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Cytosolic malate and oxaloacetate activate S-type anion channels in *Arabidopsis* guard cells

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Summary

- Intracellular malate–starch interconversion plays an important role in stomatal movements. We investigated whether malate or oxaloacetate from the cytosolic membrane side regulate anion channels in the plasma membrane of *Arabidopsis thaliana* guard cells. Physiological concentrations of cytosolic malate have been reported in the range of 0.4–3 mM in leaf cells.
- Guard cell patch clamp and two-electrode oocyte voltage-clamp experiments were pursued.
- We show that a concentration of 1 mM cytosolic malate greatly activates S-type anion channels in *Arabidopsis thaliana* guard cells. Interestingly, 1 mM cytosolic oxaloacetate also activates S-type anion channels. Malate activation was abrogated at 10 mM malate and in *SLAC1* anion channel mutant alleles Interestingly, malate activation of S-type anion currents was disrupted at below resting cytosolic free calcium concentrations ($[Ca^{2+}]_{cyt}$), suggesting a key role for basal $[Ca^{2+}]_{cyt}$ signaling. Cytosolic malate was not able to directly activate or enhance SLAC1-mediated anion currents in *Xenopus* oocytes, whereas in positive controls cytosolic $NaHCO_3$ enhanced

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Author contributions

This research was designed by J.I.S., J.W., J.Z. and C.W. C.W. and J.W. conducted patch clamp experiments with guard cells. J.Z., C.W. and D.B. conducted *Xenopus* oocyte experiments. J. I. S., J. Z. and C.W. wrote the manuscript with comments from all authors.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Cytosolic malate at 1 mM activates ionic currents in *Arabidopsis thaliana* wild-type (WT) guard cells with 0.1 mM malate showing partial activation in the depicted experimental set, whereas 10 mM malate showed no activation of currents.

Fig. S2 Cytosolic malate (1 mM) does not activate S-type anion channel currents in *Arabidopsis thaliana* wild-type guard cells at 0.08 μM free cytosolic Ca^{2+}.

Fig. S3 Cytosolic malate at 1, 10 and 20 mM does not significantly enhance or inhibit SLAC1-mediated ion currents in *Xenopus* oocytes co-expressing OST1 or CPK6.

Fig. S4 Cytosolic malate at 1 mM does not significantly enhance SLAH3-mediated ion currents in *Xenopus* oocytes.

Fig. S5 Average S-type anion channel currents recorded at −145 mV from different genotypes as shown in Figs 4 and 6 under control conditions (0 mM malate).

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SLAC1 activity, suggesting that malate may not directly modulate SLAC1. Cytosolic malate activation of S-type anion currents was impaired in *ost1* and in *cpk5/6/11/23* quadruple mutant guard cells.

- Together these findings show that these cytosolic organic anions function in guard cell ion channel regulation.

Keywords

Arabidopsis; Cl^- channel; ion channel regulation; malic acid; stomata

Introduction

Stomatal pores, which are formed by pairs of guard cells in the epidermis of aerial tissues, control gas exchange and account for loss of water, including during drought stress. Stomatal movements are regulated by several signals, including the phytohormone abscisic acid (ABA), CO_2, humidity, reactive oxygen species, light, and pathogens (Hetherington & Woodward, 2003; Roelfsema *et al.*, 2012; Murata *et al.*, 2015; Ye *et al.*, 2015). Stomatal movements are regulated by controlled transport of osmoregulatory ions through several types of ion channels. Blue light promotes stomatal opening. H^+ efflux from the cytosol of stomatal guard cells is mediated by the H^+-ATPase that hyperpolarizes the membrane potential, which consequently activates voltage-gated inward K^+ channels, causing stomatal opening (Shimazaki *et al.*, 1986, 2007; Schroeder *et al.*, 1987; Kinoshita & Hayashi, 2011). By contrast, the plant hormone abscisic acid (ABA), elevated CO_2 concentrations and ozone induce stomatal closure. These stimuli activate anion channels among regulation of several ion channels and transporters and the efflux of anions induces plasma membrane depolarization that activates outward K^+ channels, causing stomatal closure (Pandey *et al.*, 2007; Negi *et al.*, 2014; Munemasa *et al.*, 2015).

The *SLAC1* gene was genetically mapped and isolated from EMS mutant screens and plays a central role in stomatal movements (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). The *SLAC1* (SLOW ANION CHANNEL-ASSOCIATED1) gene, is required for slow anion channel activity in *Arabidopsis* guard cells and stomatal closing mediated by multiple stimuli, including abscisic acid, CO_2, ozone, H_2O_2 and Ca^{2+} (Negi *et al.*, 2008; Vahisalu *et al.*, 2008). Several protein kinases including OST1 (OPEN STOMATA1), and CPKs (Ca^{2+}-dependent protein kinases), and GHR1 (GUARD CELL HYDROGEN PEROXIDE-RESISTANT1) can cause phosphorylation and activation of SLAC1 anion channels in *Xenopus* oocytes (Geiger *et al.*, 2009, 2010; Lee *et al.*, 2009; Brandt *et al.*, 2012; Hua *et al.*, 2012; Brandt *et al.*, 2015).

S-type anion channels in guard cells and SLAC1 expressed in *Xenopus* oocytes are permeable to Cl^- and NO_3^- (Schmidt & Schroeder, 1994; Geiger *et al.*, 2011). However, SLAC1 and S-type anion channels in *Arabidopsis* guard cells are not permeable to HCO_3^- and malate (Geiger *et al.*, 2009; Xue *et al.*, 2011; Laanemets *et al.*, 2013). Intracellular bicarbonate generated by carbonic anhydrases can act as a second messenger and activate S-

type anion channels in guard cells (Hu *et al.*, 2010; Xue *et al.*, 2011; Tian *et al.*, 2015; Wang *et al.*, 2016).

R-type anion channels form a distinct type of anion channel in the plasma membrane of guard cells (Keller *et al.*, 1989; Hedrich *et al.*, 1990; Schroeder & Keller, 1992). R-type anion channels are encoded by the aluminium-activated malate transporter (ALMT) gene *ALMT12* (Meyer *et al.*, 2010b). ALMTs form a unique family of passive anion transport systems in plants. ALMTs are involved in dicarboxylic acid excretion required for aluminium tolerance (Hoekenga *et al.*, 2006) and in the efflux of inorganic and organic anions including malate during stomatal closure (Gerhardt *et al.*, 1987; Meyer *et al.*, 2010b; Sasaki *et al.*, 2010). AtALMT12 is mainly expressed in guard cells and targeted to the plasma membrane encoding for R-type anion channels (Meyer *et al.*, 2010b). Loss of *AtALMT12* impaired stomatal closure in response to ABA, darkness and high levels of CO_2 (Meyer *et al.*, 2010b; Sasaki *et al.*, 2010).

Malate exists in several cellular compartments (i.e. vacuoles, cytosol, chloroplasts and mitochondria). Malate is transported among compartments and this malate transport is important for regulation of subcellular malate concentrations (Van Kirk & Raschke, 1978; Martinoia *et al.*, 1985; Martinoia & Rentsch, 1994; Emmerlich *et al.*, 2003; Meyer *et al.*, 2010a; Hills *et al.*, 2012). The uptake of apoplastic malate is mediated by the plasma membrane AtABCB14 (ATP BINDING CASSETTE TRANSPORTER) in *Arabidopsis* guard cells (Lee *et al.*, 2008).

Plants exhibit CAM metabolism by using malic acid as a store of available CO_2 during the night; as a result malate accumulates to high levels of up to 350 mM in vacuoles (Luttge, 1987; Martinoia & Rentsch, 1994). Decarboxylation decreases the malic acid level by 200 mM during the day. C_3 and C_4 plants accumulate malic acid as salts (i.e. K-malate) at concentrations of up to 100–200 mM in vacuoles (Winter *et al.*, 1982). However, the cytosolic malate concentration is tightly controlled and its level is kept in the range from *c.* 0.4 to 3 mM in the dark and *c.* 2 to 5 mM in the light, since malate is at a central point of metabolic pathways affecting osmotic balance and pH homeostasis (Gerhardt *et al.*, 1987; Martinoia & Rentsch, 1994; Winter *et al.*, 1994; De Angeli *et al.*, 2013).

Previous studies have shown that extracellular malate can activate R-type anion channels in isolated protoplasts and intact *Vicia faba* guard cells (Marten *et al.*, 1992; Hedrich & Marten, 1993; Hedrich *et al.*, 1994). These observations suggest a ‘feedforward’ mechanism for control of R-type anion channels (Hedrich & Marten, 1993; Wang & Blatt, 2011), such that malate released from guard cells during stomatal closing (Van Kirk & Raschke, 1978), can further enhance stomatal closing. However, analysis of *cytosolic* malate concentrations on guard cell plasma membrane ion channels has thus far shown that high malate concentrations ≥ 10 mM can inhibit S-type anion channels in *Vicia faba* guard cells (Schmidt & Schroeder, 1994; Wang & Blatt, 2011). Enhancement of guard cell ion currents by millimolar malate was also observed (Wang & Blatt, 2011). Moreover, 1 mM oxaloacetic acid (OAA) inhibits anion currents in *Vicia faba* guard cells (Wang & Blatt, 2011).

In this study, we investigated whether cytosolic malate and OAA can regulate anion channels in *Arabidopsis* guard cells. Interestingly, we have found that malate and OAA cause a clear activation of S-type anion channels in *Arabidopsis* guard cells. We have found that 1 mM malate and 1 mM OAA activate S-type anion channel Cl^- currents in wild-type guard cells. Malate activation occurs at both resting and elevated cytosolic Ca^{2+} concentrations, but interestingly, physiological baseline cytosolic free Ca^{2+} concentrations are required for malate activation of S-type channels in guard cells. Furthermore, high cytosolic malate (10 mM) did not activate these channels, presumably due to the previously reported channel inhibition at high malate (Schmidt & Schroeder, 1994; Wang & Blatt, 2011). We further show that loss of *slac1*, *ost1* and *cpk5/6/11/23* impair 1 mM malate activation of S-type anion channel currents in guard cells. We also investigate reconstitution of malate regulation of the SLAC1 anion channel in *Xenopus laevis* oocytes. These experiments suggest that malate does not directly increase SLAC1-mediated anion channel activity, which in positive controls is found to be distinct from bicarbonate regulation of SLAC1.

Materials and Methods

Plant growth conditions

Arabidopsis thaliana L. Heynh. **[Author, please confirm inserted text 'L. Heynh.' is correct]** seedlings were grown on Murashige and Skoog (MS) medium (Sigma-Aldrich) containing 1% (w/v) sucrose and 0.8% (w/v) agar for 7 d and were transplanted into soil (Sunshine Professional Blend). The potted plants were kept in a growth chamber (white light of 100 μmol m^{-2} s^1 at 22°C, 70% relative humidity) for 4–5 wk.

Patch clamp analyses

Arabidopsis thaliana guard cell protoplasts were isolated as described previously (Yamamoto *et al.*, 2016). During patch clamp recordings, the membrane voltage was stepped to potentials starting from +35 to −145 mV for 5 s with −30 mV decrements with a holding potential at +30 mV. All assays were conducted at room temperature (22°C) under dim light.

The bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 10 mM MES-Tris (pH 5.6), and 1 mM $CaCl_2$, with D-sorbitol added to an osmolality of 485 mmol/kg. The pipette solution contained 3.35 mM $CaCl_2$, 6.7 mM EGTA, 2 mM $MgCl_2$, 10 mM HEPES-Tris (pH 7.1), and 150 mM CsCl, with an osmolality of 500 mmol kg^{-1}. The final free Ca^{2+} concentration in the pipette solution was 0.2 μM when indicated as calculated using Max Chelator software version 5.60 developed by Dr. Chris Patton at Stanford University. The free calcium concentration was buffered to 2 μM (5.86 mM $CaCl_2$ in the pipette solution) or to 0.01 μM free Ca^{2+} (0.3 mM $CaCl_2$ in the pipette solution) when indicated in the figures. Final osmolalities were adjusted with D-sorbitol. For analysis of malate and oxaloacetate activation of S-type anion channels, the indicated malate and oxaloacetate concentrations and 5 mM Mg-ATP were freshly added to the pipette solution and the pH was adjusted with Tris before patch clamp experiments. As reported in previous research, the time- and voltage-dependent kinetics of deactivation of S-type anion channels in guard cells and SLAC1-mediated currents in *Xenopus* oocytes (Schmidt & Schroeder, 1994; Brandt *et al.*, 2015) show variability that may reflect distinct post-translational protein modification states

that remain to be characterized. Intracellular malate and OAA activated anion currents independent of these channel states.

Two-electrode voltage-clamp recordings in *Xenopus laevis* oocytes

All constructs were cloned into the pNB1 oocyte expression vector using the USER (Uracil-Specific Excision Reagent) method (Nour-Eldin *et al.*, 2006). To investigate intracellular malate effects on anion channel activity in oocytes, SLAC1yc and OST1yn cRNA (Geiger *et al.*, 2009) or SLAC1 and CPK6 cRNA or SLAH3 and CPK21 cRNA were co-injected into oocytes and incubated in ND96 buffer at 16°C for 2 d before voltage-clamp recordings (Wang *et al.*, 2017). The extracellular recording solution contained 10 mM MES/Tris (pH 7.4), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM KCl, 24 mM NaCl, and 70 mM Na-gluconate. Osmolality was adjusted to 220 mM using D-sorbitol. To investigate effects of malate, 11.5 mM bicarbonate, 1 mM, 10 mM or 20 mM malate were injected into each oocyte as final calculated concentrations based on oocyte volume calculations (final concentration = 25 nl [injected volume] / 500 nl [oocyte volume] × injected concentration) (Wang *et al.*, 2016). To maintain the same injection volume of 25 nl in all experiments, 20 mM, 200 mM, 400 mM malate and 230 mM $NaHCO_3$ solutions were prepared to achieve final concentrations of 1 mM, 10 mM and 20 mM malate and 11.5 mM bicarbonate in oocytes. For malate and bicarbonate injections, oocytes were recorded consistently 10 min after injections (Wang *et al.*, 2016). Steady state currents were recorded starting from a holding potential of 0 mV and ranging from +40 to −160 mV in −20 mV decrements, followed by a −120 mV voltage 'tail' pulse (Geiger *et al.*, 2009; Wang *et al.*, 2016).

Results

We investigated whether physiological *cytosolic* malate affects the activity of S-type anion channels in the plasma membrane of *Arabidopsis* guard cells. Interestingly, adding 1 mM malate to the patch clamp pipette solution that dialyzes the cytoplasm of guard cells caused enhancement of whole guard cell ion currents (Fig. 1, Supporting Information Fig. S1), similar to *Vicia faba* guard cells (Wang & Blatt, 2011). Addition of 0.1 mM malate to the cytosol was not sufficient to cause a robust enhancement in ion currents (Fig. 1). In one of the experimental data sets, 0.1 mM cytosolic malate caused a significant but small enhancement of ion currents in guard cells (Fig. S1; $P < 0.02$ at −145 mV, $n = 8$ guard cells). All experiments were performed in the presence of 165.6 mM chloride ions in the pipette solution that dialyzes the cytosol, suggesting that the effect of the malate anion is unique relative to chloride ions.

Previous studies have however shown that higher cytosolic malate concentrations (i.e. ≥10 mM) can inhibit or block S-type anion channels in *Vicia faba* guard cells (Schmidt & Schroeder, 1994; Wang & Blatt, 2011) and that S-type anion channels in *Arabidopsis* guard cells are largely impermeable to malate anions (Laanemets *et al.*, 2013). We therefore tested the effect of adding 10 mM malate to the cytosol. Interestingly, at 10 mM malate, the activation of guard cell ion currents was not observed (Figs 1, S1).

Robust abscisic acid activation of S-type anion channels in *Arabidopsis* guard cells is mediated by simultaneously elevating cytosolic Ca^{2+} (Siegel *et al.*, 2009; Brandt *et al.*,

2015). Addition of 1 mM malate to the cytosol in guard cells enabled activation of ion currents when the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was buffered to 0.2 μM (Fig. 2). When $[Ca^{2+}]_{cyt}$ was buffered to an elevated level of 2 μM, anion currents were not activated in the absence of malate (Figs 2, 3), consistent with previous studies (Allen *et al.*, 2002; Brandt *et al.*, 2015). Addition of 1 mM malate to the pipette solution with $[Ca^{2+}]_{cyt}$ buffered to 2 μM led to even stronger enhancement of guard cell anion currents than at 0.2 μM $[Ca^{2+}]_{cyt}$ (Fig. 2). However, when the cytosolic free Ca^{2+} was buffered to a slightly below resting concentration of 0.08 μM and a sub-resting level of 0.01 μM, 1 mM malate did not significantly enhance anion channel currents (Figs 3, S2).

The SLAC1 gene is required for S-type anion channel activity in guard cells and in *Xenopus* oocytes (Negi *et al.*, 2008; Vahisalu *et al.*, 2008; Geiger *et al.*, 2009; Lee *et al.*, 2009). We investigated whether cytosolic malate activation is mediated by SLAC1-associated anion currents by using *slac1* mutant plants, in which R-type anion channel currents are intact (Vahisalu *et al.*, 2008). The malate activation of anion currents in guard cells was disrupted in two *slac1* mutant alleles, showing that intracellular malate activates S-type anion channels in guard cells and that SLAC1 plays a key role in this response (Fig. 4).

S-type anion currents have been shown to be activated by high intracellular concentrations of bicarbonate anions (HCO_3^-) in guard cells (Hu *et al.*, 2010; Xue *et al.*, 2011; Tian *et al.*, 2015) and the same concentrations of intracellular bicarbonate enhances the activity of SLAC1 channels activated by the protein kinases OST1, CPK6 and CPK23 in *Xenopus* oocytes (Wang *et al.*, 2016). We investigated whether SLAC1-mediated currents in *Xenopus* oocytes are also directly enhanced by intracellular malate. However, micro-injection of malate at a final concentration of 1 mM in the cytoplasm of oocytes did not enhance OST1- or CPK6-mediated SLAC1 channel activity in oocytes (Figs 5, S3a). In positive control experiments, injection of HCO_3^- enhanced SLAC1-mediated currents in the same batches of oocytes (Fig. 5), consistent with previous findings (Wang *et al.*, 2016). These data suggest that the mechanism of HCO_3^- enhancement of SLAC1 activity differs from the malate activation of S-type anion channels in guard cells found here. We also tested the SLAH3 channel that contributes to S-type anion channel function in guard cells (Geiger *et al.*, 2011). Cytosolic malate (1 mM) also did not enhance SLAH3 anion channel activity in *Xenopus* oocytes (Fig. S4). Additional experiments were conducted by injecting malate at final concentrations of 10 mM or 20 mM into oocytes expressing SLAC1 and OST1. However, these treatments did not clearly enhance or inhibit SLAC1-mediated anion currents (Fig. S3b) ($n > 3$ oocyte batches tested).

We further investigated whether the intracellular malate activation of S-type anion currents in guard cells (Figs 1, 2) depends on upstream protein kinases, using the *ost1* and *cpk5/6/11/23* mutants that impair abscisic acid activation of S-type anion channels in guard cells (Li *et al.*, 2000; Geiger *et al.*, 2009; Brandt *et al.*, 2015). The *ost1* and *cpk5/6/11/23* mutants displayed no S-type anion currents without malate consistent with previous findings in these mutants (Geiger *et al.*, 2009; Brandt *et al.*, 2015) and also with zero malate controls in wild-type guard cells (Figs 1, 4, 6, S5). We found that malate activation of S-type anion currents was impaired in the *ost1* and *cpk5/6/11/23* mutants (Fig. 6).

Oxaloacetic acid is a precursor in the malate synthesis pathway in guard cells (Martinoia & Rentsch, 1994). To test the effect of OAA on S-type anion channel currents, we added oxaloacetate (OAA) to the pipette solution. Surprisingly, 1 mM OAA was able to activate S-type anion channel currents, while this stimulation was not observed at 0.1 mM cytosolic OAA (Fig. 7). The unexpected activation of S-type anion currents by cytosolic OAA was found in independent experimental sets by J.W. and C.W.

Discussion

Previous studies have shown that *extracellular* malate enhances R-type anion channel activity but not S-type anion channel activity in guard cells (Marten *et al.*, 1992; Hedrich & Marten, 1993; Hedrich *et al.*, 1994). Moreover, intracellular malate at high concentrations ≥ 10 mM inhibits S-type anion channel activity in guard cells (Schmidt & Schroeder, 1994; Wang & Blatt, 2011) and at 1 mM malate an enhancement of anion currents occurs in *Vicia faba* guard cells (Wang & Blatt, 2011).

Cytosolic malate concentrations in the range from 0.4 to 3 mM have been reported in plant cells (Gerhardt & Heldt, 1984; Winter *et al.*, 1994; Farre *et al.*, 2001). In the present study we have found that cytosolic malate concentrations of 1 mM activate S-type anion channels in *Arabidopsis* guard cells, whereas lower malate concentrations of 0.1 mM showed either weak or no substantial activation of anion currents in guard cells (Figs 1, S1). Moreover, another cytosolic dicarboxylic acid OAA also stimulates S-type anion channels in the guard cell plasma membrane (Fig. 7). By contrast, in *Vicia faba* guard cells 1 mM intracellular oxaloacetate inhibited plasma membrane anion currents (Wang & Blatt, 2011).

The present study suggests that cytosolic OAA and malate at concentrations in the range of 1 mM, predicted to be physiological (Gerhardt & Heldt, 1984; Winter *et al.*, 1994; Farre *et al.*, 2001) can play an important role in anion channel up-regulation in *Arabidopsis* guard cells thus functioning in stomatal closing. During stomatal opening, guard cells synthesize malate from starch and transport malate into vacuoles, where malate is stored at high concentrations in the >100 mM range as osmotic counter ion to K^+ ions (Winter *et al.*, 1994). An increase in malate production in guard cells required for stomatal opening has been predicted to lead to an increase in the cytosolic malate concentration (Wang & Blatt, 2011). An increase in cytosolic malate to 10 mM would inhibit S-type anion channel activity (Figs 1, S1) (Schmidt & Schroeder, 1994; Wang & Blatt, 2011), which would favor stomatal opening. Thus high cytosolic malate concentrations may contribute to stomatal opening by inhibiting S-type anion channels. In line with this prediction, the stomatal opening signals blue light, red light and/or low CO_2 cause down-regulation of S-type anion channel activity in guard cells (Roelfsema *et al.*, 2002; Roelfsema *et al.*, 2006).

Malate enhanced S-type anion currents in guard cells both at a resting cytosolic free Ca^{2+} concentration of 0.2 μM and at 2 μM free Ca^{2+} (Fig. 2). Interestingly however, when the free calcium was clamped to 0.08 μM, a concentration that is only slightly below resting levels, or to a sub-resting level of 0.01 μM free Ca^{2+}, S-type anion channels were not activated by intracellular malate (Figs 3, S2). A previous study showed that buffering the free calcium concentration to sub-resting levels disrupts abscisic acid activation of anion channels in

Vicia faba guard cells (Levchenko *et al.*, 2005). A contribution of baseline resting Ca^{2+} levels to stimulus-induced stomatal closing has been observed (Gilroy *et al.*, 1991; Grabov & Blatt, 1998; Levchenko *et al.*, 2005; Siegel *et al.*, 2009) but sub-resting levels have been less studied. Based on the present findings and previous research (Levchenko *et al.*, 2005) further analyses of guard cell ion channel regulation at sub-baseline levels should be of interest for dissecting functions of resting calcium concentrations on ion channel regulation, in particular based on studies showing that abscisic acid and elevated CO_2 increase the sensitivity of S-type anion channels and inward-rectifying K^+ channels to cytosolic Ca^{2+} (Siegel *et al.*, 2009; Chen *et al.*, 2010; Xue *et al.*, 2011; Brandt *et al.*, 2015).

Predicted physiological cytosolic malate concentrations (0.4–3 mM) activate S-type anion channels in guard cells (Figs 1, 2, 4, 6) (Wang & Blatt, 2011). During stomatal closing, malate concentrations are reduced in guard cells through efflux and starch synthesis, (Van Kirk & Raschke, 1978; Schnabl, 1981; Schnabl *et al.*, 1982), indicating that low millimolar cytosolic malate concentrations may occur. Thus the up-regulation of anion channel activity identified here could contribute to stomatal closing. Consistent with these findings, malate activation of S-type anion channels was disrupted in the *ost1* and *cpk5/6/11/23* mutants (Fig. 6) that impair stomatal closing (Mustilli *et al.*, 2002; Yoshida *et al.*, 2002; Brandt *et al.*, 2015). The enhancement of S-type anion channel activity by intracellular malate depends on the *SLAC1* gene, as *slac1* mutants disrupted the cytosolic malate response (Fig. 4).

The ALMT9 and ALMT6 chloride channels are targeted to the tonoplast of guard cells. ALMT9 has been shown to mediate malate and fumarate currents directed into vacuoles of mesophyll cells (Kovermann *et al.*, 2007; De Angeli *et al.*, 2013; Zhang *et al.*, 2013, 2014). AtALMT6 mediates Ca^{2+} and pH dependent malate currents into guard cell vacuoles (Meyer *et al.*, 2011). Most recently, another tonoplast targeted ALMT channel, AtALMT4, was shown to mediate malate efflux from vacuoles, functioning in stomatal closure in response to ABA (Eisenach *et al.*, 2017). Interestingly, malate is not only transported into vacuoles by ALMT9, but cytosolic malate and oxaloacetate also up-regulate the ALMT9 channels that reside in the vacuolar membrane of guard cells and other plant cells, with ALMT9 channel activation occurring at *c.* 0.3 mM cytosolic malate (De Angeli *et al.*, 2013).

As ALMT6 & ALMT9 channels function in stomatal opening (Meyer *et al.*, 2011; De Angeli *et al.*, 2013) and S-type anion channels function in stomatal closing, these findings together suggest that an additional degree of regulation of these ion channels would be required to avoid futile simultaneous activation of counteracting ion channels in the guard cell vacuolar and plasma membranes. During stomatal opening S-type anion channels are directly down-regulated by type 2C protein phosphatases (Brandt *et al.*, 2015). This tight down-regulation by PP2Cs may preclude malate activation of S-type anion channels during stomatal opening, if cytosolic malate concentrations are low. Based on the present findings suggesting that malate acts further upstream of S-type anion channels rather than as a direct channel activator, these data point to the hypothesis that malate facilitates stomatal closing under permissive conditions that trigger stomatal closing, when PP2C phosphatases are inhibited. This hypothesis will require further investigation.

Consistent with the hypothesis that malate regulation of S-type anion channels depends on additional coinciding signal transduction mechanisms, cytosolic malate activation of anion channels does not appear to occur via a direct interaction with and up-regulation of SLAC1; Cytosolic malate in oocytes did not affect SLAC1- and SLAH3-mediated anion channel activity (Figs 5, S3, S4), as found in experiments conducted independently by three of the authors (C.W., J.Z. and D.B.). In control experiments, SLAC1-mediated currents were upregulated by intracellular bicarbonate in the same oocyte batches showing typical SLAC1 properties (Fig. 5). This is consistent with a recent study, in which high intracellular HCO_3^- could enhance anion currents mediated by SLAC1, when protein kinases were co-expressed, including OST1yn, CPK6 or CPK23 in *Xenopus laevis* oocytes. By contrast, direct modulation of SLAC1 or SLAH3 activity by malate was not observed (Figs 5, S3, S4). These data, together with the requirement of protein kinases for malate activation of S-type channels in guard cells (Fig. 6) indicate that malate activation of S-type anion channels is likely to occur via modulation of signaling mechanisms upstream of S-type anion channels in guard cells.

In summary, the present study reveals a new mode of S-type anion channel regulation in guard cells by cytosolic malate and oxaloacetate. This newly recognized regulation mechanism could contribute to physiological stomatal closing, as well as to the well-known regulation of stomatal movements by malate. Further research will be needed to dissect which of the many known upstream stomatal regulation mechanisms are directly regulated by cytosolic malate concentrations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

Allen GJ, Murata Y, Chu SP, Nafisi M, Schroeder JI. Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the *Arabidopsis* farnesyltransferase mutant *era1-2*. Plant Cell. 2002; 14:1649–1662. [PubMed: 12119381]

Brandt B, Brodsky DE, Xue S, Negi J, Iba K, Kangasjarvi J, Ghassemian M, Stephan AB, Hu H, Schroeder JI. Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. Proc Natl Acad Sci, USA. 2012; 109(26):10593–10598. [PubMed: 22689970]

Brandt B, Munemasa S, Wang C, Nguyen D, Yong T, Yang PG, Poretsky E, Belknap TF, Waadt R, Aleman F, et al. Calcium specificity signaling mechanisms in abscisic acid signal transduction in *Arabidopsis* guard cells. eLife. 2015; 4:e03599.

Chen ZH, Hills A, Lim CK, Blatt MR. Dynamic regulation of guard cell anion channels by cytosolic free Ca^{2+} concentration and protein phosphorylation. Plant Journal. 2010; 61(5):816–825. [PubMed: 20015065]

De Angeli A, Zhang J, Meyer S, Martinoia E. AtALMT9 is a malate-activated vacuolar chloride channel required for stomatal opening in *Arabidopsis*. Nature Communications. 2013; 4:1804.

Eisenach C, Baetz U, Huck NV, Zhang J, De Angeli A, Beckers GJM, Martinoia E. ABA-induced stomatal closure involves ALMT4, a phosphorylation-dependent vacuolar anion channel of *Arabidopsis*. Plant Cell. 2017; 29(10):2552–2569. [PubMed: 28874508]

Emmerlich V, Linka N, Reinhold T, Hurth MA, Traub M, Martinoia E, Neuhaus HE. The plant homolog to the human sodium/dicarboxylic cotransporter is the vacuolar malate carrier. Proceedings of the National Academy of Sciences, USA. 2003; 100(19):11122–11126.

Farre EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Willmitzer L. Analysis of the compartmentation of glycolytic intermediates, nucleotides, sugars, organic acids, amino acids, and sugar alcohols in potato tubers using a nonaqueous fractionation method. Plant Physiology. 2001; 127(2):685–700. [PubMed: 11598242]

Geiger D, Maierhofer T, Al-Rasheid KA, Scherzer S, Mumm P, Liese A, Ache P, Wellmann C, Marten I, Grill E, et al. Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1. Science Signaling. 2011; 4(173):ra32. [PubMed: 21586729]

Geiger D, Scherzer S, Mumm P, Marten I, Ache P, Matschi S, Liese A, Wellmann C, Al-Rasheid KA, Grill E, et al. Guard cell anion channel SLAC1 is regulated by CDPK protein kinases. Proc Natl Acad Sci, USA. 2010; 107(17):8023–8028. [PubMed: 20385816]

Geiger D, Scherzer S, Mumm P, Stange A, Marten I, Bauer H, Ache P, Matschi S, Liese A, Al-Rasheid KA, et al. Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. Proc Natl Acad Sci, USA. 2009; 106(50):21425–21430. [PubMed: 19955405]

Gerhardt R, Heldt HW. Measurement of subcellular metabolite levels in leaves by fractionation of freeze-stopped material in nonaqueous media. Plant Physiology. 1984; 75(3):542–547. [PubMed: 16663663]

Gerhardt R, Stitt M, Heldt HW. Subcellular metabolite levels in spinach leaves – regulation of sucrose synthesis during diurnal alterations in photosynthetic partitioning. Plant Physiology. 1987; 83(2): 399–407. [PubMed: 16665257]

Gilroy S, Fricker MD, Read ND, Trewayas AJ. Role of calcium in signal transduction of commelina guard-cells. Plant Cell. 1991; 3(4):333–344. [PubMed: 12324599]

Grabov A, Blatt MR. Membrane voltage initiates Ca^{2+} waves and potentiates Ca^{2+} increases with abscisic acid in stomatal guard cells. Proceedings of the National Academy of Sciences, USA. 1998; 95(8):4778–4783.

Hedrich R, Busch H, Raschke K. Ca^{2+} and nucleotide dependent regulation of voltage dependent anion channels in the plasma membrane of guard cells. EMBO J. 1990; 9:3889–3892. [PubMed: 1701140]

Hedrich R, Marten I. Malate-induced feedback-regulation of plasma-membrane anion channels could provide a CO_2 sensor to guard-cells. Embo Journal. 1993; 12(3):897–901. [PubMed: 7681395]

Hedrich R, Marten I, Lohse G, Dietrich P, Winter H, Lohaus G, Heldt HW. Malate-sensitive anion channels enable guard cells to sense changes in the ambient CO_2 concentration. Plant J. 1994; 6:741–748.

Hetherington AM, Woodward FI. The role of stomata in sensing and driving environmental change. Nature. 2003; 21:901–908.

Hills A, Chen ZH, Amtmann A, Blatt MR, Lew VL. OnGuard, a computational platform for quantitative kinetic modeling of guard cell physiology. Plant Physiology. 2012; 159(3):1026–1042. [PubMed: 22635116]

Hoekenga OA, Maron LG, Pineros MA, Cancado GM, Shaff J, Kobayashi Y, Ryan PR, Dong B, Delhaize E, Sasaki T, et al. AtALMT1, which encodes a malate transporter, is identified as one of several genes critical for aluminum tolerance in *Arabidopsis*. Proc Natl Acad Sci, USA. 2006; 103(25):9738–9743. [PubMed: 16740662]

Hu H, Boisson-Dernier A, Israelsson-Nordström M, Böhmer M, Godoski J, Ries A, Kuhn J, Schroeder J. Carbonic anhydrases are upstream regulators of CO_2-controlled stomatal movements in guard cells. Nature Cell Biol. 2010; 12(1):87–93. sup pp 81–18. [PubMed: 20010812]

Hua D, Wang C, He J, Liao H, Duan Y, Zhu Z, Guo Y, Chen Z, Gong Z. A plasma membrane receptor kinase, GHR1, mediates abscisic acid- and hydrogen peroxide-regulated stomatal movement in *Arabidopsis*. Plant Cell. 2012; 24(6):2546–2561. [PubMed: 22730405]

Keller BU, Hedrich R, Raschke K. Voltage-dependent anion channels in the plasma membrane of guard cells. Nature. 1989; 341:450–453.

Kinoshita T, Hayashi Y. New insights into the regulation of stomatal opening by blue light and plasma membrane H^+-ATPase. International Review of Cell and Molecular Biology. 2011; 289:89–115. [PubMed: 21749899]

Kovermann P, Meyer S, Hortensteiner S, Picco C, Scholz-Starke J, Ravera S, Lee Y, Martinoia E. The *Arabidopsis* vacuolar malate channel is a member of the ALMT family. Plant Journal. 2007; 52(6): 1169–1180. [PubMed: 18005230]

Laanemets K, Wang YF, Lindgren O, Wu J, Nishimura N, Lee S, Caddell D, Merilo E, Brosche M, Kilk K, et al. Mutations in the SLAC1 anion channel slow stomatal opening and severely reduce K^+ uptake channel activity via increased intracellular Ca^{2+} sensitivity of K^+ uptake channels. New Phytologist. 2013; 197(1):88–98. [PubMed: 23126621]

Lee M, Choi Y, Burla B, Kim YY, Jeon B, Maeshima M, Yoo JY, Martinoia E, Lee Y. The ABC transporter AtABCB14 is a malate importer and modulates stomatal response to CO_2. Nat Cell Biol. 2008; 10(10):1217–1223. [PubMed: 18776898]

Lee SC, Lan W, Buchanan BB, Luan S. A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. Proc Natl Acad Sci, USA. 2009; 106(50): 21419–21424. [PubMed: 19955427]

Levchenko V, Konrad KR, Dietrich P, Roelfsema MRG, Hedrich R. Cytosolic abscisic acid activates guard cell anion channels without preceding Ca^{2+} signals. Proceedings of the National Academy of Sciences, USA. 2005; 102(11):4203–4208.

Li J, Wang XQ, Watson MB, Assmann SM. Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science. 2000; 287(5451):300–303. [PubMed: 10634783]

Luttge U. Carbon-dioxide and water demand - crassulacean acid metabolism (CAM), a versatile ecological adaptation exemplifying the need for integration in ecophysiological work. New Phytologist. 1987; 106(4):593–629.

Marten I, Zeilinger C, Redhead C, Landry DW, Alawqati Q, Hedrich R. Identification and modulation of a voltage-dependent anion channel in the plasma-membrane of guard-cells by high-affinity ligands. Embo Journal. 1992; 11(10):3569–3575. [PubMed: 1382976]

Martinoia E, Flugge UI, Kaiser G, Heber U, Heldt HW. Energy-dependent uptake of malate into vacuoles isolated from barley mesophyll protoplasts. Biochimica Et Biophysica Acta. 1985; 806(2):311–319.

Martinoia E, Rentsch D. Malate compartmentation – responses to a complex metabolism. Annu Rev Plant Physiol Plant Mol Biol. 1994; 45:447–467.

Meyer S, De Angeli A, Fernie AR, Martinoia E. Intra- and extra-cellular excretion of carboxylates. Trends Plant Sci. 2010a; 15(1):40–47. [PubMed: 19913451]

Meyer S, Mumm P, Imes D, Endler A, Weder B, Al-Rasheid KA, Geiger D, Marten I, Martinoia E, Hedrich R. AtALMT12 represents an R-type anion channel required for stomatal movement in *Arabidopsis* guard cells. Plant Journal. 2010b; 63(6):1054–1062. [PubMed: 20626656]

Meyer S, Scholz-Starke J, De Angeli A, Kovermann P, Burla B, Gambale F, Martinoia E. Malate transport by the vacuolar AtALMT6 channel in guard cells is subject to multiple regulation. Plant Journal. 2011; 67(2):247–257. [PubMed: 21443686]

Munemasa S, Hauser F, Park J, Waadt R, Brandt B, Schroeder JI. Mechanisms of abscisic acid-mediated control of stomatal aperture. Curr Opin Plant Biol. 2015; 28:154–162. [PubMed: 26599955]

Murata Y, Mori IC, Munemasa S. Diverse stomatal signaling and the signal integration mechanisms. Annual Review of Plant Biology. 2015; 66:369–392.

Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J. Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell. 2002; 14:3089–3099. [PubMed: 12468729]

Negi J, Hashimoto-Sugimoto M, Kusumi K, Iba K. New approaches to the biology of stomatal guard cells. Plant and Cell Physiology. 2014; 55(2):241–250. [PubMed: 24104052]

Negi J, Matsuda O, Nagasawa T, Oba Y, Takahashi H, Kawai-Yamada M, Uchimiya H, Hashimoto M, Iba K. CO_2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. Nature. 2008; 452(7186):483–486. [PubMed: 18305482]

Nour-Eldin HH, Norholm MHH, Halkier BA. Screening for plant transporter function by expressing a normalized *Arabidopsis* full-length cDNA library in *Xenopus* oocytes. Plant Methods. 2006; 2:17. [PubMed: 17069646]

Pandey S, Zhang W, Assmann SM. Roles of ion channels and transporters in guard cell signal transduction. FEBS Lett. 2007; 581:2325–2336. [PubMed: 17462636]

Roelfsema MR, Hanstein S, Felle HH, Hedrich R. CO_2 provides an intermediate link in the red light response of guard cells. Plant J. 2002; 32(1):65–75. [PubMed: 12366801]

Roelfsema MR, Hedrich R, Geiger D. Anion channels: master switches of stress responses. Trends in Plant Science. 2012; 17(4):221–229. [PubMed: 22381565]

Roelfsema MR, Konrad KR, Marten H, Psaras GK, Hartung W, Hedrich R. Guard cells in albino leaf patches do not respond to photosynthetically active radiation, but are sensitive to blue light, CO_2 and abscisic acid. Plant, Cell & Environ. 2006; 29(8):1595–1605.

Sasaki T, Mori IC, Furuichi T, Munemasa S, Toyooka K, Matsuoka K, Murata Y, Yamamoto Y. Closing plant stomata requires a homolog of an aluminum-activated malate transporter. Plant Cell Physiol. 2010; 51(3):354–365. [PubMed: 20154005]

Schmidt C, Schroeder JI. Anion selectivity of slow anion channels in the plasma membrane of guard cells (large nitrate permeability). Plant Physiology. 1994; 106(1):383–391. [PubMed: 12232336]

Schnabl H. The compartmentation of carboxylating and decarboxylating enzymes in guard cell protoplasts. Planta. 1981; 152:307–313. [PubMed: 24301024]

Schnabl H, Elbert C, Kramer G. The regulation of the starch-malate balances during volume changes of guard cell protoplasts. Journal of Experimental Botany. 1982; 33:996–1003.

Schroeder JI, Keller BU. Two types of anion channel currents in guard cells with distinct voltage regulation. Proc Natl Acad Sci, USA. 1992; 89:5025–5029. [PubMed: 1375754]

Schroeder JI, Raschke K, Neher E. Voltage dependence of K^+ channels in guard cell protoplasts. Proc Natl Acad Sci, USA. 1987; 84:4108–4112. [PubMed: 16593851]

Shimazaki K, Doi M, Assmann SM, Kinoshita T. Light regulation of stomatal movement. Annual Review of Plant Biology. 2007; 58:219–247.

Shimazaki K, Iino M, Zeiger E. Blue light-dependent proton extrusion by guard-cell protoplasts of *Vicia faba*. Nature. 1986; 319(6051):324–326.

Siegel RS, Xue SW, Murata Y, Yang YZ, Nishimura N, Wang A, Schroeder JI. Calcium elevation-dependent and attenuated resting calcium-dependent abscisic acid induction of stomatal closure and abscisic acid-induced enhancement of calcium sensitivities of S-type anion and inward-rectifying K^+ channels in *Arabidopsis* guard cells. Plant Journal. 2009; 59(2):207–220. [PubMed: 19302418]

Tian W, Hou C, Ren Z, Pan Y, Jia J, Zhang H, Bai F, Zhang P, Zhu H, He Y, Luo S, Li L, Luan S. A molecular pathway for CO_2 response in *Arabidopsis* guard cells. Nature Communications. 2015; 6:6057.

Vahisalu T, Kollist H, Wang YF, Nishimura N, Chan W-Y, Valerio G, Lamminmaki A, Brosche M, Moldau H, Desikan R, et al. SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. Nature. 2008; 452:487–491. [PubMed: 18305484]

Van Kirk CA, Raschke K. Release of malate from epidermal strips during stomatal closure. Plant Physiol. 1978; 61(3):474–475. [PubMed: 16660318]

Wang C, Hu HH, Qin X, Zeise B, Xu DY, Rappel WJ, Boron WF, Schroeder JI. Reconstitution of CO_2 regulation of SLAC1 anion channel and function of CO_2-permeable PIP2;1 aquaporin as CARBONIC ANHYDRASE4 interactor. Plant Cell. 2016; 28(2):568–582. [PubMed: 26764375]

Wang C, Zhang J, Schroeder JI. Two-electrode voltage-clamp recordings in *Xenopus laevis* oocytes: reconstitution of abscisic acid activation of SLAC1 anion channel via PYL9 ABA receptor. Bio Protoc. 2017; 7(2):2114.

Wang Y, Blatt MR. Anion channel sensitivity to cytosolic organic acids implicates a central role for oxaloacetate in integrating ion flux with metabolism in stomatal guard cells. Biochem J. 2011; 439(1):161–170. [PubMed: 21745184]

Winter H, Robinson DG, Heldt HW. Subcellular volumes and metabolite concentrations in spinach leaves. Planta. 1994; 193(4):530–535.

Winter K, Usuda H, Tsuzuki M, Schmitt M, Edwards GE, Thomas RJ, Evert RF. Influence of nitrate and ammonia on photosynthetic characteristics and leaf anatomy of *Moricandia arvensis*. Plant Physiology. 1982; 70(2):616–625. [PubMed: 16662544]

Xue S, Hu H, Ries A, Merilo E, Kollist H, Schroeder JI. Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO_2 signal transduction in guard cell. EMBO J. 2011; 30(8):1645–1658. [PubMed: 21423149]

Yamamoto Y, Negi J, Wang C, Isogai Y, Schroeder JI, Iba K. The transmembrane region of guard cell SLAC1 channels perceives CO_2 signals via an ABA-independent pathway in *Arabidopsis*. Plant Cell. 2016; 28(2):557–567. [PubMed: 26764376]

Ye W, Adachi Y, Munemasa S, Nakamura Y, Mori IC, Murata Y. Open stomata 1 kinase is essential for yeast elicitor-induced stomatal closure in *Arabidopsis*. Plant and Cell Physiology. 2015; 56(6): 1239–1248. [PubMed: 25840086]

Yoshida R, Hobo T, Ichimura K, Mizoguchi T, Takahashi F, Aronso J, Ecker JR, Shinozaki K. ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in *Arabidopsis*. Plant and Cell Physiology. 2002; 43(12):1473–1483. [PubMed: 12514244]

Zhang J, Baetz U, Krugel U, Martinoia E, De Angeli A. Identification of a probable pore forming domain in the multimeric vacuolar anion channel AtALMT9. Plant Physiology. 2013; 163(2):830–843. [PubMed: 23918900]

Zhang J, Martinoia E, De Angeli A. Cytosolic nucleotides block and regulate the *Arabidopsis* vacuolar anion channel AtALMT9. Journal of Biological Chemistry. 2014; 289(37):25581–25589. [PubMed: 25028514]

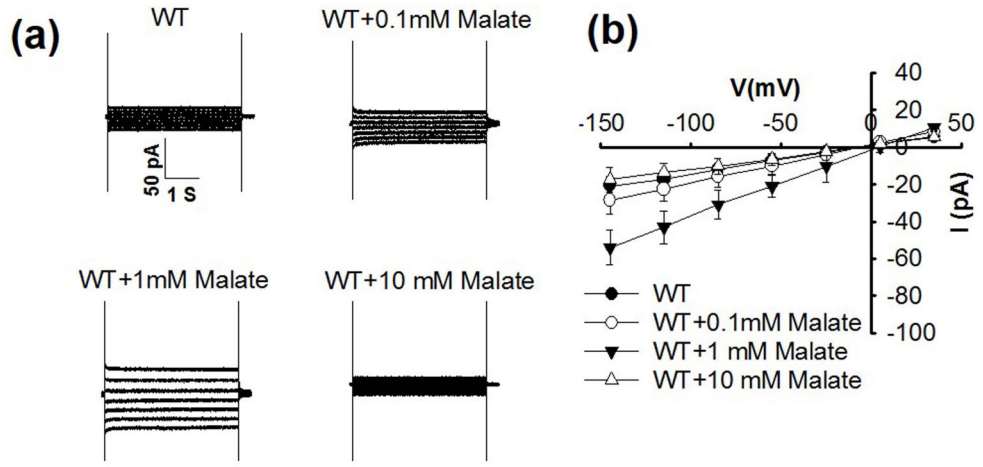

Fig. 1.

Cytosolic malate at 1 mM activates ionic currents in *Arabidopsis thaliana* wild-type (WT) guard cells, whereas 0.1 mM did not significantly enhance ion currents in these experiments and 10 mM malate showed no activation of currents. (a) Typical whole-cell recordings of ionic currents in guard cell protoplasts of wild type plants without malate or with 0.1 mM, 1 mM and 10 mM malate added to the pipette solution that dialyzes the cytosol of guard cells. (b) Steady state current-voltage relationships recorded as in (a). The number of guard cells was $n = 5–8$ for each condition. Data are mean ± SE (One-way ANOVA and Tukey's test: $P < 0.01$ for WT vs WT+1 mM malate; $P < 0.01$ for WT+10 mM malate vs WT+1 mM malate; $P < 0.05$ for WT+0.1 mM malate vs WT+1 mM malate at −145 mV).

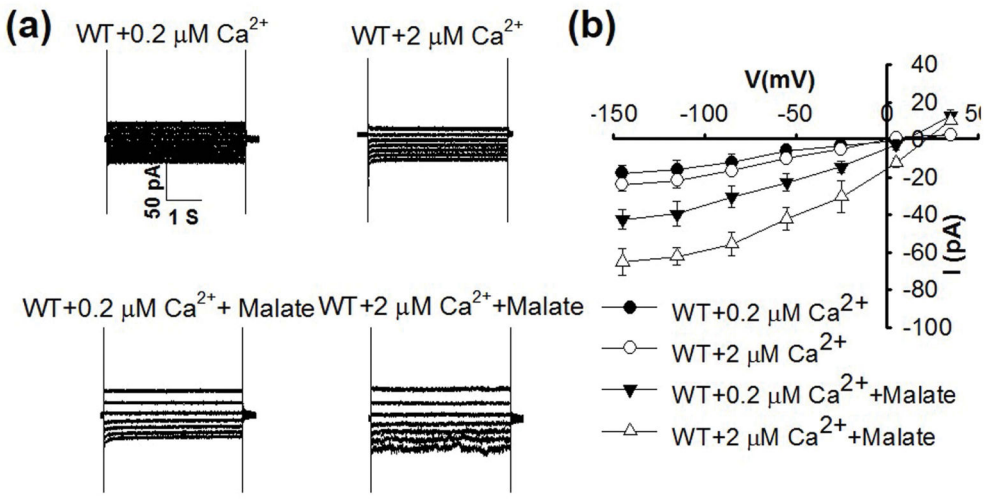

Fig. 2.

Malate activation of anion currents in *Arabidopsis thaliana* wild-type (WT) guard cells occurs at a buffered resting cytosolic free Ca^{2+} concentration of 0.2 μM, and malate activation of S-type anion channel currents is enhanced by 2 μM free Ca^{2+} compared with 0.2 μM free Ca^{2+} in the pipette solution. (a) Typical whole-cell recordings of S-type anion channel currents in guard cell protoplasts of wild type plants with 0.2 μM free Ca^{2+}, 2 μM free Ca^{2+}, 0.2 μM free Ca^{2+} + 1 mM malate and 2 μM free Ca^{2+}+1 mM malate in the pipette solution. (b) Steady state current-voltage relationships recorded as in (a). The number of guard cells was $n = 5–7$ for each condition. Data are mean ± SE ($P < 0.01$ for 0.2 μM free Ca^{2+} vs 0.2 μM free Ca^{2+} + 1 mM malate; $P < 0.01$ for 2 μM free Ca^{2+} vs 2 μM free Ca^{2+} + 1 mM malate at −145 mV).

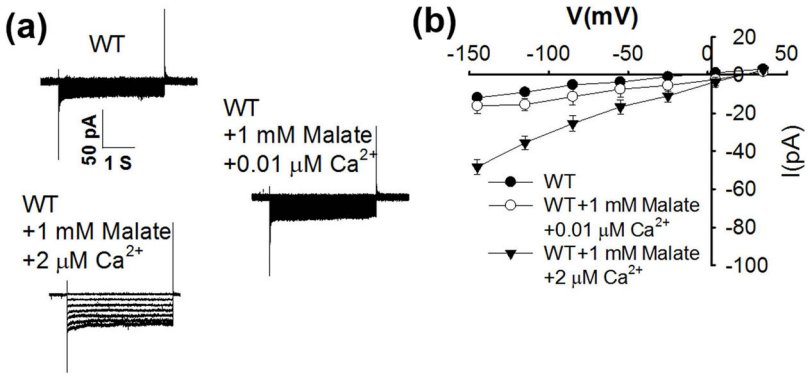

Fig. 3.

Cytosolic malate (1 mM) does not activate S-type anion channel currents in *Arabidopsis thaliana* wild-type (WT) guard cells at a low free cytosolic Ca^{2+} concentration of 0.01 μM. (a) Typical whole-cell recordings of S-type anion channel currents in guard cell protoplasts of wild type plants with or without 1 mM malate at 0.01μM and 2 μM free Ca^{2+}. Note 2 μM free Ca^{2+} was buffered with 5.7 mM $CaCl_2$ + 6.7 mM EGTA; 0.01 μM free Ca^{2+} used 0.3 mM $CaCl_2$ + 6.7 mM EGTA. (b) Steady-state current-voltage relationships of recordings as in (a). The number of guard cells was from seven to nine for each condition. Data are mean ± SE ($P < 0.01$ for WT vs WT+1 mM malate+2 μM free Ca^{2+}; $P < 0.01$ for WT+1 mM malate+0.01 μM free Ca^{2+} vs WT+1 mM malate+2 μM free Ca^{2+}).

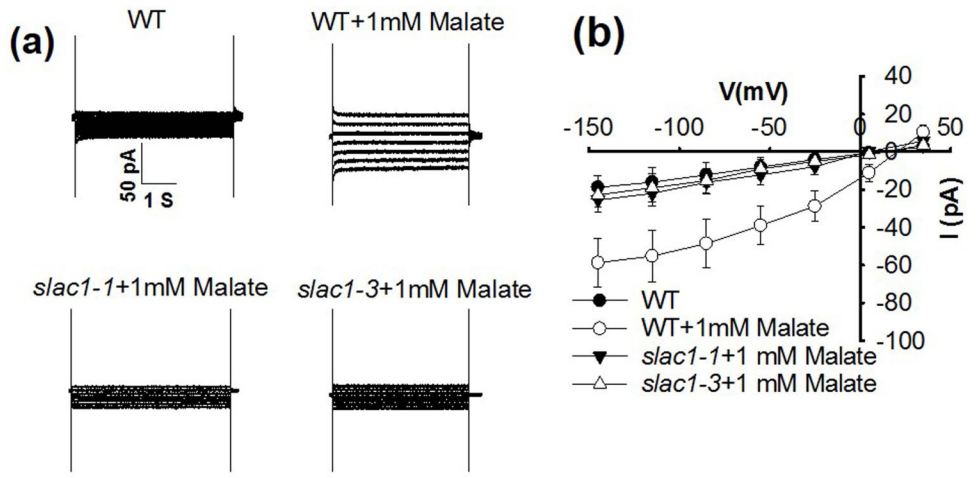

Fig. 4.

slac1-1 and *slac1-3* mutants impair 1 mM malate activation of S-type anion channel currents in *Arabidopsis thaliana* guard cells. (a) Typical whole-cell recordings of S-type anion channel currents in guard cell protoplasts of wild type (WT), *slac1-1* and *slac1-3* mutant plants with 1 mM malate and 2 μM free Ca^{2+} in the pipette solution that dialyzes the cytosol. (b) Steady-state current-voltage relationships recorded as in (a). The number of guard cells was $n = 5–7$. Data are mean ± SE ($P < 0.01$ for *slac1-1* + 1 mM malate vs WT + 1 mM malate; $P < 0.01$ for *slac1-3* + 1 mM malate vs WT + 1 mM malate at −145 mV).

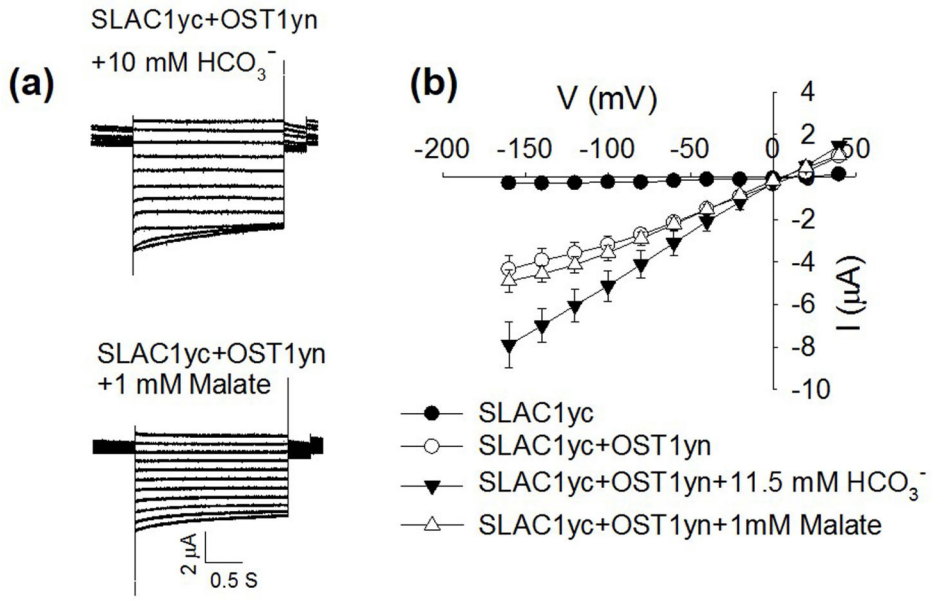

Fig. 5.

Cytosolic malate at 1 mM did not enhance SLAC1yc-OST1yn–mediated anion channel currents in *Xenopus* oocytes. (a) Whole-cell currents were recorded from oocytes expressing SLAC1yc and OST1yn after injection of the indicated final $NaHCO_3$ or malate concentrations. (b) Steady state current-voltage relationships from oocytes recorded as in (a). The number of oocytes was $n = 9–14$. Data are mean ± SE. Experiments shown here are from one batch of oocytes, with similar findings made in three independent oocyte batches. ($P < 0.01$ for SLACyc+OST1yn vs SLACyc+OST1yn +11.5 mM HCO_3^-; $P < 0.01$ for SLACyc+OST1yn +1 mM malate vs SLACyc+OST1yn +11.5 mM HCO_3^- at −160 mV.)

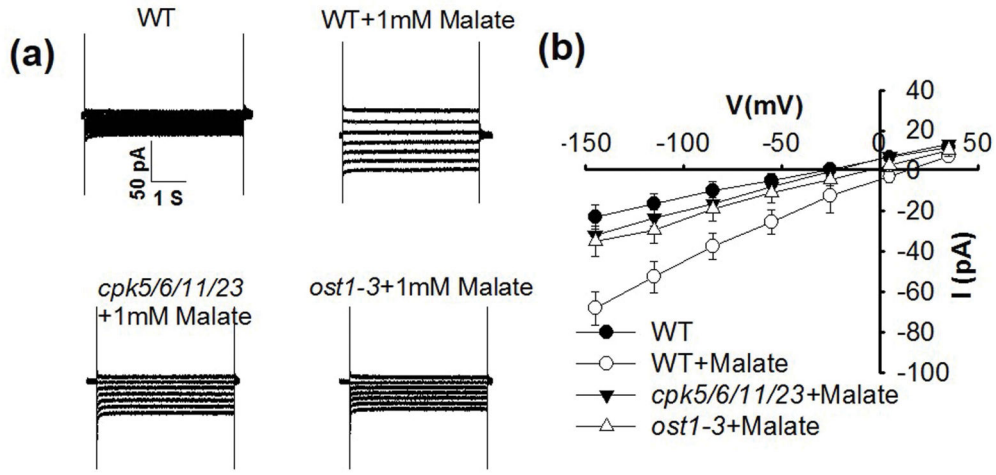

Fig. 6.

cpk5/6/11/23 and *ost1-3* mutant plants impair 1 mM malate activation of S-type anion channel currents in *Arabidopsis thaliana* guard cells. (a) Typical whole-cell recording of S-type anion channel currents in guard cell protoplasts of wild type (WT), *cpk5/6/11/23* and *ost1-3* mutant plants. (b) Steady state current-voltage relationships recorded as in (a). The number of guard cells was $n = 4–7$. Data are mean ± SE. The pipette solution contained 2 μM free Ca^{2+} ($P < 0.01$ for *cpk5/6/11/23*+1 mM malate vs WT+1 mM malate; $P < 0.01$for *ost1-3*+1 mM malate vs WT+1 mM malate at −145 mV).

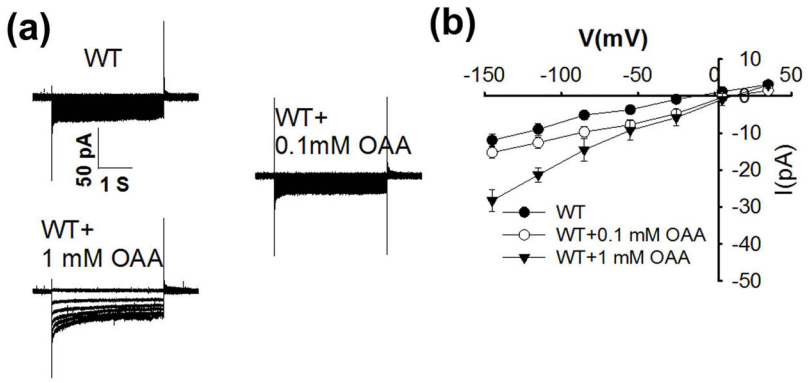

Fig. 7.

Cytosolic oxaloacetate (OAA) at 1 mM activates S-type anion channel currents in wild-type (WT) *Arabidopsis thaliana* guard cells, whereas 0.1 mM OAA does not show activation. (a) Typical whole-cell recordings of S-type anion channel currents in guard cell protoplasts of wild type (WT) plants without OAA or with 0.1mM or 1 mM OAA added to the pipette solution. The pipette solutions contained 2 μM free Ca^{2+}. (b) Steady state current-voltage relationships recorded as in (a). The number of guard cell is $n = 6–9$ for each condition. Data are mean ± SE. Note that Fig. 3 shares the same WT I–V curves, as data were obtained in the same experimental data sets ($P < 0.01$ for WT vs WT+1 mM OAA; $P < 0.01$ for WT+0.1 mM OAA vs WT+1 mM OAA at −145 mV).