Higher sterol content regulated by CYP51 with concomitant lower phospholipid content in membranes is a common strategy for aluminium tolerance in several plant species

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Received 18 August 2014; Revised 28 September 2014; Accepted 10 October 2014

Abstract

Several studies have shown that differences in lipid composition and in the lipid biosynthetic pathway affect the aluminium (Al) tolerance of plants, but little is known about the molecular mechanisms underlying these differences. Phospholipids create a negative charge at the surface of the plasma membrane and enhance Al sensitivity as a result of the accumulation of positively charged Al$^{3+}$ ions. The phospholipids will be balanced by other electrically neutral lipids, such as sterols. In the present research, Al tolerance was compared among pea (Pisum sativum) genotypes. Compared with Al-tolerant genotypes, the Al-sensitive genotype accumulated more Al in the root tip, had a less intact plasma membrane, and showed a lower expression level of PsCYP51, which encodes obtusifoliol-14α-demethylase (OBT 14DM), a key sterol biosynthetic enzyme. The ratio of phospholipids to sterols was higher in the sensitive genotype than in the tolerant genotypes, suggesting that the sterol biosynthetic pathway plays an important role in Al tolerance. Consistent with this idea, a transgenic Arabidopsis thaliana line with knocked-down AtCYP51 expression showed an Al-sensitive phenotype. Uniconazole-P, an inhibitor of OBT 14DM, suppressed the Al tolerance of Al-tolerant genotypes of maize (Zea mays), sorghum (Sorghum bicolor), rice (Oryza sativa), wheat (Triticum aestivum), and triticale (×Triticecale Wittmark cv. Currency). These results suggest that increased sterol content, regulated by CYP51, with concomitant lower phospholipid content in the root tip, results in lower negativity of the plasma membrane. This appears to be a common strategy for Al tolerance among several plant species.

Key words: Aluminium (Al) tolerance, CYP51, phospholipid, plasma membrane, sterol, uniconazole-P.
Introduction

Aluminium (Al) toxicity has been accepted to be a primary and common factor that negatively affects plant growth in acid soils. Improving the Al tolerance of crop plants would be one approach for producing sufficient foods for world agriculture. The results of many physiological studies have suggested that plants have developed various strategies of Al tolerance, including organic acid anion (OA) excretion from roots, modification of cell wall structure (Yang et al., 2010), and Al sequestration (reviewed by Horst et al., 2010; Magalhães, 2010; Ryan et al., 2011). Understanding the molecular mechanisms of these Al-tolerance strategies is essential for establishing efficient breeding programmes, e.g. using marker-assisted selection.

Recent progress in molecular genetics and physiological research has identified various genes that regulate Al tolerance. In particular, genes regulating Al-activated/induced OA excretion have been isolated from various plant species. ALMT1 (Al activated Malate Transporter 1) was first isolated from wheat (Triticum aestivum) (Yang et al., 2010), and Al sequestration (reviewed by Horst et al., 2010; Magalhães, 2010; Ryan et al., 2011). Understanding the molecular mechanisms of these Al-tolerance strategies is essential for establishing efficient breeding programmes, e.g. using marker-assisted selection.

Functional orthologues have been characterized in various other plants species, such as AtALMT1 (Hoekenga et al., 2006) and AtMATE (Liu et al., 2009) in Arabidopsis thaliana. An Al-tolerant bread wheat genotype derived from a Portuguese landrace exhibited very high basal transcript levels of both citrate and malate transporter genes, TaMATE1 and TaALMT1, respectively (Garcia-Oliveira et al., 2014). Additionally, studies on mutants have identified transcription factors that regulate the expression of these genes, including STOP1 (Sensitive TO Proton Rhizotoxicity 1) in Arabidopsis (Iuchi et al., 2007) and its homologue in rice (Oryza sativa), ART1 (Aluminium Resistance Transcription Factor 1) (Yamaji et al., 2009). Further research has identified that STOP1/ART1 regulate multiple Al-tolerance genes (Sawaki et al., 2009), including ALS3/STAR2 (Aluminium Sensitive 3 in Arabidopsis/Sensitive To Al Rhizotoxicity 2), encoding a prokaryotic-type ABC transporter, OsMGT1 (Oryza sativa Magnesium TRANSPORTER1), encoding a magnesium transporter (Chen et al., 2012), and other genes (Xia et al., 2013). The STOP1/ART1 system is probably conserved among land-plant species, including bryophytes (Ohyama et al., 2013), while several other genes that regulate Al tolerance do not belong to this system. Although studies using Al-sensitive/tolerant mutants have identified several genes regulating Al tolerance (e.g. SLOW WALKER 2) (Nezames et al., 2013), it is likely that many genes have not been identified because of the complexity of the mechanisms of Al tolerance and toxicity.

The physico-chemical and physiological properties of the plasma membrane affect Al tolerance in plants. In previous studies, loss of integrity of the plasma membrane was strongly correlated with the degree of Al damage in the sensitive cultivars after an Al treatment (Ishikawa and Wagatsuma, 1998; Wagatsuma et al., 2005). The lipid composition of the plasma membrane could explain the different responses of plasma membrane integrity to Al between Al-tolerant and Al-sensitive cultivars. Ryan et al. (2007) showed that modifying the plasma membrane lipid composition [i.e. a higher Δ⁶-sphingolipid content, and predominance of the (Z)-isomer] conferred Al tolerance in transgenic Arabidopsis. The (Z)-isomer of sphingolipids affects the fluidity of the plasma membrane and may alter the raft structure at the plasma membrane. The phospholipid contents in the plasma membrane also affect Al tolerance, which can be explained by the toxic mechanisms of Al³⁺ in solution (Deleers et al., 1985). In a previous study on Arabidopsis, simulations of Al toxicity in a complex solution and calculations of the amount of Al³⁺ at the plasma membrane surface based on GEOCHEM-EZ (Shaﬁ et al., 2010) and a speciation-based Gouy-Chapman-Stern model (SGCS) (Kinrade and Wang, 2010) identiﬁed that Al³⁺ attracted to the plasma membrane surface determined the degree of Al toxicity (Kobayashi et al., 2013). Because phospholipids are responsible for the negative charge at the plasma membrane surface, the pah1pah2 (phosphatidate phosphohydrolase 1 and 2) mutant, which accumulates phospholipids under P-starved conditions as a result of defective P-recycling, was more Al-sensitive than the wild type under P-starved conditions. These studies have highlighted the importance of plasma membrane lipid composition in Al tolerance. Zhang et al. (1996) reported that the ratio of total sterols to phospholipids in microsomal membranes isolated from 5-mm root tips was slightly higher in an Al-resistant wheat cultivar than in an Al-sensitive one. This finding provided further evidence that the phospholipid contents of the plasma membrane are an important factor in Al tolerance.

Khan et al. (2009) reported that Al tolerance was positively correlated with the ratio of sterols to phospholipids in root-tip cells of various rice cultivars. Application of uniconazole-P, an inhibitor of obtusifoliol-14α-demethylase (OBT 14DM), decreased the sterol content in root-tip cells of rice. Uniconazole-P increased the phospholipid to sterol ratio and induced Al sensitivity in an Al-tolerant cultivar. It has been suggested that CYP51, which encodes OBT 14DM, has a role in Al tolerance. In the present study, this model was tested by comparing the phospholipid to sterol ratios among various plant species. Molecular cloning and expression analyses showed that expression levels of CYP51 were lower in an Al-sensitive mutant line of pea than in an Al-tolerant cultivar. Finally, the model was tested using transgenic Arabidopsis with knocked-down CYP51 expression. The results of all of these analyses ﬁtted the model, and strongly suggested that CYP51 plays a signiﬁcant role in Al tolerance.

Materials and methods

Plant materials and growth conditions

The whole experiment consisted of three parts using different plant materials: three cultivars and one mutant of pea; the wild type and a transformant of Arabidopsis thaliana; and five plant species including six cultivars and four lines. Seeds of the gibberellin (GA) mutant line and the wild type of pea (lh and Torsdag, respectively) were harvested from the Research Farm of Teikyo University,
Japan. The *AtCYP51*-KD transgenic line-1 of *Arabidopsis*, which was transformed by Kusuhio *et al.* (2001), was used in the present experiments. The seed progenies were obtained using the single-seed descent method. Germination and preculturing of *Arabidopsis* was carried out as described by Toda *et al.* (1999). To collect *Arabidopsis* seeds for T3 progeny, seeds were harvested by one using a pipetter and germinated on Rock纤维 (Nttobco Co. Ltd, Tokyo, Japan). The seedlings were fertilized with a 1/1000 dilution of HYPONeX nutrient solution (HYPONeX Japan Ltd, Osaka, Japan) and were grown for 1 week at 22±1 °C under a 12-h light/12-h dark photoperiod. Each 1-week-old seedling was transferred from the Rockfiber to a pot filled with fertilized and sterilized peat soil (Supermix, Sakata Seeds, Yokohama, Japan). Seedlings were watered for 1 week and thereafter grown independently and covered with a transparent plastic cylinder to avoid cross-pollination. Seedlings were fertilized once weekly with 1/1000 diluted HYPONeX nutrient solution and grown under the same light conditions as those described above. Seeds were collected 3 months after germination (Supplementary Figure S1).

The *Arabidopsis* seeds collected were surface sterilized with 1% NaClO, and then kept at 4°C for 3–4 days before planting to synchronize germination. The germinated seeds were transferred to floats for experiments. Each float consisted of a nylon mesh (50 mesh per inch) supported on a plastic photo slide mount. Approximately 20 seeds were placed on each float, and 30 floats were floated on 6 l nutrient solution in the same plastic container (Kobayashi *et al.*, 2005). The basic nutrient solution consisted of 200 μM CaCl₂, 60 μM MgSO₄, and other MGRL nutrients (Fujiiwara *et al.*, 1992), without inorganic phosphate (Pi), at a strength of 1/50.

The T3 progeny were used for experiments on Al tolerance, visualization of Al accumulation and plasma membrane permeability, and analyses of phospholipids and sterols. Three lines of T3 progeny were used for real-time, quantitative reverse transcription PCR (qRT-PCR) and OA analyses. Seeds of two pea cultivars (*Pisum sativum* L. cv. Harunoka and cv. Hyougo), two sorghum cultivars (*Sorghum bicolor*) Moench cv. Super sugar and cv. Kaneko-hybrid), and two maize cultivars (*Zea mays* L. cv. KD 850 and cv. KD 520) were purchased from Kaneko Seeds (Gunma, Japan) and Takii Seeds (Kyoto, Japan). Seeds of two lines of triticale (*×Triticosecale Wittmark cv. Currency*) lines ST2 and ST22), two lines of wheat (*Triticum aestivum* L. lines ET8 and E88), and two cultivars of rice (*Oryza sativa* L. cv. Rikuu-132 and cv. Rikuu-20) were harvested from the Field Science Centre of Yamagata University, Japan. Seeds of pea, sorghum, maize, triticale, wheat, and rice were soaked in tap water overnight to allow synchronous germination. The germinated seeds were grown on floats on nutrient solution with or without 4 μM AlCl₃, (pH 5.0) under a 12-h light/12-h dark photoperiod (22±1°C). Root lengths were measured on day 7. Images of roots were acquired using Image J software. Ten seedlings were used for each measurement, and this process was repeated three times. Al tolerance was calculated as the ratio of root length in the Al treatment to that in the control.

Visualization of Al accumulation and plasma membrane permeability

After treatment with or without Al for 24 h (in the case of pea only, the duration of Al treatment was 1 h), whole roots were stained with haematoxylin (0.2% in 0.02% sodium iodide, w/w; pH 4.8) for 15 min after treatment as described by Polle *et al.* (1978). Al accumulation in the tip portion was observed by stereoscopy (SMZ-10, Nikon, Tokyo, Japan; or SZ-61, Olympus), and Al distribution in a section 2–3 mm from the root apex was observed by light microscopy (LABOPHOT; Nikon) (Ishikawa *et al.*, 1996). To visualize plasma membrane permeability, whole roots were stained with fluorescein diacetate-propidium iodide (FDA-PI) (12.5 mg 1 l⁻¹ FDA, 5 mg 1 l⁻¹ PI) as described by Ishikawa *et al.* (2001). In the case of pea only, FDA-PI staining was carried out using the following two root samples: (1) a root sample after Al treatment, and (2) a root sample after a 1-h Al treatment followed by a 3-h post-treatment with 0.2 mM CaCl₂. The stained roots were observed under a fluorescence microscope equipped with a B2 filter (excitation filter, 450–490 nm; barrier filter, 520 nm) (EFDA-2, Nikon).

Isolation of a full-length cDNA encoding *CYP51* in pea

RT-PCR was carried out using an Expand HF PCR system (Roche, Penzberg, Germany) and the primers were degenerated to clone *CYP51* from pea. Pooled cDNA (10 ng) prepared from frozen 1-cm root tips of 5-day-old pea seedlings was used as the template. The nucleotide sequences of the degenerate primers were as follows: 5' TTYAAYGTNCCNACNTTGYG-3' (sense) and 5' CCNACNNACWNGCNTTCCA-3' (antisense). To determine

Screening for Al tolerance and estimation of root growth of *Arabidopsis*

These experiments were conducted as described by Ikka *et al.* (2007) with minor modifications. Briefly, seeds were preincubated at 4°C for 3 days to allow synchronous germination. The germinated seeds were grown on floats on nutrient solution with or without 4 μM AlCl₃, (pH 5.0) under a 12-h light/12-h dark photoperiod (22±1°C). Root lengths were measured on day 7. Images of roots were acquired using a digital camera (SP-350; Olympus, Tokyo, Japan) attached to a stereoscopic microscope (SZ61; Olympus). Then, the lengths of all roots in the image were measured using Image J software. Ten seedlings were used for each measurement, and this process was repeated three times. Al tolerance was calculated as the ratio of root length in the Al treatment to that in the control.

Screening for Al tolerance in the presence or absence of a sterol metabolism inhibitor

Screening for Al tolerance was carried out as described by Khan *et al.* (2009). Briefly, roots of young seedlings with a primary root length of ~4 cm were pre-treated with 0.2 mM CaCl₂ (pH 4.9) for 6 h to allow them to adapt to low-pH conditions. Then, the roots were treated with 0.2 mM CaCl₂ with AlCl₃ (Al treatment) or without AlCl₃ (control) at pH 4.9 (or at pH 5.0 for sorghum only) for 24 h in the long-term experiments. The concentration of AlCl₃ was 20 μM for pea, triticale, and maize, 10 μM for rice, 5 μM for wheat, and 2.5 μM for sorghum. In the short-term experiments, the roots of the wild type and the GA mutant of pea were treated for 1 h with 0.2 mM CaCl₂ with or without Al in the absence of uniconazole-P [(E)-(S)-1-(4- chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazole-1-yl) pent-1-en-3-ol] (Wako Pure Chemicals, Osaka, Japan). Then, the roots were immersed in 0.2 mM CaCl₂ (pH 5.2) for 9 h to allow re-elongation. Consequently, the duration of the Al treatment was 1 h, but the duration of the whole treatment was 10 h. Root lengths were measured with a ruler. In the long-term experiments, Al tolerance was calculated as the ratio of root elongation of the longest primary root after the 24-h Al treatment to that in the control. In the short-term experiments, Al tolerance was calculated as the ratio of root elongation of the longest primary root after 1-h treatment with AlCl₃ followed by 9-h re-elongation in 0.2 mM CaCl₂ to that in the control (10-h in 0.2 mM CaCl₂ solution). Twelve seedlings were used for each set of experiments. Data shown are averages of triplicate sets. For the inhibitor experiment, Tween-20 (used to solubilize uniconazole-P) was added to control and Al solutions to a final concentration of 0.0005% (w/v). This concentration of Tween-20 did not inhibit normal root elongation (data not shown). The Al tolerance in the presence of uniconazole-P was calculated by comparing root elongation under (AlCl₃ + CaCl₂ + uniconazole-P) conditions to that under (CaCl₂ + uniconazole-P) conditions. The concentration of uniconazole-P that inhibited root elongation differed among plant species, but not between cultivars or lines (data not shown). Based on preliminary experiments to identify the concentration of uniconazole-P that did not inhibit normal elongation of roots in control solution (data not shown), 0.51 μM uniconazole-P was used for wheat and rice, and 1.02 μM uniconazole-P was used for triticale, maize, and sorghum.
the full-length cDNA sequence, 5'- and 3'-rapid amplification of cDNA ends (RACE) was performed as described previously (Toyomasu et al., 1998).

Real-time qRT-PCR

Total RNA was extracted from frozen 1-cm root tips of 5-day-old pea seedlings using an RNAqueous column with Plant RNA Isolation Aid (Ambion, Austin, TX, USA). cDNA was synthesized from 1 µg total RNA with a QuantiTect reverse transcription kit (Qiagen, Hilden, Germany). Real-time qRT-PCR using SYBR Green I was carried out using a TP800 thermal cycler (Takara Bio, Shiga, Japan) as described previously (Sawada et al., 2008) using gene-specific primers. The mean expression level of two replicates was normalized to that of 18S rRNA as the internal control. Total RNA was extracted from Arabidopsis by the method of Suzuki et al. (2004) using root tissues of plants grown hydroponically in modified MgRL medium for 1 week in the presence of 4 µM AlCl₃, as described previously (Kobayashi et al., 2007). Transcript levels of CYP51G1 and UBQ1 were quantified by real-time PCR using an Applied Biosystems 7300 instrument (Applied Biosystems, Foster City, CA, USA) and specific primer pairs, following the manufacturer’s instructions.

Malate collection and analysis

The Arabidopsis plants used in experiments on malate excretion were grown and treated as described previously (Kobayashi et al., 2007). Briefly, the roots of in vitro-grown seedlings were exposed to 0.2 mM CaCl₂ with or without 10 µM AlCl₃ at pH 5.0 for 24 h. Malate was quantified by the enzymatic cycling technique described by Hampp et al. (1984).

Extraction and analysis of phospholipids and sterols

Lipids were extracted and analysed as described previously (Khan et al., 2009). Briefly, lipids were extracted from 10-mm apical root samples. The modified Bligh and Dyer method (1959) (Uemura and Yoshida, 1984) was used to extract phospholipids [isopropanol: chloroform: H₂O (1:1:1, v/v/v)], and a modified method of Hartmann and Benveniste (1987) was used to extract sterols [dichloromethane: methanol (2:1, v/v)]. Lipids were extracted form whole plants of Arabidopsis. After purification and dehydration of the extract, P was analysed by the molybdenum blue method and A₅ Stereo-sterols were analysed using a spectrophotometric method (Zlattkis and Zak, 1969). For sterols, A₅₂₀ of the samples was measured, and the data are expressed as β-sitosterol (Wako Pure Chemicals) equivalents.

Results

Al tolerance, Al accumulation, and plasma membrane permeability of pea cultivars and a pea mutant

Using different Al treatment conditions (i.e. different durations of Al treatments), Al tolerance was compared among various cultivars, the wild type, and a mutant of pea. In one experiment, plants were exposed to toxic Al solution for 1 h, and then their root re-growth over the following 9 h was compared (Fig. 1, open bars). These conditions were used to assess the degree of constitutive Al tolerance in the cultivars, the wild type, and the mutant. In another experiment, roots were kept in Al toxic solution for 24 h, and then their root elongation was compared (Fig. 1, closed bars). These conditions were used to evaluate induced Al tolerance in the cultivars and the mutant. In both conditions, the lh mutant and cv. Hyougo were significantly more Al sensitive than were cv. Harunoka and the wild type (Torsdag). The Al-sensitive genotypes accumulated more Al in the root tips than did Al-tolerant genotypes (Supplementary Figure S4). Additionally, the Al-sensitive genotypes showed fewer intact plasma membranes than did Al-tolerant genotypes after a 1-h Al treatment followed by a 3-h post-treatment with 0.2 mM CaCl₂ (Fig. 2).

Next, the lipid composition and expression levels of CYP51 were evaluated in the Al-sensitive and Al-tolerant pea genotypes. The CYP51 homologue in pea was cloned by degenerate PCR using primers designed from CYP51 sequences in other legumes [barrel clover (Medicago truncatula L.) and soybean (Glycine max Merr.)]. A full-length cDNA, which showed high homology to orthologues in other legumes, encoded 489 amino acids. The nucleotide sequence of PsCYP51 has been deposited in the GenBank/EMBL database [Accession number AB633330]. The PsCYP51 transcript levels in the cultivar and the mutant were determined by qRT-PCR with specific primers. The highest concentration of sterols in the root-tip portion was in the Al-tolerant cv. Harunoka, while the highest concentration of phospholipids was in the most Al-sensitive line, the lh mutant (Fig. 3). In general, Al treatment decreased the sterol content (Fig. 3,
open bars) and increased the phospholipid content (Fig. 3, closed bars). The highest ratio of phospholipids to sterols was in the most Al-sensitive line, the lh mutant. The relative level of CYP51 mRNA in the root tip of pea was positively correlated with sterol content ($R^2 = 0.915$, $P < 0.05$), but negatively correlated with the ratio of phospholipids to sterols ($R^2 = 0.907$, $P < 0.05$) (Fig. 4). These results suggest that the sterol/phospholipid ratio, which is regulated by the biosynthesis of sterols, plays a role in determining Al tolerance.

**Effect of sterol biosynthetic inhibitor, uniconazole-P, on Al tolerance, plasma membrane permeability, and Al accumulation in several plant species**

Next, the effects of the sterol biosynthetic inhibitor, uniconazole-P, on Al tolerance, plasma membrane permeability, and Al accumulation, was determined in several plant species. The Al tolerance of cultivars or lines was compared in the presence or absence of uniconazole-P, which inhibits OBT 14DM (Khan et al., 2009), the product of CYP51 (Kim et al., 2005). The Al-tolerant cultivars or lines of triticale (line ST2), maize (cv. KD520), wheat (line ET8), and sorghum (cv. Super sugar) were grown in Al solutions in the presence or absence of uniconazole-P, and their root growth was compared with that of sensitive cultivars or lines of each species (triticale, line ST22; maize, cv. KD850; wheat, line ES8; sorghum, cv. Kaneko-hybrid; rice, cv. Rikuu-20). The Al-tolerant cv. Rikuu-132 was included as the reference.

The effect of uniconazole-P on the Al responses of Al-tolerant cultivars and lines was evaluated using Al at a concentration that caused similar biological responses among all species (nearly 50% growth inhibition), except for wheat (30% growth inhibition). In these conditions, the sensitive cultivars and lines showed symptoms of Al toxicity, characterized by 70–80% growth inhibition (Fig. 5). Uniconazole-P treatment suppressed the Al tolerance of all tolerant cultivars and lines, but did not affect the Al tolerance of sensitive cultivars and lines under conditions that severely inhibited root growth. Therefore, uniconazole-P affected the Al tolerance of any plant species when they were grown under moderate Al-toxicity conditions (i.e. those resulting in <50% growth inhibition). To further analyse the effects of uniconazole-P on Al tolerance, Al accumulation and intactness of the plasma membrane were compared between cultivars or lines. Red fluorescence in FDA-PI-stained cells indicated damaged plasma membranes. The cells of tolerant cultivars and lines showed stronger red fluorescence in the presence of uniconazole-P than in its absence (Fig. 6A). Additionally, haematoxylin staining indicated that uniconazole-P enhanced Al accumulation in Al-tolerant cultivars and lines (Fig. 6B). In contrast, uniconazole-P hardly affected the intactness of the plasma membrane (Fig. 6A) or Al accumulation (Fig. 6B) in Al-sensitive cultivars and lines nor did it affect their Al tolerance (Fig. 5). These results provide further evidence that a block in the
sterol biosynthetic pathway (inhibition of OBT 14DM) enhanced the Al sensitivity of Al-tolerant cultivars and lines, as a result of a loss of plasma membrane integrity and enhanced Al accumulation.

**Effect of suppression of CYP51 on Al tolerance of Arabidopsis**

The results described above suggested that sterol biosynthesis, which is regulated by *CYP51*, plays a role in Al tolerance in various plant species. To further test this possibility, the Al tolerance of a transgenic *Arabidopsis* line with knocked-down *AtCYP51* expression (*AtCYP51*-KD-1; Kushiro et al., 2001) was analysed. The root growth of *AtCYP51*-KD-1 was comparable to that of the wild-type Col-0 in control medium, but was inhibited in Al-containing medium (Fig. 7A, B). After 24 h of Al treatment, the plasma membrane at the root-tip portion of *AtCYP51*-KD-1 was damaged (Fig. 8A) and more Al accumulated in the root tissues of *AtCYP51*-KD-1 than in those of Col-0 (Fig. 8B). The ratio of phospholipids to sterols was higher in *AtCYP51*-KD-1 than in Col-0 (Fig. 9), indicating greater negativity of the plasma membrane surface in *AtCYP51*-KD-1 than in Col-0. Because malate excretion was similar in Col-0 and *AtCYP51*-KD-1 under Al-stressed conditions (Supplementary Figure S2; Fig. 10), it is likely that the plasma membrane lipid composition had been altered as a result of suppression of *CYP51*, which enhanced the Al sensitivity of *AtCYP51*-KD-1.

**Discussion**

The plasma membrane plays important roles in Al tolerance (Khan et al., 2009; Maejima et al., 2014). In previous studies, electrostatic computer modelling identified that $\{\text{Al}\}^{3+}$ at the plasma membrane surface ({$\{\text{Al}\}^{3+}$}$_{\text{PM}}$) determines Al toxicity in several plant species (Wang et al., 2011; Kobayashi et al., 2013). Greater negativity at the plasma membrane surface increases $\{\text{Al}\}^{3+}$$_{\text{PM}}$ and phospholipids are the major source of the negative charge at the plasma membrane (Wagatsuma and Akiba., 1989; Kobayashi et al., 2013). These findings indicated that a higher concentration of phospholipids in the plasma membrane increases Al toxicity. Under Pi-deprived conditions, Pi was removed from phospholipids by phosphatidate...
phosphohydrolases in the wild type (i.e. concomitant with increased amounts of electrically neutral galactolipids), while the phospholipid contents in the \textit{pah1pah2} double mutant remained at a level comparable to that under Pi-sufficient conditions (Nakamura et al., 2009). As a result, the double mutant showed Al sensitivity in Pi-deprived conditions, but not in Pi-sufficient conditions (Kobayashi et al., 2013). Lipid composition also affects the permeability of the plasma membrane under Al-stressed conditions, which could affect Al tolerance (Khan et al., 2009). The results of the present study indicate that sterol biosynthesis affected Al tolerance. This finding can be explained by the characteristics of sterols, which are electrically neutral. Because of their stereochemical structure, sterols make the plasma membrane less fluid and permeable than do several other lipid species, because the mobility of phospholipid fatty acyl chains is restricted (Hartmann, 1998).

Comparison of Al tolerance among different pea genotypes identified that the phospholipid/sterol ratio was negatively correlated with Al sensitivity (Fig. 3). The Al-sensitive phenotype was associated with greater permeability of the plasma membrane in the root-tip portion (Fig. 2) and greater Al accumulation (Supplementary Figure S4). This was correlated with suppressed expression of \textit{CYP51}, which encodes OBT 14DM, a key enzyme in sterol biosynthesis (Kushiro et al., 2001; Kim et al., 2005). The Al-sensitive \textit{lh} mutant is a low-GA mutant, because it contains a dysfunctional \textit{PsK01} gene. \textit{PsK01} encodes a cytochrome P450 in the CYP701A subfamily. \textit{PsK01} has \textit{ent}-kaurenoid oxidase (KO) activity; that is, it catalyses the three steps of oxidation of \textit{ent}-kaurene to \textit{ent}-kaurenoid acid in the GA biosynthetic pathway (Davidson et al., 2004). This raised the question as to whether GA biosynthesis might regulate Al tolerance in several plant species. GA has much stronger effects on hypocotyl elongation than on root elongation (Yaxley et al., 2001; Tanimoto, 2002) (little retardation of root elongation in the \textit{lh} mutant in the control; data not shown). Although the interaction between GA biosynthesis and sterol biosynthesis has not been clarified yet, there was repressed expression of \textit{PsCYP51} in the \textit{lh} mutant, and its phospholipid/sterol ratio was higher than that in other genotypes. These findings suggest that sterol biosynthesis, regulated by \textit{PsCYP51}, plays a critical role in Al tolerance.

This possibility was evaluated using a combination of a reverse-genetics approach in \textit{Arabidopsis} and chemical and biological approaches in various other plant species. Transgenic \textit{Arabidopsis} with knocked-down expression of \textit{AtCYP51}-\textit{KD} showed an Al-sensitive phenotype, which was characterized by greater Al accumulation and fewer intact root-tip membranes (Fig. 8). Little difference was detected in phytosterol content between \textit{Col}-0 and \textit{AtCYP51}-\textit{KD}-1 under normal conditions (Fig. 9). Similar results have already been reported by Kushiro et al. (2001) and Kim et al. (2005). They suggested that these results might indicate the presence of branch pathways, which are quiet under normal conditions and might be activated by specific growth conditions and eventually produce the final end products, i.e. phytosterols. In the present experiment, knocked-down transformation and Al treatment might correspond to such specific growth conditions. Additionally, Kushiro et al. (2001) detected a considerably higher content of obtusifoliol in \textit{AtCYP51}-\textit{KD} [0.45 in \textit{Col}-0, and 7.40 µg g$^{-1}$ fresh weight (FW) in \textit{AtCYP51}-\textit{KD}]. Obtusifoliol is a typical abnormal sterol which is accumulated by the inhibition of OBT 14DM (Burden et al., 1987), and is considered to occupy greater van der Waals volume, which induces greater plasma membrane permeability (Khan et al., 2009). Recently, a unique but less productive sterol biosynthetic pathway through lanosterol (which had been considered to be specific for fungi and mammals) was found in dicotyledonous plants in response to several stimuli (Kolesnikova et al., 2006; Suzuki et al., 2006). Further explanation cannot be given at present because of the lack of detailed knowledge on the whole sterol biosynthetic pathway in plants. Uniconazole-P suppressed Al tolerance in the Al-tolerant cultivars/lines of various plant species. Uniconazole-P is a triazole-type fungicide that inhibits OBT 14DM, a key enzyme in the post-squalene sterol biosynthetic pathway (Benveniste, 1986). In the presence of Al at a concentration
inducing 20–40% growth inhibition of tolerant cultivars/lines, uniconazole-P suppressed the Al tolerance of Al-tolerant cultivars/lines (Fig. 5). This concentration of Al almost completely inhibited root growth in Al-sensitive cultivars/lines. Each concentration of uniconazole-P (0.51 µM for wheat and rice, and 1.0 µM for triticale, maize, and sorghum) had little effect on normal root elongation and plasma membrane permeability in the control solution (data not shown). The universal effect of uniconazole-P on different plant species suggested that suppression of Al tolerance resulted from the effect of this chemical in modifying plasma membrane lipid composition, and not from its interference with different Al-tolerance mechanisms in each plant species.

The results of these experiments strongly suggest that sterol biosynthesis plays an important role in the Al tolerance of plants. Previous studies on sterol inhibitors including uniconazole-P have suggested that inhibition of OBT 14DM has multiple effects on the composition of plasma membrane lipids (Khan et al., 2009). Inhibition of OBT 14DM increased phospholipid contents, resulting in greater electrical negativity at the plasma membrane surface (Kobayashi et al., 2013), and may also have

![Fig. 6.](image)

Fig. 6. (A) Plasma membrane permeability of the root-tip portion of lines or cultivars of triticale, maize, and wheat with different degrees of Al tolerance. Plants were treated as described in Fig. 5, then roots were stained with FDA-PI to visualize plasma membrane permeability: in the experiments, reddish fluorescence indicates severe permeabilization of the plasma membrane lipid layer and greenish fluorescence indicates intactness of plasma membrane. Control seedlings (without Al) showed greenish fluorescence (data not shown). Scale bar, 1 mm. (B) Al accumulation in the root-tip portion of lines or cultivars of triticale, maize, and wheat with different degrees of Al tolerance under Al treatment with or without uniconazole-P. Plants were treated as described in Fig. 5, then roots were stained with haematoxylin to visualize Al accumulation. In the experiments, a denser purple colour indicates greater Al accumulation, seen here as darker zones. Roots in control (without Al) show a whitish yellow colour, indicating little inclusion of Al (data not shown). Scale bar, 1 mm. This figure is available in colour at JXB online.

![Fig. 7.](image)

Fig. 7. Difference in growth pattern between wild-type (Col-0) and CYP51 knocked-down line of Arabidopsis (AtCYP51-KD-1) after Al treatment (A) and Al tolerance of Col-0 and CYP51 knocked-down line-1 (B). Three-day-old synchronously germinated Arabidopsis seedlings of Col-0 and CYP51 knocked-down line-1 were treated with or without 4 µM AlCl₃ (pH 5.0) for 7 days. After acquiring images with a digital camera attached to a stereoscopic microscope, the length of each root in each image was measured using Image J software. Al tolerance was calculated as the ratio of root length in Al treatment to that in the control. Ten seedlings were used for each measurement. Values are means of three independent replicates ± standard error. Different lower case letters above each column indicate significant differences at a 5% level. Scale bar, 2 mm.
Higher sterols regulated by CYP51 for Al tolerance

increased the content of abnormal sterols, thereby increasing plasma membrane permeability (Kushiro et al., 2001; Khan et al., 2009). These changes could enhance Al sensitivity as a result of an increased Al concentration at the plasma membrane surface and increased Al permeability through the plasma membrane. Based on these findings, the suppression of CYP51 expression under conditions in which Al inhibits root growth could further enhance Al toxicity.

In control conditions without Al, the root-tip portions of all Al-tolerant cultivars and lines used in these experiments contained a higher sterol content and a lower phospholipid content than those of the Al-sensitive ones (Fig. 10). Additionally, Al treatment decreased the sterol content and increased the phospholipid contents. To date, there have been no reports on the reverse cross-talk between sterol content and phospholipid content in the root-tip portion (Supplementary Figure S4). Several nonspecific lipid transfer proteins (nsLTPs) transferring phospholipids, glycolipids, fatty acids, and steroids among membranes with wide-ranging binding affinities (Cheng et al., 2004), on lipid-transfer proteins (Peretti et al., 2008), or on other proteins (Joulet et al., 2007), may be one of the related candidates for the reverse cross-talk. Plasma membrane lipids play other roles such as stabilization of raft structures, which are important to maintain stress recognition and signal transduction through plasma membrane proteins (Lynch and Dunn, 2004). Further research should be conducted to uncover more details of the complex roles of plasma membrane lipids in Al tolerance.

![Fig. 8. Plasma membrane permeability (A) and Al accumulation (B) in root-tip portions of Arabidopsis seedlings of wild-type (Col-0) and the CYP51 knocked-down line (CYP51-KD-1).](image)

![Fig. 9. Phospholipid (PL) and sterol (S) contents in Arabidopsis seedlings of the wild type (Col-0) and the CYP51 knocked-down line (AtCYP51-KD-1).](image)
Fig. 10. Sterol content (A) and phospholipid content (B) in the root-tip portion of several plant species treated with or without Al in the absence of uniconazole-P. Young seedlings with roots ~4 cm long were treated for 24 h with 0.2 mM CaCl₂ ± 2.5 µM AlCl₃, at pH 4.9 (sorghum), and 5 µM (wheat), 10 µM (rice), or 20 µM AlCl₃ (others) at pH 5.0. Then, 10-mm apical root samples were extracted and the contents of S and PL were determined. S is expressed as β-sitosterol equivalents. Values are means of three independent replicates ± standard error.

Supplementary material

Supplementary data can be found at JXB online.

Supplementary Figure S1. Difference in growth pattern of Arabidopsis between wild-type (Col-0) and transformant (AtCYP51-KD-1).

Supplementary Figure S2. Relative transcript levels of AtCYP51 in wild-type Col-0 and CYP51 knocked-down lines of Arabidopsis.

Supplementary Figure S3. Malate release from Arabidopsis roots.

Supplementary Figure S4. Al accumulation in the root-tip portion of pea.

Supplementary Figure S5. Relationship between sterol content and phospholipid content in the root-tip portion of pea and rice.

Funding

This work was supported by Grants-in-Aid for Scientific Research (A) and (B) (Nos 18208008, 23380041) from the Japan Society for the Promotion of Science (JSPS).

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