A Defective Vacuolar Proton Pump Enhances Aluminum Tolerance by Reducing Vacuole Sequestration of Organic Acids

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Plants cope with aluminum (Al) toxicity by secreting organic acids (OAs) into the apoplastic space, which is driven by proton (H+) pumps. Here, we show that mutation of vacuolar H+-translocating adenosine triphosphatase (H+-ATPase) subunit a2 (VHA-a2) and VHA-a3 of the vacuolar H+-ATPase enhances Al resistance in Arabidopsis (Arabidopsis thaliana). vha-a2 vha-a3 mutant plants displayed less Al sensitivity with less Al accumulation in roots compared to wild-type plants when grown under excessive Al3+. Interestingly, in response to Al3+ exposure, plants showed decreased vacuolar H+ pump activity and reduced expression of VHA-a2 and VHA-a3, which were accompanied by increased plasma membrane H+ pump (PM H+-ATPase) activity. Genetic analysis of plants with altered PM H+-ATPase activity established a correlation between Al-induced increase in PM H+-ATPase activity and enhanced Al resistance in vha-a2 vha-a3 plants. We determined that external OAs, such as malate and citrate whose secretion is driven by PM H+-ATPase, increased with PM H+-ATPase activity upon Al stress. On the other hand, elevated secretion of malate and citrate in vha-a2 vha-a3 root exudates appeared to be independent of OAs metabolism and tolerance of phosphate starvation but was likely related to impaired vacuolar sequestration. These results suggest that coordination of vacuolar H+-ATPase and PM H+-ATPase dictates the distribution of OAs into either the vacuolar lumen or the apoplastic space that, in turn, determines Al tolerance capacity in plants.

In acidic soils, phytotoxic levels of active aluminum (Al) are released from Al-containing minerals when the pH drops to 5 or lower (Kochian, 1995). These active Al forms, primarily Al3+, can severely threaten root vitality and inhibit primary root elongation, thus limiting the acquisition of nutrient elements and water necessary for crop growth, which makes Al toxicity a major constraint for crop production in acid soils (Kochian, 1995; Ma, 2007). Such symptoms are generally recognized as the primary consequences from targeted actions of Al in the root apex, specifically its distal part of the transition zone (DTZ). The DTZ, located between the apical meristem zone (MZ) and basal elongation zone, accumulates most of the Al contents in the root apex under Al stress and is the primary root site of Al toxicity (Ryan et al., 1993; Sivaguru and Horst, 1998).

Plants have evolved diverse adaptive strategies to cope with Al toxicity, among which external exclusion and internal tolerance are widely considered as the major mechanisms (Kochian, 1995; Ma, 2007). Both mechanisms are related to translocation of ions. For example, the exclusion mechanism is achieved by the secretion of organic acid (OA) anions, including malate, citrate, and oxalate, the specificities of which depend on the plant species, from the roots to externally chelate Al3+ (Miyasaka et al., 1991; Delhaize et al., 1993; Larsen et al., 1998; Zheng et al., 1998; Hoekenga et al., 2003, 2006; Sasaki et al., 2004). Furthermore, the tolerance mechanism underlies the sequestration of cytosolic Al3+ or its complex into the vacuoles (Vazquez et al., 1999; Shen et al., 2002; Illés et al., 2006). In Arabidopsis (Arabidopsis thaliana), malate and citrate are the major OAs in root exudates.
under Al stress and are responsible for Al tolerance in Arabidopsis (Larsen et al., 1998; Hoekenga et al., 2003, 2006; Magalhaes et al., 2007; Liu et al., 2009). Intriguingly, although large amounts of cellular malate and citrate are originally stored in the vacuoles, considerable amounts are rapidly excreted through the PM-localized Al-activated malate transporter1 (ALMT1) and multidrug and toxic compound extrusion (MATE), respectively, after exposure to Al stress (Hoekenga et al., 2003, 2006; Kobayashi et al., 2007; Liu et al., 2009). Compared to the well-documented Al exclusion mechanism, the molecular mechanisms underlying vacuolar Al sequestration are poorly understood. Whereas several ATP-binding cassette-type transporters, including ALUMINUM SENSITIVE1 (ALS1), SENSITIVE TO ALUMINUM RHIZOTOXICITY1 (STAR1), and ALS3, have been suggested to be vital factors for Al tolerance in Arabidopsis (Larsen et al., 2005, 2007; Huang et al., 2010), the nature of these transporters remains elusive. Recent reports showed that ALS3 interacts with STAR1 to form an ATP-binding cassette transporter complex in the tonoplast and functions in phosphate (Pi) deprivation-induced root growth inhibition (Belal et al., 2015; Dong et al., 2017; Godon et al., 2019; Wang et al., 2019), suggesting that these transporters may have more functions in addition to coping with Al toxicity.

In plants, the active transport of ions is fundamentally driven by the proton (H\(^+\)) gradients established by H\(^+\) pumps, namely, plasma membrane H\(^+\)-translocating adenosine triphosphatase (PM H\(^+\)-ATPase), vacuolar H\(^+\)-ATPase (V-ATPase), and vacuolar H\(^+\)-pyrophosphatase (V-PPase). Gaxiola et al. (2007). The function of PM H\(^+\)-ATPase upon Al stress likely depends on plant species and genotypes. For example, the Al-induced activation of PM H\(^+\)-ATPase is found to coincide with secretion of OAs in soybean (Glycine max) roots (Shen et al., 2005; Liang et al., 2013), but PM H\(^+\)-ATPase in barley (Hordeum vulgare; Matsumoto, 1988) and summer squash (Cucurbita pepo) roots (Ahn et al., 2001) is inhibited by Al treatment. Consistent with the observations in soybean roots, PM H\(^+\)-ATPase is found to be activated by Al stress in Arabidopsis roots (Shen et al., 2005; Bose et al., 2010). The PM H\(^+\)-ATPase in Arabidopsis is encoded by 11 isoforms of P-type ATPases (AHA1–AHA11; Palmgren, 2001; Gaxiola et al., 2007). The AHA genes are differentially expressed in Arabidopsis tissues, and eight of them (AHA1–AHA4, AHA7, AHA8, AHA10, and AHA11) are expressed in roots (Ueno et al., 2005). Despite the fact that apoplastic acidification is generally observed upon Al stress (Zhang et al., 2017), little is known about how PM H\(^+\)-ATPase is linked to Al response in Arabidopsis. The PM H\(^+\)-ATPase activity has been hypothesized to be related to the activity of vacuolar H\(^+\) pump (Li et al., 2005; Cho et al., 2012), which is constituted by V-ATPase and V-PPase (Gaxiola et al., 2007). In Arabidopsis, two tonoplast-localized isoforms of vacuolar H\(^+\)-ATPase subunit a (VHA-a), VHA-a2 and VHA-a3, provide the H\(^+\)-translocating activity. Compared to the wild type, vha-a2 vha-a3 double mutant plants, in which the majority of V-ATPase activity is eliminated, display a retarded growth phenotype and are hypersensitive to excessive metals, including zinc (Zn) and calcium (Ca; Krebs et al., 2010; Tang et al., 2015). Another Arabidopsis vacuolar H\(^+\) pump, Arabidopsis type-1 V-PPase (AVP1), is a major vacuolar H\(^+\)-pumping pyrophosphatase and has been shown to be involved in improving plant growth (Li et al., 2005; Ferjani et al., 2011; Segami et al., 2018). To ensure that plant cells respond appropriately to constantly changing environmental ions, the PM H\(^+\)-ATPase and vacuolar H\(^+\) pumps may tightly coordinate to establish spatial H\(^+\) gradients across plasma and vacuole membranes and thus maintain cellular homeostasis of ions, including OAs. However, the functional relationship among these H\(^+\) pumps in Arabidopsis response to Al stress remains elusive, despite the fact that translocation of OAs has been established as a major event in tolerance of Al toxicity.

In this study, we report that V-ATPase, but not V-PPase, is a major vacuolar H\(^+\) pump functioning in Arabidopsis root response to Al stress. In contrast to its positive role in internal detoxification of other metals, V-ATPase was repressed by excessive Al\(^3+\) and knockout of its members AHA-a2 and VHA-a3 caused Al resistance. We further identified the relationship between V-ATPase and other H\(^+\) pumps and found that the PM H\(^+\)-ATPase was activated and contributed to Al resistance in vha-a2 vha-a3 mutant. A detailed functional analysis showed that these two types of H\(^+\) pump coordinated to drive OAs allocation through the PM-localized transporters into the root exudates for chelating external Al\(^3+\) in a low Pi-independent manner. Thus, this study uncovers a previously unrecognized mechanism underlying Al exclusion achieved by regulation of V-ATPase activity in Arabidopsis roots.

**RESULTS**

**The vha-a2 vha-a3 Mutant Is Resistant to Al\(^3+\) Stress**

Previous studies showed that VHA-a2 and VHA-a3 redundantly confer resistance in Arabidopsis to excessive metals, such as Zn\(^2+\) and Ca\(^2+\), but not magnesium (Mg\(^2+\)), through vacuolar sequestration (Krebs et al., 2010; Tang et al., 2015). Considering the importance of pH gradients across the tonoplast in metal transport into the vacuole (Schumacher and Krebs, 2010), we checked whether the vha-a2 vha-a3 mutant showed altered sensitivity to several other metals including Al\(^3+\) and manganese (Mn\(^2+\)), whose uptake by plants could be enhanced under the acidic conditions (Kochian et al., 2004). We included Zn\(^2+\), Ca\(^2+\), and Mg\(^2+\) as positive and negative controls (Krebs et al., 2010; Tang et al., 2015) in this analysis. On one-sixth strength Murashige and Skoog agar medium with pH 4.3 and pH 5.8, no significant difference was observed between vha-a2 vha-a3 and wild-type seedlings (Supplemental Fig. S1). Moreover, in the presence of toxic levels of Ca\(^2+\), Mn\(^2+\),
Zn$^{2+}$, or Mg$^{2+}$, the growth of the wild type and vha-a2 vha-a3 mutant was similar and was not affected by pH (4.3 or 5.8) of the medium (Supplemental Fig. S1). In the presence of 150 μM AlCl$_3$, however, the vha-a2 vha-a3 mutant displayed a more resistant growth phenotype with longer primary roots and more biomass on pH 4.3 medium compared with the wild type, whereas this mutant displayed similar growth as the wild type on pH 5.8 medium regardless of AlCl$_3$ supplementation (Supplemental Fig. S1). These results demonstrate that disruption of VHA-a2 and VHA-a3 function enhances the resistance to Al$^{3+}$, instead of creating a more sensitive response as shown for other metals, probably in a pH-dependent manner.

Primary root growth inhibition is a typical symptom of Al toxicity in plants (Ryan et al., 1993; Sivaguru and Horst, 1998); therefore, we decided to examine the root growth phenotypes in the wild type and vha-a2 vha-a3 mutant grown under Al stress. Supplementation of 250 μM AlCl$_3$ to pH 4.3 medium caused a 72% and 53% inhibition of primary root growth of the wild-type and vha-a2 vha-a3 seedlings, respectively (Fig. 1, A and B). We also observed the root structure and found that the root-apex structure in the wild-type and vha-a2 vha-a3 mutant roots was comparable when they grew under the normal conditions. Under 250 μM Al$^{3+}$ treatment, the DTZ of the wild-type root apex displayed notable callose formation, a sensitive indicator of Al injury to roots (Wissemeir et al., 1987; Horst et al., 1997; Sivaguru and Horst, 1998), whereas the DTZ of the vha-a2 vha-a3 mutant root apex produced much less callose (Fig. 1C), suggesting that vha-a2 vha-a3 mutant roots are more tolerant to Al toxicity than roots of the wild type. We further examined the changes in root growth under various AlCl$_3$ concentrations and found that the longer primary roots in the vha-a2 vha-a3 mutant were also observed under other AlCl$_3$ concentrations ranging from 150 to 300 μM (Fig. 1, A and B). Moreover, the vha-a2 vha-a3 mutant displayed longer primary roots than the wild type when AlCl$_3$ was replaced with (Al)$_2$(SO$_4$)$_3$ with Al$^{3+}$ concentration up to 300 μM (Supplemental Fig. S2), indicating that the resistance in the vha-a2 vha-a3 mutant to Al salts was specifically attributable to Al$^{3+}$ and not to their anions as counterions. In contrast to the Al resistance observed in the vha-a2 vha-a3 mutant, both the vha-a2 single mutant and the vha-a3 single mutant displayed similar growth as the wild type when exposed to 250 μM AlCl$_3$ (Supplemental Fig. S3),
indicating there is functional redundancy between VHA-a2 and VHA-a3 in response to Al³⁺ stress.

**vha-a2 vha-a3 Mutant Roots Contain Less Al**

To determine the mechanisms underlying Al resistance in the *vha-a2 vha-a3* mutant, we measured the Al content in wild-type and *vha-a2 vha-a3* plants using inductively coupled plasma-mass spectrometry (ICP-MS). After treatment with 250 μM Al³⁺ for 3 d, the Al content in the roots of both wild-type and mutant plants was strikingly elevated, and the Al elevation in the wild-type plants was remarkably higher than that in the mutant plants (Fig. 2A). In contrast to the considerable Al accumulation in roots, the Al content in shoots was not significantly altered, and both wild-type and *vha-a2 vha-a3* shoots displayed a similar Al content after treatment with 250 μM Al³⁺ (Fig. 2A). As Al is locally distributed in the root apex (Ryan et al., 1993; Sivaguru and Horst, 1998), we used hematoxylin, a dye shown to stain Al-rich deposits in Al-injured cell walls (Polle et al., 1978; Illés et al., 2006), to assist in the visualization of Al distribution in wild-type and *vha-a2 vha-a3* mutant root tips. After treatment with 150 μM Al³⁺ for 6 h, hematoxylin staining signals strongly expanded in the root-apex regions of the wild type, including the MZ and DTZ (Fig. 2B). By contrast, the signals in the *vha-a2 vha-a3* root apex were largely restricted at the DTZ, with much weaker signals in the remaining regions (Fig. 2B), indicating that the DTZ is still the target site of Al toxicity in *vha-a2 vha-a3* roots. We further used the fluorescence dye morin (2′,3′,4′,5,7-pentahydroxyflavone), which detects Al in the cytosol but neither cell wall bound Al nor vacuole-compartmentalized Al (Eggert, 1970; Larsen et al., 1998; Eticha et al., 2005), to examine internal Al accumulation. In the absence of Al³⁺, the morin fluorescence signals were undetectable in wild-type and *vha-a2 vha-a3* mutant root apices (Fig. 2C). In the presence of 150 μM Al³⁺, the signals abundantly appeared at the distal and proximal regions in the wild-type root apex and were stronger than those in the *vha-a2 vha-a3* mutant root apex (Fig. 2, C and D). Moreover, the DTZ in the *vha-a2 vha-a3* mutant displayed weaker fluorescence signals than the MZ (Fig. 2, C and D), which is opposite to the hematoxylin staining pattern (Fig. 2B), suggesting that there is differential distribution between external and internal Al. Taken together, these results demonstrate that the Al-resistant growth phenotype of the *vha-a2 vha-a3* mutant may be related to less Al accumulation in cell walls and inside the cells of the root apex, which are typical events in Al exclusion.

**V-ATPase and V-PPase Activity Are Repressed upon Al Stress**

The Al tolerance in the *vha-a2 vha-a3* mutant indicates a negative role of VHA-a2 and VHA-a3 in response to Al stress. To test how Arabidopsis plants regulate VHA-a2 and VHA-a3 under Al treatment, we analyzed the effect of Al stress on the expression of these two genes together with the expression of the Al stress genes under Al treatment as 1. Data in (A) and (D) are means ± SD from three independent experiments. Asterisks represent a significant difference between the mutant lines and the wild type under the same culture conditions (Student’s *t* test; ***, *P* < 0.001).
during the first 3 h after treatment and then remained at low levels (Fig. 3A). Remarkably, this down-regulation was specific to Al\(^{3+}\), because other metals, such as Ca\(^{2+}\) and Zn\(^{2+}\), rather up-regulated the expression level of VHA-a2 and VHA-a3 (Fig. 3B). Such changes in VHA-a2 and VHA-a3 transcript levels were parallel to the hypersensitive responses of the vha-a2 vha-a3 mutant to these metals (Supplemental Fig. S1; Krebs et al., 2010; Tang et al., 2015). We further measured the V-ATPase activity in microsomal membranes isolated from wild-type and vha-a2 vha-a3 mutant roots in the absence or presence of 250 \(\mu M\) Al\(^{3+}\). In the absence of Al\(^{3+}\), V-ATPase activity in the vha-a2 vha-a3 mutant was 20\% of that in the wild type (Fig. 3C), consistent with previous results (Krebs et al., 2010; Tang et al., 2012). After treatment with 250 \(\mu M\) Al\(^{3+}\) for 72 h, V-ATPase activity in the wild type was repressed by 20\%, whereas that in the vha-a2 vha-a3 mutant was unaffected (Fig. 3C). These results are consistent with the notion that VHA-a2 and VHA-a3 comprise the major V-ATPase activity, and they demonstrate that V-ATPase activity and its isoforms VHA-a2 and VHA-a3 are down-regulated under Al stress.

We also examined whether another vacuolar H\(^{+}\) pump, namely, V-PPase, is affected by Al stress. Without Al treatment, V-PPase activity was comparable between wild-type and vha-a2 vha-a3 root vacuoles (Fig. 3D), consistent with previous studies (Krebs et al., 2010; Tang et al., 2012). After treatment with 250 \(\mu M\) Al\(^{3+}\), V-PPase activity in wild-type and vha-a2 vha-a3 vacuoles was reduced by 22\% and 53\%, respectively (Fig. 3D). To examine a potential role of V-PPase activity in Al resistance, we isolated a transfer DNA insertion mutant of V-PPase avp1-4 (GK-596F06-025557; Supplemental Fig. S4, A and B; Yang et al., 2018) and found that this mutant displayed a similar phenotype as the wild type when grown under 250 \(\mu M\) Al\(^{3+}\) (Supplemental Fig. S4, C and D). To test whether AVP1 contributes to Al resistance in the vha-a2 vha-a3 mutant, we generated the avp1-4 vha-a2 vha-a3 triple mutant by crossing the avp1-4 mutant with the vha-a2 vha-a3 mutant (Supplemental Fig. S4B) and found that the avp1-4 vha-a2 vha-a3 triple mutant still displayed comparable growth to the vha-a2 vha-a3 mutant in the absence or presence of 250 \(\mu M\) Al\(^{3+}\) (Supplemental Fig. S4, C and D). We also used de-etiolated3 (det3) mutant, a vacuolar ATPase subunit C–defective mutant (Schumacher et al., 1999), and found that det3 mutant exhibited a rate of Al-induced root growth inhibition comparable to that of the wild type (Supplemental Fig. S5). These results suggest that AVP1 and DET3 may not function in Al resistance in Arabidopsis plants.

**Figure 3.** Vacuolar H\(^{+}\) pumps are repressed by Al stress. A and B, RT-qPCR analysis of expression level of VHA-a2 and VHA-a3 genes in 2-week-old wild-type (WT) roots following various metal treatment. The time after which expression levels were measured is as indicated for 150 \(\mu M\) AlCl\(_3\) treatment (A) and 12 h for 30 mM CaCl\(_2\) (Ca), 0.4 mM ZnCl\(_2\) (Zn), 10 mM MgCl\(_2\) (Mg), and 150 \(\mu M\) AlCl\(_3\) (Al) treatment (B). ACTIN2 was used as an internal standard. Data are means ± SD. n = 4. C and D, V-ATPase (C) and V-PPase (D) activity in vacuolar membranes isolated from 5-week-old wild type and vha-a2 vha-a3 mutant (vha-a2 a3) roots treated with 250 \(\mu M\) AlCl\(_3\) treatment for 72 h (250) compared to no treatment (0). Data are shown as percentage of the wild type without Al\(^{3+}\) treatment. Data are means ± SD (n = 3). Values labeled with different letters are significantly different (Student’s t test, P < 0.05).
The PM H\(^+\)-ATPase Is Activated and Contributes to Al Resistance in the vha-a2 vha-a3 Mutant

As AVP1 and DET3 are unlikely to be responsible for Al resistance in the vha-a2 vha-a3 mutant, we wondered whether other H\(^+\) pumps, such as the one located at the PM, might serve as an alternative in this response. To this end, we initially examined H\(^+\)-ATPase activity in PM vesicles isolated from wild-type and vha-a2 vha-a3 roots and found a similar activity in the vesicles from wild-type and vha-a2 vha-a3 plants under control conditions. However, in the presence of 250 \(\mu\)M Al\(^{3+}\), the H\(^+\)-ATPase in wild-type and vha-a2 vha-a3 root PM vesicles was increased by \(-20\%\) and \(40\%\), respectively (Fig. 4A), indicating that Al stress activates the PM H\(^+\)-ATPase particularly when V-ATPase is defective. In Arabidopsis roots, eight AHA genes (AHA1—AHA4, AHA7, AHA8, AHA10, and AHA11) were detected by RT-PCR assay (Ueno et al., 2005). We then analyzed the transcriptional level of these genes in wild-type and vha-a2 vha-a3 roots treated without or with 150 \(\mu\)M Al\(^{3+}\). Among these genes, the expression levels of AHA1, AHA2, and AHA7 in wild-type roots were significantly up-regulated by Al treatment. Compared to that in the wild type, the expression levels of AHA1, AHA2, and AHA7 in the vha-a2 vha-a3 mutant were further increased by 1.6-, 5-, and 1.7-fold, respectively, and expression of the remaining genes was unaffected by Al treatment (Fig. 4B). Further time-course analysis showed that AHA2 transcripts in wild-type and vha-a2 vha-a3 roots were gradually up-regulated after exposure to Al stress, and the expression level in vha-a2 vha-a3 roots was higher than that in wild-type roots after 3 h of the treatment (Fig. 4C). These results demonstrate that, in contrast to V-ATPase and V-PPase, PM H\(^+\)-ATPase activity is increased and could be further activated in vha-a2 vha-a3 roots upon Al stress.

AHA2 is a major isoform of PM H\(^+\)-ATPase in Arabidopsis roots (Harper et al., 1990). Interestingly, we found that AHA2 is the most responsive gene among AHA s to Al stress in the vha-a2 vha-a3 root. We therefore used an AHA2 mutation line, aha2-4, to evaluate the relationship between Al-induced activation of PM H\(^+\)-ATPase and Al resistance. The aha2-4 mutant displayed a similar root growth phenotype as the wild type in
the control conditions. However, the \textit{aha2-4} mutant displayed more severe growth retardation than the wild type under 250, 300, and 350 $\mu$M Al$^{3+}$ treatment (Fig. 4, D and E). Moreover, ICP-MS analysis showed that \textit{aha2-4} roots contained 28% more Al than wild-type roots after treatment with 250 $\mu$M Al$^{3+}$ (Fig. 4F). Consistent with ICP-MS data, morin fluorescence signals in \textit{aha2-4} roots were stronger compared to those in the wild type upon 150 $\mu$M Al$^{3+}$ treatment (Fig. 4G), indicating that AHA2 contributes to Al resistance in Arabidopsis roots.

To validate the role of AHA2 in Al resistance in the \textit{vha-a2 vha-a3} mutant, we constructed the \textit{aha2-4 vha-a2 vha-a3} triple mutant by genetically crossing \textit{aha2-4} with the \textit{vha-a2 vha-a3} mutant and subsequently characterizing its phenotype in the presence of various Al$^{3+}$ concentrations (Fig. 5, A and B). The wild-type, \textit{vha-a2 vha-a3}, and \textit{aha2-4 vha-a2 vha-a3} plants grew comparably on control medium. Under higher Al$^{3+}$ conditions (250, 300, and 400 $\mu$M), the \textit{vha2 vha-a2 vha-a3} triple mutant displayed shorter primary roots than the \textit{vha-a2 vha-a3} mutant, indicating that AHA2 is involved in Al resistance in the \textit{vha-a2 vha-a3} mutant. Notably, this triple mutant still had longer roots than the wild type under these treatments (Fig. 5, A and B), suggesting that

![Figure 5](https://plantphysiol.org)
AHA2 is an important PM H\(^{+}\)-ATPase isoform in the maintenance of root Al resistance when V-ATPase is defective.

To further clarify whether PM H\(^{+}\)-ATPase is a critical linker to the achievement of Al resistance through defective V-ATPase, we applied a PM H\(^{+}\)-ATPase activator and inhibitor to examine their effects on root Al response of the wild type and the \textit{vha-a2 vha-a3} mutant. As shown in Figure 5, C and D, addition of 1 \textmu M fusicoccin, an activator of PM H\(^{+}\)-ATPase (Rasi-Caldogno et al., 1986; Shen et al., 2005; Zhu et al., 2005), rescued the Al-induced inhibition of wild-type root growth, creating a phenotype that was similar to that of the \textit{vha-a2 vha-a3} mutant. On the other hand, addition of 5 \textmu M vanadate, a PM H\(^{+}\)-ATPase inhibitor (Shen et al., 2005), inhibited the root growth of the \textit{vha-a2 vha-a3} mutant and affected Al resistance of the \textit{vha-a2 vha-a3} mutant, creating Al sensitivity as in the wild type (Fig. 5, C and D). Such changes in root growth were relevant to Al response, as both additions did not affect the growth phenotypes of the wild type and the \textit{vha-a2 vha-a3} mutant in the absence of Al\(^{3+}\) (Fig. 5, C and D). We next examined Al accumulation in the wild-type and \textit{vha-a2 vha-a3} mutant roots using fluorescence and ICP-MS analyses. The morin fluorescence analysis showed that 1 \textmu M fusicoccin alleviated, whereas 5 \textmu M vanadate enhanced, the fluorescence intensity, and both treatments consequently caused comparable levels of fluorescence in wild-type and \textit{vha-a2 vha-a3} mutant roots upon Al stress (Fig. 5E). Consistent with the morin fluorescence analysis, ICP-MS analysis showed that 2 \textmu M fusicoccin reduced Al content in the wild type by 41\% (Fig. 5F). In addition, 10 \textmu M vanadate increased Al content in wild-type and \textit{vha-a2 vha-a3} mutant roots by 38\% and 72\%, respectively (Fig. 5F). These results further support the hypothesis that PM H\(^{+}\)-ATPase activity contributes to the defective V-ATPase-induced root Al resistance.

**PM H\(^{+}\)-ATPase Contributes to H\(^{+}\) and Malate Efflux under Al Stress**

The PM H\(^{+}\)-ATPase mediates H\(^{+}\) efflux into the apoplast (Gaxiola et al., 2007; Zhang et al., 2017). To examine whether PM-ATPase activity affects apoplastic pH in wild-type and \textit{vha-a2 vha-a3} mutant roots during Al exposure, we measured the pH value in the non-buffer solutions containing 250 \textmu M Al\(^{3+}\) on which the wild type or the \textit{vha-a2 vha-a3} mutant was grown, and we found that the \textit{vha-a2 vha-a3} mutant caused a more significant pH decrease than the wild type after 3 h of incubation (Supplemental Fig. S6). Next, we used the fluorescent pH indicator 5-hydroxy pyrene-1,3,6-trisulfonic acid disodium salt (HPTS; Barbez et al., 2017) to measure the apoplastic pH. We immersed 7-d-old seedlings after treatment in one-sixth strength Murashige and Skoog solution containing HPTS with initial pH 5.0 for 30 min and selected two different channels with excitation wavelengths of 405 and 458 nm to visualize the protonated and deprotonated forms of HPTS, respectively (Fig. 6A). In the absence of Al\(^{3+}\), the 458/405 ratios of the MZ epidermal cells in the wild-type and the \textit{vha-a2 vha-a3} mutant root apex were 0.36 and 0.28, respectively (Fig. 6). In the presence of 150 \textmu M Al\(^{3+}\), the deprotonated HPTS signals (excitation, 458 nm) in wild-type and \textit{vha-a2 vha-a3} mutant root apices became weaker (Fig. 6A). Moreover, the 458/405 ratios in wild-type and \textit{vha-a2 vha-a3} mutant root apex were reduced to 0.24 and 0.15, respectively (Fig. 6, B and C), suggesting a higher apoplastic pH in the wild type. These results indicate that loss of VHA-a2 and VHA-a3 contributes to root-apex H\(^{+}\) secretion under Al stress.

Given the enhanced H\(^{+}\) efflux through activated PM H\(^{+}\)-ATPase is responsible for root Al resistance, lowered external pH might also enhance root growth as observed in the \textit{vha-a2 vha-a3} mutant. To examine this possibility, we applied nonbuffered mediums with pH values of 4.3, 4.9, 5.5, and 5.8 and compared the growth phenotype of the wild type and the \textit{vha-a2 vha-a3} mutant. Without Al\(^{3+}\) treatment, we observed similar root growth between the wild type and the \textit{vha-a2 vha-a3} mutant under these acidic conditions (Supplemental Fig. S7, A–C). Moreover, in the presence of 250 \textmu M Al\(^{3+}\), the \textit{vha-a2 vha-a3} mutant displayed similar responses to pH 4.3 or pH 4.9 (Supplemental Fig. S7, D–F). We also found that these pH values did not change the differential levels of Al-induced inhibition of root growth and biomass between the wild type and the \textit{vha-a2 vha-a3} mutant (Supplemental Fig. S7, D–F), which is in agreement with the previous observations in bread wheat (Triticum aestivum; Pellet et al., 1997). We supplemented the medium with the pH buffer agent and found that growth, including biomass and primary root length, of the \textit{vha-a2 vha-a3} mutant was still comparable to that of the wild type under pHs ranging from 4.3 to 5.8 (Supplemental Fig. S8). These results demonstrate that an increase in H\(^{+}\) secretion did not mimic the enhanced Al resistance in the \textit{vha-a2 vha-a3} mutant.

In Arabidopsis root Al response, the PM H\(^{+}\)-ATPase functionally bears the exudation of malate and citrate (Zhang et al., 2017), the major Al\(^{3+}\) chelators (Ma et al., 2001). The increase in apoplastic H\(^{+}\) observed in the plants with defective V-ATPase may provide a stronger H\(^{+}\) motive force across the PM, which could facilitate the efflux of cytosolic anions, including malate and citrate. We thus examined the malate and citrate content in wild-type and \textit{vha-a2 vha-a3} root exudates using HPLC analysis (Supplemental Fig. S9). Without Al treatment, \textit{vha-a2 vha-a3} roots exuded a little more malate and citrate than wild-type roots (Fig. 7, A and C). After exposed to 250 \textmu M Al\(^{3+}\), however, \textit{vha-a2 vha-a3} roots secreted more malate and citrate (1.8- and 1.6-fold, respectively) than the wild-type roots (Fig. 7, A and C). To verify the correlation of these OAs exudation with the PM H\(^{+}\)-ATPase activity, we supplemented with the PM H\(^{+}\)-ATPase inhibitor vanadate and found that 10 \textmu M vanadate treatment inhibited malate and citrate content in \textit{vha-a2 vha-a3} root exudates by 70\% and 53\%, respectively, and diminished...
the different degrees in these OAs contents between wild-type and vha-a2 vha-a3 roots under Al stress (Fig. 7, A and C), suggesting that the activation of PM H^+-ATPase activity contributes to the increase in root OAs excretion upon Al stress.

We further examined whether this root OAs excretion is involved in Al resistance in the vha-a2 vha-a3 mutant using exogenous application of malate and citrate. Although 10 μM malate and 20 μM citrate did not affect the normal growth of the wild type and the vha-a2 vha-a3 mutant, they rescued Al-induced primary root growth inhibition in the wild type by 30% and 23% (Fig. 7, E and F). In addition, the root growth of the wild type was similar to that of the vha-2 vha-a3 mutant under these treatments (Fig. 7, E and F). We also used OAs transport inhibitors niflumic acid (NIF) and anthracene-9-carboxylic acid (9-AC), which have been found to efficiently inhibit Al-induced root malate and citrate exudation (Osawa and Matsumoto, 2002; Zhu et al., 2005; Wang et al., 2007), on the wild type and vha-a2 vha-a3 mutant. Under control conditions, 0.1 μM NIF and 0.2 μM 9-AC did not significantly affect the growth of the wild type and the vha-a2 vha-a3 mutant. When combined with 250 μM Al^{3+}, however, both inhibitors diminished the Al-resistant growth of the vha-a2 vha-a3 mutant, resulting in comparable root length between the wild type and the vha-a2 vha-a3 mutant (Fig. 7, E and F). Together, these results indicate that the increases in OAs levels released from vha-a2 vha-a3 roots contribute to the enhanced Al resistance of the vha-a2 vha-a3 mutant.

Defective V-ATPase–induced Al Resistance Is Independent of OA Synthesis and Tonoplast-Localized ALS3 and STAR1

To determine whether the increases in malate and citrate in vha-a2 vha-a3 root extrudes come from Al-induced OAs synthesis, we examined the RNA levels of malate
and citrate metabolism genes, including peroxisomal NAD-malate dehydrogenase 1 (PMDH1), mitochondrial malate dehydrogenase 1 (MMDH1), MMDH2, malate dehydrogenase 1 (MDH1), citrate synthase 1 (CSY1), and CSY3 in the wild type and the vha-a2 vha-a3 mutant. RT-qPCR analysis showed that although the transcription levels of most genes, including PMDH1, MMDH1, MMDH2, CSY1, and CSY3, but not MDH1, were up-regulated, their levels were comparable between wild-type and vha-a2 vha-a3 roots after Al stress (Supplemental Fig. S10). These results suggest that further increases in OAs within vha-a2 vha-a3 root extrude are independent of Al-induced capacities of malate and citrate synthesis, consistent with previous results obtained from Al-sensitive and Al-resistant triticale genotypes (Hayes and Ma, 2003).

In mature plant cells, the vacuoles occupy as much as 90% of the cellular volume and contain the major proportions of malate and citrate (Marty, 1999; Meyer et al., 2010). A defect in V-ATPase may impair vacuolar malate and citrate sequestration, because this process requires a well-established H⁺ gradient across the tonoplast (Sze et al., 1999; Schumacher and Krebs, 2010; Seidel et al., 2013). To check this hypothesis, we measured internal malate and citrate content in the wild type and the vha-a2 vha-a3 mutant without or with 250 μM Al³⁺ treatment. The wild type and the vha-a2 vha-a3 mutant had similar content of basal malate and citrate without Al³⁺ treatment. In the presence of Al³⁺, however, the vha-a2 vha-a3 mutant contained a similar malate content and less citrate content compared to that in the wild type after Al³⁺ treatment (Fig. 7, B and D), implying that vacuolar OAs storage is impaired in the vha-a2 vha-a3 mutant. To test this idea, we examined the expression patterns of ALS3 and STAR1, two important tonoplast-localized transporters that function in Al tolerance (Larsen et al., 1998, 2005; Huang et al., 2010; Dong et al., 2017; Wang et al., 2019), and we found that neither of these had significantly different transcripational levels between the wild type and the vha-a2 vha-a3 mutant upon Al treatment (Supplemental Fig. S11, B and C). To further examine the role of ALS3 and STAR1, we performed genetic analysis of als3 and star1 mutant plants with defective V-ATPase activity induced by concanamycin A (ConcA), a V-ATPase activity inhibitor (Supplemental Fig. S11; Gaxiola et al., 2007). Under control growth conditions, the wild type and als3 and star1 mutants grew similarly and were not affected by

**Figure 7.** Al stress redistributes malate and citrate for Al resistance. A and C, Malate (A) and citrate exudation (C) from 5-week-old wild-type (WT) and vha-a2 vha-a3 mutant (vha-a2 a3) plants incubated in normal solutions (control) or in solutions containing 250 μM AlCl₃ (Al³⁺) or 250 μM AlCl₃ and 20 μM vanadate (Al³⁺ + VA) for 72 h. Data are mean ± so (n = 3). B and D, Malate (B) and citrate content (D) in 5-week-old wild type and vha-a2 a3 roots incubated in solutions without (control) or with 250 μM AlCl₃ (Al³⁺) for 72 h. Data are mean ± so (n = 3). E and F, Growth phenotype (E) and relative root length (F) of 11-d-old wild type and vha-a2 a3 seedlings grown under normal conditions (control) or experimental conditions supplemented with 10 μM malate (malate), 20 μM citrate (citrate), 250 μM AlCl₃ (Al³⁺), 10 μM malate and 250 μM Al³⁺ (malate + Al³⁺), 20 μM citrate and 250 μM Al³⁺ (citrate + Al³⁺), 0.1 μM NIF, 0.2 μM 9-AC, 250 μM Al³⁺ and 0.1 μM NIF (NIF + Al³⁺), or 250 μM Al³⁺ and 0.2 μM 9-AC (9-AC + Al³⁺). Bars = 1 cm. Relative root length was normalized to the wild type without Al³⁺ treatment set as 100%. Data are means ± so of three independent plant samples in three replicates. Values in (A–D) and in (E) labeled with different letters are significantly different analyzed with one-way ANOVA (Student’s t test, P < 0.05) and two-way ANOVA (Tukey’s HSD test, P < 0.05), respectively.
the addition of 5 nM ConcA. As reported previously by Larsen et al., 2005, 2007; Huang et al., 2010, primary root growth in als3 and star1 mutant plants was more severely inhibited by 250 μM Al3+ compared to in the wild type. However, Al-induced inhibition of primary root growth of the wild type, als3, and star1 was rescued by ConcA applied at a similar level by ~40% (Supplemental Fig. S11, D and E). Therefore, these data not only confirm the notion that a defect in V-ATPase contributes to Al resistance but also demonstrate that ALS3 and STAR1 are not involved in Al resistance related to V-ATPase activity.

PM-Localized ALMT1 Is Activated to Mediate Al Resistance Caused by Defective V-ATPase

The finding of elevated secretion of malate and citrate in vha-a2 vha-a3 mutant root exudates suggests a connection between the PM-localized transporters, namely, malate transporter ALMT1 and citrate transporter MATE, and Al resistance in the vha-a2 vha-a3 mutant. We therefore assessed the expression patterns of ALMT1 and MATE genes under Al stress and found that these two genes in wild-type roots were significantly up-regulated in a time-dependent manner after exposure to 250 μM Al3+ (Fig. 8A; Supplemental Fig. S11A). The up-regulation of ALMT1 expression was further elevated in vha-a2 vha-a3 mutant roots (Fig. 8A). We also analyzed the transcript levels of Sensitive to Proton Rhizotoxicity1 (STOP1) and WRKY (amino acid sequences)-type transcription factor WRKY46, two well-characterized transcription factors that up- and down-regulate ALMT1 gene expression, respectively (Iuchi et al., 2007; Ding et al., 2013). We found that the vha-a2 vha-a3 mutant had a higher transcript level of STOP1 and a lower transcript level of WRKY46 than that in the wild type under Al stress (Fig. 8, B and C), findings that are consistent with the higher level of ALMT1 in the vha-a2 vha-a3 mutant compared with that in the wild type.

We next evaluated whether ALMT1 contributes to the Al resistance caused by defective V-ATPase by using the ALMT1 insertional knockout mutant line almt1 (SALK_00962). As reported previously (Hoekenga et al., 2006), almt1 displayed a hypersensitive root growth with 250 μM Al3+ treatment compared with the wild type (Fig. 8, D and E). Accordingly, Al-induced morin fluorescence signals in almt1 mutant root tips were stronger than those in wild-type root tips (Fig. 8, F and G). We then examined the effect of ConcA on wild-type and almt1 mutant responses to Al stress. We found that 5 nM ConcA rescued root growth inhibition by 40% and reduced the morin fluorescence signals by 32% in the wild type treated with 250 μM Al3+ (Fig. 8, D–G). In contrast with the changes in the wild type, the Al-induced root growth inhibition and morin fluorescence signals in almt1 mutant root tips were only slightly affected by the ConcA treatment (Fig. 8, D–G), implying that ALMT1 is an important transporter required for defective V-ATPase–induced Al resistance.

Disturbance of Malate Efflux across the PM Overrides Al-Inhibited Expression of VHA-a2 and VHA-a3

Cytosolic OAs may be sequestered into either the apoplastic space or the vacuole lumen. Our finding that the Al-induced suppressed V-ATPase activity activated ALMT1 led us to explore what may happen to V-ATPase activity when OAs efflux through the PM is impaired. To this end, we added 9-AC or vanadate, which inhibits OAs exudation from the roots (Fig. 7; Zhu et al., 2005; Wang et al., 2007) and diminishes the Al resistance in the vha-a2 vha-a3 mutant (Fig. 7), to the experimental treatment without or with 250 μM Al3+. As shown in Figure 9A, addition of 0.2 μM 9-AC or 5 μM vanadate alone weakly increased the levels of VHA-a2 and VHA-a3 transcript. When combined with 250 μM Al3+; however, addition of 9-AC overrode the Al-induced inhibition of transcript levels of both VHA-a2 and VHA-a3, which were dramatically up-regulated more than 10-fold compared with their expression levels under Al3+ or 9-AC treatment (Fig. 9A). In addition, such an override also occurred in the presence of 5 μM vanadate but to a much lesser extent (Fig. 9A). On the other hand, addition of 0.2 μM 9-AC or 5 μM vanadate severely reduced Al-induced up-regulation of ALMT1 expression (Fig. 9C). To further investigate the relationship between these Al-induced changes, we examined whether the ALMT1 mutation affects the expression patterns of VHA-a2 and VHA-a1 and found that both VHA-a2 and VHA-a3 transcripts were expressed at similar levels in the wild type and almt1 mutant without Al3+ treatment. However, the transcript levels of both genes in the almt1 mutant were much higher than those in the wild type after exposure to 250 μM Al3+ (Fig. 9B). Together, these results suggest that this Al-induced stimulation of V-ATPase activity may operate as a backup mechanism if the external Al exclusion is lost, which in turn enhances OAs or Al-OA complexes storage in the vacuoles, leading to internal Al tolerance.

Tolerance of Low Pi or Iron Toxicity Is Unlikely to Contribute to the Al Resistance in the vha-a2 vha-a3 Mutant

High concentrations of Al3+ in the external medium may cause phosphorus deficiency, which subsequently inhibits primary root growth (Kochian et al., 2004). We thus assessed whether the Al resistance in the vha-a2 vha-a3 mutant is related to a tolerance of low Pi stress. We first measured Pi content in wild-type and vha-a2 vha-a3 mutant plants using ICP-MS analysis and found no difference in the Pi content of roots and shoots of both plants grown under conditions without or with 250 μM Al3+ (Supplemental Fig. S12A). Notably, addition of Al3+ slightly increased Pi content in roots but did not significantly affect Pi content in shoots (Supplemental Fig. S12A). Together, these results suggest that enhanced Al resistance in the vha-a2 vha-a3 mutant is unlikely to be
a result of changes in low-Pi stress tolerance. To evaluate this possibility, we analyzed the expression patterns of genes involved in low-Pi resistance, including LOW PHOSPHATE ROOT1 (LPR1), NITROGEN LIMITATION ADAPTATION (NLA), PHOSPHATE TRANSPORTER1;1 (PHT1;1), PHT1;4, and transcription factor WRKY45 (Shin et al., 2004; Stefanovic et al., 2007; Svistoonoff et al., 2007; Lin et al., 2013; Wang et al., 2014). RT-qPCR data indicated that the expression levels of most of the tested genes were similar in the wild type and vha-a2 vha-a3 roots under 250 μM Al3+ treatment, except for PHT1;4 (Supplemental Fig. S12B), suggesting a comparable Pi response between the wild type and the vha-a2 vha-a3 mutant. To examine whether Al resistance in the vha-a2 vha-a3 mutant depends on Pi doses, we performed phenotypic analysis of the wild type and vha-a2 vha-a3 mutant grown under conditions of 250 μM Al3+ with various Pi levels (Supplemental Fig. S12, C and D). On medium with a high, normal, and low level of Pi (2 mM, 200 μM, and 20 μM, respectively), vha-a2

Figure 8. Defective V-ATPase activates a malate efflux system responsible for Al resistance. A to C, RT-qPCR assay of ALMT1 (A), STOP1 (B), and WRKY46 (C) transcripts in roots of 2-week-old wild-type (WT) and vha-a2 vha-a3 mutant (vha-a2 a3) plants following Al3+ treatment for the times as indicated in (A) or for 12 h (B and C). ACTIN2 was used as the internal reference gene. D and E, Growth phenotype (D) and relative root length (E) of 11-d-old wild type and almt1 mutant plants grown under normal conditions (control) or with 5 mM ConcA, 250 μM Al3+ (Al3+), or 5 mM ConcA and 250 μM Al3+ (ConcA + Al3+); F and G, Morin fluorescence signal (F) and relative fluorescence intensity (G) in 7-d-old wild type and almt1 mutant root tips incubated in solutions without (control) or with 150 μM AlCl3 (Al3+) or 5 mM ConcA and 150 μM Al3+ (ConcA + Al3+) for 6 h. Relative fluorescence was normalized to the wild type without Al3+ treatment set as 1. Data in (A–C) and (D) are mean ± SD (n = 3). Bars = 1 cm (D) and 50 μm (F). Relative root length in (E) was normalized to wild type without Al3+ treatment set as 1, and data are means ± sd of three independent plant samples in three replicates. Asterisks in (A) indicate statistically significant differences compared with the wild type (Student’s t test; ***, P < 0.001), and values labeled with different letters in (B), (C), and (G) are significantly different (Student’s t test, P < 0.05). Values labeled with different letters in (E) are significantly different analyzed with two-way ANOVA (Tukey’s HSD test, P < 0.05).
vha-a3 mutant and wild-type plants grew similarly. When combined with 250 μM Al3+, these Pi levels did not abolish the vha-a2 vha-a3 mutant phenotype of longer primary root compared to the wild type, further supporting our earlier hypothesis that enhanced Al resistance in the vha-a2 vha-a3 mutant is not dependent on tolerance of Pi deficiency.

The over-accumulation of iron (Fe) in root is a critical factor in Pi deficiency-induced inhibition of primary root growth (Ward et al., 2008; Müller et al., 2015; Balzergue et al., 2017; Dong et al., 2017; Mora-Macías et al., 2017; Godon et al., 2019; Wang et al., 2019). To determine whether Al resistance in the vha-a2 vha-a3 mutant may rather result from a tolerance of Fe toxicity, we measured the Fe content and analyzed Fe accumulation patterns in wild-type and vha-a2 vha-a3 mutant roots. Similar to results reported previously by Tang et al. (2012), our ICP-MS data showed that Fe content in vha-a2 vha-a3 roots was less than that in the wild type under normal conditions. However, in the presence of 250 μM Al3+, the Fe content in the vha-a2 vha-a3 mutant was nearly 40% higher than that in the wild type (Supplemental Fig. S13 A). Such changes appear to exclude the possibility that enhanced Al resistance in the vha-a2 vha-a3 mutant results from internal Fe deficiency. Moreover, the staining patterns detected by Perls/diaminobenzidine staining method, which stains Fe2+ and Fe3+ accumulation in the root (Roschttardtz et al., 2009; Dong et al., 2017), were similar between wild-type and vha-a2 vha-a3 root tips treated without or with 250 μM Al3+ (Supplemental Fig. S13 B), indicating similar extracellular Fe distribution in roots of the two genotypes. Although the exact role of changes in Fe accumulation needs further clarification, these results demonstrate that the Al-resistance growth phenotype of vha-a2 vha-a3 does not result from a tolerance of Fe stress.

DISCUSSION

Vacuoles are the main storage site for the majority of cellular ions and metabolites and are crucial for detoxification and nutrient homeostasis (Marty, 1999; Schumacher and Krebs, 2010). Vacuoles utilize the H+ electrochemical gradient across the tonoplast energized by vacuolar H+-pumps to regulate ions distribution (Szé et al., 1999). A defect in V-ATPase activity induced by VHA-a2 and VHA-a3 mutation causes a severely stunted growth phenotype, which largely results from impaired nutrient homeostasis crucial for plant development, including essential metal mineral nutrients, such as K+, Ca2+, and Zn2+ (Krebs et al., 2010; Tang et al., 2012). In this study, we found that, upon excessive Al3+, the vha-a2 vha-a3 mutant displayed an Al-resistance growth phenotype with longer primary roots, whereas vha-a2 and vha-a3 single mutants did not show discernible difference in Al resistance compared to wild-type plants (Fig. 1; Supplemental Fig. S1). Such phenotypes were only observed in the vha-a2 vha-a3 mutant, whereas defects in other vacuolar H+ pumps in Arabidopsis plants, including avp1-4 and det3 mutants, resulted in similar Al sensitivity as the wild type (Supplemental Figs. S4 and S5). Moreover, although V-PPase activity was reduced by Al stress when combined with defective V-ATPase (Fig. 3D), we found that V-PPase is not responsible for the Al resistance in the vha-a2 vha-a3 mutant, because AVP1 mutation did not alter vha-a2 vha-a3 mutant responses to Al3+ stress (Supplemental Fig. S4). We thus conclude that VHA-a2 and VHA-a3 are the dominant vacuolar H+ pump subunits and function redundantly in mediating Al-induced inhibition of primary root growth. Consistent with the notion that V-ATPase functions in the vacuolar sequestration of metals (Krebs et al., 2010; Tang et al., 2012), our results also showed that the vha-a2 vha-a3
mutant had less Al content in roots and similar Al content in shoots compared with that of the wild type following exposure to Al stress. Moreover, vha-a2 vha-a3 mutant roots accumulated less Al, including a decrease in cytosol Al content and reduced Al distribution in the cell wall of the root-apex MZ part (Fig. 2). Our results thus reveal that the Al-resistance growth phenotype of the vha-a2 vha-a3 mutant is unlikely achieved by internal Al tolerance.

Several findings in this study support that Al resistance induced by defective V-ATPase is related to Al exclusion, which is mainly achieved by the secretion of OAs from roots to chelate external Al (Kochian, 1995; Ma, 2007). First, malate and citrate are major and moderate contributors, respectively, in Arabidopsis root exclusion, which is mainly achieved by the secretion of malate and citrate phenocopied the effects of VHA-a2 and VHA-a3 to drive malate and citrate flux in response to Al stress. In addition to chelation of external Al³⁺ (Kochian et al., 2004; Ma, 2007), the protection provided by released OAs on active sites in the cell wall from Al binding is considered as another efficient strategy to increase Al resistance (Li et al., 2009). Interestingly, Al-induced callose accumulation and hematoxylin stain were weakened in the root-apex MZ of the vha-a2 vha-a3 mutant compared with those in the wild type, whereas they were similar in the root-apex DTZ of these plants (Figs. 1C and 2B), which is the target site of Al toxicity in roots. The local distribution patterns indicate that enhanced Al resistance in vha-a2 vha-a3 roots may be related to the protection of cell walls located at the MZ from Al³⁺ binding facilitated by secretion of malate and citrate. Second, exogenous application of malate and citrate phenocopied the effects of VHA-a2 and VHA-a3 mutation on root growth under Al stress (Fig. 7). Third, consistent with the role of VHA-a2 and VHA-a3 mutation, addition of ConcA, a vacuolar H⁺ pump inhibitor, enhanced Al resistance in the wild type. However, this addition did not rescue Al-induced inhibition of primary root growth of the almt1 mutant (Fig. 8), suggesting that ALMT1 plays a vital role in defective V-ATPase-induced Al resistance. Moreover, the vha-a2 vha-a3 mutant displayed enhanced up-regulation of ALMT1 and MATE expression under Al stress, accompanied by an increase in STOP1 expression and a decrease in WRKY46 expression (Fig. 8; Supplemental Fig. S11A). Such responses were reported to contribute to a more active PM-localized transport system responsible for root malate and citrate excretion (Hoekenga et al., 2006; Kobayashi et al., 2007; Liu et al., 2009). In addition, analysis of the expression patterns of ALS3 and STAR1 genes and the growth phenotypes of als3 and star1 mutants upon Al stress demonstrated that these two tonoplast-localized proteins are not required for defective V-ATPase–induced Al resistance, although they are crucial for Al resistance (Larsen et al., 2005, 2007; Huang et al., 2010; Dong et al., 2017; Wang et al., 2019). Finally, we proposed that Al resistance in the vha-a2 vha-a3 mutant is not related to tolerance of Pi deficiency, because limitation of Pi supply did not phenocopy nor abolish the longer primary root phenotype observed in the vha-a2 vha-a3 mutant in response to Al stress (Supplemental Fig. S12). Importantly, after exposure to Al toxicity, neither Pi content in roots and shoots nor the expression levels of representative low-Pi responsive genes of vha-a2 vha-a3 plants were higher than those of wild-type plants (Supplemental Fig. S12). In addition, Al-induced changes in Fe accumulation in vha-a2 vha-a3 mutant root tips, namely, higher internal Fe content and similar external Fe distribution pattern compared with that of the wild type (Supplemental Fig. S13), do not appear to agree with the notion that higher Fe levels contribute to root growth inhibition in Pi-deprived plants (Ward et al., 2008; Müller et al., 2015; Balzergue et al., 2017; Dong et al., 2017; Mora-Macias et al., 2017; Godon et al., 2019; Wang et al., 2019). Since Pi and Fe play important roles in root Al responses, such changes in Pi and Fe demand more in-depth examination to determine how these two ions regulate vha-a2 vha-a3 mutant responses to Al stress. Taken together, these results suggest that V-ATPase is suppressed to promote OAs exudation to detoxify external Al³⁺ and not for vacuolar Al sequestration. Although the central importance of OAs in external Al detoxification has long been recognized (Kochian et al., 2004; Ma, 2007; Meyer et al., 2010), the exact source of a burst of OAs in the root exudates after exposure to Al stress remains to be fully understood. In our study, we also collected data to determine the mechanisms underlying Al-induced elevated secretion of malate and citrate in vha-a2 vha-a3 mutant root exudates. We found that the transcriptional abundance of malate and citrate metabolism genes was similar between the wild type and the vha-a2 vha-a3 mutant (Supplemental Fig. S10). Moreover, vha-a2 vha-a3 mutant roots contained no more malate and citrate content than that in wild-type roots under Al treatment, although elevated secretion of malate and citrate was observed in vha-a2 vha-a3 mutant root exudates (Fig. 7). These results are consistent with the notion that Al-induced elevated secretion of malate and citrate in root exudates poorly associates with internal OAs metabolism (Hayes and Ma, 2003). In plants, the majority of malate and citrate is typically stored in vacuoles, where they function in osmoregulation and as counterions (Martinoia and Rentsch, 1994; Marty, 1999; Meyer et al., 2010), alongside a H⁺ electrochemical gradient across the tonoplast that is predominantly maintained by V-ATPase (Sze et al., 1999; Gaxiola et al., 2007; Schumacher and Krebs, 2010). On the other hand, we found that Al-induced OAs secretion was coupled with dramatic decreases in VHA-a2 and VHA-a3 expression. These decreases could significantly reduce OAs storage in the vacuoles as previously reported in maize (Zea mays) roots (Garzón et al., 2011). In this context, vha-a2 vha-a3 mutant roots lost the major V-ATPase activity (Fig. 3; Krebs et al., 2010; Tang et al., 2012),
suggesting that OAs transport into the vacuoles is severely impaired and that alternative pathways are required for these OAs to efflux. Indeed, we found that Al-induced up-regulation of ALMT1 and MATE was further enhanced when VHA-a2 and VHA-a3 were disrupted (Fig. 8; Supplemental Figure S11). These data also demonstrate that defective V-ATPase not only inhibits OAs transport into the vacuoles but also enhances OAs flux into the apoplastic space. Interestingly, we also found that ALMT1 mutation and addition of anion transporter inhibitor 9-AC, which damage OAs transport across the PM, overrode Al-induced suppression of VHA-a2 and VHA-a3 expression (Fig. 9). These results thus reveal an antagonistic relationship between cytosolic OAs transport across these two membrane systems. As a result, during Al toxicity-induced root growth inhibition, Arabidopsis roots strategically suppress vacuolar H\(^+\)-ATPase activity to reduce OAs storage in the vacuoles, and this coupled with increases in PM-localized OAs transporter activity thereby redirects sufficient intracellular OAs into the apoplast.

Another important factor for cytosol-apoplast OAs transport is a well-established H\(^+\) gradient across the PM, which is largely maintained by AHA-type H\(^+\)-ATPase (Zhang et al., 2017). Although low pH values were found to up-regulate ALMT1 transcripts (Kobayashi et al., 2013; Godon et al., 2019) and mediate OAs secretion from the roots (Pellet et al., 1997; Degenhardt et al., 1998; Larsen et al., 1998; Shen et al., 2005), the mechanisms underlying root growth regulation by PM H\(^+\)-ATPase under Al stress remain largely unknown. Here, we defined the role of PM H\(^+\)-ATPase in Al-induced inhibition of root growth by regulating OAs flux. We found that PM H\(^+\)-ATPase activity was activated upon Al treatment and that this activation was further enhanced when V-ATPase was defective. For example, compared to the wild type, vha-a2 vha-a3 mutant roots displayed an increase in several characteristics associated with enhanced PM H\(^+\)-ATPase activity, including higher H\(^+\) pump activity in root PM vesicles, higher expression levels of AHA1, AHA2, and AHA7 (Fig. 4), and more H\(^+\) exudation in the apoplast (Supplemental Fig. S6). In addition, visualization of local H\(^+\) distribution by fluorescent pH indicator HPTS indicated that increased H\(^+\) exudation was largely detected in the vha-a2 vha-a3 root-apex meristem part (Fig. 6), which is consistent with the observation that this part accumulated less Al (Fig. 2). Further genetic analysis of plants with altered PM H\(^+\)-ATPase activity showed that the increase in PM H\(^+\)-ATPase activity contributed to the enhanced Al resistance in vha-a2 vha-a3 mutants (Figs. 4 and 5). After determining that this increase is not sufficient to confer Al resistance (Supplemental Figs. S7 and S8), we examined possible changes in OAs transport in plants with altered PM H\(^+\)-ATPase activity. We found that, upon Al treatment, changes in malate and citrate content in vha-a2 vha-a3 root exudates positively correlated with the activity of PM H\(^+\)-ATPase. Furthermore, addition of the PM H\(^+\)-ATPase inhibitor vanadate reduced the secretion of malate and abolished the Al resistance of the vha-a2 vha-a3 mutant (Figs. 5 and 7). Interestingly, the response patterns of PM H\(^+\)-ATPase to Al-induced suppression of V-ATPase activity were similar to those of ALMT1 (Figs. 3 and 8), suggesting that PM H\(^+\)-ATPase and ALMT1 function downstream of defective V-ATPase–associated signaling. We thus propose that activated PM H\(^+\)-ATPase induces an enhanced apoplastic H\(^+\) gradient across PM that, in turn, coordinates with PM-localized transporters to efficiently deliver intracellular OAs into the apoplastic space to maintain root growth upon Al treatment.

In conclusion, the results of this study have uncovered a previously unrecognized Al exclusion mechanism facilitated by OAs allocation by vacuolar-type H\(^+\)-ATPase. As central intermediates in plant metabolism, malate and citrate are strictly synthesized and well distributed in the vacuoles. On the other hand, root internal malate and citrate have been found to be readily mobilized into soils when plants are exposed to multiple environmental stimuli (Meyer et al., 2010). Integrating our data, we proposed a model in which tonoplast- and PM-localized OAs transport systems antagonistically function in determining external Al exclusion or internal Al tolerance (Fig. 10). This model shows that, in response to Al stress, AHAs, ALMT1, and MATE transcripts are preferentially activated over
Arabidopsis Stock Centre. The mutants Center, and the mutant (Sigma-Aldrich). The pH was buffered by 2 mM Homo-PIPES (Sigma-Aldrich) Murashige and Skoog or one-sixth strength Murashige and Skoog medium Table S1, and the F3 and later generations were used for analyses. the mutant and then screened by PCR using the primers listed in Supplemental and Supplemental Table S1. For on-plate growth assays, seeds were sterilized with 75% (v/v) ethanol for 5 min, washed in sterilized water three times, and sown on half-strength Murashige and Skoog or one-sixth strength Murashige and Skoog medium containing 1% (w/v) Suc (Sigma-Aldrich) and solidified with 1% (w/v) agar (Sigma-Aldrich). The pH was buffered by 2 mM Homop-PIPES (Sigma-Aldrich) as described previously by Hoesken et al. (2006) to 4.5, unless otherwise stated. To examine effects of Pi, the Pi concentration in the normal medium was increased to 2 mM or decreased to 20 μM. The plates were incubated at 4°C in darkness for 2 d and then were positioned vertically. On day 11 after germination, the seedlings were imaged to measure primary root lengths. After 3 d of treatment, roots of the plants were collected to prepare tonoplast vesicles as described previously (Tang et al., 2012). Wavelengths of the UV spectrum were detected as described in a previous study (Barbez et al., 2017). Image analysis was performed using the Fiji software (Barbez et al., 2017). Measurements of Apoplastic pH Values To measure the pH in the solutions, 36 5-week-old seedlings were transferred into 400 mL of one-sixth strength Murashige and Skoog solutions with initial pH 5.4 supplemented with 250 μM AlCl3. After treatment, solutions were collected for pH measurement by pH indicator (FE28, Mettler Toledo). To detect apoplastic H+ secretion, HPTS staining was used to visualize apoplastic pH as described previously (Barbez et al., 2017), with modification. Briefly, 7-d-old Arabidopsis seedlings were transferred into one-sixth strength Murashige and Skoog solutions, pH 5.0, without or with 150 μM AlCl3, for 6 h and then incubated in the one-sixth strength Murashige and Skoog solutions, pH 5.0, containing 1 mM HPTS for 30 min. After rinsing with water three times, the roots of seedlings were observed under a confocal microscope (LSM-710, Leica). The protonated HPTS form (excitation, 405 nm; emission peak, 514 nm) and deprotonated HPTS form (excitation, 458 nm; emission peak, 514 nm) were detected as described in a previous study (Barbez et al., 2017). Image analysis was performed using the Fiji software (fiji.sc). The experiments described were performed in at least three biological repetitions. Measurements of Malate and Citrate Content Root malate and citrate exudation was detected as described previously by Ding et al. (2013). Briefly, 36.5-week-old Arabidopsis seedlings were transferred into 400 mL of one-sixth strength Murashige and Skoog solutions, pH 4.5, without or with 250 μM AlCl3. After 3 d of incubation, the solutions were
collected and passed through cation- and anion-exchange columns filled with 10 g of resin-001X and 5 g of Dowex 1×8 chloride form (100–200-μm mesh, Sigma-Aldrich). After elution with 10 mL of 1 M HCl, the eluate was concentrated in a rotary evaporator at 40°C. The residue was redissolved in 1 mL of deionized ultrapure water and was used to detect malate and citrate concentration by HPLC (model 1200SL, Agilent Technologies).

To measure root internal malate and citrate content, 5-week-old plants were incubated in one-sixth strength Murashige and Skoog solutions, pH 4.5, with or without 250 μM AlCl₃. After 3 d of incubation, the roots were collected to prepare crude samples. Two grams of roots was ground at 4°C with 10 mL of ethanol or with 250 μL HCl, and the residue was redissolved in 1 mL of deionized ultrapure water and centrifuged at 4,950 g.

**Supplemental Table S2.** Phenotypic analysis of 

*wrky45* (AT3G01970); *wrky46* (AT2G46400).

**Supplemental Table S1.** Primers used in this study.

**Supplemental Table S2.** Statistical analysis tables.

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**LITERATURE CITED**


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