Running Title: Detoxify Al at both apoplast and symplast

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Coordination between apoplastic and symplastic detoxification confers plant Al resistance

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ABSTRACT

Whether Al toxicity is an apoplastic or symplastic phenomenon is still a matter of debate. Here we found that three auxin overproducing mutants, yucca, sur2, and sur1-3 had increased Al sensitivity, while a T-DNA insertion mutant, xth15, showed enhanced Al resistance, accompanied by low endogenous IAA levels, implying that auxin may be involved in plant responses to Al stress. We used yucca and xth15 mutants for further study. The two mutants accumulated similar total Al in roots and had significantly reduced cell wall Al and increased symplastic Al content relative to the wild type, Col-0, indicating that altered Al levels in the symplast or cell wall cannot fully explain the differential Al resistance of these two mutants. The expression of ALS1, a gene that functions in Al redistribution between the cytoplasm and vacuole and contributes to symplastic Al detoxification, was less abundant in yucca and more abundant in xth15 than wild type, consistent with possible ALS1 function conferring altered Al sensitivity in the two mutants. Consistent with the idea that xth15 can tolerate more symplastic Al because of possible ALS1 targeting to the vacuole, morin staining of yucca root tip sections showed more Al accumulation in the cytosol than in wild type and xth15 showed reduced morin staining of cytosolic Al even though yucca and xth15 had similar overall symplastic Al content. Exogenous application of an active auxin analog, NAA, to wild type mimicked the Al sensitivity and distribution phenotypes of yucca, verifying that auxin may regulate Al distribution in cells. Together, these data demonstrate that auxin negatively regulates Al tolerance through altering ALS1 expression and Al distribution within plant cells, and plants must coordinate exclusion and internal detoxification to reduce Al toxicity.

INTRODUCTION

Aluminum (Al) toxicity is a major growth-limiting factor for crop production on acid soils worldwide (Foy, 1988; Kochian, 1995), which occupy approximately 50% of the world’s potential arable land (von Uexküll and Mutert, 1995). Ionic aluminum inhibits root elongation as well as water and nutrient uptake and results in significant loss of crop productivity (Kochian et al., 1995). Despite the increasing evidence of the functional and structural damage resulting from Al toxicity, the mechanism underlying Al-induced root growth inhibition remains unclear.

Aluminum resistant plants have developed two mechanisms to cope with Al toxicity.
One is based on the exclusion of Al from the root symplasm, whereas the other relies on the ability to tolerate symplastic Al (Kochian et al., 2004; Taylor, 1991). The well-documented exclusion mechanism is to prevent Al from entering root cells by secretion of organic acid anions by the root apex, resulting in the formation of stable non-phytotoxic chelates with Al (Kochian, 1995; Ryan et al., 2001; Ma and Furukawa, 2003). The internal detoxification of Al is primarily based on the storage of Al in the vacuole as Al-oxalate (Ma et al., 1997a; Shen et al., 2002) or Al-citrate (Ma et al., 1997b) complexes, thus changing the distribution of Al within cells (Ma 2000; Ma and Hiradate 2000). Recently, some transporters involved in the Al distribution within cells have been identified. In Arabidopsis, the tonoplast localized ABC transporter \( ALS1 \) is thought to transport Al into the vacuole (Larsen et al., 2007), while \( ALS3 \) is responsible for movement of Al away from sensitive tissues for sequestration in more tolerant tissues (Larsen et al., 2005, 2007). Huang et al. (2012) identified a rice tonoplast-localized Al transporter, encoded by \( OsALS1 \), which is responsible for sequestration of Al into vacuoles and thus contributes to the internal detoxification of Al in rice. In addition, some plants have been found to harbor multiple strategies for Al detoxification. Buckwheat, for example, can secrete oxalate to detoxify external Al and accumulate large amounts of Al within vacuoles by forming Al-oxalate complexes in a molar ratio of 1:3 (Ma et al., 1997c). Rice, which is the most Al resistant cereal crop, employs multiple strategies to achieve high Al resistance. The rice Al stress-responsive transcriptional factor, \( ART1 \), regulates expression of 31 downstream genes (Yamaji et al., 2009). In addition to gene-based Al tolerance strategies, some external factors can also affect Al resistance, such as external phytohormone application. For example, exogenous application of cytokinin can alleviate Al-induced inhibition of lateral root growth in Al-sensitive soybean (Pan et al., 2001). Several studies have demonstrated that Al may interact with auxin signalling pathways, leading to alterations of auxin accumulation and distribution in roots (Kollmeier et al., 2000; Doncheva et al., 2005; Shen et al., 2008). A recent study indicated that \( \text{Al}^{3+} \) induced alteration of auxin distribution in roots, leading to arrest of root elongation while naphthylphthalamic acid (NPA, an auxin polar transport inhibitor) applied exogenously substantially alleviated the Al-induced inhibition of root elongation (Sun et al., 2010). However, these previous studies only present circumstantial evidence on the disruption of the accumulation and polar transportation of auxin, which may be a primary cause of the Al-induced inhibition of root growth, the main mechanism of
how auxin contributes to Al sensitivity remains unclear.

Cell walls are not only a critical site for Al storage in plants, but also serve as the first barrier to cellular Al uptake. For example, 85-90% of the total Al accumulated by barley roots is tightly bound to the cell walls (Clarkson et al., 1967), and almost 90% of the cellular Al is associated with the cell walls of cultured tobacco cells (Chang et al., 1999). Accumulating evidence demonstrates that the cell wall plays important roles in the manifestation and perception of Al toxicity (Horst et al., 2010). The cell wall is very complex in structure and composition. The binding of Al changes cell wall structure, makes the wall more rigid, and reduces mechanical extensibility and cell expansion (Tabuchi and Matsumoto, 2001; Ma et al., 2004). An Al-sensitive rice cultivar accumulates higher Al in the cell wall than an Al-resistant cultivar (Yang et al., 2008). Higher pectin content is partially attributed to higher Al accumulation in the cell wall (Eticha et al., 2005a; Liu et al., 2008; Yang et al., 2011a). Furthermore, Al stress also results in an increase of not only pectin but also hemicellulose content in wheat (Tabuchi and Matsumoto, 2001), triticale (Liu et al., 2008) and rice (Yang et al., 2008). Recently, Yang et al. (2011b) reported that hemicellulose, not pectin, is the major cell wall component that binds Al in Arabidopsis. Al inhibits XET (xyloglucan endotransglucosylase) action, an enzyme that may cut and rejoin xyloglucan chains leading to cell wall loosening (Fry et al., 1992; Nishitani and Tominaga, 1992; Thompson and Fry, 2001), and down-regulates the expression of XTH14, 15 and 31. Further study showed that Arabidopsis with XTH31 knocked out has lower xyloglucan content and cell wall Al binding capacity, and higher Al resistance (Zhu et al., 2012). Al-induced secretion of organic acid anions decreases Al retention in the cell wall (Zheng et al., 2004), and, as a consequence, Al content in the root apex is decreased, which renders plants more Al resistant. Together these data support the view that higher Al retention in the cell wall results in greater Al sensitivity. However, an important question remains open; if the total Al content in the roots remains constant, but the Al retention in the cell wall is decreased, will plants be more Al sensitive or more Al resistant?

In the present study, we used two Arabidopsis mutants that accumulate similar Al levels in the roots, but one mutant, yucca, is Al sensitive whereas the other, xth15, is Al resistant. We explored the underlying mechanisms leading to this difference in Al sensitivity. Although both mutants accumulate similar Al levels in cell wall, xth15 may sequester more Al into vacuoles than yucca. These data suggest the importance
of coordination between external and internal detoxification mechanisms in Arabidopsis.

RESULTS

To investigate the effect of auxin on Al sensitivity, we used three high endogenous auxin mutants, the recessive mutants superroot1 (sur1-3; Boerjan et al., 1995) and superroot2 (sur2; Barlier et al., 2000; Delarue et al., 1998), and the dominant activation-tagged yucca1, which overexpresses the flavin monooxygenase-like YUCCA proposed to be involved in tryptophan-dependent indole-3-acetic acid (IAA) biosynthesis (Zhao et al., 2001).

Inhibition of root elongation is the most typical symptom of Al toxicity in plants. When Arabidopsis seedlings were treated with 50 µM Al for 24 h, root elongation was inhibited by 63% in an auxin over-producing mutant, yucca, while 41% in WT (Fig. 1A). In longer-term (7 d) experiments, root growth was also more inhibited by exogenous Al in yucca than in WT (Fig. 1C). To confirm that the Al sensitive phenotypes observed in yucca are caused by the overproduction of auxin, we analyzed the Al sensitivity of the other two high endogenous auxin mutants, sur2 and sur1-3, and found that they showed similar Al sensitivity as yucca (Fig. 1A, 1C). As the increased endogenous auxin levels had similar effects on Al resistance, we used yucca as plants with “high levels of endogenous auxin” for the following experiments. WT plants treated with NAA (an active auxin) also are more Al sensitive (Fig. 1B, 1C), confirming that the Al sensitivity of yucca is likely due to auxin overaccumulation. We also measured the solution pH when 0.05 µM NAA was added to the non-buffered Al solution and found that there was no decline of pH. Furthermore, when the solution was buffered at pH 4.5 with MES, the relative root elongation showed no significant difference with the non-buffered solution (Fig. 1B, Supplement Fig. 1). Moreover, if NAA increases the activity of Al, the inhibition of the root elongation should also be more profound in NAA+Al treatment than Al treatment alone, but there was almost no difference of Al sensitivity in yucca between Al and NAA+Al treatment (Supplement Fig. 1). Therefore, the effect of NAA+Al on root growth was caused by the synergetic action of Al and NAA. Furthermore, Figure 1A shows that xth15, mutants with a T-DNA insertion in the XTH15 locus, treated with 50 µM Al for 24 h showed only 13 % root elongation inhibition compared to WT root inhibition of 41% (Fig. 1A). Similarly, xth15 seedlings grown for 7 days on agar medium containing 50 µM Al3+.
had longer roots than similarly treated WT seedlings (Fig. 1C) and the root growth of
xth15 was also inhibited by NAA+Al (Fig. 1B, 1C). Interestingly, the IAA levels in
xth15 roots were also lower than WT (Fig. 2) and correlated with enhanced Al
resistance (Fig. 1). All these results demonstrate that higher levels of endogenous or
exogenous auxin correlate with increased Arabidopsis sensitivity to Al.

Al content is a critical index to indicate whether exclusion or internal
detoxification mechanism underlies Al resistance. To distinguish between these
mechanisms in the altered Al sensitivity of yucca and xth15 mutants, we determined
Al content in roots of seedlings exposed to 50 µM Al for 24 h. Surprisingly, WT,
yucca, xth15, and WT and xth15 treated with NAA all accumulated similar levels of
Al (Fig. 3). These results suggest that elevated auxin or loss of XTH15 function does
not affect overall Al accumulation levels but may cause alterations in Al sensitivity by
changing the distribution of Al within root cells.

To address cellular distribution, we first measured Al content in symplast and root
cell walls according to Xia et al. (2010). The purity of the apoplastic solution was
reported by the absence of detectable malic dehydrogenase (about 35 KDa) (Fig. 4A).
Al accumulation in the symplast of untreated WT was lower than that in yucca, xth15,
and WT treated with exogenous NAA (Fig. 4B), whereas the Al content in the cell
wall was higher in WT than in WT treated with NAA or in xth15 or yucca (Fig. 4C).
Although the reduction in cell wall Al content of xth15 relative to WT may correlate
with the enhanced Al resistance of xth15, the reduction in cell wall Al of yucca and
NAA-treated WT is unexpected given the enhanced Al sensitivity of yucca, and
NAA-treated WT and xth15. These data prompted us to investigate whether
differential subcellular localization of symplastic Al may impact the Al sensitivity
phenotypes of yucca, xth15 and WT treated with exogenous NAA.

ALS1 is reported to be a root tip tonoplast transporter and responsible for Al
redistribution between the cytoplasm and vacuole (Larsen et al., 2007). To determine
whether differential expression abundance of ALS1, predicted to correlate with
function levels, could underlie the differential sensitivity of yucca and xth15, we
monitored ALS1 expression in roots with and without Al stress. Although ALS1
expression was not Al inducible (Fig. 5), in accordance with Larsen et al. (2007), root
ALS1 expression was about 50% lower in yucca and WT treated with exogenous NAA
than in WT (Fig. 5), but was about 50% higher than WT in xth15 under control
conditions (Fig. 5). Therefore relative ALS1 expression correlated with resistance in
these two mutants, suggesting that high auxin levels may impair ALS1-dependent Al detoxification, whereas loss of XTH15, which also resulted in a low auxin level, may enhance this subcellular detoxifying mechanism. Furthermore, we found that als1-2 sensitivity to Al was not exacerbated by NAA treatment (Fig. 6A, 6B), although NAA-treatment of als1-2 led to lower cell wall Al content (Fig. 6C, 6D), consistent with the idea that auxin enhances Al toxicity by down-regulating the expression of ALS1.

To gain further evidence for possible vacuole compartmentalization of Al, we localized the Al that enters cells with morin staining. Morin can detect Al in the cytosol but not cell wall-bound Al or vacuole-compartmentalized Al (Eticha et al., 2005b; Huang et al., 2012). The lack of morin staining in vacuole may be attributed to two reasons according to Huang et al. (2012): (a) morin is not permeable to the tonoplast, and (b) vacuolar Al is chelated by organic reagents, such as malic and citric acids, and morin cannot detect complexed Al forms, similar to cell wall-bound Al (Eticha et al., 2005b). Therefore, strong Al-dependent green fluorescence represents Al present in the cytosol and nucleus. The green fluorescence of morin was only faintly detected when seedlings were treated with 0.5 mM CaCl2 in the absence of Al, whereas after exposure to 0.5 mM CaCl2 combined with 50 μM Al, yucca and NAA-treated WT displayed stronger Al-dependent green fluorescence than WT in root cells (Fig. 7), and xth15 displayed relatively weaker morin staining (Fig. 7). These results are consistent with the conclusion that elevated auxin results in more Al accumulation in the cytosol whereas loss of XTH15 function, with a lower level of endogenous auxin, accumulates less Al in the cytosol and therefore may target more Al to the vacuole.

DISCUSSION

To survive in an Al toxic environment, Al resistant plant species adopt strategies to either restrict Al uptake (exclusion of Al from the root symplasm) or cope with internalized Al (tolerate symplastic Al) (Kochian et al., 2004; Taylor, 1991). The typical exclusion mechanism is generally associated with lower Al content in the roots or fixation of Al in the apoplast. However, in the present study, although there is no significant difference in the total Al content among the roots of yucca, xth15, NAA-treated WT and non-NAA-treated WT, there are large differences in Al resistance. To elucidate the possible mechanisms leading to the differential Al
sensitivity, we found that although less Al retention in the cell wall may contribute to enhanced Al resistance, when similar amounts of Al are present in the roots, more exclusion of Al from the more susceptible sites such as cytoplasm and nucleus may also be fundamental for plant resistance to Al. This work provides solid evidence for the importance of cooperation between Al exclusion and internal detoxification in plants.

Increasing evidence has shown that binding of Al in cell wall appears to be closely related to Al sensitivity, as fixation of Al in the cell wall will affect the proper functioning of cell wall (Horst et al., 2010). For example, Horst (1995) reported that Al bound to cell wall components increases wall rigidity, affects cell wall loosening, and thus ultimately inhibits root elongation. Ma et al. (2004) demonstrated that Al decreases cell wall viscosity and elasticity, thus reducing cell wall extensibility and, as a consequence, cell elongation is inhibited. Therefore, higher Al sensitivity is correlated with more Al accumulation in the cell wall, as demonstrated in maize suspension cells (Schmohl and Horst, 2000) and intact root apices (Eticha et al., 2005a), rice (Yang et al., 2008), triticale (Liu et al., 2008) and rice bean (Zhou et al., 2012). In Arabidopsis, there is also a large difference in Al resistance among different ecotypes (Hoekenga et al., 2006). Although there is no report on the difference of Al content in roots among the different ecotypes, several studies demonstrated that Al resistant mutants (Larsen et al., 1998) or transgenic lines (Ezaki et al., 2007) usually accumulate significantly less Al in the roots as compared with wild type. Recently, we identified another Arabidopsis mutant xth31 that is highly Al resistant. xth31 accumulates significantly less Al in cell walls due to less xyloglucan content, as xyloglucan is responsible for Al binding in the hemicellulose of cell wall (Zhu et al., 2012). However, in some Arabidopsis mutants, Al sensitivity is not related to Al content. For example, als7 and als4 roots both accumulate less Al than wild type after exposure to Al-containing solutions, yet root growth of the mutants is significantly inhibited (Larsen et al., 1996). Nezames et al. (2012) found that the increase in root growth seen for alt2-1als3-1 compared with als3-1 and Col-0 under Al stress is not related to the root Al accumulation because alt2-1als3-1 roots accumulate wild-type levels of Al. Here we screened a number cell wall associated mutants for Al sensitivity and found a mutant, xth15, that displayed enhanced Al resistance. In this mutant, T-DNA is inserted into the first exon of XTH15 (110 bp downstream of the translation initiator ATG codon; Supplement Fig. 2A). XTH15 transcripts were not detected in the
homozygous line (Supplement Fig. 2B), indicating that the xth15 allele is likely a null mutation. XTH15 is likely to function in seedling roots because strong root staining is found in transgenics harbouring a GUS reporter gene driven by the XTH15 5’ potential regulatory region (Becnel et al., 2006). We examined the two different mutants, the auxin overproducing mutant, yucca, and the cell wall mutant, xth15, which showed large differences in Al sensitivity despite having similar root Al content. Furthermore, both mutants had moderate reductions of Al retention in the cell wall (Fig. 4C), suggesting that exclusion of Al from the cell wall is not necessarily sufficient to confer elevated Al resistance. Therefore, there must exist other mechanisms that contribute to differential Al sensitivity.

A fundamental mechanism of internal detoxification is sequestration of Al into the vacuoles. Ma et al. (1997a, c) identified forms of Al-organic acids compounds in the cell sap of hydrangea and buckwheat leaves and proposed that these compounds might be sequestered in vacuoles. Later, Shen et al. (2002) demonstrated that most Al and oxalate in the protoplasts of buckwheat leaves is present in the vacuoles. These reports provide physiological evidence to demonstrate the compartmentalization of Al into the vacuole in the forms of Al-organic acids and the contribution of compartmentalization to internal Al detoxification. With the studies of various mutants altered in Al sensitivity, molecular mechanisms underlying resistance can be uncovered. Larsen et al. (2005) identified ALS3, which encodes a transporter that is localized to the phloem, leaf hydathodes, and root epidermis, and is predicted to transport Al away from sensitive tissues for sequestration in more tolerant tissues. Indeed, als3 displays severe Al-inhibition of root growth. In addition, ALS1, which encodes a transporter localized to the root tip and the vasculature, has been implicated in Al sequestration to more tolerant tissues (Larsen et al., 2007). Recently, Huang et al. (2012) identified OsALS1, which is expressed ubiquitously in rice with the encoded protein localizing to the tonoplast, is responsible for sequestration of Al into the vacuoles and is required for internal detoxification of Al in rice. Although both ALS1 and ALS3 have critical roles in Al resistance, ALS3 is a plasma membrane transporter that moves Al away from the root tip and is not involved in redistribution between the cytoplasm and vacuole. We found, however, that ALS1 expression was lower in yucca and NAA-treated WT, but higher in xth15 (Fig. 5), thus correlating with Al resistance. Moreover, the expression of ALS1 was also down-regulated when xth15 was treated with NAA (Fig. 5). However, there was lower IAA content in xth15 roots, suggesting
that auxin accumulation may affect \textit{ALS1} expression and altered \textit{ALS1} expression may affect the efficacy of internal detoxifying mechanisms of Al in plants, which is in accordance with the no effect of NAA in the \textit{als\_1-2} mutant (Fig. 6). However, it is interesting that the NAA applied exogenously had no effect on WT and \textit{xth\_15} growth while it inhibited the \textit{als\_1-2} growth (Fig. 1, 6); this may be attributed to differential sensitivity to NAA, and \textit{als\_1-2} might have a lower suitable NAA level to promote the root growth. Furthermore, the morin staining reported more Al present in the cytosol in \textit{yucca} and less cytosolic Al in \textit{xth\_15} than WT (Fig. 7). Therefore, when similar amounts of Al are present in the symplasm, there may be enhanced Al redistribution into the vacuoles facilitated by enhanced expression of \textit{ALS1}, which may lead to more Al resistance. These results lead to the next question of how might auxin regulate the expression of \textit{ALS1}. As there are no auxin response elements (AuxREs) TGTCTC in the 2 kb-promoter regions of \textit{ALS1} genes (Hagen and Guilfoyle, 2002), the effect may be indirect, needing further investigation.

In the present study, we also demonstrated that auxin is a negative factor in plant Al resistance, as both endogenous auxin overproduction, as in the \textit{yucca} mutant, and exogenous application of NAA resulted in higher Al sensitivity in Arabidopsis, whereas \textit{xth\_15} with low levels of endogenous auxin exhibited higher Al resistance (Fig. 1). Indeed, we have used a series of auxin concentrations and found that with each incremental increase in the auxin concentration, \textit{ALS1} expression was progressively repressed accompanied by increasing inhibition of root growth (Supplement Fig.3). Kollmeier et al. (2000) found that Al alters auxin accumulation and distribution in roots possibly due to effects on the auxin polar transport system while application of exogenous IAA to the elongation zone significantly alleviated the Al induced inhibition of root growth in maize. Recently, Sun et al. (2010) demonstrated that Al affects auxin distribution through Al-induced changes in ethylene production; however, application of an IAA polar transport inhibitor can partially alleviate the inhibition of root growth in Arabidopsis under Al stress. The role of auxin in Al resistance is therefore complex. Effects may be influenced by species-specific responses or experimental treatment conditions. However, previous studies reported a relationship between Al-induced inhibition of root elongation and the disruption of auxin accumulation or distribution; the underlying physiological and molecular mechanisms remained undefined. Here, we demonstrate that auxin may exacerbate Al sensitivity by modifying the expression of \textit{ALS1} and therefore Al
redistribution as seen in *xth15*, *yucca* and wild type supplied with exogenous NAA (Fig. 5; Supplement Fig. 3). Therefore, auxin may have a role in altering Al distribution within cells.

In conclusion, our study focusing on the Al resistance of WT, *yucca* and *xth15*, demonstrates that auxin negatively regulates Al tolerance through altering *ALS1* expression and Al distribution within plant cells, providing evidence for the importance for plant coordination of apoplastic and symplastic detoxification of Al to withstand Al toxicity.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

The Columbia ecotype (Col-0) of Arabidopsis (*Arabidopsis thaliana*) served as the wild type (WT) and the background for all mutants, including *xth15*, *yucca*, *sur2*, *sur1-3* and the Al sensitive mutant *als1-2* used in this study. For short-term treatments (24 h), 0.5 mM CaCl$_2$ solution (pH 4.5) was used as control media (“CK”), while for longer duration treatments (7 d), the nutrient solution (pH 4.5) was used as control media and 50 μM Al and 0.05 μM NAA were directly added for Al, NAA or NAA+Al treatments. Seeds were surface sterilized and germinated on an agar-solidified nutrient medium in Petri dishes. The nutrient medium consisted of the following macronutrients: 6.0 mM KNO$_3$, 4.0 mM Ca(NO$_3$)$_2$, 1 mM MgSO$_4$, 0.1 mM NH$_4$H$_2$PO$_4$ and the following micronutrients: 50 μM Fe(III)-EDTA, 12.5 μM H$_3$BO$_3$, 1 μM MnSO$_4$, 0.5 μM CuSO$_4$, 1 μM ZnSO$_4$, 0.1 μM H$_2$MoO$_4$, 0.1 μM NiSO$_4$ according to Murashige-Skoog salts (1962). The final pH was adjusted to 4.5. The seeds were vernalized at 4 ºC for 2 d. Petri dishes were placed into a growth chamber, positioned vertically and kept under controlled environmental conditions at 24 ºC, 140 μmol photons m$^{-2}$ s$^{-1}$ and a 16/8 h day/night rhythm.

For hydroponic culture, seedlings were first aseptically germinated on the above solid Murashige and Skoog medium. After 2 weeks, the young plantlets were placed on vermiculite for additional 3 weeks in an environmental controlled growth chamber. Seedlings of similar rosette diameters were then transferred to the nutrient solution containing Murashige-Skoog salts for another one week. Then the plants were subjected to the following treatments: CK (0.5 mM CaCl$_2$, pH 4.5), Al (50 μM Al in the 0.5 mM CaCl$_2$, pH 4.5), NAA (adding 0.05 μM NAA to the above mentioned
“CK” solution), NAA+Al (adding 0.05 μM NAA to the above mentioned “Al” solution). After 24 h, the roots were excised for RNA extraction or for Al content analysis. When for Al content analysis, the seedlings were washed three times with deionized water, and the fresh weight was recorded.

**Effect of Al on Root Growth**

Seedlings with root lengths of 1 cm were selected and transferred to Petri dishes containing agar-solidified CaCl$_2$ (0.5 mM) medium with different Al concentrations (0, and 50 μM total concentration of Al in the form of AlCl$_3$·6H$_2$O). Root length measurements were performed using a digital camera connected to a computer. Data were quantified and analyzed by Photoshop 7.0 (Adobe Systems). For long duration experiments, seedlings with a root length of 1 cm were selected and then transferred to Petri dishes containing agar-solidified nutrient solution medium with different Al concentrations (0 and 50 μM total concentration of Al).

**Gene Expression Analysis**

Total RNA was isolated using TRIzol (Invitrogen). cDNA was prepared from 1 μg of total RNA using the PrimeScript RT reagent kit (Takara). For real-time RT-PCR analysis, 1 μL of 10-fold-diluted cDNA was used for the quantitative analysis of gene expression performed with SYBR Premix ExTaq (Takara) with the following pairs of gene-specific primers (*ALS*1: forward: 5'-GACCGTTGGAGCACTCACTTC-3'; reverse: 5'-CAGGATTACCGACTGGACACT-3' and for *tubulin*: forward: 5'-AAGTTCTGGGAAGTGGTT-3'; reverse: 5'-CTCCCAATGAGTGACAAA-3). Each cDNA sample was run in triplicate. Expression data were normalized with the expression level of *tubulin* gene. For the Semi-Quantitative RT-PCR analysis, the primers used for *18S*: forward: 5'-ATGATAACTCGACTCGACG-3'; reverse: 5'-CTTGGATGTGGTAGCCGTTT-3' and for *XTH15*: forward: 5'-CCGCTCGAGAAGAGAAGCAACTTCTTCGACGAGT-3'; reverse: 5'-GCTCTAGAGACTCTGGACTTCT-3'.

**Al Content Measurement**

After treatment, the roots were excised after washing three times with 0.5 mM
CaCl₂ and then put in a Ultra free-MC Centrifugal filter units (Millipore) and centrifuged at 3,000 × g for 10 min at 4 °C to remove apoplastic solution. The roots were then frozen at −80 °C overnight. The root-cell sap solution was obtained by thawing the samples at room temperature, and then centrifuging at 20,600 × g for 10 min. The residual cell walls were washed with 70% ethanol three times before being immersed in 0.5 mL of 2 N HCl for 36 h with occasional vortexing, according to Xia et al. (2010). For root Al content analysis, materials were digested with HNO₃/HClO₄ (4:1, v/v). The Al in the root, symplastic solution and cell wall extracts was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES; IRIS/AP optical emission spectrometer).

**SDS-PAGE**

The protein from the apoplastic and root-cell sap fractions and malic dehydrogenase (*Thermus flavus*; Sigma, M7032) were heated at 100 °C for 10 minutes in 2×SDS-loading buffer to denature the proteins. 20 μL of the protein samples were loaded in each well.

SDS-PAGE was conducted using a 12% resolving gel and 4% stacking gel. The resolving gel, to a total volume of 10 mL, was consisted of 3.35 mL water (distilled), 4 mL 30% acrylamide/bis-acrylamide stock, 2.5 mL 1.5 M Tris-HCl (pH 8.8), 0.1 mL 10%(w/v) SDS, 0.05 mL 10% ammonium persulfate and 0.005 mL TEMED. Then carefully pour into glass plate and overlay gel with ddH₂O to ensure a flat surface and to exclude air. After the gel has set more than half an hour, get rid of the ddH₂O, mix the 4% stacking Gel: 3.05 mL water (distilled), 0.65 mL 30% acrylamide/bis-acrylamide stock, 1.25 mL 0.5 M Tris-HCl (pH 6.8), 0.05 mL 10%(w/v) SDS, 0.025 mL 10% ammonium persulfate and 0.005 mL TEMED, pour onto top of set resolving gel, insert comb. The electrophoresis buffer was consisted of 3.03 g/L Tris base, 18.77 g/L glycine and 1 g/L SDS. The gel runs at 80V for about 3 h. The gel was stained with 0.1% Coomassie Brilliant Blue (R250) in methanol/water/acetic acid (45:45:10, v/v/v) for 2 to 3 hours at room temperature with agitation and then destained in ethanol/water/acetic acid (45:45:10, v/v/v). Finally, pictures were taken using a digital camera.

**Morin staining**
About 1 cm long seedlings of WT (Col-0) and the yucca and xth15 mutants were exposed to 0.5 mM CaCl₂ solution (pH 4.5) containing 50 µM Al for 12 h. Roots were stained in 0.01% morin for 30 min, then excised and embedded in 5% agar. Root tips were transversely sectioned from the apex, and the green fluorescence signal was observed using a laser-scanning confocal microscope (LSM510; Zeiss).

IAA Measurement

For analysis of the IAA concentration in roots, the whole root (about 20 mg) was collected for each sample. Four replicates of the samples were purified after addition of 250 pg ¹³C6-IAA internal standard and analyzed by gas chromatography-selected reaction monitoring mass spectrometry as described (Ljung et al., 2005).

Statistical Analysis

Each experiment was repeated at least three times. Data were analyzed by one-way ANOVA procedure and the means were compared by Duncan’s multiple range test. Different letters on the histograms indicate that the means were statistically different at P<0.05 level.

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Figure Legends

Figure 1. The effect of Al and NAA on root growth of Arabidopsis. (A) 1 cm long seedlings were grown on 0.5 mM CaCl$_2$ medium containing 0 ("CK") or 50 µM Al for 24 h. Data are means ± SD (n = 10). Columns with different letters are significantly different at P < 0.05. (B) 1 cm long seedlings were grown on 0.5 mM CaCl$_2$ medium containing 0 or 50 µM Al in the presence or absence of NAA. Root elongation was measured before and after treatment for 24 h. Data are means ± SD (n = 10). Columns with different letters are significantly different at P < 0.05. (C) The effect of Al and NAA on the root growth of Arabidopsis. 1 cm long seedlings were grown on nutrient plates containing 0 or 50 µM Al for 7 d. All the experiments were done at pH 4.5. Pictures were taken using a digital camera.

Figure 2. Accumulation of IAA in Col-0, yucca, xth15 and als1-2 without Al treatment. Data are means ± SD (n = 4). Columns with different letters are significantly different at P < 0.05.

Figure 3. The Al content in the plant roots. Six-week-old plants were treated with 0.5 mM CaCl$_2$ solution containing 50 µM Al or 50 µM Al combined with 0.05 µM NAA for auxin applied exogenously treatment. The pH was adjusted to 4.5. Data are means ± SD (n = 4). Columns with different letters are significantly different at P < 0.05.

Figure 4. Purity of apoplastic solution (A), Al content in symplast (B) and cell wall (C). Six-week-old plants were grown on 0.5 mM CaCl$_2$ media containing 50 µM Al or 50 µM Al combined with 0.05 µM NAA for 24 h. The pH was adjusted to 4.5. Data are means ± SD (n = 4). Columns with different letters are significantly different at P < 0.05. MD: malic dehydrogenase; Marker: protein standards.

Figure 5. Effect of Al on ALS1 expression. Six-week-old plants were treated with 0.5 mM CaCl$_2$ solution containing 0 or 50 µM Al in the presence or absence of 0.05 µM NAA for 24 h. The pH was adjusted to 4.5. Total RNAs were extracted from roots and subjected to reverse transcription followed by real-time PCR. Expression levels without Al treatment of WT were normalized to the expression level of tubulin under control conditions (-Al) were assigned as expression level of 1. Data are means of
three independent biological replicates. Columns with different letters are significantly different at P < 0.05.

Figure 6. The effect of Al and NAA on the root growth and Al content in root symplast and cell wall of als1-2. (A) 1 cm long seedlings were grown on 0.5 mM CaCl₂ medium containing 0 or 50 µM Al in the presence or absence of NAA for 24 h. The pH was adjusted to 4.5. Data are means ± SD (n = 10). Columns with different letters are significantly different at P < 0.05. (B) The effect of Al and NAA on the root growth of Arabidopsis. 1 cm long seedlings were grown on nutrient plates containing 0 or 50 µM Al for 7 d. The pH was adjusted to 4.5. Pictures were taken using a digital camera. Al content in symplast (C) and cell wall (D). Six-week-old plants were grown on 0.5 mM CaCl₂ solution containing 50 µM Al or 50 µM Al combined with 0.05 µM NAA for 24 h. The pH was adjusted to 4.5. Data are means ± SD (n = 4). Columns with different letters are significantly different at P < 0.05.

Figure 7. Subcellular distribution of Al stained with morin (green). About 1 cm long seedlings were exposed to 0.5 mM CaCl₂ solution with or without 50 µM Al in the presence or absence of NAA for 12 h. The pH was adjusted to 4.5. Roots were transversely sectioned at 5 and 10 mm from the apex for morin staining and fluorescence observation. Scale bar = 10 µm.

Supplement Figure 1. The effect of Al on root growth of Arabidopsis. 1 cm long seedlings were grown on 0.5 mM CaCl₂ solution buffered with 10 mM MES in the presence or absence of 50 µM Al and 0.05 µM NAA for 24 h. The pH was adjusted to 4.5. Data are means ± SD (n = 10). Columns with different letters are significantly different at P < 0.05.

Supplement Figure 2. (A) Schematic structure of the xth15 mutant carrying a single copy of the T-DNA insert in the first exon of the XTH15 gene. The black and white boxes represent the coding and untranslated regions (UTR), respectively. (B) Semi-Quantitative RT-PCR study of XTH15 expression.
Supplement Figure 3. The effect of Al and NAA on root growth of Arabidopsis. (A) 1 cm long seedlings were grown on 0.5 mM CaCl$_2$ medium containing 50 µM Al in the presence or absence of NAA for 24 h. The pH was adjusted to 4.5. Relative root growth under Al treatment in the absence of NAA was assigned as 100%. Data are means ± SD ($n = 10$). Columns with different letters are significantly different at $P < 0.05$. (B) The effect of Al and NAA on the expression of **ALS1** in Arabidopsis. Six-week-old plants were grown on 0.5 mM CaCl$_2$ solution containing 50 µM Al in the presence or absence of NAA for 24 h. The pH was adjusted to 4.5. Expression levels with Al treatment in the absence of NAA was assigned as expression level of 1. Data are means ± SD ($n = 3$). Columns with different letters are significantly different at $P < 0.05$. 
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