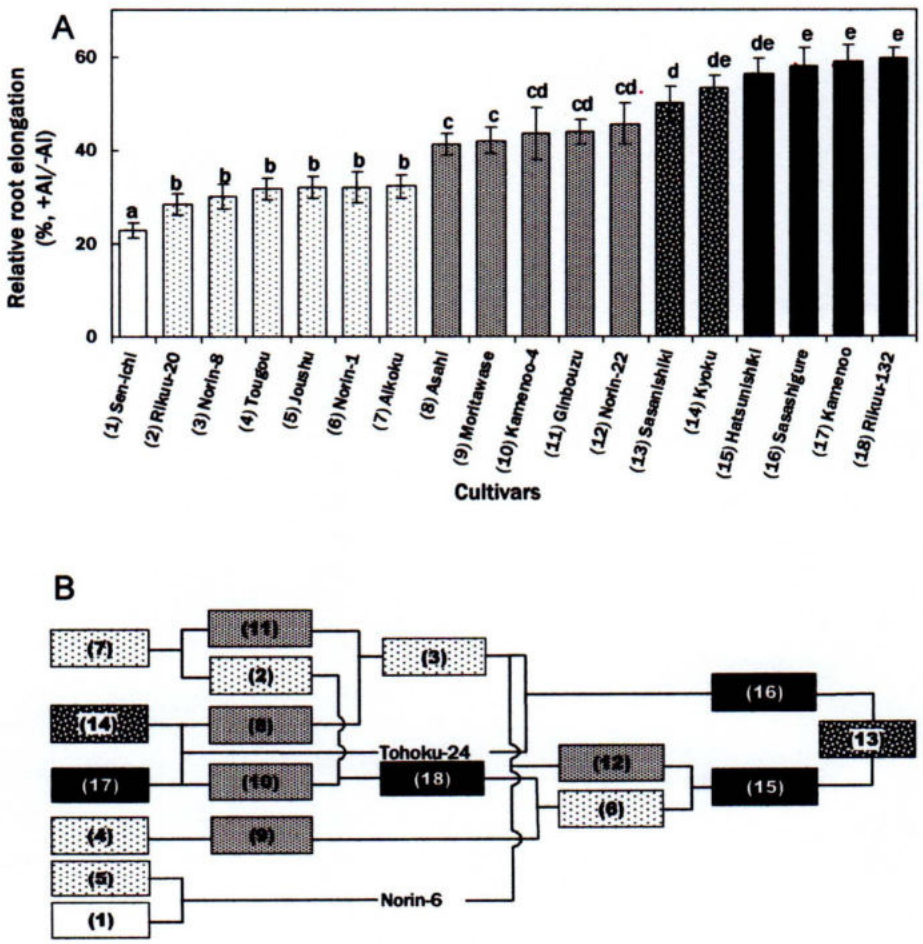


Fig. 3.1: Relative root elongation of ancestor cultivars of the same family line of Sasanishiki (A) and family tree of cv. Sasanishiki shown with graded black densities basically correspondent to differential Al tolerances among cultivars in Fig. 1A (B). Four-d-old seedlings were pretreated in 0.2 mM $CaCl_2$ for 6h (pH 4.9) and then transferred to 0.2 mM $CaCl_2$ with (Al treatment) or without (control) 20 μM $AlCl_3$ (pH 4.9) for 24h. Al tolerance is calculated as the ratio of net root elongations of the longest root in Al treatment to that in control. Values are means ±SE ($n \geq 10$). Average values with same letter(s) are not significantly different at 5% level of significance by Fisher's LSD.

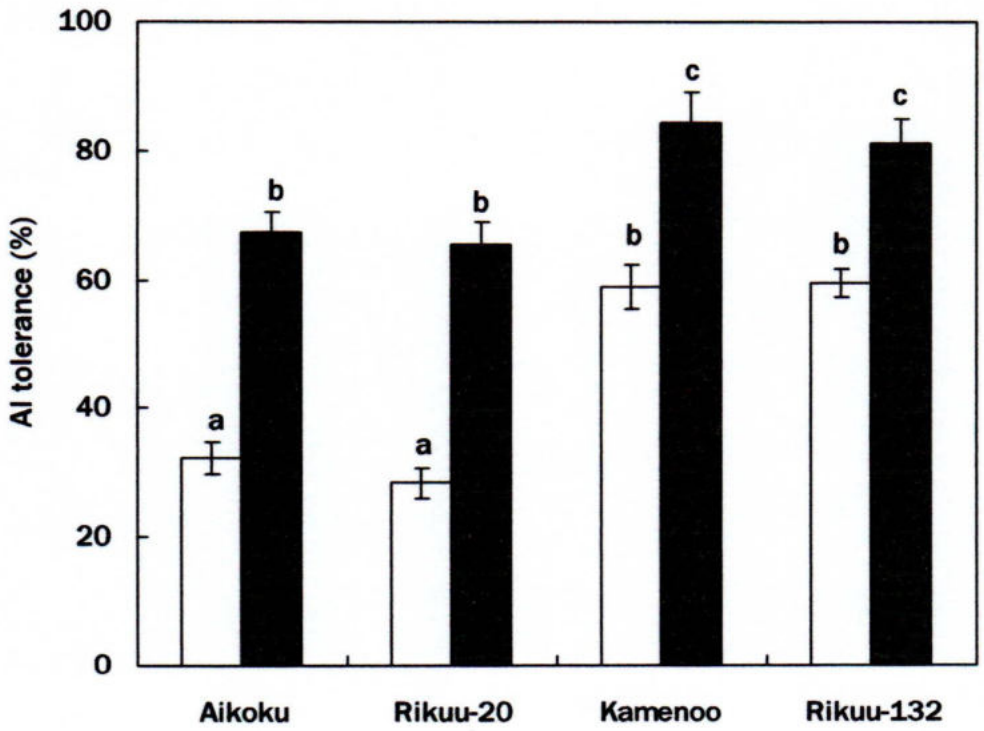


Fig. 3.2: Al tolerance of the selected Sasanishiki pedigree cultivars for 24h (white bar) and for 1h Al treatment followed by 12h reelongation (closed bar). Al tolerances were calculated as described in materials and methods of previous chapter.

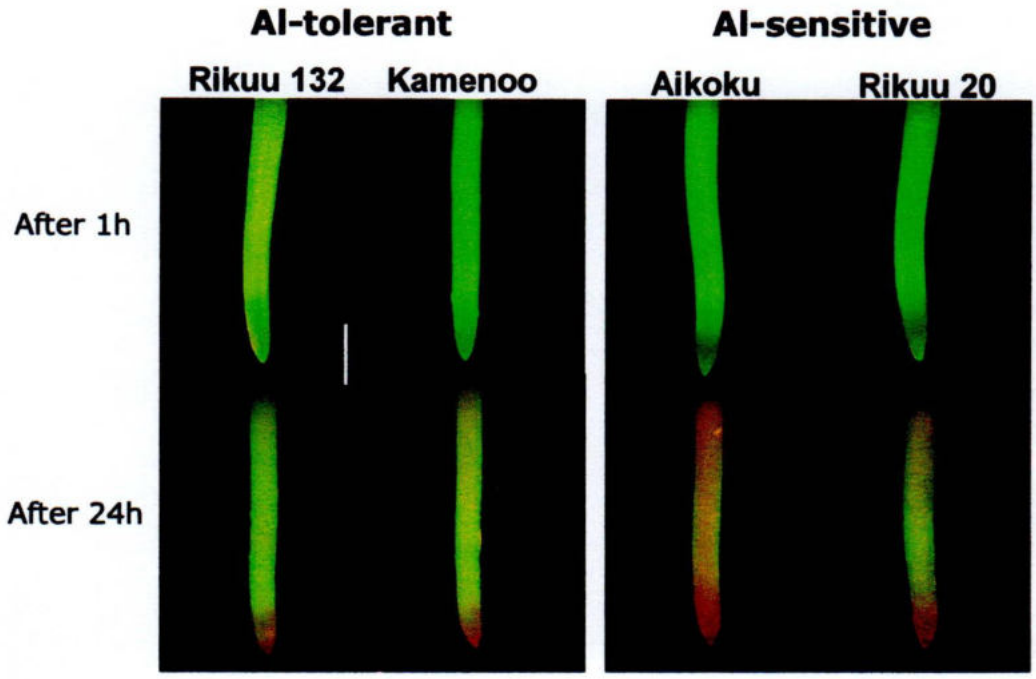


Fig. 3.3: Plasma membrane permeabilization in root of tolerant and sensitive cultivars which were selected from Sasanishiki pedigree by FDA-PI fluorescence microscopy.

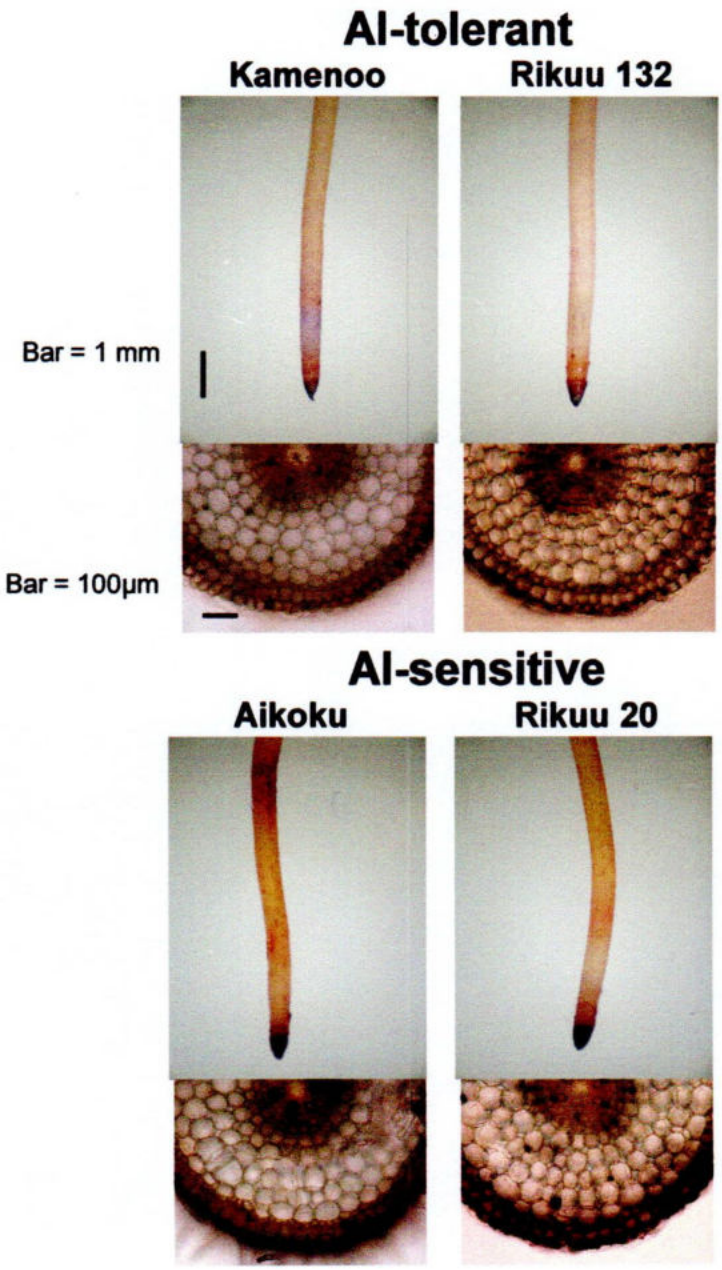


Fig. 3.4: Al accumulation in root-tips and root-tip sections in tolerant and sensitive cultivars which were selected from Sasanishiki pedigree by hematoxycilin staining technique after 24h 20μM AlCl3 treatment in 0.2mM CaCl2 at pH 4.9.

3.4 Discussion

In a previous study, we found that some Japonica cultivars, such as Sasanishiki, are highly Al tolerant (Khan et al. 2005, Chapter 1). In the present study, I 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 (Fig. 3.1B). Ancestor cultivars showed a wide range of Al tolerance, and originated from Al-tolerant and -sensitive ancestors. The family tree suggests that Al tolerance of Kamenoo was transmitted to Rikuu-132.

Differential Al accumulation was found after 24 h of Al treatment, i.e., less Al accumulated in the Al-tolerant cultivars Rikuu-132 and Komenoo (Fig. 3.4). Al accumulation was associated with permeabilization of the PM at the root tip, which was greater in the sensitive cultivar Rikuu-20 (Fig. 3.3). It was considered that, the sensitive cultivar Rikuu-20 had a greater proportion of phospholipids in the PM than the tolerant cultivar Rikuu-132. Greater phospholipids (consisting the negative site in the PM) confers higher sensitivity to Al. Ishikawa and Wagatsuma (1998) suggested greater negative site (phospholipids) in the sensitive cultivars. Wagatsuma and Akiba (1991) also reported greater negative sites in the Al-sensitive crop plant species while studying Al tolerance in 4 crops having variation in Al tolerance. Recently, Wagatsuma et al. (2005b) reported greater negative sites in Al-sensitive crops ascribed as greater methylene blue stainability using 18 cultivars or lines of several crops. Their results are in accordance with the present study. Higher negative sites in the PM (ascribed as the higher phospholipids) binds with Al and makes cluster of phospholipids and finally PM become cracked. Normal PM do not permit PI to enter due to its hydrophobic nature and large

size (molecular weight of PI is 668.39). On the other hand FDA can enter through the PM due to hydrophilic nature and relative small size (molecular weight of FDA is 416.38). This covalent bonding of phospholipids and subsequent cracking could further increase greater Al accumulation. Finally it could be concluded that primary exclusion mechanism of Al tolerance is also underlie in the cultivars of Sasanishiki pedigree. Representative tolerant and sensitive cultivars were selected from this experiment, i.e., Aikoku and Rikuu-20 were sensitive and Kamenoo and Rikuu-132 were tolerant, for further clarification of Al tolerance mechanism.

Chapter 4

Study on organic acid exudation for Al tolerance

Chapter 4

Study on organic acid exudation for Al tolerance

4.1 Introduction

Al tolerance mechanism has been suggested by many researchers. Among those, most deliberate clarification was based on organic acid (OA) exudation. For example, malate from wheat (Delhaize et al. 1993, Sasaki et al. 2004), oat (Zheng et al. 1998a), rye (Li et al. 2000a), triticale (Ma et al. 2000), sunflower (Saber et al. 1999), radish (Zheng et al. 1998), rape (Zheng et al. 1998b), *Arabidopsis* (Hoekenga et al. 2003); citrate from maize (Kidd et al. 2001), sorghum (Magalhaes et al. 2007), tobacco (Delhaize et al. 2001); and oxalate from taro (Ma and Miyasaka 1998), and buckwheat (Zheng et al. 1998a) has been reported as probable Al tolerance mechanism. Secreted OA in the rooting bath may bind with Al and become unavailable.

Resistance in certain wheat and maize genotypes has been correlated with the ability to release organic acids, such as malic and citric acid, in response to Al (Delhaize et al. 1993b, Ryan et al. 1995). Released organic acids are thought to complex with Al^{3+} and prevent its uptake. Citrate is much more effective at detoxifying Al than is malate, and there are distinct advantages to employing citrate exudation to exclude Al, compared with malate as formation constant for Al:citrate of 9.6 compared to 5.7 for Al:malate. Polle et al. (1978) and Ryan et al. (1995) found in wheat genotypes that a range of Al sensitivities were correlated with their capability to release malate. They also found that Al resistance generally correlated with the release of malate from roots in the presence of Al. Wheat genotypes rank order with respect to malate release suggests that malate

release is an important mechanism by which wheat genotypes differ in the capability of resisting the growth-inhibiting effects of Al.

Internal detoxification by organic acid anions has also been reported as an Al tolerance mechanism in some crop species. Zheng et al. (2005) reported higher Al accumulation in Al-tolerant buckwheat (cv. Jiangxi) than in Al-sensitive one (cv. Shanxi) and suggested that the greater Al resistance in buckwheat is due to immobilization and detoxification of Al by phosphorus in the root tissue. Ma et al. (1998) also reported that oxalate is involved in both external and internal detoxification of Al in buckwheat.

Considering these points, experiment was conduced whether this OA exudation mechanism also existing in rice or not.

4.2 Materials and Methods

Rikuu-132 and Rikuu-20 seeds 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. A picture of experimental procedure has been shown in Fig. 4.1. 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.

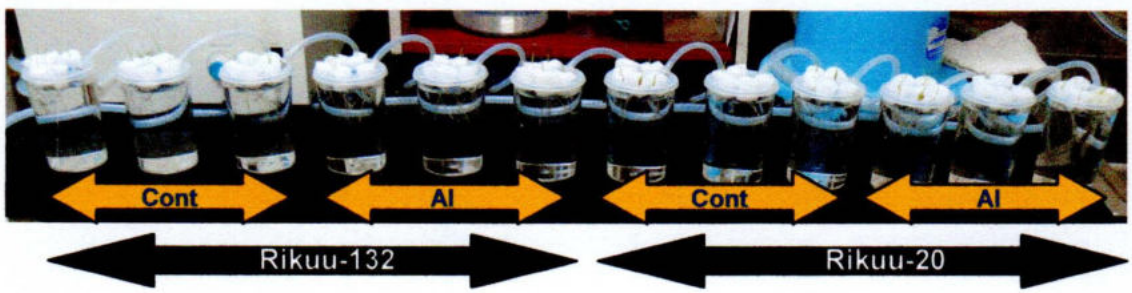


Fig. 4.1 Treatment procedure for control and Al treatment for organic acid exudation. Only selected cultivars of rice were treated.

4.3 Results

To examine whether OA release could account for differential Al tolerance, major OA acid released from rice, citrate, was quantified for both contrasting Al tolerance cultivars with Al treatment. Greater exudation of citrate (1.82nmol h^{-1} $seedling^{-1}$) was detected in Al treatment in Al-sensitive Rikuu-20 than that in Al-tolerant Rikuu-132 (0.46nmol h^{-1} $seedling^{-1}$) (Fig. 4.2A). Exudation of malate was less than that of citrate, however the tendency was same as citrate (Fig. 4.2B). These results suggested that OA release cannot explain Al tolerance difference between these cultivars.

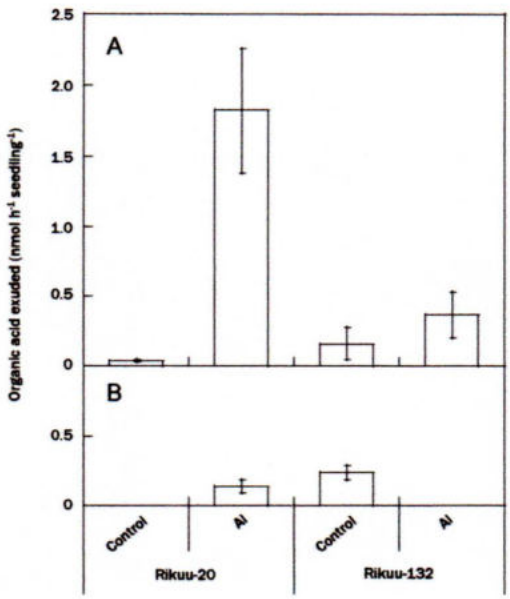


Fig. 4.2: Citrate (A) and malate (B) exuded from rice cultivars Rikuu-20 and Rikuu-132. Five- day-old seedlings were treated for 5h with or without Al (20μM) in 0.2mM $CaCl_2$ (pH 4.9) following 5h pretreatment with 0.2mM $CaCl_2$ (pH 4.9). Exudates were collected during the 5h treatment. Values are means ±SE (n = 3)

4.4 Discussion

Organic acid excretion has been described as one of the major Al tolerant mechanisms of several crop plant species, this may not fit to explain Al tolerance variation between Rikuu-20 and Rikuu-132. Though Kikui et al. (2007) recently reported greater OA exudation from tolerant rice than sensitive rice, in this case, sensitive cultivar Rikuu-20 excreted greater amount of citrate than Rikuu-132, while no difference was observed in malate excretion (Fig. 4.2). This indicated that other Al tolerant mechanisms would make differential Al tolerance between these cultivars. This would account for previous research in rice Al tolerance variation that was not associated with OA release (Ishikawa et al. 2000, Ma et al. 2002, Yang et al. 2008). Contrary to this result, Hayes and Ma (2003) found that tolerant triticale line exude more OA than sensitive line (ST2, tolerant line exuded 19.8nmol malate and 9.6nmol citrate per plant whereas, ST22, Al-sensitive line exuded 3.8nmol malate and 3.1nmol citrate per plant over 20 h). Moreover, Ryan et al. (1995) showed a close correlation between the degree of Al resistance and the magnitude of Al-activated root malate release in 36 different wheat genotypes differing in Al resistance.

Resistance to Al can be achieved via exclusion of Al from the root apex and/or via intracellular tolerance by sequestration of Al in the plant's symplast. In a study, Shen et al. (2004) observed internal OA exudation (citrate and oxalate in the leaves) in buckwheat, a highly Al-resistant crop species. Although recent evidence for an Al-resistance mechanism involving internal detoxification and sequestration is starting to emerge, the most compelling evidence has focused on a resistance mechanism based on chelation and exclusion of extracellular based on chelation and exclusion of extracellular

Al via Al-activated root organic acid release (Kochian et al. 2004). Although, in the present study, I did not measure internal OA exudation in shoot or leaves or rice cultivars, but in another study (Chapter 7 of this thesis) it was observed that leaves of rice did not accumulate Al. Therefore, it could be refer that internal detoxification is not the mechanism for Al tolerance of rice. Finally, I would like to suggest both internal and external detoxification is not involved for Al tolerance in rice. These results led me to clarify the role of PM lipid composition for Al tolerance mechanism in rice.

Chapter 5

Study on the lipid composition of pharmaceutically changed PM

Chapter 5

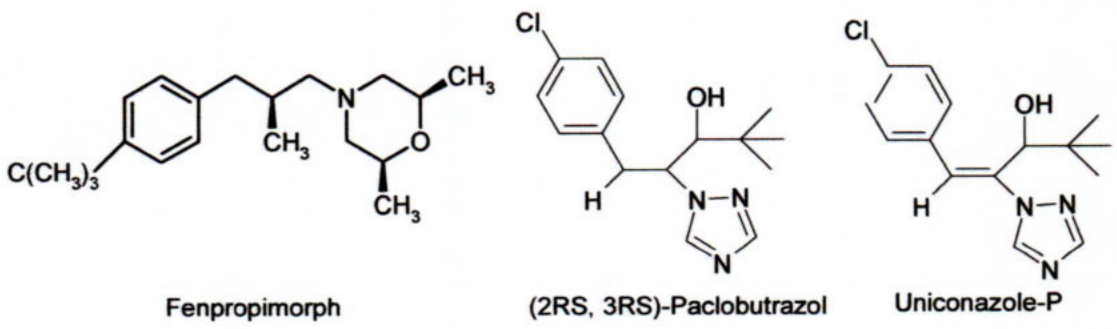
Study on the lipid composition of pharmaceutically changed PM

5.1 Introduction

Higher plants predominantly contains mixtures of cholesterol, stigmasterol and sitosterol. Burden et al. (1987) found that sterol biosynthesis-inhibiting fungicides increases membrane permeability in barley. Grandmougin et al. (1989) found that fenpropimorph treated maize roots are lack of Δ^5- sterols and are replaced by 9β,-19-cyclopropyl sterols such as cycloeucalenol and 24-methyl pollinastanol which were absent in control plant. Due to lack of absolute substrate specificity of many enzymes involved in the cycloartenol to Δ^5- sterol pathway, the blocking of cycloeucalenol-obtusifoliol isomerase (COI) causes the accumulation of abnormal sterols (Grandmougin et al. 1989, Cerdon et al. 1996). Blocking the immediate general sterol synthesis process is not the only factor, but also of sterols derived from it that are not in the normal pathway of sterol biosynthesis. This unusual newly synthesized sterol mainly accumulates in membrane fraction and regulate the membrane fluidity and consequently the activity of membrane bound enzymes. This kind of change in membrane fraction changes the rigidity of the membrane which in turn regulates permeation of Al.

There are several sterol metabolism inhibitors which can reduce the Δ^5-sterols in the PM. In the present study, 3 sterol metabolism inhibitors from two groups were selected. Fenpropimorph is a morpholine type of fungicide which primary use is to reduce rust and powdery mildew of cereal crops. Chemical composition of sterol metabolism inhibitors used in the present study has been shown in Fig. 5.1. Fenpropimorph inhibits

cycloeucalenol-obtusifoliol isomerase (COI) (Cerdon et al. 1996). On the other hand, (2RS,3RS)-paclobutrazol and uniconazole-P are triazole type fungicide with high plant growth regulatory activity on a wide variety of crops (Sugavanam 1984). Although there are several stereoisomers of paclobutrazol of paclobutrazol but (2RS,3RS)-diasterioisomer is most effective for plant growth regulatory activity (Sugavanam 1984). Both 2RS-3RS-paclobutrazol and uniconazole-P inhibit obtusifoliol isomerase (Burden et al. 1987). Though all 3 inhibitors decrease Δ^5-sterols in the PM but ultimate accumulation of abnormal sterols and their role in PM permeabilization are different among the species. Fenfpropimorph accumulates cycloeucalenol, 24-methylpollinastanol and 24-dihydrocycloeucalenol and these effect on PM permeabilization are moderate (Dahl et al. 1980). On the other hand, (2RS,3RS)-paclobutrazol and uniconazole-P accumulates obtusifoliol, dihydroobtusifoliol and 14α-methyl-Δ^8-ergostenol and these effect on PM permeabilization are severe (Dahl et al. 1980). Both 2RS-3RS-paclobutrazol and uniconazole-P also have a inhibitory effect on *ent*-kaurene (CYP51A2).



Fenpropimorph (2RS, 3RS)-Paclobutrazol Uniconazole-P

Fig. 5.1: Chemical composition of the sterol metabolism inhibitors used in the present study

In this experiment, these sterol metabolism inhibitors were used to change the lipid composition in the PM of roots. It was observed in the previous study (Chapter 1 and 2) that PM intactness of leakiness would be the Al tolerance strategy in rice. Phospholipids and sterols are the major component of the PM and may have greater contribution for PM lipid permeabilization. It was my intension to know what happened on the PM lipid permeability after changing the sterol content. After checking the PM permeability and Al accumulation, further, major lipids were measured to know the actual contributions of each lipid classes.

5.2 Materials and methods

5.2.1 Effect of sterol metabolism inhibitors on root elongation and PM permeabilization

Fenpropimorph ((2R,6S)-rel-4-[3-[4-(1,1-dimethylethyl)phenyl]-2-methylpropyl]-2,6-dimethylmorpholine; Schott Duran, Germany) was solubilized with water, but 0.0005% Tween 20 was used for preparation of 1.7μM 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) and uniconazole-P ((E)-(RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pent-1-en-3-ol; Wako Pure Chemical Industries Ltd., Japan). Four-d-old seedlings were treated for 24h with graded concentrations up to 5, 1.7, or 1.7μM of fenpropimorph, paclobutrazol, or uniconazole-P, respectively, in the existence of 0.2mM $CaCl_2$ 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.

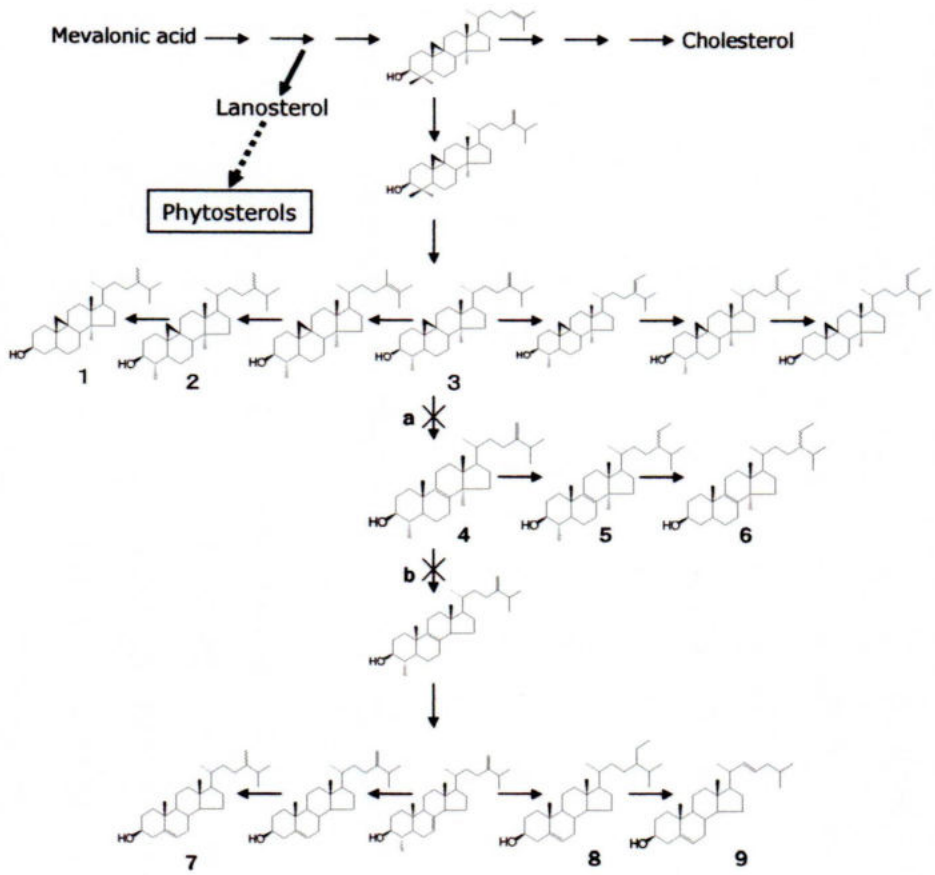


Fig. 5.2: Biosynthetic pathway of sterols compiled from Burden et al. (1987), Grandmougin et al. (1989) by ChemDraw Ultra 7.0 a: Site of action for fenpropimorph which inhibits cycloeucalenol obtusifoliol isomerase (COI); b: Site of action for paclobutrazol or uniconazole-P which inhibits obtusifoliol-14α-demethylase (OBT 14DM). 1, 24-methylpollinastanol; 2, 24-dihydrocycloeucalenol; 3, cycloeucalenol; 4, obtusifoliol; 5, dihydroobtusifoliol; 6, 14α-methyl-Δ^8-ergostenol; 7, campesterol; 8, sitosterol; 9, stigmasterol. Lanosterol pathway beginning at the step of cyclization of 2,3-oxidosqualene specifically in dicotyledonous plants was recently found by Kolesnikova et al. (2006).

Four-d-old seedlings were treated with 0.2mM $CaCl_2$ (Cont), 0.66μM fenpropimorph, 0.68μM paclobutrazol or 1.02μM uniconazole-P in 0.2mM $CaCl_2$ (pH 4.9). These concentrations of the respective inhibitors induced the greatest difference in the inhibition of root elongation based on the former experiment using graded concentrations of each inhibitor. Root elongation was measured before and after the treatments. No less than ten seedlings were used for each experiment. PM permeability was also observed before and after the treatments. Al tolerance in presence of inhibitors were measured following the equation below.

Tolerance to X inhibitors (%) =

$$\frac{\text{Root elongation in inhibitor X during 24h (cm)}}{\text{Root elongation in control without inhibitor 24h (cm)}} \times 100$$

5.2.2 PM permeability and Al accumulation in Al treatment with inhibitors

After germination and preculturing, seedling of the selected cultivars were treated with inhibitors in 0.2mM $CaCl_2$ for 24h (pH 4.9). Concentration of each inhibitor was 0.66μ fenpropimorph, 0.68μM paclobutrazol and 1.02μM uniconazole P. In parallel way, roots were treated with inhibitors in Al solution (20μM $AlCl_3$ in 0.2mM $CaCl_2$) for 24h (pH 4.9). Afterwards, roots were washed and treated with FDA-PI for 5 min and observed under microscope equipped with UV light.

Having treatments in similar way, roots were stained with hematoxylin solution and observed under light microscope.

All these experiments were repeated 3-4 times.

5.2.3 Preparation of root samples for lipid analysis

Roots of 4-d-old seedlings were treated with control (0.2mM Ca), Al (20μM Al in 0.2mM Ca), (2RS-3RS)-fenpropimorph (0.66μM fenpropimorph in 0.2 mM Ca), uniconazole-P (1.02μM uniconazole in 0.2mM Ca), Al+fenpropimorph (20μM Al, 0.66μM fenpropimorph in 0.2mM Ca) and Al+uniconazole (20μM Al, 1.02μM uniconazole, 0.2mM Ca) for 24 h at pH 4.9. Treatment solution was changed every 8 hours 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.

5.2.4 Extraction and measurement of phospholipids and glucocerebrosides

To extract PLs and cerebosides, 2.5, 2.5 and 1.25 ml of *n*-propanol, chloroform and water was added, respectively with the root samples and was homogenized. After standing for 10 minutes, extra 2ml of chloform were added and filtered through filter paper No. 6. This extraction was repeated two more times and collected together. Extracted filtrate was shaken with similar volume 0.1M KCl to remove protein and water soluble molecules (e.g. ATP) and then separated. Ten gram of $NaSO_4$ per 100 ml extract was added and shaken vigorously (to remove water molecules from the extract) and was filtered. The extract was concentrated by rotary evaporator, transferred to a vial and dried with N_2 gas. Finally it was resolubilized with 400μL of chloroform.

50μl of extracted phospholipids were transferred in a beaker and was dried. Digestion was carried out with HNO_3:$HClO_4$ (5:3, v/v) mixture followed by heating on the sand bath. Just after drying, 1 ml of 1M HCl was added and was heated again. Then it was

transferred to a test tube. Deionized water (1 ml) was poured into the beaker, heated and transferred to the same test tube and was pooled. This procedure was repeated two more times. Two drops of α-dinitrophenol indicator wad added to the test tube. pH adjustment was carried out (just below pH 3.3) by adding 1M NH_4OH. Afterwards, P was measured with molybdenum blue method spectrophotometrically at A_{660}.

Statistical analysis of Fisher's least significance difference (LSD) was carried out using KaleidaGraph 4.0 (Synergy Software, USA).

Phospholipids and glucocerebosides were extracted basically by Bligh-Dyer method (1959) modified partially by Uemura and Yoshida (1984). Each portion of 2.5mL *n*-propanol, 2.5mL chloroform and 1.25mL 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 (10g per 100mL solution), concentrated at 40°C, and finally purged with N_2 gas. This concentrate was resolubilized with 200mL per gram of fresh root weight with chloroform and stored at –18°C before measurement. An aliquot of resolubilized solution was evaporated and, digested with acid mixture (HNO_3:60%$HClO_4$ = 5:3, v/v). Phospholipids were estimated after the analysis of phosphorus by molybdenum blue spectrophotometric method.

5.2.5 Phospholipids and cerebrosides class analysis by HPTLC

Extracted phospholipids and glucocerebrosides were developed on HPTLC (Silica gel 60 F_{254}, Merck Ltd., Japan) and using a development solvent mixture of

chloroform:methanol:acetic acid = 65:25:8 following the procedure of Uemura et al. (2004). Amount of each lipid species were quantified qualitatively by developing color with 20% H_2SO_4 in methanol and heating.

5.2.6 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 resolubilized with 200µl of chloroform.

Δ^5-Sterol measurement was carried out following the procedure of Zlatkis and Zak (1969) method. Shortly, 5µl of extracted sample was taken in a vial and added 95µl of acetic acid. Two ml of *o*-pthelaldehyde solution (3.73mM in glacial acetic acid) was added followed by 1 ml conc. H_2SO_4 and was shaken vigorously. After 10 minutes, spectrophotometric measurement was carried out at A_{550}. Concentration of sterols was calculated by comparing with the standard ones.

Statistical analysis of Fisher's LSD was carried out using KaleidaGraph 4.0 (Synergy Software, USA).

5.2.7 Sterol class analysis by HPTLC

Extracted phospholipids and glucocerebrosides were devloped 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.

5.2.8 Al tolerance of the selected cultivars in presence of inhibitors

Four-d-old seedlings were treated with 0.2mM $CaCl_2$ (Cont), 0.66μM fenpropimorph, 0.68μM paclobutrazol or 1.02μM uniconazole-P in 0.2mM $CaCl_2$ 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 8h. 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.

5.3 Results

As two sterol metabolism inhibitors were solubilized in Tween 20, previous checking was done whether it makes any harmful effect on root growth or not. The concentration of 0.0005% of Tween 20 exhibited similar greater relative root elongation (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 was studied using these sterol metabolism inhibitors. Greater root elongation inhibition was observed in the tolerant cultivars irrespective of the species of inhibitor (Fig. 5.3). Concentration of inhibitor which makes the greatest difference in root elongation was selected for next stage of experiment. Here, 0.66μM fenpropimorph, 0.68μM paclobutrazol and 1.02μM uniconazole-P was selected for screening and other experiments with inhibitors. At this stage effect of the sterol metabolism inhibitors on PM permeabilization was also studied. It can be observed that all the cultivars showed mostly green fluorescence which ascribed intact PM (Fig. 5.3).

All rice cultivars showed an increase in PM permeabilization after Al-treatment in presence of lipid metabolism inhibitors (Fig. 5.4). 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 accumulation after Al-treatment in presence of lipid metabolism inhibitors (Fig. 5.5). The results shows almost similar greater Al accumulation in Al treatment with lipid metabolism inhibitors irrespective of the Al tolerance among the cultivars. It also can be observed that increase in Al accumulation was greater in Al tolerant cultivars.

Among the phospholipids, PC, PE content was greater in control of Rikuu-20 (Al-sensitive) than that of Rikuu-132 (Al-tolerant) (Fig. 5.6). 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. 5.6). This result indicates a less contribution of cerebrosides for Al tolerance in rice.

Δ^5-Sterol content was greater in control of Rikuu-132 than that of Rikuu-20, however, differentiation was not clear due to similar d.f. in the development solvent used in the present study (Fig. 5.7). Several other unknown sterols (possibly abnormal sterols) were also detected.

One possible mechanism underlying the variation in Al tolerance between these two cultivars is differential composition of lipids in the PM, as lipid composition can affect surface negativity and membrane tightness. To test this possibility, I quantified the major neutral lipid Δ^5-sterols and negatively charged lipids phospholipids in the root-tips of cultivars with contrasting Al tolerance. In the control treatment, Rikuu-20 had significantly less Δ^5-sterols (2.63±0.05 μmol g^{-1} FW of root-tips) than Rikuu-132 (2.94±0.20 μmol g^{-1} FW of root-tips), while Rikuu-20 had significantly more phospholipids (1.33±0.01 μmol g^{-1} FW of root-tips) than Rikuu-132 (1.20±0.02 μmol g^{-1} FW of root-tips) (Fig. 5.4 A, B). In the Al treatment, the same tendency was observed, although Al treatment decreased Δ^5-sterols and increased phospholipids in both cultivars. These results suggest that Rikuu-132's PM is less negative than that of Rikuu-20, with or without Al treatment.

As it was evident that negatively charged abundance of phospholipids in the PM makes more Al-sensitive and, on the contrary, neutral Δ^5-sterols makes more intact PM, therefore, I calculated the lipid ratio to make a meaningful clarification on these lipid

classes. Lipid ratio (phospholipids/Δ^5-sterols) showed a significant negative exponential relationship with Al tolerance ($y = 33.8x^{-0.67}$, R2 = - 0.667*) (Fig. 5.7).

To make the general clarification on the effect of root elongation, I measured relative root elongation (Al tolerance) in presence of sterol metabolism inhibitors. In presence of inhibitors, Al tolerance did not change significantly for Rikuu-20 (31 and 29% for Al+Fen and Al+Uni, respectively) whereas significant decrease was observed for Rikuu-132 (48 and 35% for Al+Fen and Al+Uni, respectively) (Fig. 5.8A). Although, Al tolerance in presence fenpropimorph decreased severely but fur severe decrease was observed in presence of uniconazole. In fact, Al tolerance in Al+Uni treatment was almost similar to that in Rikuu-20. Therefore, Al tolerance in Al+Uni treatment was also studied in other cultivars within the pedigree. Similar significant decreasing tendency was observed in other tolerant cultivars, i.e., Sasanishiki, Kyoku and Kamenoo (Fig. 5.8B). On the other hand, Al tolerance of other Al-sensitive cultivar (Aikoku) did not differ among Al and Al+Uni treatments.

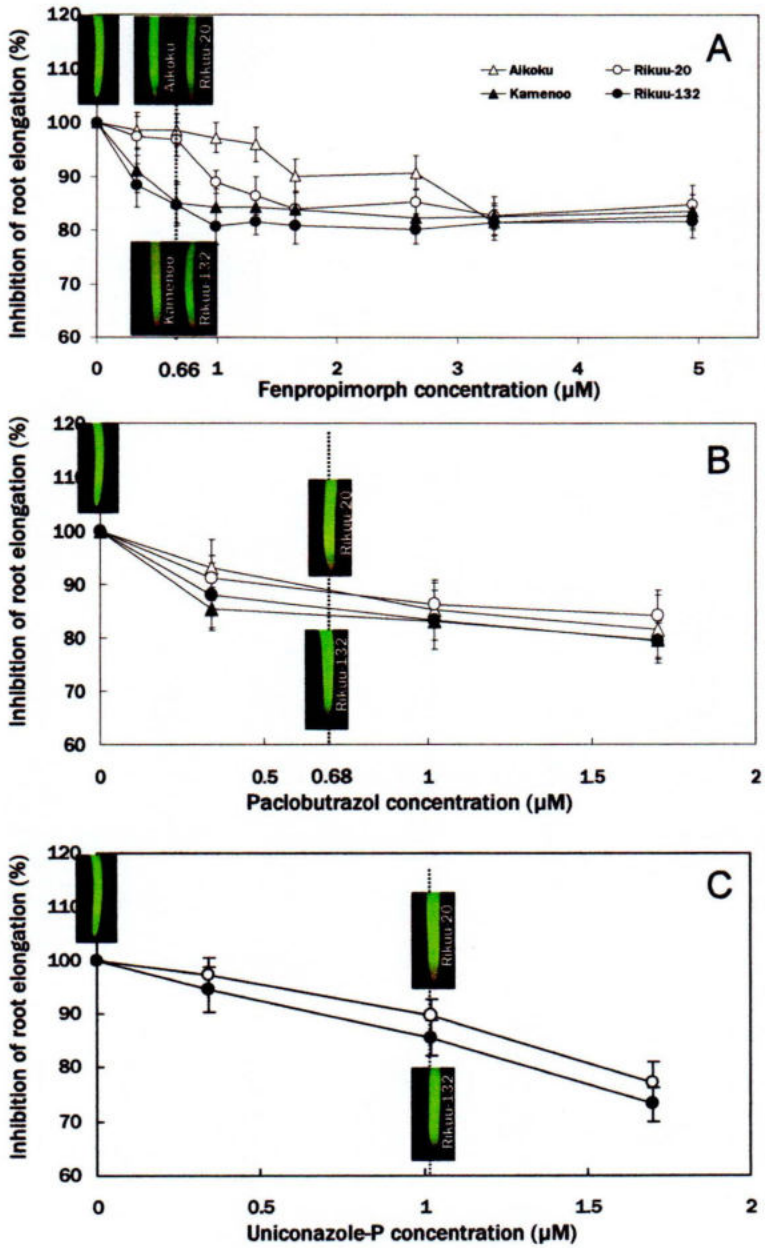


Fig. 5.3: Inhibition of root elongation with the concentration of sterol metabolism inhibitor fenpropimorph (A), paclobutrazol (B) and uniconaxole (C). Figures of roots whows the PM permeabilization of the roots in selected concentration of the inhibitos.

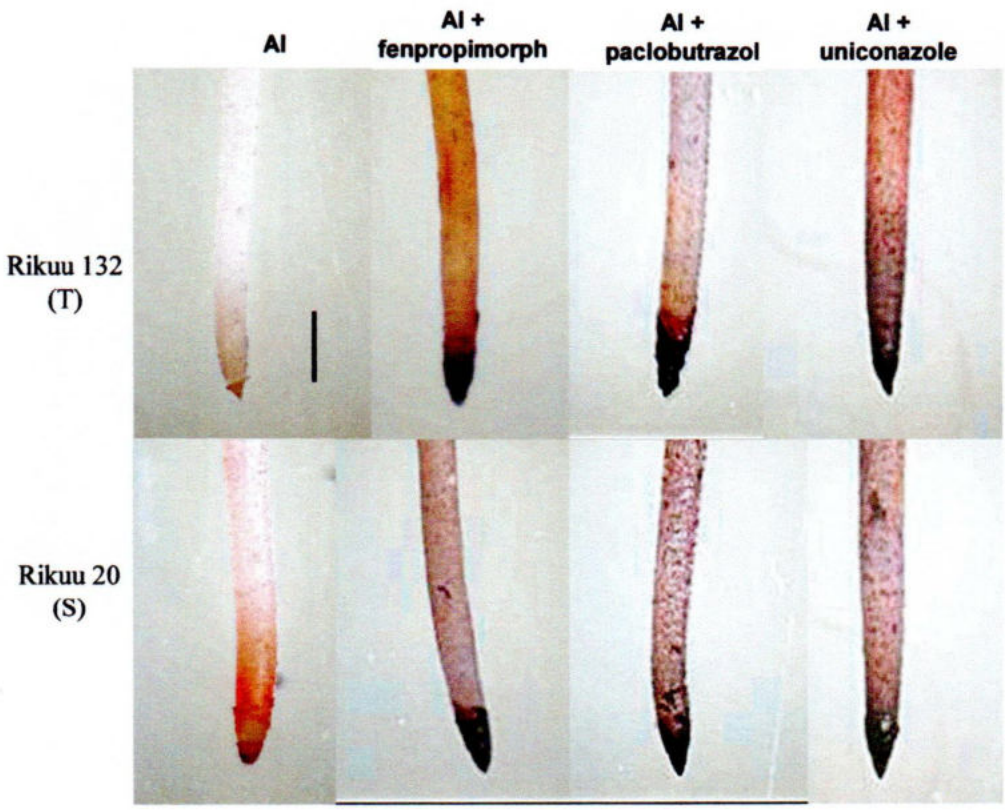


Fig. 5.4: Al accumulation visualized by hematoxylin staining after 24 h Al treatment with sterol metabolism inhibitors. Each of the treatment solutions contained 0.2mM $CaCl_2$ for 24h at pH 4.9, 20μM $AlCl_3$, 0.66μM fenpropimorph, and 1.02μM uniconazole-P, according to the following treatments: Al, Al+Fen and Al+Uni. Photographs are representative of at least three independent observations. Bar = 1 mm.

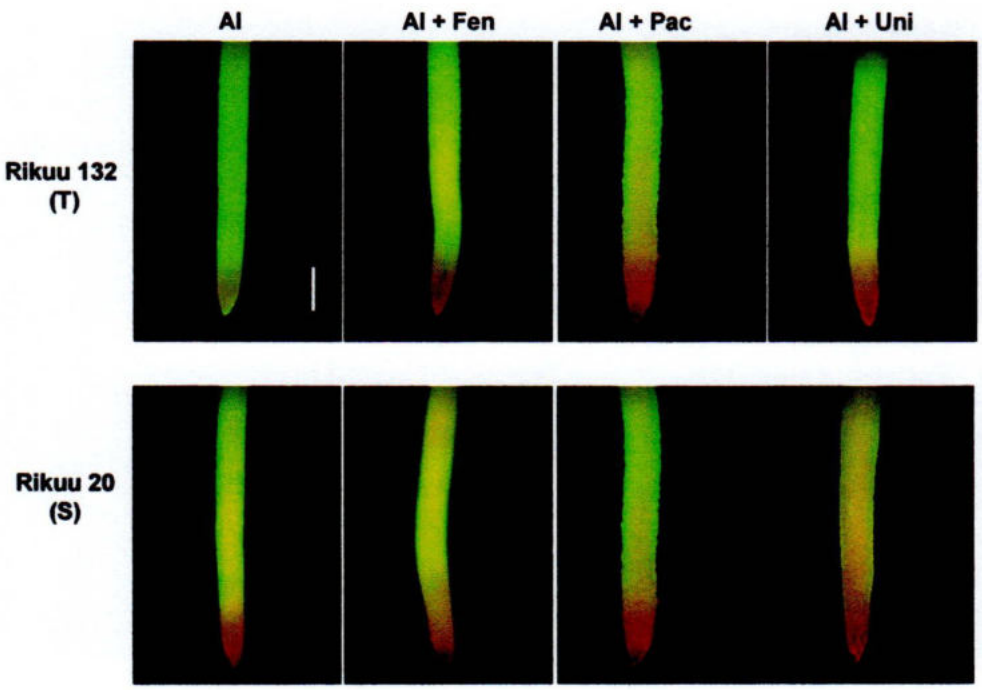


Fig. 5.5: PM permeability visualized using FDA-PI fluorescence microscopy after 24 h Al treatment with sterol metabolism inhibitors. Each of the treatment solutions contained 0.2mM $CaCl_2$ for 24h at pH 4.9, 20µM $AlCl_3$, 0.66µM fenpropimorph, and 1.02µM uniconazole-P, according to the following treatments: Al, Al+Fen and Al+Uni. Photographs are representative of at least three independent observations. Bar = 1 mm.

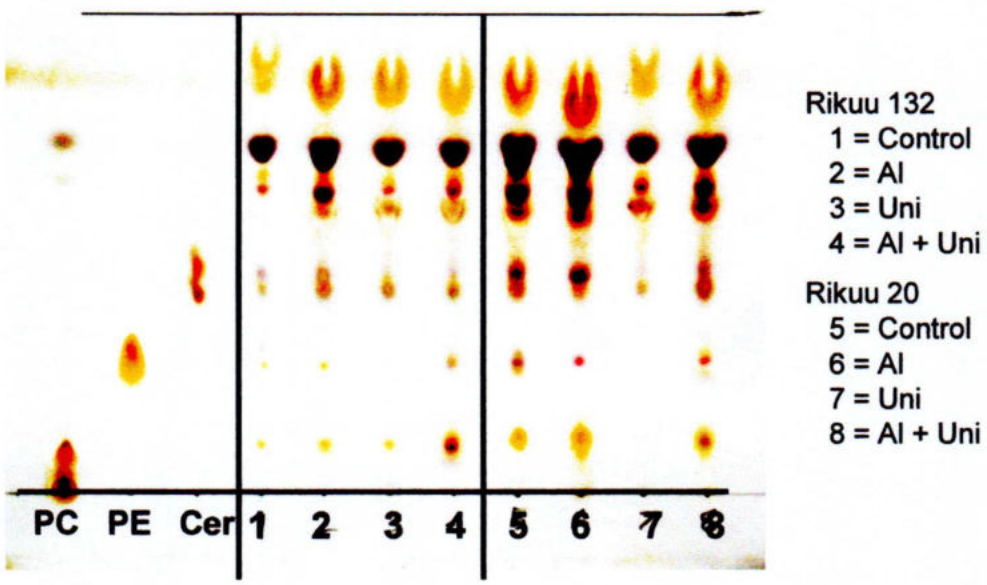


Fig. 5.6: Phospholipid class analysis by HPTLC after treatment in control, Al, uniconazole-P and Al+uniconazole-P for Rikuu-20 and Rikuu-132. Samples were extracted as described in Materials and Methods. Development solvent: chloroform:methanol:acetic acid = 65:25:8, color development by 20% H_2SO_4 in methanol and heating.

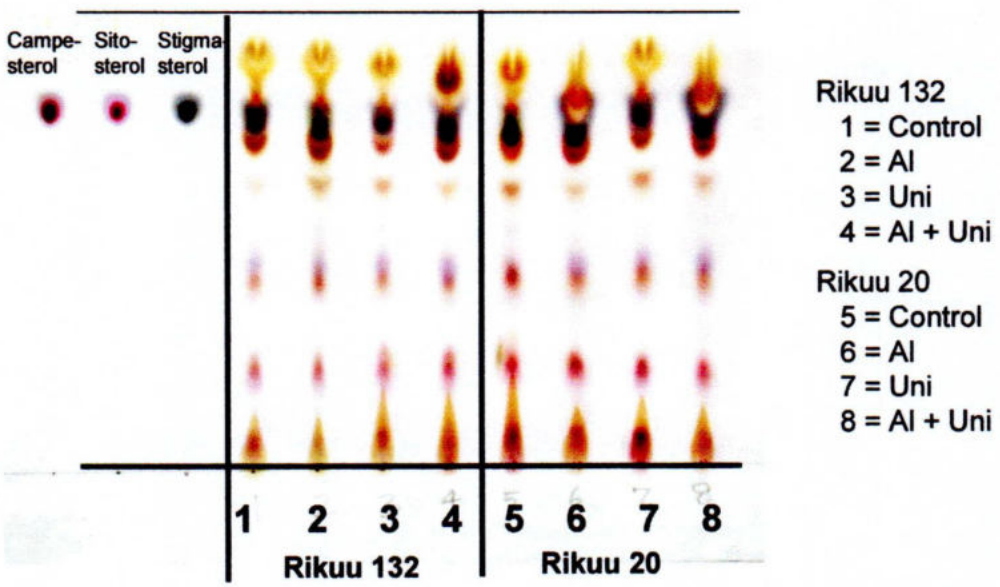


Fig. 5.7: Δ^5-Sterol analysis by HPTLC after treatment in control, Al, uniconazole-P and Al+uniconazole-P for Rikuu-20 and Rikuu-132. Samples were extracted as described in Materials and Methods. Development solvent: dichloromethane:methanol = 85:15, color development by 20% H_2SO_4 in methanol and heating.

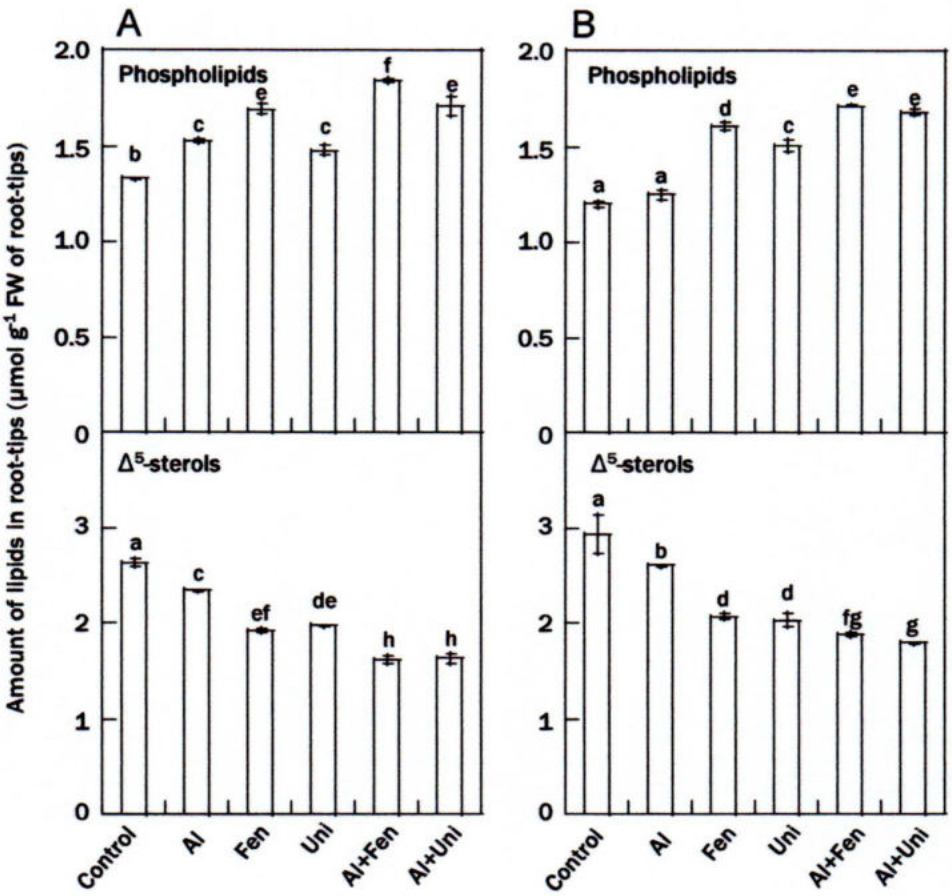


Fig. 5.8: Phospholipids and Δ^5-sterols in root-tips (0-1 cm from root-tip portion) of 4-day-old Rikuu-20 (A) and Rikuu-132 (B) seedlings. Each of the treatment solutions contained 0.2mM $CaCl_2$ for 24h at pH 4.9, 0 or 20μM $AlCl_3$, 0 or 0.66μM fenpropimorph, and 0 or 1.02μM uniconazole-P, according to the following treatments: control, Al, Al+Fen and Al+Uni. Values are means ± SE (n = 2). Values with same letter(s) in the same lipid classes are not significantly different at 5% significance level (Fisher's LSD).

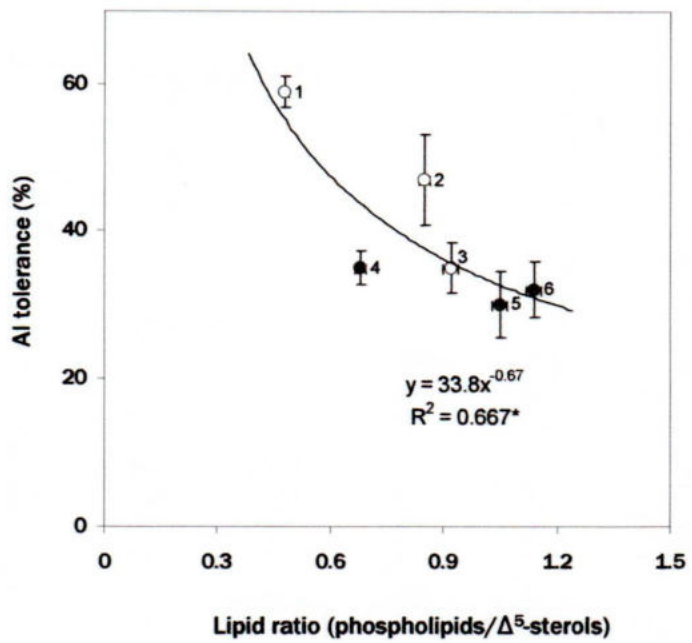


Fig. 5.9: Relationship between lipid ratio (phospholipids/Δ^5-sterols) and relative root elongation (%, +Al/-Al), i.e., Al tolerance of cv. Rikuu-132 (open circles) and cv. Rikuu-20 (closed circles). Each of the treatment solutions contained 20µM $AlCl_3$ in 0.2mM $CaCl_2$ for 24h at pH 4.9, 0.66µM fenpropimorph, and 1.02µM uniconazole-P, according to the following treatments: Al, Al+Fen and Al+Uni. 1 & 4, Al treatment of Rikuu-132 and Rikuu-20, respectively; 2 & 5, Al+Fen treatment of Rikuu-132 and Rikuu-20, respectively; 3 & 6, Al+Uni treatment for Rikuu-132 and Rikuu-20, respectively. Values are means ± SE ($n \geq 10$ for Al tolerance, $n = 2$ for lipid ratio). Circles without SE indicate small SE values.

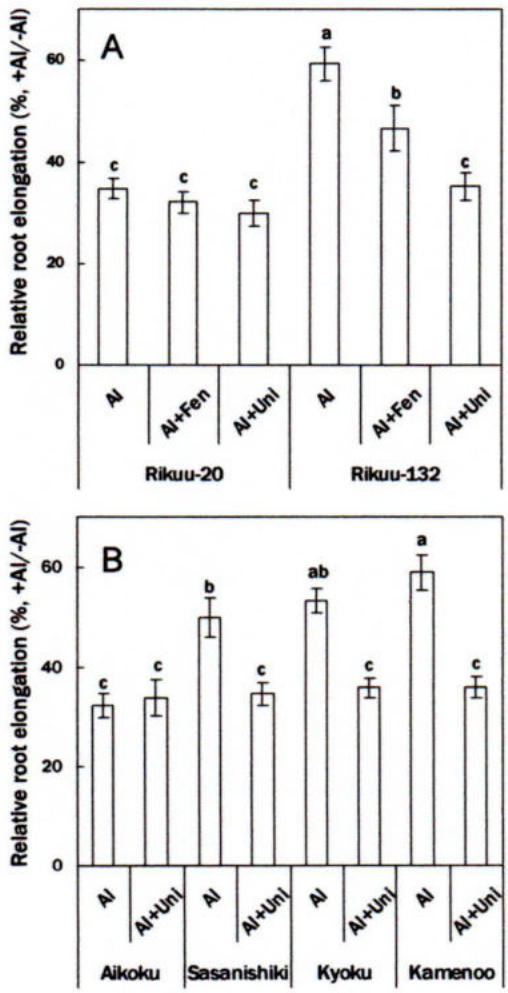


Fig. 5.10: Relative root elongation (%, +Al/-Al), i.e., Al tolerance of rice cultivars Rikuu-20 and Rikuu-132 after Al treatment with two sterol metabolism inhibitors (fenpropimorph and uniconazole-P) (A). Cultivars Aikoku, Kyoku, Sasanishiki and Kamenoo under uniconazole-P treatment (B). Values are means ± SE ($n \geq 10$). Average values with same letter(s) within all cultivars and treatments are not significantly different at 5% level of significance (Fisher's LSD).

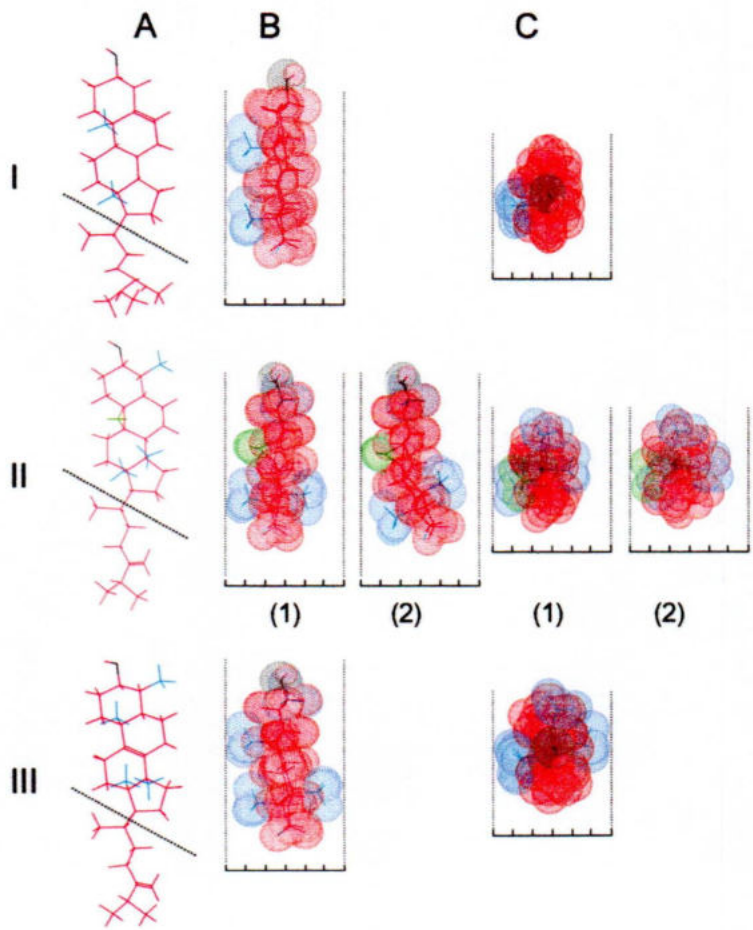


Fig. 5.11: Chemical structures of three typical sterols (A) and van der Waals conformations of these sterols without fatty acyl chains (constructed using Chem3D Ultra 7.0) (B, C). I, sitosterol; II, cycloeucalenol; III, obtusifoliol. B, side view. C, upper view. Cycloeucalenol has straight (1) and bending (2) conformations that coexist (Milon et al. 1989). Each sterol structure is separated from fatty acyl chain by dotted line. 3β-Hydroxyl group shown in black; α-methyl groups shown in blue; 9,19-cyclopropane ring shown in green. Scale bar shows the arbitrary unit.

5.4 Discussion

Fenpropimorph, N-substituted morpholine, is a systemic fungicide for controlling powdery mildew and rust in cereal crops, and inhibits cycloeucalenol obtusifoliol isomerase (COI) as primary target: As a result, abnormal sterols, i.e., 24-methylpollinastanol, 24-dihydrocycloeucalenol, and cycloeucalenol which can be detected only a trace amount in normal conditions are highly enriched in PM, but on the contrary, normal final sterol products (phytosterols), i.e., campesterol, sitosterol and stigmasterol are considerably decreased (Burden et al. 1987, Grandmougin et al. 1989) (Fig. 5.2). On the other hand, (2RS,3RS)-paclobutrazol and uniconazole-P, triazole-type heterocycle compounds, are plant growth regulators with antifungal activity, and inhibit obtusifoliol-14α-demethylase (OBT 14DM) (Haughan et al. 1988, Rademacher 2000). Though there are several isomers of paclobutrazol, in this experiment I decide to use (2RS,3RS)-paclobutrazol which has been reported to inhibit sterol metabolism more severely than other isomers. After inhibition in specific stage, considerable decrease in normal phytosterols with simultaneous increase in abnormal sterol, i.e., obtusifoliol, dihydroobtusifoliol and 14α-methyl-Δ^8-ergostenol and these abnormal sterols mostly accumulated in the PM. Kolesnikova et al. (2006) reported for the first time a new dicotyledonous phytosterol metabolic pathway, from 2,3-oxidoqualene to lanosterol, which had been considered as the specific pathway for fungi and animals.

Fenpropimorph inhibited root elongations for Aikoku, Rikuu-20, Kamenoo and Rikuu-132, and inhibition was greater for Al-tolerant two cultivars than for Al-sensitive two cultivars (Fig. 5.3A). At 0.66μM fenpropimorph which induced the greatest differences in the inhibition of root elongations between average values of each two

cultivars with different Al tolerance. In this stage, PM of both the cultivars was almost intact irrespective of the inhibitors. Although only one picture is placed on Y-axis, PM intactness was common for all cultivars, and therefore other three pictures were omitted. (2RS,3RS)-paclobutrazol tends to inhibit root elongations greater for Al-tolerant two cultivars than for Al-sensitive two cultivars and at 0.68μM (2RS,3RS)-paclobutrazol which induced the greatest difference in the inhibition of root elongations, similar PM permeabilization was recognized for all cultivars (Fig. 5.3B).

Uniconazole-P tends to inhibit root elongations greater for Al-tolerant Rikuu-132 than for Al-sensitive Rikuu-20 and at 1.02μM uniconazole-P which induced the greatest difference in the inhibition of root elongations similar PM permeabilization was recognized for all cultivars (Fig. 5.3C).

Although PM permeabilization was greater or least for Rikuu-20 or Rikuu-132 without inhibitors respectively, those were greater for both cultivars after Al treatment irrespective of inhibitors to exhibit similar permeabilizations (Fig. 5.5). Corresponding similar results were observed in Al accumulations in root-tip portions: Al accumulation was greater or less for Rikuu-20 or Rikuu-132 after Al treatment without inhibitors respectively, however those were greater for both cultivars after Al treatment irrespective of inhibitors to exhibit similar Al accumulations.

In later stage of experiments, I selected Rikuu-132 as the representative of Al-tolerant cultivar and Rikuu-20 as the representative of Al-sensitive cultivar. Al tolerances in Rikuu-20 in the presence of all inhibitors were not different but on the contrary, Al tolerance for Rikuu-132 without inhibitors was greatest followed by that in the presence of fenpropimorph or (2RS,3RS)-paclobutrazol, and that in the presence of uniconazole-P

was least (Fig. 5.8A). Inhibitory effect of fenpropimorph, however, was not severe like in case of uniconazole-P. Al tolerance for Rikuu-132 in the presence of uniconazole-P was not different significantly from that for Rikuu-20 without inhibitors.

Significantly lesser Δ^5-sterols were measured in control of Rikuu-20 (2.63μM g^{-1} FW of root-tip) than that of Rikuu-132 (2.94μM g^{-1} FW of root-tips) (Fig. 5.8). For both cultivars Δ^5-sterols was decreased significantly by each treatment in the following order: Al > inhibitor without Al > inhibitor with Al. Reverse general tendencies were observed in phospholipids. Phospholipids were greater in control of Rikuu-20 (1.33μM g^{-1} FW of root-tip) than that of Rikuu-132 (1.20μM g^{-1} FW of root-tip). In Al treatment, although phospholipids were increased for Rikuu-20, those were not increased for Rikuu-132. Inhibitors increased phospholipids for Rikuu-132 significantly, however the greatest increase was observed for both cultivars in the treatments with Al. Although no significant relationships were recognized between Al tolerance and Δ^5-sterols or phospholipids in root-tips of both cultivars among all 6 samples, i.e., treatments with (1) Al, (2) Al with fenpropimorph, or (3) Al with uniconazole-P (data not shown), significant negative exponential relations was recognized between Al tolerance and molar lipid ratio (phospholipids/Δ^5-sterols) ($R^2 = 0.668$*) (Fig. 5.9).

All inhibitors decreased the amount of Δ^5-sterols and increased phospholipids in the membranes of both cultivars (Fig. 5.6). These changes in the tolerant cultivar Rikuu-132 were relatively larger than in the sensitive cultivar Rikuu-20. Treatment with inhibitors resulted in an increase in the ratio of phospholipids to Δ^5-sterols in Rikuu-132, to a level similar to that of Rikuu-20 (Fig. 5.4). This made the root-tip cells of Rikuu-132 leaky during Al treatment, as shown by FDA-PI staining, and it increased Al accumulation to a

level similar to that of sensitive Rikuu-20 (Fig. 5.5). Al tolerance of Rikuu-132, judged by root elongation, was also suppressed by both inhibitors, while that of Rikuu-20 was unchanged after inhibitor treatments (Fig. 5.8A). Of the two inhibitors, the OBT 14DM inhibitor uniconazole-P decreased Al tolerance of Rikuu-132 to a greater extent, resulting in similar Al tolerance to the Al-sensitive Rikuu-20. A similarly strong inhibitory effect was observed in other tolerant cultivars (Sasanishiki, Kyoku, and Kamenoo) in Al+uniconazole-P treatment (Fig. 5.8B). However, the sensitive cultivar Aikoku was not inhibited in these conditions, similarly to Rikuu-20 (Fig. 58B).

Differences in capacities to induce cooperative intermolecular van der Waals interactions with neighbouring phospholipids within membranes have been pointed out (Milon et al. 1989). Figure 5.11 shows the chemical structures and corresponding van der Waals conformations of typical sterols calculated and drawn by Chem 3D Ultra 7.0 computer program. Sitosterol (in Fig. 5.11) is an example for typical and normal phytosterols (7 in Fig. 5.2), cycloeucalenol (II in Fig. 5.11) is an example for abnormal sterols which are enriched after treatment with fenpropimorph (3 in Fig. 5.2), and obtusifoliol (III in Fig. 11) is an example for abnormal sterols which are enriched after treatment with (2RS,3RS)-paclobutrazol or uniconazole-P (4 in Fig. 5.2). Two conformations II(1) and II(2) were drawn on cycloeucalenol as two conformations II(1) and II(2) coexist, differing mainly at ring C (Milon et al. 1989). The sterol ring system of cycloeucalenol is bent, forced into a non-planar conformation by the 9,19-cyclopropyl group on the β-face of this molecule (Dahl et al. 1980). As α-face is curvilinear, the 14α-methyl group is not exposed. Finally, comparing each van der Waals volume, obtusifoliol is greatest followed by cycloeucalenol, and sitosterol was confirmed to be least.

Conclusively it can be said that obtusifoliol-14α-demethylase (OBT-14DM) is one of the main target for Al tolerance in rice.

Chapter 6

Clarification of lipid composition for Al tolerance in other crop species

Chapter 6

Clarification of lipid composition for Al tolerance in other crop species

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 (20μM $AlCl_3$ 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°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 2.5μM $AlCl_3$ 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.

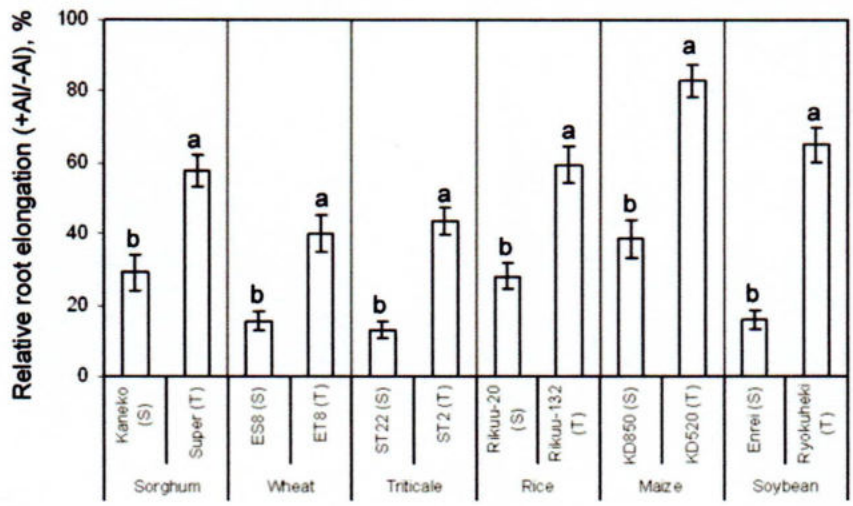


Fig. 6.1 Al tolerance of the representative cultivars of sorghum, wheat, triticale, rice maize and soybean. Al treatment for sorghum was 2.5μM $AlCl_3$, in all other cases Al tolerance was in 20μM $AlCl_3$. Al tolerance of sorghum, maize and soybean based on Afrin et al. (2009). Al tolerance of wheat, triticale were based on Wagatsuma et al. 2005 and Al tolerance of rice was based on Khan et al. (2009).

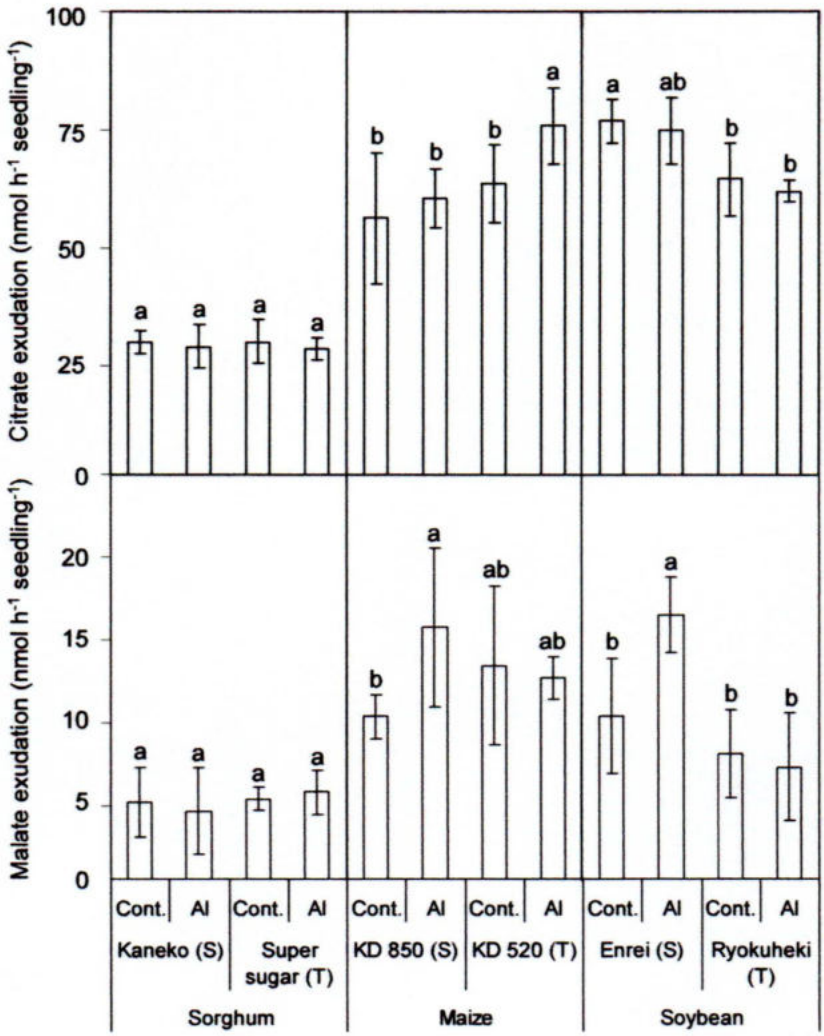


Fig. 6.2. Citrate (A) and malate (B) exuded from sorghum, maize and soybean. Five-day-old seedlings were treated for 5 h with or without Al in 0.2 mM $CaCl_2$ (pH 4.9) following 5 h of pretreatment with 0.2 mM $CaCl_2$ (pH 4.9). Exudates were collected during the 5 h treatment. Values are means ± SE (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.

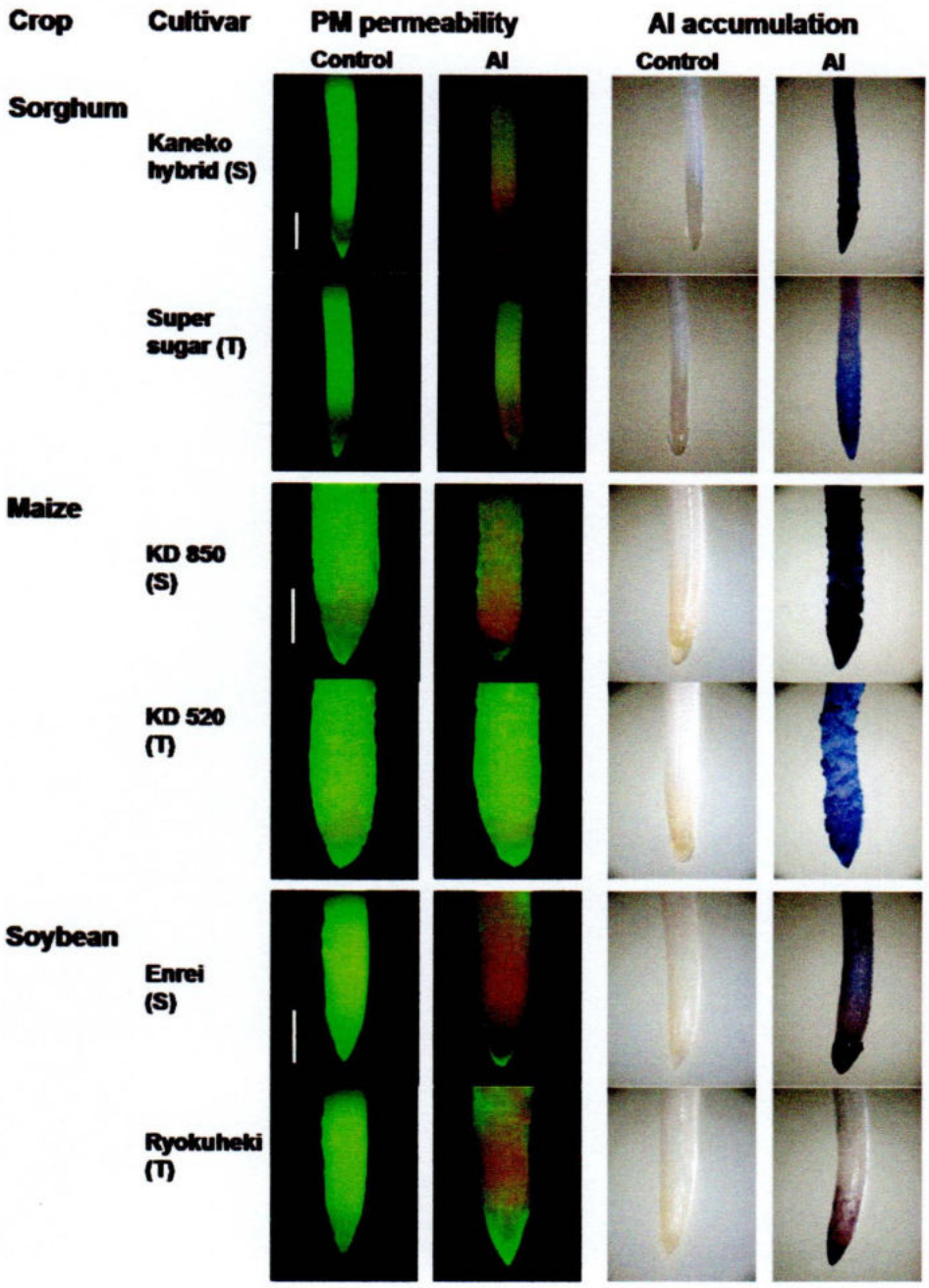


Fig. 6.3: Plasma membrane (PM) permeability by FDA-PI fluorescence microscopy and Al accumulation by hematoxylin staining light microscopy. Al treatment conditions were same as in Fig. 6.1. Green fluorescence in FDA-PI staining indicates intact PM, red fluorescence indicates permeabilized PM. Dark purple 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.

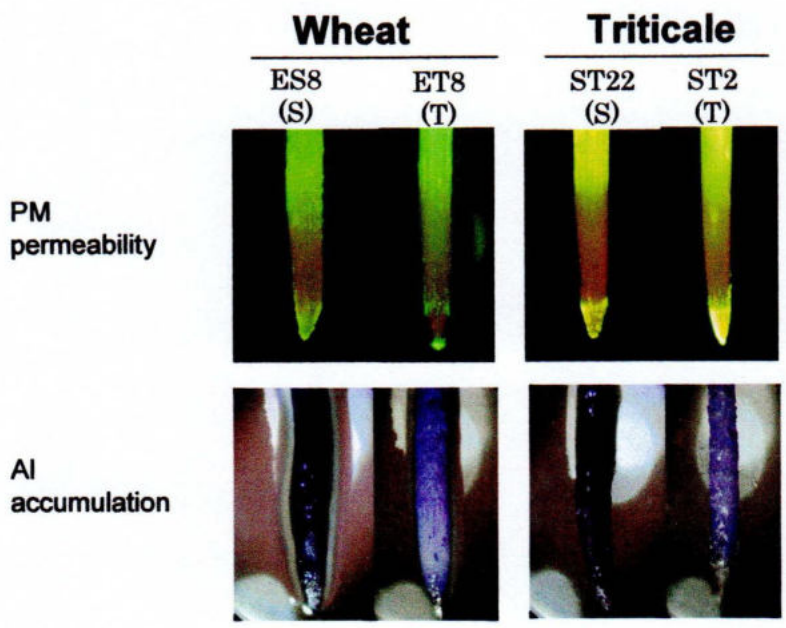


Fig. 6.4: PM permeability and Al accumulation of wheat (Wagatsuma et al. 2005) and triticale (Wagatsuma et al. 2005). Treatment conditions were same as in Fig. 6.1. Photograph are representative of at least three independent observations.

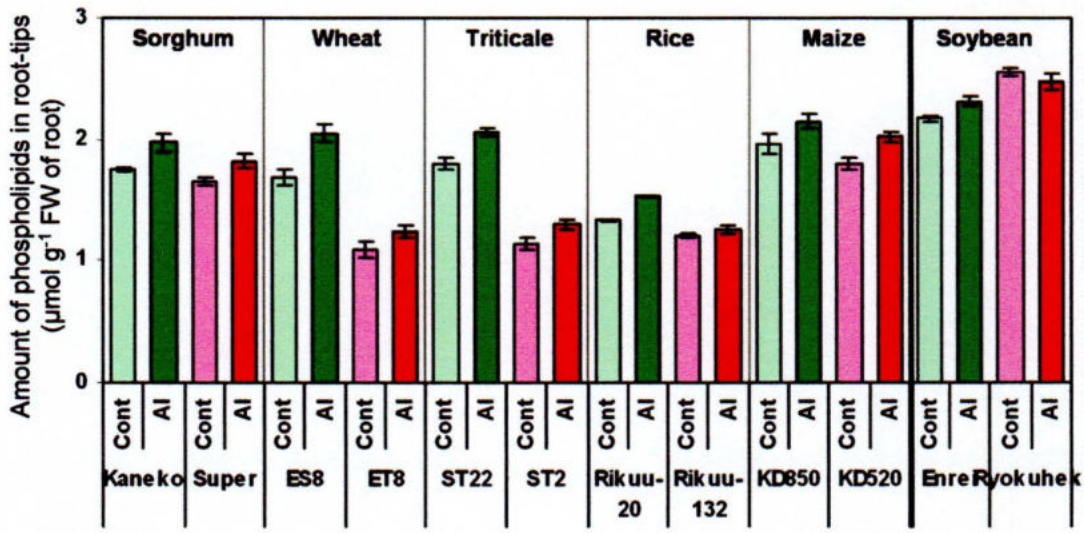


Fig. 6.5: Amount of phospholipids in the root tips for control and Al treatment of the selected cultivars. Treatment conditions were same as described in Fig. 6.1. Data are average ±SE (n = 2).

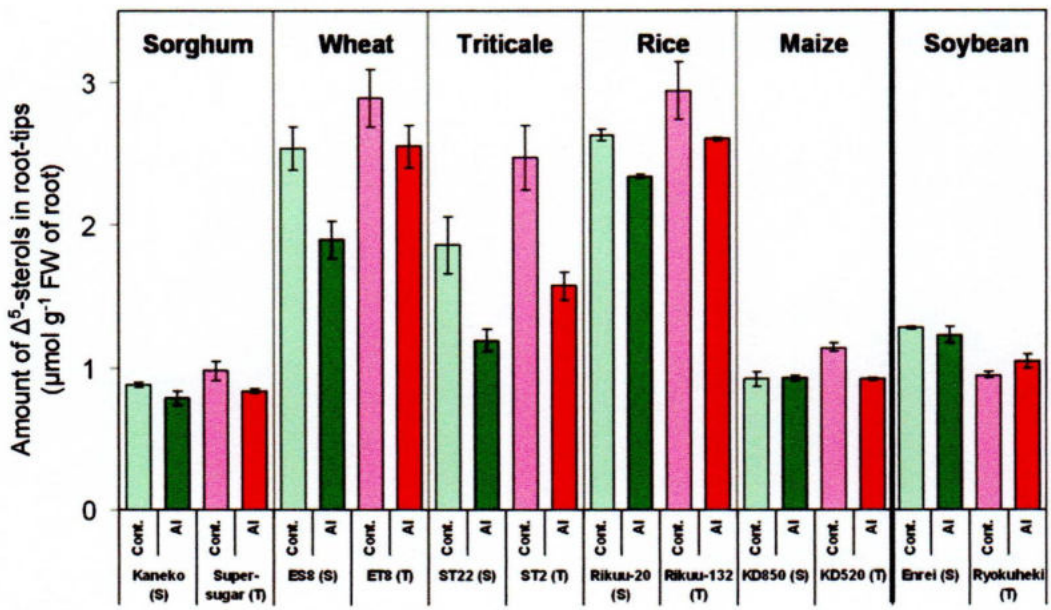


Fig. 6.6: Amount of Δ^5-sterols in the root-tips (0-10mm) for control and Al treatment. Treatment conditions were same as described in Fig. 6.1. Data are mean±SE (n = 2).

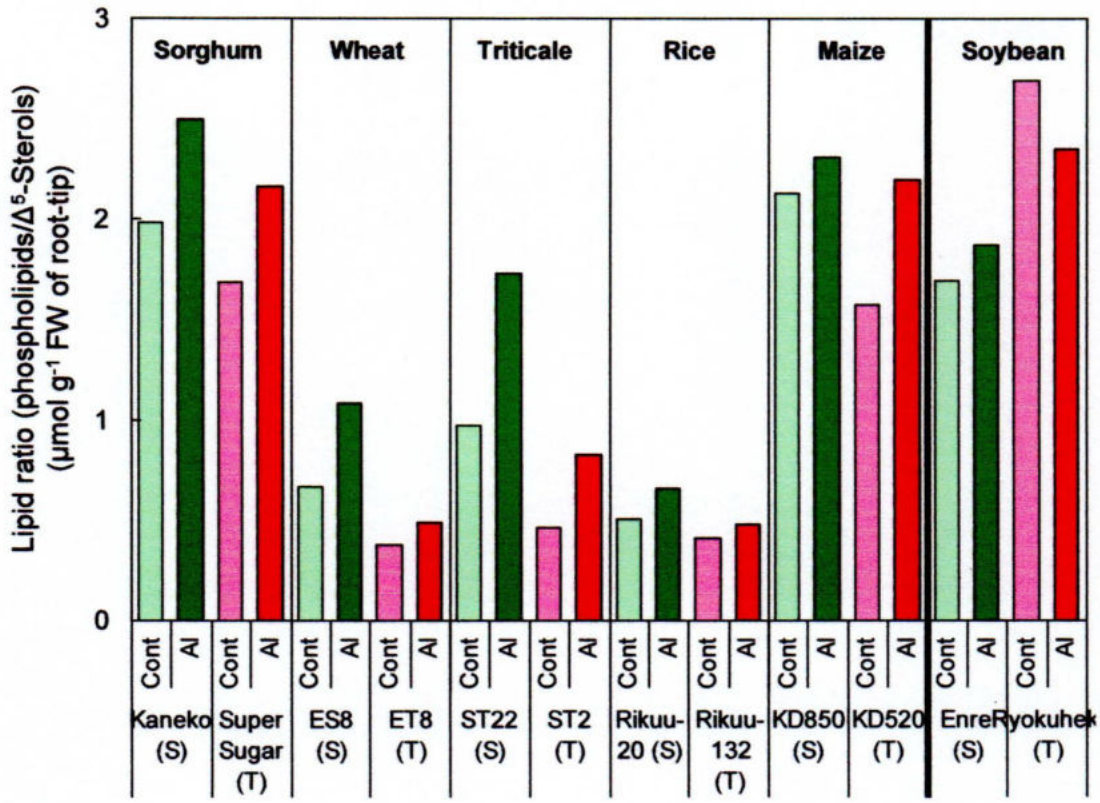


Fig. 6.7: Lipid ratio (phospholipids/Δ^5-sterol) in root tip portion. Data are ratio of average values obtained in Fig. 6.4 and 6.5.

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 1/5th 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) (1/5th 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, Kamenoo>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, Kamenoo>>> Domannaka> Aikoku>Rikuu-20, BR34 and whole plant Al tolerance in LN was in the order of BR41 > Rikuu-132, Sasanishiki> Domannaka > Kamenoo >>> 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.

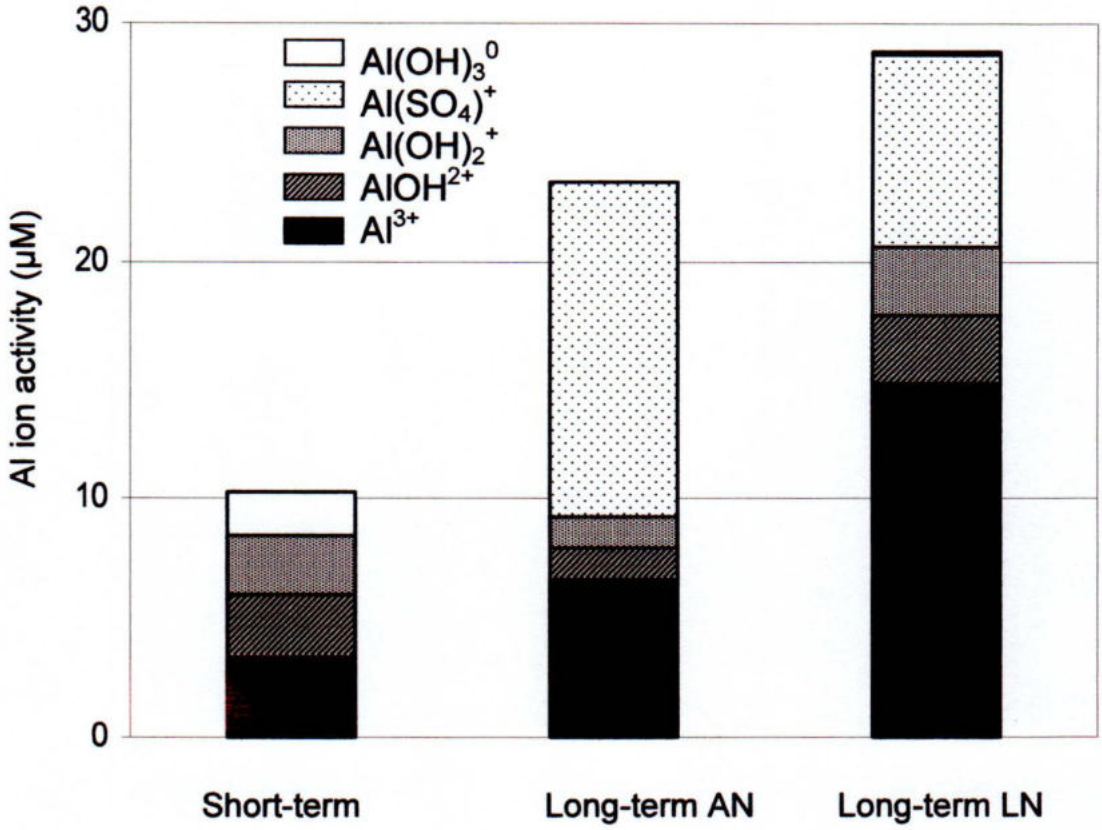


Fig. 7.2: Al ion activity in the culture solution. In short-term (24 h) added Al concentration was 20μM. In long-term experiments added Al and P concentration was 370μM and 230μM respectively. AN, adequate nutrient; LN, low nutrient (added nutrient concentration is $1/5^{th}$ of AN).

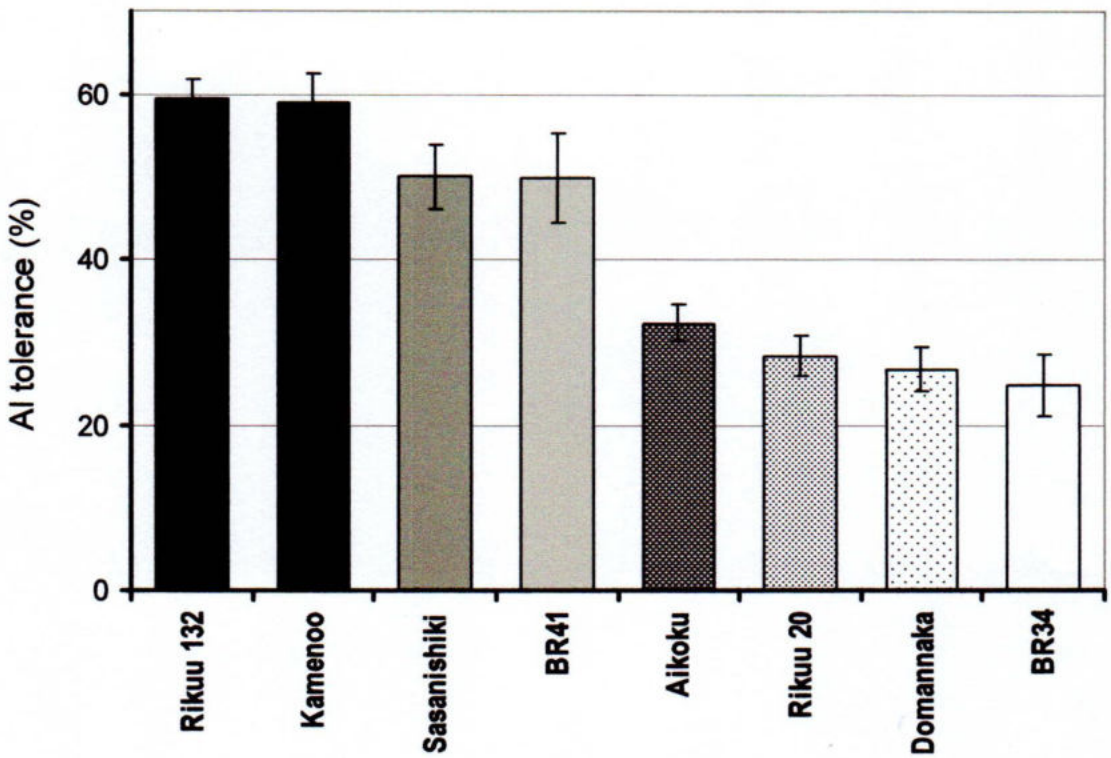


Fig. 7.3: Short-term Al tolerance of the rice cultivars. Control treatment: 0.2mM $CaCl_2$ at pH 4.9 for 24h. Al treatment: 20μM $AlCl_3$ in 0.2mM $CaCl_2$ at pH 4.9 for 24h. Relative net root elongation was used as Al tolerance. Dotted line indicates average Al tolerance in all cultivars. Denser color indicates higher Al tolerance. Bar indicate ±SE, n = 12.

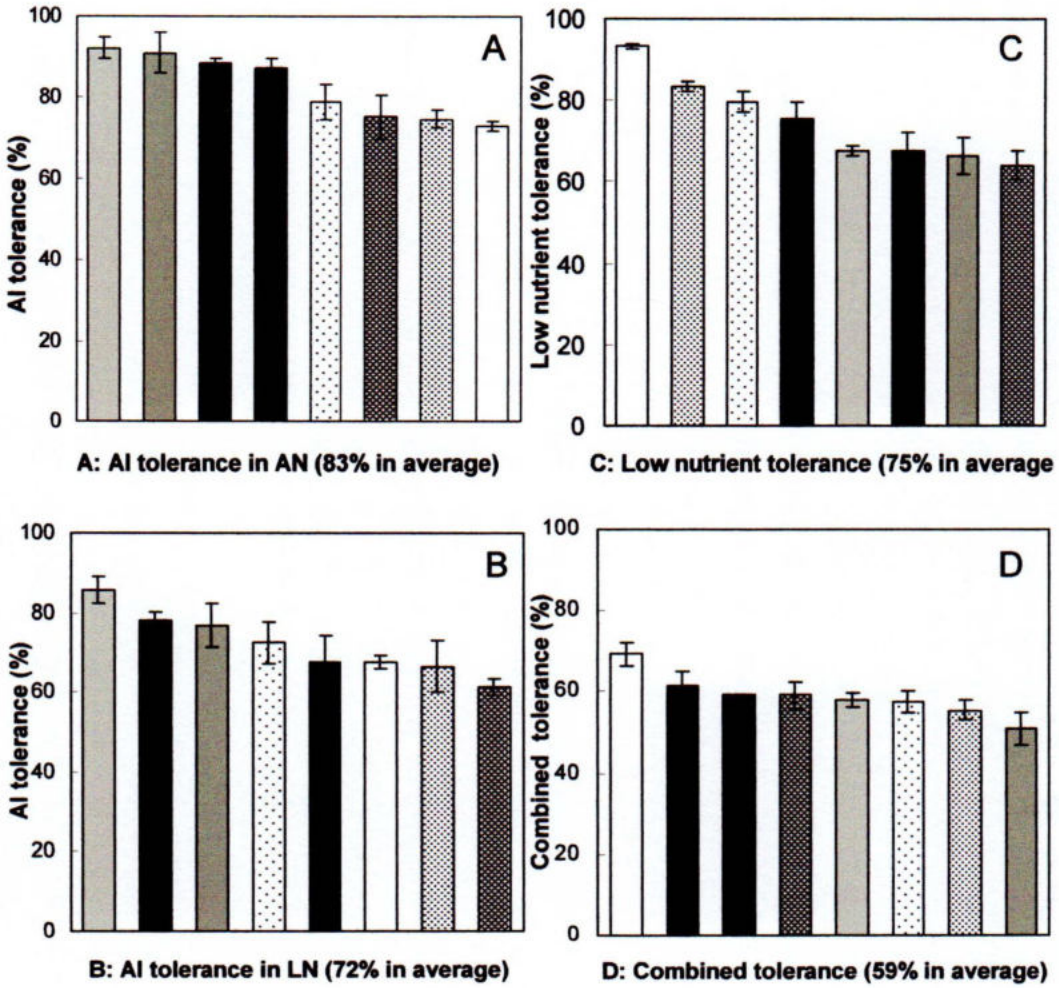


Fig. 7.4: Relative tolerances in long-term (35 d) hydroponics culturing experiment. A. Al tolerance in AN conditions, B. Al tolerance in LN conditions, C. Low nutrient tolerance and D. Combined tolerance. Definitions of the tolerances were described in the chapter Materials and Methods. Color of each 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

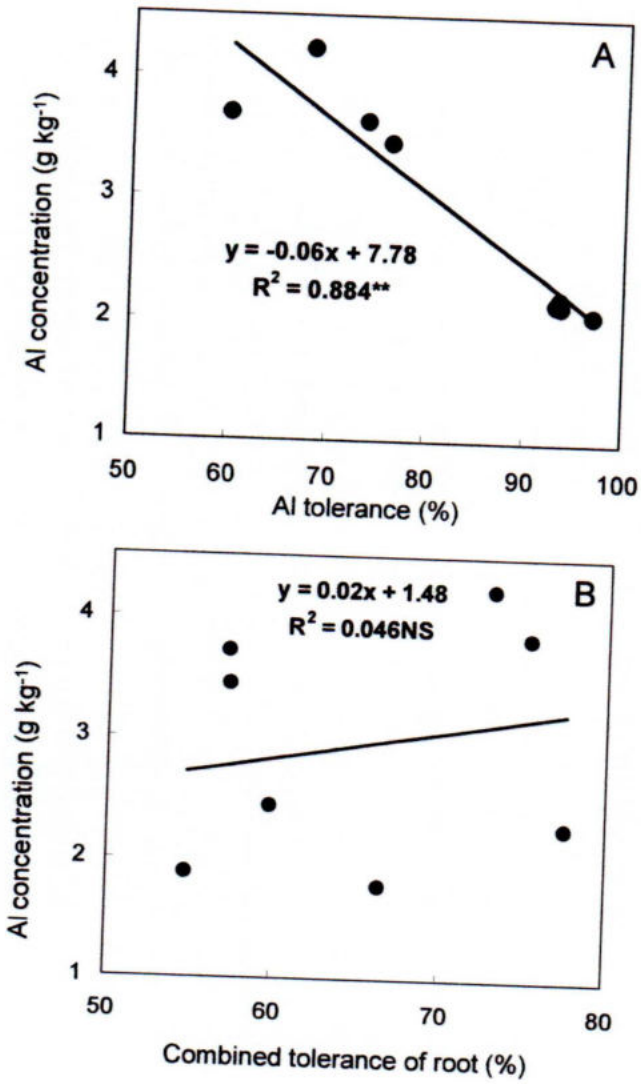


Fig. 7.5: Relationship of Al concentration with Al tolerance (A) and combined tolerance (B)

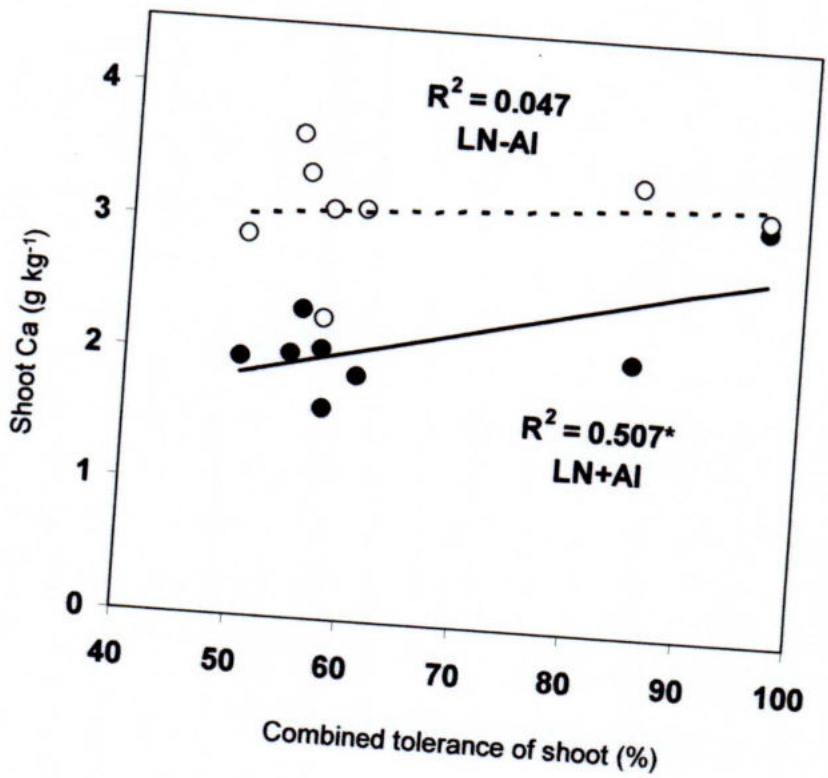


Fig. 7.6: Relationship of shoot Ca concentration with combined tolerance. Open circles are in LN condition, closed circles in LN+Al conditions

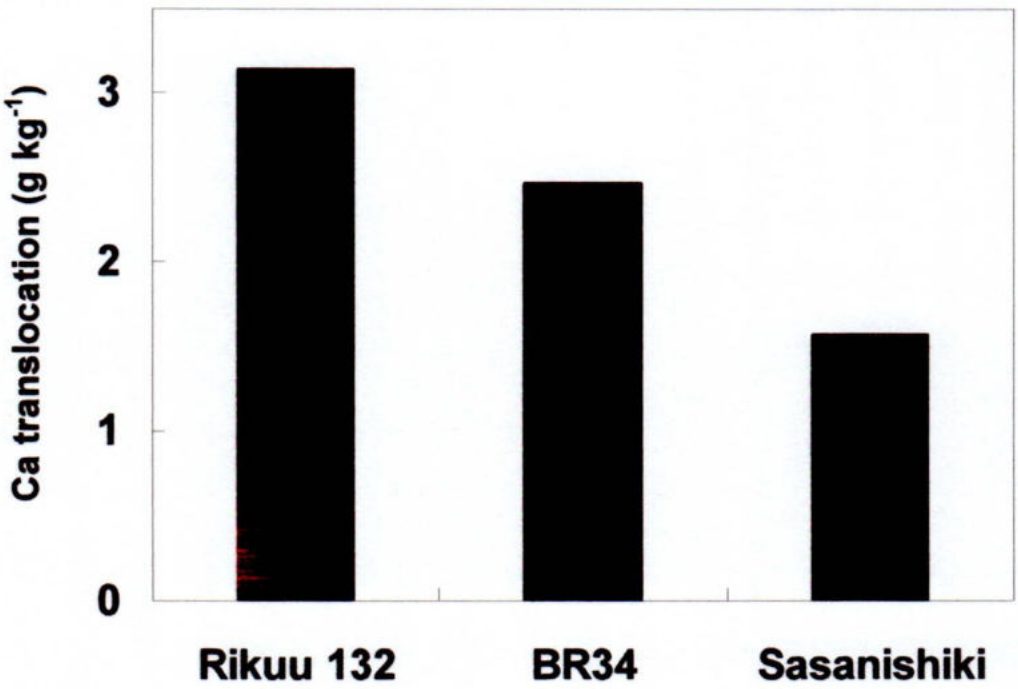


Fig. 7.7: Ca translocation from root to shoot of typical cultivars. Values are mean of 3 replicated samples

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).

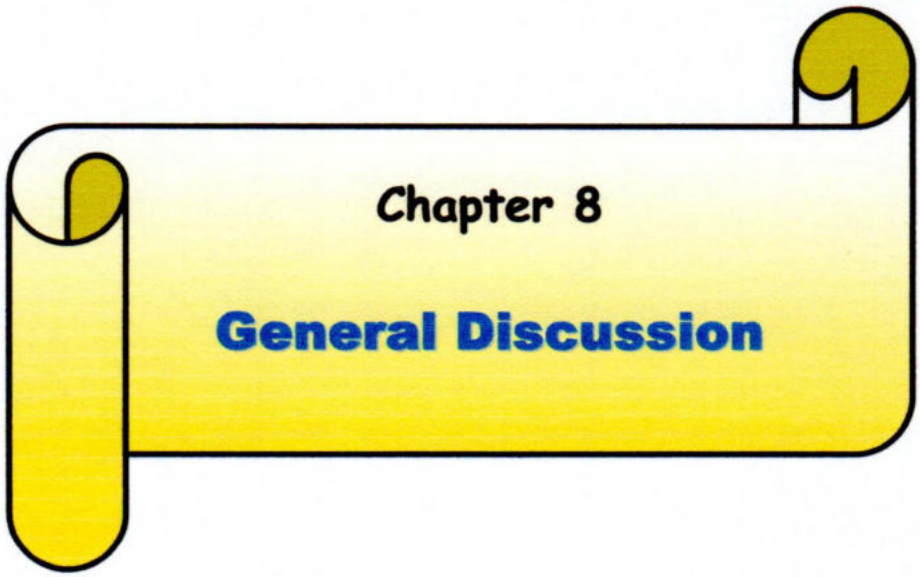
Although, Al tolerance is related mainly with the Al status in the plant in any nutrient condition, in the present experiment while using only rice crop for Al tolerance study, Al toxicity was not found as a main factor in combined condition (Figure 7.5B. For combined tolerance, Ca status can be ascribed 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

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.

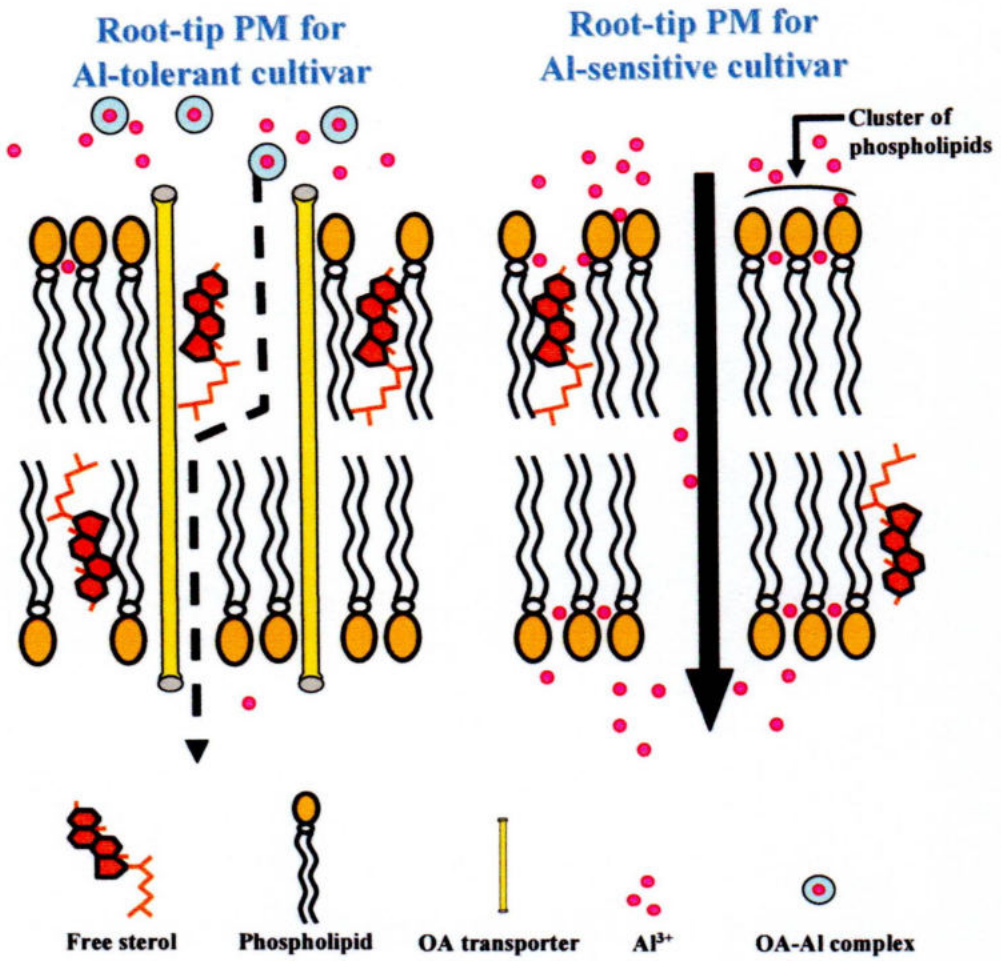


Fig. 8.1. Schematic representation of Al-tolerance strategy by PM lipid bilayer with less PM negativity, less permeability and OA exudation in wheat and maize.

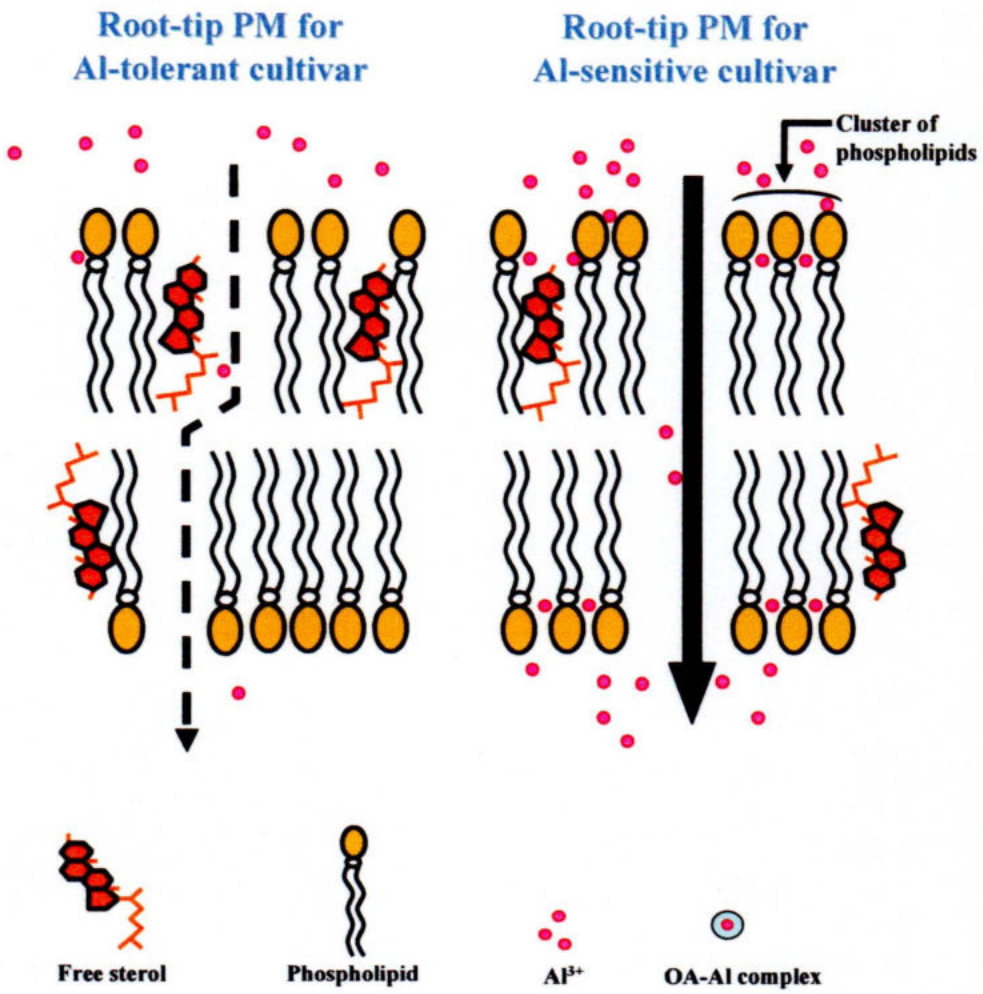


Fig. 8.2. Schematic representation of Al-tolerance strategy by PM lipid bilayer with less PM negativity and less permeability in sorghum, triticale and rice.

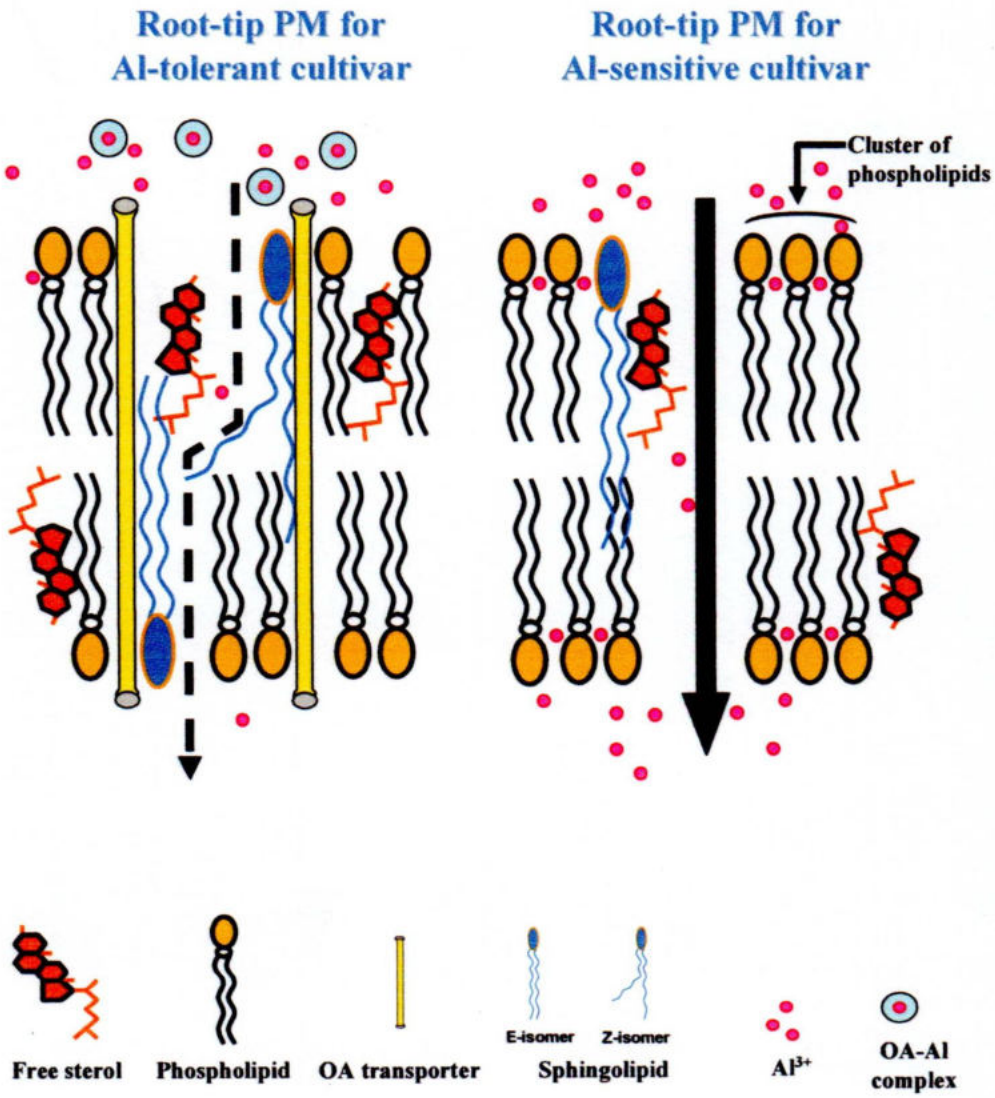


Fig. 8.3. Schematic representation of Al-tolerant PM to be prepare in the future with less PL, less permeability, greater Z-isomer of sphingolipids and greater OA transporter.

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).

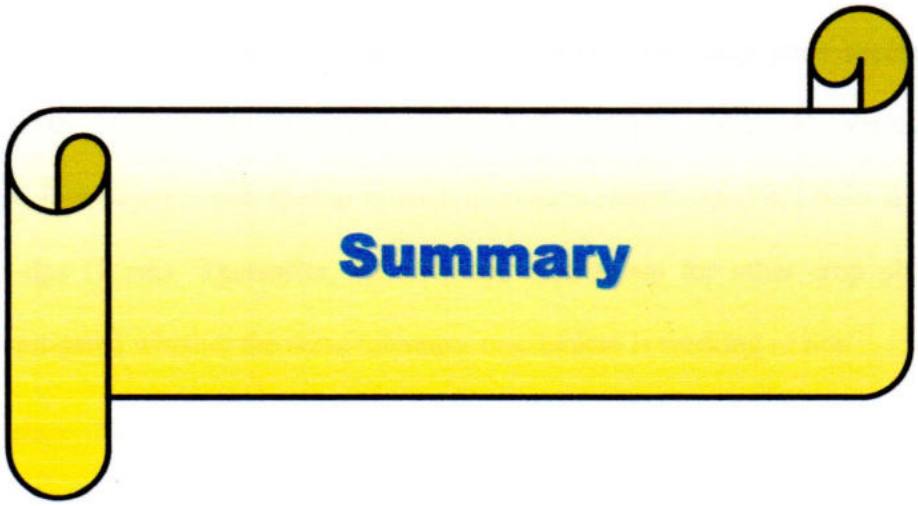
In the present study, root Al content showed significant positive correlation with Al tolerance (Figure 7.5A). Clear differential Al content was also found in tolerant and sensitive group of cultivars. In AN nutrient condition Al tolerance increases with the decrease in root Al connect which was also 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

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、

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、Ａｌ含有率、膜透過性のいずれもが増大し、Ａｌ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Ａｌ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、Ａｌ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Ａｌの同時処理のデータを総合した結果、PL/S 比とＡｌ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Ａｌ感受性品種である陸羽２０号、愛国のＡｌ耐性値は変わらないのに対し、Ａｌ耐性品種である亀の尾、旭、ササニシキのＡｌ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Ａｌ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、Ａｌ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Ａｌ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、Ａｌ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、Ａｌ耐性品種間差と根端のＡｌ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Ａｌ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、Ａｌ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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