Research Article

Alleviation by ABA of Al toxicity in rice bean is not associated with citrate efflux but depends on ABI5-mediated signal transduction pathways

Running title: Cross-talk between Al and ABA signals

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Abstract
Under conditions of aluminum (Al) toxicity, which severely inhibits root growth in acidic soils, plants rapidly alter their gene expression to optimize physiological fitness for survival. Abscisic acid (ABA) has been suggested as a mediator between Al stress and gene expression, but the underlying mechanisms remain largely unknown. Here, we investigated ABA-mediated Al-stress responses, using integrated physiological and molecular biology approaches. We demonstrate that Al stress caused ABA accumulation in the root apex of rice bean (*Vigna umbellata*), which positively regulated Al tolerance. However, this was not associated with known Al-tolerance mechanisms. Transcriptomic analysis revealed that nearly one-third of the responsive genes were shared between the Al-stress and ABA treatments. We further identified a transcription factor, ABI5, as being positively involved in Al tolerance. *Arabidopsis abi5* mutants displayed increased sensitivity to Al, which was not related to the regulation of *AtALMT1* and *AtMATE* expression. Functional categorization of ABI5-mediated genes revealed the importance of cell wall modification and osmoregulation in Al tolerance, a finding supported by osmotic stress treatment on Al tolerance. Our results suggest that ABA signal transduction pathways provide an additional layer of regulatory control over Al tolerance in plants.

INTRODUCTION

Aluminum (Al) is the third most abundant element in the earth’s crust and is present in the form of oxides and aluminosilicates, which are non-toxic to plants. At soil pH values below 5, ionic species of Al (mainly Al$^{3+}$) are released into solution from the soil minerals, resulting in a rapid inhibition of root elongation, even at micromolar concentrations (Kochian 1995). Unfortunately, nearly 50% of potentially arable soils, worldwide, are acidic (von Uexkull and Mutert 1995). In addition, human activities have contributed to soil acidification through, for example, the use in agriculture of ammonium-containing fertilizers (Guo et al. 2010). Therefore, Al toxicity represents one of the major limiting factors for crop production in acidic soils.

During the past several decades, a great deal of research on this topic has improved our understanding of the response of plants to Al toxicity in acidic soils (Kochian et al. 2015). With regards to the mechanisms of Al toxicity, a number of molecular and physiological processes, such as cell wall biogenesis and modification,
mitochondria dysfunction, disruption to Ca\(^{2+}\) homeostasis, and hormone imbalance have been proposed to cause cellular dysfunction, leading to growth inhibition (Barcelo and Poschenrieder 2002; Rengel and Zhang 2003; Ma 2007). However, the nature of Al toxicity is still contested.

By contrast, substantial progress has been made towards elucidating Al resistance mechanisms (Kochian et al. 2004; Delhaize et al. 2012). For example, Al-activated organic acid secretion has been widely documented as a very important mechanism by which the root apex prevents toxic Al\(^{3+}\) ions from entering into cells. However, the manner in which the root tip senses and transduces the Al-stress signal, leading to gene expression and the eventual physiological responses, remains poorly understood (Liu et al. 2014).

The target of Al toxicity is mainly confined to the root apex, where phytohormones such as gibberellin, cytokinin, and abscisic acid (ABA) are synthesized. Therefore, as might be expected, phytohormones have been implicated in the Al-stress response. Several studies have reported that Al stress induces the accumulation of indole-3-acetic acid (IAA) in maize (Zea mays) (Kollmeier et al. 2000), Arabidopsis (Arabidopsis thaliana; Sun et al. 2010; Zhu et al. 2013; Yang et al. 2014), wheat (Triticum aestivum; Yang et al. 2011), and rice (Oryza sativa; Wu et al. 2014) roots. A similar situation has been shown for ethylene in maize (Gunse et al. 2000), Arabidopsis (Sun et al. 2010), and wheat (Tian et al. 2014), and for ABA in soybean (Glycin max; Shen et al. 2004; Hou et al. 2010), barley (Hordeum vulgare; Kasai et al. 1993a, 1993b), and buckwheat (Fagopyrum esculentum Moench; Reyna-Llorens et al. 2015), and for cytokinin in common bean (Massot et al. 1994, 2002) and Arabidopsis (Yang et al. 2017a).

It still remains controversial whether the changes in phytohormone homeostasis are involved in the plant Al-stress response. For example, in maize it was shown that the production of ethylene was related neither to Al toxicity nor tolerance mechanisms (Gunse et al. 2000). However, in wheat, ethylene production was shown to negatively regulate Al-induced malate efflux from the roots, which resulted in accumulation of Al and subsequent inhibition of root elongation (Tian et al. 2014). A similarly contradictory scenario, involving Al-induced accumulation of endogenous IAA, content seems to be associated with the Al-induced efflux of malate in wheat root apices (Yang et al. 2011).

In Arabidopsis, the Al-induced inhibition of root elongation is associated with the
accumulation of IAA, which acts downstream of ethylene (Sun et al. 2010). It was further demonstrated that ethylene signaling up-regulates expression of a Trp aminotransferase gene, \textit{TAA1}, specifically at the root tip transition region, between the meristem and the elongation zone, thereby resulting in local IAA biosynthesis and subsequent inhibition of root elongation (Yang et al. 2014). A recent study also showed that Al-induced IAA biosynthesis can further activate adenosine phosphate isopentenyltransferase (IPT) gene expression, which promotes local cytokinin biosynthesis and the inhibition of root growth (Yang et al. 2017a). However, root growth inhibition was shown to be regulated by COI1-mediated jasmonic acid signaling, supporting the hypothesis that this inhibition is independent from auxin signaling, and provides novel insight into phytohormone-mediated root growth inhibition in response to Al stress (Yang et al. 2017b).

ABA plays primary regulatory roles in the initiation and maintenance of seed and bud dormancy, and in plant stress responses, particularly water stress. The elucidation of the roles of ABA in freezing, salt, and water stress led to a characterization of ABA as a stress hormone. Indeed, more than half of transcriptional changes are common to ABA, drought, and salinity (Shinozaki et al. 2003). Several lines of evidence suggest a signaling role for ABA in the Al-stress response. For example, exogenous ABA enhanced Al-induced citrate secretion from soybean roots (Shen et al. 2004). Furthermore, as an ABA-dependent increase in citrate secretion was sensitive to a protein kinase inhibitor, K-252a, it would appear that ABA is involved in the early response to Al stress. In buckwheat, absence of Al, ABA triggered the secretion of oxalate from the roots (Ma et al. 2001), implying that ABA mimics Al signaling by activating anion channels that are permeable to oxalate. Recently, it was reported that both Al and ABA induce the expression of buckwheat \textit{FeALS3} (Reyna-Llorens et al. 2015), a homolog of the \textit{Arabidopsis} \textit{AtALS3} and is implicated in Al tolerance by facilitating Al compartmentation away from sensitive sites. While the role of ABA in the plant Al-stress response has been well characterized, the relationship between Al stress and ABA signal transduction pathways remains largely unknown.

Rice bean is an Al-tolerant leguminous species that is well adapted to acidic soils. One of its Al-tolerance mechanisms relies on Al-activated citrate secretion from the root tip, which is mediated by the expression of \textit{VuMATE1}; a member of the multidrug and toxic compound extrusion family (Yang et al. 2011; Liu et al. 2013). Expression of the functional homologous genes, \textit{AtMATE} in \textit{Arabidopsis} and \textit{OsFRDL4} in rice (\textit{O.}
are regulated by Cys2His2-type zinc finger transcription factors (TFs), STOP1 and ART1, respectively (Liu et al. 2009; Yokosho et al. 2011).

In rice, ART1 regulates *OsFRDL4* expression by directly binding to the GGNVS motif in the promoter region (Tsutsui et al. 2012). Interestingly, a yeast one-hybrid analysis revealed that rice bean VuSTOP1, a homolog of ART1, binds to an unidentified, but not ART1 *cis*-acting element, of the *VuMATE1* promoter, suggesting that the situation is more intricate with regards to the regulation of *VuMATE1* expression in rice bean (Fan et al. 2015). Recently, it was reported that phytohormones, such as ABA and IAA could induce *AtALMT1* expression, thereby relating Al signaling to phytohormone-mediated signal transduction pathways (Kobayashi et al. 2013). Interestingly, an *in silico* analysis also identified an ABA-responsive element in the promoter region of *VuMATE1* (Liu et al. 2016). The potential involvement of ABA, in regulating *VuMATE1* expression is, thus, of great interest.

Identification of early Al-responsive genes, in the root tip of rice bean, led to the discovery of a set of genes associated with water and ion transport, suggesting that Al stress may be physiologically relevant to osmotic stress (Fan et al. 2014). This led us to speculate as to whether ABA is a mediator of Al stress signaling, as ABA plays a pivotal role in osmotic regulation. In this study, we performed a global transcriptome analysis of the rice bean root tip in response to treatment with either Al or ABA. We report that nearly one-third of the differentially expressed genes (DEGs) were common to ABA and Al, demonstrating the potential for cross-talk between Al and ABA signal transduction pathways. Furthermore, our studies established that exogenous ABA application resulted in a remarkable alleviation of Al-induced root elongation inhibition, suggesting that ABA is positively involved in Al resistance. We also determined that a previous well-documented external Al-exclusion mechanism, relying on *VuMATE1*-mediated citrate secretion, and other known Al-resistance genes, were not related to ABA-mediated Al resistance. Our results support the hypothesis that ABA signal transduction pathways provide an additional layer of regulatory control over Al resistance in rice bean.

**RESULTS**

**Al-induced rapid accumulation of ABA in rice bean**

To examine whether ABA is involved in the Al-stress response, in rice bean, we
monitored endogenous ABA content in the root tip under Al stress. The endogenous ABA content increased with the increase of external Al concentration, ranging from 5 to 25 µM (Figure 1A). However, further increase in Al concentration did not increase the ABA content (Figure 1A). Interestingly, endogenous ABA content increased rapidly within 3 h of exposure to 25 µM Al, and remained relatively constant thereafter during the entire treatment period (Figure 1B).

It was previously reported that ABA is rapidly transported between roots and shoots, and that Al stress accelerates this process (Hou et al. 2010). ABA is very effective in inducing stomatal closure (Li et al. 2000), thereby decreasing stomatal conductance. To further assess the involvement of ABA in the Al stress response, we next examined changes in leaf stomatal conductance under Al stress. In agreement with the rapid increase in endogenous ABA content in root apices, leaf stomatal conductance was rapidly decreased after 3 h of exposure to 25 µM Al, and became relatively stable thereafter (Figure S1).

**ABA is positively involved in rice bean Al tolerance**

To assess the relationship between Al stress and the changes in endogenous ABA, a bioassay experiment was performed to ascertain whether elevated ABA content is related to Al tolerance or Al toxicity in rice bean. At a concentration of 5 µM, ABA displayed the best ameliorative effects on Al-induced root growth inhibition in rice bean (Figure S2). Thus, an ABA concentration of 5 µM was used to establish a time-course effect on root growth. As shown in Figure 1C, Al stress rapidly inhibited root elongation, by some 60% after 3 h of exposure, and this situation remained relatively constant during the remainder of the treatment. By contrast, addition of 5 µM ABA to the nutrient solution, in the presence of 25 µM Al, resulted in a full restoration of root elongation. These results indicated that elevated endogenous ABA content is associated with Al-tolerance mechanisms, rather than Al toxicity in rice bean.

Specificity of the ameliorative effect of ABA, on rice bean Al tolerance, was further assessed by testing the effect of ABA on root elongation inhibition induced by other metals. Both 10 µM cadmium (Cd) and 15 µM lanthanum (La) could significantly inhibit rice bean root elongation. However, ABA failed to restore both the Cd- and La-induced root elongation inhibition (Figure S3). This result suggested that the ameliorative effect of ABA on Al toxicity is a specific response.
VuMATE1-dependent citrate secretion is not responsible for ABA-mediated Al tolerance

The ameliorative effects of exogenous ABA on Al toxicity in soybean have previously been proposed to relate to a stimulation of citrate efflux from the root apices (Shen et al. 2004). To evaluate whether a similar mechanism operates in rice bean, we investigated the effects of exogenous ABA on Al-induced root apices citrate efflux. In the absence of Al, citrate was almost undetectable in the treatment solution bathing the root apices. Al stress resulted in citrate secretion, in a time-dependent manner. However, the amount of secreted citrate was significantly lower in the presence of ABA than an Al treatment alone, during the entire duration of the experiment (Figure 2A).

We next assessed VuMATE1 expression, as the encoded protein is responsible for Al-induced citrate secretion in rice bean (Yang et al. 2011; Liu et al. 2013). As expression of VuMATE1 was maximal, and the ameliorative effect of ABA on Al toxicity had emerged at 6 h of Al exposure (Liu et al. 2013; Figure 1C), a 6 h treatment was selected for the following experiments. In the absence of Al, expression of VuMATE1 was not detected, by quantitative real-time (qRT)-PCR analysis. ABA, itself, slightly increased VuMATE1 expression in the absence of Al. By contrast, Al treatment significantly increased VuMATE1 expression, but addition of ABA caused a significant decrease in the Al-induced expression of VuMATE1 (Figure 2B). These results clearly suggested that the ameliorative effects of exogenous ABA, on Al-induced root growth inhibition, were not related to VuMATE1-dependent citrate efflux.

ABA-mediated Al tolerance is not related to Al exclusion from the root tip

To assess whether external exclusion, or internal tolerance mechanisms are involved in ABA-dependent Al tolerance, we next analyzed both the cell wall and cell sap Al content in the root apices of rice bean. We detected low levels of Al in both the cell wall and cell sap, in the absence of Al, irrespective of treatment with or without ABA. Al treatments resulted in an approximately 6-fold increase in the root tip Al content, either in the apoplast or in the symplast (Figure 2C, D). Approximately 15% of the total accumulated Al was present in the cell sap, suggesting that apoplastic and symplastic Al toxicity might co-exist. However, in the presence of Al, there were no
significant differences in both cell wall and cell sap Al content, irrespective of ABA treatment (Figure 2C, D). These findings support an Al tolerance, rather than an Al exclusion mechanism that is involved in ABA-mediated Al resistance. Given that VuMATE1-mediated citrate efflux contributes considerably to the exclusion of Al, this result also suggested that a decrease in Al-induced citrate efflux, by ABA, could be a consequence of an increase in Al tolerance, mediated by ABA.

**De novo transcript assembly and annotation**

Based on the rapid (within 3 h) accumulation of ABA in the root tip under Al stress, we speculated as to whether ABA might act as a signaling agent that interacts with the Al stress signal. To this end, a genome-wide transcriptome analysis, based on RNA-sequencing (RNA-Seq), was performed to compare the DEGs in the root tip of rice bean, in response to a 6 h treatment with either Al (25 µM) or ABA (5 µM). Following adaptor trimming, we obtained 40.4 to 45.1 million reads, depending on the treatment (NCBI accession number: SRR3999442). Using Trinity software, these reads were then assembled into contigs, ranging from 73,915 to 79,534 bp, with a mean length of ~ 495 bp. After filling the gaps, using paired-end reads, these contigs were further assembled into a total of 59,589 unigenes (Table S1). The size distribution of the unigenes is shown in Figure S4. For annotation, unigene sequences were searched using BLASTx against the NCBI non-redundant protein (Nr) database and the Swiss-Prot protein database, with a cut-off E-value of $10^{-5}$. As a result, 48,751 unigenes had good comparability to known gene sequences (Table S1).

**ABA does not regulate known Al-resistance gene homologs**

The expression levels of transcripts from the RNA-Seq data were determined using the method of reads per kilobase of exon per million mapped reads (RPKM) (Mortazavi et al. 2008). To identify genes displaying significant transcriptional changes, in response to the treatments, DEGs were evaluated using an absolute value of log$_2$FC (fold change) ≥1 and a FDR (false discovery rate) ≤0.001. On this basis, a total of 2,801 (2,209 up-regulated and 592 down-regulated), and 3,257 (2,482 up-regulated and 775 down-regulated) DEGs were detected in the Al- and ABA-treated roots, respectively (Table S2; Figure 3).

To confirm our RNA-Seq data, we randomly selected 18 genes and tested their expression in response to either Al or ABA treatment, by qRT-PCR analysis (Table
S3). A significant correlation ($r^2=0.822$) was obtained between these two datasets, although the overall expression level was slightly higher in the qRT-PCR analysis than that calculated from the RNA-Seq data (Figure S5). Thus, the present result indicated that our RNA-Seq data were deemed reliable.

A number of genes responsible for Al resistance have been identified and characterized in rice and Arabidopsis (Delhaize et al. 2012). To explore the molecular basis by which ABA enhances Al resistance, in rice bean, we next surveyed the Al resistance gene homologs from our RNA-Seq dataset. Here, three rice bean homologs of STOP1/ART1 were identified, all of which were up-regulated by Al stress, but not affected by ABA (Figure 3). Expression of Unigene13261_All was much higher than the other two genes, either in the absence or presence of Al. Sequence alignment and BLAST searches revealed that Unigene13261_All was the same as VuSTOP1, a gene we previously identified as an Al tolerance gene (Fan et al. 2015).

We identified three STAR1 and two ALS3/STAR2 homologs, in the rice bean root tips, which were up-regulated by Al stress, but only the expression levels of CL3060.Contig2_All (a STAR1 homolog) and CL5267.Contig1_All (a ALS3/STAR2 homolog) were highly elevated. Although ABA could induce expression of CL3060.Contig2_All and CL3060.Contig3_All, the levels were lower than that induced by Al.

Treatment with Al greatly up-regulated three genes encoding STEROL 4-ALPHA-METHYL OXIDASE 1, which is a key enzyme that catalyzes sterol biosynthesis (Darnet and Rahier 2004), but again, these were only weakly induced by ABA. Recent evidence indicates that sterol biosynthesis is important for avoiding the binding of Al ions to phospholipids in the plasma membrane, thereby increasing Al tolerance (Wagatsuma et al. 2015). A total of six MATE efflux transporters was identified, and with the exception of Ungene8017_All, these homologs were all up-regulated by Al stress. However, only the expression level of CL3862.Contig1_All was substantially increased following Al stress.

Sequence alignment established that CL3862.Contig1_All and Unigene20395_All are VuMATE1 and VuMATE2, respectively, two previously identified Al tolerance genes responsible for Al-stimulated citrate efflux (Liu et al. 2013; 2018). In line with our previous work, ABA could slightly induce VuMATE1 expression, but both the intensity and localization of the expression differed from that induced by Al (Liu et al. 2016). These results suggested that ABA was not the major mediator responsible for
the expressional-induction of these Al-resistance genes, under Al stress.

**Overlap of the Al- and ABA-responsive genes**

Nearly one-third (773 and 207 up- and down-regulated, respectively) of the DEGs that responded to Al stress also responded to ABA, indicating an overlap in Al and ABA signaling (Figure 3). To further assess the genetic function of these co-upregulated genes, we first excluded those having RPKM values lower than 10, after treatment; the remainder were interrogated, using a BLASTx search in the *Arabidopsis* Information Resource (TAIR) database (http://www.arabidopsis.org/Blast/index.jsp), for biological process annotation. To this end, a total of 205 genes were considered to be co-expressed with relatively high transcription under Al and ABA treatment (Table S4).

Out of the 131 genes having assigned biological processes, some were predicted to be directly involved in the ABA response, and a substantial proportion was associated with ‘water relation’ and ‘cell wall structure and modification’ processes (Table S4). These findings reinforced the relationship between Al and ABA signaling, and suggested an involvement of water relations in Al stress.

With respect to ABA-dependent gene transcription, ABI3, ABI4, and ABI5 constituted the topmost TFs in the ABA signal transduction pathways, followed by several other TFs, including members of the bZIP, MYB, and MYC families. To further clarify whether ABA-dependent signal transduction pathways are involved in Al-stress response in rice bean, we searched and compared the corresponding TFs in our RNA-Seq data. A total of 33 and 41 genes encoding these TF proteins were identified as differentially regulated by Al and ABA, respectively (Table S5). Among them, 12 were shown to be both Al- and ABA-responsive. Furthermore, they were all up-regulated, with the exception of a gene (CL4652.Contig3_All) that was down-regulated by ABA treatment. Although the function of these TFs, with respect to Al tolerance, has yet to be investigated, the present analysis provided additional evidence for cross-talk between the Al and ABA signaling pathways.

**ABI5 involvement in Al tolerance is independent of STOP1**

To further examine the mode by which ABA might alleviate Al toxicity, we used an *Arabidopsis abi5* mutant carrying a G-to-A point mutation in the fifth nucleotide before the first putative ATG (Ren et al. 2010). This mutant was also previously
confirmed to be insensitive to exogenous ABA (Ren et al. 2010; Lei et al. 2014). In the standard growth medium, root growth did not differ significantly between the wild-type (WT) and abi5 mutant plants. However, the abi5 mutant displayed severe primary root growth arrest in comparison to the WT, in response to Al stress (Figure 5A). Whereas relative root elongation of the primary root was inhibited by nearly 20% in the WT plants, that of the abi5 mutant was inhibited by 50%, in response to Al stress (Figure 5B). This result indicated that ABA signal transduction pathways are positively involved in Al tolerance.

As known Al-tolerance genes are not involved in ABA-mediated Al tolerance, in rice bean, we next investigated whether the same applies in Arabidopsis. Here, we compared the gene expression of AtALMT1 and AtMATE in the abi5 and stop1 mutant plants with that in the WT plants. While expression of both genes was induced by Al stress, in the WT plants, no significant differences were observed between the WT and abi5 mutant, either in the absence or presence of Al (Figure 5C, D). By contrast, expression of both AtALMT1 and AtMATE was dramatically repressed in the stop1 mutant, in the presence of Al stress. Furthermore, expression of AtALMT1 was dramatically repressed even in the absence of Al stress, although the expression of AtMATE was not affected (Figure 5C, D).

We next selected 55 Arabidopsis genes, homologous to those in rice bean, in which each gene was regulated by both Al and ABA, and examined the expression patterns in either the WT or abi5 mutant, in response to Al stress (Table S6; Figure 6). Among these, 47 genes (85%) displayed similar expression patterns between Arabidopsis WT plants and rice bean with respect to Al stress. Notably, 23 genes (46%) displayed decreased expression levels in the abi5 mutant in comparison with the WT, under Al stress, with At5g66390 (Peroxidase 72), At3g28210 (Stress-associated protein12), At4g25810 (XTH23), and At3g09270 (GLUTATHIONE S-TRANSFERASE TAU 8) exhibiting the greatest differences.

It was recently reported that Stress-associated protein12 (At3g28210) is involved in osmotic stress (Ma et al. 2015), and Peroxidase 72 (At5g66390) and XTH23 (At4g25810) are involved in cell wall structure and modification (Herrero et al. 2013). Thus, ABI5 may regulate Al tolerance through cell wall modification and osmotic regulation.

Accumulating evidence suggests that cell wall modification is actually interrelated with osmotic regulation, and both are implicated in Al tolerance (Yang et al. 2013).
For example, polyethylene glycol (PEG) 6000-induced osmotic stress alleviated Al-induced root growth inhibition, in common bean, which was attributed to cell wall assembly and modification (Yang et al. 2011). Thus, we next examined the role of PEG 6000-induced osmotic stress in Al tolerance. Here, we ascertained that Al treatment strongly inhibited root elongation, in rice bean, in the absence of PEG 6000 (Figure S6). However, the addition of PEG 6000 significantly alleviated Al-induced root elongation inhibition, although PEG 6000 inhibited root elongation in the absence of Al (Figure S6).

DISCUSSION
ABA is a plant hormone involved not only in fundamental physiological processes, but also in many stress responses in higher plants. Furthermore, ABA has been shown to be involved in Al tolerance mechanisms via, for example, the regulation of Al-stimulated citrate and malate efflux from soybean and Arabidopsis roots, respectively (Shen et al. 2004; Kobayashi et al. 2013). In the present study, we demonstrate that ABA was positively involved in the Al tolerance mechanism in rice bean (Figure 1C; Figure S2), and further, that Al stress could rapidly increase internal ABA content (Figure 1A, B; Figure S1). However, the improvement in Al tolerance by ABA, in rice bean, was not associated with Al-stimulated citrate secretion. This conclusion is based on experimental evidence that Al-stimulated citrate efflux, from rice bean root tips, was not enhanced by ABA application. Rather, the level of Al-stimulated citrate secretion was attenuated by ABA (Figure 2A).

A similar situation was reported for wheat where exogenous ABA application had no effect on Al-induced malate secretion from the roots (Ryan et al. 2003). We have also demonstrated that expression of VuMATE1 plays an important role in Al-induced citrate secretion in rice bean (Yang et al. 2011; Liu et al. 2013). In accordance with the attenuated secretion of citrate, the expression of VuMATE1 was also decreased by ABA, compared with Al treatment alone (Figure 2B). In addition, the extra protection against Al stress, afforded by exogenously applied ABA, was not related to Al exclusion (Figure 2C, D). This is in contrast to an increase in Al-induced citrate secretion, by exogenous ABA application, that was accompanied by a decrease in Al accumulation in the root tips of soybean (Shen et al. 2004). Taken together, these findings suggest that the underlying basis of ABA-mediated Al tolerance cannot simply be attributed to the regulation of Al exclusion that relies on Al-stimulated
organic acid anions efflux.

We here present a large-scale transcriptomic analysis unraveling the underlying basis of ABA-mediated Al tolerance. A substantial overlap was observed between Al-responsive and ABA-responsive genes, suggestive of the existence of cross-talk between the Al and ABA signaling pathways (Figure 3). Based on our transcriptome analysis, Al stress-regulated genes could be classified into two major groups. One was involved in ABA-dependent gene expression. At least three types of TFs are known to be involved in ABA-dependent gene expression. The bZIP TF family binds to an ABRE element to activate ABA-dependent gene expression (Uno et al. 2000). A MYC TF, AtMYC2, and a MYB TF, AtMYB2, cooperatively bind cis-elements in the promoter of RD22, a drought-inducible gene, to activate RD22 expression (Abe et al. 1997).

We identified 33 and 41 DEG, encoding these types of TFs, that were regulated by Al and ABA, respectively, and 11 of these TFs were shown to be up-regulated by either Al or ABA treatment (Table S5). These results indicate that the ABA signal transduction pathways are involved, at least in part, in the Al stress signal. The increased Al sensitivity, in the Arabidopsis abi5 mutant, offered further support for this hypothesis (Figure 5).

The TFs in the other group was implicated in ABA-independent gene expression. We previously characterized the function of VuMATE1 and VuSTOP1 with respect to Al tolerance in rice bean (Liu et al. 2013; Fan et al. 2015). Apparently, the expression of both genes is not regulated by ABA (Figure 2B; Figure 4). Analysis of potential Al tolerance genes, such as VuSTAR1 and VuALS3, revealed that their expression is also independent of ABA signaling. Consistently, the expression of AtALMT1 and AtMATE was not affected in the Arabidopsis abi5 mutant line (Figure 5C, D). Thus, our findings are consistent with the notion that ABA signal transduction pathways are involved in an Al tolerance mechanism in plants.

Concerning the TFs involved in the induction of Al-tolerant gene expression, AtSTOP1 in Arabidopsis and OsART1 in rice are two homologs of the C2H2-type zinc-finger TF family. OsART1 regulates at least 31 downstream genes, some of which have been demonstrated to be involved, at different cellular levels, in Al tolerance (Yamaji et al. 2009). However, these downstream genes differ from those regulated by AtSTOP1 (Sawaki et al. 2009). For example, evidence is lacking that OsART1-regulated genes are involved in proton tolerance, whereas AtSTOP1 is
clearly associated with proton tolerance. In addition, whereas induction of both OsSTAR1 and OsSTAR2 expression requires OsART1 (Huang et al. 2009), AtSTAR1 expression is not regulated by AtSTOP1 (Sawaki et al. 2009).

Recently, in Arabidopsis, the AtWRKY46 and CALMODULIN BINDING TRANSCRIPTION ACTIVATOR2 TFs were shown to be negatively and positively involved, respectively, in the regulation of AtALMT1 expression (Ding et al. 2013; Tokizawa et al. 2015). It has also been shown that the Al-responsive expression of OsSTAR1, in rice, requires not only OsART1, but also the Abscisic acid, Stress, and Ripening 5 TFs (Arenhart et al., 2014).

Our findings established that expression of rice bean ABI5 homologous transcripts was induced by Al stress (Table S5). Here, induction of ABI5 expression may have resulted from an increase in ABA level in response to Al stress (Figure 1A, B). As ABA signal transduction pathways, in both rice bean and Arabidopsis, are not associated with STOP1/ART1-regulated Al tolerance gene expression (Figure 4; Figure 6), ABI5 thus appears to represent a novel TF that is positively involved in Al tolerance.

Our studies also suggest that ABA-mediated Al tolerance may relate to an adjustment of osmotic stress and cell wall modification. Strong support for this notion is provided by our finding that a substantial number of genes, co-regulated by Al and ABA, were functionally-assigned to water relation and cell wall structure and modification (Figure S3). Furthermore, simulating osmotic pressure with PEG 6000 resulted in an increase in Al tolerance in rice bean (Figure S6), providing circumstantial evidence that ABA-mediated Al tolerance is associated with osmotic adjustment. Similar observations were previously reported in common bean (Yang et al. 2011).

The loss-of-function mutation of ABI5 resulted in a down-regulation of a subset of genes involved in osmotic adjustment and cell wall modification (Figure 6), which is in accordance with an increased sensitivity to Al toxicity in the abi5 mutant plants (Figure 5). Interestingly, ABA was shown to be positively involved in Al tolerance in buckwheat, where it regulated FeALS3 expression (Reyna-Llorens et al. 2015). STAR2/ALS3 plays a positive role in Al tolerance by interacting with STAR1 to modify cell wall properties (Huang et al. 2009), reinforcing our notion that ABA involvement in Al tolerance relates to the regulation of water relations and cell walls.

Earlier we characterized an AtSTOP1 homolog from rice bean, VuSTOP1, which
was shown to be more relevant to proton than Al tolerance (Fan et al. 2015). An in planta complementation assay indicated that VuSTOP1 plays a minor role in the regulation of VuMATE1 expression, in response to Al stress (Fan et al. 2015). B-glucuronidase (GUS) activity assays, carried out in transgenic Arabidopsis lines carrying a deleted VuMATE1 promoter further demonstrated that cis elements involved in the Al-inducible and root apex-specific expression of VuMATE1 are situated between -820 and -555 bp of the VuMATE1 promoter (Liu et al. 2016). Interestingly, whereas VuSTOP1 did not interact with this promoter region, an ART1 cis-acting element was present (Liu et al. 2016).

In our study, at least three STOP1/ART1 homologs were induced by Al stress in rice bean (Figure 4). Recent research has shown that, in Arabidopsis, AtSTOP2 partners with AtSTOP1 in regulating expression of some Al- and proton-tolerance genes (Kobayashi et al. 2014). Thus, it would be interesting to explore whether other STOP1-like genes are involved in the regulation of VuMATE1 expression, and whether there is functional redundancy in terms of Al and proton tolerance among the different members of this C2H2-type zinc-finger TF family.

In conclusion, we here demonstrate that ABA, as a signaling agent, is positively involved in Al tolerance mechanisms, in rice bean, and is independent of known Al tolerance mechanisms, including Al-mediated organic acid anions efflux. In addition, we provided the first genome-wide transcriptome analysis of ABA-mediated Al tolerance mechanisms, and report that Al triggers ABA signal transduction pathways, independent from STOP1/ART1-mediated Al tolerance mechanisms. Furthermore, we identified ABI5 as a transcriptional regulator that mediates Al tolerance by regulating genes involved in osmotic adjustment and cell wall structure and modification.

MATERIALS AND METHODS

Plant materials and growth conditions
Seeds of rice bean (Vigna umbellata) were soaked in deionized water overnight, and germinated at 26°C in the dark. After germination, the seeds were transferred to a net tray floating on 5 l of 0.5mM CaCl$_2$ solution (pH 4.5); this solution was renewed daily. On day 3, seedlings were transplanted into a 1.2 l plastic pot (8 seedlings per pot) containing a low ionic strength nutrient solution with the following composition (µM): CaSO$_4$ (200), CaCl$_2$ (200), MgSO$_4$ (100), KNO$_3$ (400), NH$_4$NO$_3$ (300), NaH$_2$PO$_4$ (5), H$_3$BO$_3$ (3), MnCl$_2$ (0.5), ZnSO$_4$ (0.4), CuSO$_4$ (0.2), Fe-EDTA (10), and
(NH₄)₆Mo₇O₂₄(1). Plants were first acclimatized for one day in an environmentally-controlled growth room (14 h/26°C day and a 10 h/22°C night regime, irradiance of 300 μmol photons m⁻²s⁻¹ and 65% RH), prior to experimental treatments.

ABA measurement
Three-day-old seedlings were transferred to low ionic strength nutrient solution containing 0, 5, 10, 25, or 50 μM Al for 12 h, or were treated with 25 μM Al for 3, 6, 12, or 24 h. After treatments, root apices (apical 0-1 cm) were cut with a sharp razor, weighted, and immediately frozen in liquid nitrogen, then stored at -80°C until extraction. The extraction procedure was as previously described (Hou et al. 2010). ABA was determined using an immunoassay detection kit, according to the manufacturer’s instructions (Sigma), incorporating an automatic enzyme-linked immunoassay system (Bio-Tek Elx800).

Root growth assay
Three-day-old seedlings of rice bean were transferred to low ionic strength nutrient solution, at pH 4.5, containing 0 or 25 μM AlCl₃, either in the presence or absence of 5 μM ABA, for 3, 6, 12, or 24 h. The primary root length was measured before and after the treatment. Relative root elongation was used as an Al tolerance index, by calculating the percentage the root elongated during the treatment (Al, ABA, or Al+ABA) to that in the control (-Al) conditions. For Arabidopsis, germinated seedlings having roots of 1 cm length were exposed to 1/5-strength Hoagland nutrient solution, at pH 5.0, containing 0 (control), or 100 μM Al for 7 d. Root elongation was measured before and after treatment.

Measurement of stomatal conductance
Seedlings were cultured in nutrient solution for one week, at which the true leaves were fully expanded, then subjected to nutrient solution, with or without 25 μM AlCl₃ for different times. Stomatal conductance was measured with a portable photosynthesis system (Licor-6400; LICOR Inc. Lincoln NE, USA), equipped with an LED red blue light source. All measurements were carried out at a photon flux density of 1200 μmol m⁻² s⁻¹, a leaf temperature of 25°C and CO₂ of 390 ± 5 μmol L⁻¹ in the sample chamber.
Measurement of cell wall and cell sap Al

After 6 h of 25 µM Al treatment, seedling roots were briefly washed with deionized water to decontaminate surface Al. Excised root apices (0-1 cm, 30 tips for each sample) were put in Ultra free-MC centrifugal filter units (Millipore) and centrifuged at 3,000 × g for 10 min at 4°C to remove apoplastic solution. After freeze/thawing, three times, the root cell sap solution was obtained by centrifuging at 20,600 × g for 10 min. Residual cell walls were washed with 70% ethanol, three times, and immersed in 0.5 mL of 2 N HCl for at least 24 h. The Al in the symplastic solution and cell wall extracts was determined by inductively-coupled plasma atomic emission spectrometry (Thermo Jarrel Ash, San Jose, CA).

Citrate determination

Three-day-old seedlings were subjected to nutrient solution containing 0 or 25 µM Al, with or without 5 µM ABA, for 3, 6, 9, or 12 h. After treatment, root exudates were collected and citrate levels determined, as previously described (Liu et al. 2013).

RNA isolation and solexa sequencing

Total RNA was extracted from frozen root apices, treated by either 25 µM Al or 5 µM ABA for 6 h, using an RNeasy Mini Kit (Qiagen) and digested with RNase-free DNAase I (Qiagen). A cDNA library was constructed following the Illumina manufacturer’s instructions. In brief, polyA+ RNA was purified from total RNA, using Oligo(dT) magnetic beads, and broken into short fragments using divalent cations at 94°C for 5 min. Using these short fragments as templates, random hexamer-primer was used to synthesize the first-strand cDNA, followed by the synthesis of second-strand cDNA using DNA polymerase I and RNaseH. Short fragments were purified with a QiaQuick PCR Extraction Kit (Qiagen) and ligated to sequencing adapters. The products were amplified by PCR to create a cDNA library. The cDNA library was sequenced using Illumina HiseqTM 2000 system.

Sequence assembly and annotation

The sequencing-received raw image data were transformed by base calling into raw reads. Reads were assembled using Trinity software (Grabherr et al. 2011). The longest assembled sequences were referred to as contigs. Reads were then mapped
back to contigs with paired-end reads to detect contigs from the same transcript and the distances between these contigs. N was used to connect each two contigs to represent unknown sequences, and then for Scaffold. Finally, sequences were obtained that lacked N and could not be extended on either end, and were defined as unigenes. These unigene sequences were aligned by BLASTx to protein databases, including the NCBI and the Swiss-Prot database (E-value ≤ 10^5).

**Differential gene expression analysis**
Gene expression levels were calculated according to the Reads Per kb Million reads (PRKM) method (Mortazavi et al. 2008). To identify DEGs by different treatments, the false discovery rate (FDR) value less than 0.01 and Log2 (fold change) ≥1 were used as the threshold to judge the significance of gene expression difference.

**Quantitative RT-PCR Analysis**
Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen), and cDNA was then synthesized from ~1 µg of total RNA, using SuperScript™ reverse transcriptase (Takara, Dalian, China). After appropriate dilution of cDNA samples, 1 µL of cDNA (100 ng µL^⁻¹) was used, as a template, for the qRT-PCR in a total volume of 10 µL. Primers for qRT-PCR analysis are listed in Table S2 and S5. The primers used for VuMATE1 expression analysis were as used previously (Liu et al. 2013). PCR amplification conditions were as follows: 94°C for 5 min; 45 cycles of 94°C for 10 s, 55°C for 15 s and 72°C for 20 s. For each candidate gene, the PCR reactions were carried out in triplicate, and expression data were normalized with expression level of 18S rRNA.

**Statistical Analysis**
Data shown are the mean ± standard deviation of three independent experiments. Mean differences were compared using the statistical software data processing system (SPSS 17.0), followed by the Student’s t test and the differences between group means were considered significant at P < 0.05.

**ACKNOWLEDGEMENTS**
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AUTHOR CONTRIBUTIONS
J.L.Y and S.J.Z conceived the research plans and designed the experiments; W.F., J.M.X., P.W. and H.Q.L performed most of the experiments; W.W.C provided technical assistance to W.F; W.F and J.M.X analyzed the data; J.L.Y. wrote the manuscript.

REFERENCES
Cell Environ 37: 1586-1597


Ma JF, Zhang W, Zhao Z (2001) Regulatory mechanisms of Al-induced secretion of


root apex. **Plant Cell Environ** 34: 2138-2148


**Plant Soil** 346: 215-230


Yokosho K, Yamaji N, Ma JF (2011) An Al-inducible MATE gene is involved in external detoxification of Al in rice. **Plant J** 68: 1061-1069


**FIGURE LEGENDS**

**Figure 1. Influence of Al treatment on endogenous ABA content in the rice bean root apex and time-course effects of exogenous ABA on Al-induced root elongation**

(A) Rice bean seedlings (3-d-old) exposed for 12 h to nutrient solution containing different Al concentrations. (B) Rice bean seedlings (3-day-old) exposed to nutrient...
solution containing 0 or 25 µM Al for different times. (C) Rice bean seedlings (3-d-old) exposed to nutrient solution containing 0 or 25 µM Al, with or without 5 µM ABA, for different times. Root elongation was measured before and after treatment. Data are mean ± SD (n=3). Asterisks indicate significant differences between treatments.

Figure 2. Effect of ABA on citrate secretion, *VuMATE1* expression, cell wall Al content and cell sap Al content

(A) Rice bean seedlings (3-d-old) exposed to nutrient solution containing 0 or 25 µM Al, with or without 5 µM ABA, for the indicated times. Root exudates were analyzed, enzymatically for citrate. (B) RNA extracted from root tips of 3-d-old rice bean seedlings exposed to nutrient solution containing 0 or 25 µM Al, with or without 5 µM ABA for 6 h, and root tips (0-1 cm) was analyzed for *VuMATE1* expression. (C, D) Root tips of 3-d-old rice bean seedling exposed to nutrient solution containing 0 or 25 µM Al, with or without 5 µM ABA for 6 h, were used to extract cell wall and cell sap. Data are mean ± SD (n=3). n.s. represents no statistically significant difference between treatments.

Figure 3 Number of genes up-regulated (>2-fold) and down-regulated (<0.5-fold) by Al and ABA in rice bean root tips

Figure 4. Expression profiles of Al tolerance gene homologs determined by RNA-seq analysis

Gene expression levels are represented by the reads per kilobase Million reads (PRKM) method.

Figure 5. Comparative Al tolerance and gene expression analyses between wild-type and *abi5* mutant plants

(A) Al tolerance phenotype of wild-type (WT) and *abi5* mutant seedlings. Seven-d-old seedlings with 1-cm root length were transferred to agar plates containing 1/5 Hoagland nutrient solution (10 µM P) with 0 (-Al) or 100 µM Al (+Al) for 7d. Bar, 1 cm. (B) Relative root elongation of WT and *abi5* mutant plants. Root elongation was measured before and after treatment. Data are mean ± SD (n=3). (C, D) Relative expression of *AtALMT1* (C) and *AtMATE* (D) in 3-week-old *Arabidopsis* seedlings of
WT, *abi5* and *stop1* mutants, grown hydroponically, subjected to 1/5 Hoagland nutrient solution (10 µM P) with 0 or 25 µM Al for 24 h. Data are mean ± SD (n=3). Asterisks indicate statistically significant difference between WT and mutants.

**Figure 6. Heatmap (RNA-Seq data) for selected rice bean and *Arabidopsis* gene sets exhibiting Al and ABA co-expression patterns in response to Al stress**

Genes showed different expression patterns between rice bean and *Arabidopsis* in response to Al are marked by an x, and genes with expression levels repressed in *abi5* in comparison with WT are marked by an *.

**SUPPORTING INFORMATION**

**Figure S1.** Effect of Al stress on stomatal conductance of fully expanded true leaves of rice bean

**Figure S2.** Effect of different exogenous ABA concentrations on Al-induced root growth inhibition in rice bean

**Figure S3.** Effect of exogenous ABA on rice bean root elongation inhibition induced by other metals

**Figure S4.** Distribution of unigene lengths

**Figure S5.** qRT-PCR validation of RNA-Seq expression data

**Figure S6.** Effect of PEG 6000 on Al-induced root elongation inhibition in rice bean

**Table S1.** Summary for transcriptome of rice bean root apex under different treatments

**Table S2.** DEGs in rice bean root tips in response to Al and ABA

**Table S3.** List of genes and primer pairs used for qRT-PCR analysis

**Table S4.** Extraction of genes whose expression was co-regulated by Al and ABA

**Table S5.** Transcription factors putatively involved in ABA-dependent gene expression

**Table S6.** List of genes and primer pairs used for qRT-PCR analysis in *Arabidopsis*. 
Figure 1

(A) ABA content (pg g⁻¹ FW) at different Al concentrations (μM).

(B) ABA content (pg g⁻¹ FW) over time with and without Al treatment.

(C) Root elongation (cm) over time with different treatments: -Al, +ABA, +Al, and +Al+ABA.
Figure 2
Figure 3

Up-regulated genes

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Down-regulated genes

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