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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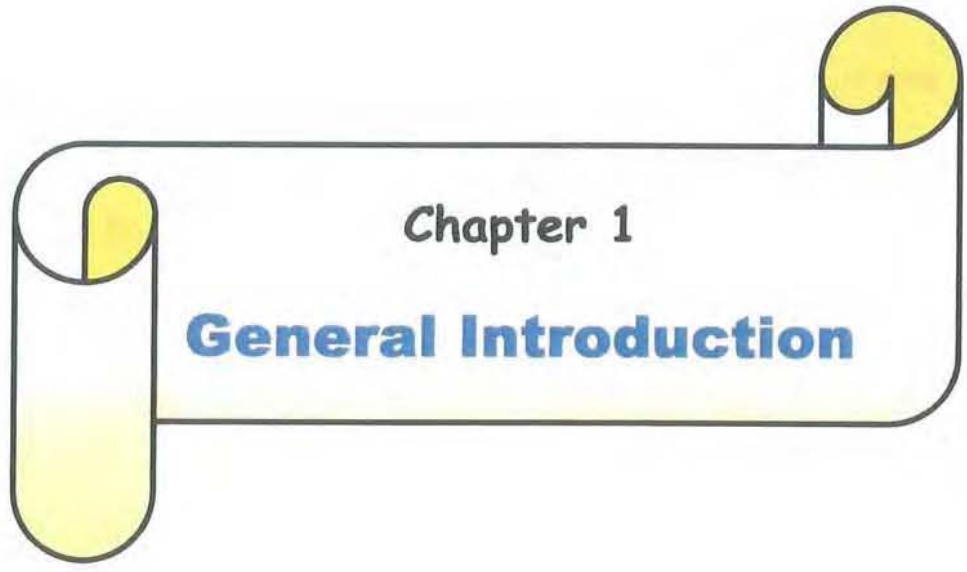

Chapter 1

General Introduction

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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

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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.

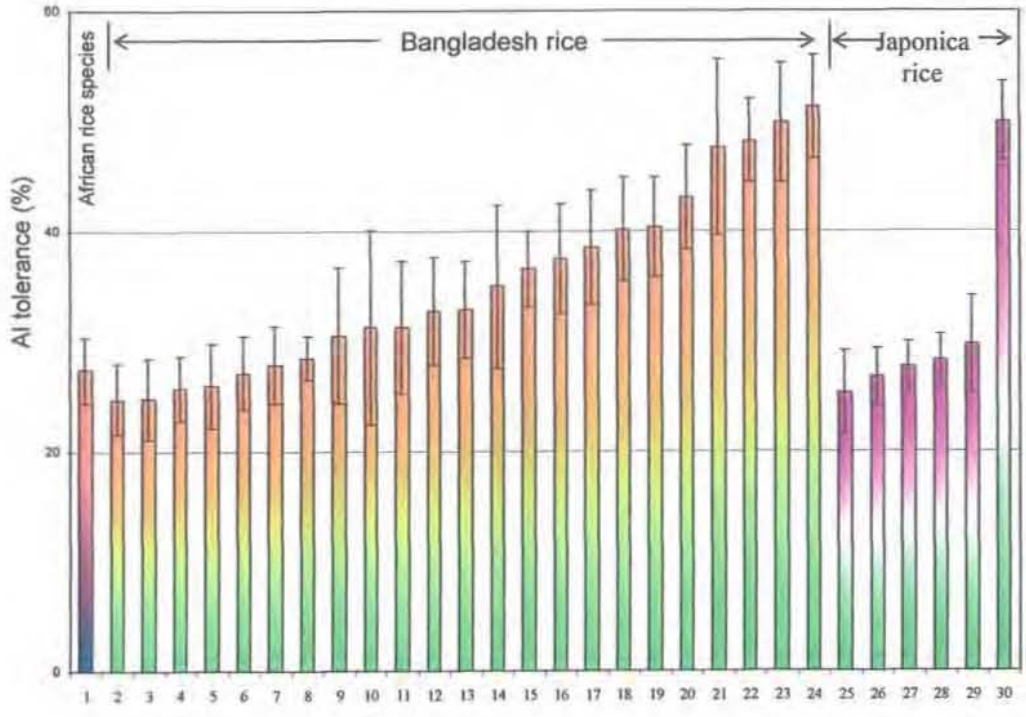


Figure 2.1: Aluminum tolerance screening with 20μM $AlCl_3$ in 0.2 mM $CaCl_2$ (pH 4.9, 24h). 1, *Oryza glaberrima sp.*; 2, cv. Moyna; 3, cv. BRRIdhan34 4, cv. Asha; 5, cv. Dulabhog; 6, cv. BRRIdhan27; 7, cv. Chandina; 8, cv. Niamat; 9, cv. BRRIdhan37; 10, cv. Kiron; 11, cv. Naya Pajam; 12, cv. Asha; 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.

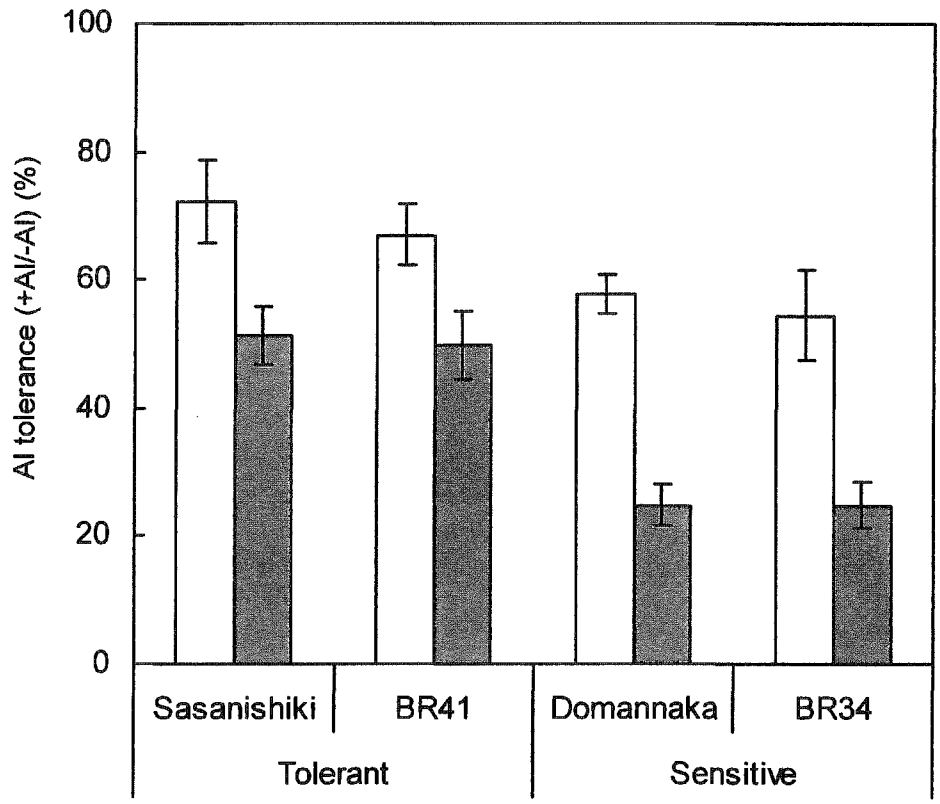


Figure 2.2: Al tolerance tolerances for 1h (open bar) and 24h Al treatments. 1h Al treatment, 1h treatment in ±20μM $AlCl_3$ in 0.2mM $CaCl_2$ (pH 4.9) and reculturing in Al free medium (0.2mM $CaCl_2$ at pH 5.2); 24h Al treatment, continuous treatment with ±20μM $AlCl_3$ in 0.2mM $CaCl_2$ (pH 4.9).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

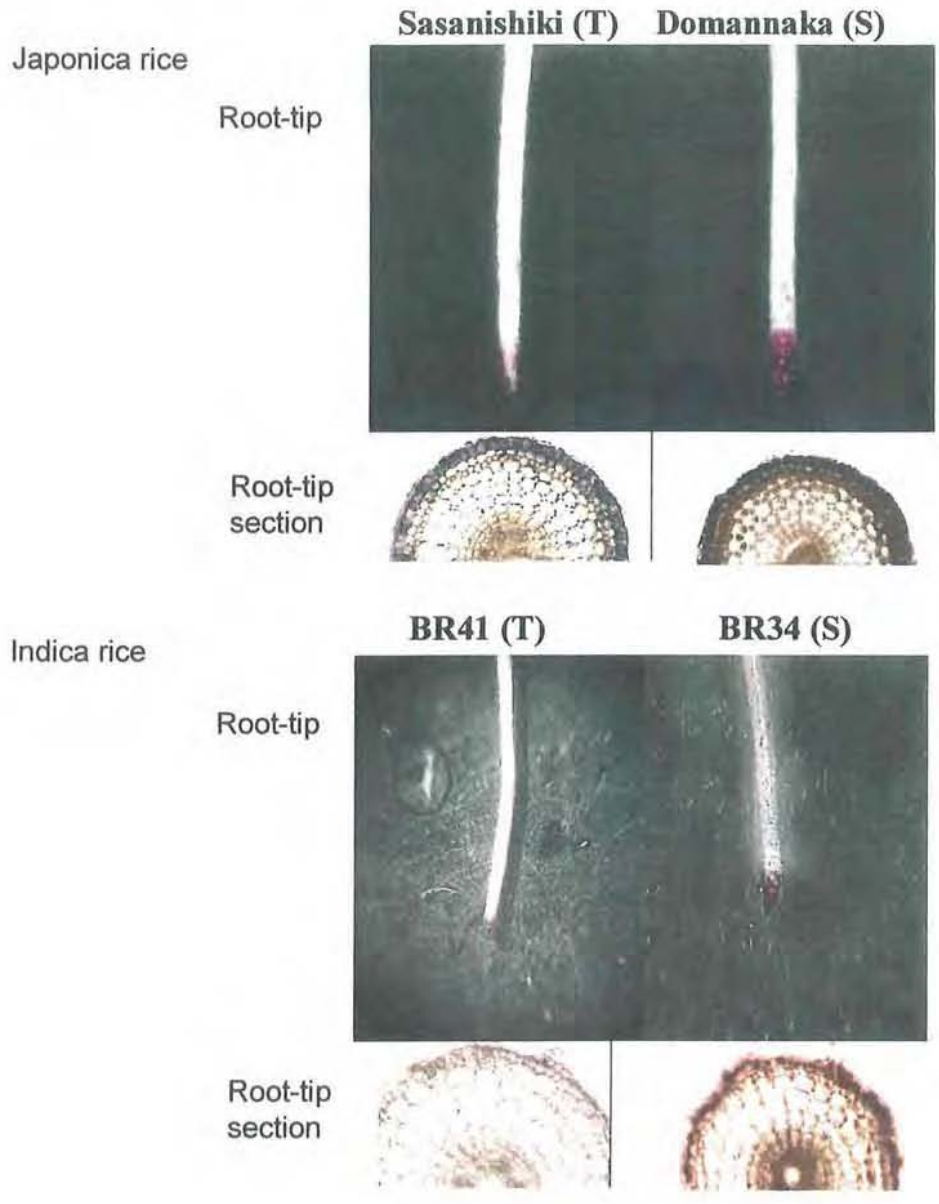


Figure 2.3: Localization of Al in 1-cm root-tip and 2-3 mm sections from apex detected by hematoxylin staining method. Deeper brownish purple color indicates higher Al accumulation

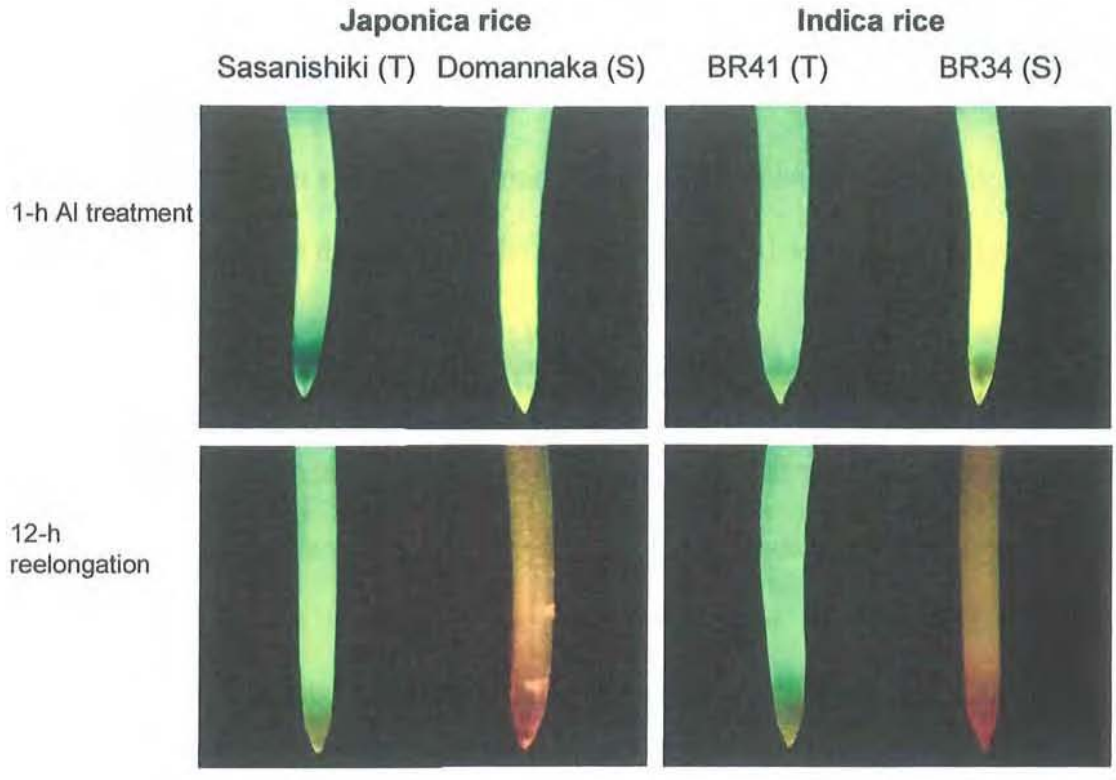


Figure 2.4: Permeability of root-tip cells after 1h Al treatment (50μM $AlCl_3$ in 0.5mM $CaCl_2$, pH 4.5) and after reelongation in Al free medium was observed by FDA-PI staining

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).

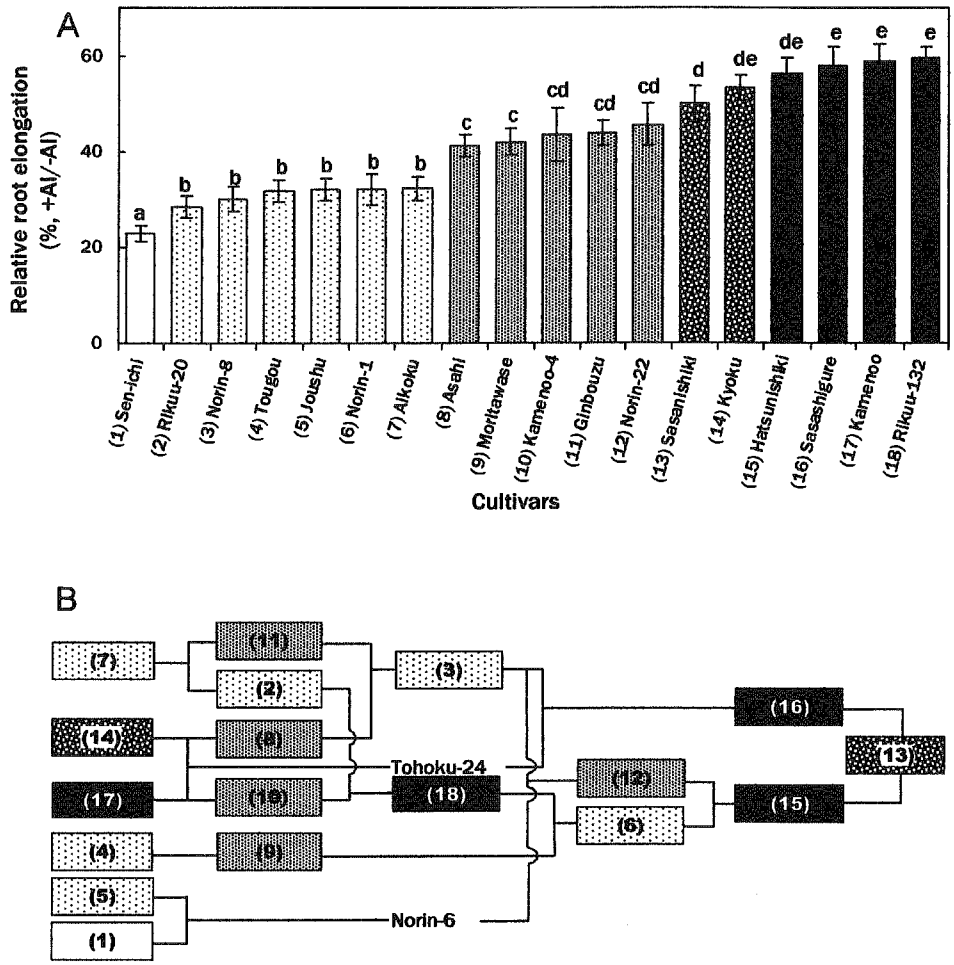


Fig. 3.1: Relative root elongation of ancestor cultivars of the same family line of Sasanishiki (A) and family tree of cv. Sasanishiki shown with graded black densities basically correspondent to differential Al tolerances among cultivars in Fig. 1A (B). Four-d-old seedlings were pretreated in 0.2 mM $CaCl_2$ 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.

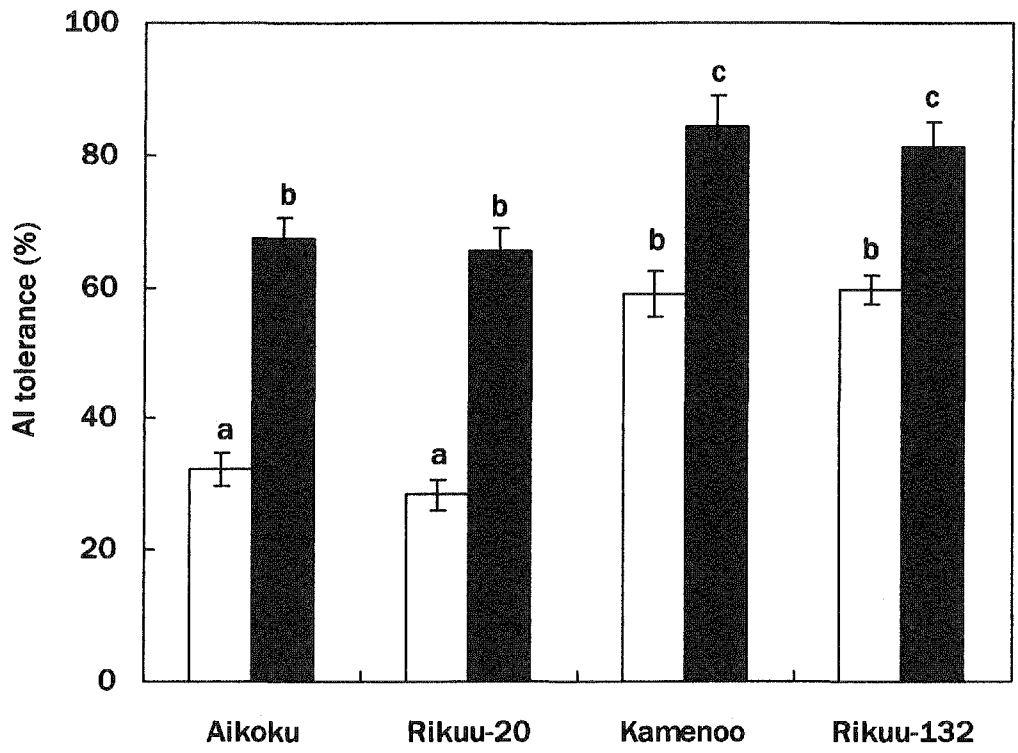


Fig. 3.2: Al tolerance of the selected Sasanishiki pedigree cultivars for 24h (white bar) and for 1h Al treatment followed by 12h reelongation (closed bar). Al tolerances were calculated as described in materials and methods of previous chapter.

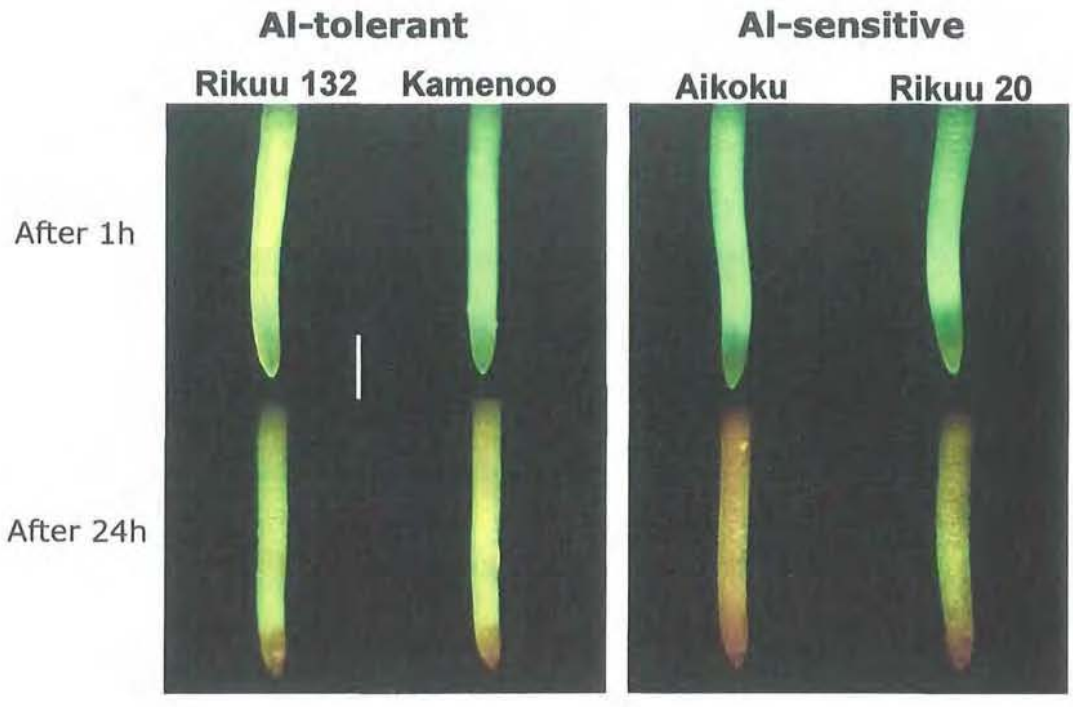


Fig. 3.3: Plasma membrane permeabilization in root of tolerant and sensitive cultivars which were selected from Sasanishiki pedigree by FDA-PI fluorescence microscopy.

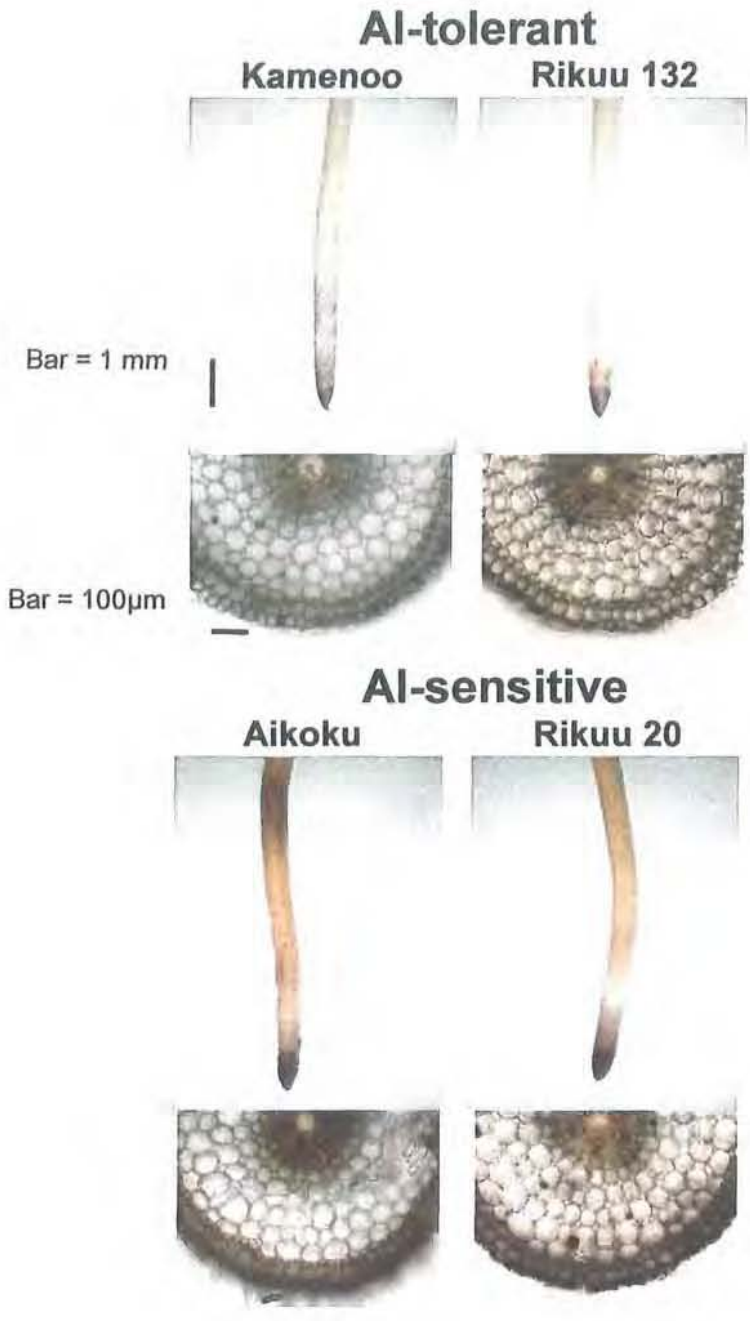


Fig. 3.4: Al accumulation in root-tips and root-tip sections in tolerant and sensitive cultivars which were selected from Sasanishiki pedigree by hematoxycilin staining technique after 24h 20μM AlCl3 treatment in 0.2mM CaCl2 at pH 4.9.

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.

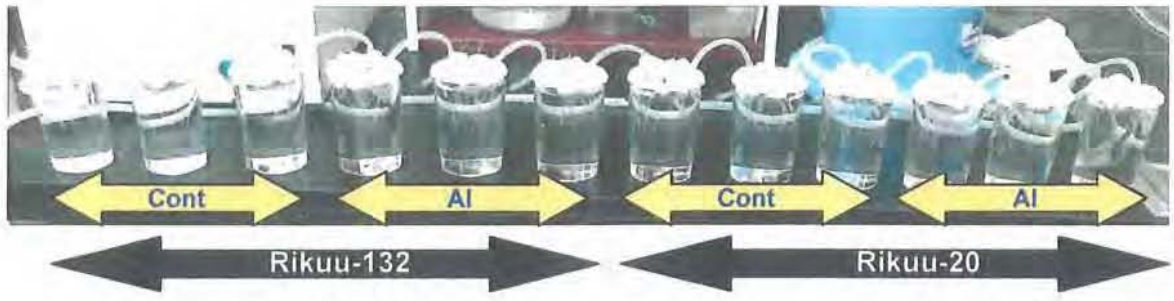


Fig. 4.1 Treatment procedure for control and Al treatment for organic acid exudation. Only selected cultivars of rice were treated.

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.

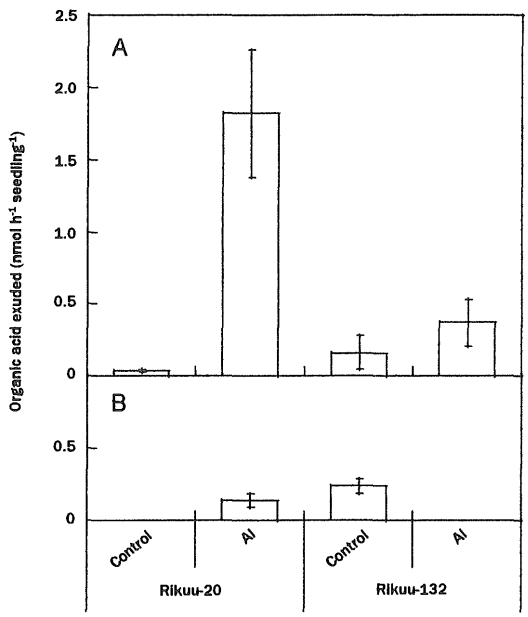


Fig. 4.2: Citrate (A) and malate (B) exuded from rice cultivars Rikuu-20 and Rikuu-132. Five- day-old seedlings were treated for 5h with or without Al (20μM) in 0.2mM $CaCl_2$ (pH 4.9) following 5h pretreatment with 0.2mM $CaCl_2$ (pH 4.9). Exudates were collected during the 5h treatment. Values are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

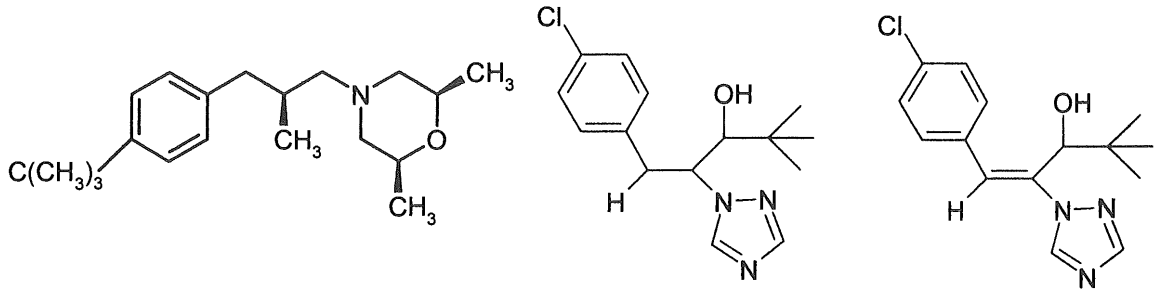
Study on the lipid composition of pharmaceutically changed PM

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.

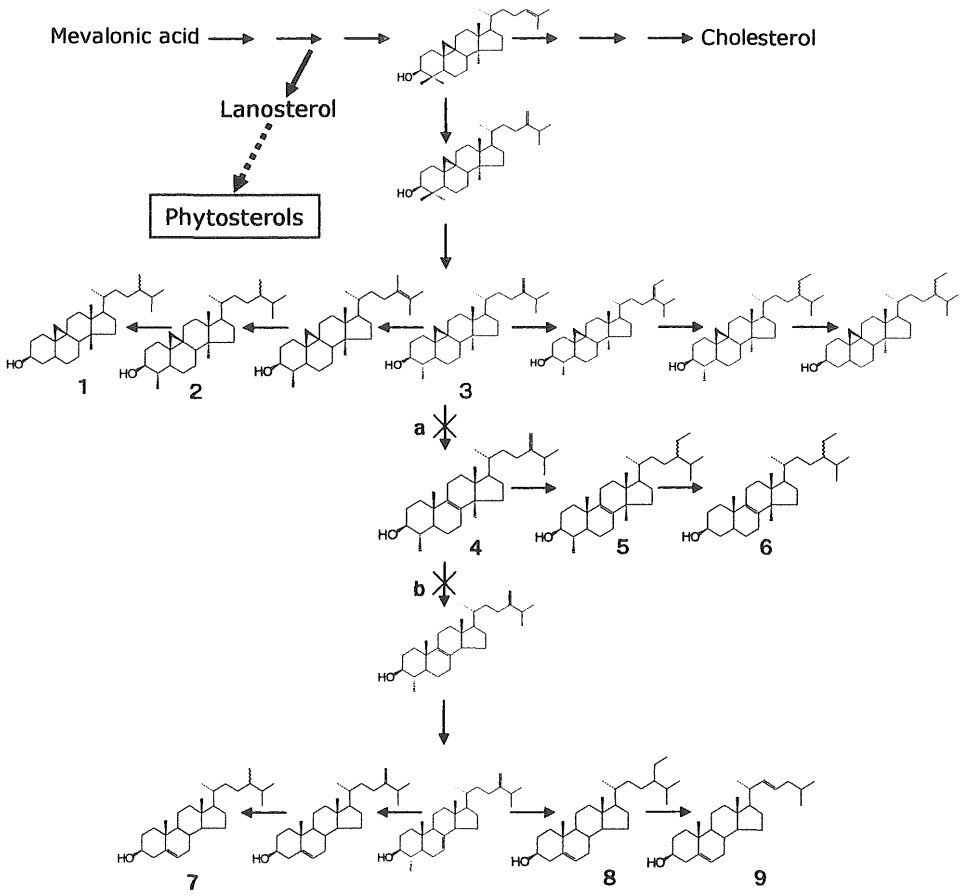


Fig. 5.2: Biosynthetic pathway of sterols compiled from Burden et al. (1987), Grandmougin et al. (1989) by ChemDraw Ultra 7.0 a: Site of action for fenpropimorph which inhibits cycloeucalenol obtusifoliol isomerase (COI); b: Site of action for paclobutrazol or uniconazole-P which inhibits obtusifoliol-14α-demethylase (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.

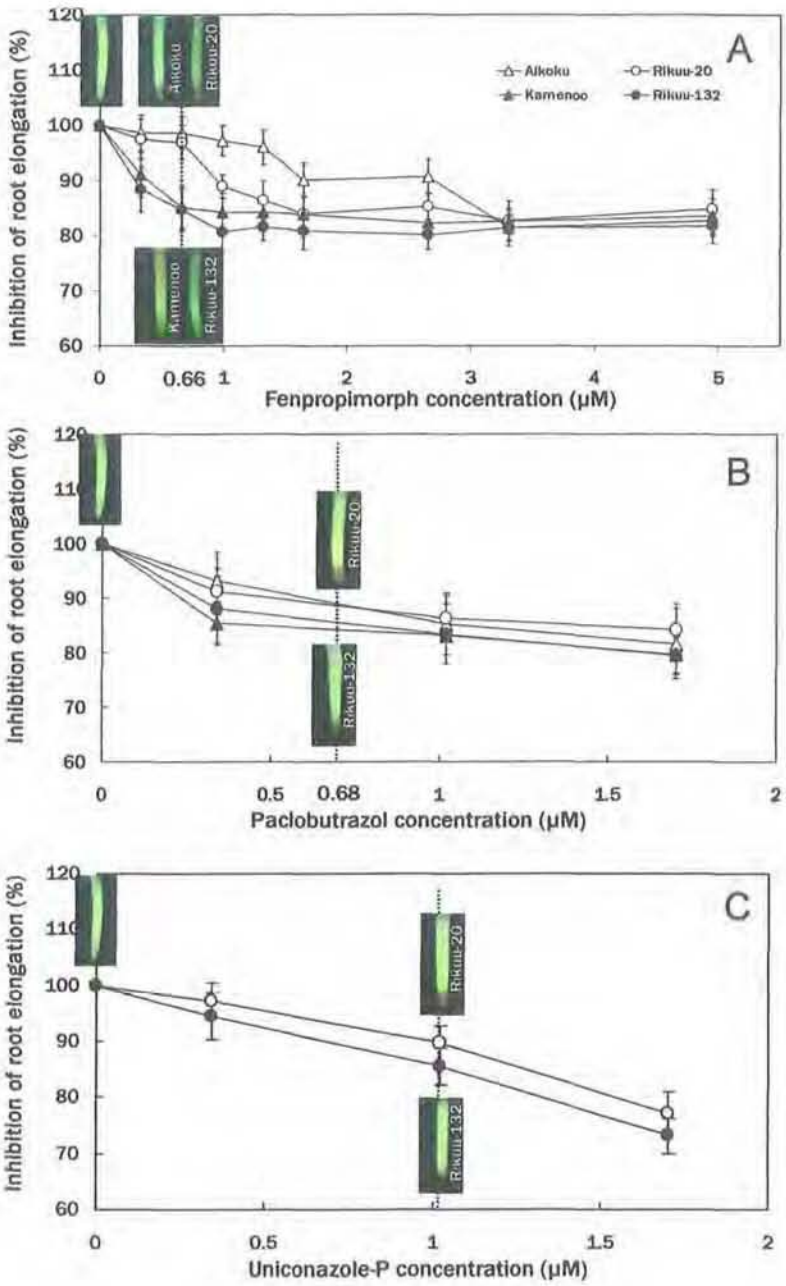


Fig. 5.3: Inhibition of root elongation with the concentration of sterol metabolism inhibitor fenpropimorph (A), paclobutrazol (B) and uniconaxole (C). Figures of roots whows the PM permeabilization of the roots in selected concentration of the inhibitos.

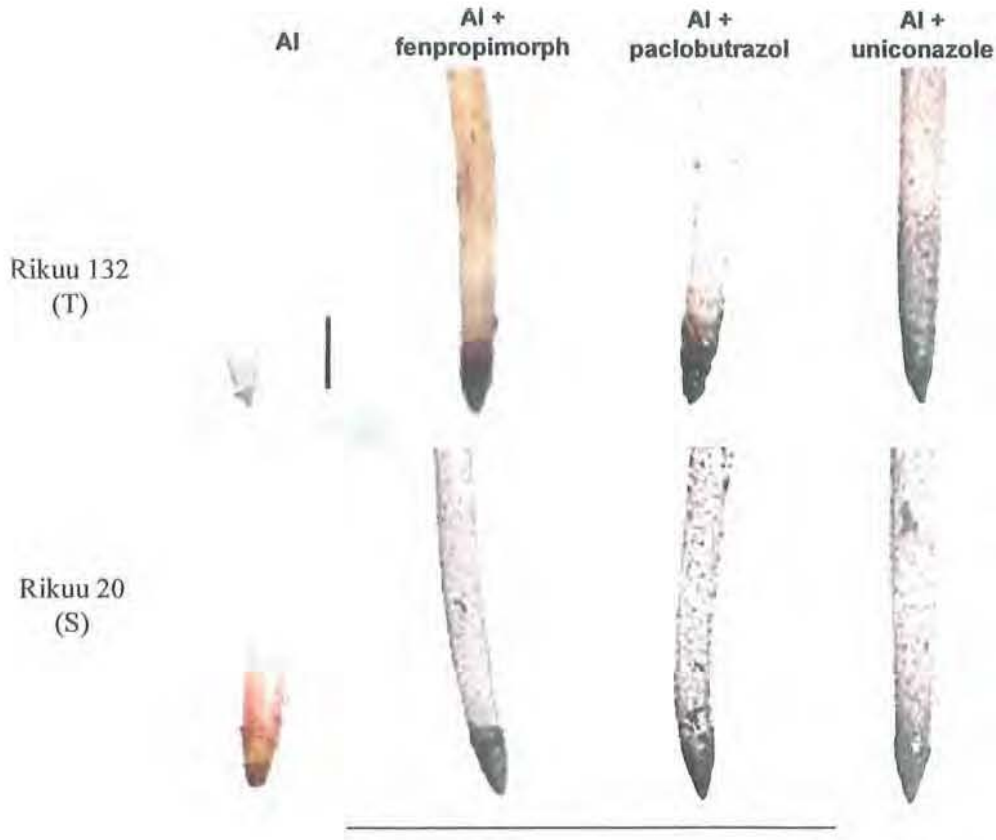


Fig. 5.4: Al accumulation visualized by hematoxylin staining after 24 h Al treatment with sterol metabolism inhibitors. Each of the treatment solutions contained 0.2mM $CaCl_2$ for 24h at pH 4.9, 20µM $AlCl_3$, 0.66µM fenpropimorph, and 1.02µM uniconazole-P, according to the following treatments: Al,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

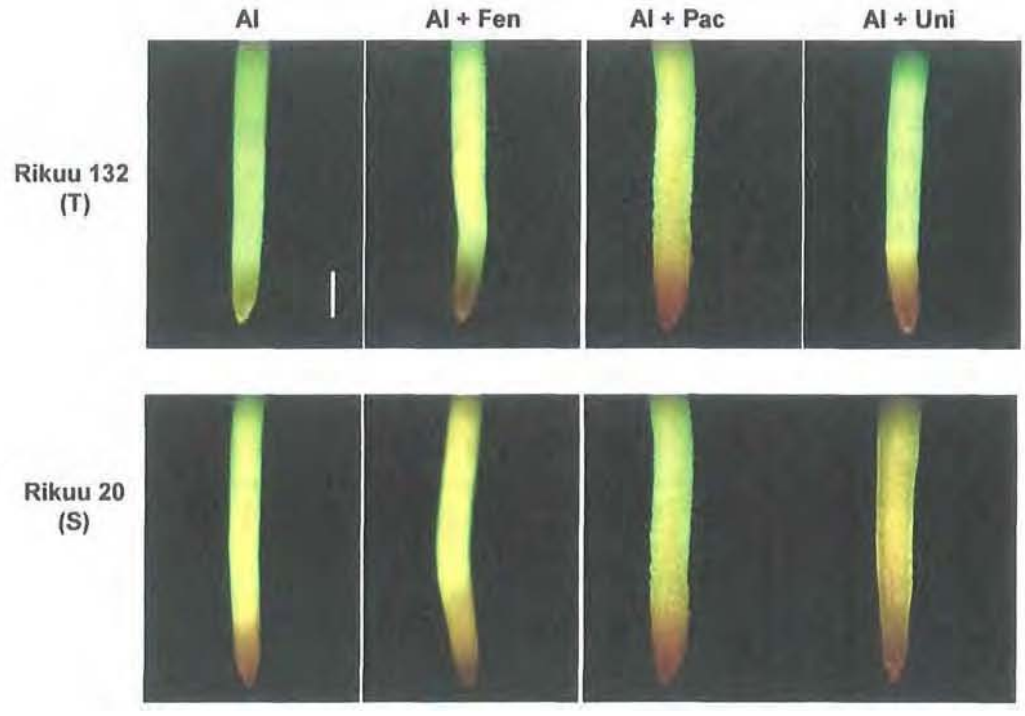


Fig. 5.5: PM permeability visualized using FDA-PI fluorescence microscopy after 24 h Al treatment with sterol metabolism inhibitors. Each of the treatment solutions contained 0.2mM $CaCl_2$ for 24h at pH 4.9, 20μM $AlCl_3$, 0.66μM fenpropimorph, and 1.02μM uniconazole-P, according to the following treatments: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

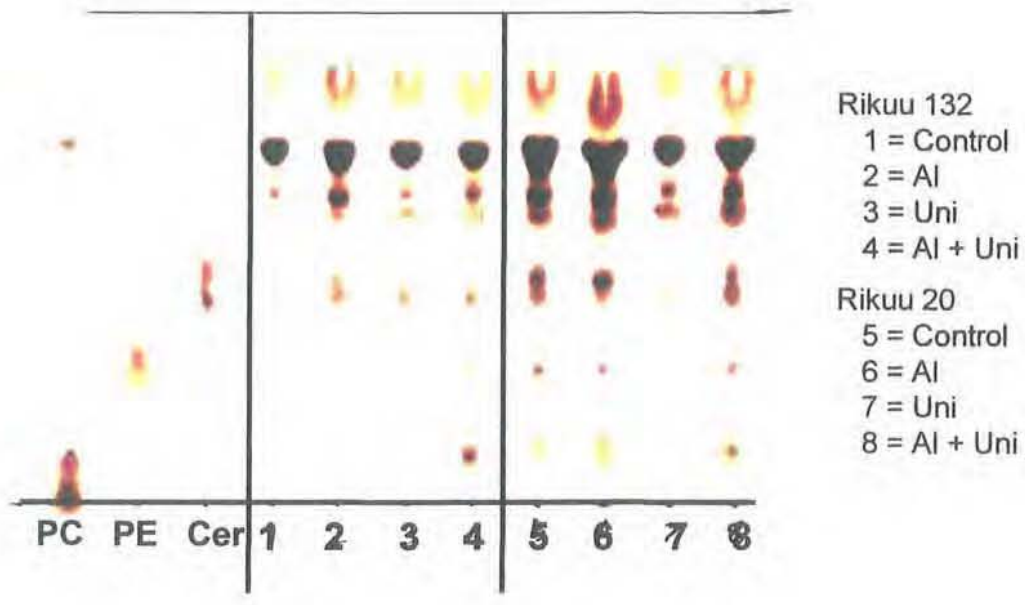


Fig. 5.6: Phospholipid class analysis by HPTLC after treatment in control, Al, uniconazole-P and Al+uniconazole-P for Rikuu-20 and Rikuu-132. Samples were extracted as described in Materials and Methods. Development solvent: chloroform:methanol:acetic acid = 65:25:8, color development by 20% H_2SO_4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

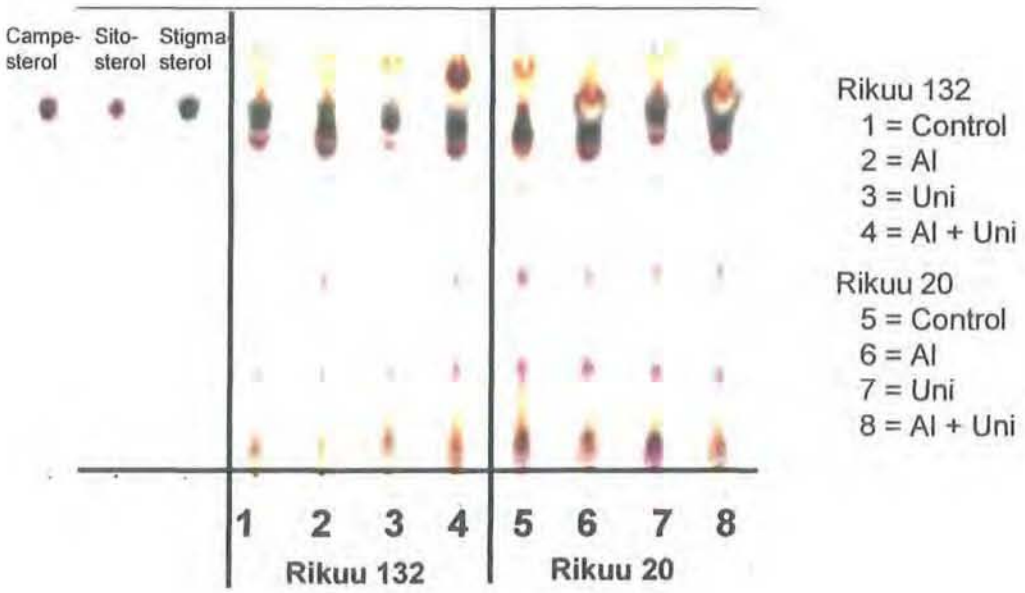


Fig. 5.7: Δ^5-Sterol analysis by HPTLC after treatment in control, Al, uniconazole-P and Al+uniconazole-P for Rikuu-20 and Rikuu-132. Samples were extracted as described in Materials and Methods. Development solvent: dichloromethane:methanol = 85:15, color development by 20% H_2SO_4 in methanol and heating.

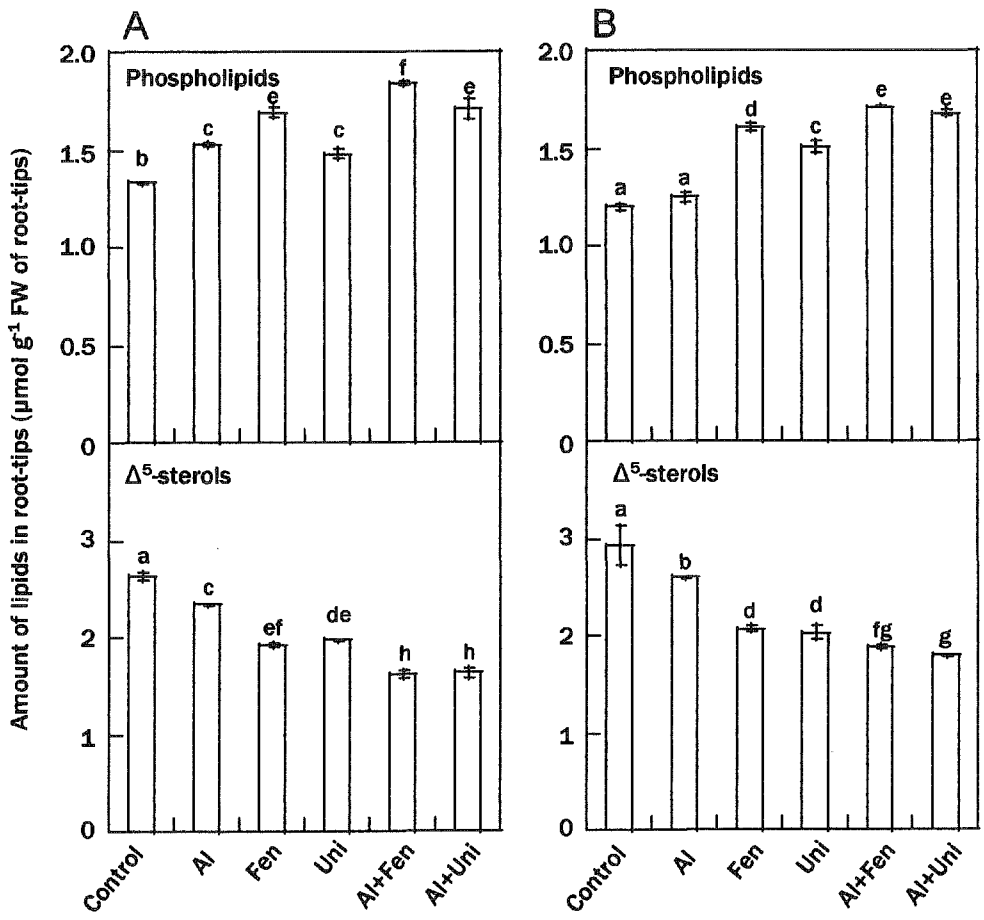


Fig. 5.8: Phospholipids and Δ^5-sterols in root-tips (0-1 cm from root-tip portion) of 4-day-old Rikuu-20 (A) and Rikuu-132 (B) seedlings. Each of the treatment solutions contained 0.2mM $CaCl_2$ for 24h at pH 4.9, 0 or 20μM $AlCl_3$, 0 or 0.66μM fenpropimorph, and 0 or 1.02μM uniconazole-P, according 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).

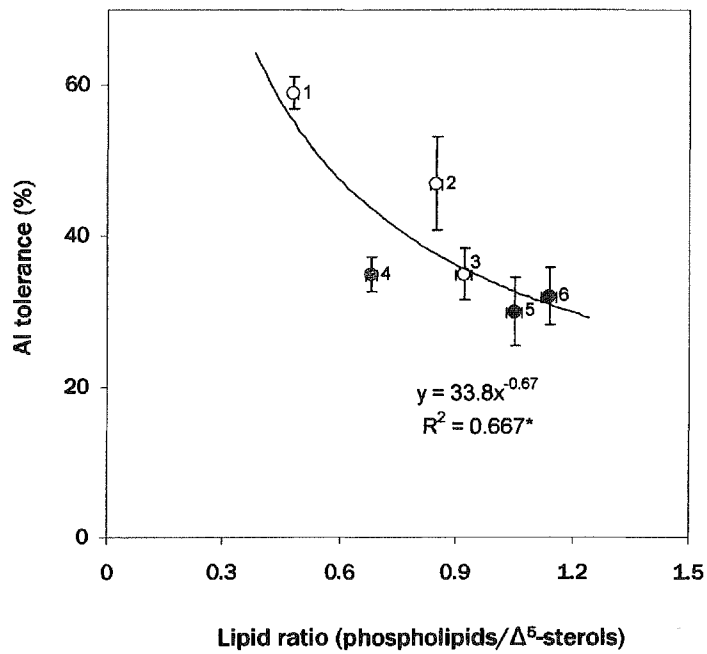


Fig. 5.9: Relationship between lipid ratio (phospholipids/Δ^5-sterols) and relative root elongation (%, +Al/-Al), i.e., Al tolerance of cv. Rikuu-132 (open circles) and cv. Rikuu-20 (closed circles). Each of the treatment solutions contained 20μM $AlCl_3$ in 0.2mM $CaCl_2$ for 24h at pH 4.9, 0.66μM fenpropimorph, 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.

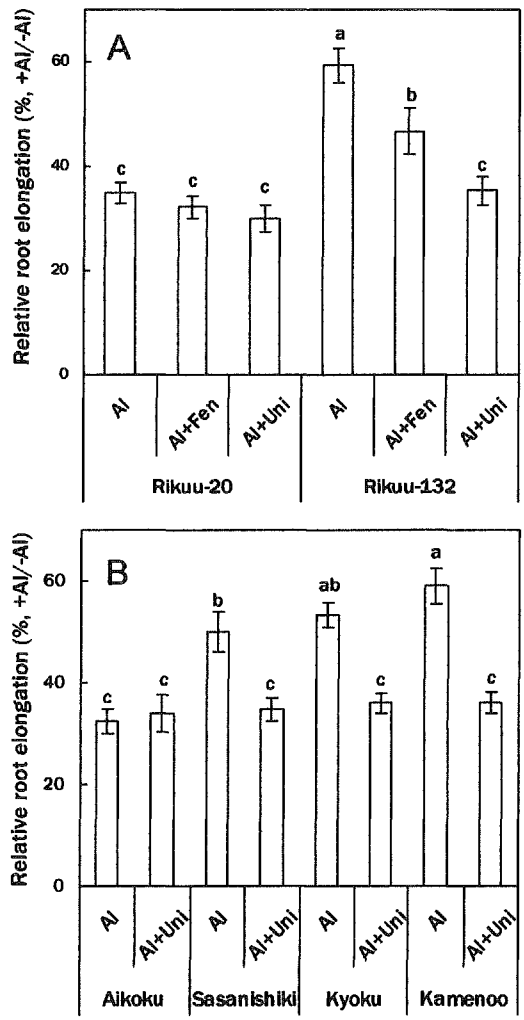


Fig. 5.10: Relative root elongation (%, +Al/-Al), i.e., Al tolerance of rice cultivars Rikuu-20 and Rikuu-132 after Al treatment with two sterol metabolism inhibitors (fenpropimorph and uniconazole-P) (A). Cultivars Aikoku, Kyoku, Sasanishiki and Kamenoo under uniconazole-P treatment (B). Values are 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).

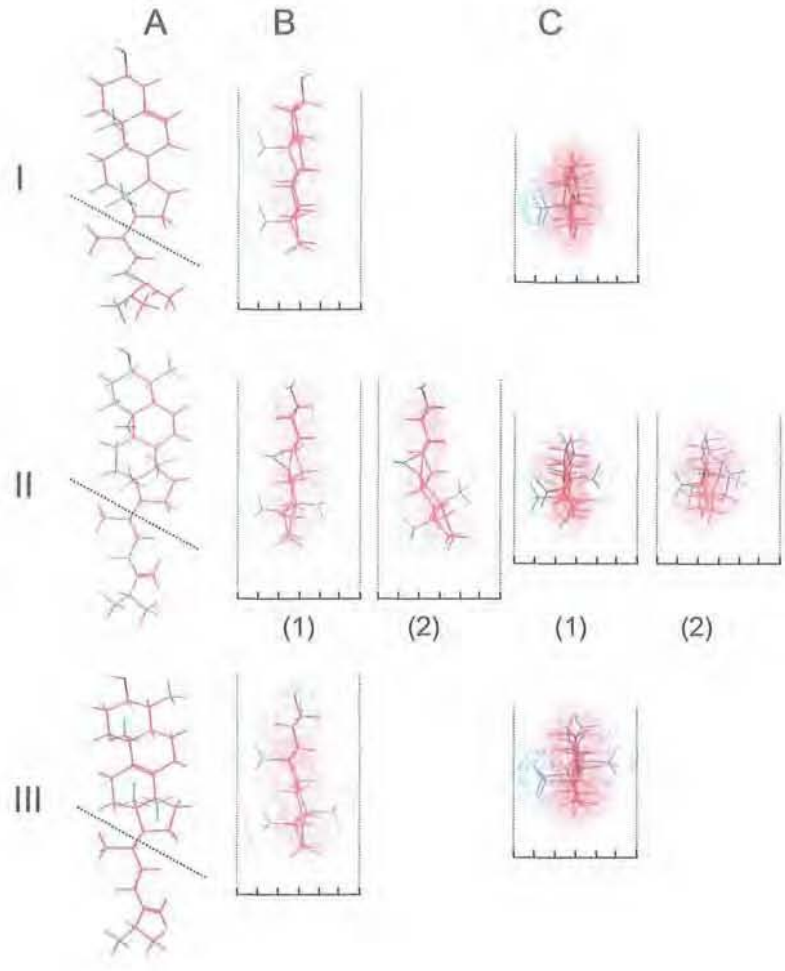


Fig. 5.11: Chemical structures of three typical sterols (A) and van der Waals conformations of these sterols without fatty acyl chains (constructed using Chem3D Ultra 7.0) (B, C). I, sitosterol; II, cycloeucalenol; III, obtusifoliol. B, side view. C, upper view. Cycloeucalenol has straight (1) and bending (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.