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Characterization of the TaALMT1 Protein as an Al³⁺-activated Anion Channel in Transformed Tobacco (*Nicotiana Tabacum* L.) Cells

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TaALMT1 encodes a putative transport protein associated with Al³⁺-activated efflux of malate from wheat root apices. We expressed TaALMT1 in Nicotiana tabacum L. suspension cells and conducted a detailed functional analysis. Protoplasts were isolated for patch-clamping from cells expressing TaALMT1 and from control cells (empty vector transformed). With malate $^{2-}$ as the permeant anion in the protoplast, an inward current (anion efflux) that reversed at positive potentials was observed in protoplasts expressing TaALMT1 in the absence of Al^{3+} . This current was sensitive to the anion channel antagonist niflumate, but insensitive to Gd^{3+} . External AlCl₃ (50 μ M), but not La³⁺ and Gd^{3+} , increased the inward current in TaALMT1-transformed protoplasts. The inward current was highly selective to malate over nitrate and chloride $(P_{mal} >> P_{NO3} \ge P_{Cl}, P_{mal})$ $P_{Cl} \ge 18$, $+/-Al^{3+}$), under conditions with higher anion concentration internally than externally. The anion currents displayed a voltage and time dependent deactivation at negative voltages. Voltage ramps revealed that inward rectification was caused by the imposed anion gradients. Single channels with conductances between 10 and 17 pS were associated with the deactivation of the current at negative voltages, agreeing with estimates from voltage ramps. This study of the electrophysiological function of the TaALMT1 protein in a plant heterologous expression system provides the first direct evidence that TaALMT1 functions as an Al³⁺activated malate²⁻ channel. We show that the Al³⁺-activated currents measured in TaALMT1-transformed tobacco cells are identical to the Al³⁺-activated currents observed in the root cells of wheat, indicating that TaALMT1 alone is likely to be responsible for those endogenous currents.

Keywords: ALMT1 — Anion channel — Aluminium — Malate — Al³⁺-gated — Patch-clamp.

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; BSA, bovine serum albumin; EGTA, glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid; E_{rev} , reversal potential; ET8, aluminium tolerant line of wheat; G, conductance; GHK, Goldman–Hodgkin–Katz; I/V curve,

current voltage curve; I_f, final current; I_i, initial current; I_m, membrane current; P_{Cl}, permeability to Cl⁻; P_{mal}, permeability to malate^{2–}; P_{NO3}, permeability to NO₃⁻; PVP, polyvinylpyrrolidone; TEA, tetraethylammonium; V_m, membrane voltage

Introduction

Aluminum (Al) is the most abundant metal element in the earth's crust. When the soil pH is above 6.0 the concentration of free Al³⁺ cations in the soil solution is low because they either precipitate as insoluble gibbsite or become bound to anions or ligands on mineral surfaces. In acidic conditions Al solubility increases and the presence of the phytotoxic species Al³⁺ becomes a major factor limiting crop growth and yield (Kochian, 1995). A number of plant species and genotypes within species display an inheritable resistance to A1³⁺ stress. Exudation of organic anions from roots has been identified to be an important mechanism for resistance in plants (Ma et al. 2001, Ryan et al. 2001, Kochian et al. 2004). The organic anions released from the root apices bind with the Al³⁺ cations to form non-toxic complexes, thus protecting root apices from Al^{3+} toxicity. Those organic anions released from plant roots in response to Al³⁺ treatment include malate, citrate and oxalate (Ryan et al. 2001). A positive correlation exists between the Al^{3+} resistance of a range of wheat genotypes and Al³⁺-activated malate efflux (Ryan et al. 1995a, Raman et al. 2005). Early studies demonstrated that malate efflux was unrelated to intracellular malate content of the root tissues or to the activities of enzymes responsible for malate synthesis (PEP carboxylase and malate dehydrogenase), suggesting that the transport of malate across the plasma membrane, rather than synthesis, is the key regulator of efflux from roots (Ryan et al. 1995b).

A gene encoding an Al^{3+} -activated malate transporter (*TaALMT1*) was isolated from wheat (Sasaki et al. 2004). *TaALMT1* encodes a membrane-bound protein with six

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putative membrane spanning regions (Motada et al. 2008) that localizes to the plasma membranes of root cells (Yamaguchi et al. 2005). The expression of TaALMT1 in Xenopus oocytes, rice (Oryza sativa), cultured tobacco (Nicotiana tabacum L.) cells and barley (Hordeum aestivum) confers a similar phenotype: an Al³⁺-activated efflux of malate, which enhances the Al^{3+} resistance of some of these transgenic plant cells (Sasaki et al. 2004, Delhaize et al. 2004). TaALMT1 is a member of a novel protein family (Delhaize et al. 2007) and homologues identified in Arabidopsis (AtALMT1, AtALMT9), Brassica napus (BnALMT1), and maize (ZmALMT1) have also been shown to encode Al³⁺-activated malate transport proteins (Hoekenga et al. 2006, Ligaba et al. 2006, Kovermann et al. 2007, Piñeros et al. 2008a). However, when ZmALMT1 was expressed in Xenopus oocytes the increase in current magnitude associated with Al³⁺ treatment was small and relatively non-selective for many anions (Piñeros et al. 2008a). Another ALMT targeted to the tonoplast and present in mesophyll cells of Arabidopsis (AtALMT9), shows Al³⁺ activation in *Xenopus* and is relatively nonselective anion transport, and was thought to contribute to malate homeostasis (Kovermann et al. 2007). None of the previous studies have established whether any of these ALMT proteins function as a channel.

Malate exists predominately as the divalent anion in the cytoplasm, and movement of malate²⁻ out of the root cells is likely to be passive due to the negative electrical potential gradient across the plasma membrane. The observation that Al³⁺-activated efflux of malate from wheat roots is sensitive to several anion-channel blockers (Ryan et al. 1995b) is consistent with TaALMT1 being a channel. The first electrophysiological studies showed that Al³⁺ could activate a Cl⁻ permeable channel in protoplasts prepared from Al-tolerant root apical cells (Ryan et al. 1997). Zhang et al. (2001) subsequently showed that the Al³⁺-activated currents carried by malate efflux were manyfold more permeable to malate^{2–} than to Cl[–]. Furthermore, the magnitude of the malate currents was greater in protoplasts prepared from an Al³⁺-resistant genotype of wheat than those from a near-isogenic but Al³⁺-sensitive genotype. Al³⁺-activated currents have now been described in maize (Kollmeier et al. 2001, Piñeros and Kochian 2001, Piñeros et al. 2002) confirming that similar responses occur in other plant species.

Physiological studies published to date support the hypothesis that *TaALMT1* encodes a malate-permeable anion channel and that its activation by Al^{3+} to release malate²⁻ underlies Al^{3+} resistance in wheat. However, no direct experimental evidence has been presented to demonstrate whether *TaALMT1* encodes an ion channel or how the protein is activated by Al^{3+} . Expression studies in *Xenopus* oocytes have demonstrated that TaALMT1

generates Al³⁺-activated currents (Sasaki et al. 2004, Piñeros et al. 2008b). Expression of ZmALMT1 in Xenopus oocytes enhances outward and inward currents carried by anion influx and efflux respectively, and Al³⁺ only slightly stimulates the inward current (Piñeros et al. 2008a), suggesting that ZmALMT1 is unlikely to underpin all the Al³⁺-dependent efflux of organic anions from maize. TaALMT1 expressed in Xenopus oocytes has also revealed some interesting characteristics not revealed in previous studies including the ability to carry anion currents without activation by Al³⁺, and to carry outward currents (anion influx) (Piñeros et al. 2008b). However, there has been no detailed electrophysiological study on the function of ALMT1 proteins in a plant expression system, and utilizing the advantages of patch-clamp techniques. Xenopus expression studies using two electrode voltage clamping are constrained by the relative inability to define the cytoplasmic concentration of permeable anions making assessment of selectivity more difficult for an efflux transporter, and with the added uncertainty of native anions in the egg cytoplasm being able to permeate the transporter (Piñeros et al. 2008b). In the present study, we investigated the electrophysiology of TaALMT1 function in a plant expression system that allowed direct comparison with the native transport signature in protoplasts derived from wheat roots. The relatively silent electrical background of the expression cells has enabled us to provide evidence that the currents evoked by TaALMT1 expression are carried by an anion channel with voltage- and time-dependent deactivation at negative membrane potentials.

Results

Malate efflux from tobacco suspension cells

Malate efflux was measured from tobacco cells transformed with *TaALMT1* or transformed with an empty vector. A small efflux was detected from both cell types in an AI^{3+} -free solution. Although some variations existed between experiments the efflux from transformed cells was consistently greater than from control cells

Table 1Malate efflux over 20 h from tobacco suspensioncells in the Al^{3+} -free control solution. Data are mean $\pm SE$ of three replicates

Experiment	Malate efflux (nmol g FW ⁻¹ 20 h ⁻¹) \pm SE		Ratio of fluxes
	Vector control cells	<i>TaALMT1</i> - expressing cells	<i>TaALMT1</i> / control
1	21 ± 3	50 ± 9	2.4
2	49 ± 9	339 ± 40	6.9
3	26 ± 10	91 ± 15	3.5

(Table 1). When $100 \,\mu\text{M}$ Al³⁺ was added to the media, malate efflux from cells transformed with *TaALMT1* increased by more than 20-fold, while control cells showed little change. This increase was not observed when similar concentrations of La³⁺ were added to the media (Fig. 1). The Al³⁺-activated efflux of malate from *TaALMT1*-transformed cells was inhibited by 25 μ M niflumate.

Inward currents in tobacco protoplasts transformed with TaALMT1

Protoplasts were isolated from the tobacco cells and examined with the whole-cell patch-clamp technique. With malate²⁻ as the main anion in the patch-pipette and a bath solution consisting of 0.2 mM CaCl₂ and 10 mM tetraethylammonium chloride (TEACl) (pH 4.0), a small inward current of about 20 mA m^{-2} at -174 mV was measured in all protoplasts transformed with either TaALMT1 or an empty vector immediately after formation of the whole-cell configuration (Fig. 2A). During the initial 20-30 min period in the whole-cell configuration when the pipette contents equilibrated with the protoplast cytoplasm, hyperpolarization from $+6 \,\mathrm{mV}$ (near to the resting potential) activated larger inward currents in TaALMT1 protoplasts compared to empty vector controls (Fig. 2B, C). The inward current in TaALMT1 protoplasts displayed a small timedependent decay during the voltage pulse at negative voltages. We accounted for this by measuring both initial currents (at the very beginning of the pulse) and final currents (at the end of the pulse). This decay during



Fig. 1 A^{3+} -activated malate efflux from tobacco suspension cells. Flasks containing suspension cells stably transformed with TaALMT1 and empty vectors were placed on a shaker and after 60 min with the various treatments (Nif = niflumate with μ M levels indicated) the suspensions were centrifuged and malate concentrations measured in the supernatant with an enzyme assay as described by Ryan et al. (1995b). AlCl₃ and LaCl₃ concentrations were 100 μ M. Values are means of two experiments (error bars denote range) for empty vector cells (black bars) and *TaALMT1* transformed cells (grey bars).

hyperpolarizing voltage pulses is further characterized for Al³⁺-activated currents. In protoplasts transformed with an empty vector the inward currents decreased by approximately 60% (n = 16) during the 30 min following whole-cell formation (Fig. 2A). By contrast, the initial and final currents in *TaALMT1*-transformed protoplasts remained unchanged over the same period (54% of protoplasts, n = 24) or increased in magnitude (46% of protoplasts) (Fig. 2A, C).

The initial and steady-state inward currents in protoplasts transformed with TaALMT1 were inhibited by the anion channel blocker niflumate but not by Gd³⁺, a blocker of non-selective cation channels (Fig. 2D). Thus the currents are more likely to be generated by anion efflux rather than cation uptake. The current-voltage relationship for the niflumate-sensitive current was obtained by subtracting the whole-cell initial currents measured after niflumate addition from the initial currents measured prior to niflumate addition (data not shown). The reversal potential (E_{rev}) of the resulting difference curve was 56 mV which is consistent with the very positive reversal potential expected for malate^{2–}. There was no difference in the E_{rev} using steady-state (after decay) or initial currents. If we assume that the niflumate-sensitive current was mainly caused by malate²⁻ efflux, the relative permeability of the transport to malate²⁻ and Cl⁻ (P_{mal}/P_{Cl}) is greater than about 20 (this value is very sensitive to small changes in estimated reversal potential). These calculations used the modified Goldman-Hodgkin-Katz equation (cf. Zhang et al. 2001, 2004) taking into account ionic strength.

Al³⁺ activates an inward current in tobacco protoplasts transformed with TaALMT1

The effect of adding Al^{3+} to the bath solution was examined after the whole-cell currents had stabilized. Addition of 50 µM AlCl₃ to the bath solution increased the inward current in 94% (n=32) of the protoplasts examined that were transformed with TaALMT1 (e.g. Fig. 3A). The average initial inward current density at -174 mV increased from $-40 \pm 15 \text{ mA} \text{ m}^{-2}$ to $-10 \pm 21 \text{ mA}$ $m^{-2}~(n\!=\!23)$ and the $E_{\rm rev}$ shifted from $-6\pm15\,mV$ to $+38 \pm 9 \text{ mV}$ (n = 30) (Fig. 3B). The activation of current by Al^{3+} was rapid (less than 2 min) in 60% of protoplasts (n=30) (e.g. Fig. 3A) while 80% exhibited an increase within 5 min of adding Al³⁺. Current density stabilized after approximately 10 min and remained relatively constant if Al³⁺ was retained in the solution. Decay of the inward current at negative membrane potentials was more apparent with Al³⁺ present in the bath (Fig. 3C cf Fig. 2C). By contrast, the whole-cell currents for all the control protoplasts (n = 16) was reduced by Al³⁺ addition with current density decreasing from -22.4 ± 5.1 to -14.0 ± 3.7 mA m⁻² (n = 16) after 10 min (Fig. 3D).



Fig. 2 Inward currents in $A|^{3+}$ -free solution after whole-cell formation and blockade by niflumate. (A) Combined data of inward current (at -174 mV) with time after formation of whole-cell configuration. In *TaALMT1* transformed protoplasts an inward

To test whether activation of the inward current in the protoplasts transformed with *TaALMT1* is specific to Al^{3+} treatment, additional trivalent cations were applied. Exposure of *TaALMT1*-transformed tobacco protoplasts to 100 µM La³⁺ or Gd³⁺ did not induce an increase in inward current (Supplementary Fig. S1). Furthermore, when 50 µM AlCl₃ was added to the bath solution already containing La³⁺ or Gd³⁺ the inward current increased significantly as before indicating that neither La³⁺ nor Gd³⁺ inhibit the Al³⁺-activated current (Supplementary Fig. S1).

The Al^{3+} -activated inward currents are generated by malate efflux

We investigated whether the Al³⁺-activated inward currents were generated by an influx of cations or efflux of anions. The only cations in the bath solutions included 10 mM TEA⁺ and 0.2 mM Ca²⁺ while the anions in the pipette solution were 40 mM malate²⁻ and 0.1 mM Cl⁻. The current-voltage relationship of the Al³⁺-activated current reversed near +60 mV, which is clearly more positive than the theoretical equilibrium potentials for either TEA⁺ or Cl⁻ (approximately -60 mV and -110 mV, respectively). This indicates that TEA⁺ and Cl⁻ fluxes are unlikely to be contributing significantly to the currents. We tested this by measuring the changes in E_{rev} with changes in TEACl concentration in the bath. The E_{rev} was $+40.6 \pm 7.5$ with 10 mM TEACl in the bath solution and $+42.1 \pm 6.6$ mV with 50 mM TEACl (n=3) demonstrating that the Al³⁺ activated current is unaffected by a 5-fold change in the external concentrations of TEA⁺ or Cl⁻. This is also consistent with a high relative permeability of malate²⁻ over Cl⁻. The GEOCHEM program (Parker et al. 1987) was used in these experiments to calculate the concentration of AlCl₃ required for maintaining constant activity of Al^{3+} .

The theoretical equilibria potential for Ca^{2+} and malate²⁻ in these experiments are both very positive. Therefore the Al³⁺-activated current could be generated by Ca^{2+} influx and/or by malate²⁻ efflux. Normally these ion movements could be differentiated from one another by altering the malate or Ca^{2+} concentrations in the bath solution and monitoring the shift in E_{rev} . However, malate cannot be added to the bathing solution without chelating the Al³⁺ required for maximum current activation

current increased while in empty vector controls inward currents diminished. Data are mean \pm SE for *TaALMT1* (n = 40) and empty vector (n = 16) transformed protoplasts, respectively. (B, C) Superimposed series of current versus time responses to voltage clamps (to -174 mV plus 20 mV increments) for a representative empty vector protoplast (B), and *TaALMT1* transformed protoplast (C) after 20 min in the whole cell configuration. (D) Current (initial) versus voltage curves showing lack of effect of 0.1 mM GdCl₃ and inhibition by 100 μ M niflumate on a subset of *TaALMT1* transformed protoplasts. Data are mean \pm SE of three protoplasts.



Fig. 3 Effect of $50 \,\mu$ M Al³⁺ on the whole-cell currents at pH 4.0 in *TaALMT1* transformed tobacco protoplasts and empty vector controls. (A) Time course of activation of inward current in a tobacco protoplast transformed with *TaALMT1* after the addition of $50 \,\mu$ M AlCl₃ (arrow). The clamped membrane voltage is indicated. (B) Initial (circles) and steady (squares) current versus voltage curves of combined data for *TaALMT1* before and after addition of Al³⁺. Data are mean ± SE (n = 20). (C) Superimposed series of current versus time responses to voltage clamps (as in Fig. 2B, C) for a representative *TaALMT1* protoplast exposed to $50 \,\mu$ M AlCl₃ for 10 min. (D) Current versus voltage curves of combined data for empty vector protoplasts before and after addition of Al³⁺ for comparison with B. Data are mean ± SE (n = 11).

(Ryan et al. 1997, Zhang et al. 2001). Instead we relied on a range of antagonists to differentiate between Ca²⁺ influx and malate efflux. The contribution of Ca²⁺ fluxes was tested with Gd³⁺ and La³⁺, which are wide-spectrum Ca²⁺ channel blockers (White 2000). The Al³⁺-activated currents were unaffected by 100 μ M of either of these treatments suggesting that the inward currents are not generated by Ca²⁺ influx. Niflumate is an anion channel antagonist which inhibited the malate²⁻ efflux from the intact tobacco cells (Fig. 1). Addition of 100 μ M niflumate to the bath solution rapidly inhibited the Al³⁺-activated inward current by 73.4 ± 4.1% (*n*=6) (Fig. 4).

Relative permeability of TaALMT1 to other anions

The relative permeability of the TaALMT1 protein to malate^{2–} and Cl[–] was obtained using two alternative methods. Firstly, we calculated the current–voltage curve for the Al³⁺-activated current by subtracting the currents

measured in control solution from the currents measured after addition of Al^{3+} . The current–voltage curve resulting from this subtraction reversed at a membrane potential greater than $57.3 \pm 7.7 \text{ mV}$ (n=10) and the modified Goldman-Hodgkin-Katz voltage equation (Lewis et al. 1979) estimated P_{mal}/P_{Cl} to be greater than 18. This calculation accounted for the effect of ionic strength on activities of the ions present in the system and assumed that malate^{2–} carried the current. A similar value for P_{mal}/P_{Cl} was obtained by using reversal potential of niflumatesensitive current (Fig. 4C). These reversal potentials were similar to that obtained for the niflumate-sensitive current before Al^{3+} was added.

The permeability of TaALMT1 to NO_3^- was investigated by replacing malate²⁻ in the pipette solution with NO_3^- . In these conditions the inward currents remained small and similar in control protoplasts to those expressing *TaALMT1* (Fig. 5A, D). Addition of Al³⁺ activated a small

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Fig. 4 Effect of niflumate on Al³⁺-activated currents in *TaALMT1*transformed protoplasts. (A, B) Before and after the addition of niflumate (100 μ M) of a superimposed series of current versus time response (voltage protocol as in Fig. 2B, C) in the presence of 50 μ M AlCl₃ (pH 4.0). (C) Current (initial) versus voltage curves of combined results before and after addition of niflumate. Data are mean \pm SE (*n*=3).

increase in inward current in the control and *TaALMT1*transformed protoplasts and shifted E_{rev} in the positive direction (Figs. 5B and E, C and F). Although the magnitude of these currents was significantly less than those measured when malate²⁻ was the anion, the magnitude of the increases were greater in the *TaALMT1*-transformed protoplasts than in the vector controls (Fig. 5C, F). Addition of Al³⁺ increased the current density in the *TaALMT1*transformed protoplasts by $8.8 \pm 3.9 \text{ mA m}^{-2}$ (n=6) at -174 mV (from -13.9 ± 2.9 to $-22.7 \pm 2.7 \text{ mA m}^{-2}$) compared to the $4.4 \pm 1.6 \text{ mA m}^{-2}$ (n = 6) change in vector controls (from -6.9 ± 0.8 to $-10.3 \pm 1.2 \text{ mA m}^{-2}$). The intersection of the current voltage curves before and after addition of Al³⁺ was greater than 60 mV for *TaALMT1*transformed protoplasts, indicating that with this gradient of activities ($[NO_3^-]_i = 62 \text{ mM}$, $[CI^-]_o = 9.3 \text{ mM}$) the P_{NO3}/P_{CI} was equal to or greater than 1. The Al³⁺-activated inward current at hyperpolarized potentials displayed a more pronounced and rapid decay than was observed with malate in the pipette solution in the *TaALMT1*-transformed protoplasts. By contrast, no decaying current was observed in the control protoplasts when challenged by Al³⁺ (Fig. 5E).

Voltage dependency of the Al^{3+} *-activated malate*²⁻ *inward current*

A feature of the Al³⁺ activated malate²⁻ currents was voltage dependent decay at negative membrane potentials (Figs. 3C, 4A, 5B). This decay was slow and could be fitted to the sum of two exponential components. The time constants were not strongly voltage dependent (Supplementary Fig. S2) and averaged 128 ms and 2008 ms for the fast and slow component, respectively. The decay in inward current was up to 50% of the initial current at the most negative membrane potentials imposed (Fig. 6A, B). The decay of inward current accounted for a characteristic saturation of the final (steady state) inward current with increasingly negative membrane voltage as observed in the current-voltage curves (Figs. 3B, 6A). There was a very close resemblance in current-voltage curves of Al³⁺-activated malate currents in TaALMT1-transformed tobacco protoplasts and protoplasts of the Al-tolerant ET8 line of wheat (Fig. 6A). The similarity between the voltage dependence of the decay of inward current in TaALMT1-transformed tobacco and Al-tolerant wheat root protoplasts is also illustrated in Figure 6B.

The question arises regarding the nature of the decay of inward current at negative voltages. There are three alternatives: (i) malate depletion and diffusion effects; (ii) inactivation referred to as decay of current due to progressive closure of channels at a constant voltage after the current has initially been activated by hyperpolarization; (iii) voltage-dependent deactivation, a decay of current resulting from changing the channels from open to closed state in response to a negative going voltage change, from a fully activated state.

(i) Decay of the inward current might reflect the depletion of malate in the protoplast during long hyperpolarizing voltage clamps. Alternatively, malate efflux to the external medium may chelate the AI^{3+} so reducing transport activation. Both these options depend on protoplast geometry. Malate depletion would be more rapid in smaller protoplasts and therefore these would be expected to show a



Fig. 5 Whole-cell currents in response to $50 \,\mu$ M AlCl₃ measured with NO₃⁻ as main permeant anion in the pipette solution. (A–E) Superimposed series of current versus time responses in response to voltage clamps (as in Fig. 4). (C, F) Current (initial value) versus voltage curves of combined data for *TaALMT1a* and empty vector tobacco protoplasts respectively. Data are mean ± SE of six protoplasts for TaALMT1 and empty vector protoplasts. The dashed line is for current difference between –Al and +Al.

greater proportion of current decay. Similarly, malate diffusion away from the external surface of the protoplast into the bulk medium will also be a function of protoplast diameter. The decay of the current was not dependent on the size of the protoplast over a 2-fold range in diameters (Supplementary Fig. S3) and calculations from the integrated currents over time during a voltage clamp to -174 mV, indicated that only a small fraction of internal malate concentration (2 mM out of 40 mM) would be depleted after 20 s. Stirring of the medium would also affect the degree of decay of the current if malate concentration was elevated in the external unstirred layer. We have



Fig. 6 Voltage-dependent inactivation of the Al³⁺-activated malate^{2–} currents. (A) Examples of whole-cell current–voltage curves showing initial and final currents measured in tobacco protoplasts expressing *TaALMT1* (*Nt TaALMT1*) and protoplasts prepared from root apices of the wheat genotype ET8 (*Ta*). (B) Final current relative to initial current (I_f/I_i) as a function of voltage, comparing *TaALMT1* in tobacco (solid squares), with ET8 (open squares). The fitted Boltzmann equations were not significantly different between the two data sets with values *Nt TaALMT1* (*Ta ET8*) of V₅₀ = –180 (–195) mV and slope = –44 (37) mV. (C) Time-dependent inactivation of inward current (at –174 mV, left) of Al³⁺-activated malate^{2–} currents and tail currents (at 6 mV, right) comparing a *TaALMT1* transformed tobacco protoplast (*Nt TaALMT1*) and ET8 root tip protoplast (*Ta*). (D) Amplitude of tail currents as a function of the change in conductance relative to maximum conductance after inactivation for eight separate *TaALMT1* transformed tobacco protoplasts. Tail amplitude was measured from the amplitude of a sum of two exponentials fitted to the kinetics at 6 mV.

previously found that flowing medium in the same chamber used here has a dramatic effect on the concentration profile of an ion within 10–50 μ m of the protoplast (Gilliham et al. 2006). However, there was no change observed in decay of inward current with or without flowing solution. Thus we conclude that the decay in the inward current is an intrinsic property of the underlying transport in response to voltage.

(ii) and (iii) From examination of the first several milliseconds after a negative going voltage clamp we were not able to detect any time-dependent activation of the inward current as has been observed with an anion channel in *Pisum sativum* leaf mesophyll protoplasts (Elzenga and Van Volkenburgh 1997). This would suggest that the current was already fully activated at depolarized potentials before hyperpolarization. Thus inward rectification of the initial current versus $V_{\rm m}$ curves was likely to be the result of non-symmetrical distribution of malate rather than

voltage-dependent gating. Using a voltage ramp protocol to capture the initial current before any decay we were able to analyse the rectification and curvature over a continuous voltage range (Fig. 7A). The initial current–voltage curves were better predicted by the GHK current equation (Hille 1991) for a divalent inward current (inward current $t = malate^{2-}$ efflux, solid line Fig. 7A). This further supports the conclusion that the malate²⁻ current is fully activated at the holding potential, normally near 0 mV. Thus we conclude that the decay in current is a voltage dependent deactivation from a fully activated state.

From this it would be expected that after deactivation of the current at negative potentials, there would be currents at depolarized potentials corresponding to the reactivation of the transport. An example is shown in Fig. 6C, which also serves as a comparison with the malate^{2–} currents observed under identical conditions in a protoplast of the



Fig. 7 Voltage ramp analysis of whole-cell currents of *TaALMT1* expressing protoplasts and a root tip protoplast from Al-resistant wheat (ET8). Voltage was ramped from a holding potential of -180 mV (-200 mV in C) over 5 s. (A) *TaALMT1*-expressing tobacco protoplast that has been fully activated by Al³⁺. Rectification can be accounted for by the solute gradients according to the GHK current equation. The current–voltage curve is shown for the original data (filled circles) and the fit to the GHK current equation (solid line) for an internal divalent anion (malate) with an activity of 13 mM (concentration 40 mM), and an external univalent anion (Cl⁻) with an activity of 9.3 mM (concentration 10.4 mM) as used in the experiments. The P_{mal}/P_{Cl} was 18 and $r^2 = 0.998$. Shown for comparison is a fit to the data using only univalent anions on either side of the membrane of the same activities in which case a more gentle curvature is observed (dashed line). (B) Sequence of ramps obtained during early stages of activation of current by Al³⁺ in a *TaALMT1* protoplast. (C) Sequence of ramps obtained in a *TaALMT1* protoplast before addition of Al³⁺. (D) Sequence obtained for an ET8 wheat root protoplast during the period of current decay in the presence of Al³⁺ with identical solutions to B. (E) Analysis of the data in B and D where conductances in the voltage range of -180 to -100 mV have been plotted in ascending order. The slope of the linear fits (shown with 95% confidence limits) is an upper limit of likely channel conductances (solid squares = *TaALMT1* tobacco, open squares = ET8 wheat protoplast).

Al resistant ET8 line of wheat. We observed that the magnitude of currents, which activate slowly towards more inward current (increasing malate^{2–} efflux), were correlated with the degree of inactivation in the previous pulse, both between protoplasts and as a result of different voltages in the pre-pulse for an individual protoplast (Fig. 6D).

Current fluctuations attributable to channel activity

During voltage ramps we often observed discrete conductance levels at negative voltages (Fig. 7B), particularly during progressive activation of the current after addition of Al^{3+} or during ramps performed during the decay of inward current after a negative going V_m pre-pulse.

This provides a hint that channel activity is at least a component of the Al³⁺-activated currents. Similar discrete changes in conductance were also observed occasionally in TaALMT1-transformed protoplasts before exposure to Al³⁺ (Fig. 7C). Voltage ramps performed on a protoplast of the Al resistant ET8 line of wheat under identical solution conditions to the data shown in Fig. 7B is shown for comparison in Fig. 7D. Discrete conductance levels were also observed. We analyzed this data by measuring the conductances in the more linear portion of the curve (-180)to -100 mV) and arranging them in ascending order of conductance (Fig. 7E). The slope of the straight line fit to conductance versus ramp number will provide at least an upper limit on the channel conductance (i.e. conductance per increment increase in ramp conductance), assuming that channel activity is the cause of the changes in ramp conductance. For the data shown in Fig. 7, we obtain an estimate of 24 pS for TaALMT1, and 11 pS for Al-resistant ET8 wheat.

Single-channel events were also evident in some cases during current deactivation (Fig. 8A, B) especially for protoplasts with smaller overall currents. Single steps could be observed indicating that deactivation was due to the progressive closure of channels in the whole cell patch when either malate or NO_3^- is included as the main permeant anion in the pipette solution (Fig. 8A, B). It was possible to analyze these currents using the Clampfit (pClamp 9) software to obtain an estimate of fluctuation currents as a function of voltage (Fig. 8D). In most cases (n=5)protoplasts) there was good agreement between whole-cell current and single-channel events in terms of reversal potential, and the slope of current versus voltage gave a conductance of 10 pS. In one case it appeared that the current levels were a multiple, and a higher conductance of 17 ps was obtained. In both cases the extrapolated straight line crossed the voltage axis very close to, and not significantly different from, the reversal potentials from whole-cell difference currents. Since we demonstrated that



Fig. 8 Channel fluctuations of TaALMT1. (A) *TaALMT1* tobacco protoplast giving current vs time response to voltages (-154, -174 mV) in which current steps can be observed. In some cases single-channel events were observed and these were compared against events in detached outside-out patches after Al³⁺ activation (inset, scale bar = 3 pA). (B) Similar steps in current were observed with NO₃⁻ as the intracellular anion. The levels shown were determined with a transient analysis technique (see text). (C) Single-channel steps accounting for inactivation in an ET8 wheat root tip protoplast. (D) Current versus voltage curves for current fluctuations in whole-cell and a detached outside-out patch. Square filled symbols are combined (n=4 protoplasts, conductance = 9.7 pS, reversal potential = 33 mV). One protoplast gave higher currents (filled triangles, conductance 16.7 pS, reversal potential = 38 mV). Open squares are levels detected in an outside-out patch.

the whole-cell currents in cells expressing TaALMT1 were generated from malate²⁺ fluxes, and not other ions, we conclude that these single-channel events are caused by the TaALMT1 protein. Using a transient analysis technique (Tyerman et al. 1992), we were also able to detect long-lived levels (>15 ms) within the whole cell records, that agreed with the data obtained from Clampfit fluctuation analysis. The levels detected by the transient analysis are shown superimposed in Fig. 8B, which is an example with NO_3^- as the anion in the pipette. When outside-out patches were obtained before addition of Al³⁺ no channel activity was detected after Al^{3+} addition to the bath solution (n=8). Outside-out patches were also pulled from protoplasts already Al³⁺ activated. In this case it appeared that the channel activity rapidly decreased, and only in a couple of instances could single channels be captured. Single channel amplitudes could be measured at -174 mV before they inactivated. These also showed two apparent levels, the larger of which is shown in Fig. 8A, and they agreed with the analysis carried out on the whole-cell patches (Fig. 8D, open symbols). For comparison Fig. 8C shows malate currents observed under identical conditions in a protoplast from an Al³⁺-resistant genotype of wheat, ET8, where current fluctuations are observed of similar size to those observed in tobacco cells expressing TaALMT1 (cf. Fig. 8A).

Discussion

The cloning of TaALMT1 (Sasaki et al. 2004) has greatly boosted our understanding of the mechanisms of Al³⁺ resistance in plants. Until now electrophysiological analysis of TaALMT1 function has been completed in Xenopus oocytes (Sasaki et al. 2004, Piñeros et al. 2008b). Although these studies have revealed some novel properties of the protein, they have not provided direct evidence that TaALMT1 encodes an Al³⁺-activated anion channel that controls the Al³⁺ resistance phenotype in wheat. There has been no electrophysiological evidence for ALMT1 as a channel protein, nor has ALMT1 been investigated electrophysiologically in a plant expression system. In the present study, we conducted the first electrophysiological investigation of TaALMT1 in a transgenic plant-cell system and compared the characteristics of the currents with the original malate²⁻ currents observed in Al³⁺ resistant wheat (Zhang et al. 2001).

Two types of whole-cell currents were observed in tobacco protoplasts expressing *TaALMT1* that were not present in the cells transformed with an empty vector. The first of these currents was independent of Al^{3+} and occurred soon after the whole-cell configuration was obtained. This current was generated by malate^{2–} efflux because: (i) it was observed when malate^{2–} was in the pipette solution, (ii) it

was insensitive to the cation channel blocker Gd³⁺ but significantly inhibited by niflumate, and (iii) the E_{rev} for the niflumate-sensitive current was consistent for a malate²⁻ conductance. Since the current was only observed in protoplasts transformed with TaALMT1 we further conclude that this Al³⁺-independent malate²⁻ current is mediated by the TaALMT1 protein. This is consistent with TaALMT1-expressing cells exhibiting greater malate efflux than the controls (Table 1). It is possible that TaALMT1 functions at a low level in the absence of Al^{3+} , perhaps even in wheat, but that this response only becomes measurable when the gene is highly expressed. Alternatively, the TaALMT1 protein might be activated to a small degree by the high concentrations of malate used in the pipette solution. Recently Piñeros et al. (2008b) also demonstrated that TaALMT1 can carry anion currents without activation by Al³⁺ when expressed in *Xenopus* oocytes. Furthermore the activation by Al³⁺ did not appear to change the selectivity of the transporter (Piñeros et al. 2008b), a result consistent with our observations (see below). Transport activity without Al^{3+} has also been reported for *AtALMT1*. a homolog of *TaALMT1* that contributes to the Al^{3+} resistance of Arabidopsis (Hoekenga et al. 2006). In that study, malate efflux was greater from Xenopus oocytes expressing AtALMT1 than from control oocytes in the absence of external Al^{3+} (Hoekenga et al. 2006).

The second inward current novel to protoplasts transformed with *TaALMT1* was detected when Al^{3+} was added to the bath. The Al^{3+} -activated inward current was insensitive to cation channel blockers but sensitive to niflumate. Niflumate was previously shown to be an effective inhibitor of the Al^{3+} -activated malate efflux from wild-type wheat roots, of the Al^{3+} -activated inward currents in wheat-root protoplasts (Ryan et al. 1995b, Ryan et al. 1997, Zhang et al. 2001) and of the Al^{3+} -activated malate efflux from transgenic tobacco cells (present study).

The Al³⁺-activated current was much larger than that observed without Al³⁺ present, and tended to dominate the whole-cell ionic conductances resulting in a positive shift in whole-cell reversal potential. However, subtraction of I/V curves before and after Al³⁺ addition, or before and after inhibition with niflumate (with or without Al³⁺ present) showed that the selectivity of malate²⁻ over Cl⁻ was not substantially changed by Al³⁺ activation. We calculated the permeability of the Al³⁺-activated current to be approximately 18- to 20-fold greater for malate²⁻ than for Cl⁻ and NO₃⁻. This is also consistent with a measurable Cl⁻ and NO₃⁻ permeability of *TaALMT1* expressed in *Xenopus* (Piñeros et al. 2008b).

The Al^{3+} -activated malate²⁻ currents in the tobacco cells transformed with *TaALMT1* are similar to the Al^{3+} activated currents described previously in wheat (Ryan et al.

1997, Zhang et al. 2001). Both currents are activated by Al^{3+} treatment but not by La^{3+} , both currents show voltage dependent deactivation at negative membrane potentials, both currents reverse at very positive potentials and both currents are inhibited by niflumate but are insensitive to La³⁺. However some differences are also apparent: (i) activation of the inward current by Al^{3+} occurred in almost all of the tobacco cells examined whereas only one third of cells showed the response in wheat. This may not be surprising since we expect all of the tobacco cells to be expressing TaALMT1 but it is unknown whether all cells in the apices of wheat roots express TaALMT1; (ii) activation of the current by Al³⁺ occurred more rapidly in tobacco with 80% of protoplasts showing an increase in current density within $5 \min$ of Al^{3+} addition. In wheat, by comparison, and with malate in the pipette solution, Zhang et al. (2001) calculated the average time for the current to be activated was 9 min and Ryan et al. (1997) estimated the half-time of activation to be 14 min with Clin the pipette solution. Furthermore, the magnitude of the Al³⁺-activated current in the tobacco protoplasts $(-128.8 \pm 22.4 \text{ mA m}^{-2})$ was almost 2-fold greater than the current measured in the wheat protoplasts $(-68.8 \pm 7.6 \text{ mA m}^{-2})$ at the same voltage. These differences may be explained by the high level of TaALMT1 expression expected in the transgenic tobacco cells since expression is driven by the CaMV 35S promoter. Perhaps more perplexing was the finding that the permeability of the transporter/ channels for malate²⁻ relative to Cl⁻ (P_{mal}/P_{Cl}) is 2-fold different in the two systems. Using the niflumate-sensitive current in both cases, we calculated $P_{\rm mal}/P_{\rm Cl}$ to be approximately 18-20 in the tobacco cells compared to 7.8 in the wheat cells. Note that the estimate for $P_{\rm mal}/P_{\rm Cl}$ of 7.8 for wheat is revised from the value of 2.6 reported by Zhang et al. (2001) since that previous calculation ignored the effect of ionic strength on malate^{2–} activities in the solution. It is possible that in intact wheat roots Al³⁺ activates more than one type of anion transport protein or channel and that these exhibit variable characteristics such as permeability for malate^{2–}. For example, it was recently shown that ZmALMT1, when expressed in Xenopus, was poorly selective between anions and could show some stimulation by Al^{3+} (Piñeros et al. 2008a).

Small Al^{3+} -activated currents were also detected when NO_3^- was the main anion in the pipette solution. The kinetics of this current differed from those associated with malate²⁻ efflux due to the relatively large and more rapid deactivation. Perhaps the accumulation of malate in the unstirred layer outside the plasma membrane influences TaALMT1 function as has been reported for the slow-anion channel in guard cells (Hedrich et al. 1994). Interestingly the kinetics of the Al³⁺-activated inward current generated with NO_3^- were comparable to those measured in maize root

cells when Cl⁻ was used as the main permeant anion (Piñeros and Kochian 2001).

An important previously unresolved question regarding TaALMT1 physiology is whether ALMT1 functions as an anion channel or some other type of transport protein. The pharmacology of the Al³⁺-activated malate efflux in wild-type wheat and other transgenic cells expressing TaALMT1 is consistent with channel function and this point has been made in many previous reports (Ryan et al. 1995b, Zhang et al. 2001, Ryan et al. 2001). Furthermore, Ryan et al. (1997) reported some Al³⁺-activated singlechannel activity in wheat root cells when Cl⁻ was the main anion in the pipette solution. Attempts to activate singlechannels in excised outside-out patches were unsuccessful. In outside-out patches excised from whole-cells already showing the Al³⁺-activated response, single channels were only briefly observed before inactivating. We found that single-current fluctuations in the whole-cell records corresponded to channels turning off and that these could account for deactivation at negative membrane potentials. The estimate of single-channel conductance was in the range of 10-24 pS which agreed with the rarer events in outsideout patches pulled from already activated protoplasts. The dependence of the channel activity on "whole-cell" conditions, indicates that other components, disassociated in outside-out patch formation, may be required for channel activity. Furthermore, because TaALMT1 shows Al³⁺activated malate efflux in a variety of expression systems, including Xenopus oocytes, it suggests that the components required are ubiquitous. Other explanations include the possibility that membrane patches induce conformational changes in the protein or that TaALMT1 function depends on the formation of multimeric complexes that are less likely to be maintained in isolated patches.

Al³⁺-activated anion channel activity has been reported in the excised patches of maize roots (Piñeros and Kochian 2001) suggesting that Al³⁺-activated anion channels are present in these cells and that activation by Al^{3+} results from a direct interaction between Al^{3+} and the channel protein. A recent study by Piñeros et al. (2008a) indicated that ZmALMT1 does not underlie Al tolerance in maize. Therefore, other membrane proteins may exist in maize root cells to account for the Al-activated currents in maize. Recent studies reveal that Al-activated citrate efflux from barley roots (Furukawa et al. 2007, Wang et al. 2007) and sorghum roots (Magalhaes et al. 2007) occurs through an entirely different class of transporter belonging to the multidrug and toxic compound extrusion (MATE) family. This transporter (HvAACT1) when expressed in *Xenopus* oocytes shows citrate selective (relative to malate) inward currents activated by Al^{3+} .

It is unlikely that *TaALMT1* encodes a receptor that leads to the activation of a different channel rather than

encoding an anion channel itself. Zimmermann et al. (1994) identified an anion channel (TSAC) in a different line of tobacco suspension cells than that used here that could pass large currents when Cl⁻ was the main anion. TSAC showed slow inactivation at negative potentials and had a singlechannel conductance of 15 pS (150 mM Cl⁻). Despite these apparent similarities to the TaALMT1 currents observed here, we did not observe any anion channel behaviour corresponding to TSAC in the empty vector control protoplasts under our imposed solution gradients. It is also unlikely that TSAC would show such high $P_{\rm mal}/P_{\rm Cl}$ since this property of TaALMT1 is novel amongst plant plasma membrane anion channels (Schmidt and Schroeder 1994, Frachisse et al. 1999), though not compared to vacuolar channels (Cerana et al. 1995, Cheffings et al. 1997, Hafke et al. 2003). ZmALMT1, in contrast, shows low selectivity between anions when expressed in Xenopus oocytes (Piñeros et al. 2008a). It remains unclear whether this reflects the in planta selectivity of the ZmALMT1 transporter.

The possibility that TaALMT1 acts as a receptor that interacts with a native anion channel is also inconsistent with the observation that a small but measurable amount of malate efflux occurs from *TaALMT1*-expressing tobacco cells in the absence of Al^{3+} . Furthermore, if *TaALMT1* encodes a receptor protein rather than a transport protein then we would need to conclude that an, as yet, unidentified highly selective malate channel is present in all the animal and plant cells that have been used to examine its function, since TaALMT1 has generated similar phenotypes in *Xenopus* oocytes, barley, rice and tobacco suspension cells (Delhaize et al. 2004, Sasaki et al. 2004, Piñeros et al. 2008b).

With regard to voltage dependence of the TaALMT1 channel, our results are most consistent with the channel being fully activated by Al³⁺ at depolarized membrane potentials. The inward rectification of the currents is the result of the concentration gradients of permeating anions rather than voltage-dependent activation of the channel at negative potentials. This conclusion is consistent with outward rectification being observed for TaALMT1 when expressed in Xenopus with the appropriate anion gradients (Piñeros et al. 2008b). Upon hyperpolarization the channel undergoes a transition to a partially deactivated state. This is reversed by depolarization, and tail currents corresponding to channel opening were correlated with the degree of deactivation. This behaviour is qualitatively similar to other slow-type anion channels (Schroeder and Keller 1992, Dietrich and Hedrich 1998, Frachisse et al. 2000, Zhang et al. 2004) suggesting a common voltagedependent gating mechanism that may be revealed by structural analysis of the TaALMT1 protein. The ZmALMT1 and TaALMT1 protein when expressed in *Xenopus* oocytes shows similar behaviour (Piñeros et al. 2008a, 2008b).

Measurements in the elongation zone of intact wheat roots indicate that the membrane potential settles near – 100 mV after addition of Al^{3+} (Wherrett et al. 2005). Therefore, a consequence of deactivation is that malate efflux would become less dependent on voltage gradient for membrane potentials in the normal physiological range. The effect can only be observed in patch-clamped protoplasts when the currents become saturated at increasingly negative membrane potentials. There is remarkable similarity in behaviour of deactivation of malate currents between TaALMT1 expressed in tobacco and the Al^{3+} resistant (ET8) line of wheat (Zhang et al. 2001).

This study has highlighted strong similarities between the Al³⁺-activated malate efflux in wheat roots and the electrophysiological behaviour of TaALMT1 in transgenic tobacco cells. The results present the strongest evidence so far that the TaALMT1 protein is an Al³⁺-activated malate channel responsible for both the malate efflux from intact wheat roots and the Al³⁺-dependent inward currents described previously in those cells. Finally, we provide new insights into the physiology of TaALMT1 by demonstrating that it is not an inwardly-rectifying anion channel, as first believed, but an anion channel that is active at depolarized potentials.

Materials and Methods

Plant materials and protoplast preparation

Tobacco suspension cells (*Nicotiana tabacum* L. cv. Samsun, a cell line SL) transformed with the *TaALMT1* gene from wheat (cell line 4) or an empty vector (cell line 9) (Sasaki et al. 2004) were grown in MS medium that contained (for 0.4 liters): 1.7 g Murashige and Skoog salts, 12 g sucrose, 4 ml Gamborgs salts (100 × solution), 0.2 ml 2,4-D (3 mM) with 100 μ g ml⁻¹ kanamycin. Protoplasts were prepared from these cells by incubating the suspension cells in an enzyme solution of 0.4% [w/v] cellulase (Onozuka RS, Yakult Pharma. Ind.) and 0.04% [w/v] pectolyase (Sigma-Alridch) PVP, 0.5% [w/v] BSA, 1 mM CaCl₂, 500 mM sorbitol, 2 mM ascorbic acid, 10 mM Mes/Tris (pH 6.0) for 1 h at 25°C. Clean protoplasts were collected with a sucrose density gradient (Shachtmann et al. 1991).

Seeds of the ET8 $(Al^{3+}$ -resistant) genotype of wheat (*T. aestivum* L.) were surface sterilized with 0.5% sodium hypochlorite and grown for 4–5d in 0.2 mM CaCl₂ solution (pH 4.5) as described previously (Ryan et al. 1997). Protoplasts of the terminal 2–3 mm of roots were isolated using the procedure of Schachtman et al. (1991). Freshly isolated protoplasts were kept on ice until required for the electrophysiological experiments.

Malate efflux from tobacco suspension cells

Transgenic tobacco suspension cells were grown in MS media solution on a rotary shaker until the logarithmic phase of growth. For data shown in Table 1 aliquotes of the suspension containing about 1 g of cells were centrifuged and cells gently re-suspended in 15ml of Ca media consisting of 3mM CaCl₂ and 3mM sucrose (pH 4.5). This was repeated and the re-suspended cells were sealed in 50 ml tubes and placed on a shaker for 20 h (~100 rpm). Malate concentrations in the supernatant were measured after centrifugation with an enzyme assay (Delhaize et al. 1993). For data presented in Figure 1, cells were washed in Ca media as before. Aliquots were then collected and cells resuspended in the same media with or without added treatments at approximately 0.15 g FW/10 ml. Treatments included 100 μ M AlCl₃, 100 μ M LaCl₃ or 100 μ M AlCl₃ with niflumate. The flasks were placed on a shaker and after a further incubation for either 60 min or 20 h the suspensions were centrifuged and malate assayed in the supernatant. Niflumic acid solutions were prepared in concentrated stocks with ethanol and so equivalent amounts of ethanol were added to control treatments to account for the effect of this solvent on malate efflux.

Electrophysiology

Patch pipettes pulled from borosilicate glass blanks (Clark Electromedical, Reading, UK) were coated with Sylgard (184 silicone elastomer kit, Dow Corning Co., Midland, MI, USA). Pipettes filled with the pipette solutions (see below) had resistances ranging from 10 to $18 \text{ M}\Omega$ in sealing solution (10 mM CaCl_2). The voltage across the patch was controlled and current measured using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). Series resistance was compensated to approximate 50% and capacitance was compensated. Pulse and ramp protocols with sampling frequencies of 2kHz and corresponding filtering frequencies of 0.5 kHz were used to elicit the current from a holding potential that was close to the resting membrane potentials. Records were stored and analyzed using pClamp 8.0 and pClamp 9 (Axon Instruments, Foster City, CA, USA). Junction potentials for each change in bathing solution were calculated and corrected for using the program JPCalc (P.H. Barry, University of New South Wales, Kensington, NSW, Australia). All the experiments were carried out at room temperature (20-22°C). Current-voltage curves were constructed using either initial current values that were measured after approximately 50 ms of voltage pulses or steadystate currents measured after the currents had stabilized in time at a particular voltage. The current-voltage curves were fitted with third order polynomials using Prism program (Graph Pad Software, San Diego, CA, USA). When means are presented error bars represent SEM.

Experimental solutions

All pipette solutions unless otherwise specified were composed of (mM): 40 malic acid, 0.05 CaCl₂, 1.95 Ca-gluconate₂, 2 Mg-ATP, 10 EGTA, 10 N-2-hydroxyethylpiperazine-N-2- ethanesulphonic acid (HEPES), pH 7.2 adjusted with tetraethylammonium hydroxyl. Osmolality of the pipette solution was adjusted to 700 mOsmol kg⁻¹ with sorbitol. The protoplasts were first placed in a chamber filled with a 'sealing solution' composed of (mM): 10 CaCl₂, 5 MES, pH 6.0 and osmolality of 700 mOsm kg⁻¹ adjusted with TRIS and sorbitol, respectively. After the whole-cell patchconfiguration was achieved, the bath solution was replaced by control solution (0.2 mM CaCl₂, 10-50 mM TEACl, pH = 4.0). An identical solution containing 50-110 µM AlCl3 was used to examine the response of whole-cell conductance to Al³⁺. Bath solutions containing AlCl₃ were prepared from 10-mM stock in 0.1 mM HCl. To prevent the formation of triskaidekaaluminum, the pH of solutions was raised slightly prior to the addition of Al^{3+} and then adjusted down with HCl if required (Ryan et al. 1997). All solutions were kept at 4°C until used and filtered through a 0.2 µm Millipore filter before use. When required the chemical speciation program GEOCHEM (Parker et al. 1987) was used to compute the free activities of ions. All chemicals used in the present study were purchased from Sigma.

Supplementary material

Supplementary material mentioned in the article is available online to subscribers at the journal website www.pcp.oxford journals.org.

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