An exclusion mechanism is epistatic to an internal detoxification mechanism in aluminum resistance in Arabidopsis

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Abstract

Background: In *Arabidopsis*, the aluminum (Al) exclusion mechanism is mainly facilitated by ALMT1-mediated malate exudation and MATE-mediated citrate releases from the root. Recently, we have demonstrated that coordinated functioning between the ALMT1-mediated exclusion mechanism, via exudation of malate from the root tip, and a NIP1;2-facilitated internal detoxification mechanism, via removal of Al from the root cell wall and subsequent root-to-shoot Al translocation, plays critical roles in achieving overall Al resistance. However, the genetic relationship between *ALMT1* and *NIP1;2* in these processes remained unclear.

Results: Through genetic and physiological analyses, we demonstrate that unlike *ALMT1* and *MATE*, which function independently and additively, *ALMT1* and *NIP1;2* show an epistatic relationship in Al resistance in Arabidopsis. These results indicate that *ALMT1* and *NIP1;2* function in the same biochemical pathway, whereas *ALMT1* and *MATE* in different biochemical pathways.

Conclusion: The establishment of the epistatic relationship and the coordinated functioning between the ALMT1 and NIP1;2-mediated exclusion and internal detoxification mechanisms are pivotal for achieving overall Al resistance in the non-accumulating Arabidopsis plant. We discuss and emphasize the indispensable roles of the root cell wall for the implementation of the Al exclusion mechanism and for the establishment of the epistatic relationship between the ALMT1-mediated exclusion mechanism and the NIP1;2-facilitated internal detoxification mechanism.

Background

Aluminum (Al) is the most abundant metal element in the earth crust [1]. Under neutral or alkalescent conditions, Al is present in the soil as forms that are non-toxic to plants [2]. However, at low pH (<5.0), aluminum ions (Al^{3+}) are dissolved and released from the soil clays into the soil solutions, which are highly toxic to root growth, development and function, causing severely impaired water and nutrient uptakes by the root and, as a result, reduced yields for crops grown on acidic soils [1, 3].

Plants have adopted several strategies to cope with Al stresses, including Al exclusion and internal detoxification mechanisms [4, 5]. The exclusion mechanism relies on root exudation of organic matters to the rhizosphere, where the organic matters and Al^{3+} ions form stable complexes that are unable to enter the root cell, including the root apoplast, and thus non-toxic to the plant [6]. Organic acids (OAs), e.g. malate, citrate and oxalate, are the most common organic matters for fighting against Al toxicity, used by various plant species, including wheat, barley, sorghum, Arabidopsis, peanut, etc. [7–16]. Recently, Al and salicylic acid (SA)-activated root exudation of benzoxaxinoids has been recognized as an important exclusion mechanism for Al resistance in Maize [17].

The internal tolerance mechanism is involved in detoxification of the Al^{3+} ions that enter the cytosol of the plant cell. It has been postulated that the internal mechanism facilitates the sequestration of Al into the vacuole of the root cell and translocation of Al from the sensitive root tissues to the less sensitive leaf

tissues for further sequestration into the vacuole of the leaf cells [18, 19]. However, the functional and regulatory components underlying these processes remain largely unclear.

In *Arabidopsis thaliana,* the exclusion mechanism plays key a role in Al resistance [12, 13], which mainly relies on Al-activated root exudation of malate and citrate via the plasma membrane (PM)-localized malate transporter, ALMT1, and the citrate transporter MATE from the multidrug and toxic compound extrusion family, respectively [12, 13]. ALMT1facilitates the exudation of a large amount of malate from the root tip, while MATE mediates the release of a smaller amount of citrate from the more mature root region upon Al exposure [14]. The expression of both *ALMT1* and *MATE* is under the control of a master transcription factor, STOP1, i.e., sensitive to proton rhizotoxicity 1 [13, 20, 21].

In *Arabidopsis,* Al^{3+} ions in the rhizosphere can freely enter and be retained in the root cell wall at low pH (<5.0) [6]. The Al^{3+} ions in the root cell wall directly or indirectly activate the PM-localized malate and citrate transporters, leading to OA releases from the cytosol of the root cell and formation of Al-OA complexes in the rhizosphere as well as in the root cell wall. Although it has been demonstrated that Al-OA complexes in the rhizosphere cannot enter the root cell, including the root cell wall [6], whether the Al-OA complexes retained in the root cell wall are toxic to the plant remained unclear previously.

Our recent studies have demonstrated that the Arabidopsis nodulin 26-like intrinsic protein 1;2 (*NIP1;2)* gene encodes a PM-localized transporter that specifically transports Al-malate (Al-Mal) complexes but not charged Al^{3+} ions or other forms of Al-ligand complexes from the root cell wall into the root symplast [6, 22]. As the transport substrate of NIP1;2 is the Al-Mal complex but not the Al^{3+} ion, the ALMT1-mediated malate release into the root cell wall is a prerequisite for the NIP1;2-facilitated removal of Al from the root cell wall and subsequent translocation from the sensitive root tissues to the less sensitive shoot tissues [6]. Thus, the coordinated activities between the exclusion mechanism facilitated by ALMT1-mediated malate releases and the NIP1;2-mediated internal detoxification mechanism are essential for achieving overall Al tolerance in Arabidopsis [6, 22].

In genetics, the terms dominant and recessive are used to describe the effects of different alleles at a genetic locus on determining the expression of a trait. Dominant alleles ultimately determine the expression of the trait, whereas recessive alleles are much less likely to be expressed. When a dominant allele is paired with a recessive one, the dominant allele determines the trait. Recessive traits only manifest when both alleles in the locus are recessive in an individual. In contrast, the term of epistasis is used to describe interactions between genes located in different genetic loci. It is referred to a situation where the actions of one locus mask the allelic effects of another locus, in the same way that dominant alleles mask the effects of recessive ones at the same locus [23, 24]. In other words, epistasis describe a situation where the phenotypic expression at on locus depends on the genotype at a different locus.

Here, we provide further genetic evidence for the existence of an epistatic relationship between *ALMT1* and *NIP1;2.* We demonstrate that such an epistatic relationship is required for orchestrating the functions of different Al resistance mechanisms in Arabidopsis. We emphasize the essential role of the root cell

wall in establishing the epistatic relationship between the ALMT1-mediated exclusion mechanism and the NIP1;2-facilitated internal detoxification mechanism in Arabidopsis. We also discuss possible relationships between the exclusion and the internal detoxification mechanisms for Al accumulating plants under Al stresses.

Results

Generation of an amlt1_nip1;2 double mutant line

Three independent T-DNA insertion mutants of *NIP1;2,* i.e., *nip1;2–1* (SALK_126593), *nip1;2–2* (SALK_147353) and *nip1;2–3* (SALK_076128), displayed comparable hypersensitive phenotypes to Al stresses at pH 4.2 (Additional file 1: Fig. S1), confirming previously reported [6]. To further study the functional and genetic relationships between *NIP1;2* and *ALMT1,* a homozygous *almt1_nip1;2* double mutant line was generated through a cross between *almt1* (SALK_009629) and *nip1;2–3* (hereafter *nip1;2)*„ followed by selection from the F2 population of this cross of the mutant plant with homozygous *almt1* and *nip1;2* alleles.

Quantitative real-time qRT-PCR analyses indicated that in the wild type (WT, *Col–0)*„ the expression of *ALMT1* and *NIP1;2* in the root was both induced by Al treatment although the levels of *ALMT1* transcripts were about 4-fold higher than those of *NIP1;2* (Fig. 1). Under the *nip1;2* mutant background, the level of *ALMT1* expression in the root was comparable with that in the WT (Fig. 1a), whereas *NIP1;2* expression was greatly suppressed (Fig. 1b). In contrast, under the *almt1* background, although the level of the *NIP1;2* expression in the root were comparable with that in the WT, the Al-induced *ALMT1* expression in the root was barely detectable (Fig. 1a). These results confirmed that both *almt1* and *nip1;2* are knockout (KO) mutants [6, 12] and the expression of *ALMT1* and *NIP1;2* is independent of the each other [22]. Under the *almt1_nip1;2* background, the expression of *ALMT1* and *NIP1;2* in the root was both barely detectable (Fig. 1a, b), indicating that *almt1_nip1;2* is a double KO mutant line.

Comparable sensitivity between almt1_nip1;2 and almt1 to Al stresses

To evaluate Al resistance of individual lines, relative root growth (RRG%) was calculated for 5-d-old plants of *WT, nip1;2, almt1* and *almt1_nip1;2* treated with a range of Al concentrations (0–50 μM) at pH 4.2 (Fig. 2). Root growth of both the *almt1* and *nip1;2* single mutants was more severely inhibited by Al stresses than did the WT over the range of Al concentrations tested (Fig. 2). Nevertheless, root growth was more strongly inhibited in *almt1* than in *nip1;2* (Fig. 2). For instance, at Al concentration of 20 μM, root growth was inhibited by 82 and 69 % in *almt1* and *nip1;2,* respectively (Fig. 2 and Additional file 1: Fig. S2). These results indicate that *ALMT1* plays a larger role in contribution to overall Al resistance than does *NIP1;2* in Arabidopsis.

In contrast, no significant differences in root growth were observed between the *almt1_nip1;2* double mutant and the *almt1* single mutant over the entire range of Al concentrations tested (Fig. 2). Taken

together, these results indicate that the Al-resistant phenotype of *almt1_nip1;2* resembled that of *almt1,* but not *nip1;2.*

Genetic analysis of the allelic effects of ALMT1 and NIP1;2 on Al resistance

To examine the combinational effects of different genotypes at the *ALMT1* and *NIP1;2* loci on Al resistance, 215 plants randomly selected from the F2 population derived from the cross of *almt1* and *nip1;2* were evaluated for their genotypes and root-growth phenotypes under 20 μM Al treatment. On the basis of their sensitivity to Al stresses, the F2 plants could be classified into three distinct groups (Table 1): *a)* plants with at least one wild-type allele at both the *ALMT1* and *NIP1;2* loci (Group A); *b)* plants with homozygous *almt1/almt1* mutant alleles (Group B); and *c)* plants with homozygous *nip1;2/nip1;2* mutant alleles and at least one wild-type allele of *ALMT1,* i.e., *almt1/ALMT1* or *ALMT1/ALMT1* (Group C).

Root growth of the F2 plants with at least one wild-type *ALMT1* allele and one wild-type *NIP1;2* allele was comparable with that of the wild-type WT background, i.e., *ALMT1/ALMT1 NIP1;2/NIP1;2* (Group A, Table 1). In contrast, homozygous *almt1* and/or *nip1;2* mutant plants in Groups B and C were more sensitive to Al stresses than did the plants in Group A (Table 1). These results indicate that a single wild-type allele of *ALMT1* or *NIP1;2* could mask the phenotypic effects of the corresponding T-DNA insertion mutant alleles, indicating that the wild-type alleles of *ALMT1* and *NIP1;2* are both completely dominant ones.

In contrast, although homozygous mutations of *almt1* or *nip1;2* caused significant root growth inhibition (Fig. 2 and Table 1), the effects of genotypic variation at one locus on the phenotypic expression of the other locus were quite different between *ALMT1* and *NIP1;2* (Table 1). For instance, under a homozygous *almt1/almt1* background (i.e., Group B in Table 1), root growth was solely determined by the homozygous *almt1* mutant alleles regardless of the genotypic variation at the *NIP1;2* locus. In contrast, under a homozygous *nip1;2/nip1;2* background, the degrees of root growth inhibition by Al were strongly affected by the genotypic variation at the *ALMT1* locus: plants with homozygous *almt1/almt1* alleles, i.e., the *almt1_nip1;2* double mutant plants, displayed greatly enhanced root-growth inhibition compared with those with one or two copies of the wild-type *ALMT1* allele, i.e., plants in Group C, Table 1. The fact that the homozygous *almt1* mutation at the *ALMT1* locus could mask/override the effects of genotypic variation at the *NIP1;2* locus indicates that there exist interactions between the *ALMT1* and *NIP1;2* loci in which *ALMT1* is genetically epistatic to *NIP1;2.*

Additive effects of ALMT1 and MATE and epistatic relationship between ALMT1 and NIP1;2 in Al resistance

In Arabidopsis, the Al-activated and ALMT1-facilated malate exudation from the root-tip region plays a major role in Al resistance, while the Al-activated and MATE-mediated citrate release from more mature root regions plays a smaller but significant role [12–14]. Although both the *almt1* and *mate* single mutants are more sensitive to a range of Al concentrations (0–50 μM) tested than did the WT, *almt1*

consistently displayed significantly stronger root-growth inhibition than did the *mate* mutant (Fig. 3a and Additional file 1: Fig. S2).

Compared with the *almt1* and *mate* single mutants, an *almt1_mate* double mutant showed significantly more severe root-growth inhibition phenotypes over the entire range of Al concentrations tested (Fig. 3a and Additional file 1: Fig. S2). For instance, at 20 μM Al, root growth of *almt1_mate* was inhibited by 93%, whereas root growth of *almt1* and *mate* by 82 and 72%, respectively (Fig. 3a). Thus, the effects of *ALMT1* and *MATE* on Al resistance are additive, suggesting that ALMT1 and MATE function in different biochemical pathways, which is consistent with our previous observation that ALMT1 and MATE function independently in achieving overall Al resistance in Arabidopsis [13].

In contrast, the *almt1_nip1;2* double mutant did not display stronger mutant phenotypes than did the *almt1* mutant (Fig. 2). Instead, root growth was comparable between *almt1* and *almt1_nip1;2* over the entire range of Al concentrations tested (Fig. 2). Thus, when *almt1_nip1;2* was directly compared with *almt1_mate,* the latter displayed consistently more severe root-growth-inhibition phenotypes than did the former (Fig. 3b and Additional file 1: Fig. 2S). In conclusion, our experimental conditions could distinguish the additive effect of *ALMT1* and *MATE* from the epistatic relationship between *ALMT1* and *NIP1;2.*

ALMT1-mediated root exudation of malate is independent of the NIP1;2 function

To evaluate the effects of different genotypes on root OA exudation, Al-activated root exudation of malate and citrate was examined for *WT, almt1, nip1;2* and *almt1_nip1;2.* Under the control condition (-Al), comparable basal levels of root exudation of malate and citrate were observed among individual lines (Fig. 4a, b). Al exposure triggered releases of large and comparable amounts of malate from the roots of *WT* and the *nip1;2* mutant (Fig. 4a). In contrast, both *almt1* and *almt1_nip1;2* lacked detectable Al-activated root malate exudation (Fig. 4a). These results indicate that Al-activated malate exudation from the root is mainly facilitated by the ALMT1 malate transporter in Arabidopsis and the Al-activated and ALMT1-mediated root malate exudation is independent of the NIP1;2 function.

Compared with root malate exudation, Al exposure also triggered smaller, but significant, increases in citrate exudation from the root (Fig. 4b). In contrast, no significant differences were observed in the amounts of citrate in the root exudates from all lines examined upon Al exposure (Fig. 4b). These results indicate that the Al-activated and MATE-facilitated root citrate exudation is independent of the ALMT1 and NIP1;2 functions in Arabidopsis (Fig. 4b). Taken together, these results indicate that the phenotypes of OA exudation of the *almt1_nip1;2* double KO line resemble those of the *almt1* mutant, but not the *nip1;2* mutant.

ALMT1 functions upstream of NIP1;2 in the process of Al removal from the root cell wall

To test the relationship between *ALMT1* and *NIP1;2* in the processes of Al removal from the root cell wall, Al contents in the root cell wall and cell sap were measured for the *WT, almt1, nip1;2* and *almt1_nip1;2* plants treated with 50 μM $AlCl_3$ at pH 4.3 for 2 d (Fig. 5). Compared with the *WT* plants, the *almt1* and

nip1;2 plants accumulated significantly higher and lower concentrations of Al in the root cell walls (Fig. 5a) and root cell sap (Fig. 5b), respectively. These results confirmed that both ALMT1-mediated malate releases and a functional NIP1;2 are required for Al removal from the root cell wall into the root cytosol [6]. However, compared with the *nip1;2* mutant, the *almt1* mutant also accumulated significantly higher concentrations of Al in the root cell wall (Fig. 5a) and lower concentrations of Al in the root cell sap (Fig. 5b). These results suggest that besides the ALMT1 and NIP1;2-dependent process, there exists ALMT1-depenedent but NIP1;2-indenpent processes for Al removal from the root cell wall in Arabidopsis.

The Al concentrations in the root cell wall (Fig. 5a) and root cell sap of the *almt1_nip1;2* double mutant (Fig. 5b) were comparable with those of the *almt1* single mutant, which were significantly different from those in the *nip1;2* single mutant. These results indicate that *ALMT1* is genetically epistatic to *NIP1;2* in the biochemical pathway leading to Al removal from the root cell wall into the root symplasm in Arabidopsis.

Externally supplied malate partially restored NIP1;2-facilitated Al uptakes from the root cell wall in almt1 but not in almt1_nip1;2

To evaluate the effects of externally supplied malate on Al uptakes from the root cell wall for *almt1, nip1;2* and *almt1_nip1;2,* plants of these lines were treated with 50 μM $AlCl_3$ (pH 4.2) for 8 h, allowing Al to get into and be retained in the root cell walls (Fig. 6a) [6], followed by addition of 0 or 200 μM malate for another 8 h.

Between these two treatments, no statistically significant differences in Al contents in the root cell wall (Fig. 6a) and the root cell sap (Fig. 6b) were observed in the *nip1;2* single mutant and the *almt1_nip1;2* double mutant. In contrast, in *almt1,* compared with those under the Al treatment alone, external supplementation of malate after Al treatment led to significantly decreased Al concentrations in the root cell wall (Fig. 6a) and significantly increased concentrations in the root cell sap (Fig. 6b). These results indicate that even though the *almt1* mutant has a functional NIP1;2 transporter [6], the presence of malate in the root cell wall is essential for NIP1;2-facilitated Al removals from the root cell wall. Taken together, these results indicate that the ALMT1-mediated releases of malate to the root cell wall function in an earlier step in the NIP1;2-facilitated process for Al uptakes from the root cell wall to the root cytosol.

Discussion

ALMT1 is genetically and functionally epistatic to NIP1;2

In Arabidopsis, coordinated activity of ALMT1 and NIP1;2 is required for NIP1;2-facilitated Al removal from the root cell wall into the root symplasm and subsequent root-to-shoot Al translocation, which are critical steps in the internal detoxification mechanism [6, 19, 22]. In this process, first, the Al^{3+} ions enter the root apoplast from the rhizosphere and then activate the PM-localized ALMT1 transporter, leading to malate exudation from the root tip cell into the root apoplast and rhizosphere [12–14]. In the root apoplast, the released malate interacts with the Al^{3+} ions to form Al-Mal complexes, which are

subsequently transported from the root cell wall into the root cytosol by the PM-localized NIP1;2 [6]. As the transport substrate of the NIP1;2 transporter is the Al-Mal complex, but not the Al^{3+} ion, the ALMT1-mediated malate exudation into the root cell wall and subsequent formation of Al-Mal complexes in the root apoplast is a prerequisite for NIP1;2-facilitated Al removal from the root cell wall (Fig. 5) [6]. Thus, ALMT1 plays a key role in both the exclusion mechanism, via facilitating malate exudation to the rhizosphere to chelate toxic Al^{3+} ions, and the internal detoxification mechanism, through facilitating the NIP1;2-mediated removal of the Al-Mal complex from the root cell wall and translocation from the root to the shoot [6].

Phenotypic examination of root growth indicated that the *almt1_mate* double mutant was more sensitive to Al stresses than did either of the *almt1* or *mate* single mutants, indicating that the effects of *almt1* and *mate* mutations are additive (Fig. 3a). In contrast, no additive or synergistic effects were observed between the *almt1* and *nip1;2* mutations (Figs. 2–5). Instead, examination of root-growth phenotypes (Fig. 2 and Additional file1: Fig. S1), Al-activated root OA exudation (Fig. 4) and NIP1;2-facilitated Al removal from the root cell wall (Fig. 5) indicated that the phenotypes of the *almt1_nip1;2* double KO mutant resembled those of *almt1,* but not *nip1;2.* Moreover, externally supplied malate could partially compensate the loss of the ALMT1-mediated malate exudation for the NIP1;2-facilitated function in the *almt1* mutant (Fig. 6). Taken together, these results indicate that ALMT1 and NIP1;2 function in a single biochemical pathway, where ALMT1 functions upstream of NIP1;2.

The significance of the epistatic relationship between ALMT1 and NIP1;2 in Al tolerance for the non-accumulating Arabidopsis

On acid soils, most plants, i.e., the so-called non-accumulators, limit the uptake of Al from the soil and accumulate no more than 0.2 mg Al g^{-1} dry weight of the plant [19]. In contrast, a few Al accumulator plant species can accumulate much higher concentrations of Al in the shoot/leaf. For instance, hydrangea (*Hydrangea macrophylla)* plants can accumulate up to 3 mg Al g^{-1} dry weight of the plant [19], while buckwheat (*Fagopyrum esculentum)* plants 1.7 mg Al g^{-1} dry weight without showing any sings of toxicity [25, 26].

In both the accumulating and non-accumulating plant species, the overall Al tolerance can be achieved by the exclusion and the internal tolerance mechanisms [5, 19, 27]. However, these two mechanisms are not mutually exclusive but they are coordinately functioned as in the case of the ALMT1- and NIP1;2-mediated Al tolerance in Arabidopsis [6, 22].

Arabidopsis is a non-accumulating species for Al. Therefore, Al tolerance in Arabidopsis is mainly dependent on the exclusion mechanism via the ALMT1- and MATE-mediated root exudation of malate and citrate, respectively, to the rhizosphere and the root apoplast where the OAs detoxify the Al^{3+} ions [12–14]. As ALMT1 facilitates the release of a large amount of malate from the root tip region [14], a major target for Al toxicity [28–31], ALMT1 makes a significantly larger contribution to overall Al resistance in Arabidopsis than does MATE [13, 14].

In the root apoplast, the released malate anions chelate the Al^{3+} cations and thus reduce the concentration of free Al^{3+} cations, which minimizes the harmful interactions of these cations in the cell wall. However, the simple binding of Al and malate in the apoplast of the root cell is not enough to provide full protection against Al toxicity [6]. Instead, the Al-Mal complexes in the root apoplast need to be removed for achieving higher degrees of Al tolerance [6].

Here, we have further demonstrated that ALMT1 is genetically and functionally epistatic to NIP1;2. Such an epistatic relationship allows the Arabidopsis plant to be protected by the exclusion mechanism first, which effectively prevents or limits the entry of toxic Al^{3+} cations into the root cell, including the root cell wall, before the NIP1;2-facilitated internal detoxification mechanism, i.e., uptakes of Al from the root cell wall, start to function. Without the establishment of such an epistatic relationship between the exclusion and internal detoxification mechanisms, high levels of Al could be accumulated in the plant, which is harmful to the non-accumulator like Arabidopsis.

On the bases of these observations, we postulate that for the non-accumulating plant species, a dominant exclusion mechanism is essential for Al resistance via continuously preventing the entry of Al to the root cell. After the exclusion mechanism is established, the internal detoxification mechanism will play a secondary and scavenging role for removing the toxic Al^{3+} ions from the root apoplast and cytosol for further sequestration into the vacuole of the root cell and/or for translocation from the root to less sensitive shoot.

In contrast, for the accumulating plants to take up large amounts of Al^{3+} ions from the root and accumulate them in the shoot, the exclusion mechanism must be suppressed. Interestingly, Al-activated release of oxalic acid is required for the protection of root growth and function for young seedlings of buckwheat, an Al accumulator, under Al stresses [32]. Thus, it is likely that at the early developmental stage, the exclusion mechanism is also essential for Al accumulators to withstand the initial shocks of Al toxicity. Once the internal detoxification mechanism is established at later developmental stages, the exclusion mechanism will be suppressed or discontinued to function, allowing the accumulation of large amounts of Al in the shoot via the internal detoxification mechanism.

Essential roles of the root cell wall in overall Al resistance in Arabidopsis

As mentioned above, the significance of the epistatic relationship between the exclusion and internal detoxification mechanisms for overall Al resistance in Arabidopsis is that the ALMT1-mediated exclusion mechanism provides a shield/barrier for the root against the toxic Al^{3+} ions in the rhizosphere before the NIP1;2-facilitated internal detoxification mechanism is allowed to function. However, the pivotal roles of the root cell wall are less well recognized in this system.

The root cell wall is highly negatively charged and as a result, Al^{3+} cations in the rhizosphere can freely enter and be retained in the root aploplast at low pH (<5.0) [6]. The Al-activated and ALMT1-mediated malate release to the rhizosphere results in the formation of Al-Mal complexes in the rhizosphere. As the

Al-Mal complex is unable to enter the root cell wall [6], with a functional ALMT1-mediated malate exudation to the rhizosphere, it is the root cell wall that acts as a shield/barrier that separates the Al in the rhizosphere from the root symplasm. Furthermore, the presence of such a shield ensures that only the Al-Mal complex in the root cell wall, but not in the rhizosphere, is accessible to the NIP1;2 transporter localized to the PM of the root cell. In conclusion, the root cell wall plays pivotal roles in the establishment of the exclusion mechanism as well as the epistatic relationship between the exclusion and the internal detoxification mechanisms for overall Al resistance and for the prevention of over-accumulation of Al in Arabidopsis plants.

Conclusion

ALMT1 is genetically epistatic to NIP1;2 for achieving coordinated functions between an exclusion and an internal tolerance mechanism and overall Al resistance in the non-accumulating Arabidopsis plant. This elegant system ensures that an exclusion mechanism is established before an internal tolerance mechanism is stepped in to achieve overall Al resistance in the non-accumulating Arabidopsis.

Methods

Materials and culture conditions

Arabidopsis T-DNA insertion lines *nip1;2–1* (SALK_126593), *nip1;2–2* (SALK_147353), *nip1;2–3* (SALK_076128), *mate* (SALK_081671) and *almt1 (*SALK_009629C) were acquired from the Arabidopsis Biological Resource Center (https://abrc.osu.edu/). The *almt1_nip1;2* double mutant was generated by crossing *almt1* and *nip1;2–3* (*nip1;2)* single mutants, followed by selection of F2 plants with homozygous T-DNA insertions at both the *ALMT1* and *NIP1;2* loci by PCR-based genotyping [6]. The *almt1_mate* double mutant was generated previously [13].

Primer sequences for genotyping the *ALMT1* locus were: 5'-CGCAGCTGCACATATATCACA–3' (*ALMT1* gene specific primer) and 5'-GCTGTTGCCCGTCTCACTGGTG–3' (T-DNA left boarder primer) for detection of the T-DNA insertion; and 5'-CGCAGCTGCACATATATCACA–3' and 5'-CGAAGTGCAACGCACCACTA–3' for amplification of the sequence encompassing the T-DNA insertion region. Primer sequences for genotyping the NIP1;2 locus for *nip1;2–3* were: 5'-GCTCGCATCTAGATCCTAAT–3' (*NIP1;2* gene specific primer) and 5'-GCTGTTGCCCGTCTCACTGGTG–3' (T-DNA left boarder primer) for detection of the T-DNA insertion; 5'- GCTCGCATCTAGATCCTAAT–3' and 5'-CGAAGTGCAACGCACCACTA–3' for amplification of the sequence encompassing the T-DNA insertion region.

For growth experiments, seeds of the wild type (WT, *Col–0)*,, individual mutant lines or the F2 population from the cross of *almt1* and *nip1;2* were surface-sterilized and cold stratified at 4°C in the dark for 3 d for synchronization of germination. Seeds were subsequently sown onto a 250 μM polypropylene mesh in a Magenta box containing a hydroponic growth solution, supplemented with 2.0 mM Homo-PIPES to maintain pH to 4.2. The hydroponic solution consisted of the following macronutrients in mM: $MgCl_2$, 3.0;

$(NH_4)_2SO_4$, 0.25; $Ca(NO_3)_2$, 1.0 M; KCl, 2.0; $CaCl_2$, 2.75; KH_2P0_4, 0.18; and the following micronutrients in μM: H_3BO_3, 50.0; $MnSO_4$, 10.0; $CuSO_4$, 0.5; $ZnSO_4$, 2.0; Na_2MoO_4, 0.1; $CoCl_2$, 0.1; 1% sucrose. Plants were grown in a growth chamber under a 16/8- h day/night cycle at 22℃.

For gene expression analysis, ~ 500 seeds (~10 mg) were germinated in a control hydroponic solution (-Al) for 5 days. Then, seedlings were transferred to a hydroponic solution (pH 4.2) containing 20 μM $AlCl_3$ for 24 h before collection of the root samples for RNA extraction. Three replicates (Magenta boxes) were included for each line and each treatment.

For Al treatment, seeds of the WT and individual mutant lines were germinated and grown in the above-mentioned hydroponic solution (pH 4.2) supplemented with 0, 5, 10, 20, 30, 40 or 50 μM of $AlCl_3$ for 7 days. Relative root growth (RRG%) was calculated according to the following formula: RRG% = root growth of individual plants under Al treatment/mean root growth under the control (-Al) condition.

For phenotyping and genotyping the F2 individuals derived from the cross of *almt1* and *nip1;2,* F2 seeds were germinated and grown in the hydroponic solution (pH 4.2) supplemented with 20 μM $AlCl_3$ for 7 d. Root length of 215 randomly selected plants was measured before the plants were transferred to the soil for growth for 2 weeks. Then, DNAs were extracted from leaves of each plant. T-DNA insertions at the *ALMT1* and *NIP1;2* loci were evaluated by PCR followed the procedures mentioned above.

RNA isolation and quantitative real-time RT-PCR

Total RNAs were extracted from Arabidopsis roots using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg DNaseI-digested total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR was performed on a 7500 Fast Real-Time PCR System with Power SYBR Green PCR Master Mix according to manufacturers' protocols (Applied Biosystems, Inc.) The relative expression levels of the target genes were referred to an endogenous calibrator gene, *18S rRNA,* for each real-time qRT-PCR experiment. The real-time primers were as follows: *NIP1;2,* 5'- GGTTCGATATACTGATAAGCCA–3' and 5'-GATACAACTTAACCTCCGATGAC–3'; *ALMT1,* 5'-TTCCCGATTCCGAGCTCATT–3' and 5'-CTCAGATTTTCAGATCCCAGTGGAC–3'.

Detection of organic acid exudation from roots

Surface-sterilized seeds (~2–3 mg) from each line were germinated in Magenta boxes containing the sterile hydroponic growth solution (pH 4.2) for 6 days, and then the seedlings were transferred to 20 ml of filter-sterilized exudation solutions (pH 4.2) with or without 50 μM Al^{3+} in a sterile Petri dish for 2 days. The exudation solution consisted of the following macronutrients in μM: $MgCl_2$, 275; $CaCl_2$, 275; KCl, 275; $Ca(NO_3)_2$, 33.4; $MgSO_4$, 33.4; K_2SO_4,16.7; and the following micronutrients in μM: H_3BO_3, 50.0; $MnSO_4$, 10.0; $CuSO_4$, 0.5; $ZnSO_4$, 2.0; Na_2MoO_4, 0.1; $CoCl_2$, 0.1; and 1% sucrose, supplemented with 3.0 mM Homo-PIPES (pH 4.2); Then the exudation solutions were collected and the numbers of plants were

counted. Then the exudation solutions were passed through anionic and cationic chromatography columns to remove Al^{3+} and other inorganic anions that could interfere with the measurement of organic acid anions. Subsequently, the eluate was concentrated to dryness using a rotary evaporator at 40°C. The residue was re-dissolved in 1 ml of Milli-Q water. Malate and citrate concentrations were then measured according to the enzymatic method previously described [10].

Root cell sap and cell wall preparation and Al determination.

Arabidopsis lines were firstly germinated and grown in the hydroponic solution (pH 4.2) for 7 days, then treated in a fresh hydroponic solution (pH 4.2) supplemented with 50 μM $AlCl_3$ for 2 d. After the treatment, the roots were cut and washed three times with deionized water and then put into an Ultra free-MC Centrifugal filter unit (Millipore) and centrifuged at 3,000 rpm for 10 min at 4 °C to remove the apoplastic solution. The root samples were then frozen in a –80°C freezer overnight. The root cell sap solution was obtained by thawing the root samples at room temperature and then centrifuging at 13,000 rpm for 10 min. The residual cell wall was washed with 70% ethanol three times and then digested in 1 mL of 2 N HCl for at least 24 h with occasional shaking. Al contents in the symplastic solution and cell wall extract were determined by inductively coupled plasma mass spectrometry (ICP-MS).

For testing the effects of sequential Al^{3+} and malate treatment on Al accumulation, ~150 7-d-old seedlings of the WT, *almt1, nip1;2–3* and *almt1_nip1;2* lines were pretreated with the hydroponic solution (pH 4.2) supplemented with 50 μM $AlCl_3$ for 8 h, washed three times with 0.5 mM $CaCl_2$, then transferred to hydroponic solutions (pH 4.2) supplemented with or without 200 μM malate for 8 h. Al concentrations in cell sap and cell wall were measured as mentioned above. Three biological replicates (Magenta boxes) with the same setting were prepared for each plant line and each treatment.

Abbreviations

Al-Mal: aluminum-malate

ALMT: Al-activated malate transporter

KO: knock-out

MATE: multidrug and toxic compound extrusion

NIP: nodulin 26-like intrinsic protein

OA: organic acid

RRG: relative root growth.

Declarations

Availability of data and materials

All relevant data are within this article and its Additional files.

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

J. L. conceived and supervised the research. Y. W. and J. L. designed the experiments. Y. W., W. Y., Y. Cao, Y. Cai, S. M. L., W. W., Y. K. and C. L. performed the experiments and analyzed the results. J. L. and Y. W. wrote the paper.

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Table 1

Table 1. Allelic effects of *ALMT1* and *NIP1;2* on Al resistance.

Genotypes at *the ALMT1* locus	Genotypes at the *NIP1;2* locus		
	nip1;2/nip1;2	*nip1;2/NIP1;2*	*NIP1;2/NIP1;2*
almt1/almt1	2.9 ± 0.5^{B}	2.7 ± 0.9^{B}	3.1 ± 0.7^{B}
almt1/ALMT1	6.3 ± 1.4^{C}	12.1 ± 2.4^{A}	10.9 ± 1.6^{A}
ALMT1/ALMT1	6.8 ± 1.8^{C}	11.7 ± 1.9^{A}	11.5 ± 2.8^{A}

Seeds were germinated and grown in hydroponic growth medium supplemented with 20 mM $AlCl_3$ (pH 4.2) for 5d. Data are mean (mm) ± SD (n= 10). *Letters* represent groups with significant differences ($P \leq 0.05$) as determined by Fisher's LSD test.

Figures

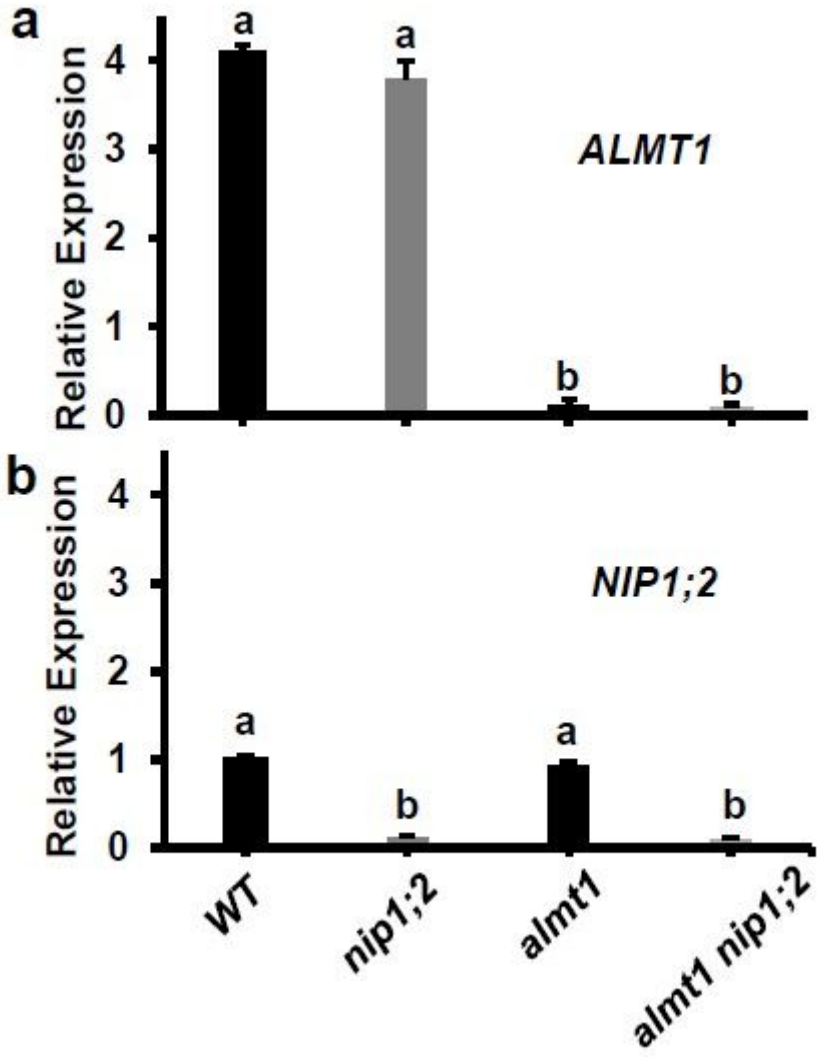

Figure 1

ALMT1 (a) and NIP1;2 (b) expression patterns in the root. RT-qPCR analysis of ALMT1 and NIP1;2 gene expression in the roots of 7-d-old seedlings of WT, almt1, nip1;2 and almt1_nip1;2 treated with 20 μM AlCl3 (pH 4.2) for 24 h. Data are represented as mean ± SD (n=3); Different letters indicate significant differences between individual lines.

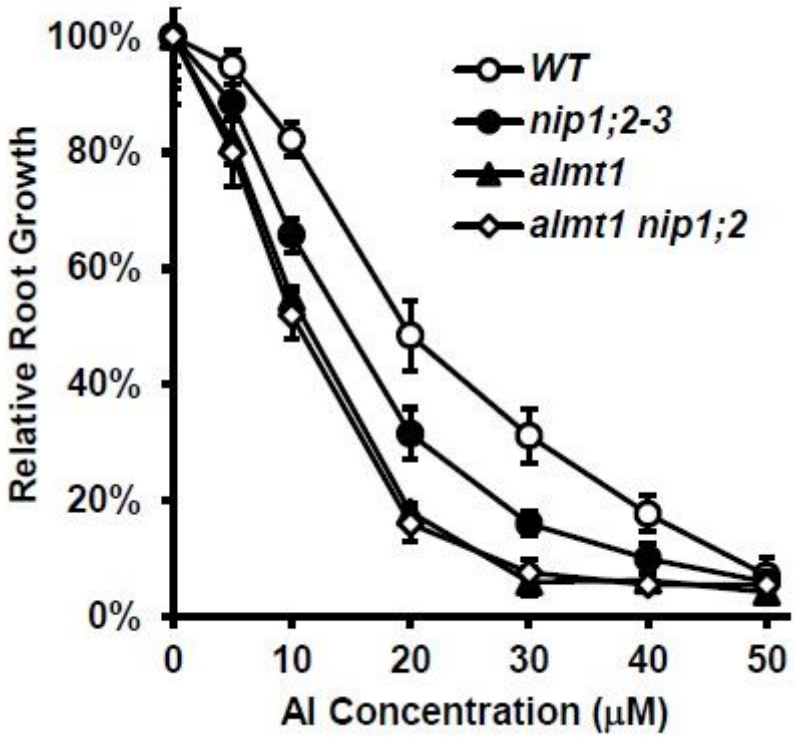

Figure 2

Relative root growth of WT and almt1, nip1;2 and almt1_nip1;2 mutants. Seeds were germinated and grown in hydroponic solution (pH 4.2) supplemented with 0, 5, 10, 20, 30, 40, 50 μM of AlCl3 for 5 days. Relative root growth (RRG%) was calculated according to the following formula: RRG% = root growth of individual plants under Al treatment/mean root growth under the control (-Al) condition. Data are mean ± SD of 10 seedlings.

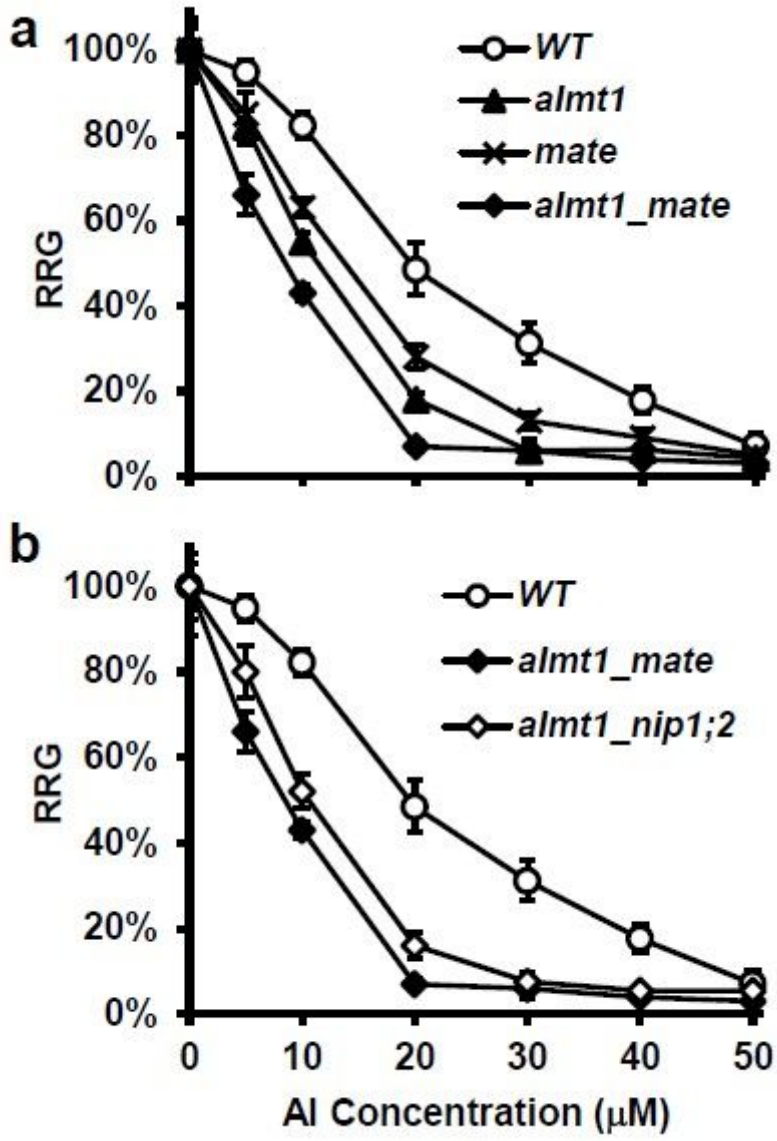

Figure 3

a Relative root growth (RRG%) of WT and almt1, mate and almt1_mate mutants and b RRG% of WT and almt1_mate, almt1_nip1;2 double mutants. Seeds were germinated and grown in hydroponic solution (pH 4.2) supplemented with 0, 5, 10, 20, 30, 40, 50 μM of AlCl3 for 5 days. Data are mean ± SD of 10 seedlings.

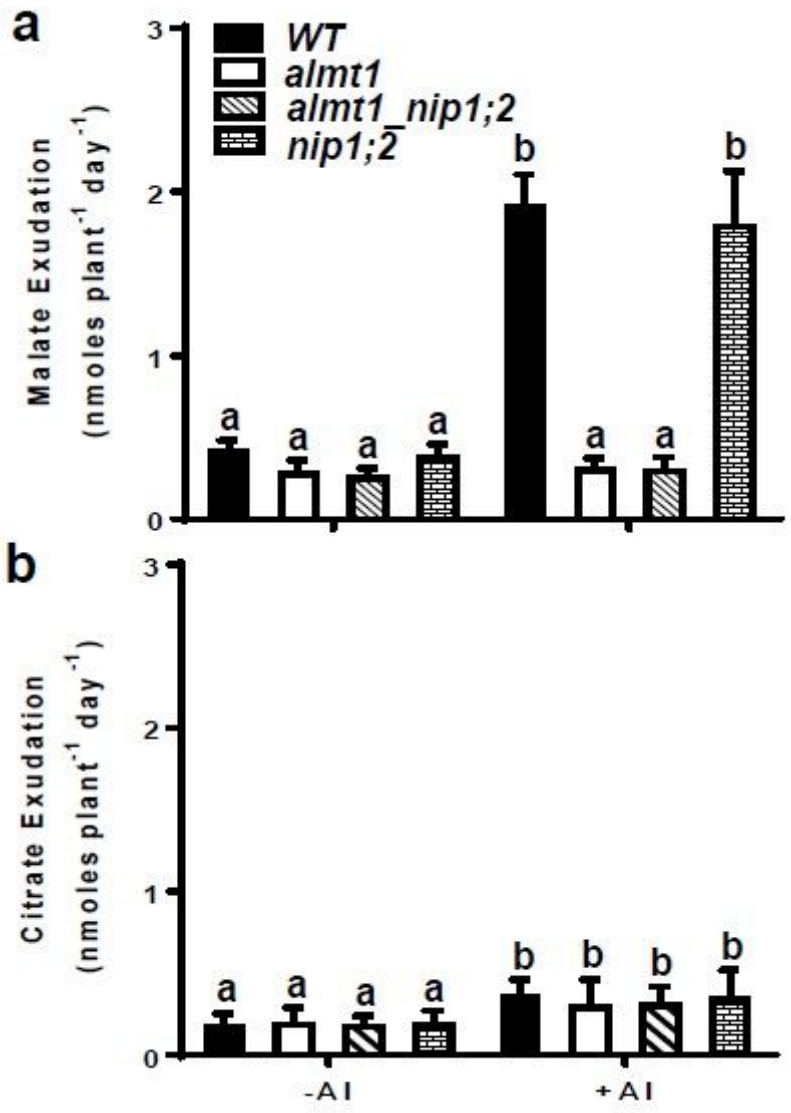

Figure 4

Root exudation of malate (a) and Citrate (b). Here, 6-d-old seedlings of WT, almt1, nip1;2 and almt1nip1;2 were treated in 20 ml of exudation buffers (pH 4.2) supplemented without (-) or with (+) 50 μM AlCl3 for 2 d before the concentrations of malate and citrate in the exudation buffer were determined. Data are mean ± SD of three biological replicates. Letters represent groups with significant differences ($P \leq 0.05$).

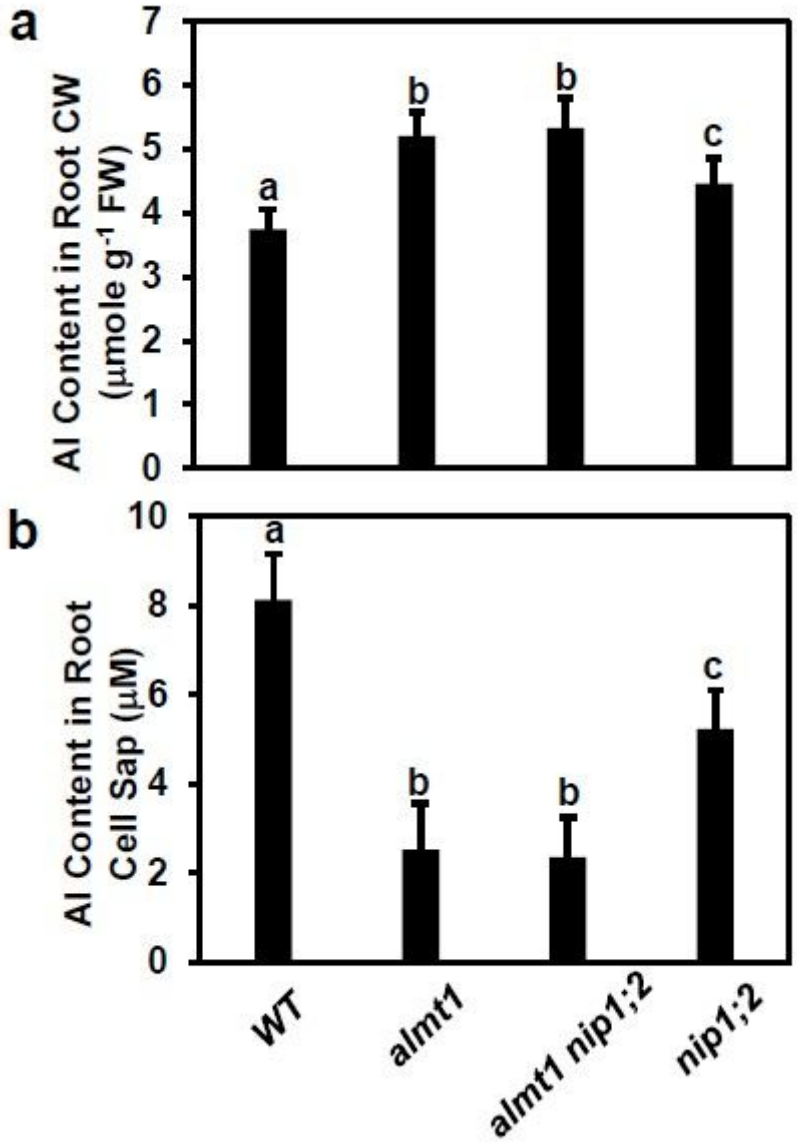

Figure 5

Aluminum content in the root cell wall (a) and the root cell sap (b). Seven-day-old seedlings of WT, almt1, nip1;2 and almt1_nip1;2 were exposed to 50 μM AlCl3 (pH 4.2) for 2 days. Al concentrations in the root cell wall (a) and the root cell sap (b) were determined by ICP-MS. Data are mean ± SD of three biological replicates from three magenta boxes. Letters represent groups with significant differences ($P \leq 0.05$).

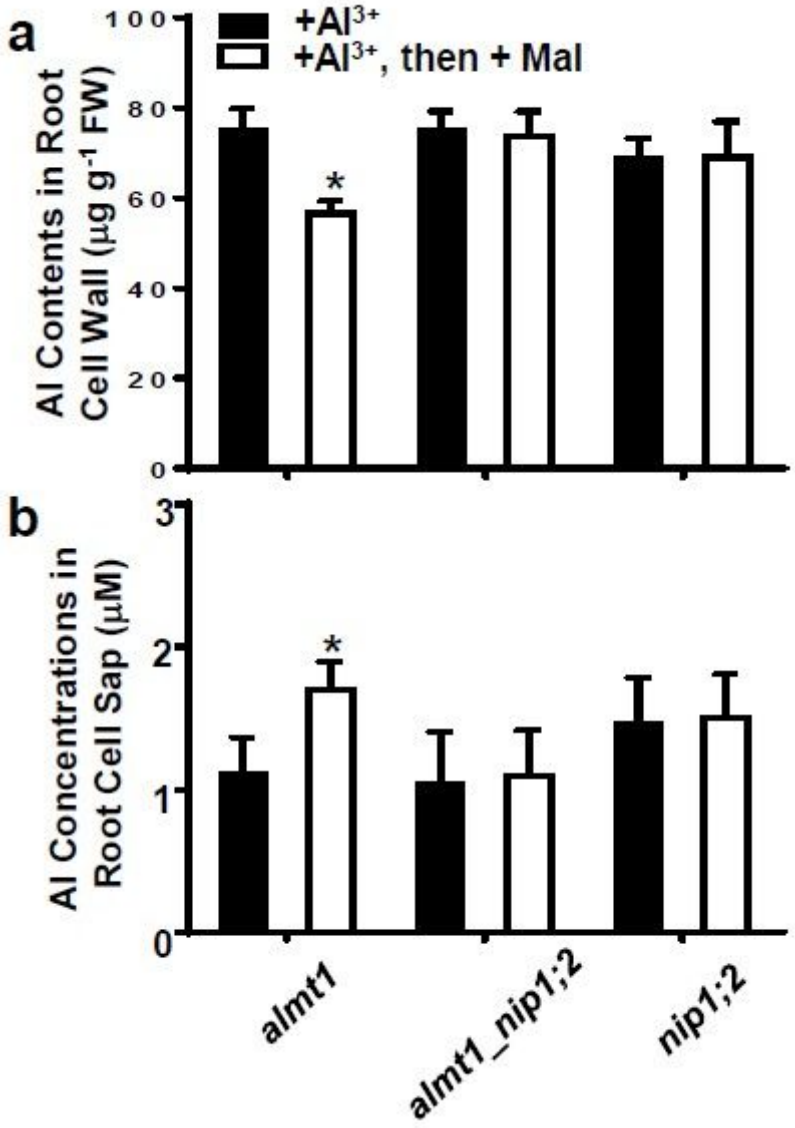

Figure 6

Effects of externally supplied malate in NIP1;2-mediated Al uptake in the almt1, almt1_nip1;2 and nip1;2 lines. Here 7-d-old seedlings were pretreated with AlCl3 (pH 4.2) for 8 h, washed three times with 0.5 mM CaCl2, and then treated with 200 μM malate (−Al) for 8 h. a Al concentrations in the root cell wall and b root cell sap were determined by ICP-MS. Data are mean ± SD of three sample replicates from three magenta boxes. *, significant differences ($P \leq 0.05$) between the − and the + malate treatment.

Supplementary Files

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