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Phosphorylation at S384 regulates the activity of the TaALMT1 malate transporter that underlies aluminum resistance in wheat

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SUMMARY

In this study we examined the role of protein phosphorylation/dephosphorylation in the transport properties of the wheat (*Triticum aestivum*) root malate efflux transporter underlying Al resistance, TaALMT1. Pre-incubation of *Xenopus laevis* oocytes expressing TaALMT1 with protein kinase inhibitors (K252a and staurosporine) strongly inhibited both basal and Al^{3+}-enhanced TaALMT1-mediated inward currents (malate efflux). Pre-incubation with phosphatase inhibitors (okadaic acid and cyclosporine A) resulted in a modest inhibition of the TaALMT1-mediated currents. Exposure to the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA), enhanced TaALMT1-mediated inward currents. Since these observations suggest that TaALMT1 transport activity is regulated by PKC-mediated phosphorylation, we proceeded to modify candidate amino acids in the TaALMT1 protein in an effort to identify structural motifs underlying the process regulating phosphorylation. The transport properties of eight single point mutations (S56A, S183A, S324A, S337A, S351-352A, S384A, T323A and Y184F) generated in amino acid residues predicted to be phosphorylation sites and examined electrophysiologically. The basic transport properties of mutants S56A, S183A, S324A, S337A, S351-352A, T323A and Y184F were not altered relative to the wild-type TaALMT1. Likewise the sensitivity of these mutants to staurosporine resembled that observed for the wild-type transporter. However, the mutation S384A was noticeable, as in oocytes expressing this mutant protein TaALMT1-mediated basal and Al-enhanced currents were significantly inhibited, and the currents were insensitive to staurosporine or PMA. These findings indicate that S384 is an essential residue regulating TaALMT1 activity via direct protein phosphorylation, which precedes Al^{3+} enhancement of transport activity.

Keywords: Aluminum tolerance, malate transporter, protein phosphorylation/dephosphorylation, site-directed mutagenesis, TaALMT1, TEVC, *Xenopus* oocyte.

INTRODUCTION

Aluminum (Al)-toxicity is the primary factor limiting plant growth and, ultimately, crop productivity on acidic soils that comprise up to 50% of the world's potential arable lands. Many of these acid soils are located in the developing countries of the world where the ever-growing population pressure on agricultural land is very high, leading to continuing food insecurity in those regions. When the soil pH falls below 5.0, soluble Al in the soil solution exists predominantly as the toxic trivalent cation Al^{3+} that can inhibit root growth in many species, even at low micromolar concentrations (Kochian *et al.*, 1995). In response, both monocotyledonous and dicotyledonous plant species have developed a unique mechanism of Al resistance that involves the release of Al-chelating organic anions such as citrate, malate and oxalate from the roots, resulting in the chelation and exclusion of phytotoxic forms of Al from the root tip (Miyasaka *et al.*, 1991; Delhaize *et al.*, 1993; Kochian, 2005; Ryan *et al.*, 1995; Ma *et al.*, 1997).

Electrophysiological studies in protoplasts isolated from root tips of Al-resistant wheat (*Triticum aestivum*; Ryan *et al.*, 1997; Zhang *et al.*, 2001) and maize (*Zea mays*; Kollmeier *et al.*, 2001; Piñeros and Kochian, 2001; Piñeros *et al.*, 2002) led to the discovery and characterization of plasma membrane anion-permeable channels which were specifically activated by extracellular Al^{3+}. The remarkable similarities between the transport properties of these

channels and the Al-activated malate and citrate exudation responses reported in intact roots strongly indicated that this type of transporter was probably mediating the release of Al-activated organic acid observed at the whole root level. The first gene encoding an Al-activated malate transporter, *TaALMT1*, was identified in wheat by Sasaki *et al.* (2004). *TaALMT1* encodes a hydrophobic membrane protein that when expressed heterologously localizes to the plasma membrane of root cells, and more importantly confers Al-activated malate efflux and increased Al resistance in plants (Delhaize *et al.*, 2004; Yamaguchi *et al.*, 2005). Recent electrophysiological studies using *Xenopus* oocytes and tobacco protoplasts as heterologous expression systems have established that TaALMT1 mediates a selective efflux of malate over other anions (Piñeros *et al.*, 2008b; Zhang *et al.*, 2008). However, it is also permeable to other physiologically relevant anions such as Cl^- and NO_3^-. The similarities between the transport characteristics of TaALMT1 expressed in *Xenopus laevis* oocytes and those described for Al^{3+}-activated anion channels recorded in root cell protoplasts, as well as tobacco cell protoplasts overexpressing TaALMT1 (Zhang *et al.*, 2008), suggest that TaALMT1 encodes a novel type of anion channel. Most unique is the fact that the activity of TaALMT1 is greatly enhanced by the high-affinity binding of Al^{3+} to the transporter ($K_{m1/2}$ of approximately 5 μM Al^{3+}). Homologs of TaALMT1 encoding Al^{3+}-enhanced malate transporter proteins involved in Al resistance have also been identified in Arabidopsis (AtALMT1; Hoekenga *et al.*, 2006) and rape (*Brassica napus*; BnALMT1 and BnALMT2; Ligaba *et al.*, 2006). Although being structurally very similar, other homologs such as the maize ZmALMT1 (Piñeros *et al.*, 2008a) and the Arabidopsis AtALMT9 (Kovermann *et al.*, 2007) have been implicated in physiological processes not associated with Al resistance, such as mineral nutrition and malate homeostasis.

Although malate efflux underlies the Al-resistance phenotype observed in Al-tolerant wheat (Delhaize *et al.*, 1993; Ryan *et al.*, 1995), physiological studies have established a lack of strong correlation between intracellular organic acid content and the magnitude of the organic anion efflux. This suggests that transport across the plasma membrane (presumably via TaALMT1), rather than organic acid biosynthesis, is the rate-limiting step for this mechanism (Ryan *et al.*, 2001). Therefore, the elucidation of potential regulatory pathways modulating TaALMT1 activity warrants further investigation. Reversible phosphorylation is an appealing candidate for regulation, as it involves the post-translational protein modification underlying the regulation of many fundamental processes including cellular signaling, proliferation, differentiation or apoptosis, via transient and highly dynamic events regulated by kinases and phosphatases (Morandel *et al.*, 2006). In animal cells, reversible phosphorylation has been implicated in modulating the activity of ion channels (Levitan, 1994; Levin *et al.*, 1995; Catterall, 2000; Davis *et al.*, 2001; Thomas *et al.*, 2003). Similarly, in plant cells the guard cell plasma membrane slow anion channel (Schmidt *et al.*, 1995), inward- and outward-rectifying K^+-channels (Armstrong *et al.*, 1995), Ca^{2+}-channels (Köhler and Blatt, 2002) and slow vacuolar ion channels (Bethke and Jones, 1997) are reported to be modulated by reversible phosphorylation.

Indirect evidence from studies using protein kinase and phosphatase inhibitors have suggested that reversible protein phosphorylation may be involved in the Al-activated malate efflux from intact wheat and Arabidopsis roots (Osawa and Matsumoto, 2001; Kobayashi *et al.*, 2007). However, it remains unclear whether the latter is due to direct phosphorylation of ALMT1-type transporters or if phosphorylation events play a role upstream of the signaling cascade that triggers malate efflux. In this study we combined the use of kinase and phosphatase inhibitors and site-directed mutagenesis of putative protein kinase C (PKC) phosphorylation candidate motifs to elucidate the potential role of protein phosphorylation in regulating the transport activity of TaALMT1. We present evidence of specific amino acid sites that are involved in direct phosphorylation modulating TaALMT1 transport protein activity.

RESULTS

Modulation of TaALMT1 transport activity

We recently reported that the TaALMT1 transporter mediates a selective efflux of malate over other anions when expressed in *Xenopus* oocyte cells (Piñeros *et al.*, 2008b). Aside from its permeability characteristics, the regulation of the transport activity of TaALMT1 is unique compared with other animal and plant anion channels. As shown in Figure 1(a,b), TaALMT1-expressing oocytes are functionally active (i.e. they mediate an inward current) in the absence of extracellular Al^{3+}. However, the addition of extracellular Al^{3+} significantly enhances the transport mediated by TaALMT1. Enhancement of TaALMT1 activity by the high-affinity binding of Al^{3+} to the transporter ($K_{m1/2}$ of approximately 5 μM Al^{3+}) results in an increase in its permeability rather than in an alteration of its selectivity properties. In the present study we focused on characterizing additional regulatory pathways which could modulate the activity of this transporter.

Analysis of the TaALMT1 amino acid sequence using PROSITE indicated the existence of six putative PKC phosphorylation motifs containing serine, threonine and/or tyrosine residues throughout the protein (Figure 1c). The first site is predicted to be located in the extracellular N-terminus, prior to the first transmembrane domain. The following site is located on the intracellular side of the protein, on the hydrophilic loop between the fifth and six transmembrane domains. The remaining four motifs were identified

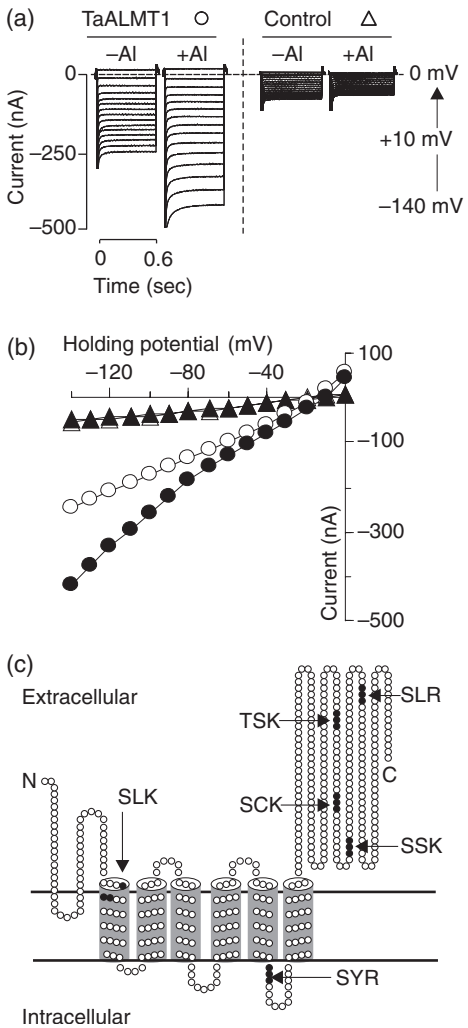

Figure 1. Functional characteristics and hypothetical topology of the TaALMT1 transporter.

(a) Electrophysiological characterization of inward currents mediated by *Xenopus laevis* oocytes expressing TaALMT1 (left panel) and control oocytes (i.e. not injected with cRNA, right panel) recorded in ND96 solution with or without 100 μM $AlCl_3$. Oocyte treatment before electrophysiological measurements and the voltage protocol used for recording are described in the Experimental Procedures.

(b) Mean current/voltage (*I/V*) curves were constructed from the average of current amplitude at the end of each test pulse for TaALMT1-expressing (circle) and control (triangle) oocytes in the presence (closed symbols) or absence (open symbols) of 100 μM $AlCl_3$ (n = 10).

(c) Distribution of the six putative protein kinase C phosphorylation sites predicted by PROSITE (http://ca.expasy.org/prosite: Rost *et al.*, 2003). The six transmembrane spanning domains and the extracellular orientation of the N- and C-termini are based on the topology model proposed by Motada *et al.* (2008).

throughout the long extracellular C-terminus hydrophilic region which comprises about half of the protein (Figure 1c). Interestingly, according to the TaALMT1 topology proposed by Motada *et al.* (2007), four out of the six motifs face the extracellular side of this plasma membrane transporter. Given that reversible phosphorylation is implicated in modulating the activity of ion channels, among various other processes, we evaluated the possibility that direct protein phosphorylation of these putative motifs plays a role in the regulation of TaALMT1 transport activity.

TaALMT1 transport activity is sensitive to kinase inhibitors

We first adopted a pharmacological approach by evaluating the effect of several compounds that modify the activity of protein kinases on the TaALMT1-mediated inward currents. One hour's incubation of TaALMT1-expressing oocytes in a solution containing broad-range inhibitors of protein kinases resulted in severe inhibition of the TaALMT1-mediated currents (Figure 2). Incubation of oocytes with the protein kinase inhibitor K252a (1 μM) for 1 h strongly inhibited the basal (i.e. Al^{3+}-independent) TaALMT1-mediated currents (from –221 to –84 nA at –140 mV), as well as the Al-enhancement of the TaALMT1-mediated currents (from –392 to –83 nA at –140 mV). Incubation of TaALMT1-expressing cells with a different protein kinase inhibitor, staurosporine (5 μM), also suppressed the basal and Al-enhanced components of the TaALMT1-mediated inward currents. Addition of extracellular Al^{3+} in the perfusion solution only weakly enhanced the magnitude of already K252a-or staurosporine-inhibited inward currents. To rule out any effect of DMSO (the solvent used to prepare the inhibitor stock solutions), currents from TaALMT1-expressing oocytes incubated in DMSO alone (0.1% final concentration) were also examined. The DMSO had no effect on TaALMT1 transport activity, as the magnitude of the basal and Al-enhanced currents in TaALMT1-expressing cells treated with DMSO was equivalent to those recorded in cells incubated without DMSO. Likewise, pre-incubation of control oocytes (i.e. not injected with cRNA) with staurosporine or K252a had no significant effect on the small endogenous inward currents.

To further address the specificity of the kinase inhibition, we studied the concentration dependence of staurosporine-induced inhibition of TaALMT1-mediated currents. Oocytes pre-incubated for 1 h in ND96 solution containing a series of staurosporine concentrations that ranged from 0 to 5 μM resulted in TaALMT1-mediated inward currents that were inhibited in a concentration-dependent manner (Figure 3a,b). Treatment of oocytes with 0.5, 1.25, 2.5 and 5 μM staurosporine inhibited the Al-enhanced inward currents by 15, 25, 45 and 70%, respectively (at a holding potential of –140 mV relative to untreated cells; Figure 3c). Analysis of the dose–response curve indicated a high affinity, with an IC_{50} (the concentration that causes 50% inhibition) of about 3 μM staurosporine. Likewise, the inhibition induced by staurosporine occurred in a time-dependent fashion. Inhibition of Al-enhanced TaALMT1-mediated whole-cell currents by staurosporine could be detected within 30 min of exposure. Longer periods of exposure (i.e. 1 h) resulted in further inhibition (about 80%).

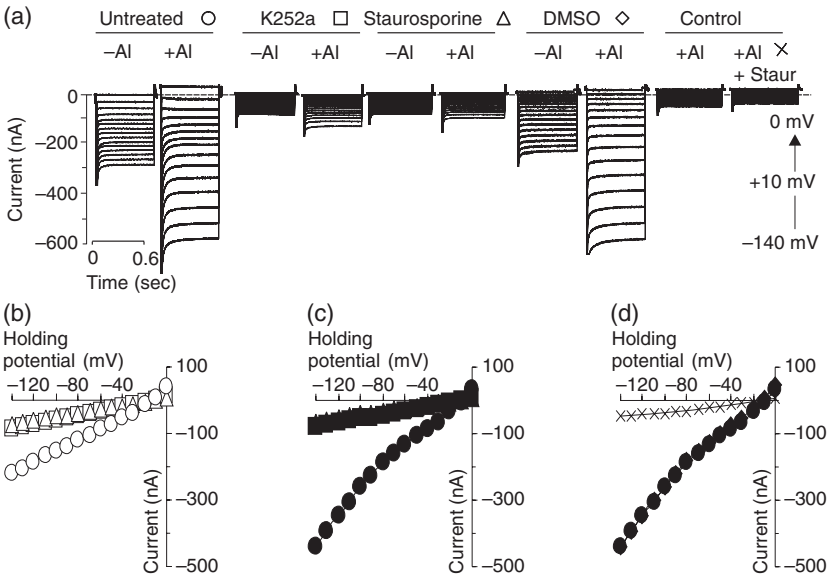

Figure 2. TaALMT1 transport activity is sensitive to kinase inhibitors.
(a) TaALMT1-expressing cells were pre-incubated in ND96 solution containing 1 μM K252a, 5 μM staurosporine or just 0.1% DMSO for 1 h prior to electrophysiological measurements. Measurements were conducted in the presence (right panel) or absence (left panel) of 100 μM $AlCl_3$. Control cells (oocytes not injected with cRNA) shown as reference were pre-incubated with 5 μM staurosporine.
(b, c) Mean current/voltage (*I*/*V*) relationship from untreated cells, K252a- or staurosporine-treated cells without (open symbols, b) or with 100 μM $AlCl_3$ (closed symbols, c) (n = 8).
(d) *I*/*V* curve showing the mean currents recorded in the presence of 100 μM $AlCl_3$ for untreated cells and cells treated with 0.1% DMSO (closed symbols) (n = 8). Note that *I*/*V* curves of mean currents in K252a- and staurosporine-treated cells (b, c), and untreated and DMSO-treated cells (d) are overlapping.

The effect of phosphatase inhibitors on TaALMT1 transport activity

Given the sensitivity of TaALMT1 to protein kinase inhibitors, we subsequently evaluated the effect of phosphatase inhibitors in order to address the potential role of reversible phosphorylation in the modulation of transport activity. TaALMT1-expressing or control oocytes were pre-treated with okadaic acid (1 μM; a broad-range inhibitor of protein phosphatase 1 and 2A) or cyclosporine A (5 μM; a specific inhibitor of protein phosphatase 2B). TaALMT1 activity (both basal and Al-enhanced currents) was relatively insensitive to pre-incubation in either phosphatase inhibitor (Figure 4a–c). Although TaALMT1-mediated inward currents were highly insensitive to okadaic acid, cyclosporine A inhibited TaALMT1-mediated inward currents by about 50%. Nonetheless, the magnitude of this inhibition was significantly smaller than those resulting from pre-incubation in kinase inhibitors. Interestingly, pre-incubation in okadaic acid led to a negative shift in E_{rev} (from about −15 to −31 mV), increasing the outward currents at less negative potentials. This observation was recorded in both TaALMT1-expressing and control cells (data not shown), suggesting that incubation in okadaic acid has an additional effect on an endogenous *Xenopus* oocyte transporter(s). In contrast, cyclosporine A had no significant effect on the small endogenous inward currents recorded in control oocytes (data not shown). Similar to the kinase inhibition, the inhibitory effect of okadaic acid occurred in a time-dependent fashion (Figure 4e,f). However, okadaic acid treatments of up to 1 h only resulted in a small inhibition of inward currents. These results indicate that TaALMT1 activity is significantly more sensitive to kinase inhibitors than phosphatase inhibitors.

TaALMT1-mediated currents are enhanced by phorbol 12-myristate 13-acetate (PMA)

Since the analysis of the TaALMT1 sequence predicted several PKC phosphorylation motifs, we evaluated the effect of the PKC activator, phorbol 12-myristate 13-acetate (PMA), on TaALMT1 transport activity. Currents were first recorded from oocytes bathed in ND96 solution containing Al^{3+}, which was then replaced by an identical bathing solution containing 1 μM PMA. Currents were recorded 15 min after incubation. As shown in Figure 5, PMA treatment led to a significant increase in the TaALMT1-mediated inward currents, with the increase being more noticeable at more negative holding potentials. The enhancement in TaALMT1 activity by PMA treatment reinforced the corroborated inference that TaALMT1 is regulated via PKC phosphorylation.

S384 mutation abolishes TaALMT1 transport activity

Further evidence for the role of direct phosphorylation of TaALMT1 in malate efflux mediated by TaALMT1 was provided by examining the functional characteristics of

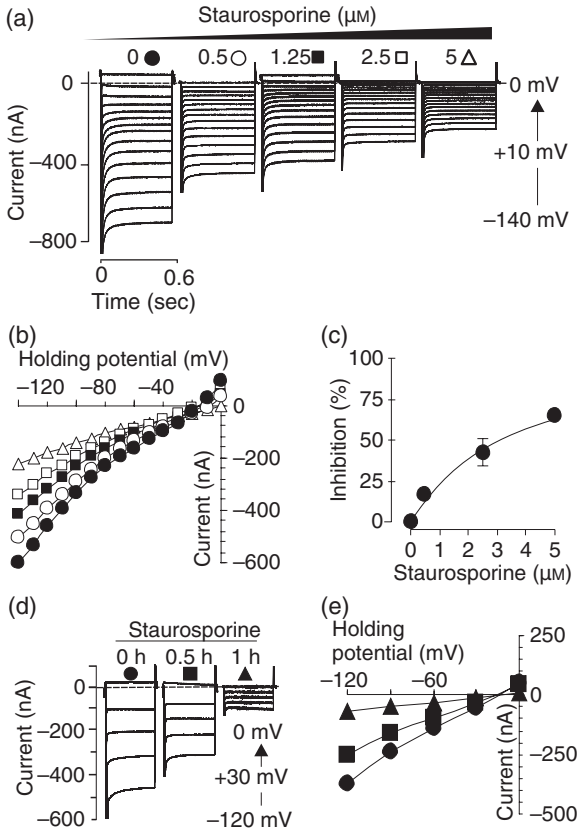

Figure 3. TaALMT1 transport activity is inhibited by staurosporine in a concentration-dependent manner.
(a) TaALMT1-expressing cells were pre-incubated in ND96 containing 0.5, 1.25, 2.5 or 5 μM staurosporine for 1 h prior to recording in the presence of 100 μM $AlCl_3$.
(b) Mean current/voltage (*I*/*V*) relationship from recordings similar to those shown in (a) (n = 5). Symbols corresponding to each treatment are shown in (a).
(c) Inhibition of inward currents at each staurosporine concentration as a percentage of currents in untreated cells. Data values were normalized to currents in untreated cells and fitted to the Hill equation: $\%\ \text{Inhibition} = V_{max} \times x^n/(k^n + x^n)$. Best fitting was obtained by fixing V_{max} inhibition at 100%, resulting in an IC_{50} value of 3.1 ± 0.2 μM (Hill coefficient = 1.1 ± 0.1 and R^2 = 0.979).
(d) Time-dependent effect of staurosporine on TaALMT1 transport activity. TaALMT1-expressing cells were pre-incubated in ND96 solution containing 5 μM staurosporine for 0, 0.5 and 1 h prior to recording in the presence of 100 μM $AlCl_3$.
(e) Mean *I*/*V* relationship from recordings similar to those shown in (d) (n = 7). Corresponding symbols are shown in (d).

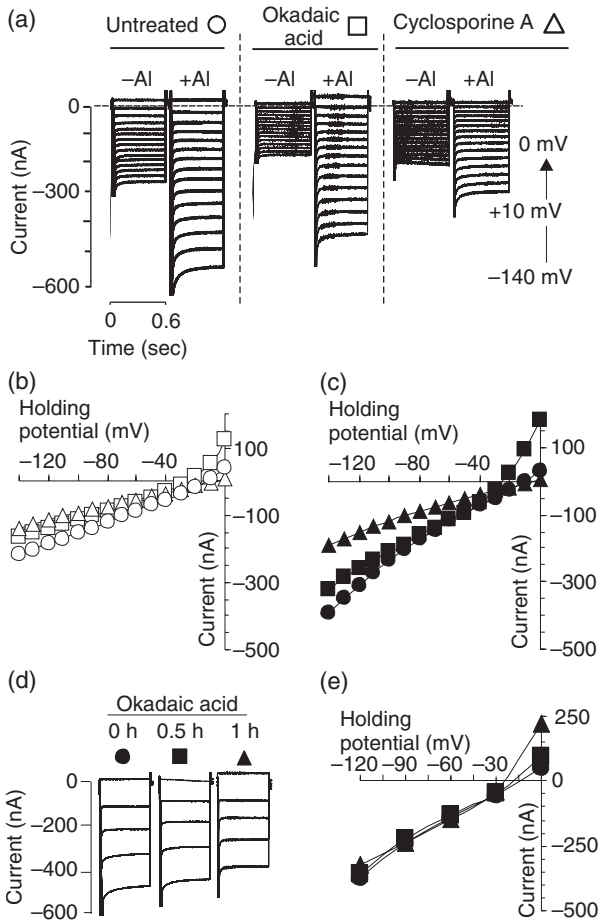

Figure 4. TaALMT1 transport activity has lower sensitivity to phosphatase inhibitors.
(a) TaALMT1-expressing cells were pre-incubated in ND96 solution or ND96 solution containing 1 μM okadaic acid or 5 μM cyclosporine A an hour before recording in the presence (right panel) or absence (left panel) of 100 μM $AlCl_3$.
(b, c) Mean current/voltage (*I*/*V*) relationship from untreated cells, okadaic acid or cyclosporine A treated cells recorded in the absence (b) or presence (c) of 100 μM $AlCl_3$ (n = 6). Symbols corresponding to the inhibitors are described in (a).
(d) Time-dependent effect of okadaic acid on TaALMT1 transport activity. TaALMT1-expressing cells were pre-incubated in ND96 solution containing 1 μM okadaic acid for 0, 0.5 and 1 h prior to recording.
(e) Mean *I*/*V* relationship from recordings as those shown in (d) recorded in the presence of 100 μM $AlCl_3$ (n = 7). Corresponding symbols are shown in (d).

TaALMT1 mutants, where the serine, threonine and/or tyrosine (residues in the predicted phosphorylation consensus motifs) were replaced with alanine or phenylalanine (see Figure 1c). Based on their physicochemical and functional properties, serine and threonine (small, polar residues) were substituted by alanine, a similar small, non-reactive amino acid. Tyrosine residues (an aromatic, partially hydrophobic, amino acid) were substituted by a phenylalanine, the same type of amino acid only differing in the lack of a hydroxyl group in the *ortho*- position on the benzene ring. Four diagnostic parameters were compared between wild-type TaALMT1 and mutants: (i) the kinetics of the inward current which in wild-type TaALMT1 activates instantaneously followed by a minor time-dependent inactivation; (ii) the conductivity of the inward current (i.e. current magnitude); (iii) the ability of the protein to enhance its transport (i.e. enhanced inward currents) upon exposure to extracellular Al^{3+}; and (iv) selectivity, as the inward

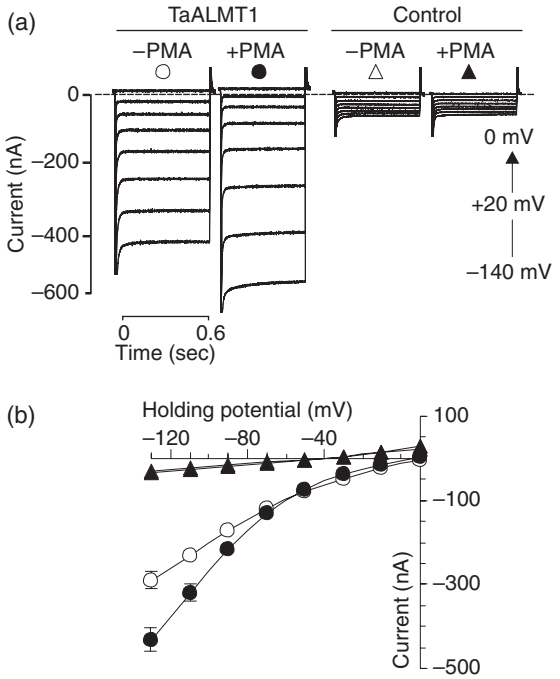

Figure 5. Phorbol 12-myristate 13-acetate (PMA) activates TaALMT1-mediated currents.
(a) Currents recorded from TaALMT1-expressing and control cells in ND96 solution containing 100 μM $AlCl_3$ prior to (left panel) and 15 min after (right panel) the addition of 1 μM PMA to the bath medium.
(b) Mean current/voltage (*I*/*V*) relationship from recordings as those shown in (a) (n = 6). Corresponding symbols are shown in (a).

current mediated by wild-type TaALMT1-expressing cells consistently reversed (reversal potential E_{rev}) at about −12 mV under this set of ionic conditions (i.e. pre-loaded with malate and bathed in ND96 ± Al^{3+}).

Figure 6 shows the functional characteristics of three TaALMT1 mutants for those two PKC phosphorylation motifs predicted to be located along the first half of the TaALMT1 protein, the region containing the transmembrane domains, and presumably the pore region of the transporter. Substitution of the first serine by alanine in either of the two first motifs (i.e. S56A and S183A), and tyrosine by phenylalanine in the second motif (i.e. Y184F), resulted in a transporter with similar transport properties to those seen in the wild-type TaALMT1. That is, the mutant proteins exhibited similar kinetics, conductance, permeability and a significant enhancement of the inward currents upon exposure to extracellular Al^{3+} compared with wild-type protein. Figure 7 depicts the functional characteristics of four TaALMT1 mutants for the next three PKC phosphorylation motifs predicted to be located along the second half of the extracellular hydrophilic region of the TaALMT1 protein. Substitution of the first threonine by alanine at the third motif (i.e. T323A) resulted in TaALMT1 variants which retained functional characteristics comparable to those described for wild-type TaALMT1 (i.e. similar reversal potentials and the enhancement in transport activity upon exposure to extracellular Al^{3+}). However, the conductivity of this variant was significantly increased relative to that recorded in wild-type TaALMT1. The basal transport activity (i.e. current magnitudes in the absence of Al^{3+}), as well as that recorded after Al^{3+}-enhancement for the mutant T323A, were about three times higher than those recorded in wild-type TaALMT1 in identical ionic conditions (Figures 7 and 9). Substitution of the second serine by alanine in this third motif, as well as substitution of the first serine in the fourth motif and double substitution of the serine residues by alanine at the fifth motif (i.e. S324A, S337A, S351-352A) resulted in TaALMT1 variants which retained functional

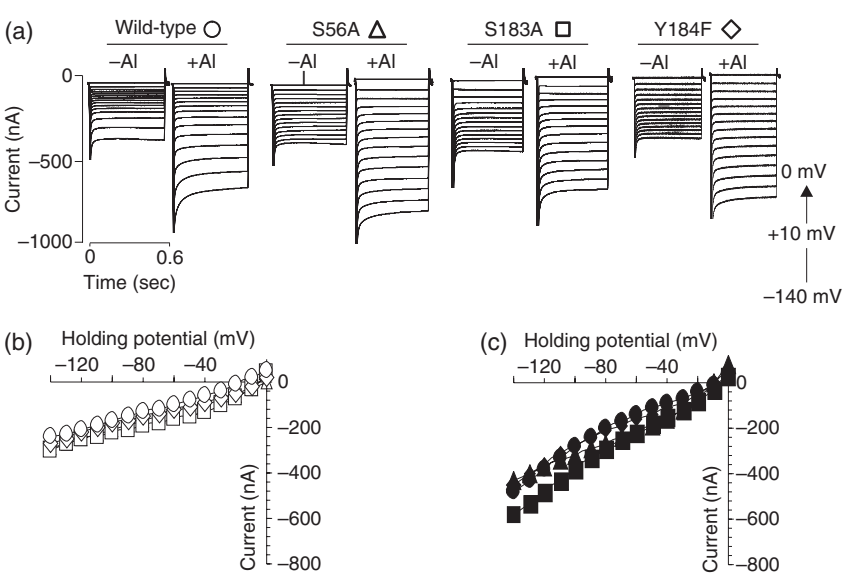

Figure 6. Mutation of serine and tyrosine residues in the first two putative protein kinase C phosphorylation motifs does not alter basic transport properties of TaALMT1 (malate permeation and activation by Al; see Figure 1c).
(a) Representative currents from cells expressing the wild-type TaALMT1 and mutants (S56A, S183A and Y184F), recorded in ND96 solution with (right panel) or without (left panel) 100 μM $AlCl_3$. Mean current/voltage (*I*/*V*) relationships from recordings like those shown in (a) recorded in the absence (open symbols, b) or presence (closed symbols, c) of 100 μM $AlCl_3$ (n = 8). Corresponding symbols are shown in (a).

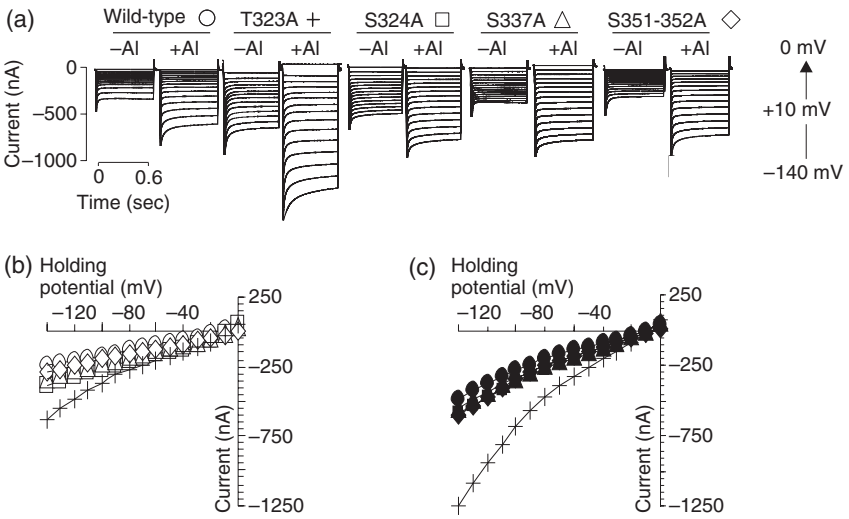

Figure 7. Mutation of threonine and serine residues in the third to fifth putative protein kinase C phosphorylation motifs does not alter the basic transport properties of TaALMT1 (see Figure 1).
(a) Representative currents recorded from cells expressing the wild-type TaALMT1 and the mutants (T323A, 324A,S337A and S231-352A), recorded in ND96 solution with (right panel) or without (left panel) 100 μM $AlCl_3$. Mean current/voltage (*I*/*V*) relationships from recordings as those shown in (a) recorded in the absence (open symbols, b) or presence (closed symbols, c) of 100 μM $AlCl_3$ (n = 8). Corresponding symbols are shown in (a).

characteristics comparable to those described for wild-type TaALMT1. Substitution of serine by alanine in the sixth and last motif (i.e. S384A), which is closest to the end of the C-terminus of the protein, yielded a TaALMT1 variant that was significantly different from any of the mutants described above. The S384A mutation significantly inhibited basal and Al-enhanced transport activity (Figures 8 and 9), with the magnitude of the currents being only slightly larger than currents from control cells not expressing TaALMT1 cRNA. Treatment with PMA was ineffective at restoring or enhancing the activity of the S384A mutant. Furthermore, inward currents mediated by S384A were not sensitive to staurosporine, unlike the wild-type TaALMT1 or the other mutant versions of TaALMT1 (Y184F, T323A, S324A, S351-352A; data not shown). The lack of transport activity and insensitivity to staurosporine and PMA indicates that the S384A substitution rendered this TaALMT1 variant functionally inactive. Overall, these mutations resulted in either no change, an increase in conductance (T323A), or significant inhibition of the transporter (S384A).

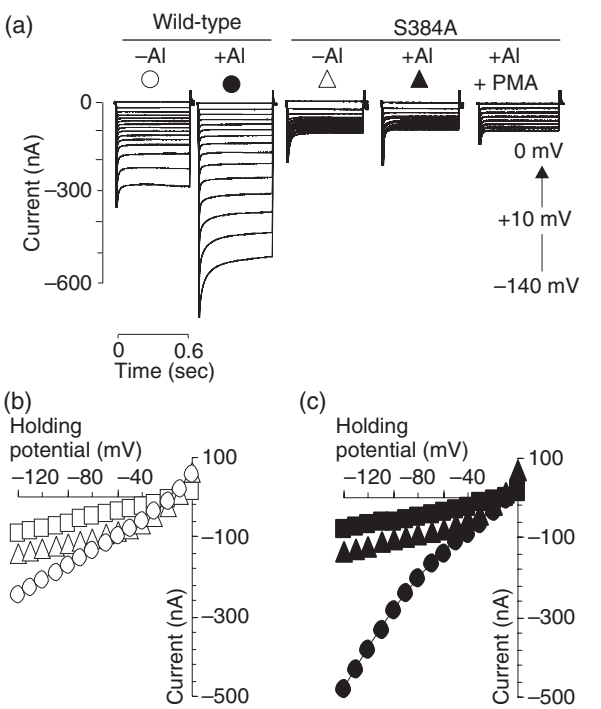

Figure 8. Mutation of serine residues in the sixth putative protein kinase C phosphorylation motif located at the end of the C-terminal tail impairs TaALMT1 transport activity (see Figure 1).
(a) Representative currents recorded from cells expressing the wild-type TaALMT1 and the mutant (S384A) in ND96 solution in the presence (right panel) or absence (left panel) of 100 μM $AlCl_3$. Phorbol 12-myristate 13-acetate (PMA) treatment was performed in ND96 solution with 100 μM $AlCl_3$ containing 1 μM PMA. Mean current/voltage (*I*/*V*) relationships from recordings are as those shown in (a) recorded in the absence (open symbols, b) or presence (closed symbols, c) of 100 μM $AlCl_3$ (n = 8). Corresponding symbols are shown in (a). Currents from control cells (i.e. not injected with cRNA) are shown for reference (square symbol).

DISCUSSION

When exposed to toxic levels of Al^{3+}, many plant species, including wheat, transport malate ions out of the root tip, which functions to chelate and detoxify Al^{3+} in the rhizosphere (Delhaize *et al.*, 1993; Ryan *et al.*, 1995). Malate efflux from wheat roots is mediated by TaALMT1, a plasma membrane Al-activated malate transporter (Sasaki *et al.*, 2004). Molecular and electrophysiological findings have provided substantial evidence that *TaALMT1* is a key gene underlying the Al resistance phenotype observed in wheat (Delhaize *et al.*, 2004; Sasaki *et al.*, 2004; Piñeros *et al.*, 2008b). Other studies have also indicated that the Al-inducible malate release mediated by ALMT proteins in wheat and Arabidopsis roots are sensitive to pharmacological compounds that disrupt protein phosphorylation (Osawa and Matsumoto, 2001; Kobayashi *et al.*, 2007). Pre-treatment of root apexes of Al-resistant wheat plants with 1 μM K252a or 10 μM staurosporine decreased Al-activated malate efflux by 60 and 40%, respectively (Osawa and Matsumoto, 2001).

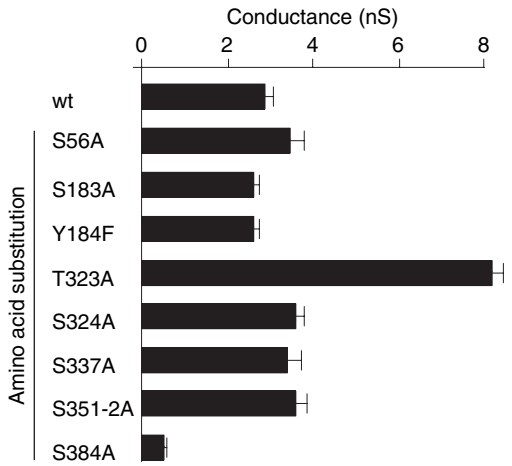

Figure 9. Whole-cell conductance of wild-type TaALMT1 and TaALMT1 variants containing serine, threonine and tyrosine mutations.
The conductance was estimated by linear regression of the test potentials between −100 to −20 mV from the current/voltage (*I*/*V*) relationships obtained after subtracting the endogenous currents recorded in control oocytes. Values represent the average from the conductance estimated from at least eight different cells. Relative to wild-type TaALMT1, the whole-cell conductance of T323A was enhanced by three-fold by while S384A resulted in a six-fold decrease.

Similarly, in Arabidopsis Al-activated malate release was significantly reduced in roots pre-treated with K-252a or staurosporine (Kobayashi *et al.*, 2007). However, exactly where along the signaling cascade protein phosphorylation events were involved was not elucidated in these studies. Recent detailed electrophysiological studies examining the functional properties of TaALMT1 expressed in heterologous systems have established that TaALMT1 is a channel-type protein capable of mediating anion influx and efflux. The transport activity of TaALMT1 is modulated by high-affinity binding of Al^{3+} to the transporter ($K_{m1/2}$ of approximately 5 μM Al^{3+}), significantly enhancing the transport activity (Piñeros *et al.*, 2008b; Zhang *et al.*, 2008). Given the fact that reversible phosphorylation is implicated in modulating various processes, including the activity of ion channels, we investigated the potential role of protein phosphorylation in the direct regulation of TaALMT1-mediated malate efflux. For this purpose we first performed heterologous expression of TaALMT1 in *Xenopus* oocytes and studied the effect of protein kinase and phosphatase inhibitors on TaALMT1-mediated inward current (malate efflux). Our result established that treatment with potent inhibitors of protein kinases (K252a and staurosporine) strongly inhibited TaALMT1-mediated transport (i.e. inward currents) in a concentration- and time-dependent manner (Figures 2 and 3). In addition to being consistent with the Al-induced inhibition of malate efflux by protein kinase inhibitors observed in intact plant cells, our results indicated that the TaALMT1 activity is modulated by protein kinase-mediated phosphorylation. TaALMT1 mediates an anion efflux (i.e. inward current) even in the absence of Al^{3+}, with the transport activity being significantly enhanced upon exposure to extracellular Al^{3+} (Piñeros *et al.*, 2008b). The fact that both K252a and staurosporine significantly suppressed TaALMT1-mediated currents recorded in the absence of Al^{3+} (Figure 2a,b) indicates that phosphorylation and enhancement by Al constitute two independent pathways of TaALMT1 regulation, with protein phosphorylation being a prerequisite for TaALMT1 basal activity and subsequent enhancement by Al^{3+}. These observations are in agreement with previous electrophysiological studies where the use of general protein kinase antagonists has indicated that cation channels such as the inward- and outward-rectifying K^+ channels, as well as the slow anion channels of the guard cell plasma membrane involved in stomatal closure, are also modulated by protein phosphorylation (Armstrong *et al.*, 1995; Schmidt *et al.*, 1995). Unlike kinase inhibitors, the effect of phosphate inhibitors on TaALMT1-mediated currents was not as pronounced, such that pre-treatment of oocytes expressing TaALMT1 with okadaic acid (a strong inhibitor of protein serine/threonine phosphatase 1, 2A and 2B) or cyclosporine A (an inhibitor of protein phosphatase type 2B) only led to a minor reduction of the TaALMT1-mediated inward current amplitudes recorded in the absence and presence of Al^{3+} (Figure 4). A similar small inhibition of Al-induced wheat root malate efflux by phosphatase inhibitors (okadaic acid, calyculin A and cyclosporine A) has been previously reported (Osawa and Matsumoto, 2001). In contrast, Kobayashi *et al.* (2007) reported that exposure of Arabidopsis roots to calyculin (a protein phosphatase 2A inhibitor) resulted in reduction of the Al-induced malate efflux, followed by a reduction in *AtALMT1* gene expression level. This led the authors to suggest that the calyculin A-induced reduction in malate release was probably due to a reduction in *AtALMT1* gene expression. However, in light of the phosphatase inhibitor (cyclosporine A) results described in the present study, although long-term regulation of malate exudation may also be regulated at the transcript level, the modulation of TaALMT1-transport activity by protein dephosphorylation is most likely to involve a post-translational modification of the transporter.

The significant difference in sensitivity of the transport activity of TaALMT1 to protein kinase versus phosphatase inhibitors suggests that protein phosphorylation plays a major role in regulating TaALMT1 transport activity, while protein dephosphorylation may only have a minor role. It has previously been shown that both Ca^{2+}-dependent phosphorylation and dephosphorylation modulate the activity of an inward K^+ channel (KAT1) in guard cells (Luan *et al.*, 1993; Li *et al.*, 1998). However, as Li *et al.* (1998) suggested, the two events may not necessarily happen concurrently or at the same motif site on the protein. The okadaic acid-induced inhibition of inward- and outward-rectifying K^+-channels in *Vicia faba* guard cells

has also been interpreted as inhibition of phosphatase activity leading to a net increase of protein phosphorylation (Thiel and Blatt, 1994).

Ion channel conductance can also be modulated by agents that change the activity of protein kinases. For example, activators of PKC such as PMA, oleyl-acetyl-glycerol (OAG) and phorbol 12, 13-didecanoate (PDD) can modulate the activity of Na^+ and K^+ channels in animal cells (West *et al.*, 1991; Bush *et al.*, 1992; Boland and Jackson, 1999; Thomas *et al.*, 2003). To further establish the role of PKC in modulating TaALMT1 transport activity, we measured the changes in the transporter's activity upon addition of PMA (an unspecific PKC activator; Thomas *et al.*, 2003). The inclusion of PMA in the bath medium resulted in an enhancement of the TaALMT1 transport activity (Figure 5). Regulation of ion channels by PKC has usually been attributed to processes involving direct phosphorylation (Zhu *et al.*, 1999; McHugh *et al.*, 2000; Po *et al.*, 2001; Karle *et al.*, 2002), pathways involving intermediate signal transduction pathways (Thomas *et al.*, 2003) or pathways involving the interaction between PKC and PKA (Lo and Numann, 1998). Similarly, agents that activate protein kinase A by elevating the intracellular cAMP concentration, such as 8-bromoadenosine-cyclic monophosphate (8-Br-cAMP) and forskolin can modulate the activity of K^+-channels (Blumenthal and Kaczmarek, 1992; Levin *et al.*, 1995; Thomas *et al.*, 1999). The heterologous protein expression and pharmacological approaches employed in the present study strongly indicated that the PKC-mediated direct phosphorylation of TaALMT1 underlies its modulation of transport activity. However, given that we have examined the activity of TaALMT1 in *Xenopus* oocytes, an endogenous oocyte kinase capable of phosphorylating TaALMT1 may be required. Levin *et al.* (1995), Ivanina *et al.* (1994), Scott *et al.* (1994) and Rehm *et al.* (1989) have suggested the existence of an unidentified endogenous kinase in *Xenopus* oocytes capable of phosphorylating animal voltage-gated K^+-channels at PKA consensus sequences. Earlier, Huganir *et al.* (1984) reported the phosphorylation of a nicotinic acetylcholine receptor by an endogenous tyrosine-specific protein kinase in *Torpedo californica*. Aluminum induced protein kinases have been reported in the wheat root apex (Osawa and Matsumoto, 2001) and in *Coffea arabica* suspension cells (Arroyo-Serralta *et al.*, 2005). Whether these kinases are analogous to the endogenous kinase(s) that phosphorylate TaALMT1 in oocytes, and could potentially modulate TaALMT1 activity *in planta*, remains an open question.

Modulation of ion channel activity by direct phosphorylation has been fairly well studied. Studies involving site-directed mutagenesis have established that the activity of multiple K^+ (Zhu *et al.*, 1999; Po *et al.*, 2001), Ca^{2+} (McHugh *et al.*, 2000), Na^+ (West *et al.*, 1992) and chloride channel 3 (CLC-3) volume-regulated Cl^- (Duan *et al.*, 1999, Finn *et al.*, 1992) channels in animal cells are reported to be modulated by direct protein phosphorylation at PKC phosphorylation sites. Similarly, in plants mutation of selected protein phosphatases (Armstrong *et al.*, 1995; Pei *et al.*, 1997) and protein kinases (Li *et al.*, 2000) suppressed the responses of ion channels to ABA and stomatal closure. Johansson *et al.* (1998) showed that water channel activity of a major intrinsic protein of spinach PM28A expressed in *Xenopus* oocytes is also regulated by phosphorylation of two serine residues (S115 and S274). In the present study, using site-directed mutagenesis we disrupted six putative PKC phosphorylation motifs containing serine, threonine and tyrosine residues within the TaALMT1 protein (see Figure 1c). Among these sites, the first two are located in the first half of the protein, which contains the transmembrane domain regions which are strongly conserved among ALMTs from different plant species (see Figure 4 in Piñeros *et al.*, 2008a). Substitution of serine residues in these two sites by alanine (S56A and S183A), or tyrosine by phenylalanine (Y184F) at the second site resulted in transporters that have similar functional characteristics (such as malate permeation and Al-activation) as the wild type (Figure 6). Substitution of serine to alanine in the third, fourth and fifth sites (S324, S337A and S351-352A) also did not alter the functional characteristics of the transporter (Figure 7). In contrast, substitution of threonine by alanine at residue 323 (T323A) resulted in a transporter that, while it exhibits similar Al enhancement and ion permeation properties as the wild type protein, exhibited a significant increase in whole-cell conductance. Interestingly secondary structure predictions using the Protein Homology/analogY Recognition Engine (PHYRE V0.2) indicate that this site is located within a loop region joining two adjacent helixes (http://www.sbg.bio.ic.ac.uk/~phyre/). Unlike most amino acids, which contain only one non-hydrogen substituent attached to their C-beta carbon, threonine is one of the three C-beta branched amino acids, consequently containing two non-hydrogen substituents attached to their C-beta carbon. Within this context, substitution of threonine may have a significant structural impact on the TaALMT protein, as its presence results in a higher degree of restriction in the conformation of the main-chain, hampering alpha-helical conformations. We can only speculate that alterations of the structural rigidity upon substitution of threonine by alanine may underlie the significant increase in ion conductance. In contrast, substitution of serine by alanine at the sixth and most distal site (S384) rendered the TaALMT1 transporter in an impaired state (Figure 8). The greatly reduced activity of mutant S384A was confirmed as this mutated transporter was insensitive to PMA, such that PMA treatment was ineffective at restoring or enhancing the activity of the S384A mutant. Together, these findings suggest that the S384A mutation disrupts the direct phosphorylation by PKC at this site, impairing TaALMT1 transport activity. Nonetheless, we cannot entirely rule out the possibility that impairment of the S384A mutant

is not due to disruption of the direct phosphorylation process, but rather is due to a different alteration(s) such as protein expression (e.g. protein trafficking) or changes in protein structure (e.g. protein–protein interactions). Mutations in protein phosphorylation motifs have been shown to alter a wide variety of processes including channel gating, subcellular trafficking, degradation and protein–protein interactions (Levin *et al.*, 1995; Shih *et al.*, 1996;. O'Connell *et al.*, 2005; Anderson *et al.*, 2006). For example, changes in the phosphorylation state of the mammalian renal outer-medullary K^+ channel (ROMK; Kir1.1) altered channel expression by increasing its cell surface delivery due to a suppression of the C-terminal endoplasmic reticulum retention signal (O'Connell *et al.*, 2005). Although similar alterations could underlie the impairment of TaALMT1 activity by the S384 mutation, the fact that the other serine to alanine mutations did not alter TaALMT1 transport, as well as the non-reactive nature of the substitution (i.e. alanine is usually present in all non-critical protein contexts), suggests these alternative explanations are unlikely.

Recently, immunocytological techniques applied to TaALMT1 expressed in a different heterologous system (i.e. mammalian cells) have suggested a protein topology where both the N- and C-termini of the protein are located on the extracellular side of the plasma membrane (Motada *et al.*, 2007). Thus, assuming the folding and orientation of TaALMT1 in this system occurs as *in planta*, the most intriguing finding in our study is the realization that the S384 residue (distal from the pore-forming region and quite probably involved in the direct phosphorylation) is located within the extracellular C-terminal tail of the protein. Thus, it would be necessary to presume the existence of a serine kinase that can phosphorylate the TaALMT1 protein in the root cell wall matrix. In line with this assumption, He *et al.* (1999) reported on five Arabidopsis WAK genes (*Wak1–5*), all of which encode a cytoplasmic serine/threonine protein kinase, a transmembrane domain and an extracytoplasmic region with several epidermal growth factor (EGF) repeats. Except for Wak2, which was weakly expressed in the root, the other four WAK proteins studied by He *et al.* (1999) were expressed in green organs, flowers and siliques. On the other hand, the report by Sivaguru *et al.* (2003) showed that the Al-induced cell wall-associated receptor kinase 1 (WAK1) is abundantly expressed in roots and shoots of Arabidopsis. Moreover, transgenic Arabidopsis plants overexpressing WAK1 showed enhanced Al tolerance. WAK-like kinases (WAKL) that are highly expressed in roots and flowers have also been reported in Arabidopsis (Verica *et al.*, 2003). WAK and WAKL belong to the receptor-like kinase (RLK) subfamily whose members are candidates for a signaling molecule that mediates the communication between cell wall and cytoplasm (He *et al.*, 1996; Kohorn, 2000; Verica *et al.*, 2003). Besides Al stress (Sivaguru *et al.*, 2003), WAK or WAKL genes may also be involved in cell elongation and morphogenesis (Lally *et al.*, 2001, Wagner and Kohorn, 2001), wounding or pathogen infection (He *et al.*, 1998; Verica *et al.*, 2003), as well as mineral nutrition responses (Hou *et al.*, 2004). Thus, it is not unreasonable to suggest that phosphorylation of TaALMT1 at S384 could potentially be mediated by WAK or WAKL isoform(s) with serine kinase activity. However, which of the WAK/WAKL mediate TaALMT1 phosphorylation and how phosphorylation takes place in the extracellular matrix remains unknown.

In conclusion, we have provided substantial pharmacological evidence indicating that direct protein phosphorylation by PKC is a prerequisite for the activation of the TaALMT1 transporter and subsequent enhancement of malate efflux transport activity by extracellular Al^{3+}. In addition, functional characterization of TaALMT1 mutants with altered putative PKC phosphorylation sites revealed that residue S384 is critical for activation of TaALMT1, and quite probably is involved in TaALMT1 phosphorylation. We carried out a comprehensive study integrating pharmacology, electrophysiology and site-directed mutagenesis as means to demonstrate the role of protein phosphorylation in the regulation of Al-activated anion channels. Future studies will focus on the structural and functional analysis of the ALMT-type transporters.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis

Six PKC phosphorylation motifs (amino acids 56–58, 183–185, 323–325, 337–339, 351–353 and 384–386) were identified in the TaALMT1 protein sequence using the PROSITE program (http://ca.expasy.org/prosite; Rost *et al.*, 2003). A total of eight mutations were generated by site-directed mutagenesis of serine, threonine and tyrosine residues in the predicted phosphorylation motifs. Serine and threonine residues were substituted by alanine, and a tyrosine residue by phenylalanine (Figure 1c). Single point mutations were generated using the overlap extension PCR method, involving two rounds of PCR. In the first round of PCR the two fragments (N and C halves) of the mutant were generated with primers that introduce the mutated codon on both sense and antisense strands and provide overlapping regions on both fragments (see Appendix S1). During the first PCR the primer 5′-CGCGCAGATCTATGGATATTGATCACGGC-3′ (containing a *Bgl*II adaptor upstream of the initiation codon) was used as the sense primer to amplify the N half of first fragments in combination with an antisense primer containing each mutant codon and overlapping region (see Appendix S1). The C half fragments were generated using in combination of the antisense primer 5′-CGCACTAGTTTACAAAATAACCACGTCAGGCAA-3′ (containing a *Spe*I adaptor downstream of the initiation codon) with sense primer containing each mutant codon and overlapping region (see Appendix S1 in Supporting Information for primer sequences). The resulting PCR products were purified from gel using a gel purification kit (Qiagen, http://www.qiagen.com/). The respective N and C half products were used as templates on a second round of PCR using the sense and antisense primers described above (containing the *Bgl*II and *Spe*I adaptors, respectively). All PCR reactions were carried out using Phusion Hot Start DNA polymerase (Finnzymes, http://www.finnzymes.com/) following the manufacturer's

instructions. The incorporated *Bgl*II and *Spe*I restriction sites flanking the amplified full coding region of each mutant were used for directional cloning into the *Bgl*II and *Spe*I site of the T7TS vector, which are flanked by 5′ and 3′ untranslated regions of a *Xenopus* β-globin gene. All constructs were fully sequenced and checked for sequence accuracy.

In vitro transcription and cRNA injection

Complementary RNA for wild-type TaALMT1 and the eight mutated coding regions was synthesized from 1 μg of linearized (*Bam*HI) plasmid DNA template using a mMessage mMachine *in vitro* transcription kit (Ambion, http://www.ambion.com/) according to the manufacturer's recommended procedures. The cRNA was eluted in RNase-free water, divided in 2 μl aliquots and stored at −80°C until injection. Harvesting of stage V–VI *X. laevis* oocytes was performed as described previously (Golding, 1992; Piñeros *et al.*, 2008b). Defolliculated oocytes were maintained in ND88 solution (supplemented with 50 μg ml^{-1} gentamycin) overnight prior to injections. Oocytes were injected with 48 nl water containing 25–50 ng of a cRNA (or 48 nl of water as a control), and incubated in ND88 at 18°C for 2–3 days prior to the electrophysiological measurements.

Electrophysiological measurements

Electrophysiological measurements were performed as described in Piñeros *et al.* (2008a). Prior to electrophysiological measurements, control oocytes or those expressing wild-type and TaALMT1 mutants were injected with 50 nl of water with 0.1 M D-malic acid (pH 7.5, adjusted with NaOH). Three to four hours after malate was injected, whole-cell currents were recorded under constant perfusion with a GeneClamp 500 amplifier (Axon Instruments, http://www.moleculardevices.com/home.html) using the two-electrode voltage-clamp (TEVC) technique. The recording electrodes were filled with 0.5 M K_2SO_4 and 30 mM KCl, and had resistances between 0.5 and 1.5 MΩ. Cells were bathed in ND96 solution containing (in mM) 96 NaCl, 1 KCl, 1.8 $CaCl_2$ and 0.1 $LaCl_3$, with or without 100 μM $AlCl_3$ (pH 4.5). Currents were elicited by voltage pulses stepped between −140 and 0 in 10-mV intervals with a 6-sec rest at 0 mV between each voltage pulses. The output signal was digitized and analyzed using a Digidata 1320A Pclamp 9 data acquisition system (Axon Instruments). The steady-state current–voltage (*I*/*V*) relationships were constructed by measuring the current amplitude at the end of the test pulse. Mean current values represent the average of at least *n* (indicated in each figure legend) oocytes and two to three donor frogs. Error bars denote SEM and are not shown when they are smaller than the symbol.

Inhibitors and activator treatments

K252a and okadaic acid were purchased from Calbiochem (http://splash.emdbiosciences.com/). Staurosporine, cyclosporine A and PMA were purchased from Sigma (http://www.sigmaaldrich.com/). All compounds were dissolved in DMSO at concentrations that ensured that no more than 0.1% DMSO was present in the incubation media. This concentration of DMSO had no effect on TaALMT1 transport activity (see Figure 2). Stock solutions of 1 mM (K252a, okadaic acid and PMA) or 5 mM (staurosporine and cyclosporine A) were stored at −20°C. Three hours after malate injection, cells were incubated in ND96 solution with or without 1 or 5 μM inhibitor for 1 h prior to the electrode impalement. For experiments involving PMA, oocytes were first impaled in ND96 solution without Al. The bath solution was replaced by ND96 solution containing Al and inward currents were recorded. The bath solution was then replaced by the same solution containing 1 μM PMA and currents were recorded 15 min after initiating incubation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplemental materials and methods.

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