Molecular mechanisms of Al detoxification in Al-tolerant plant species

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Chapter 1 Introduction

With increasing global population and food demand, enhancing crop production is highly required to face the coming crisis of food deficiency. On the other hand, plants are suffering from various biotic and abiotic stresses, such as pathogens, salinity and aluminum (Al) toxicity, which significantly inhibit crop growth and productivity. Therefore, enhancing tolerance of crops to various stresses is an important approach to increase crop productivity in future.

1. Al toxicity in plants

Aluminum (Al) toxicity has been recognized as a major factor limiting crop productivity on acid soil, which comprises about 40% of the arable land in the world (Ma 2000). Soluble ionic aluminum (mainly Al^{3+}) in acid soils rapidly inhibits root elongation at the micromolar level, subsequently affecting the uptake of water and nutrients, resulting in low crop productivity on these soils (Kochian 1995; Ma 2007; Ryan et al. 2011). The targeting site of Al toxicity is the root apex (Ryan et al. 1993). During the initial stages of Al toxicity, Al inhibits cell expansion (Kochian 1995). The cell wall is the first Al contacting compartment when plants are exposed to Al toxicity, and it is the major site for Al accumulation (Ma 2007). Al binding to the cell wall reduced its extensibility (Ma et al. 2004). The major Al binding site in the cell wall is considered to be the pectin, because Al bounds to cation exchange sites provided by the negative charged pectin fraction (Horst 1995; Chang et al. 1999). The Al accumulation by root is closely related to the pectin content in the apical root sections of

maize and faba bean (Horst et al. 2010; Fig. 1.1). Recently, it was reported that methylation degree of pectin as well as methylation patterns are critical for Al adsorption into cell wall and consequently Al sensitivity not only in monocot plant, rice (Yang et al. 2008), but also in dicot plant species, buckwheat (Yang et al. 2011a). The property (both degree and pattern of methylation) of pectin is regulated by pectin methylesterase (PME) gene.

Recently, the hemicellulose in the cell wall is thought to be another major Al binding site. The amount of Al accumulated in hemicellulose (mainly xyloglucan) is much more than that in pectin in Arabidopsis (Yang et al. 2011b). Furthermore, a gene named *XTH31* (*xyloglucan endotransglucosylase-hydrolase*), which is involved in xyloglucan modification, is required for Al accumulation in cell wall (Zhu et al. 2012). Besides, some proteins such as the hydroxyproline rich glycoprotein (HRGP) in the cell wall may play a role in inhibition of root cell elongation through cross-linking of HRGPs directly or indirectly by Al (Poschenrieder et al. 2008).

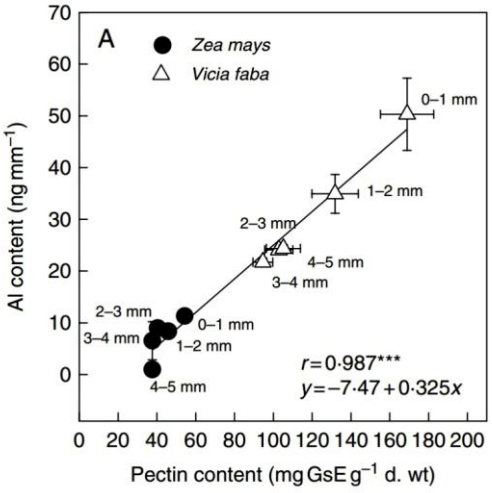


Figure 1.1 Relationship between pectin and Al content in root sections. (Horst et al. 2010)

Al rapidly affects the properties not only of the cell wall but also those of the plasma membrane. Al modifies the fluidity and permeability of membrane lipids and proteins

(Wagatsuma et al. 2005; Khan et al. 2009). Binding of Al to the plasma membrane alters its surface negativity (Kinraide et al. 1992; Kinraide 2006). Besides, Al rapidly induces membrane depolarization due to inhibition of H^+-ATPase activity (Ahn et al. 2001). These changes by Al affect the function of membrane ion transport such as K^+ and Ca^{2+} (Horst et al. 1992; Rengel et al. 2003).

Once Al enters into the cytoplasm, Al will strongly bind with the oxygen donor compounds such as inorganic phosphate, nucleotides, RNA, DNA, proteins, carboxylic acids, phospholipids, polygalacturonic acids, heteropolysaccharides, lipopolysaccharides, flavonoids and anthocyanins (Martin 1988). The binding of Al with these substances may result in structural and functional damage to the roots. Besides, the symplastic Al induces alteration of the cytoskeleton, affects endocytosis and vesicle recycling (Frantzios et al. 2005; Illéš et al. 2006; Amenós et al. 2009).

2. Molecular mechanisms for Al tolerance in plants

Plants have developed their strategies to cope with Al, which can be broadly divided into internal and external mechanisms (Kochian et al. 2004; Ma 2007; Delhaize et al. 2012).

2.1 Internal mechanisms

Al is thought to be detoxified in vivo by complexation and sequestration when it enters into cytoplasm (Kochian 1995). Especially in Al-accumulating plants, such as buckwheat (*Fagopyrum esculentum*) and hydrangea (*Hydrangea macrophylla*), the forms of A1 complexed with organic compounds have been identified (Ma et al.

1997a;c). Buckwheat can accumulate Al as high as 15 mg g^{-1} (dry weight) in its leaves (Ma et al. 2001). The process of Al accumulation in buckwheat was well studied: Al is first taken by roots and chelated with internal oxalate in root cells. The form of Al-oxalate complex at a 1:3 ratio is stable and non-phytotoxic, protecting the cytosol against Al injury. Then Al-oxalate complex changes to Al-citrate (1:1 ratio) complex for xylem loading to the leaves. In the leaves, Al is sequestered in the vacuoles in the form of both Al-oxalate and Al-citrate (Shen et al. 2002; 2003). However, transporters involved in these processes have not been identified. Recently, the plasma membrane- and tonoplast-localized transporters for Al were identified in rice, named Nrat1 and OsALS1, respectively (Xia et al. 2010; Huang et al. 2012). Nrat1 functions as a transporter for trivalent Al, which protects cell wall from binding with high concentration of Al. OsALS1 encodes a half-size ABC transporter that is thought to be responsible for internal Al detoxification by sequestration of Al from cytosol into the vacuole in rice. However, it remains to be examined whether Al-accumulating plant species have similar transporters.

In Arabidopsis, *AtALS3* (originally called *ALS3*) also encodes an ABC transporter-like protein that confers Al tolerance (Larsen et al. 2005). AtALS3 may function to redistribute accumulated Al away from sensitive tissues, with knockout of *AtALS3* resulting in accumulation of Al in inappropriate tissues, which subsequently leads to the extreme inhibition of root and shoot growth (Larsen et al. 1997; 2005). Besides, *AtALS1* (originally called *ALS1*) encodes a half type ABC transporter localized at the tonoplast (Larsen et al. 2007), suggesting its similar function to OsALS1. Although AtALS1 is the closest homologue to OsALS1, the expression pattern and tissue localization are different from each other. AtALS1 is primarily localized to the

root tip and the vasculature, and its expression is not induced by Al. Plants carrying knockout mutations in *AtALS1* results in Al hypersensitivity, but the mechanisms are unknown. One possibility is that AtALS3 mediates Al uptake across the plasma membrane and AtALS1 subsequently sequester Al into vacuoles, similar to Nrat1 and OsALS1 in rice. However, no change in Al uptake and yeast growth can be observed when they are expressed in yeast, suggesting that they may have unknown functions.

2.2 External mechanisms

In contrast to internal detoxification, the external mechanism is to protect root cell from Al invasion. Plants have developed many strategies to detoxify Al externally: 1) secretion of Al-chelating substances from the roots including organic acid anions, phenolic compounds and mucilage (Kochian et al. 2004; Ma 2007; Li et al. 2000a); 2) increasing the pH of rhizosphere to reduce the level of toxic Al^{3+} (Degenhardt et al. 1998); 3) increasing methylation of pectin to reduce Al binding to cell wall (Yang et al. 2008; Yang et al. 2011a); 4) decreasing the ratio of phospholipids to Δ^5-sterols in the plasma membrane to lower the negative binding site for Al binding (Khan et al. 2009).

Among above mechanisms for the external detoxification, the most-studied one is the secretion of organic acid anions including malate, citrate and oxalate from the roots in response to Al stress. However, individual species varied in their type of organic acid anions involved in Al detoxification. In wheat, Al can induce malate secretion from root, and Al-tolerant genotypes (e.g. Atals66) secreted much more amount of malate than Al-sensitive genotypes (e.g. Scout. Kitagawa et al. 1986; Delhaize et al. 1993b; Pellet et al. 1996; Li et al. 2000b). In barley, the Al-resistant cultivars can rapidly secrete citrate from the roots in response to Al. Further, a positive correlation between

citrate secretion and Al resistance in differential barley varieties was obtained (Zhao et al. 2003). In rye, exudation of both malate and citrate in a time-dose-dependent and Al-specific manner, has been reported to detoxify Al (Li et al. 2000b; 2002). On the other hand, oxalate is secreted from the roots of buckwheat in response to Al (Ma et al. 1997; Zheng et al. 1998).

There are two patterns of organic acid anion release differing in the timing of secretion (Ma, 2000; Ma et al. 2001). In Pattern I, no discernible delay is observed between the addition of Al and the onset of secretion, while in Pattern II, organic acid anion secretion is delayed for several hours after exposure to Al. Different mechanisms seem to be involved in the two secretion pattern. It is suggested that both activation of the organic acid anion channel and induction of gene are required in Pattern II, but gene induction is not involved in Pattern I. Recent molecular studies support these different patterns as described below.

The secreted type and amount of organic acid anions (citrate, malate or both) from individual species depend on respective Al-activated and membrane-localized transporters (Table 1.1). They belong to two different gene families: *ALMT* (Aluminum-activated malate transporter) and *MATE/AACT* (Multidrug and toxic compound extrusion/Aluminum-activated citrate transporter) (Ryan et al. 2011; Delhaize et al. 2012). *ALMT1* gene has been identified in wheat (Sasaki et al. 2004), Arabidopsis (Hoekenga et al. 2006), oilseed rape (Ligaba et al. 2006), rye (Collins et al. 2008), maize (Kirll et al. 2010) and soybean (Liang et al. 2013), although the expression patterns differ among plant species. These proteins encoded by *ALMT1*s transport malate and are localized to the plasma membranes of root cells. On the other hand, *MATE1/AACT1* gene has been identified in barley (Furukawa et al. 2007), sorghum

(Magalhaes et al. 2007), Arabidopsis (Liu et al. 2009), rye (Yokosho et al. 2010), maize (Maron et al. 2010; Ligaba et al. 2012), rice bean (Yang et al. 2011c), rice (Yokosho et al. 2011) and wheat (Tovkach et al. 2013). These proteins encoded by *MATE1/AACT1* are also localized to the plasma membranes of root cells, but transport citrate.

Table 1.1 Plant species for which the efflux of organic acid anions from roots contributes to Al resistance. (Partially from Ryan and Delhaize 2012)

Species	Organic anion released	Gene	Reference
Monocotyledons			
Barley (*Hordeum vulgare*)	citrate	*HvAACT1*	Zhao *et al.* (2003); Furukawa *et al.* (2007)
	malate	*HvALMT1*	**Gruber *et al.* (2010)**
Maize (*Zea mays*)	citrate	*ZmMATE1*	Pellet *et al.* (1995); Maron *et al.* (2010)
	malate	*ZmALMT2*	**Kirll *et al.* (2010)** **Ligaba *et al.* (2012)**
Rye (*Secale cereale*)	malate & citrate	*ScALMT* gene cluster *ScMATE2*	Collins *et al.* (2008) Yokosho *et al.* (2010)
Sorghum (*Sorghum bicolor*)	citrate	*SbMATE1*	Magalhaes *et al.* (2007)
Triticale (× Triticosecale)	malate & citrate		Ma *et al.* (2000)
Wheat (*Triticum aestivum*)	malate	*TaALMT1*	Sasaki *et al.* (2004)
	citrate	*TaMATE1*	Ryan *et al.* (2009)
Rice (*Oryza Sativa*)	citrate	***OsFRDL4***	**Yokosho *et al.* (2011)**
Dicotyledons			
Arabidopsis thaliana	malate	*AtALMT1*	Hoekenga *et al.* (2006)
	citrate	*AtMATE1*	Liu *et al.* (2009)
Aspen (*Populus tremula*)	malate & formate		Qin *et al.* (2007)
Buckwheat (*Fagopyrum esculentum*)	oxalate		Zheng *et al.* (1998)
Cassia tora	citrate		Ma *et al.* (1997)
Citrus (*Citrus sinensis*)	citrate & malate		Yang *et al.* (2011)
Common bean (*Phaseolus vulgaris*)	citrate		Shen *et al.* (2002)
Lespedeza bicolor	malate & citrate		Dong *et al.* (2008)
Oat (*Avena sativa*)	malate & citrate		Zheng *et al.* (1998)
Radish (*Raphanus sativus*)	malate & citrate		Zheng *et al.* (1998)
Rapeseed (*Brassica napus*)	citrate	*BnALMT1* and *BnALMT2*	Ligaba *et al.* (2006)
Rice bean (*Vigna umbellata*)	citrate	*VuMATE*	Yang *et al.* (2011)
Snapbean (*Phaseolus vulgaris*)	citrate		Miyasaka *et al.* (1991)
Soybean (*Glycine max*)	citrate		Yang *et al.* (2000); Silva *et al.* (2001)
	malate	***GmALMT1***	Liang *et al.* (2013)
Stylosanthes sp.	citrate		Li *et al.* (2009)
Taro (*Colocasia esculenta*)	oxalate		Ma and Miyasaka (1998)
White lupin (*Lupinus albus*)	citrate		Wang, B.L. *et al.* (2007)

The efflux of Al-activated organic acid anions mediated by ALMTs and MATE/AACTs is the major Al tolerance mechanism in some plant species, such as wheat and barley (Sasaki et al. 2006; Furukawa et al. 2007). However, among small

grain cereals, rice owns higher Al resistance but lower amount of Al-induced citrate efflux from roots (Ma et al. 2002), suggesting other mechanisms contributing to Al tolerance. Recently, *STAR1* and *STAR2* (*for sensitive to Al rhizotoxicity 1and 2*) are identified as Al tolerance genes, because knockout of either *STAR1* or *STAR2* results in Al hypersensitivity (Huang et al. 2009). STAR1 interacts with STAR2 to form a complex that localizes to the cytosolic vesicles, but the mechanism for STAR1-STAR2 involved in Al detoxification is unknown. It has been proposed that the delivery of UDP-glucose by STAR1-STAR2 complex may be involved in cell wall modification, which prevents Al accumulation and reduces damage. Furthermore, the closest homologue of *OsSTAR1* in Arabidopsis, named *AtSTAR1,* is shown to contribute to Al tolerance (Huang et al. 2010). Since *OsSTAR2* is a homologue of *AtALS3* in Arabidopsis, the possible interaction between AtSTAR1 and AtALS3 has been proposed (Huang et al. 2010).

2.3 Transcriptional regulation of Al tolerance genes

Induction of the expression of Al tolerance genes makes a significant contribution to plant growth under Al stress. The Al-induced gene expression has been found to be regulated by C2H2 zinc finger transcription factors (Fig. 1.2). In Arabidopsis, STOP1 (for sensitive to proton rhizotoxicity 1) is involved in signal transduction pathways regulating the expression of genes responsible for Al and proton stress (Iuchi et al. 2007). Knockout of *STOP1* results in increased sensitivity to Al and low pH. The Al hypersensitivity of the *stop1* mutant is mainly attributed to down-regulation of *AtALMT1*, *AtMATE1* and *AtALS3* (Sawaki et al. 2009; Liu et al. 2009). *AtALS3* encodes an ABC transporter-like protein that may function to redistribute accumulated

Al away from sensitive tissues, as mentioned above. AtALMT1 and AtMATE1 are Al-activated transporters involved in the efflux of malate and citrate, respectively. However, microarray analyses showed that various genes were also regulated by STOP1 (Sawaki et al. 2009). It would be interesting to explore other Al tolerance genes and proton tolerance genes in future.

Recent works in rice have shown that multiple genes are involved in high Al tolerance in rice. The expression of these Al tolerance genes are regulated by a transcription factor ART1 (for Al resistance transcription factor 1, Yamaji et al. 2009), a rice homologue of *AtSTOP1*. ART1 regulates the expression of at least 31 genes. Some of its downstream genes include *STAR1/2*, *Nrat1*, *OsFRDL4* and *OsALS1*, which have been functionally characterized. STAR1/2 forms an ABC transporter, which is implicated in cell wall modification (Huang et al. 2009). OsFRDL4 functions as a citrate transporter, which secretes citrate from the roots to chelate Al outside (Yokosho et al. 2011). On the other hand, Nrat1 is an Al transporter localized at the plasma membrane that mediates Al influx into the cell (Xia et al. 2010) and OsALS1 is a tonoplast ABC transporter for Al sequestration (Huang et al. 2012). The cis-element of ART1 has been identified to be [GGN(T/g/a/C)V(C/A/g)S(C/G)], which is present in the promoter region of 29 out of the 31 genes (Tsutsui et al. 2011).

More recently, it was reported that transcriptional regulation by STOP1-like proteins was evolutionarily conserved among land plants. The complementation assays in *Atstop1* mutant revealed that STOP1-like proteins in other plant species, including tobacco (NtSTOP1), black poplar (PnSTOP1), tea (CsSTOP1), *Lotus japonicas* (LjSTOP1), and *Physcomitrella patens* (PpSTOP1), can enhance proton and Al tolerance by activating several proton and Al-tolerance genes (Ohyama et al. 2013).

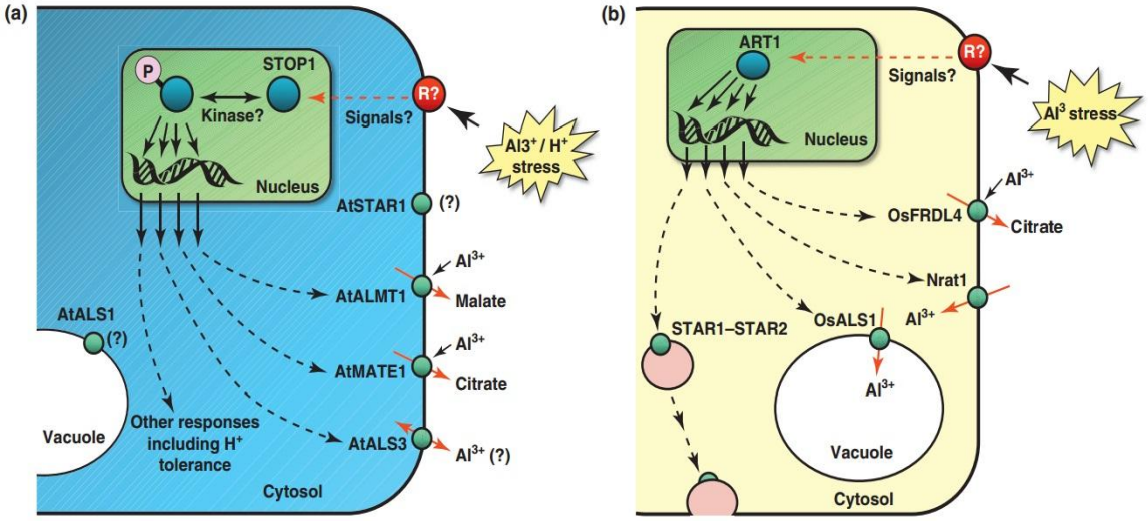


Figure 1.2 Models illustrating the coordinated induction of genes involved in Al tolerance. (a) STOP1-regulated Al^{3+}/proton tolerance in Arabidopsis. Al^{3+} and/or proton interact with a receptor (R) on the plasma membrane to initiate a signal transduction pathway. (b) ART1-regulated Al tolerance in rice. ART1 is activated by Al through an unknown pathway and binds to the promoter regions of multiple genes involved in Al tolerance. (Delhaize et al. 2012)

3. Role of malate transporter in Al tolerance

3.1 ALMT family

The ALMT proteins implicated in mechanisms of Al resistance belong to a larger group of related proteins that share an uncharacterized protein family (UPF0005; pfam01027; Delhaize et al. 2007). Since the first malate transporter gene (*ALMT1*) was identified in wheat (Sasaki et al. 2004), its homologous genes have been found in both monocots and dicots (Magalhaes 2006; Ryan et al. 2011). A phylogenetic tree of ALMT members is shown in Fig. 1.3 and all of these proteins share 19 fully conserved amino

acids mostly located in the N-terminal region (Delhaize et al. 2007). Although considerable studies focus on their function in Al-activated malate transport, the roles of the ALMT family are not restricted to Al resistance. For example, ZmALMT1 functions as an anion-selective transporter but not mediate malate or citrate transport (Pineros et al. 2008). AtALMT12 is an R-type anion channel required for stomatal movement in Arabidopsis guard cells (Meyer et al. 2010). AtALMT9 functions as a malate-activated vacuolar chloride channel required for stomatal opening in Arabidopsis (Angeli et al. 2013).

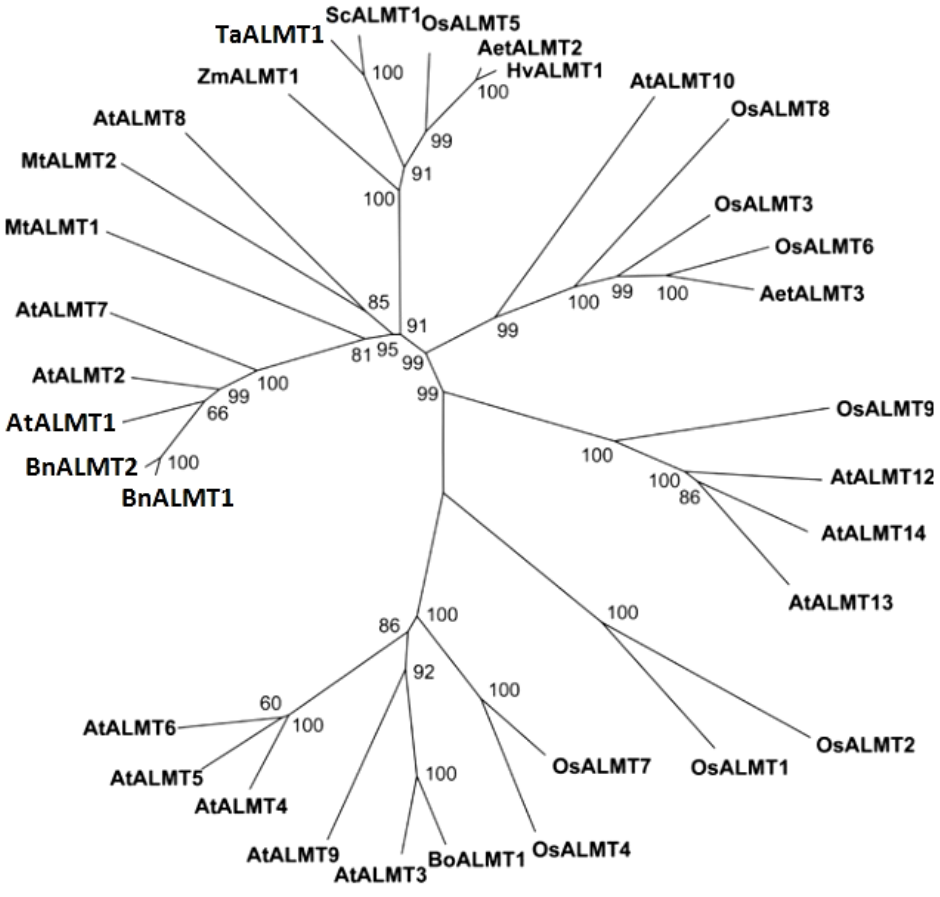


Figure 1.3 Unrooted phylogenetic tree of the ALMT family. (Delhaize et al. 2007)

3.2 Contribution of *ALMT1* genes to Al tolerance in plants

It was reported that *ALMT1* genes in many plant species mediate malate transport and confer Al tolerance. The major Al tolerance locus *Alt*, which co-segregated with an Al-induced malate efflux from root apices, was observed in wheat (Delhaize et al. 1993a; Riede and Anderson 1996, Raman et al. 2005; Zhou et al. 2007). The malate efflux transporter gene *TaALMT1* was demonstrated to co-localize with *Alt* locus (Roman et al. 2005; Zhou et al. 2007). TaALMT1 encodes an Al^{3+}-activated malate efflux channel on the plasma membrane of root apices (Yamaguchi et al. 2005). Transgenic plants expressing *TaALMT1* conferred an Al-activated malate secretion and increases their Al resistance (Sasaki et al. 2004, Delhaize et al. 2004; Pereira et al. 2010). Furthermore, different species share the common Al tolerance mechanism through ALMT1-mediated malate efflux, suggesting that with regard to mediating Al tolerance, ALMT1 family is conserved between monocots and dicots (Magalhaes 2006).

All the identified ALMT1 proteins require external Al to activate their function and efflux malate from roots (Ryan et al. 2010). It is more likely that Al directly triggers efflux by interacting with ALMT1 proteins, because heterologous expression of *ALMT1* in oocytes or tobacco suspension cells increased malate efflux just in the condition of Al (Sasaki et al. 2004; Zhang et al. 2008). This Al-dependent transport activity requires three acidic residues at the C-terminal domain (Furuichi et al. 2010). In some species, Al not only activates *ALMT1* encoded protein, but also induces its expression. *TaALMT1* (wheat), *ZmALMT2* (maize) and *HvALMT1* (barley) are constitutively expressed, whereas *AtALMT1* (Arabidopsis), *BnALMT1/2* (rape), *ScALMT1* (rye), *GmALMT1* (soybean) are up-regulated by Al (Sasaki et al. 2004; Ligaba et al. 2012;

Gruber et al. 2010; Hoekenga et al. 2006; Ligaba et al. 2006; Collins et al. 2008; Liang et al. 2013). The different Al-regulated pattern of gene expression determines the different pattern of organic acid anions secretion (Ma et al. 2001), but it is still not very clear how Al regulate these genes expression. Since Al-induced expression of *AtALMT1* is regulated by STOP1 (Iuchi et al. 2007), the common mechanisms might be involved in other plants. However, the process of sensing Al to transcriptional activation remains to be resolved. Besides, *ALMT1* genes are not only regulated by Al, but also induced by IAA, ABA, low pH, and hydrogen peroxide (Kobayashi et al. 2013). *BnALMT1/2* in oilseed rape is induced by metal ions other than Al (Ligaba et al. 2006). These findings suggest ALMT1's multiple functions in response to general stresses.

4. Mg transporters in plants and their role in Al tolerance

Magnesium (Mg) is an essential macronutrient for plant growth, which has diverse biological functions. The Mg requirement for optimal plant growth is 1.5-3.5 g kg^{-1} in vegetative parts (Marschner 2012). To maintain this level in different tissues, plants need to establish transport and regulation systems for Mg through the plants. However, compared with other cations, molecular mechanisms on uptake, translocation, distribution and storage of Mg are still poorly understood. On the other hand, Mg is able to alleviate Al toxicity in a number of plant species, but the mechanisms underlying this alleviation are not well understood.

4.1 Mg transporters in plants

There are two parallel ways for radial movement of Mg from soil solution to the stele

(Marschner 2012). One is symplasmic pathway, passing from cell to cell in the cytoplasm throughout plasmodesmata. The other is apoplasmic pathway, Mg quickly passing through the cell wall and extracellular space of epidermis and cortex, and converting to symplasmic pathway when it moves to the stele. Since entry of Mg from soil solution to the endodermis through the apoplasmic pathway is two-orders of magnitude faster than that through the symplasmic pathway, Kuhn et al. (2000) considered Mg uptake from the rhizosphere by passive diffusion. However, a kinetic experiment performed by Tanoi et al. (2011) showed that there are at least two separate transport systems involved in Mg transfer from external solution to xylem in rice.

The membrane protein-mediated transport of Mg has been studied. The first Mg transporter (CorA) was identified in bacteria by screening of Co resistance (Hmiel et al. 1986). CorA assembles as a pentamer protein complex and owns two closely spaced transmembrane domains near the C-terminus (Lunin et al. 2006; Niegowski and Eshaghi 2007). In the genome of Arabidopsis, there are 10 CorA homologues (Schock et al. 2000; Li et al. 2001; Gebert et al. 2009). All of them are characterized by a conserved Gly-Met-Asn (GMN) tripeptide motif at the end of the first transmembrane domain (Knoop et al. 2005). Except for pseudogene *AtMGT8*, all members show transport activity for Mg when expressed in yeast (Gebert et al. 2009). In the rice genome, there are nine *CorA*-like homologues. Although none of them is functionally identified, Tanoi et al. (2011) showed that Mg transfers from external solution to xylem was inhibited by Co-Hex, a known inhibitor of CorA transport, suggesting that rice CorA family is involved in Mg transport. However, information for Mg transporter system in other plant species is little known.

Among them, only *AtMGT1* and *AtMGT7* show higher expression in the roots,

suggesting that these genes may be involved in root Mg uptake. Knockout of *AtMGT7* results in a growth defect on low Mg nutrient solution (Gebert et al. 2009). However, AtMGT7 is localized to the endoplasmatic reticulum (ER), raising a question on its implication in Mg uptake from soil solution to the root cells. AtMGT1 seems more promising because it is localized to the plasma membrane (Li et al. 2001); however, further study is still needed to clarify its role in Mg uptake in plant.

In leaves, free Mg^{2+} is primarily stored in the vacuoles for adjustment of osmotic potential. Mg^{2+} influx into the vacuoles is mediated by an Mg^{2+}/H^{+} exchanger such as AtMHX (Shaul et al. 1999). Recently, AtMGT2 and AtMGT3 are reported to be involved in Mg partitioning in Arabidopsis mesophyll vacuoles (Conn et al. 2011). Both of them are localized to the tonoplast. It is speculated that Mg as a key osmoticum is required to maintain growth in low calcium concentrations in Arabidopsis.

Mg is also required for pollen development. *AtMGT9* is reported to be highly expressed in the mature anthers, leaves and young roots (Chen et al. 2009). Disruption of this gene results in abortion of mature pollen grains (Chen et al. 2009), indicating the involvement of AtMGT9 in Mg transport to anthers.

In addition to AtMGT family, there are several other transporter families involved in Mg transport. For example, AtMHX in Arabidopsis, MgtA, MgtB and MgtE in bacteria, and TRPM6/TRPM7 in human cells (Shaul et al. 1999; Smith and Maguire 1998; Townsend et al. 1995; Monteilh-Zoller et al. 2003; Chubanov et al. 2004), all of which showed no any similarity to AtMGT family. *AtMHX* is expressed throughout the plant but strongly expressed in the vascular tissue (Shaul et al. 1999). Since it is localized at vacuolar-membrane, *AtMHX* is likely to store Mg in these tissues for later release when needed. Unlike CorA, MgtA and MgtB are P-type ATPases that

primarily mediate Mg influx, and the expression of both *MgtA* and *MgtB* are highly induced by low Mg concentration (Tao et al. 1995; Soncini et al. 1996). In Arabidopsis, similar to *CorA*, all the *AtMGT* genes cannot be induced by short- or long-term Mg deficiency (Waters 2011), which gives us a speculation that there are other transporter families participated in Mg transport in plant.

4.2 Mg-mediated alleviative effect on Al toxicity

Al toxicity could be alleviated by supply of Mg in a number of plant species including sorghum (Tan et al. 1992), soybean (Silva et al. 2001a), wheat (Ryan et al. 1994), rice (Watanabe and Okada 2005) and rice bean (Yang et al. 2007). The Mg-mediated alleviative effect on Al toxicity differs with plant species. In some species such as soybean and rice bean, Mg at micromolar concentrations is able to alleviate Al toxicity (Silva et al. 2001a; Yang et al. 2007), but millimolar concentrations are required to have the alleviative effects in other species such as wheat and rice (Ryan et al. 1997; Watanabe and Okada 2005). The latter has been attributed to increased ionic strength of the solutions (Noble and Sumner 1988), reduction in Al saturation at the apoplastic exchange sites (Grauer and Horst 1992) and decreased Al activity at the root cell plasma membrane surface (Kinraide 2003; Kinraide et al. 2004). Al toxicity is caused by its binding to different cellular components including cell wall and plasma membrane (Ma 2000). The Al^{3+} and Mg^{2+} ions have similar hydrated radius (Bose et al. 2011). Therefore, high Mg concentrations at millimolar range displace or compete with Al from binding sites on the root cell wall, plasma membrane, and other components, protecting the roots from Al toxicity.

In plants, some possible mechanisms for Mg (micromolar range)-alleviated Al

toxicity were proposed: 1) addition of Mg enhances secretion of organic acid anions from plant roots, which chelate Al into non-toxic complexes and reduces Al toxicity (Silva et al. 2001b; Yang et al. 2007). 2) Al and Mg compete for membrane cation transporters and metal-binding sites of enzymes (Bose et al. 2011). 3) regulation of H^+-ATPase activity and cytoplasmic pH by addition of Mg (Ahn et al. 2001; Yang et al. 2007; Bose et al. 2011; Marty 1999; Babourina and Rengel 2009). In spite of these, there is still little evidence interpreting the mechanism of Al alleviation by Mg in higher plants.

It was found that some Mg transporters play an important role in Mg-alleviated Al toxicity. Overexpression of several Mg transporter genes is able to increase Al resistance (Deng et al. 2006; Gebert et al. 2009; Bose et al. 2011). In rice, a *CorA*-like transporter gene (*Os01g0869200*) was highly induced by Al, suggesting its possible contribution to Al tolerance (Yamaji et al. 2009).

Chapter 2 Functional analysis of a magnesium transporter gene *OsMGT1* involved in Al tolerance in rice

1. Introduction

Mg has been reported to affect the uptake of other cations in the rhizosphere. Addition of Mg is able to alleviate the rhizotoxicity induced by Cu, Zn, Al, Na and low pH (Kinraide et al. 2004). In contrast, Mg deficiency is able to be induced by supply of high concentrations of K, Ca, NH_4^+-N and Al (Ding et al. 2006; Tanaka et al. 1991; Tsutsumi and Takahashi 1988; Tan et al. 1992). The mechanisms underlying this interaction have been proposed as follows:

1) competition for apoplast binding (Grauer and Horst 1992; Kinraide et al. 2004). Mg displaces or competes with other cations from binding to the targets (cell wall, plasma membrane, etc.) at the extracellular space, reducing cations uptake by roots.
2) competition for ion transporters. Some ion transporter families are capable of transporting several cations (Piñeros and Tester 1995; Guo et al. 2010; Horie et al. 2011). The elevated Mg uptake may inhibit transport of other cations.
3) The sensitivity of Mg transporters to other cations. It was suggested that Mg transporter proteins could be molecular targets for toxic metal cations, resulting in the decrease of Mg uptake by inhibiting the Mg transporter activity (Li et al. 2001).

Mg-mediated alleviation of Al toxicity has been observed in a number of plant species (Tan et al. 1992; Silva et al. 2001a; Ryan et al. 1994; Watanabe and Okada 2005; Yang et al. 2007), but the mechanisms underlying the alleviation are still poorly

understood. Enhanced secretion of organic acid anions is likely involved in the Mg-alleviated Al toxicity at micromolar range. In soybean, addition of 50 μM Mg enhanced the citrate concentration in the root tips and stimulated citrate secretion from the roots exposed to toxic Al level (Silva et al. 2001b), resulting in increased Al tolerance by forming non-toxic citrate-Al complex in the rhizosphere. Addition of 10 μM Mg in the Al treatment solution also increased citrate secretion in rice bean (Yang et al. 2007). This increase has been associated with restoring plasma membrane H^+-ATPase activity. However, the exact mechanisms leading to the increased citrate secretion by Mg is still poorly understood.

On the other hand, increasing internal Mg seems to be also required for conferring Al tolerance. Overexpression of yeast Mg transporters (ALR1 or ALR2) confers Al tolerance in yeast (MacDiarmid and Gardner 1998). Overexpression of *AtMGT1* alleviates Al toxicity in *Nicotiana benthamiana*, which is associated with the increased Mg uptake (Deng et al. 2006). Overexpression of *AtMGT7* in *Arabidopsis thaliana* conferred higher Al tolerance, while knockout of *AtMGT7* showed an increased sensitivity to Al (Bose et al. 2011).

Rice is the most Al-tolerant crops among small grain cereal. This high level of tolerance has been attributed to multiple Al tolerance genes, which are regulated by a transcription factor, ART1 (Al resistance transcription factor 1). Some of ART1 downstream genes including *STAR1/STAR2*, *Nrat1*, *OsFRDL4* and *OsALS1* have been functionally characterized. However, most ART1-regulated genes have not been characterized. In the present study, an ART1-regulated gene, *OsMGT1* (*Oryza sativa* magnesium transporter 1; *Os01g0869200*) encoding a putative Mg transporter, was functionally characterized. It was found that rice is able to up-regulate this gene under

Al stress, conferring Al tolerance in rice.

2. Materials and methods

2.1 Plant materials and growth conditions

Two Tos-17 insertion lines of rice (*Oryza sativa* L.), NF0595 and NE4528 for *OsMGT1*, were obtained from the Rice Genome Resource Center in Japan. The homozygous lines was screened by PCR using specific primers (5′-AACACGCATCTAAAAGTTTCACC-3′ and 5′-TTCGATTATTATTGCTCCCACA-3′) for NF0595 and specific primers (5′-TAGGGATGAGCTGGAGCACT-3′ and 5′-CATCAGTTGACCGAGAGCTG-3′) for NE4528, with a left-border Tos-17 primer (5′-ATTGTTAGGTTGCAAGTTAGTTAAGA-3′). Seeds of wild type rice (cv. Nipponbare) and two Tos-17 homozygous lines were soaked in deionized water at 30 °C in the dark for two days, and then transferred to a net floating on a 0.5 mM $CaCl_2$ solution in a 1.5-L plastic container. Seedlings were grown for 3 to 7 d at 25 °C before being used for various experiments. Al and Mg were applied as $AlCl_3 \cdot 6H_2O$ and $MgCl_2 \cdot 6H_2O$, respectively. All experiments were repeated at least twice with three replicates each.

2.2 RNA isolation, cloning and gene expression analysis

Total RNA from rice roots was extracted using the RNeasy Mini Kit (Qiagen). One microgram of total RNA was used for first strand cDNA synthesis using a SuperScript II kit (Invitrogen) following the manufacturer's instructions. The cDNA fragment

containing an entire *OsMGT1* open reading frame was amplified by RT-PCR using the primers 5′-GGTACCAAAATGGAGCGGAGGGCGCA-3′ and 5′-TCACTGCAGGATCTTGCTCTTCC-3′. The fragment was cloned into the pGEM-T vector (Promega) for sequence confirmation using the ABI PRISM 310 Genetic Analyzer and the BigDye Terminators v3.1 cycle sequencing kit (Applied Biosystems).

For expression analysis, seedlings of WT were exposed to a solution containing 0 or 50 μM Al. At different time (0, 2, 4, or 6 h), the roots and shoots were sampled for RNA extraction and expression level determination. Seedlings exposed to different pH (4.5 or 5.6), or to Cd and La were also sampled. Root tips (0–1 cm) and basal roots (1–2 cm) were excided from the seedlings exposed to 50 μM Al for six hours. Samples were immediately frozen in liquid nitrogen. RNA extraction and cDNA preparation were performed as described above. The gene expression level was determined by real-time RT-PCR using Thunderbird SYBR qPCR Mix (TOYOBO) on Mastercycler ep realplex (Eppendorf). The primers used were 5′-GGCGCGTGCAGAAGATTAGGG-3′ and 5′-CGCGTATTCACGGATATGGTACAGGG-3′ for *OsMGT1*. *Histone H3* (Forward primer, 5′-AGTTTGGTCGCTCTCGATTTCG-3′; Reverse primer, 5′-TCAACAAGTTGACCACGTCACG-3′) was used as an internal control. Normalized relative expression was calculated by the ΔΔCt method.

2.3 Phylogenetic analysis

Alignment was performed with Clustal W using default setting (http://www.genome.jp/tools/clustalw/), and the phylogenetic tree was constructed using the neighbor-joining algorithm with 1000 bootstrap trials by MEGA software.

2.4 Subcellular localization

The subcellular localization was investigated by introducing 35S:OsMGT1-GFP or 35S: GFP-OsMGT1 into rice leaf protoplasts. The rice leaf protoplasts were prepared from 2-week-old rice (cv. Nipponbare) seedlings grown hydroponically, and used for transformation by the polyethylene glycol method as described by Chen et al. (2006). The GFP signal was observed using an LSM700 laser scanning microscope (Zeiss).

2.5 Evaluation of Al tolerance

To compare Al sensitivity, 5-day-old seedlings of both wild-type rice and knockout lines were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing various $AlCl_3$ concentrations (0, 10, 30, and 50 μM) for 24 h. Root length was measured by a ruler before and after the Al treatment, and relative root elongation (RRE = (root elongation with Al) / (root elongation without Al)) was calculated.

To investigate the tolerance to metals, 5-day-old seedlings were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 μM Al, 10 μM Cd, or 5 μM La for 24 h, and the root length was measured and RRE was calculated.

The alleviative effect of Mg on Al toxicity was investigated by exposing WT and knockout lines (5-day-old) to a 0.5 mM solution (pH 4.5) containing 0 or 50 μM Al in the presence of different Mg concentrations (0, 1, 2, 5, 10, 50, 100, and 500 μM), Ba (10 μM), or Sr (10 μM) for 24 h. The root length was measured and RRE was calculated.

To evaluate growth on acid soil, germinated seeds were sowed on acidic soil (Andsol, pH 4.5) or neutral soil (pH 6.5). After one-week growth, the plants were removed from soil and photographed.

2.6 Root cell sap collection and Mg determination

Five-day-old seedlings of both wild type rice and *OsMGT1* knockout lines were exposed to a 0.5 mM $CaCl_2$ solution containing 50 μM Al (pH 4.5) for 8 h. After the treatment, the root segments (0-1 cm) were excised and cell sap was extracted according to Xia et al (2010). Briefly, the root segments (15 roots each per sample) were washed three times with 0.5 mM $CaCl_2$ and then put in a Ultra free-MC centrifugal filter units (Millipore) and centrifuged at 3,000 × g for 10 min at 4°C. After frozen at -80 °C overnight, the samples were thawed at room temperature for 30 min and the root cell sap solution was obtained by centrifuging at 20,600 × g for 10 min. The residual cell wall were washed with 70% ethanol three times and then immersed in 0.5mL of 2 N HCl for at least 24 h with occasional vortex. The Mg element in the symplastic solution and cell wall extracts were determined by ICP-MS using an Agilent 7700 mass spectrometer (http://www.agilent.com).

2.7 Collection of root exudates and organic acid determination

To collect root exudates, the seedlings of both WT and knockout line were grown in a 1/2 strength Kimura B nutrient solution for 14 days as described in Yamaji and Ma (2007). Before collection, the seedlings were exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution overnight and then transferred to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 50 μM Al. After 24 h, the root exudates were collected and passed through a cation-exchange column (16 × 14 mm) filled with 5 g Amberlite IR-120B resin (H^+ form), followed by an anion-exchange column (16 × 14 mm) filled with 2 g AG 1×8 resin (100-200 mesh, formate form). Organic acid anions retained on the

anion-exchange resin were eluted by 2 N HCl, and the eluate was concentrated to dryness at 40°C using a rotary evaporator. After the residue was redissolved in 1 ml of milli-Q water, the concentration of organic acids was analyzed by enzymatic method according to Delhaize et al. (1993).

2.8 ^{25}Mg uptake experiment

A time-course experiment of ^{25}Mg uptake was conducted by exposing 7-d-old seedlings to a 0.5 mM $CaCl_2$ solution (pH 4.5) labeled with ^{25}Mg at 10 μM. ^{25}Mg (99%) was purchased from Nippon Sansho (Tokyo, Japan). At different time points (0, 0.5, 2, 6, 18, and 24 h), the roots and shoots were sampled. The roots were washed with 10 mM iced $CaCl_2$ solution for three times.

To compare the effect of Al on Mg uptake, a short-term uptake experiment was conducted. Seven-day-old rice seedlings were first exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 0 or 50 μM Al for 6 h to induce *OsMGT1* expression and subsequently subjected to a solution containing different Mg concentrations ranging from 0 to 500 μM ^{25}Mg. After 30 min, the roots were sampled as described above.

The samples were dried in 70°C oven for two days. After digested with concentrated HNO_3, the concentration of ^{24}Mg and ^{25}Mg was determined by ICP-MS with isotope mode on an Agilent 7700 mass spectrometer (http://www.agilent.com).

2.9 Microarray analysis

Seedlings (5-day-old) of both the wild type rice and *osmgt1* knockout line (NE4528) were exposed to a 0.5 mM $CaCl_2$ solution containing 50 μM Al. After 6 hours, the roots were sampled for RNA isolation. Total RNA was extracted using the RNeasy

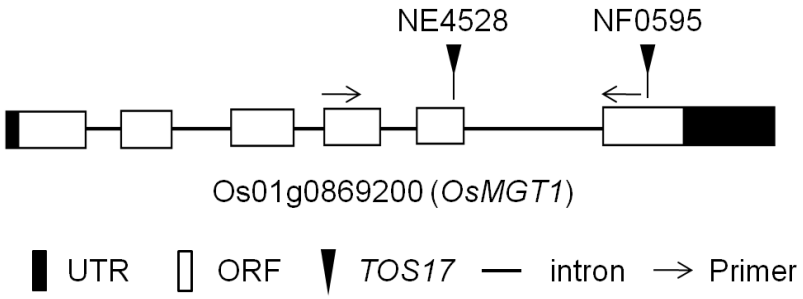
Plant Mini Kit (Qiagen). Microarray analysis was performed using the Rice Oligo DNA Microarray 44 K RAP-DB (Agilent Technologies) with three biological replicates. The hybridized slides were scanned using a DNA microarray scanner (Agilent Technologies). Signal intensities were extracted by Feature Extraction software (Agilent Technologies). For data analysis, genes with signal intensities below 100 (average of three biological replicates in WT) were excluded and genes up- or down-regulated by 2 folds were selected. Genes were categorized into 9 classes according to the OryzaExpress database (http://bioinf.mind.meiji.ac.jp/OryzaExpress/)

3. Results

3.1 Sequence analysis of *OsMGT1* in rice

OsMGT1 (*Os01g0869200*) cloned from rice contains 6 exons and 5 introns (Fig. 2.1A), encoding a peptide of 418 amino acids. Using the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/), OsMGT1 is predicated to be a membrane protein with two transmembrane domains near the C-terminus (Fig. 2.1B). OsMGT1 showed the similarity ranging from 63-81% to Arabidopsis Mg transporter AtMRS2/AtMGT family at the amino acid level (Fig. 2.1C). Like other Arabidopsis members, there is a conserved Gly-Met-Asn (GMN) tripeptide motif at the end of the first transmembrane domain (Fig. 2.1D) (Knoop et al. 2005).

A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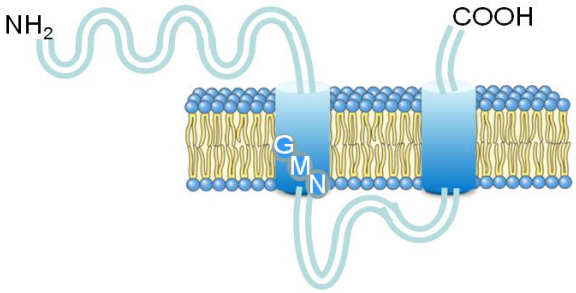
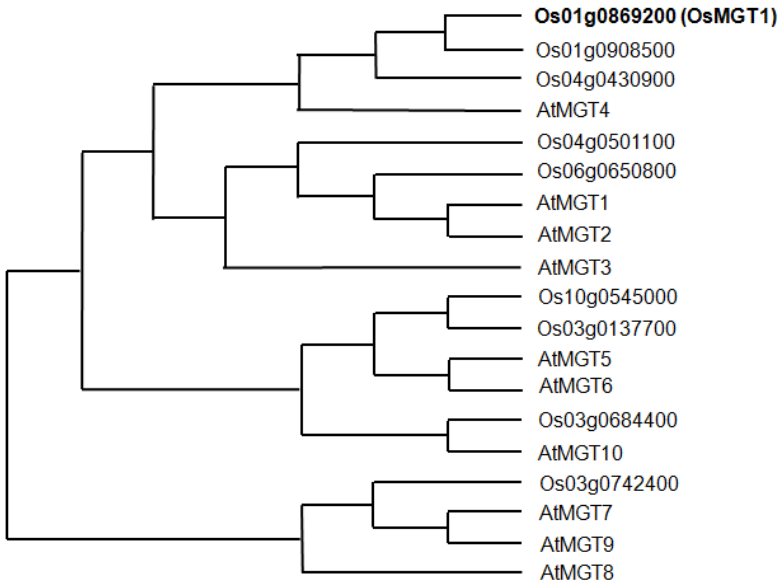
Os01g0869200 (*OsMGT1*)
UTR
ORF
TOS17
intron
Primer

B


NH2
COOH
G
M
N

C


Os01g0869200 (OsMGT1)
Os01g0908500
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AtMGT4
Os04g0501100
Os06g0650800
AtMGT1
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AtMGT3
Os10g0545000
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AtMGT7
AtMGT9
AtMGT8

D

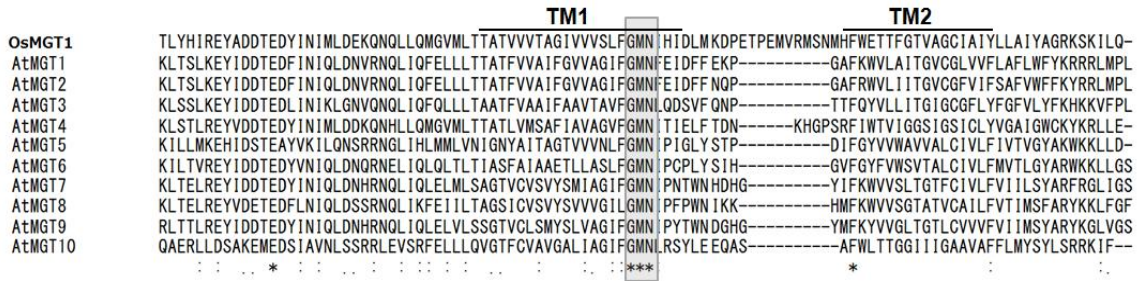


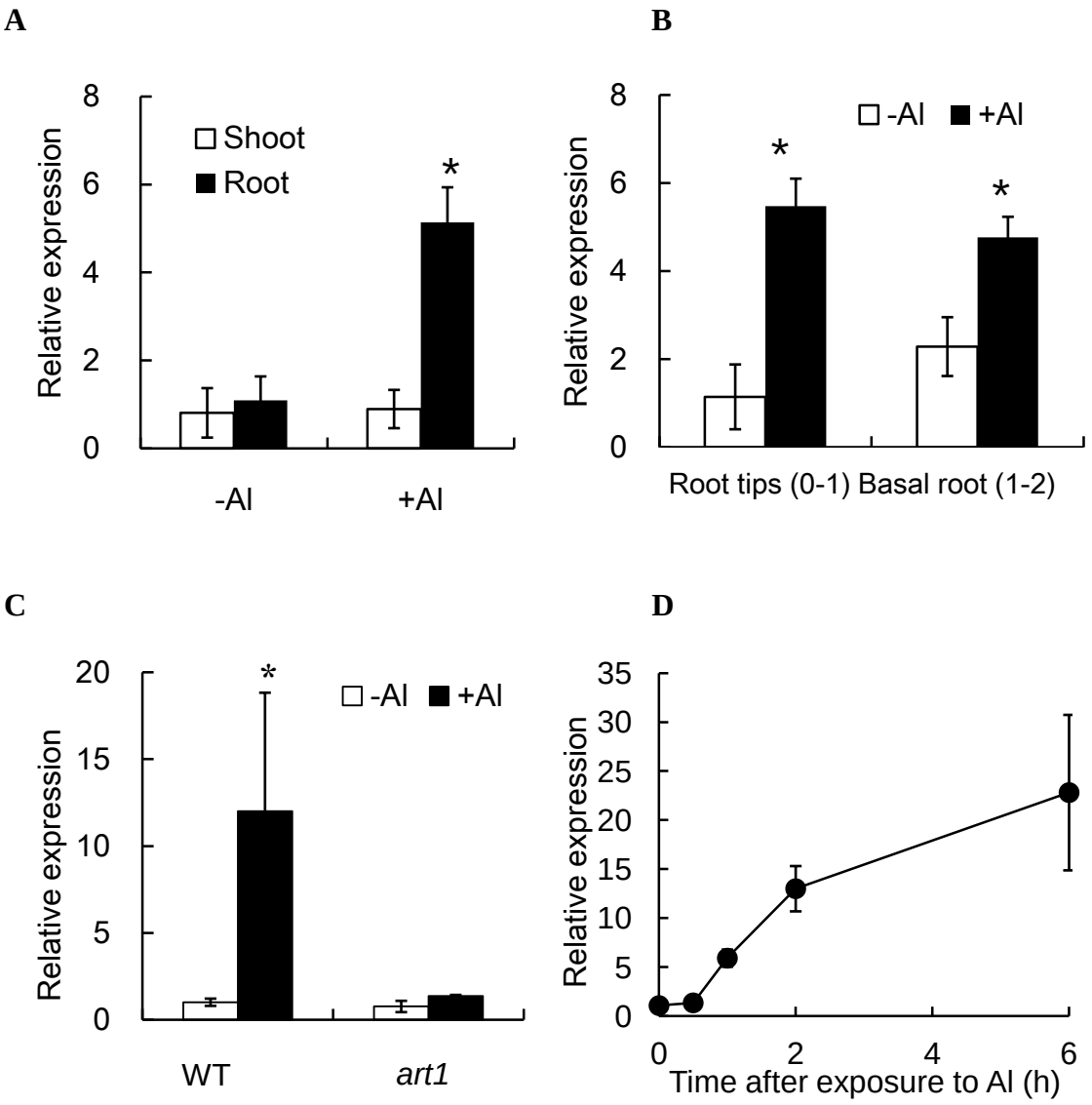
Figure 2.1 Gene structure (A), predicated membrane domains (B), phylogenetic tree (C) and alignment of OsMGT1 with its homologs (D). TM, predicated transmembrane domain. The phylogenetic tree was constructed using the Tree View program with the amino acid sequences.

3.2 Expression pattern of *OsMGT1*

Real-time RT-PCR analysis revealed that *OsMGT1* was expressed in both the shoots and roots at similar level in the absence of Al (Fig. 2.2A). However, in the presence of Al, the expression in the roots was up-regulated by 5 times, but that in the shoots was unaffected. Root spatial analysis showed that *OsMGT1* was expressed in both the root tips (0-1 cm) and mature region (1-2 cm) and the expression in both regions were up-regulated by Al (Fig. 2.2B). However, in *art1* (a mutant defective in the Al response C2H2-type zinc finger transcription factor), the Al-induced expression was not observed (Fig. 2.2C), confirming that *OsMGT1* expression was regulated by ART1 (Yamaji et al. 2009).

A time-course experiment showed that the Al up-regulated expression of *OsMGT1* occurred within one hour and kept at a high level thereafter (Fig. 2.2D). Neither low pH nor other metals including Cd and La affected the expression of *OsMGT1* in the

roots (Fig. 2.2E), indicating Al-specific response of the expression.



E

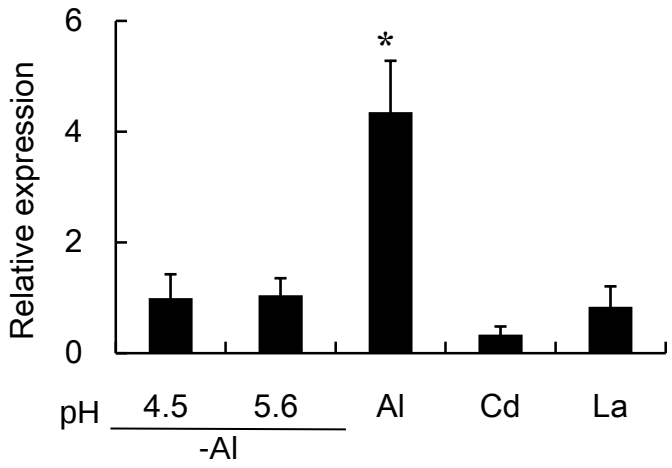


Figure 2.2 Expression pattern of *OsMGT1* in rice. A, Expression of *OsMGT1* in the roots and shoots. Rice seedlings were exposed to a solution containing 50 μM Al at pH 4.5 for 6 h and the roots and shoots were sampled for analysis. B, Root spatial expression. After exposing to 50 μM $AlCl_3$ for 6 h, root segments (0–1 cm and 1–2 cm) were excised with a razor. C, Expression of *OsMGT1* in the *art1* mutant. Both wild type rice and the *art1* mutant were exposed to 20 μM $AlCl_3$ for 4 h. D, Time-dependent expression of *OsMGT1*. Rice seedlings were exposed to a solution containing 10 μM $AlCl_3$ for different time. E, Metal- and pH-dependent expression. Rice seedlings were exposed to a solution containing 0, 30 μM Cd, 10 μM La, or 50 μM Al at pH 4.5 or containing 0 μM Al at pH 5.6 for 6 h. The expression level was determined by real time RT-PCR and relative expression to *Histone H3* (internal standard) is shown. Data are means ± SD (n=3). The asterisk shows a significant difference ($P<0.05$ by Tukey's test).

3.3 Subcellular localization of OsMGT1

The subcellular localization of OsMGT1 was investigated in rice protoplasts by transiently expressing OsMGT1 fused with GFP at both C-terminal and N-terminal

under the control of 35S promoter. Either construct gave a signal at the plasma membrane (Figs. 2.3A and 2.3B), in contrast to GFP alone, which signal was observed in cytosol and nuclei (Fig. 2.3C).

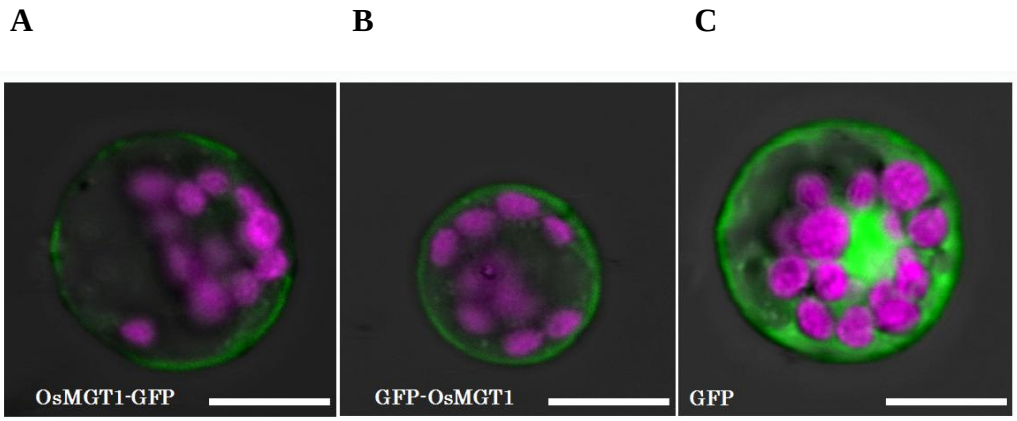


Figure 2.3 Subcellular localization of OsMGT1. OsMGT1-GFP (A), GFP-OsMGT1 (B) constructs, or GFP alone (C) were transformed into rice leaf protoplasts by polyethyleneglycol method. Pink color shows chloroplast autofluorescence. Scale bar = 10 μm.

3.4 Knockout of *OsMGT1* resulted in increased sensitivity to Al

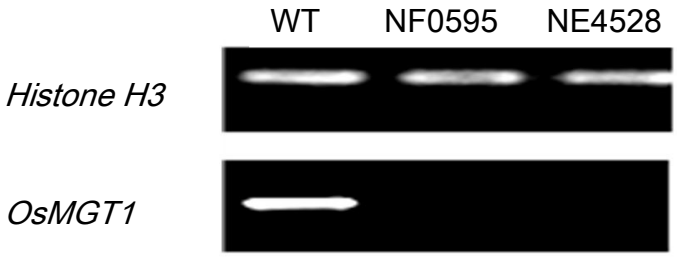
To investigate the physiological role of *OsMGT1* in Al tolerance, two independent retrotransposon *Tos-17* insertion lines, NE4528 and NF0595 were obtained. *Tos-17* was inserted at the 5^{th} and 6^{th} exon, respectively (Fig. 2.1A). RT-PCR analysis showed that there was no entire mRNA expression of this gene in two lines (Fig. 2.4A), indicating that they are knockout lines of *OsMGT1*.

We first compared the Al tolerance between WT and two knockout lines. In the absence of Al and low Al concentration (10 μM), the root elongation was similar between WT and the two knockout lines (Fig. 2.4B). However, in the presence of 30 μM and 50 μM Al, the mutants showed higher inhibition of root elongation than WT (Fig.

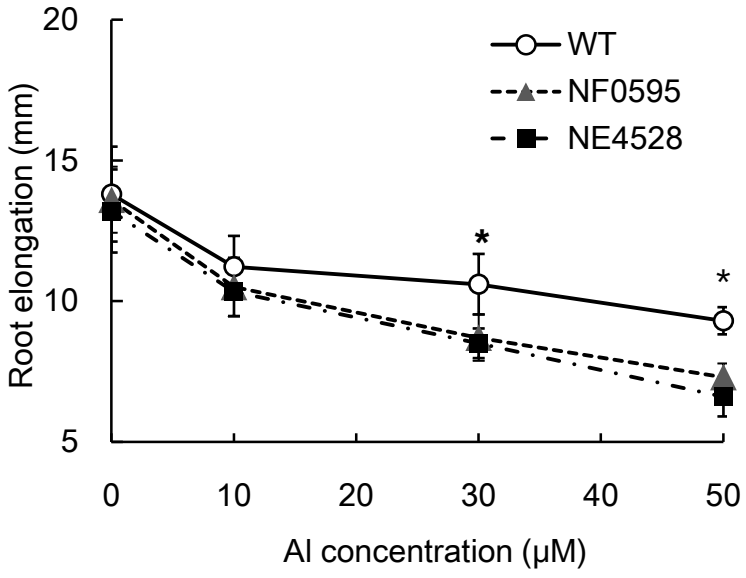
2.4B). There was no difference in the tolerance to other metals including Cd and La between WT and the two knockout lines (Fig. 2.4C), indicating that knockout of *OsMGT1* only specifically affects Al sensitivity.

To test the tolerance on acid soil, both WT and knockout lines were grown on acid soil (Andosol, pH 4.5) and neutral soil (pH 6.5). On neutral soil there was no difference in root growth (Fig. 2.4D), but the growth was inhibited in the knockout lines compared with WT on acid soil (Fig. 2.4D). These results further indicate that *OsMGT1* is required for Al tolerance in rice.

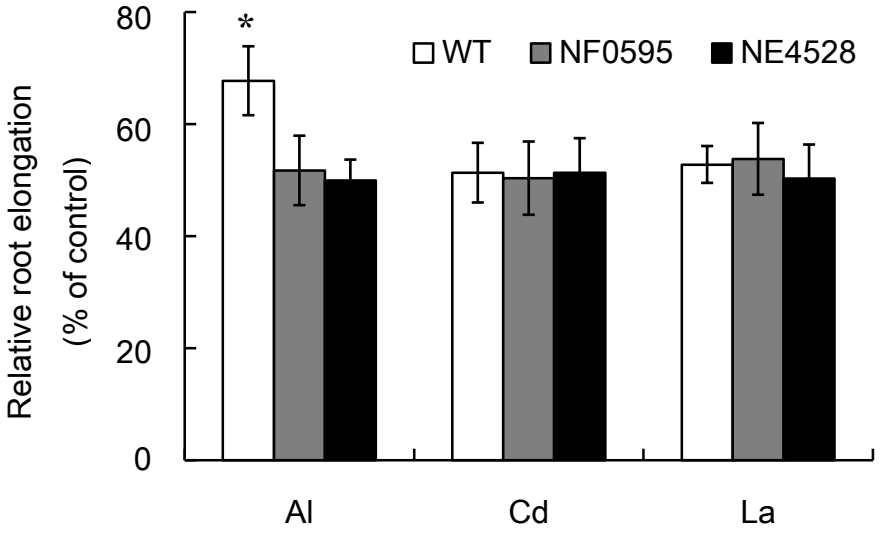
A



B



C



D

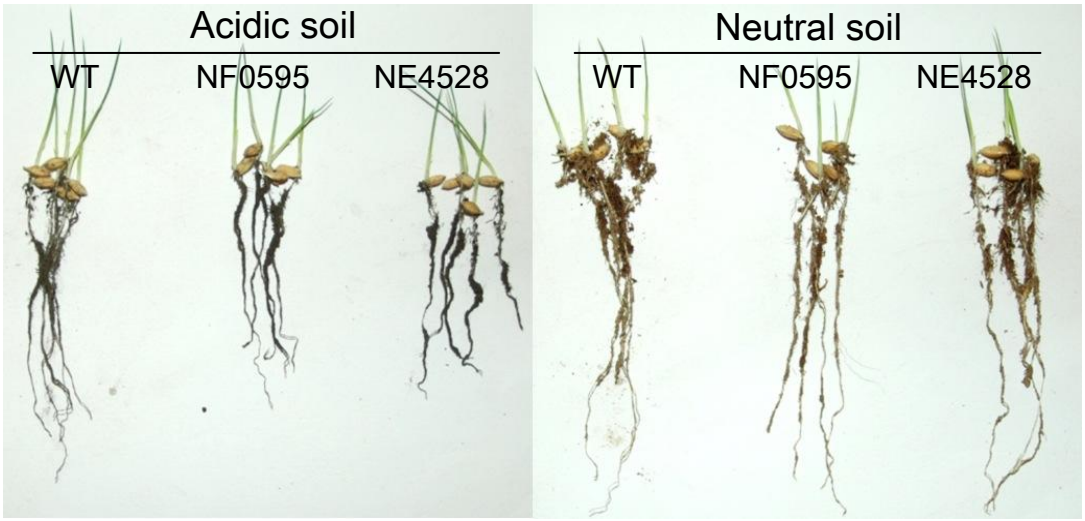


Figure 2.4 Phenotypic analysis of *OsMGT1* knockout lines. A, mRNA expression of Tos-17 lines of *OsMGT1*. A pair of specific primers was designed to examine the expression of *OsMGT1*. B, Sensitivity of *OsMGT1* knockout lines to Al. Seedlings of wild-type rice (WT) and two *OsMGT1* knockout lines (NF0595 and NE4528) were exposed to a solution containing different concentration of Al (0, 10, 30 and 50 μM) for 24 h. The root length was measured before and after the treatment and elongation relative to the root growth without Al

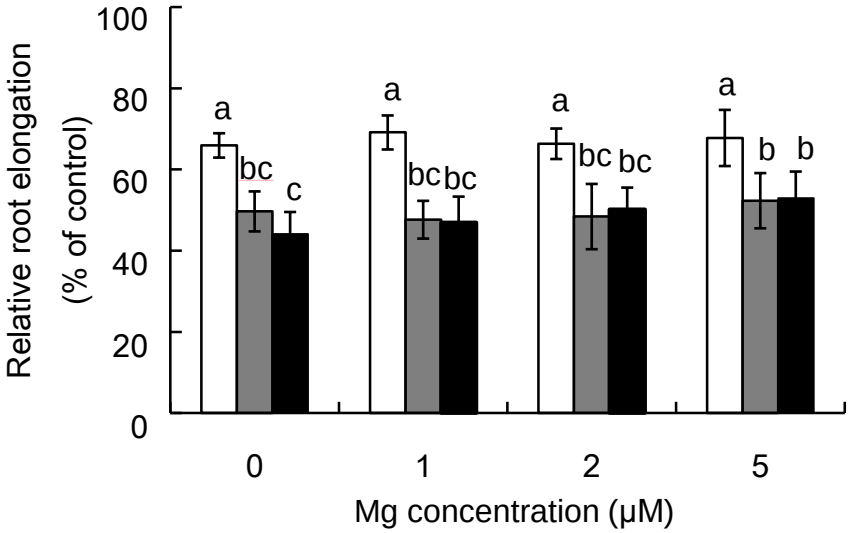
was shown. Data are means ± SD (n=10). The asterisk shows a significant difference at P<0.05 by Tukey's test. C, Sensitivity of *OsMGT1* knockout lines to other metals. WT and two knockout lines were exposed to a solution containing 50 μM Al, 10 μM Cd, or 5 μM La for 24 h. Data are means ± SD (n=10). The asterisk shows a significant difference (P<0.05 by Tukey's test). D, Growth on acidic soil. Germinated seeds were sowed on acidic soil (Andsol, pH 4.5) or neutral soil (pH 6.5) and grown for 1 week.

3.5 Micromolar Mg rescued Al-induced inhibition of root elongation

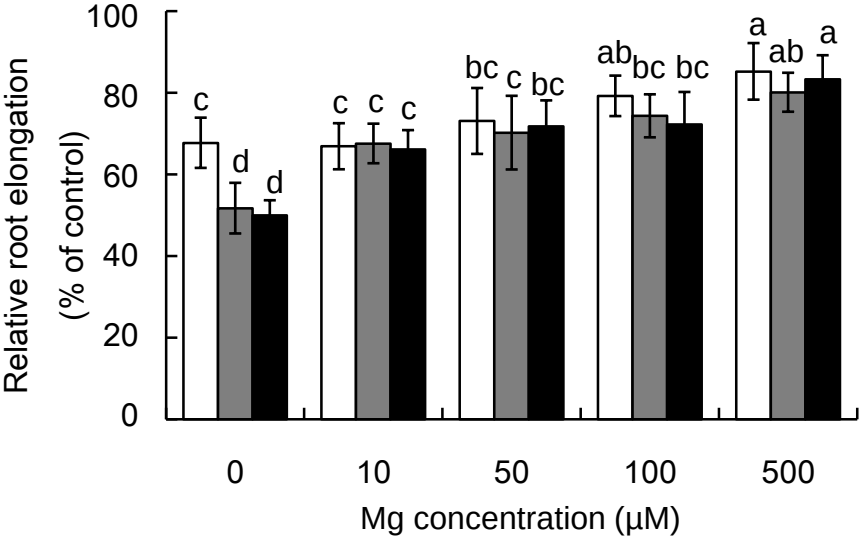
To investigate whether Al-induced inhibition of root elongation in the knockout lines can be alleviated by Mg, the seedlings were exposed to a solution containing a range of Mg concentrations in the presence of 50 μM Al. At low Mg concentrations (1 to 5 μM), addition of Mg did not alleviate Al-induced inhibition of root elongation in both WT and two knockout lines (Fig. 2.5A). However, at 10 μM and higher Mg concentrations, the root elongation in the knockout lines reached to the same level of WT (Fig. 2.5B). In the WT, Mg concentration below 50 μM did not improve the root elongation, but at Mg concentration higher than 100 μM, the Al-inhibited growth was also alleviated (Fig. 2.5B). This alleviative effect of Mg at higher concentration in both WT and knockout lines could be attributed to protective effect of Mg by preventing binding of Al to the cell components.

To test whether this alleviation is specific to Mg, Mg with two other divalent cations, Ba and Sr were compared. In the presence of the same concentration (10 μM), unlike Mg, neither Sr nor Ba showed alleviative effect on Al tolerance (Fig. 2.5C).

A


Relative root elongation
(% of control)
100
80
60
40
20
0
a
bc
c
a
bc
bc
a
bc
bc
a
b
b
0
1
2
5
Mg concentration (μM)

B


Relative root elongation
(% of control)
100
80
60
40
20
0
c
d
d
c
c
c
bc
c
bc
ab
bc
bc
a
ab
a
0
10
50
100
500
Mg concentration (μM)

C

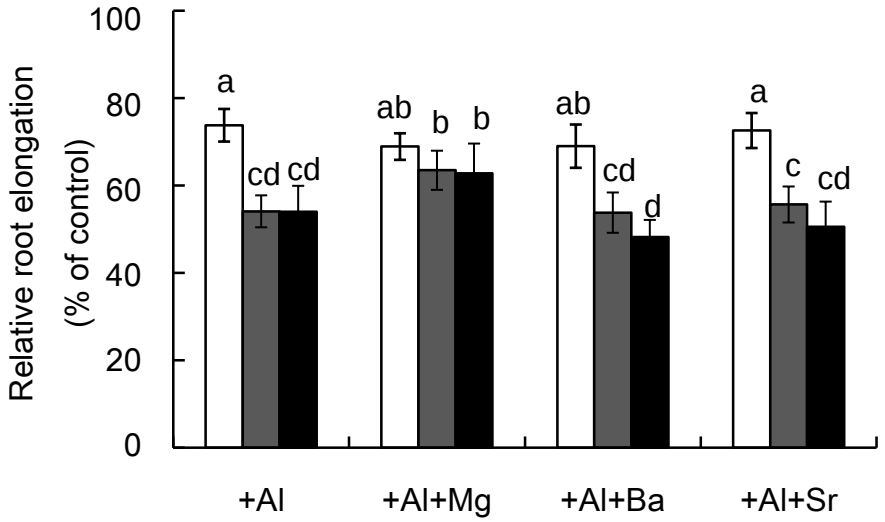


Figure 2.5 Alleviation of Mg on Al toxicity in *OsMGT1* knockout lines. A-B, Alleviative effect on Al toxicity by low (A) and high concentrations (B) of Mg. Seedlings of WT and two *OsMGT1* knockout lines were exposed to a solution containing 50 μM Al with different concentrations of Mg for 24 h. The root length was measured before and after the treatment and elongation relative to the root growth without Al was shown. Data are means ± SD (n=10). C, Alleviation of Al toxicity in *OsMGT1* knockout lines by divalent metals. Seedlings of WT and knockout lines were exposed to a solution containing 50 μM Al with 10 μM of Mg, Ba or Sr for 24 h. Data are means ± SD (n=10). Means with different letters are significantly different ($P<0.05$ by Tukey's test).

3.6 Knockout of *OsMGT1* did not affect Al-induced citrate secretion

Rice secretes citrate from the roots in response to Al (Ma et al. 2002; Yokosho et al. 2011). Since Mg-mediated alleviation of Al toxicity was reported to be attributed to enhanced organic acid anions secretion from the roots in soybean and rice bean (Silva et al. 2001b; Yang et al. 2007), the citrate secretion between WT and the knockout line

(NF0595) was compared. There was no difference in Al-induced secretion of citrate between WT and the knockout line (Fig. 2.6). This result indicates that different from other plant species, the Mg-mediated alleviation in the knockout line does not result from difference in organic acid secretion.

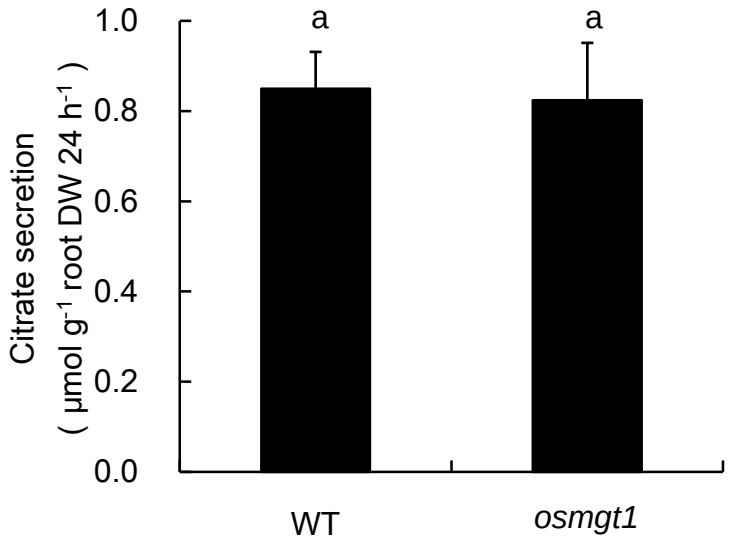


Figure 2.6 Al-induced citrate secretion in *OsMGT1* knockout line. Seedlings (21-day-old) of wild type rice (WT) and a knockout line (NF0595) were exposed to a solution containing 50 μM Al. Root exudates were collected for 24 h after Al treatment. Citrate was determined by an enzymatic method. Data are means ± SD (n=3). DW: dry weight. Means with different letters are significantly different ($P<0.05$ by Tukey's test).

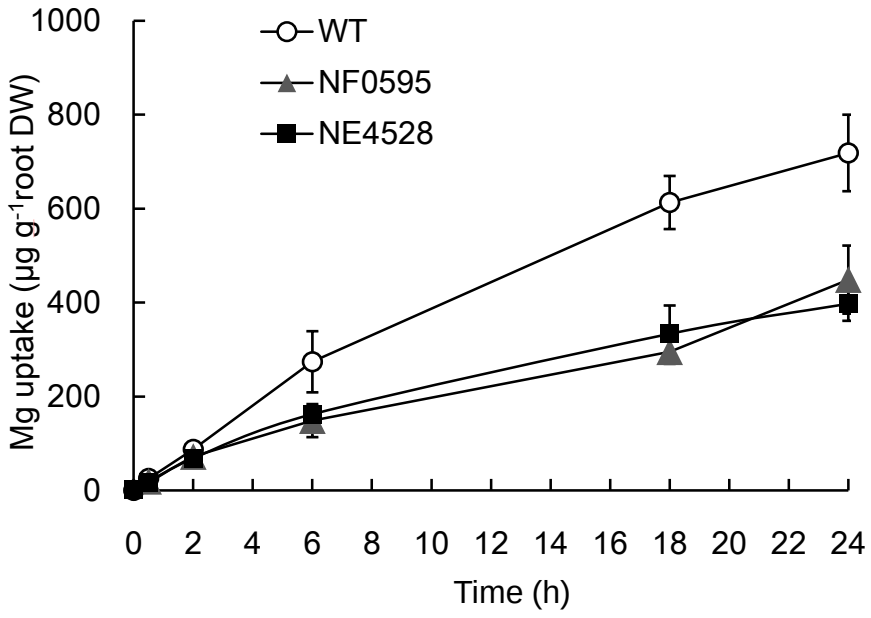
3.7 Al enhanced Mg uptake in WT, but not in *OsMGT1* knockout lines

To investigate whether OsMGT1 is involved in Mg uptake or not, the uptake experiments using stable isotope ^{25}Mg was performed. A time-course experiment showed that WT showed a higher ^{25}Mg uptake than two knockout lines in the absence of Al (Fig. 2.7A), indicating the involvement of OsMGT1 in Mg uptake in the roots. A

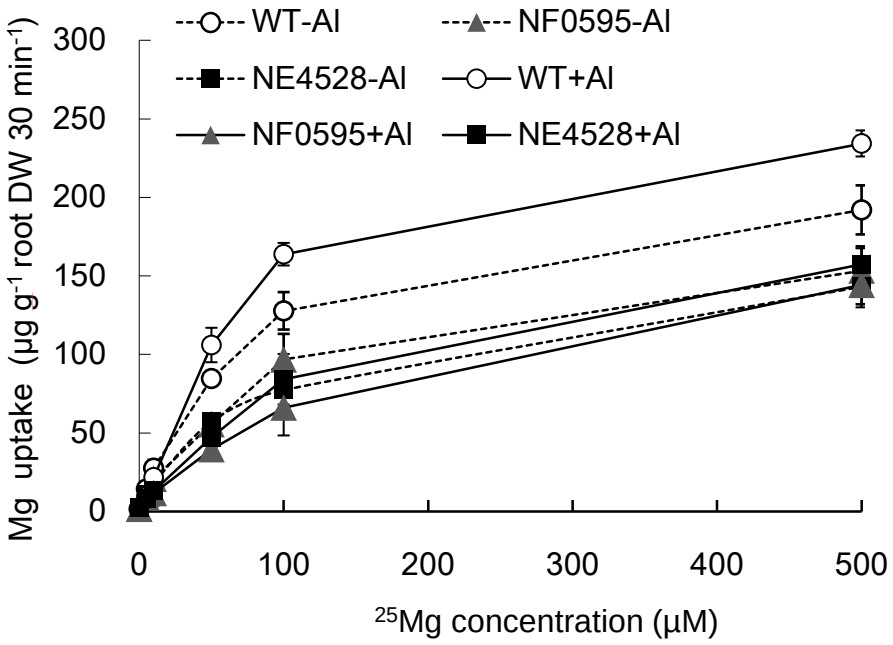
kinetic experiment with short-term (30 min) showed that the Mg uptake was increased by 6 h Al pretreatment in WT, but unaffected in the knockout lines (Fig. 2.7B). OsMGT1-mediated Mg uptake was calculated by subtracting Mg uptake of knockout lines from that of WT (Fig. 2.7C). The Km value was estimated to be 30.5 and 30.4 μM Mg, respectively, in plants exposed to Al or not. However, the Vmax was double in the Al-treated plants than in non Al-treated plants (84 μg g^{-1} root dry wt. 30 min^{-1} vs 41 μg g^{-1} root dry wt. 30 min^{-1}) (Fig. 2.7C).

The Mg concentration in the cell sap and cell wall of root tips (0-1cm) was also compared. In the absence of Al, the Mg concentration in the cell sap was similar between WT and knockout lines (Fig. 2.8A). However, in the presence of Al, the WT showed a higher Mg concentration in root cell sap than the knockout lines (Fig. 2.8A). By contrast, there was no difference in the cell wall Mg content (Fig. 2.8B).

A



B



C

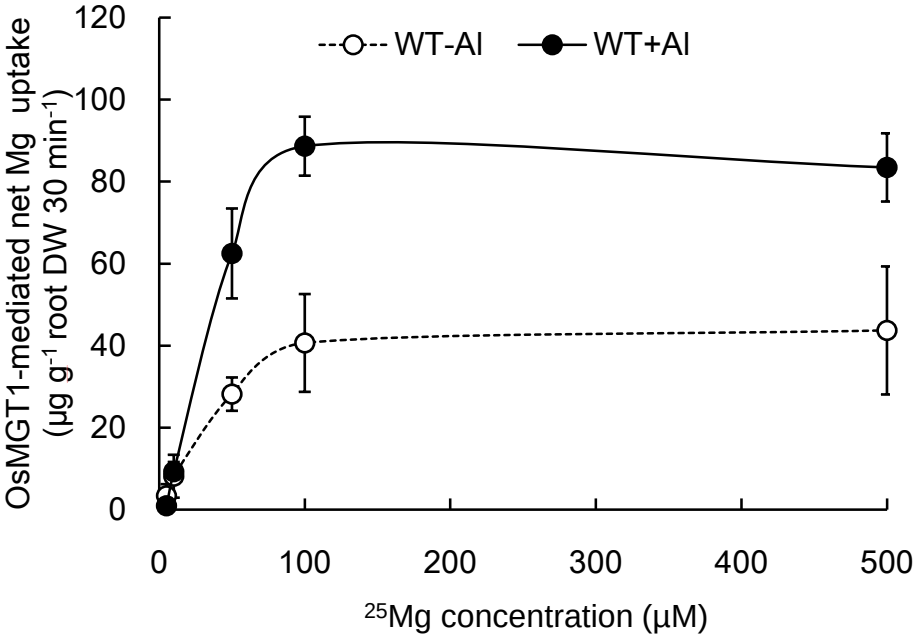
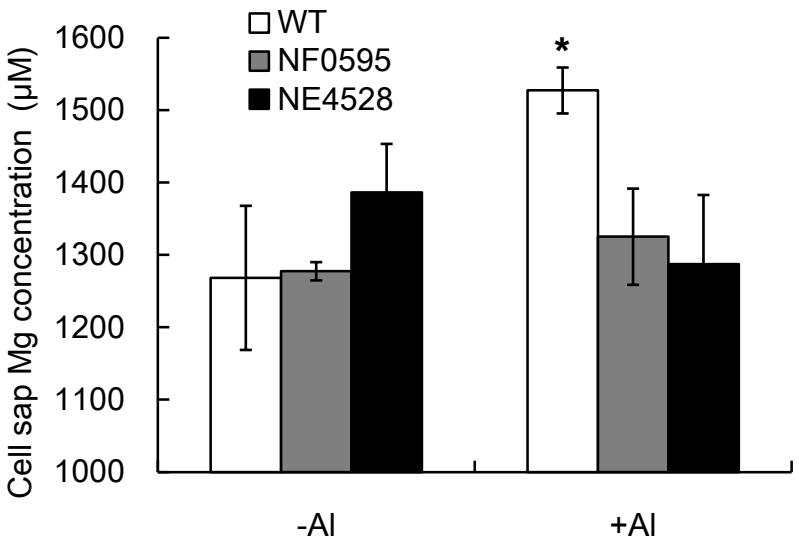


Figure 2.7 ^{25}Mg uptake in *OsMGT1* knockout lines. A, A time-dependent uptake of ^{25}Mg**.** Seedlings of WT and knockout lines were exposed to a solution containing 10 µM ^{25}Mg. At

different time points, the roots and shoots were sampled for determination of ^{25}Mg with ICP-MS. B, Effect of Al on ^{25}Mg uptake. Seedlings of WT and knockout lines were pretreated with or without Al (0 or 50 μM, pH 4.5) for 6 h and subsequently subjected to an uptake solution containing different concentrations of ^{25}Mg. After 30 min, the roots were sampled for determination of ^{25}Mg. Data are means ± SD (n=3). C, OsMGT1-mediated Mg uptake. The net uptake was calculated by subtracting Mg uptake of knockout lines from that of wild type.

A



B

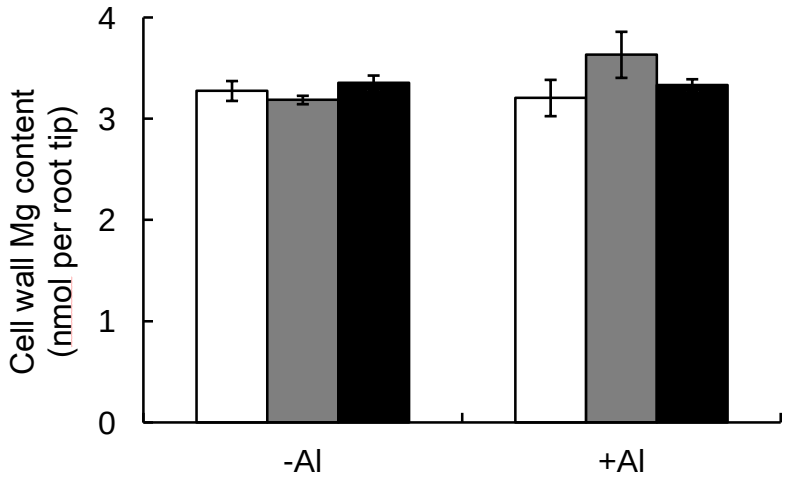


Figure 2.8 Effect of Al on Mg concentration in the cell sap and cell wall. Mg concentration in the cell sap (A) and cell wall (B) of root tips (0-1cm). Seedlings of both wild type rice (WT) and two knockout lines were exposed to a solution containing Al (0 or 50 μM) for 8 h. The root tips (0-1 cm) were excised and fractionated into cell sap and cell wall. The Mg concentration was determined by ICP-MS. Data are means ± SD (n=3). The asterisk shows a significant difference ($P<0.05$ by Tukey's test).

3.8 Transcriptomic analysis of *OsMGT1* knockout line

A comparative analysis of DNA microarray in Al-tolerant and Al-sensitive soybean genotypes revealed that Mg enhanced Al tolerance in the Al-tolerant genotype by down-regulating genes commonly induced in response to Al toxicity (Duressa et al. 2010). To examine the gene expression changes caused by knockout of *OsMGT1*, a genome-wide transcriptome analysis was carried out by comparing WT and *OsMGT1* knockout line exposed to Al for six hours using the rice 44 K oligo microarray. A total of 64 genes were down-regulated (≥2 fold) including *OsMGT1* and 132 genes were up-regulated (≥2 fold) in the knockout line. Among down-regulated genes, 38% of them are unknown genes, 21% of them are related to other metabolism and 19% are stress-related genes (Fig. 2.9). On the other hand, 34% of up-regulated genes are unknown genes, 16% are related to other metabolism and 28% are stress-related (Fig. 2.9). These genes are supposed to be induced by either Al toxicity or Mg deficiency. For example, 5 down-regulated (*Os01g0847600*, *Os03g0235000*, *Os05g0111300*, *Os06g0695300*, and *Os08g0302000*) and 4 up-regulated (*Os01g0370900*, *Os01g0374000*, *Os07g0638300*, and *Os09g0367700*) genes are involved in oxidative stress and ROS (reactive oxygen species) scavenging (Turóczy et al. 2011; Hu et al.

2011; Soranzo et al. 2004). Al is known to induce peroxidation and ROS formation in rice roots (Meriga et al. 2004; Sharma and Dubey 2007). Mg deficiency also can induce ROS generation in many plant species including rice (Cakmak and Kirkby 2008; Chou et al. 2011). One gene (*Os09g0416500*) encoding double-stranded RNA binding protein and one gene (*Os05g0270500*) encoding ribonuclease P-related protein were respectively downregulated and upregulated in the knockout line. The RNase activity and its dsRNA binding activity are Mg-dependent (Cerritelli and Crouch 1995; Smith and Pace 1993; Beebe et al. 1996). Furthermore, the Mg concentration seems critical for switching between these two activities (Cerritelli and Crouch 1995).

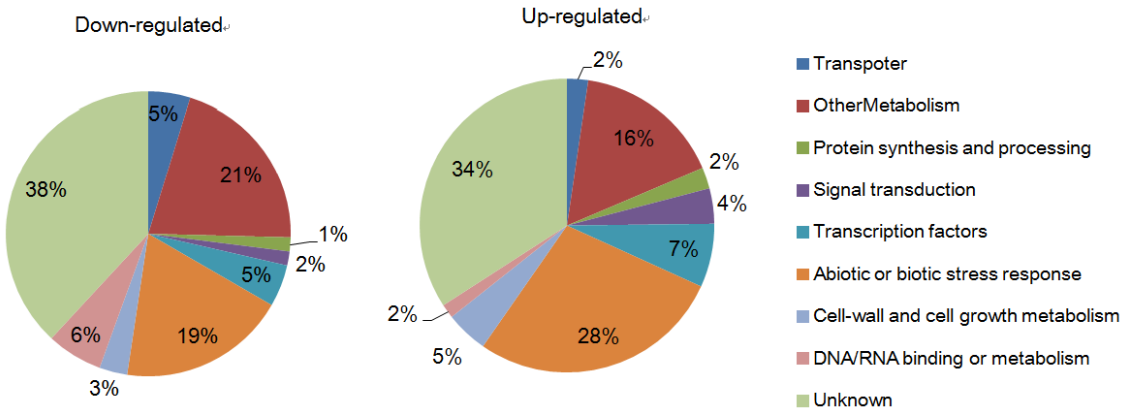


Figure 2.9 Functional classification of down- or up-regulated genes in *OsMGT1* knockout line. Micorarray analysis was conducted with roots of wild-type (WT) rice and a knockout line (NE4528) exposed to 50 μM (pH 4.5) Al for six hours. Genes significantly down-regulated or up-regulated more than 2 folds in the knockout line compared with WT were selected. Genes were categorized into 9 classes according to the OryzaExpress database (http://bioinf.mind.meiji.ac.jp/OryzaExpress/).

4. Discussion

4.1 OsMGT1 is a transporter for Mg uptake

In the genome of Arabidopsis, there are 10 CorA homologues (Schock et al. 2000; Li et al. 2001; Gebert et al. 2009). Among them, only *AtMGT1* and *AtMGT7* show higher expression in the roots, but so far no evidence has showed that they are involved in Mg uptake. In rice genome, there are nine CorA homologues (Gebert et al. 2009). However, none of them has been functionally characterized. In the present study, a plasma-localized transporter OsMGT1 for root Mg uptake in rice was found. A kinetic experiment using stable isotope ^{25}Mg with 30 min showed that knockout of *OsMGT1* decreased Mg uptake in the roots (Fig. 2.7), which suggests the direct involvement of OsMGT1 in Mg uptake in the roots. Besides, since *OsMGT1* is ART-regulated (Yamaji et al. 2009), OsMGT1 seems to be localized at all root cells as ART1 and other ART1-regulated downstream genes (Huang et al. 2009; Xia et al. 2010; Yokosho et al. 2011; Huang et al. 2012), suggesting that OsMGT1 mainly function as a Mg transporter for root Mg uptake, but not for xylem loading.

A puzzling thing on CorA-like Mg transporters in plants is that their expression is not induced by Mg deficiency. This characteristic is different from other essential elements. For example, *HvYS1*, a Fe-phytosiderophore transporter gene for Fe uptake in barley, is up-regulated by 25-fold upon Fe deficiency (Ueno et al. 2009). This phenomenon leads us to propose that other Mg transporters except for CorA-like Mg transporters are involved in Mg transport in the plants. In bacteria, there are Mg repressible and non-repressible Mg uptake systems. The non-repressible Mg uptake is mediated by the CorA protein (Hmiel et al. 1986), while the repressible Mg uptake is

mediated by the MgtA and MgtB protein (Hmiel et al. 1989). Unlike CorA, MgtA and MgtB are P-type ATPases that primarily mediate Mg influx, and the expression of both *MgtA* and *MgtB* are highly induced by low Mg concentration (Tao et al. 1995; Soncini et al. 1996). P-type ATPase is a large group of enzymes that use ATP to pump a wide range of cations across membrane against their electrochemical gradient (Williams and Mills 2005). There are 46 and 43 members, respectively, in the Arabidopsis and rice genome (Baxter et al. 2003). P-type ATPases in plants were reported to transport Na^+, K^+, H^+, Ca^{2+}, and heavy metals (Axelsen and Palmgren 1998), however, there is no report on transport of Mg by P-type ATPases. It would be interesting to explore whether these proteins also transport Mg in future. At least in rice, knockout of *OsMGT1* is unable to inhibit Mg uptake completely, suggesting other transporters also implicated in the Mg uptake.

4.2 OsMGT1-mediated alleviative effect on Al toxicity

On acidic soil, plants are apt to suffer Mg deficiency (Aitken et al. 1999). The existence of considerable Al in acidic soil is supposed to be one of the major constraints for Mg uptake in planta (Rengel and Robinson 1989; Tan et al. 1991). In Arabidopsis, two high-affinity Mg transporters (AtMGT1 and AtMGT10) activity was seriously inhibited by Al (Li et al. 2001). Overexpression of Mg transporter genes in both yeast and plant to increase internal Mg concentrations seems to be another effective way to enhance Al tolerance (MacDiarmid and Gardner 1998; Deng et al. 2006). However, as the most Al-resistant species among small grain cereals, rice seems to have developed a distinct strategy to overcome Al-induced inhibition of Mg uptake by up-regulating *OsMGT1*. In the presence of Al, the Mg uptake was significantly enhanced in the

wild-type rice, but not in the *OsMGT1* knockout lines (Figs. 2.7 and 2.8). Further analysis of the OsMGT1-mediated net Mg uptake by a kinetic experiment showed that, Vmax was double in the Al-treated plants compared with non Al-treated plants, but Km was similar (Fig. 2.7C), indicating that Al did not alter the OsMGT1 transport affinity for Mg, but up-regulated the expression of *OsMGT1*, resulting in more OsMGT1 participated in Mg transport. Therefore, increasing Mg concentrations in the cytosol by OsMGT1 contributes to higher Al tolerance in rice.

The mechanism on detoxifying Al by increasing internal Mg concentrations looks complicated as the unclear intracellular Mg and Al distribution at the subcellular level. It is known that Al in the cytosol has strong affinity for oxygen donor compounds such as inorganic phosphate, ATP, RNA, DNA, and proteins (Martin 1988). On the other hand, most Mg^{2+} in the cytosol is complexed with various ligands such as ATP and enzymes (Shaul 2002). Since Al^{3+} and Mg^{2+} ions have similar hydrated radius (Bose et al. 2011), increased Mg concentration in the cytosol prevents Al from binding to the cellular components, thus decreasing cell damage in Mg-dependent metabolism. Furthermore, since Al inhibits Mg uptake (Li et al. 2001; Rengel and Robinson 1989; Tan et al. 1991), up-regulation of Mg transporter can compensate influx of Mg into the cells.

As a conclusion, OsMGT1 is plasma membrane-localized transporter for Mg in rice and up-regulation of this transporter gene is required for conferring Al tolerance by increasing Mg uptake into the cells. In future, identification of novel Mg transporters and their functional characterization are required for better understanding of Mg transport system in plants. Investigation on regulation mechanism of Mg transporters (either transcriptional or post-translational) will also be necessary.

Chapter 3 Molecular mechanism of Al tolerance in an accession of *Holcus lanatus* adapted to acid soil

1. Introduction

Al-induced organic acid secretion from roots has been observed in a number of plant species (Ryan et al. 2001; Kochian et al. 2004; Ma 2005; Magalhaes 2006; Ma 2007). Genes responsible for the secretion have been identified, which belongs to *ALMT* and *MATE/AACT* families, respectively, for Al-induced malate and citreate transporters (Ryan et al. 2011; Delhaize et al. 2012). The variation in expression level of these genes has been observed among species and accessions. The high expression levels of these genes are associated with high Al tolerance in plant such as wheat and barley (Sasaki et al. 2006; Furukawa et al. 2007). Recent studies have revealed that the Al-tolerant genotypes have evolved pathways to enhance the expression of genes responsible for the efflux of organic acid anions by modifying the promoter sequence (Sasaki et al. 2006; Magalhaes et al. 2007; Ryan et al. 2010; Tovkach et al. 2013).

Compared with the progresses made in important crops and model plant species, little is known about the molecular mechanisms of Al tolerance in other plant species that grow in their natural habitats. Yorkshire Fog (*Holcus lanatus*), which belongs to the Poaceae family, is a close relative of the agronomic crop oat (*Avena sativa*). It is a highly adaptable grass species distributed in wide environmental conditions (Grime et al. 1988). For example, difference in arsenate tolerance in different accessions of *H. lanatus* has been reported. The higher tolerance to As seems to be achieved by suppression of the high affinity phosphate/arsenate uptake system (Meharg and Macnair

1992a). The high degree of polymorphism has been observed through investigating the arsenate tolerance and phosphate uptake in *H. lanatus* (Naylor et al. 1996, Meharg and Macnair 1992b). The difference in tolerance of *H. lanatus* to lead, zinc and cadmium was also found (Coughtrey and Martin 1978).

H. lanatus is also regarded as a relatively Al-tolerant species among grass species (Edmeades et al. 1991; Wheeler et al. 1992). For example, *H. lanatus* is distributed in most plots in the long-term (>150 years) Park Grass Experiment, Rothamsted, which vary greatly in the nutrient availability and in soil pH (from 3.6 to 7.5) (Silvertown et al. 2006). In the most acidic plots due to the long-term applications of ammonium sulphate, *H. lanatus* is the most dominant species (producing >90% of the total above-ground plant biomass in the plots), presumably due to its strong tolerance to Al toxicity. Yet, it is also found in the plots receiving the same fertilizer treatments but limed to the neutral range, where its biomass production decreases to <10% of the total due to the competition of other plant species.

In an effort to understand how *H. lanatus* adapts to highly acidic soils, two accessions of plants from an acidic plot (pH 3.6, designated as HL-A) and a limed plot (pH 7.1, designated as HL-N) was compared in their Al tolerance, organic acid anion secretion and the expression of related genes. It was found that the high Al tolerance in the accession from the acidic plot has an increased number of the cis-acting element of *HlALMT1* gene for a transcription factor HlART1, resulting in increased expression of *HlALMT1* and subsequently a higher secretion of malate from the roots.

2. Materials and methods

2.1 Plant materials and growth conditions

Two accessions of *H. lanatus* were obtained from the Park Grass Experiment (established in 1856), Rothamsted Research, UK: one from an acidic soil plot (plot 9/2d, soil pH 3.6; designated as HL-A) and the other from a limed plot of the same fertilizer treatment (plot 9/2a, soil pH 7.1, designated as HL-N). Seeds of *H. lanatus* were soaked in deionized water for 2 h, and then germinated in a plastic Petri dish with a filter paper saturated with distilled water in darkness at 25°C for 2 days. Seedlings were then transferred to a 3.5-L plastic pot containing continuously aerated one-fifth Hoagland solution at 25°C. For experiments described below, tillers from one plant of HL-N and HL-A each were used. The nutrient solution was changed once every two days.

2.2 Evaluation of pH response and Al tolerance

To investigate the effect of pH on root elongation, seedlings of both HL-N and HL-A were exposed to a 0.5 mM $CaCl_2$ solution buffered with 10 mM Homo-PIPES at pH ranging from 3.5 to 6.5 for 24 h. To compare Al sensitivity, seedlings were exposed to a 0.5 mM $CaCl_2$ solution (pH 4.5) containing 50 μM $AlCl_3$ for 0, 6, 12 and 24 h. Root length was measured with a ruler before and after the treatment, and the root elongation was calculated. To compare Al tolerance between rice and two accessions of *H. lanatus*, the seedlings were exposed to 50 μM $AlCl_3$ for 24 h and relative root elongation (RRE = (root elongation with Al) / (root elongation without Al)) was calculated. For Al staining, the roots were exposed to 100 μM $AlCl_3$ for 24 h and then stained with 0.1% Eriochome cyanine R for 15 min. The stained roots were observed under a microscope and photographed.

To determine the Al accumulation, seedlings of both HL-N and HL-A were exposed to a 0.5 mM $CaCl_2$ solution containing 25, 50 and 100 μM Al (pH 4.5). After 24 h, the roots were washed three times with 0.5 mM $CaCl_2$, and then the root segments (0-1 cm) were excised. The fractionation of cell wall and cell sap was performed according to Chen et al. (2012). Aluminum in different fraction was determined by atomic absorption spectrophotometer (Z-8270; Hitach, Tokyo, Japan).

2.3 Collection of root exudates and organic acid determination

Seedlings of both HL-N and HL-A (one-month old) were used for root exudate collection. Before collection, the seedlings were placed in a 0.5 mM $CaCl_2$ (pH 4.5) solution overnight and then transferred to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing different concentrations of Al (25, 50 and 100 μM) for different time (0, 3, 6, 9, 12 and 24 h). Seedlings were also exposed to a 0.5 mM $CaCl_2$ (pH 4.5) solution containing 50 μM Cd or La. Root exudate collection and organic acid determination followed the methods described in Yokosho et al. (2011).

2.4 Gene cloning and sequencing

Total RNA was extracted from *H. lanatus* roots using the RNeasy mini kit (Qiagen). One microgram of total RNA was used for first-strand cDNA synthesis using a SuperScript II kit (Invitrogen) following the manufacturer's instructions. To clone *HlALMT1*, a *HlALMT1* cDNA fragment of 635 bp was amplified using primers (5'-ACCGTCGTCGTCGTCATGGAGTA-3' and 5'-TTCTGGTATTGGCTCCATGGGTG-3') designed from the conserved sequences of *AtALMT1* (Arabidopsis), *TaALMT1* (wheat) and *BnALMT1/2* (rape). Then a 5'- and

3’-RACE (Smart RACE cDNA amplification kit; Clontech) was performed to amplify the full-length cDNA sequence of *HlALMT1*. The open reading frame (ORF) of *HlALMT1* cDNA was amplified by RT-PCR using primers 5’-ATGGATGTTGAGCACAACAGA-3’ and 5’-ACCACTCTGCTCTGCACCAT-3’. To clone *HlART1*, a *HlART1* cDNA fragment of 360 bp was amplified using primers (5’-ACGCGAACCTGCGGATGCACATG-3’ and 5’-CGTGGGCGAAGAGCTTGTCCTT-3’) designed from the conserved sequences of *ART1* and *ART1*-like genes in rice and Arabidopsis. The full-length cDNA of *HlART1* was generated by RACE method as described above. The open reading frame (ORF) of *HlART1* cDNA was amplified by RT-PCR using primers 5’-CAAATACGCGACTCTATAGAAGTT-3’ and 5’-GTACATCTGGAACTTTCCTGGTGAAAA-3’.

To clone the promoters of *HlALMT1*, SEFA-PCR (Wang et al. 2007) was performed to amplify the 2 kb upstream sequence of *HlALMT1* in the *H. lanatus* genome (SP1: 5’-AACCGCAAAAAGGTAGCCGCCGAT-3’; SP2: 5’-GCGAGCGATATGGCTTCTCCTA-3’; SP3: 5’-TTCTGCCAACTTATGGGNNNNNNNNNCGATGC-3’). The amplified fragments were cloned into the pGEM-T Easy vector (Promega, http://www.promega.com). The sequence was confirmed using the ABI PRISM 310 genetic analyzer and the BigDye terminators v3.1 cycle sequencing kit (Applied Biosystems).

2.5 Phylogenetic analysis

Peptide sequence alignment was analyzed by Clustal W using default settings (http://clustalw.ddbj.nig.ac.jp/). The phylogenetic tree was constructed using the Tree

View program with the amino acid sequences.

2.6 Expression pattern of *HlALMT1* and *HlART1*

For expression analysis, seedlings of *H. lanatus* were exposed to a solution containing 0, 25, 50, and 100 μM Al at different time (0, 3, 6, 9, 12 and 24 h), the roots (including 0-1, 1-2 and 2-3 cm segments) and shoots were sampled for RNA extraction and expression level determination. Samples were immediately frozen in liquid nitrogen. RNA extraction and cDNA preparation were performed as described above. The gene expression level was determined by real-time RT-PCR using Thunderbird SYBR qPCR mix (TOYOBO) on Mastercycler ep realplex (Eppendorf). The relative expression was normalized to the expression level of the *actin* gene (internal control). The primers used for *HlALMT1* were 5'-AGAGAGCAGCGACGAGATGGTCGG-3' and 5'-TTACTCAGCGTTGCTCCGACGG-3'. The primers used for *HlART1* were 5'-ACGCGAACCTGCGGATGCACATG-3' and 5'-GTGGCTCTTCTCACAGTGGCT -3'. The primers used for *actin* were 5'-TTGGATTCTGGTGATGGTGT-3' and 5'-GGAAGCTCGTAGCTCTTCTC-3'. The expression level of actin was unaffected by Al treatment and did not differ between two accessions.

To investigate tissue-specificity of *HlALMT1* expression, seedlings of *H. lanatus* were exposed to a solution containing 50 μM Al for 6 h, roots of both HL-N and HL-A were dissected at 7.5 to 12.5 mm according to Takahashi et al. (2010). The samples were immediately immersed in a fixing solution containing ethanol/acetic acid at a 3:2 ratio. Inner and outer tissues were collected from the root tissue sections using a Veritas Laser Microdissection System LCC1704 (Molecular Devices) and used for total RNA extraction as described above. The relative expression was normalized to the

expression level of the *actin* gene.

2.7 Copy number determination

To investigate *HlALMT1* copy number in the genomic DNA, the same primers and PCR conditions was used as described in the section 'Expression Pattern of *HlALMT1*' above, except that 100 ng of genomic DNA was used as the template, instead of cDNA for each reaction. The genomic copy number of *HlALMT1* in the two accessions was normalized by the cycle threshold (C_T) value of *Lsi1* (*Low silicon rice 1*; *Os02g0745100*). The *Lsi1* fragment was amplified using primers 5'-CGGTGGATGTGATCGGAACCA-3' and 5'-CGTCGAACTTGTTGCTCGCCA-3'.

To estimate transcript copy number, the standard curves for the absolute quantification for *HlALMT1* was generated. A series of dilutions (from 1×10^{-1} to 1×10^{-6} ng) of plasmids was made and then subjected to real-time PCR. Amplification efficiency was calculated to be 99.1% (*HlALMT1*). The C_T values for each sample were converted into absolute copy numbers using the standard curves.

2.8 Subcellular localization

The subcellular localization was investigated by introducing 35S:GFP-HlALMT1 or 35S:HlALMT1-GFP into onion epidermal cells. Primer pairs used for amplification and introduction of restriction sites were 5'-CTCGAGGGATCCCCCGGGATGGATGTTGAGCACAAC-3' (sense), 5'-CTCGAGCCCGGGCTCTGCACACTGAATAAC-3' (anti-sense for 35S: HlALMT1-GFP), and 5'-CCCGGGATCCGCGGCCGCTTACTCTGCACACTGAAT-3' (anti-sense for 35S:GFP-HlALMT1). Gold particles with a diameter of 1 μm coated

with 35S:GFP-HlALMT1, 35S: HlALMT1-GFP, or 35S:GFP and 35S:DsRed were introduced into onion epidermal cells using particle bombardment (PDS-1000/He particle delivery system; Bio-Rad) using 1100 p.s.i. rupture disks. After incubation at 25ºC for 18 h, the GFP signal was observed using an LSM700 laser scanning microscope (Zeiss, http://www.zeiss.com/).

2.9 Malate transport activity assay in *Xenopus* oocytes

The open reading frame (ORF) of the *HlALMT1* cDNA fragment was amplified using the following primers: 5'-CTCGAGGGATCCCCCGGGATGGATGTTGAGCACAAC-3' and 5'-CCCGGGATCCGCGGCCGCTTACTCTGCACACTGAAT-3'. The fragment that contained the ORF was inserted into a *Xenopus laevis* oocyte expression vector, pXbG–ev1 (Preston et al. 1992). cRNA preparation, injection and radioactivity measurement were performed as described previously (Yokosho et al. 2011).

For electrophysiological studies, the *HlALMT1* cRNA or water injected oocytes were incubated in the Modified Barth's Saline (MBS) solution at 18℃. After a 1 day incubation, 50 nl of 25 mM sodium citrate was injected into the oocytes and then incubated for 0.5-2 h in ND96 buffer containing 0 or 100 μM Al at pH 4.5 (Furuichi et al. 2010). The net current across the oocyte membrane was measured using the two-electrode voltage clamp syste4m with the amplifier (MEZ-7200 and CEZ-1200, Nihon Kohden, http://www.nihonkohden.co.jp/) at different membrane voltages. The electrical potential difference across the membrane was clamped from 0 mV to -120 mV. Replicates of 5-9 for each measurement were made.

2.10 Yeast one-hybrid assay

The yeast one-hybrid assay was performed using MATCHMAKER One-Hybrid Library Construction and Screening Kit (Clontech). The ORF of *HlART1* was amplified by PCR and cloned in frame after transcriptional activation domain of yeast GAL4 transcription factor (without DNA binding domain) in pGADT7 (pGADT7-HlART1). Primer pairs used for amplification and introduction of restriction sites were 5'-ACTGTCGACATGGACCGCGGCAAGAAT-3' and 5'-TAATGCGGCCGCCGGTACATCTGAAATTAT-3'. pGADT7-ART1 was constructed by Yamaji et al. (2009). The promoter regions of *HlALMT1* (-1986 to -139 bp from the start codon in HL-N and -1981 to -128 bp from the start codon in HL-A) were amplified from the genomic DNA of HL-A and HL-N, and cloned into the upstream of the HIS3 reporter gene in pHIS2.1 vector. Primer pairs used for amplification and introduction of restriction sites were 5'-TAGAATTCGCGCAGTGGCAACCTGG-3' and 5'-TATACGCGTTAGGCTGGCAGACAAACA-3'. The plasmid (pGADT7, pGADT7-HlART1 or pGADT7-ART1) together with the plasmid (N: pHIS2.1 + *HlALMT1* promoter from HL-N, or A: pHIS2.1 + *HlALMT1* promoter from HL-A) were introduced into yeast strain Y187 and cultured on SC medium (-His) containing 0, 20 or 50 mM 3-amino-1, 2, 4-triazole (3AT, a competitor of HIS3) at 30ºC according to the manufacturer's manual. After 3 days, the yeast growth was photographed.

2.11 Transient assay in tobacco protoplasts

Tobacco (*Nicotiana tabacum*) plants were cultivated hydroponically as described before (Tsutsui et al. 2011). Young and fresh leaves were cut into small pieces with a razor and protoplasts were isolated according to Tsutsui et al. (2011). For transient assay in

tobacco protoplasts, *GFP* was used as a reporter gene. The promoter regions of *HlALMT1* (-1986 to -1 bp from the start codon in HL-N and -1981 to -1 bp from the start codon in HL-A) were amplified from the genomic DNA, and cloned into the upstream of [CaMV 35S minimal promoter (-46) + *GFP* + NOS terminator] in pBluescript vector constructed by Tsutsui et al. (2011). Primer pairs used for amplification and introduction of restriction sites were 5'-CGTCTAGAGCGCAGTGGCAACCTGG-3' (forward), 5'-CGTCTAGAAGCAGAAGTGCAGAACCA-3' (reverse for HL-N) and 5'-CGTCTAGAGGCAGAACCAATGGTGGC-3' (reverse for HL-A). To construct the effector vector, The ORF of *HlART1* was amplified by PCR and cloned between CaMV 35S promoter and NOS terminator in pBluescript vector by using primer pairs: 5'-ACTGTCGACATGGACCGCGGCAAGAAT-3' and 5'-TAATGCGGCCGCCGGTACATCTGAAATTAT-3'. *DsRed* was used as an internal standard. The internal control vector was constructed by Tsutsui et al. (2011). The reporter vector, effector vector and internal control vector were co-transformed into tobacco protoplasts by PEG method according to Tsutsui et al. (2011). The transformed protoplasts were incubated with Murashige and Skoog medium (0.22% [w/v] Murashige and Skoog, 400 mM mannitol, 10 mM MES-KOH, pH 5.4) in the dark for 17 h at 22ºC, followed by exposure to 200 μM $AlCl_3$. After 6 h, the protoplasts were collected by centrifugation at 100g for 5 min and the pellet was sampled using liquid nitrogen for RNA extraction. The quantitative real-time RT-PCR was performed by using specific primers: *GFP*, 5'-AGGAGCGCACCATCTTCTTCAA-3' and 5'-GCTGTTGTAGTTGTACTCCAGC-3'; *DsRed*, 5'-GGACAACACCGAGGACGTCATC-3' and

5’-CGCCCTTGGTCACCTGCAGCTT-3’.

2.12 Generation of transgenic rice

The *HlALMT1* promoter regions from HL-N (-1986 bp) and HL-A (-1981 bp) were amplified from genomic DNA, and fused with *HlALMT1* ORF by overlap PCR. These fragments containing *HlALMT1* promoters and *HlALMT1* ORF were ligated into pPZP vector (Fuse et al. 2001) and then transformed into *Agrobacterium tumefaciens* (strain EHA101). Primer pairs used for amplification and introduction of restriction sites were 5’-ATGGGCCCGCGCAGTGGCAACCTGGA-3’ and 5’-GCTCTAGACGCTCTCTAGACAGCTGG-3’. To transform these plasmids into rice, callus was induced from mature embryos of rice cultivar Nipponbare for *Agrobacterium*-mediated transformation (Hiei et al. 1994).

The genomic copy number of *HlALMT1*, expression of *HlALMT1* in the roots of transgenic lines, citrate and malate in the root exudates were determined as described above.

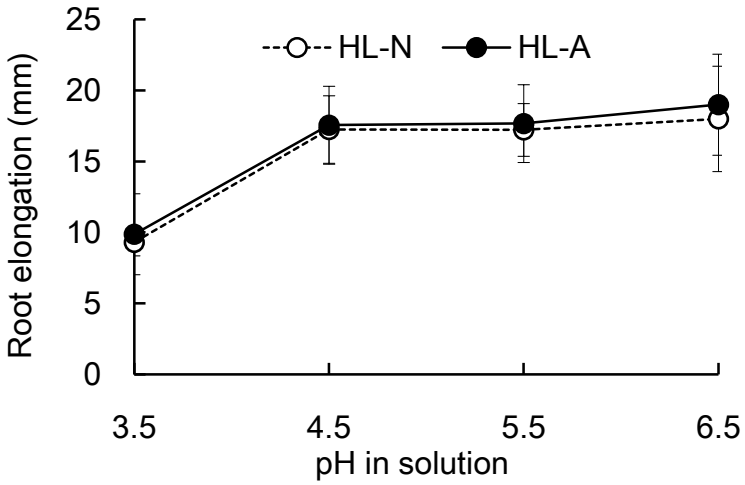
3. Results

3.1 Comparison of tolerance to low pH and Al between two accessions of *H. lanatus*

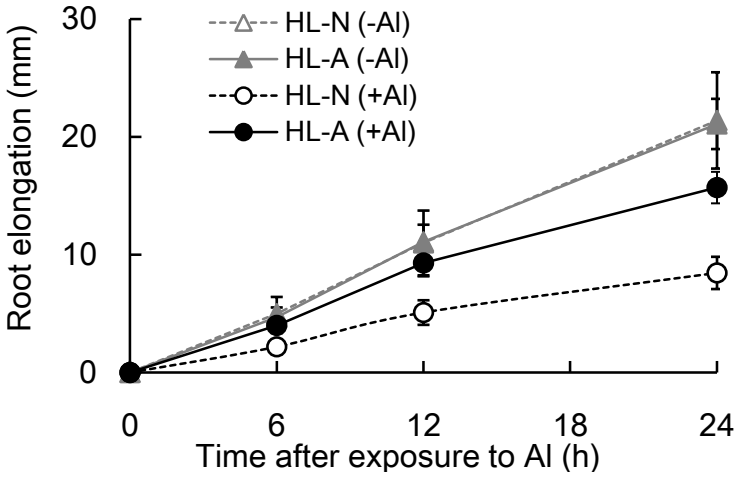
The root elongation at different pHs was first compared between HL-A from an acidic plot and HL-N from a neutral plot. However, there was no difference in the root elongation between two accessions at pHs ranging from 3.5 to 6.5 (Fig. 3.1A). Then Al tolerance between two accessions was compared. In the presence of 50 μM Al, root elongation of HL-N was more inhibited than that of HL-A in a time-dependent manner

(Fig. 3.1B). The Al tolerance of HL-A was as high as a japonica rice cultivar, Nipponbare, the most Al-tolerant species among small grain cereal crops (Fig. 3.1C). Eriochome cyanine R staining showed that HL-N accumulated more Al in the root tips than HL-A (Fig. 3.1D). Consistent with this result, the Al contents in the cell sap and cell wall of the root tips (0-1 cm) were also significantly higher in HL-N than in HL-A at each Al concentration (Figs. 3.1E and 3.1F), indicating that an exclusion mechanism is involved in the higher Al tolerance of HL-A.

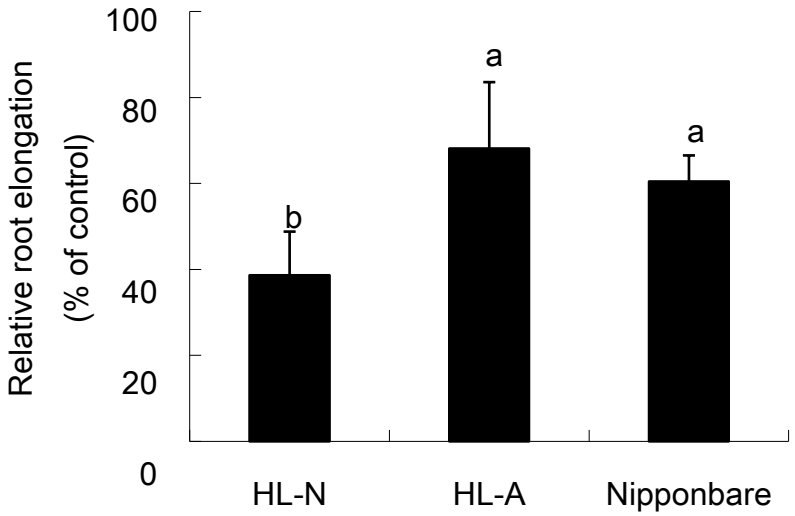
A



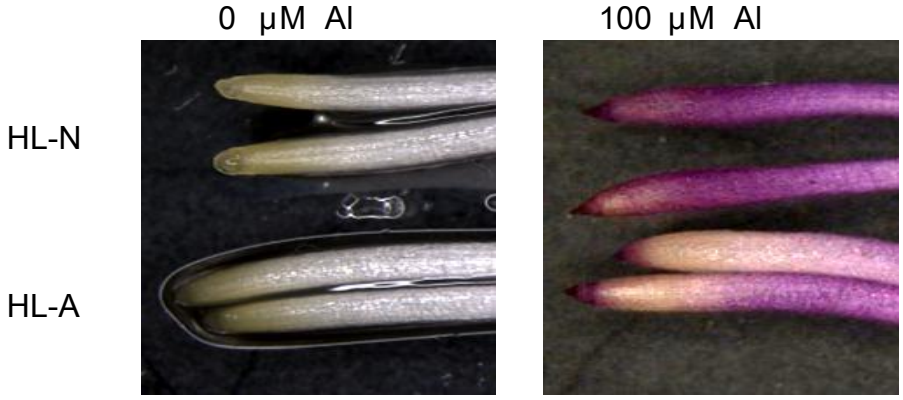
B



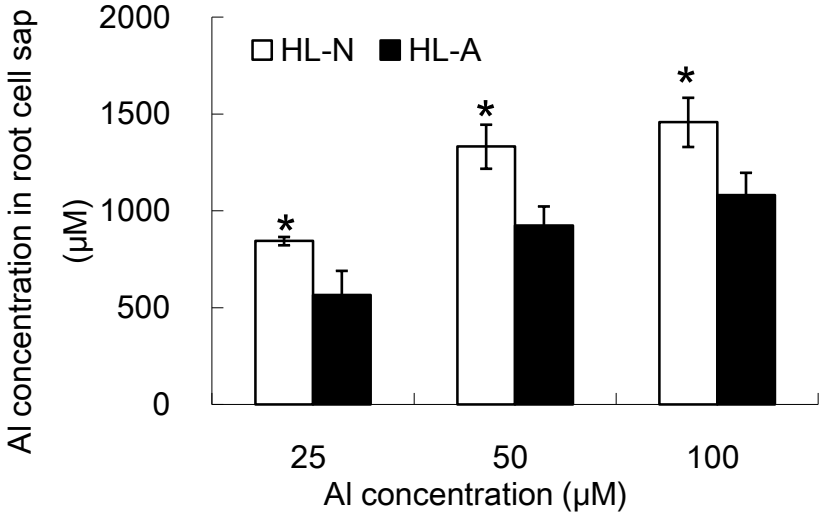
C


Relative root elongation
(% of control)
100
80
60
40
20
0
a
a
b
HL-N
HL-A
Nipponbare

D


0 μM Al
100 μM Al
HL-N
HL-A

E


Al concentration in root cell sap
(μM)
2000
1500
1000
500
0
HL-N
HL-A
*
*
*
25
50
100
Al concentration (μM)

F

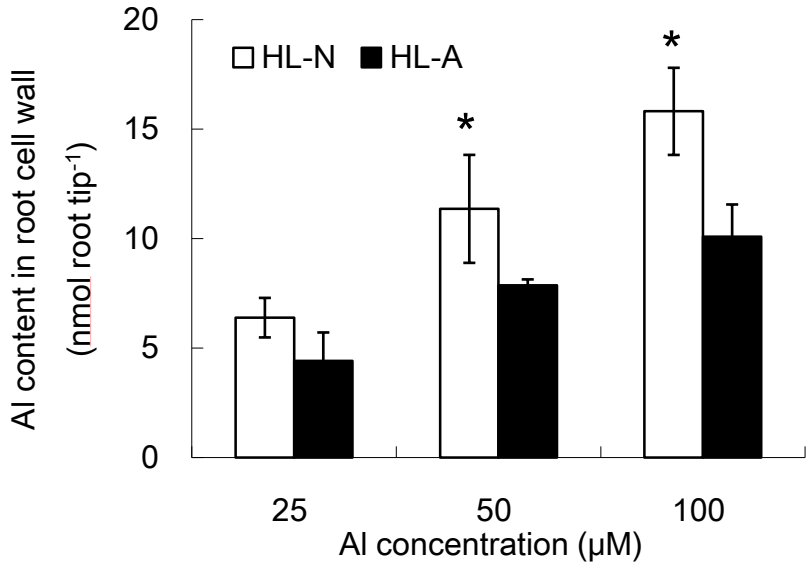


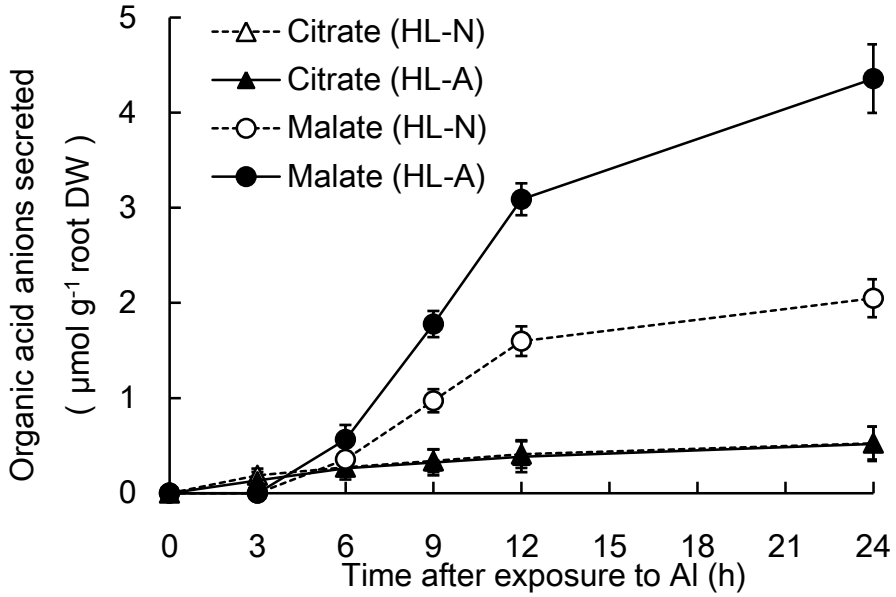
Figure 3.1 Physiological analysis of tolerance to low pH and Al in two *Holcus lanatus* accessions. A-B, Response to different pHs (A) and Al (B). Seedlings were exposed to a buffered solution at different pHs for 24 h or a solution containing 50 μM Al for 0, 6, 12, 18 and 24 h. The root length was measured before and after the treatment. Data are means ± SD (n = 10). C, Comparison of Al tolerance between rice and *H. lanatus*. Seedlings were exposed to a solution containing 50 μM Al for 24 h. Root length was measured by a ruler before and after the Al treatment, and relative root elongation (RRE; = [root elongation with Al]/[root elongation without Al]) was calculated. The means with different letters are significantly different ($P<0.05$ by Tukey's test). D, Eriochome cyanine R staining. Roots exposed to 0 and 100 μM Al for 24 h were stained in 0.1% Eriochome cyanine R for 15min. Scale bar = 10 mm. E-F, Al concentration in the cell sap (E) and Al content in the cell wall (F) of root tips (0–1 cm). Seedlings of both accessions were exposed to a solution containing 25, 50 and 100 μM Al for 24 h. The root tips were excised and fractionated into cell sap and cell wall. The Al concentration was determined by AAS. Data are means ± SD (n = 3). The asterisk shows a significant

difference between different treatments ($P<0.05$ by Tukey's test).

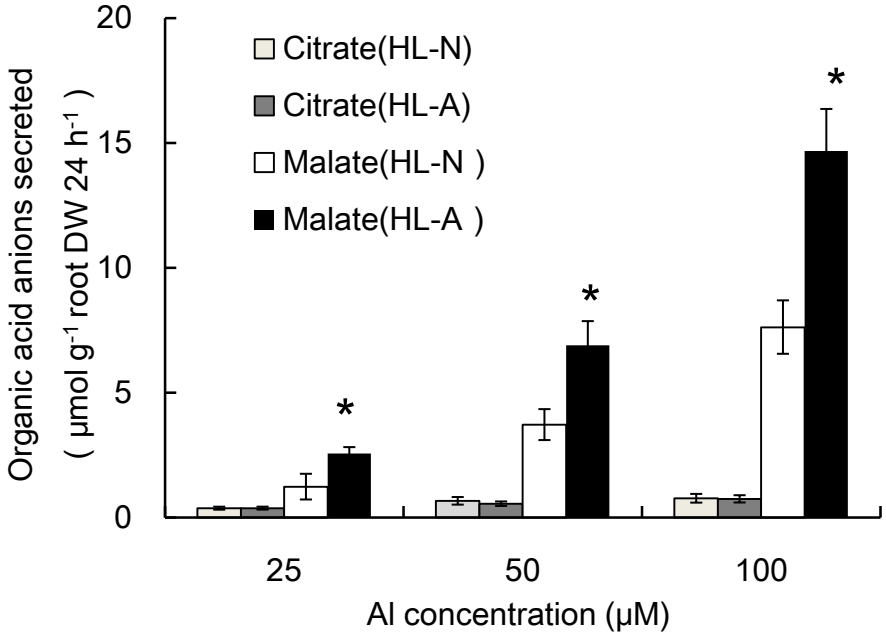
3.2 HL-A secreted more malate than HL-N in response to Al

To investigate whether the Al-induced secretion of organic acid anions is involved in higher Al tolerance in the HL-A accession, the secreted organic acid anions from roots were collected. Both accessions secreted malate and citrate from the roots in response to Al (Fig. 3.2A), but the amount of citrate secreted was small and did not differ significantly between two accessions. In contrast, HL-A secreted approximately double the amount of malate compared with HL-N (Fig. 3.2A). The amount of Al-induced malate secretion in HL-A was comparable to that in rye and wheat, which employ organic acid anion secretion for Al detoxification (Fig. 3.2; Li et al. 2000b). A dose-response experiment showed that the amount of malate secreted increased with increasing Al concentrations in both accessions, but HL-A secreted more malate than HL-N at each Al concentration (Fig. 3.2B). Furthermore, the secretion was only induced by Al, but not by Cd or La, indicating that the organic acid anion secretion is specific to Al (Fig. 3.2C).

A


Citrate (HL-N)
Citrate (HL-A)
Malate (HL-N)
Malate (HL-A)
Organic acid anions secreted
(μmol g-1 root DW)
0
1
2
3
4
5
0
3
6
9
12
15
18
21
24
Time after exposure to Al (h)

B


Citrate(HL-N)
Citrate(HL-A)
Malate(HL-N)
Malate(HL-A)
Organic acid anions secreted
(μmol g-1 root DW 24 h-1)
0
5
10
15
20
*
*
*
25
50
100
Al concentration (μM)

C

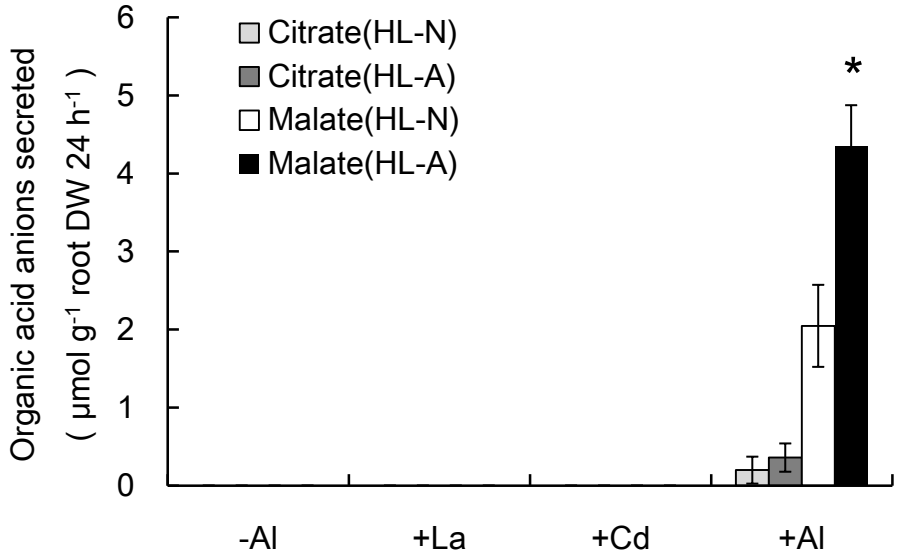


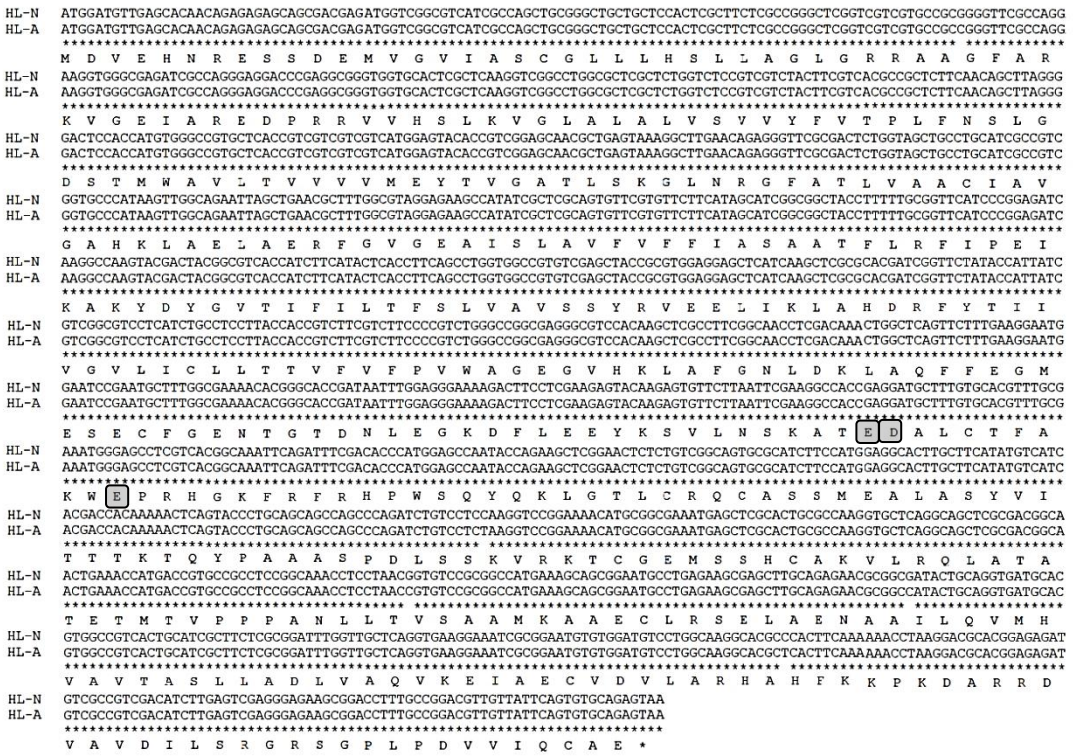
Figure 3.2 Time- and dose-dependent Al-induced secretion of organic acid anions in two *Holcus lanatus* accessions. A, Time-dependent secretion of organic acid anions. Seedlings were exposed to a solution containing 50 μM Al for 0, 3, 6, 9, 12, and 24 h. B, Dose-response. Seedlings were exposed to a solution containing 25, 50, and 100 μM Al for 24 h. C, Metal specificity. Seedlings were exposed to a solution containing 50 μM of Al, La or Cd for 24 h. Root exudates were collected from each treatment and organic acid anions were determined by an enzymatic method. Data are means ± SD (n = 3). The asterisk shows a significant difference between different treatments (P<0.05 by the Tukey's test).

3.3 Cloning of a gene responsible for malate secretion, *HlALMT1*

Al-induced secretion of malate has been reported to be mediated by ALMT1 (Sasaki et al. 2004; Ligaba et al. 2006, Hoekenga et al. 2006). Based on the sequence information of these *ALMT1*s in other species, RT-PCR at the highly conserved region and a 5'- and 3'-RACE were performed to obtain the full-length cDNA of the putative

malate transporter gene in both accessions of *H. lanatus*. The gene (designated as *HlALMT1*) isolated consists of a 1532 bp long cDNA including a 1389 bp ORF. There are five single nucleotide polymorphisms between HL-A and HL-N (Fig. 3.3A), but the peptides (463 amino acids) encoded were the same (Fig. 3.3A). HlALMT1 shared 81%, 43%, 44% and 42% identity, respectively, with TaALMT1, GmALMT1, BnALMT1 and AtALMT1 (Fig. 3.3B).

A



B

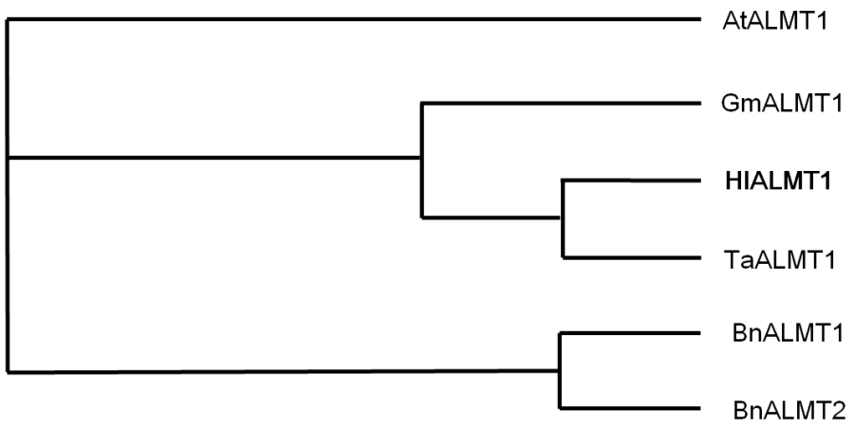


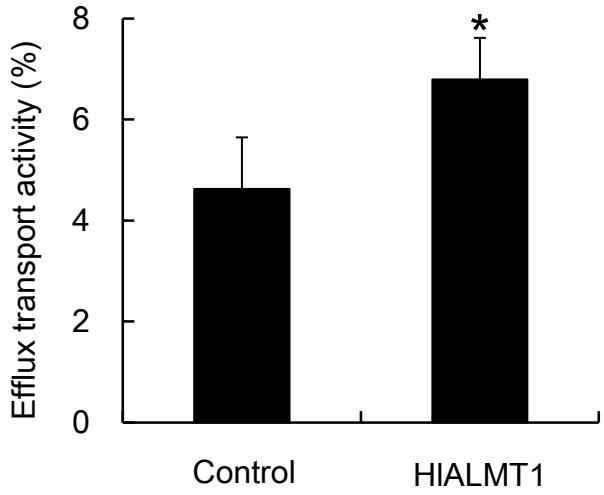
Figure 3.3 Sequence of HlALMT1 in two accessions of *H. lanatus*. A, Alignment of *HlALMT1* in HL-A and HL-N. The predicted amino acid residuals essential for Al-activated transport activity was indicated in frame. B, Phylogenetic tree of HlALMT1. The tree was constructed by Clustal W with the amino acid sequences of HlALMT1 and its homologs in other species. TaALMT1, wheat; AtALMT1, Arabidopsis; BnAlMT1/2, rape; GmALMT1, soybean.

3.4 Transport activity of HlAMLT1

To investigate the transport activity of HlALMT1 for malate efflux, *HlALMT1* was expressed in *Xenopus* oocytes injected with ^{14}C-labeled malate. Measurement of the ^{14}C radioactivity showed that oocytes expressing *HlALMT1* had a significantly higher efflux activity for malate than the control in the absence of Al (Fig. 3.4A), indicating that HlALMT1 is able to transport malate out of the cells.

To examine whether HlALMT1 is activated by Al, two-electrode voltage clamp analysis was performed. The result showed that the current of oocytes expressing *HlALMT1* was much higher in the presence of Al than that in the absence of Al (Fig. 3.4B). This result indicated that HlALMT1 is activated by Al.

A



B

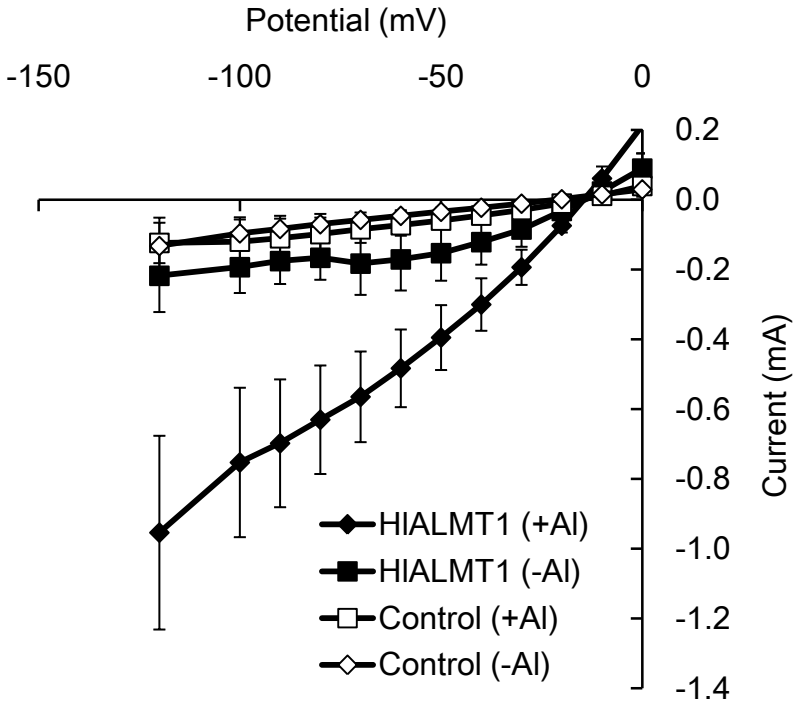


Figure 3.4 Transport activity of HlALMT1 in *Xenopus* oocyte. A, Basal transport activity for malate efflux in *Xenopus* oocytes. *HlALMT1* cRNA, or water (control) was injected into *Xenopus* oocytes. After 1-day cultivation, the oocytes were injected with ^{14}C-labeled malate. The release of ^{14}C-labeled malate from the oocytes in the absence of Al was determined after 1 h.

Percentage of total malate injected is shown. Data are means ± SD (n = 3-4). The asterisk shows a significant difference between different treatments (P<0.05 by Tukey's test). B, Al-activated transport activity for malate efflux. Malate was injected into oocytes with or without *HlALMT1* expression, and the inward current was recorded at -120 mV to 0 mV membrane potential in the presence and absence of 100 μM Al. Data are given as means ± SD (n = 5-9).

3.5 Subcellular localization of HlAMLT1

The subcellular localization of HlALMT1 was investigated by transiently expressing *GFP-HlALMT1* or *HlALMT1-GFP* fusion gene together with the red fluorescence protein gene *DsRed* as a cytosolic and nuclear marker in onion epidermal cells. When GFP alone was expressed, the fluorescence was observed in the cytosol and nucleus, which was completely overlapped with the DsRed signal (Fig. 3.5, upper panel). By contrast, the green fluorescence of both GFP-HlALMT1 (Fig. 3.5, middle panel) and HlALMT1-GFP (Fig. 3.5, lower panel) fusion was only observed at the cell periphery outside of the DsRed signal. These results indicate that HlALMT1 is localized to the plasma membrane.

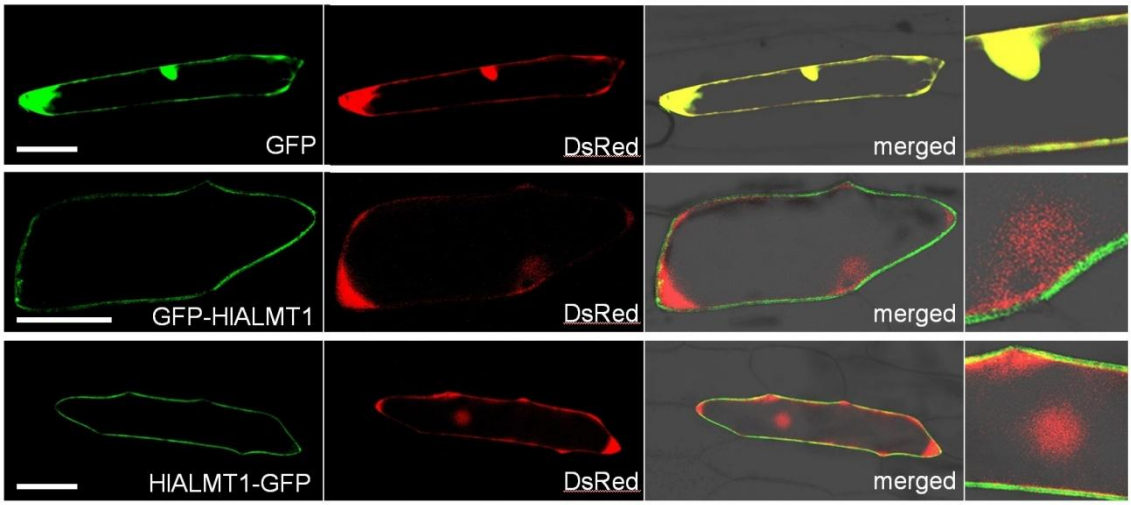


Figure 3.5 Subcellular localization of HlALMT1. *GFP* alone (upper panel) or fusions between *HlALMT1* and green fluorescent protein gene (*GFP*) at both N-terminal (middle panel) and C-terminal (lower panel) were introduced into onion epidermal cells together with *DsRed*. Scale bars = 100 μm.

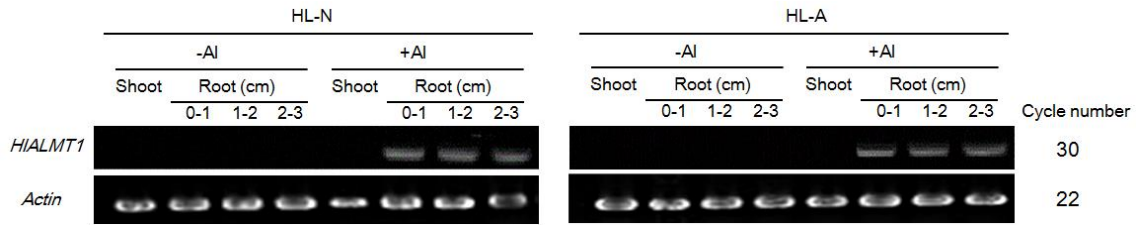
3.6 Expression pattern of *HlALMT1* in *H. lanatus*

The expression of *HlALMT1* was hardly detected in both roots and shoots of two accessions in the absence of Al (Fig. 3.6A). However, the expression in the roots, but not in the shoots was greatly induced by Al in both accessions (Fig. 3.6A). Spatial analysis showed that *HlALMT1* expressions in both the root tips (0–1cm) and the mature regions (1-3 cm) were up-regulated by Al (Fig. 3.6A). A time-course experiment showed that this induction occurred after 3-h exposure to Al (Fig. 3.6B). The expression level of *HlALMT1* was 2-fold higher in HL-A than in HL-N during the Al exposure between 6 h to 24 h (Fig. 3.6B). The expression of *HlALMT1* increased with increasing Al concentrations in both accessions, but the expression level in HL-A was higher than that in HL-N at each concentration (Fig. 3.6C).

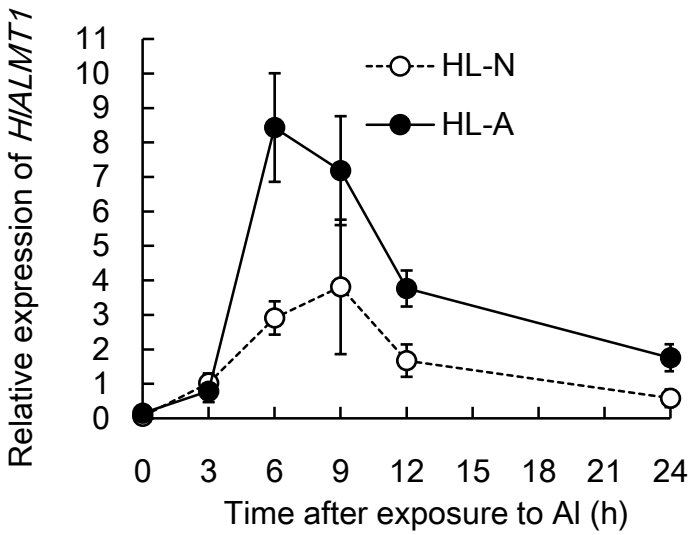
To investigate the tissue specificity of *HlALMT1* expression, laser micro dissection

(LMD) was used to separate the root tip tissues into outer and inner parts (Figs. 3.6D and 3.6E). The expression of *HlALMT1* in the outer tissues was higher than that in the inner tissues in both accessions (Fig. 3.6F). There was no difference in the expression level in the inner tissues between two accessions, but the expression in the outer tissues was much higher in HL-A than HL-N (Fig. 3.6F). These results indicate that the difference in *HlALMT1* expression between the two accessions arises from the expression in the outer tissues.

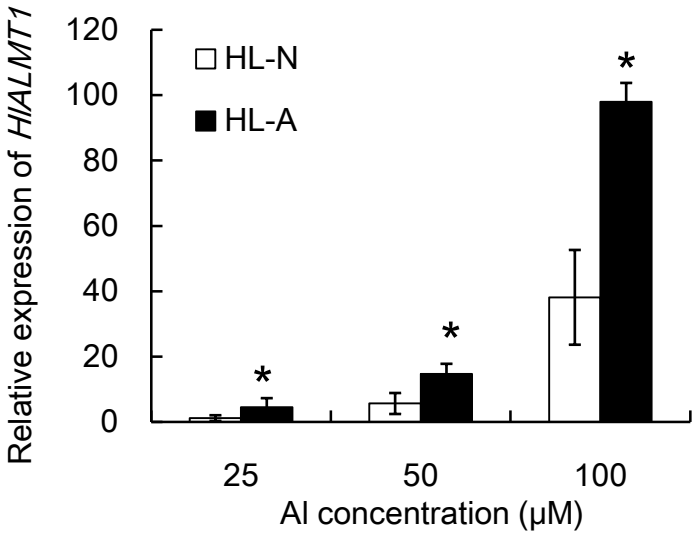
A



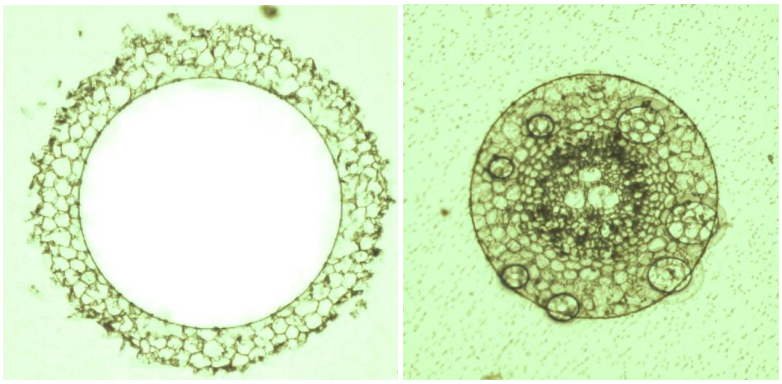
B



C


Relative expression of HIALMT1
120
100
80
60
40
20
0
HL-N
HL-A
*
*
*
25
50
100
Al concentration (μM)

D **E**



F

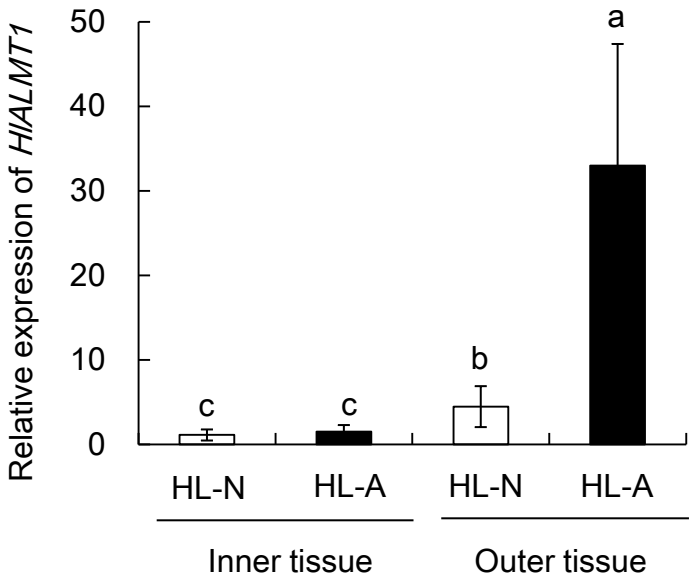


Figure 3.6 Expression pattern of *HlALMT1* in *H. lanatus.* A, Organ-specific expression. Expression of *HlALMT1* in the different root segments and shoots in the two accessions treated with or without Al. Cycle number of PCR is shown on the right side. B, Time-dependent expression of *HlALMT1*. Seedlings were exposed to a solution containing 50 μM Al for 0, 3, 6, 9, 12 and 24 h. Expression level relative to HL-N at 3 h is shown. C, Dose-responsible expression of *HlALMT1*. Seedlings were exposed to a solution containing 25, 50 and 100 μM Al for 24 h. Expression level relative to HL-N at 25 μM is shown. D-E, Outer tissues (D) and inner tissues (E) of the roots (7.5-12.5 mm) after laser microdissection. F, Tissue-specificity of *HlALMT1* expression. Expression level relative to the inner tissues of HL-N is shown. *Actin* was used as an internal standard. Data are means ± SD (n = 3). The asterisk in (C) and means with different letters in (F) are significantly different ($P<0.05$ by Tukey's test).

3.7 Copy number of *HlALMT1* gene

The differential expression of *HlALMT1* in the two accessions may be due to a difference in the genomic copy number of *HlALMT1*. However, this possibility was ruled out because the two accessions have the same genomic copy number of *HlALMT1* (the values were normalized by a silicon transporter gene *Lsi1, Low silicon rice 1*; *Os02g0745100*) (Fig. 3.7A, Ma et al. 2006).

Then the absolute mRNA level of *HlALMT1* was compared. The transcript copy number calculated from the standard curves using C_T values was 3 times higher in HL-A than in HL-N (Fig. 3.7B). These results indicate that different expression levels of *HlALMT1* in the two accessions are attributed to the transcript copy number, but not to the genomic copy number.

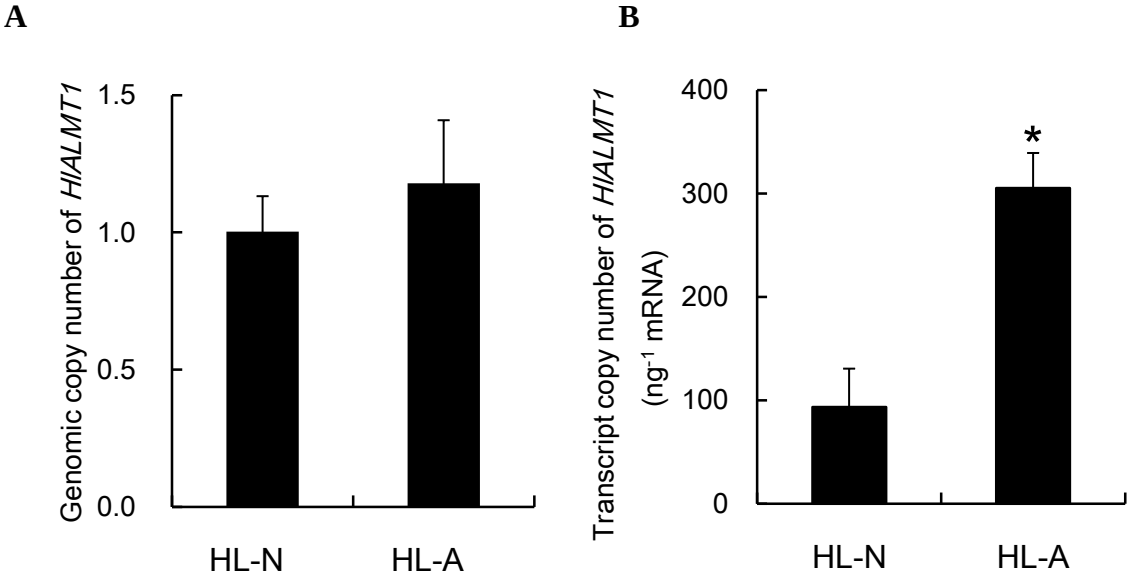


Figure 3.7 Genomic and transcript copy number of *HlALMT1* in *H. lanatus*. A, Genomic copy number in the two accessions. The values were normalized with the C_T values of *Lsi1*. B, Transcript copy number. The seedlings were exposed to 50 μM Al for 24 h prior to determination of the absolute expression level of *HlALMT1* in the root tips (0-1 cm) by

quantitative real-time RT-PCR. The transcript copy number was calculated from the standard curves using C_T values. Data are means ± SD (n = 3). The asterisk indicates a significant difference (P<0.05 by the Tukey's test).

3.8 Comparison of the promoter region of *HIALMT1* in the two accessions

One of the possible mechanisms for different expression of *HlALMT1* in the two accessions could be attributed to different promoter activities. To test this possibility, the promoter (-2 kb) region of the two accessions were isolated and compared. Although there was no difference in the amino acid sequence of *HlALMT1* between the two accessions (Fig. 3.3A), variations in the promoter region were found (Fig. 3.8). However, these differences are not due to large deletion or insertion in the promoter region. In Arabidopsis, the expressions of *AtMATE1* and *AtALMT1* are regulated by a transcription factor, STOP1 (Sawaki et al. 2009; Liu et al. 2009). In rice, the expression of *OsFRDL4* (*MATE*) is regulated by ART1 (Yamaji et al. 2009) and its cis-acting element sequence has recently been identified as [GGN(T/g/a/C)V(C/A/g)S(C/G)] (Tsutsui et al. 2011). Search of the high-affinity ART1 cis-acting element (GGTCCT, GGCCCT, GGTACT, GGTCGT, GGGCCT, GGACCT, and GGTGCT) in the promoter region of *HlALMT1* revealed five in HL-A but only three in HL-N (Fig. 3.8). The difference in the cis-acting element number between the two accessions is caused by single nucleotide substitutions.

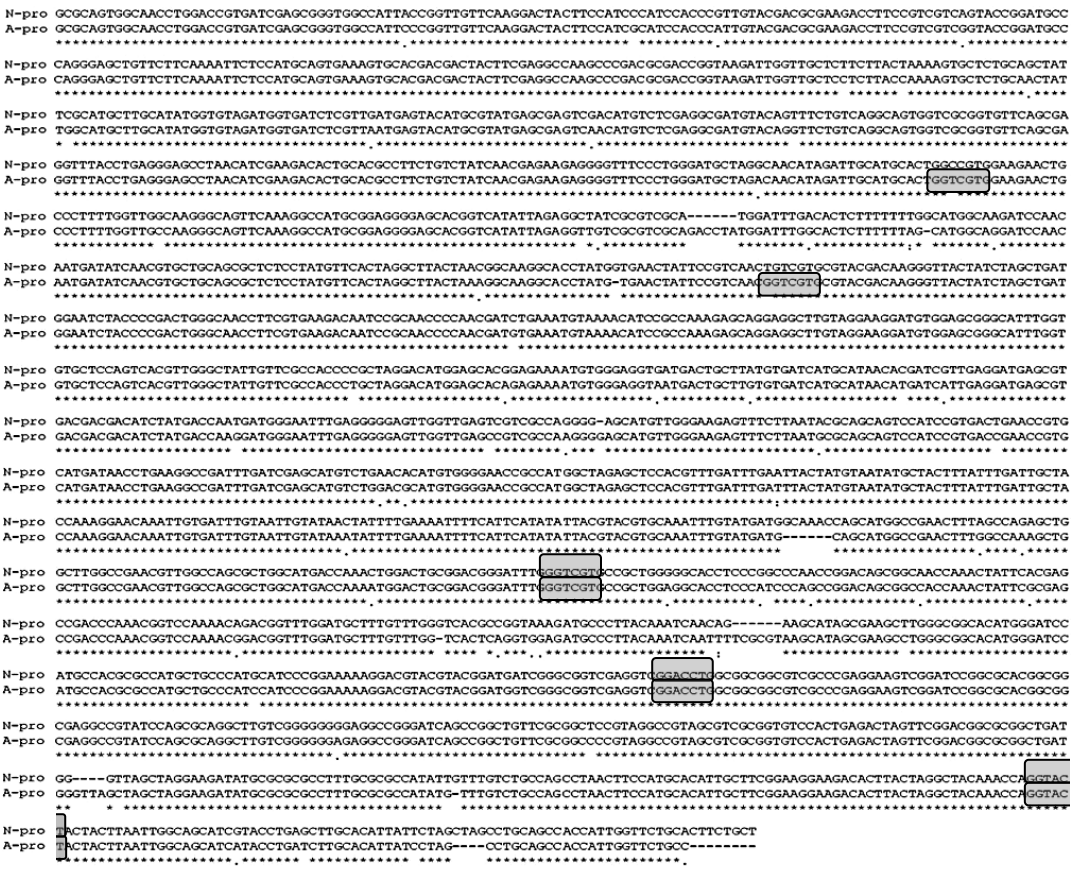


Figure 3.8 Alignment of 2-kb promoter region from HL-A and HL-N accessions of *H. lanatus*. The cis-acting element of ART1 was indicated in frame.

3.9 Cloning and expression analysis of *HlART1*

To examine whether the difference in the cis-acting element number can explain the different expression levels of *HlALMT1*, an *ART1* homolog (*HlART1*) in *H. lanatus* was first cloned based on the conserved sequences of *ART1* and *ART1*-like genes in rice and Arabidopsis. A cDNA fragment of 360 bp was amplified using a pair of primers, subsequently a 1697 bp long full-length of *HlART1* including a 1443 bp ORF was acquired from the cDNA of the two accessions of *H. lanatus* roots using 5'- and 3'-RACE. There was no difference in the nucleotide sequence of *HlART1* between

two accessions (Fig. 3.9A). *HlART1* encodes a peptide of 481 amino acids (Fig. 3.9A). HlART1 shares 51.8 % and 46 % identity, respectively, with rice ART1 and STOP1 (Fig. 3.9B). HlART1 has four conserved C2H2 domains (Fig. 3.9A), suggesting its role in the regulation of gene transcription.

The expression level of *HlART1* was similar in the roots of HL-A and HL-N, and was not affected by different concentrations of Al in either accession (Fig. 3.10).

A

B

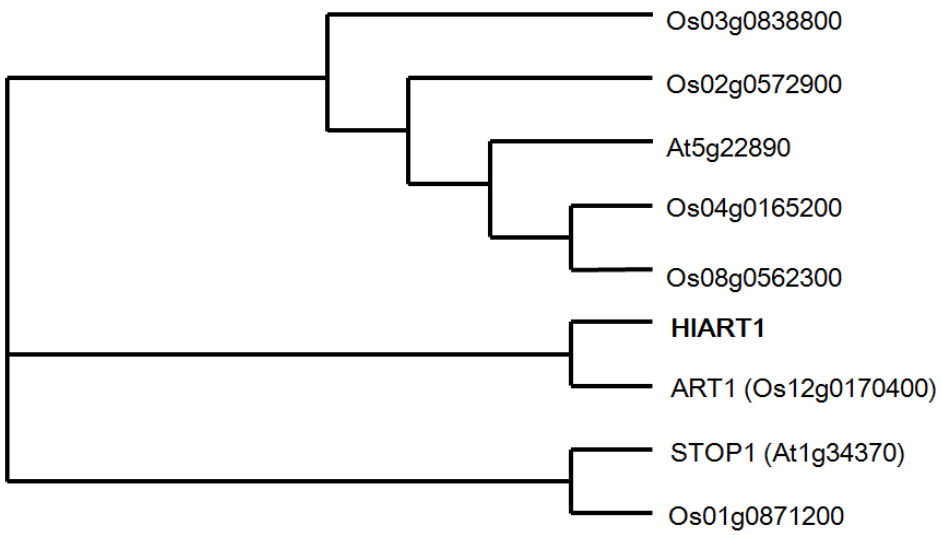


Figure 3.9 Phylogenetic analysis of HlART1. A, Alignment of ART1, STOP1 and HlART1 from HL-N and HL-A. Four conserved C2H2 domains were indicated in grey frame with upper line. B, Phylogenetic tree of HlART1. The tree was constructed by Clustal W with the amino acid sequences of HlART1 and its homologs in rice and Arabidopsis.

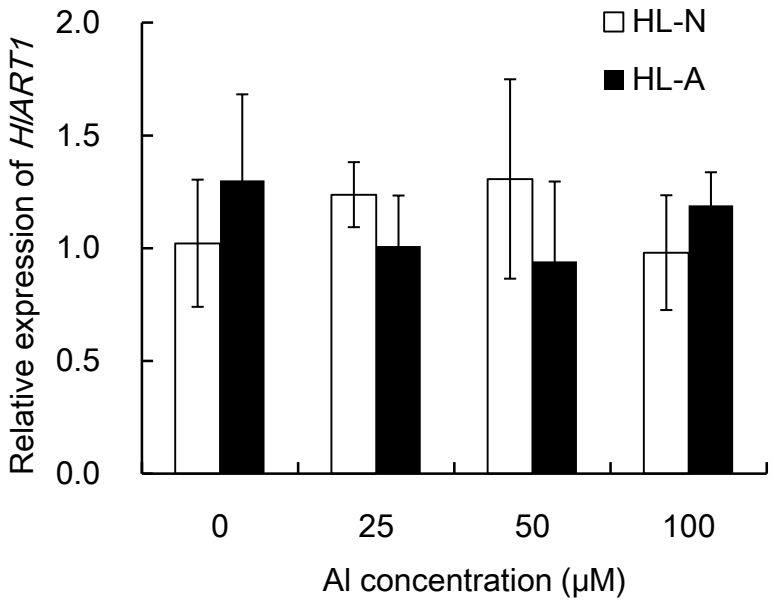


Figure 3.10 Expression pattern of *HlART1*. Seedlings of both accessions were exposed to a solution containing 0, 25, 50 or 100 μM Al for 24 h, and then the root tip (0-1cm) were excised for expression analysis. Expression level relative to HL-N (-Al) is shown. *Actin* was used as an internal standard. Data are means ± SD (n = 3).

3.10 Promoter binding and transcriptional activation assay

To investigate whether HlART1 can interact with the promoter of *HlALMT1*, a yeast one-hybrid assay was performed. In the absence of 3AT (a competitor of HIS3), there was no difference in the growth among the transformed yeast cells (Fig. 3.11).

However, in the presence of 20 mM or 50 mM 3AT, the *HlART1*-expressing cells showed better growth than the *pGADT7*-expressing (control vector) cells (Fig. 3.11). Furthermore, the growth of yeast cells containing the HL-A promoter was evidently better than those containing the HL-N promoter. Rice ART1, included as a positive control, showed similar results to HlART1, suggesting that both rice ART1 and HlART1 probably bind to the same cis-acting element in the promoter region of *HlALMT1*. These results suggest that HlART1 does interact with the promoter region of *HlALMT1* and that the cis-acting element number is involved in the regulation of the expression level of *HlALMT1*.

To quantify the promoter activity from different accessions, a transient assay in tobacco leaf protoplasts was performed. The *HlALMT1* promoter from HL-A or HL-N was fused with cauliflower mosaic virus (CaMV) 35S minimal (-46) promoter (Fang et al., 1989) and *GFP* as a reporter gene and then transformed into tobacco (*Nicotiana tabacum*) mesophyll protoplasts. *HlART1* as an effector gene and *DsRed* as an internal control were also transformed together (Fig. 3.12A). The expression of *HlART1* normalized by *DsRed* showed a similar level in all transformed protoplasts (Fig. 3.12B). In contrast, the expression of the reporter gene *GFP* normalized by *DsRed* was higher in the protoplasts carrying *HlALMT1* promoters from either accession than those carrying the CaMV 35S minimal promoter (control) when introduced together with HlART1, indicating the HlART1-dependent activity of *HlALMT1* promoter. Furthermore, the *GFP* expression was two times higher in the protoplasts carrying the HL-A promoter than those carrying the HL-N promoter (Fig. 3.12C). These results show that the *HlALMT1* promoter from HL-A has a higher activity than that from HL-N.

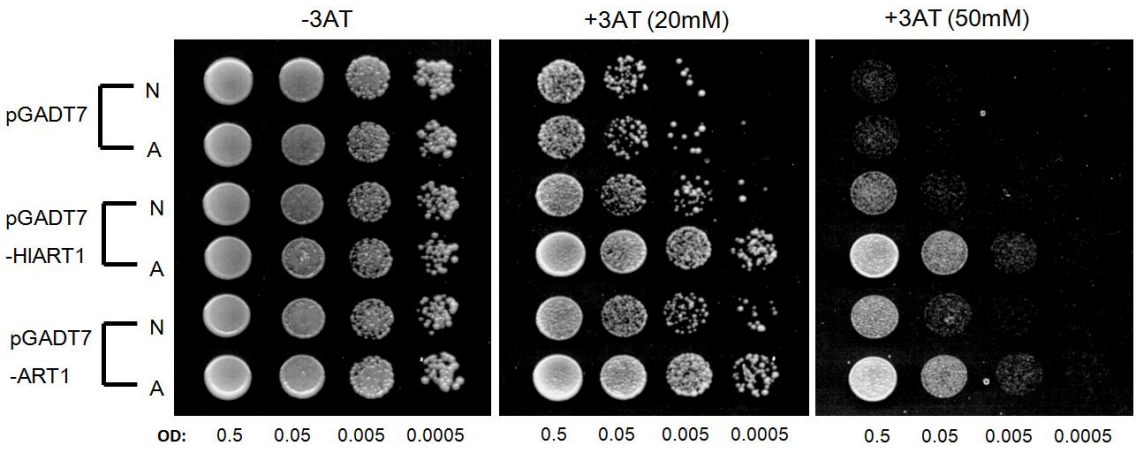
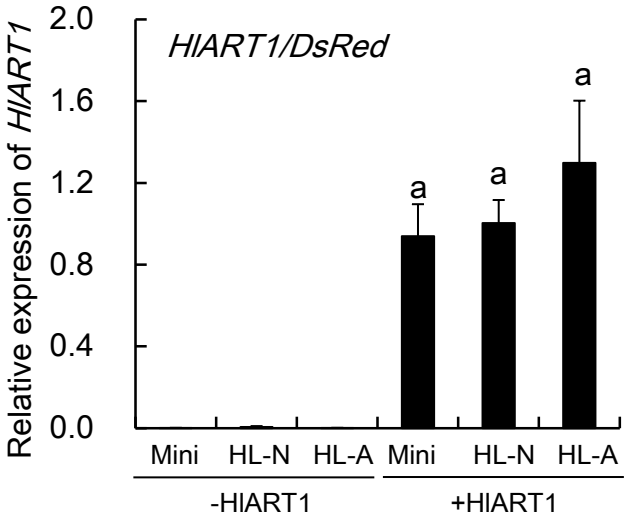


Figure 3.11 Yeast one-hybrid assay. The plasmid (pGADT7, pGADT7-HlART1 or pGADT7-ART1) together with the plasmid (N: pHIS2.1 + *HlALMT1* promoter from HL-N, or A: pHIS2.1 + *HlALMT1* promoter from HL-A) were introduced into yeast strain Y187 and cultured on SC medium (-His) in the presence of 0, 20 mM or 50 mM 3-amino-1, 2, 4-triazole (3AT, a competitor of HIS3). Four serial 1: 10 dilutions (from left to right) of yeast cell suspensions starting from OD_{600} = 0.5 were spotted on plates. The yeast was allowed to grow at 30ºC for 3 d.

A

B



C

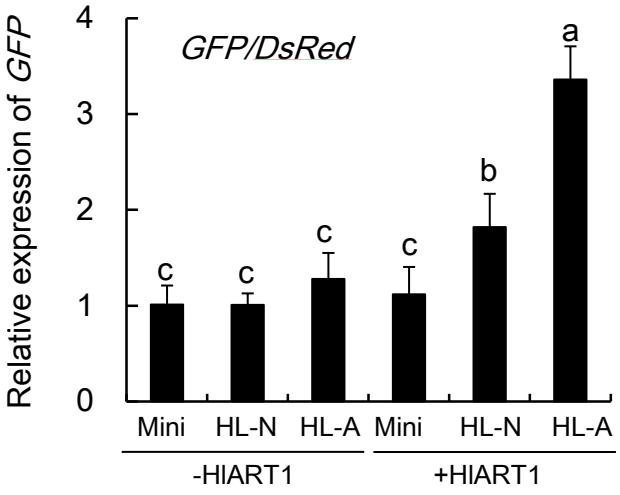


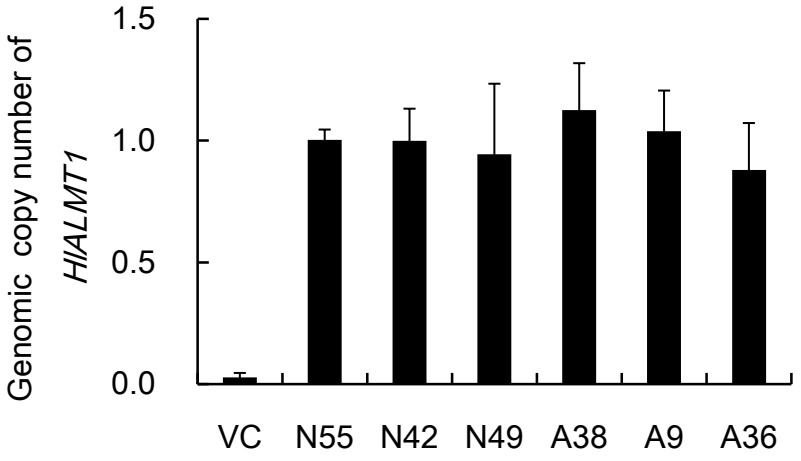
Figure 3.12 Promoter activity assay in tobacco protoplasts. A, Schematic diagram of the reporter, effector, and internal control plasmids used in transient expression analysis. B-C, Expression level of effector gene (*HlART1*) (B) and reporter gene (*GFP*) (C) driven by CaMV 35S minimal promoter (Mini), *HlALMT1* promoters from HL-N and HL-A. The reporter vector, effector vector and internal control vector were co-transformed into tobacco protoplasts by PEG method. The expression level was determined by quantitative RT-PCR. Expression level relative to Mini-GFP (+HlART1) is shown. Data are means ± SD (n = 3). Means with

different letters are significantly different (P<0.05 by the Tukey's test).

3.11 Functional analysis of *HlALMT1* promoter in transgenic rice

To further examine the role of the promoter region of *HlALMT1* in the regulation of the gene expression, *HlALMT1* driven by promoters from the two accessions were introduced into rice. a number of transgenic lines were obtained and three independent lines with the single genomic copy number of *HlALMT1* were selected for further analysis (Fig. 3.13A). The expression level of *HlALMT1* was higher in the lines driven by the HL-A promoter than that by the HL-N promoter (Fig. 3.13B). Rice itself secretes citrate, but not malate in response to Al (Ma et al. 2002). There was no difference in the citrate secretion between transgenic lines and vector control when exposed to Al (Fig. 3.13C). However, the amount of malate secreted from the roots was higher in the transgenic lines carrying the p*HlALMT1-HlALMT1* from either accession compared with the vector control (Fig. 3.13D), indicating that *HlALMT1* functions as a transporter of malate. Moreover, the amount of malate secreted was significantly higher in the transgenic lines driven by the HL-A promoter than that by the HL-N promoter (Fig. 3.13D). The Al tolerance in the transgenic lines was not altered due to high Al tolerance in rice (data not shown). These results further indicate that the different expression levels of *HlALMT1* in the two accessions results from the difference in the promoter region.

A


Genomic copy number of
HlALMT1
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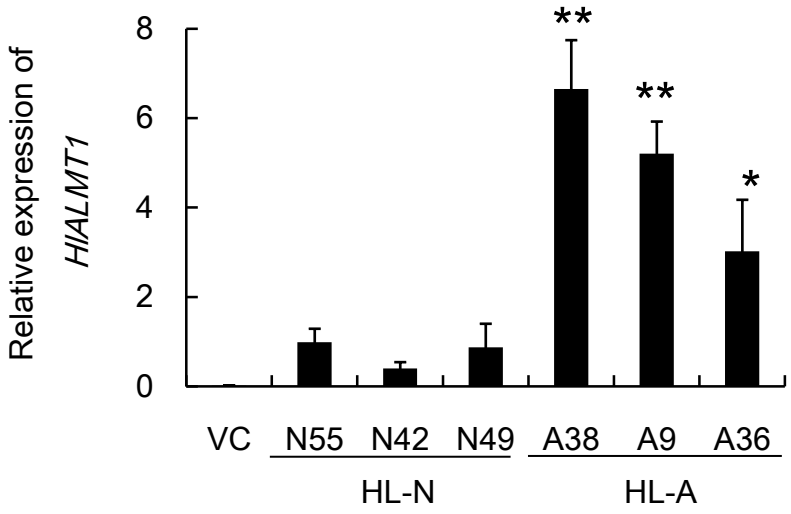
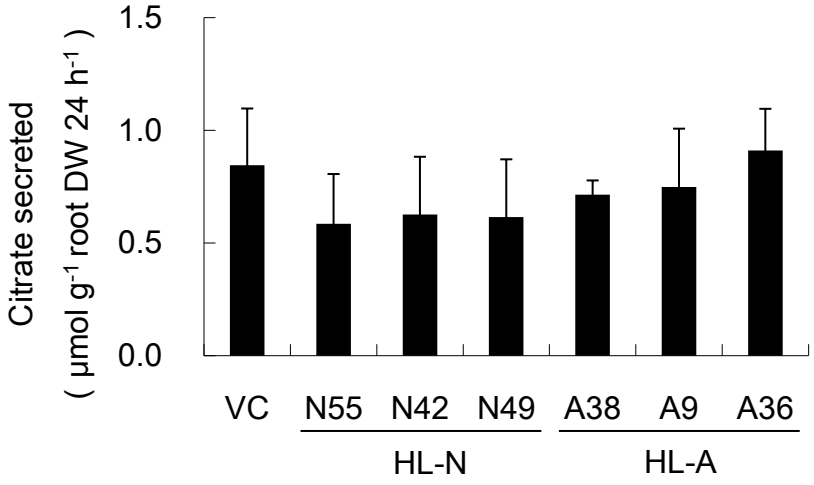
1.0
0.5
0.0
VC
N55
N42
N49
A38
A9
A36

B


Relative expression of
HlALMT1
8
6
4
2
0
**
**
*
VC
N55
N42
N49
A38
A9
A36
HL-N
HL-A

C


Citrate secreted
(μmol g-1 root DW 24 h-1)
1.5
1.0
0.5
0.0
VC
N55
N42
N49
A38
A9
A36
HL-N
HL-A

D

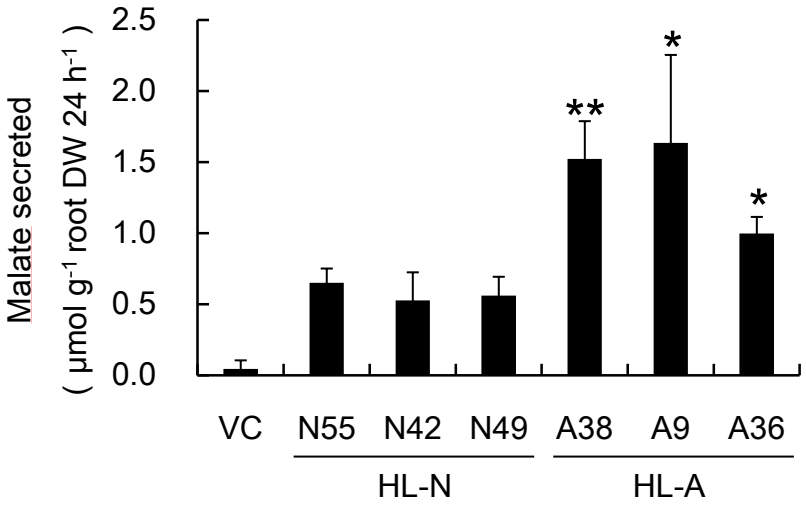


Figure 3.13 Functional analysis of *HlALMT1* promoter in transgenic rice. A, Genomic copy number of *HlALMT1* in transgenic rice. The values were normalized by the C_T value of a silicon transporter gene *Lsi1*. B, Expression level of *HlALMT1* in transgenic rice. Seedlings were exposed to a solution containing 50 μM Al for 24 h. Expression level relative to N49 is shown. C-D, Al-induced secretion of citrate (C) and malate (D) from transgenic rice. Root exudates were collected after exposing transgenic seedlings to a solution containing 50 μM Al for 24 h. VC: vector control. Data are means ± SD (n = 3). The asterisk in B and D indicates a significant difference (**P*<0.05 and ***P*<0.01 by the Tukey's test).

4. Discussion

4.1 HlALMT1 is an Al-activated malate transporter in *H. lanatus*

HlALMT1 belongs to the ALMT family and showed very high similarity to TaALMT1 (Fig. 3.3B), which has been identified as a plasma membrane-localized malate transporter in wheat. Like other ALMT1s, HlALMT1 is localized to the plasma membrane and mediates malate efflux when expressed in *Xenopus* oocytes (Figs. 3.4

and 3.5). The expression of *HlALMT1* in *H. lanatus* was specifically induced by Al in the roots (Fig. 3.6A) and limited to the outer cells of the roots (Fig. 3.6F). Furthermore, expression of *HlALMT1* in rice resulted in malate efflux from the roots (Fig. 3.13D). All these results indicate that *HLALMT1* isolated in the present study is a gene responsible for Al-induced secretion of malate in *H. lanatus*.

In addition to the induction of *HlALMT1* (Fig. 3.6), the protein encoded was activated by Al in *Xenopus* oocytes (Fig. 3.4B). This feature is the same as other ALMT1 from different species such as wheat (Sasaki et al. 2004) and Arabidopsis (Hoekenga et al. 2006). The mechanisms underlying Al-induced activation of ALMT1 is unknown, but the extracellular C-terminal is required for both basal and Al^{3+}-dependent transport activity of TaALMT1 in wheat and AtALMT1 in Arabidopsis (Furuichi et al. 2010). Further studies with site-directed mutagenesis showed that three acidic amino acids (E274, D275 and E284) in the C-terminal region are essential for Al^{3+}-activated transport activity of TaALMT1. In *H. lanatus*, these three residues are also present in HlALMT1 (Fig. 3.3A), suggesting that they are conserved in different plant species.

4.2 Differential expression level of *HlALMT1* is responsible for natural variation in Al tolerance of *Holcus lanatus*

Analysis of the HL-A accession of *Holcus lanatus*, which has adapted to acidic soil, revealed a higher Al tolerance than the HL-N accession collected from a near neutral soil (Fig. 3.1B). Physiological studies showed that this accession accumulated less Al in the roots and secreted more malate in response to Al exposure (Figs. 3.1E, 3.1F and 3.2). Malate secretion from the roots has been established as a mechanism for Al tolerance in many plant species (Ma 2000; Ma et al. 2001). The two accessions differ

in the amount of Al-induced secretion of malate (Fig. 2), indicating that this difference is at least one of the reasons responsible for the natural variation in Al tolerance between the two accessions although its contribution to Al tolerance is unknown.

There was no difference in the amino acid sequence of *HlALMT1* between the two accessions differing in Al tolerance and malate secretion (Fig. 3.3A), however HL-A showed a higher expression of *HlALMT1* than HL-N in a time-dependent and dose-dependent manner after Al treatment (Figs. 3.6B and 3.6C). Furthermore, the higher expression of *HlALMT1* in HL-A was only found in the outer root tissues (Fig. 3.6F). This difference in the expression level is not due to genomic copy number of *HlALMT1*, but to the transcript number (Fig. 3.7). These findings indicate that the differential expression levels of *HlALMT1* are responsible for the different malate secretion, and therefore different Al tolerance in the two accessions.

4.3 Expression of *HlALMT1* is determined by cis-acting element number for ART1

ART1 is a transcription factor for Al tolerance identified initially in rice (Yamaji et al. 2009). It regulates the expression of at least 30 genes. Since *H. lanatus* belongs to Gramineae like rice, a homolog of ART1 in *H. lanatus* from both accessions was isolated (Fig. 3.9). Both HlART1 and rice ART1 interact with the promoter region of *HlALMT1*, indicating that HlART1 functions as a transcription factor like ART1 (Fig. 3.11). HlART1 did not differ in the amino acid sequence or the expression level between the two accessions (Figs. 3.9A and 3.10). Furthermore, the expression level was also not affected by Al, as has been found for ART1 in rice (Fig. 3.10; Yamaji et al. 2009). This means that the differential expression of *HlALMT1* in the two accessions is not caused by variations in ART1.

However, the differential expression of *HlALMT1* was neither due to the amino acid sequence nor the genomic copy number of *HlALMT1* (Figs. 3.3A and 3.7A). Since rice ART1 binds to the cis-acting element of [GGN(T/g/a/C)V(C/A/g)S(C/G)] present in the promoter in rice (Tsutsui et al. 2011), this cis-acting element was also found in the promoter region of *HlALMT1* in the two accessions, but with different numbers: five in HL-A in contrast to three in HL-N (Fig. 3.8). The increased number of cis-element of ART1 in the promoter region enhanced promoter activity in HL-A (Fig. 3.12C). The enhanced expression of *HlALMT1* in roots results in larger amount of malate efflux to protect roots from Al toxicity, which helps HL-A survive on very acidic soils.

The environmental selection reinforced the heritable differences in many traits among the different treated plots of the Park Grass Experiments (Silvertown et al. 2005, Silvertown et al. 2006). On the acid plot, the environmental selection pressures forced the Al-tolerant genotype of *H. lanatus* (HL-A) grows better than others. Through the years, the HL-A becomes dominant due to the loss of genetic variation through genetic drift (Silvertown et al. 2006). Similar variation also occurred in *Anthoxanthum odoratum* (Davies and Snaydon 1973). Populations of *A. odoratum* collected from unlimed plots of the Park Grass Experiments were more tolerant of high Al concentrations than those from limed plots. This study proved that the rule of evolution is applicable to the *H. lanatus* growing on Park Grass plots.

In conclusion, our results show that an accession of *H. lanatus* adapted to acid soil has evolved a mechanism of Al tolerance by enhancing malate secretion mediated by a plasma membrane-localized transporter, HlALMT1. The higher *HlALMT1* expression is achieved by increasing the number of cis-acting element for a transcription factor, HlART1 in the promoter region.

Chapter 4 General discussion

In this study, molecular mechanisms of Al detoxification were investigated in two Al-tolerant species, rice (*Oryza sativa*) and Yorkshire Fog (*Holcus lanatus*). Two transporter genes (*OsMGT1* and *HlALMT1*) related to Al tolerance were functionally characterized. The mechanisms of OsMGT1 and HlALMT1 involved in Al tolerance were investigated and discussed, respectively.

OsMGT1 identified in the present study encodes a transporter for Mg. Compared with other cations, molecular mechanisms on uptake, translocation, distribution and storage of Mg are still poorly understood. Our results indicate that OsMGT1 is required for Mg uptake in rice.

It is reported that Al can inhibit Mg uptake and induce Mg deficiency in plant (Keltjens 1988; Rengel and Robinson 1989; Tan et al. 1992). In Arabidopsis, the activities of two high-affinity Mg transporters (AtMGT1 and AtMGT10) were seriously inhibited by Al (Li et al. 2001). However, this study showed that the physiological role of OsMGT1 in Al tolerance was to enhance root Mg influx when suffering from Al stress. This can partially explain why rice has higher Al tolerance than other species. Besides, Al did not alter the OsMGT1 transport affinity for Mg, but enhanced its expression level. However, none of *AtMGT* family genes is up-regulated by Al in Arabidopsis based on published microarray data (Zhao et al. 2009; Sawaki et al. 2009; Hoekenga et al. 2003). Up-regulation of Mg transporter gene *OsMGT1* to enhance Al tolerance is supposed to be a unique character in rice, which makes rice highly tolerant to Al and well adapted in acidic soil. The mechanism of Mg-mediated alleviation of Al toxicity in rice has been proposed (Fig. 4.1): Al is taken up through Nrat1 into the cells

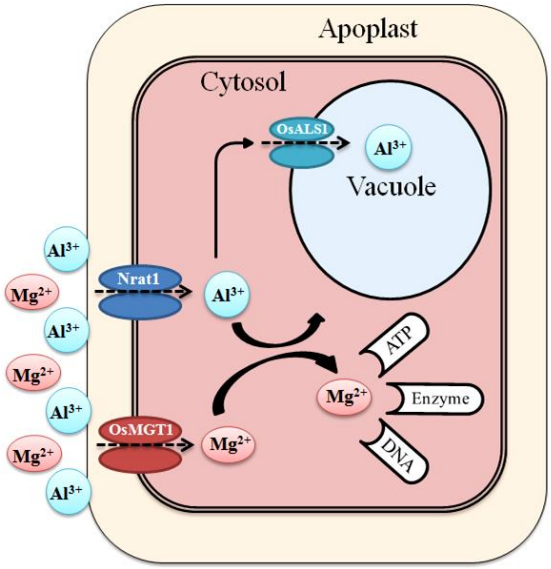
and subsequently sequestrated into the vacuole by OsALS1. Since Al^{3+} and Mg^{2+} have similar hydrated radius, the Al remained in the cytosol will compete with Mg for binding to oxygen donor compounds such as ATP, DNA, and enzymes. Al-induced up-regulation of *OsMGT1* enhances Mg influx to the cells, which prevents Al binding to the critical cellular sites.

To adapt to acid soil, many plants have developed various strategies for detoxification of Al. In this study, it was found that *H. lanatus* adapted to acid soil secretes more malate to the rhizosphere to detoxify Al. A gene responsible for Al-induced malate secretion was isolated and functionally characterized. Further studies showed that the expression level of this gene is higher in the accession adapted to acid soil, which is regulated by the number of cis-acting element for ART1 in the promoter region. Recently, several studies have reported regulation mechanisms of transporter genes involved in organic acid secretion. For example, in wheat, high expression level of *ALMT1* is achieved by generating tandem repeated elements in the promoter region in most Al-tolerant lines (Sasaki et al. 2006; Ryan et al. 2010). A transposon-like element insertion into the promoter contributes to high expression of *TaMATE1B* in Al-tolerant genotype (Tovkach et al. 2013). In sorghum, the number of tourist-like miniature inverted repeat transposable element (MITE) in the promoter of *SbMATE* results in the high level of expression and Al resistance (Magalhaes et al. 2007). In barley, a 1-kb insertion in the 5'UTR region of the *HvAACT1* coding region enhances its expression level in the Al-tolerant accessions (Fujii et al. 2012). In this study, it was found that an Al-tolerant accession of *H. lanatus* has evolved a different mechanism to enhance expression of *HlALMT1*. It is not achieved by insertion of long-sequence elements, but simply by increasing the number of cis-acting elements of ART1 in the

promoter region. It appears that amplification of transcription factor binding sites may be the root cause for functional differences in both wheat and *H. lanatu*s, but the scale of these changes is far more specific in *H. lanatu*s than the variation of longer sequences that occurs in wheat. Besides, the response of their *ALMT1* genes to Al is different: *TaALMT1* is not induced by Al, but *HlALMT1* is induced by Al in the presence of ART1, suggesting the different mechanisms of transcriptional regulation between the two species. Therefore, the increased number of cis-acting elements may result in enhanced expression of *ALMT1*, which contributes to Al tolerance (Fig. 4.2).

Enhancing crop stress resistance is one of the strategies to raise crop production. Al toxicity as the primary factor inhibiting plant growth in acidic soil is intensively investigated for long time. Recently, the discovery of *ART1*-like genes in several plant species has shed light on the molecular mechanism for Al detoxification. In this study, a new ART1-regulated gene *OsMGT1* was identified in rice, which functions as a Mg transporter conferring Al tolerance by increasing Mg uptake into the cells. In *H. lanatus*, HlART1 also mediates Al tolerance through enhancing the expression level of a malate transporter gene *HlALMT1*, by increasing its cis-acting element number in the promoter region. However, the ART1 downstream genes should be further studied because only a handful of them have been characterized. Besides, the future work should also focus on the signal transduction pathway involved in the post-transcriptional activation of ART1 in plant.

A



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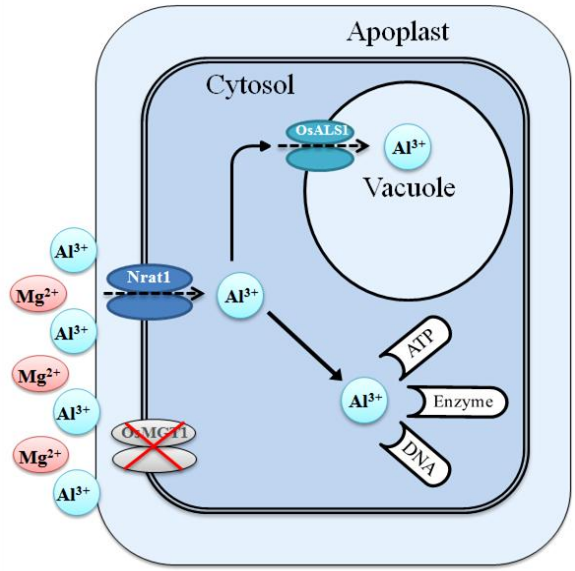

Figure 4.1 Proposed mechanism of Mg-mediated alleviation of Al toxicity in rice.

A, wild-type rice; B, *OsMGT1* knockout line.

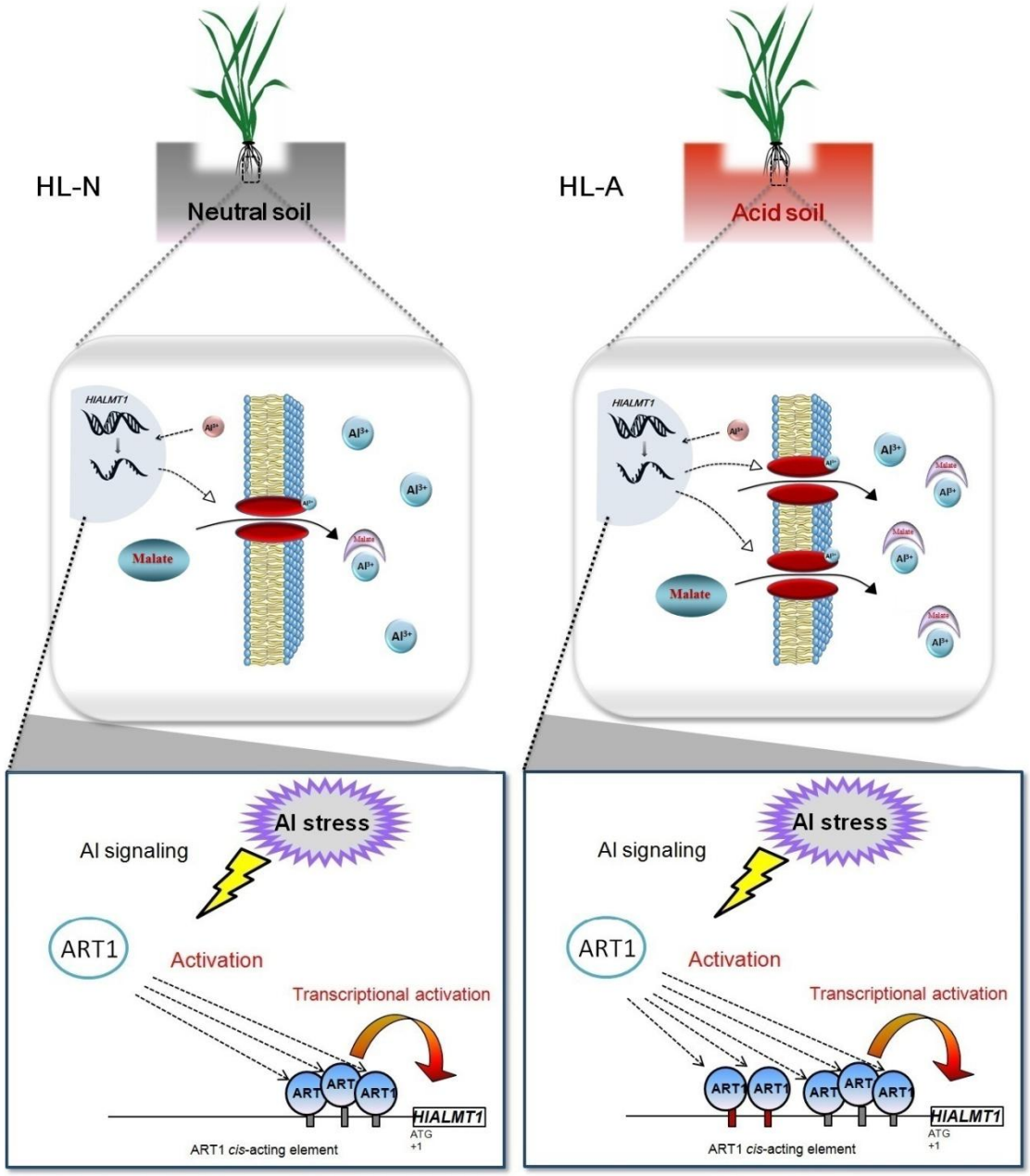

Figure 4.2 The model of regulation mechanism for *ALMT1* expression in two accessions of *Holcus lanatus* differing in Al tolerance.

Summary

Aluminum (Al) toxicity has been recognized as a major factor limiting crop productivity on acid soil, which comprises about 40% of the arable land in the world. Al rapidly inhibits root growth at low concentrations, subsequently affects the uptake of water and nutrients. However, some plant species have developed various strategies to cope with Al toxicity. In this study, molecular mechanisms of Al detoxification were investigated in two Al-tolerant species, rice (*Oryza sativa*) and Yorkshire Fog (*Holcus lanatus*), focusing on transporter genes.

1. Functional analysis of a magnesium transporter gene involved in Al tolerance in rice.

Rice is the most Al-tolerant species among small grain cereals. Recent identification of a transcription factor ART1 for Al tolerance revealed that the high Al tolerance is achieved by multiple genes implicated in detoxification of Al. ART1 regulates at least 31 genes, but only few of downstream genes have been functionally characterized. This study focused on an uncharacterized ART1-regulated gene, *OsMGT1,* which encodes a putative Mg transporter.

OsMGT1 showed the similarity ranging from 63-81% to Arabidopsis Mg transporter AtMRS2/AtMGT family at the amino acid level, and contains a conserved Gly-Met-Asn (GMN) tripeptide Mg transport motif at the end of the first transmembrane domain. *OsMGT1* was expressed in both the roots and shoots in the absence of Al, but the expression only in the roots was rapidly up-regulated by Al. Furthermore, the expression did not respond to low pH and other metals including Cd

and La. In the *art1* mutant, the expression of *OsMGT1* was not induced by Al, confirming that OsMGT1 is regulated by ART1. Transient expression in the rice protoplasts showed that OsMGT1 was localized to the plasma membrane. To investigate the physiological role of this gene in Al tolerance in rice, two independent knockout lines were obtained. When *OsMGT1* was knocked out, the tolerance to Al, but not to Cd and La, was decreased. However, this inhibition could be rescued by addition of 10 μM Mg, but not by the same concentration of Ba or Sr. In addition, knockout of *OsMGT1* did not affect Al-induced citrate secretion. A short-term (30 min) kinetic uptake experiment with ^{25}Mg was performed to compare the Mg uptake between WT and knockout lines. In the absence of Al, WT showed a higher Mg uptake than the mutants, indicating that OsMGT1 is involved in Mg uptake. In the presence of Al, the Mg uptake was enhanced in the WT, but not in the mutants. The Km value for OsMGT1-mediated Mg uptake did not change in plants exposed to Al, but the Vmax was double in the Al-treated plants compared with non Al-treated plants. The concentration of Mg in the cell sap of the root tips was also increased in the wild-type rice, but not in the knockout lines in the presence of Al. Taken together, these results indicate that OsMGT1 is a transporter for Mg uptake in the roots and that up-regulation of this gene is required for conferring Al tolerance in rice by increasing Mg concentration in the cell. OsMGT1 is one of the components of high Al tolerance in rice.

2. Molecular mechanism of Al tolerance in an accession of *Holcus lanatus* adapted on acid soil

Yorkshire Fog (*Holcus lanatus*), which belongs to Poaceae family and is a close relative

of the agronomic crop oat (*Avena sativa*), is a widely adaptable grass species that is able to grow on highly acidic soils with high level of Al, but the mechanism underlying the high Al tolerance is unknown. In the present study, two accessions of *H. lanatus* collected from an acid plot (soil pH 3.6, HL-A) and a neutral plot (pH 7.1, HL-N) was characterized in terms of Al tolerance, organic acid anion secretion and related gene expression. There was no difference in the tolerance to low pH between two accessions; however, root elongation of HL-N was inhibited by Al to a greater extent than that of HL-N. The Al concentration of the cell sap and cell wall in the root tips (0-1 cm) was significantly higher in HL-N than in HL-A. In response to Al (pH 4.5), the HL-A roots secreted approximately double the amount of malate of the HL-N roots, but there was no difference in the citrate secretion. Cloning of the gene (*HlALMT1*) responsible for malate secretion showed that the amino acid sequence encoded did not differ between two accessions. HlALMT1 was localized to the plasma membrane and showed Al-activated malate efflux activity in *Xenopus* oocytes. The expression of *HlALMT1* in *H. lanatus* was specifically induced by Al in the roots and was significantly higher in the outer layers of the HL-A roots than that of the HL-N roots. This difference was not due to the genomic copy number, but to the number of cis-acting element for an Al-responsive transcription factor (HlART1) in the promoter region of *HlALMT1*, which was demonstrated by both yeast one hybrid assay and transient assay in tobacco protoplasts. Furthermore, introduction of *HlALMT1* into rice driven by the HL-A promoter resulted in significantly higher expression level of *HlALMT1* and more Al-induced malate secretion than that by the HL-N promoter. These findings indicate that the adaptation of *H. lanatus* to acidic soils is achieved by increasing number of cis-acting element for ART1 in the promoter region of *HlALMT1*

gene, enhancing the expression of *HlALMT1* and subsequently increasing secretion of malate for Al detoxification.

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