The malate-activated ALMT12 anion channel in the grass _Brachypodium distachyon_ is co-activated by Ca\(^{2+}\)/calmodulin

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Running Title: _BdALMT12 activation requires calcium/calmodulin_

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**ABSTRACT**

In plants, strict regulation of stomatal pores is critical for modulation of CO\(_2\) fixation and transpiration. Under certain abiotic and biotic stressors, pore closure is initiated through anionic flux, with calcium (Ca\(^{2+}\)), playing a central role. The aluminum-activated malate transporter 12 (ALMT12) is a malate-activated, voltage-dependent member of the aluminum-activated malate transporter family that has been implicated in anionic flux from guard cells, controlling stomatal aperture. Herein, we report the characterization of the regulatory mechanisms mediating channel activities of an ALMT from the grass _Brachypodium distachyon_ (BdALMT12) that has highest sequence identity to _Arabidopsis thaliana_ ALMT12. Electrophysiological studies in a heterologous cell system confirmed that this channel is malate and voltage dependent. However, this was shown to be true only in the presence of Ca\(^{2+}\). Although a general kinase inhibitor increased the current density of BdALMT12, a calmodulin (CaM) inhibitor reduced the Ca\(^{2+}\)-dependent channel activation. We investigated the physiological relevance of the CaM-based regulation *in planta*, where stomatal closure, induced by exogenous Ca\(^{2+}\) ionophore and malate, was shown to be inhibited by exogenous application of a CaM inhibitor. Subsequent analyses revealed that the double substitutions R335A/R338A and R335A/K342A, within a predicted BdALMT12 CaM-binding domain (CBD), also decreased the channels ability to activate. Using isothermal titration calorimetry and CBD-mimetic peptides, as well as CaM-agarose affinity pulldown of full-length recombinant BdALMT12, we confirmed the physical interaction between the CBD and CaM. Together, these findings support a co-regulatory mechanism of BdALMT12 activation by malate, and Ca\(^{2+}\)/CaM, emphasizing that a complex regulatory network modulates BdALMT12 activity.

In plants, the control of CO\(_2\) uptake (assimilation) and loss of water vapour (transpiration) are controlled by pores in the epidermal layer, referred to as stomata. Strict regulation of stomatal function is therefore critical for mediation of plant primary metabolism, growth and development generally, as well as plant responses to both biotic (pathogenic) and abiotic (environmental) stress.

The opening and closing of stomata is directly controlled by the release and uptake of ions by guard cells that form the stomatal pore (1,
In the case of stomatal closure, upstream signalling is known to mediate the phosphorylation of select anion channels, activating them and leading to Cl− and malate efflux. This anion efflux causes depolarization of the plasma membrane, which stimulates voltage-dependent potassium channels and additional K+ efflux. Overall, this mass ion efflux decreases turgor pressure, reducing swelling of the guard cells, causing the pore to close.

Upstream of the anion channels, signal transduction events stimulated by environmental cues lead to activation of downstream Ca2+-dependent and Ca2+-independent response pathways (3). In the case of the Ca2+-independent pathway, binding of abscisic acid (ABA) to its receptor(s) leads to the sequestration and inhibition of type 2C protein phosphatases (PP2C’s), such that their target, a sucrose non-fermenting (SNF)-related kinase (SnRK; e.g. OST1 in Arabidopsis thaliana) becomes activated through auto-phosphorylation (4). The SnRK subsequently phosphorylates the anion channels (5, 6). On the other hand, abiotic stress and ABA are also known to stimulate the production of reactive oxygen species (ROS) through activation of an NADPH oxidase (7). ROS stimulates Ca2+-release into the cytosol, which in turn leads to the activation of Ca2+-dependent kinases (e.g. CPK6 and CPK21/23), which specifically phosphorylate the same anion channels as the Ca2+-independent SnRKs, leading to channel activation and ultimately the same stomatal closure (8–10).

At the channel level, working in concert, two types of anion channels presenting in the plasma membrane of guard cells, are known to mediate anion efflux and stomatal closure: the rapid (R-type) and the slow (S-type) activating anion channels (11, 12). S-type anion channels are encoded by the slow anion channel 1 (SLAC1) gene in A. thaliana (13, 14) and its homologues (e.g. SLAH3; (13)). While SLAC1 has been shown to be stimulated by SnRK, CPK and calcineurin B-like (CBL) calcium sensors and their CBL interacting protein serine-threonine type kinases (i.e. Ca2+-independent and Ca2+-dependent pathways (2, 15, 16)), SLAH3, to date has only been shown to be stimulated by the Ca2+-dependent kinase pathway (16, 17).

In the A. thaliana guard cells the R-type anion channel is encoded by the ALMT12 gene (AtALMT12; (18)). AtALMT12 is one member of a larger family of fourteen aluminum-activated malate transporter (ALMT) channels in A. thaliana, better known for releasing malate from the root tip for chelation of aluminum (19, 20). More recently, reports have shown that AtALMT12 is in-sensitive to Al3+, activated by malate, with very specific voltage-dependent properties, and its deletion selectively impairs stomata closure (18, 21). As such it was renamed A. thaliana Quick Activation Anion Channel 1 (AtQUAC1) to avoid confusion with other ALMT channels. The secondary structure of AtQUAC1 has been predicted to have six transmembrane segments at its N-terminus and a large cytoplasmic C-terminal domain. Similar to SLAC1, AtQUAC1 activation has recently been shown to be controlled by the Ca2+-independent, but phosphorylation-dependent SnRK pathway (6). However, that AtQUAC1 activity was reduced by only 50 % with deletion of OST1 (a SnRK), suggests other mechanisms of regulation may also be in play.

Although, the ALMT gene family was first identified in wheat (19), the model monocot Brachypodium distachyon ALMT12 has yet to be investigated. A BLAST search yielded seven putative ALMTs in B. distachyon, with one sequence having significant (59 %) amino acid identity to AtQUAC1. Using a recombinant expression system, patch clamp analysis was applied to investigate channel activity and regulation. The observation of Ca2+-sensitivity led to further evaluations of the effect of select kinase and calmodulin (CaM) inhibitors, with results suggesting a regulatory role for CaM in BdALMT12 activity. The relationship between malate, Ca2+, CaM and stomatal function was investigated in planta. Subsequent electrophysiological evaluation of amino acid substitutions targeting a putative BdALMT12 CaM binding domain (CBD), along with isothermal titration calorimetry and CaM-agarose affinity pull-down experiments confirmed the role of the CBD and its ability to mediate a direct interaction between CaM and BdALMT12. The general physiological context and relevance are discussed.

Results
**BRADI_3g33980v3 encodes a putative B. distachyon ALMT12 and is expressed in shoot tissue.**

A BLAST search of the Ensembl Plants *B. distachyon* sequence database yielded six unique amino acid sequences with 30 - 36 % identity (BRADI_5g09690v3; BRADI_1g43810v3; BRADI_3g51480v3, BRADI_5g18622v3; BRADI_3g51470v3; BRADI_3g57050v3), and a single sequence with 59 % amino acid identity (BRADI_3g33980v3; NCBI protein accession# XP_003574370.1; putative BdALMT12), to AtALMT12/ATQUAC1 (Figure 1A). This latter putative BdALMT12 sequence is 529 amino acids long, with an expected molecular weight of ~ 56 KDa. It was found to be ~ 90 % identical to *Hordeum vulgare* (gene id HORVU1Hr1G049820) and *Triticum aestivum* (gene id TraesCS1D01G194000) closest homologues, and 82 % identical to *Zea mays* ALMT12 (Genbank accession# PWZ19427.1). A phylogenetic analysis emphasizes that this particular putative *B. distachyon* ALMT is the only one of the seven to cluster in clade 3, with *A. thaliana* ALMTs 11-14 (Figure 1B). Direct sequence alignments of the *A. thaliana* clade 3 ALMTs with putative BdALMT12 shows that it does in fact maintain highest amino acid sequence identity with AtALMT12 (59 %), having only 39, 54 and 55 % identities respectively to AtALMTs 11, 13 and 14. Thus we refer to this *B. distachyon* protein as BdALMT12 going forward.

Further to this, expression analyses showed expression of transcripts arising from the gene BRADI_3g33980v3, encoding BdALMT12, in green leaf tissue taken from both seedlings and adult *B. distachyon* plants. Relative expression was found to be 0.313 ± 0.026 (n=4) and 0.120 ± 0.019 (n=5) respectively for young and old plants, compared to the house keeping UBC18 gene. This is consistent with previous data showing expression of AtALMT12/ATQUAC1 (as well as AtALMT6 and AtALMT9) throughout shoot tissue, compared to AtALMT1 which is expressed in roots (20, 22). Whether BRADI_3g33980v3 is specifically expressed in the guard cells remains to be demonstrated.

**Electrophysiological characteristics of a putative BdALMT12 shows co-dependent activation by malate and calcium**

To assess the electrophysiological properties of BdALMT12, whole cell patched clamp of heterologously expressed BdALMT12 in HEK293 cells was performed. Both HEK293 and Xenopus oocytes expression systems have been previously used to assess the electrophysiological properties of plant ion channels as they have relatively few endogenous ion channels, making them relatively ‘silent’ and permitting the expression and evaluation of genes from many different organisms with reproducible outcomes (18, 22–29). Notably, neither system expresses any endogenous, malate-sensitive ion channel. Heterologous HEK293 cell expression was chosen for this study as it allows whole cell electrophysiological recordings, which enables control of both internal and external cellular buffer solution, whereas the oocyte model does not.

Surprisingly, initial patch clamp recordings on BdALMT12, in the absence of any Ca\(^{2+}\), or agonist to increase internal Ca\(^{2+}\) (as per experiments performed previously on AtQUAC1; (18)), yielded no channel activation with or without malate added to the bath solution (Figure 2A and 2C). However, taking advantage of the ability to control the cytosolic Ca\(^{2+}\) concentration via the whole cell pipette solution during patch clamp experiments, the malate-dependent activation of BdALMT12 was shown to be dependent on the presence of cytosolic Ca\(^{2+}\). Furthermore, increased cytosolic Ca\(^{2+}\) concentrations were found to increase the current activation, measured at maximum transient activation, in a dose-dependent manner in cells expressing BdALMT12 (Figure 2A and 2C; Supplemental Figure 1). The activation is followed by rapid inactivation at prolonged voltage stimulation, where no difference is observed in the inactivation current (at steady state) between different cytosolic Ca\(^{2+}\) concentrations (Supplemental Figure 2). No significant increases (regardless of Ca\(^{2+}\) and malate) were observed for the GFP expressing controls (Supplemental Figure 3). Interestingly, there was no voltage shift in peak-normalized conductance between Ca\(^{2+}\) concentrations (Figure 2B), suggesting cytosolic Ca\(^{2+}\) is regulated only in activation of the channel conductance and not the voltage dependence of the activation.
**Electrophysiological analyses suggest BdALMT12 is regulated by CaM**

To determine if the Ca\(^{2+}\)-activation of the BdALMT12 channel is mediated through a Ca\(^{2+}\)-activated kinase or CaM mechanism, the effects of applying associated pharmacological inhibitors to the system was assessed. Indeed, HEK293 cells are well known to maintain expression of many of the canonical eukaryotic regulatory mechanisms, including arrays of protein kinases and CaMs that have been implicated in an array of other biophysical studies (23–29). Herein, the contributions of Ca\(^{2+}\)-activated kinases were investigated, based in part on the prediction of putative phosphorylation sites on the carboxyl terminus of the channel (e.g. BdALMT12 residues Ser391, Ser375, Ser394; (30, 31). In particular the non-specific kinase inhibitor staurosporine, which prevents ATP binding to the kinase and thus inhibits any phosphorylation events from occurring, was used (32). Unexpectedly, addition of 60 nM staurosporine to the patch-clamp pipette resulted in an increase in current density (Figures 2D and 2E), rather than a loss of activity as would be expected if the activating Ca\(^{2+}\) effect was mediated by Ca\(^{2+}\)-activated kinases.

To examine whether the cytosolic Ca\(^{2+}\)-activation effect involves CaM, the activity of BdALMT12 was assessed in the presence of W-7, a naphthalene sulfonamide derivative, which has been shown to inhibit CaM-dependent phosphodiesterase and myosin light chain kinase activities with reported IC\(_{50}\)'s of ~ 28 and 51 µM, respectively (33). In the case of BdALMT12, the peak currents were observed to decrease significantly when even as little as 1 µM W-7 was used in the pipette solution, and were reduced to base-line values with 10 µM W-7 (Figures 2F and 2G), similar to observed channel conductance in the absence of any Ca\(^{2+}\). Only a slight increase in voltage of activation (Figure 2H) and no change in the rate of inactivation (data not shown) was observed with the addition of W-7. Thus, there does not appear to be much, if any, residual Ca\(^{2+}\)-dependent activity that is un-affected by W-7, suggesting that CaM is a primary modulator of the BdALMT12 Ca\(^{2+}\)-dependent channel activation under the influence of malate.

**Pharmacological studies link BdALMT12 and CaM to calcium + malate-sensitive stomatal regulation in planta**

To validate the role of BdALMT12 and CaM in stomatal regulation *in planta*, the widths of stomatal apertures of *B. distachyon* leaves were measured after being treated with A23187 (a Ca\(^{2+}\) ionophore that leads to higher Ca\(^{2+}\) in the cytosol) in the presence and absence of malate. While neither malate nor the ionophore showed any significant effect on stomatal widths on their own, stomatal pores treated with both malate and the ionophore together were significantly smaller, consistent with the combined and very unique role of malate and Ca\(^{2+}\) in activation of BdALMT12 (Figure 3A). The inclusion of increasing concentrations of W-7 in the malate/A23187 containing buffer negated this effect, yielding concentration-dependent increases in stomatal pore widths, compared to malate/A23187 alone (Figure 3A). Indeed by 10 µM W-7, in the presence of malate/A23187, stomatal widths returned to untreated sizes. Notably, the application of W-7 alone did not increase stomatal widths significantly beyond untreated levels, emphasizing that the W-7 is most likely reversing the effect of the malate/A23187, and not simply modulating a different mechanism of stomatal regulation independently; although this possibility cannot be strictly eliminated.

Toward further investigating the biological function of the BdALMT12 gene, almt12 RNAi knockdown plants were generated in *B. distachyon*. Initially nine lines were confirmed with significant knockdown of the gene at the T\(_0\) generation (Figure 3B). Of these, only one line (KD2) still showed a significant knockdown as a young seedling (3-5 weeks old) at the T\(_1\) generation (Figure 3C). Analysis of stomatal pore widths of the KD2 seedlings yielded a small but significant increase in stomatal opening compared to wild type plants (Figure 3C).

Interestingly, by the time the T\(_1\) KD2 plants had matured to adults (8-10 weeks old), they had not only lost any knockdown, the expression level of BdALMT12 had rebounded to levels significantly higher than wild type adult plants, essentially creating a ‘BdALMT12 overexpressing condition’ (as assessed by PCR; Figure 3D). Assessment of adult KD2 line
stomatal function yielded pore widths significantly smaller than those of wild-type, consistent with the increased stomatal closure that would be expected from BdALMT12 overexpression (Figure 3D). That these effects arose due to the changes in BdALMT12 expression levels was further confirmed by investigating the response of the adult ‘BdALMT12 overexpressing’ KD2 T1 generation plants, to the exogenous application of the Ca2+ ionophore A23187, along with malate. Consistent with the proposed role of BdALMT12, stomatal pore widths were significantly more responsive to A23187 and malate in the KD2 line than the wild-type line, with widths decreasing by approximately 11% and 5% respectively, compared to untreated controls (Figure 3D; Table 1). Together this data provides important correlative validation of a possible role for BdALMT12 in mediation of stomatal function, with transient knockdown in KD2 T1 seedlings leading to stomatal opening, and the over-expression state of BdALMT12 in adult KD2 T1 plants leading to stomatal closure.

Mutational analyses identify a putative calmodulin binding domain in BdALMT12

Towards identification of putative CaM binding domains (CBDs), the BdALMT12 amino acid sequence was analyzed using MI-1 (34), Calmodulation Meta-analysis (35) and Jpred3 (36) algorithms. Only a single region (BdALMT12 residues 334-351 (Figure 1A)) met the criteria, yielding both the second highest CBD motif prediction score and displaying a strongly basic and amphiphilic alpha-helix (Figure 4G). Toward evaluation of the biological relevance of this putative CBD region, codons encoding three of the amino acids (Arg 335, Arg 338 and Lys 342 in BdALMT12) included in the basic portion of the helix, were mutated to encode Ala in double and triple substitution variants. These residues were selected on the basis that basic residues in the CBD are known to be involved in the primary interactions between the CBD and CaM (37).

Patch clamp analysis of these variants showed that the triple variant (335/338/342) lacked malate activation altogether, and that two double variants including 335/338 and 335/342 had significantly smaller peak activated current increases (Figure 4A-C). However, no significant difference between the double variant 338/342 and wildtype was detected, highlighting a particularly notable role for the 335 site in the BdALMT12 putative CBD. To confirm that reduced activities were not caused by low expression of variants on the cell surface, surface biotinylation of transfected cells expressing Myc-tagged BbQUAC1, or the Myc-tagged double or triple variants of BdALMT12, was performed to isolate surface proteins, for subsequent Western blot analysis using anti-Myc-tag antibody. While variants with significantly smaller activated current also had somewhat lower protein expression on the membrane surface (Figure 4E); the overall decrease is not sufficient enough to explain the almost complete lack of current detected for the 335/338 variant in particular. Indeed, following normalization of the activity data to the surface expression data, currents of the deactivated triple variant and the two affected double variants 335/338 and 335/342 were still significantly smaller than wildtype (Figure 4F). Additionally, the two partially deactivated double variants 335/338 and 335/342 also demonstrated a significant 10 ± 2 mV depolarizing shift at 50% whole cell conductance activation increasing the voltage of activation, thereby decreasing the voltage change needed for activation (Figure 4B). These variants also had a longer inactivation time constant in comparison to wild type (Figure 4D). Together these data show that the decrease in peak current densities is not due to the changes in voltage sensitive activation or inactivation processes, as the observed changes in kinetics should have resulted in larger peak current densities in this event. As such, it would seem that the CaM control over activation supersedes the minor impact on the voltage-gated kinetics of activation and rates of inactivation found with mutational intervention (Figures 4B and 4D and see discussion).

Interaction analyses show CaM binding to the predicted CBD domain of BdALMT12

With the electrophysiological results highlighting the involvement of the putative CBD domain in regulation of BdALMT12 activity, the physical interaction between the predicted CBD of BdALMT12 and CaM was evaluated using
CaM-agarose affinity pull-down and isothermal titration calorimetry (ITC).

Initial ITC binding studies between a wildtype BdALMT12 CBD peptide (residues 334–355) and CaM demonstrated a physical interaction as evidenced by the significant release of heat during titration of the peptide into the cell containing CaM, with quantitative analyses highlighting a Kd1 in the low nM range, and secondary weaker binding event with a Kd2 in the low µM range (Figure 5A; Table 2). While elimination of Ca2+ from the titration conditions led to a significant reduction in binding, in fact some residual interaction was still detected (Figure 5A). Quantitative analysis of this residual affinity yielded a Kd in the low µM range, with complete loss of the low nM range interaction (Table 2), raising the possibility of some Ca2+-independent binding of BdALMT12 to CaM. Notably, this effect of removing Ca2+ is in contrast to the effect of including the CaM inhibitor W-7 in the reaction, which eliminated all release of heat, implying loss of all interactions between CaM and BdALMT12 (Figure 5A; Table 2).

Subsequent evaluation of the binding affinities of CaM for double and triple alanine variant CBD peptides, representative of the variants tested by patch clamp, was unsuccessful due to substitution of the charged residues by hydrophobic residues making the peptides prone to formation of aggregates at higher concentrations. Indeed the Ala substituted peptide variants were shown to form oligomeric complexes of large particle sizes by dynamic light scattering (data not shown). As such a new set of more soluble and charge reversed double and triple variant peptides were synthesized with Asp substitutions at the same representative 335, 338 and 342 sites instead of Ala. Evaluation of the interaction of these Asp substituted variant peptides showed that the triple mutation peptide 335/338/342 eliminated all binding to CaM (Figure 5B). Similarly, the two variants 335/338 and 335/342 showed decreased binding affinities with Kd1’s in the low µM range, and complete loss of the secondary Kd2 binding event (Figure 5B and Table 2). The double variant peptide 338/342 had little impact on interactions, showing similar binding affinities compared to wildtype. These results are consistent with the electrophysiological analysis of BdALMT12 variants, and together demonstrate the existence of a functional CaM binding domain in BdALMT12, and its involvement in the regulation of BdALMT12 activities.

Toward investigating whether these interactions detected by ITC, using BdALMT12 CBD-mimetic peptides, translate to the more biologically relevant full-length BdALMT12, a CaM-agarose affinity pull-down (CAP) experiment was carried out. Western blot analysis of eluted fractions yielded a strong band at the expected Mw for WT BdALMT12 compared to samples arising from un-transfected cells (Figure 5C). The intensity of this band was dramatically reduced for cell lysates arising from transfected cells expressing the double Ala variant 335/342 (Figure 5C). This variant was selected for analysis by CAP based on its low channel activity (Figure 4A) but WT-like expression surface levels (Figure 4E). Quantification of the Western analysis shows a ~ 75% reduction (after subtraction of un-transfected background) in the amount of 335/342 variant eluted, compared to WT BdALMT12 (Figure 5D), consistent with the significant (100-fold) reduction in binding affinity detected for this variant by ITC (Table 2).

Discussion

Validating prior comments (8), one of the more salient aspects of this study is demonstration of a requirement for cytosolic Ca2+ for BdALMT12 activation. Further evaluation demonstrated that the effect of Ca2+ was dependent on CaM. While such regulation or ‘calmodulation’ of other anion channels has been demonstrated previously (38), this is the first report of the calmodulation of a member of the broader ALMT family.

Following up on initial experiments where BdALMT12 was found to be completely unresponsive to malate activation, the addition of Ca2+ demonstrated that both elevated cytosolic Ca2+ as well as external malate were in fact required for its activation. While it was subsequently shown that the Ca2+ response was dose-dependent at a fixed concentration of malate, previous studies on AtQUAC1 showed malate-activation was itself dose dependent (18). Thus how cytosolic Ca2+ concentration would affect the
activation of the BdALMT12 channel with respect to malate dose-dependence, and vice versa, remains to be determined.

With respect to a more detailed comparison of BdALMT12 and AtQUAC1 electrophysiology, the raw current traces of BdALMT12 (Figure 2C) are very similar to what has been reported for AtQUAC1 (18). However BdALMT12 appears to be activated at a lower voltage potential. Indeed, for BdALMT12, a bell shaped IV curve that starts to approximate that observed for AtQUAC1, would likely only be achieved at considerably more negative voltage pulses. However, such low membrane potential would be non-physiological and is not realistically achievable by the patch clamp system. Also worth noting, AtQUAC1 malate-dependent depolarized potentials were found to elicit outward currents (anion uptake), in keeping with AtQUAC1 being activated upon depolarization, while malate-dependent hyperpolarized potentials elicited inward currents (anion release), in keeping with the channel being closed under this condition, which, as emphasized by the authors thereof, follows with the original hallmarks of R-type channels (18). In contrast, and despite the noted amino acid sequence homology documented herein, malate-dependent BdALMT12 currents are only shown to be activated upon hyperpolarization (eliciting inward currents, representing anion release) in a Ca\textsuperscript{2+}-dependent manner. Thus, it would appear that BdALMT12 becomes more active upon hyperpolarization, emphasizing different voltage-dependent gating behavior between AtQUAC1 and BdALMT12. In this context, the possibility that other factors may be influencing the observed activities, cannot be ignored. For example, the lack of observed activation of BdALMT12 at depolarized voltages could be due to the blockage/suppression of outward anionic currents, as previously reported in guard cells (6). In this case, the hyperpolarization-induced instantaneous transient inward currents could then reflect the deactivation, rather than transient activation, of the BdALMT12 anion channels. Alternatively, that the electrophysiological differences highlighted here are arising due to differences in recombinant hosts (HEK293 cells versus oocytes) should also be kept in mind. Nonetheless, that no outward currents (anion uptake) arose from positive potentials in BdALMT12 is consistent with the primary role of BdALMT12 in stomata closure being the release of anions from guard cells (1).

Interestingly, evaluation of a possible Ca\textsuperscript{2+}-dependent kinase mechanism of activation showed that application of the non-specific kinase inhibitor (staurosporine) yielded increased whole cell current density (Figure 2E). This implies that phosphorylation of the BdALMT12 channel may decrease channel activity, and is thus more likely to serve as a feedback inhibition process, rather than a mechanism by which the channel is co-activated by Ca\textsuperscript{2+}. Interestingly, this is in contrast to what was observed previously for the wheat root TaALMT1, where Ca\textsuperscript{2+}-activated kinase inhibition led to loss of activity (30). Although not investigated further here, this observation highlights how tightly regulated BdALMT12 channel activity likely is, and how this regulation likely varies with physiological roles within the broader ALMT family.

In the absence of a kinase-associated mechanism for Ca\textsuperscript{2+}-linked activation, a role for CaM, which classically binds Ca\textsuperscript{2+} to adopt an interacting conformation, was evaluated based on previous reports highlighting a role for CaM in mediating stomatal function more generally (39–42), as well as computational predictions highlighting a putative CBD in the C-terminal domain of the channel (Figure 4). The observed drastic reduction in activated BdALMT12 currents, upon inclusion of the strong CaM inhibitor W-7 in the pipette solution, validated this hypothesis (Figure 2D).

Following up on these studies, the pharmacological effect of W-7 on malate + Ca\textsuperscript{2+}-induced closure of stomata in planta was evaluated (Figure 3). Notably, the Ca\textsuperscript{2+} signalling events were initiated through the use of a Ca\textsuperscript{2+} ionophore (A23187), which is known to allow Ca\textsuperscript{2+} entry into the guard cell cytoplasm and induce stomatal closure (42). Indeed A23187 has also been used to elevate intracellular calcium in many other cellular biological systems as well (43). The use of a Ca\textsuperscript{2+} ionophore was selected as it circumvents the ABA receptor signalling pathway, which might otherwise initiate Ca\textsuperscript{2+}-independent events also related to stomatal closure (44). Using this model, W-7 was found to inhibit Ca2+/malate-induced stomatal closure in
B. distachyon leaves, demonstrating a role for CaM-regulation of stomatal closure in B. distachyon, consistent with previous observations in Commelina species (39–42). Importantly here, that the effect of W-7 was to specifically reverse malate + Ca$^{2+}$-stimulated stomatal closure, showing no significant effect on its own compared to controls, emphasizes the likelihood of BdALMT12 being a primary player in the CaM-associated stomatal responses documented herein and elsewhere (39–42).

In this context, it is notable that the B. distachyon almt12 RNAi knockdown and rebound over expression results do provide some limited-evidence of a potential role for BdALMT12 in stomatal closure, with cells expressing less of the gene having larger stomatal openings on average and vice versa (Figure 3). This is similar to the role demonstrated for AtQUAC1 in A. thaliana (17, 20). Notably, that the adult ‘overexpressing state’ was more responsive to malate-induced stomatal closure is, furthermore, consistent with the proposed model. However, while RNAi knockdown rebound events are not uncommon (45), a detailed understanding of the mechanism leading to such phenomenon remains enigmatic. As such any conclusive findings regarding the biological relevance of BdALMT12 await future, more in depth, knockout, localization and transcriptional expression analyses. In this context of a role for CaM in stomatal regulation, it is notable that the effect was originally documented over 30 years ago. Indeed the very first report linking CaM to modulation of stomatal function showed significant increases in stomatal aperture widths with the exogenous application of CaM-binding drugs trifluoperazine and compound 48/80 (39). Interestingly, this effect was only relevant at µM Ca$^{2+}$ concentrations, with higher exogenous Ca$^{2+}$ overwhelming the effects of the CaM inhibitors (39). This is consistent with the low µM Ca$^{2+}$-sensitivity detected for BdALMT12 herein. As well, the pharmacological inhibitor, W-7, in particular has been applied, and shown to increase the average aperture width of stomata in Commelina communis L. and Commelina benghalensis species in multiple reports since then, linking CaM to ABA, vanadate, blue-light and light/dark transition -mediated stomatal closure events (40–42).

With respect to an interaction with CaM, that decreased whole cell current densities were observed upon mutation of the leading CBD basic residue (Arg 335), in combination with other site changes in the basic portion of the predicted helix, further validates this hypothesis (Figure 4). Interestingly the Arg335Ala variants also demonstrated a small depolarizing shift in the activation voltage of the normalized conductance, such that these required less membrane depolarization to achieve a conductance similar to WT. Additionally, the voltage dependent process of kinetic inactivation was slowed significantly. These two changes in kinetics suggest a link between the BdALMT12 CBD and voltage dependence, as reported previously in the literature for other channels (38). However, the altered BdALMT12 kinetics did not correlate with a larger current, and thus activation by CaM would seem to be occurring independently of the voltage-gated activation and inactivation processes. Likely, the structural changes in the Arg335Ala containing variants that affect the CaM interaction, also somehow globally affect the structure of the channel, slowing its kinetics. This is supported by absence of any change in inactivation kinetics (data not shown) and only a very minor shift in activation with pharmacological inhibition of CaM by W-7 on the wild type channel (Figure 2G). Additionally, the Ca$^{2+}$-dose response on the wild type channel had no effect on inactivation (data not shown) or activation kinetics (Figure 2B). Together these findings support a model where mutationally-derived kinetic changes are likely due to modification of the voltage dependence of the channel, and not necessarily related to the CaM interaction with the channel. Nonetheless, the decrease in whole cell current in these variants indicates BdALMT12 activation is dependent on CaM.

This CaM-linked effect, arising as a result of a direct interaction between the putative BdALMT12 CBD and CaM, was confirmed in vitro by ITC, and then for full-length BdALMT12 by CAP (Figure 5). The ITC derived two-state binding model, with $K_d$’s of 34 nM and 3.9 µM respectively, for the WT CBD peptide-CaM interaction, is consistent with other functional CBD-CaM interactions reported in the literature (46). Under these conditions, two of the isolated
CBD peptides are expected to bind to a single CaM molecule. Interestingly, that a low µM affinity interaction was still observed even in the absence of Ca²⁺, but not in the presence of W-7, might also suggest that this low affinity interaction represents a Ca²⁺-independent BdALMT12-CaM interaction, similar to that documented previously for a potassium channel (47). The role of a Ca²⁺-independent interaction would likely be related to keeping signalling pathway members in close proximity to each other, such that upon introduction of Ca²⁺, activation of the channel is not delayed by belated protein translocation events. That said, the possibility of some residual bound Ca²⁺ contaminating the system cannot be strictly eliminated.

Further investigation demonstrated that the Arg335Asp mutation in combination with changes in other sites lowers binding affinities as detected by ITC, and likely results in the loss of a conformational change that normally allows binding of the two CBD peptides to a single CaM molecule. Consistent with the patch clamp data, mutations at sites not including 335, such as the double variant 338/342, did not affect either binding affinities or functional channel properties. These results are consistent with the CAP results, in which full-length WT BdALMT12 was found to be effectively pulled-down by CaM. That the full length double Arg335/342Ala- variant lead to a dramatic reduction in pull-down, emphasizes that the physical interaction is mediated by the identified CBD, in full-length BdALMT12. Together the ITC and CAP show that both Asp and Ala substitutions for R335 are eliciting similar effects related to interfering with the BdALMT12 CBD-CaM interaction.

Together these results show that BdALMT12 variants including substitutions at R335 in the putative CBD affect both CaM binding, as well as whole cell currents, lending important support to the ideas that BdALMT12 function is dependent on CaM, the presence of a functional CBD domain in BdALMT12, a physical interaction between CaM and BdALMT12, and finally emphasizes possible biological relevance of the interaction in terms of modifying channel activity.

However, the possibility that CaM is working via alternate or multiple mechanisms that are needed for stomatal closure cannot be ignored. Indeed a recent report highlighted an interaction between CaM and a novel IQM protein that modulates stomatal function by an as yet unknown, albeit Ca²⁺-independent mechanism (48). This suggests that while CaM can regulate BdALMT12 by direct interaction in a Ca²⁺-dependent manner, other BdALMT12/Ca²⁺-independent CaM-linked mechanisms that may be contributing to the overall modulation of stomatal function more broadly, cannot be strictly eliminated. Non-the-less, the findings reported herein show that the effect of CaM on BdALMT12 activity in vitro translates to an expected response in planta, suggesting the possibility of physiological relevance.

In this context, further comparison of sequence motifs (35) and predicted helical amphipathic character (49) of the identified BdALMT12 CBD region (residues 334 – 351 in BdALMT12) in other ALMT12 homologs emphasizes that while this particular regulatory CBD may be conserved in a number of close monocot relatives such as Brachypodium distachyon, Zea mays, Oryza sativa and Sorghum bicolor, the predicted absolute values of the helical hydrophobic moment vectors of this region, of more distantly related species such as Aegilops tauschii, Ananas comosus and Asparagus officinalis are lower by ~ 0.3 fold, with the later species also reduced to only two basic residues within the motif (Table 3). Evaluation of a small cross section of ALMT12 sequences in dicot plants including Solanum lycopersicum, Spinacia oleracea, Nicotiana tabacum, Glycine max, Arabidopsis thaliana and Vitis vinifera all showed ~ 0.3 – 0.5 fold reduced amphipathic character compared to BdALMT12 in this same region, with the later three species also reduced to only two basic residues (Table 3). Finally analysis of TaALMT1, a more distantly related ALMT family member, highlighted ~ 0.7 fold reduction in amphipathic character, and maintains only a single basic residue in the region. While there are no strict limits assigned to these values for the prediction of functional CBDs, this analysis highlights that there could be variation in the extent of the relevance of this particular CBD region and CaM-associated activation mechanism across ALMT’s and across species. However, this analysis does not preclude
the possibility of functional CBD’s existing at other locations in the ALMT12 sequences, in other species. Indeed our analysis of the Arabidopsis sequence in particular, highlighted a second potential CBD region between residues 475 and 492 with predicted absolute values of the helical hydrophobic moment vectors approximately comparable to the BdALMT12 CBD characterized herein, with several putative conserved basic residues within this region, for future characterization.

Overall this work raises novel hypotheses regarding Ca\(^{2+}\) and CaM-based co-activation mechanisms for the malate-sensitive BdALMT12. It remains to be determined how the Ca\(^{2+}\) dose response might modify malate dose-responses, and whether CaM’s interaction with BdALMT12 is strictly Ca\(^{2+}\)-dependent or possibly related to other Ca\(^{2+}\)-independent pathways. More broadly, whether ALMT12’s from other plant species are also sensitive to Ca\(^{2+}\) and/or CaM remains to be tested experimentally.

**Experimental procedures**

**Chemicals** - All chemicals were obtained from Sigma-Aldrich unless otherwise stated.

**Plant materials and growth conditions** (Bd21) wild-type plants were grown in chambers with conditions: 60 % humidity, with 16-hour photoperiod, at 25 °C in the light and 20 °C in the dark.

Plant transformations were carried out essentially as described previously (50). A 400bp fragment of the BdALMT12 cDNA (nucleotide 919 to 1293) was subcloned into pDonor221 (Invitrogen) using Gateway BP clonase II enzyme mix (Invitrogen). The pDonor221-QUAC1 was then amplified with the universal primer M13 and cloned into pANIC-8D vector (ABRC (50)) with primer F-RNAi and R-RNAi (Table 4) using LR clonase II enzyme mix (Invitrogen). The vector pANIC-8D carrying a fragment of QUAC1 was then transformed into Agrobacterium strain EHA105. Subsequently, immature Brachypodium seeds were collected. After removal of the lemma, seeds were sterilized by soaking in a solution of 10 % bleach and 0.1 % triton X-100 for 4 minutes and washed three times in sterile water. Embryos were then dissected out of immature seeds and placed on callus initiation media (CIM) (4.43 g Linsmaier and Skoog Medium, 30 g sucrose, 1 mL of 0.6 mg/mL CuSO\(_4\), pH 5.8 with KOH, 2 g phytagel, 0.5 mL of 5 mg/mL 2,4-D stock solution (2000x) and distilled water to 1 liter) and incubated at 28 °C in the dark. Calluses produced from embryos were spread onto petri plates every 2 weeks until there were enough calluses for transformation. Transformed Agrobacterium was culture in 20 mL of CIM at 28 °C to 0.6 OD. Then 200 μL of 10 % Synperonic PE/F68 (Sigma) and 20 μL of 200 mM acetoryrinone were added to the Agrobacteria suspension and 6 grams of callus pieces were then added to the suspension and incubated for 5 minutes. The calluses were then poured onto petri dishes with filter papers and incubated at room temperature in the dark for 3 days, at which time the calluses were transferred to CIM plates containing 150 mg/L Timentin and 10 mg/L Phosphinothricin (so that only transformed calluses are selected) and incubated at 25 °C. Calluses with green shoots were considered T\(_0\) plants and transferred to soil and propagated as per growth conditions described above.

**qPCR expression analysis** - BdALMT12 gene expression was assessed by qPCR using the RNeasy Plant mini kit (Qiagen). The RNA was reverse transcribed for cDNA synthesis using Superscript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s protocol. The expression of BdALMT12 was determined with real-time PCR. Each cDNA was amplified using PerfeCTa SYBR Green supermix low ROX (QuantaBio) on the Stratagene Mx3000P qPCR system (Agilent Genomics). The Brachypodium UBC18 gene was used as housekeeping. Primers for UBC18 (F-UBC18 and R-UBC18) and BdALMT12 (F-BdALMT and R-BdALMT) qPCR are listed in Table 4.

**Heterologous BdALMT12 expression plasmid construction, cell culture and transfection** - Total RNA was extracted and cDNA synthesized from fresh Brachypodium leaves using the RNeasy Plant mini kit (Qiagen) and Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturers’ protocol. Full length BdALMT12 coding region gene amplification was performed using High Fidelity Phusion polymerase (New England Biolab). The primers for amplification (P1F and P1R) we designed for Gateway (Invitrogen) cloning or traditional cloning with restriction sites NheI and
SacII (Table 4). The amplified PCR products were cloned into pDONOR221 using Gateway BP Clonase II enzyme mix according to the manufacturer’s protocol. pDONOR221 vector carrying BdALMT12 and pIREs2-eGFP vector (Clontech) were digested with NheI and SacII enzymes (New England Biolab). The BdALMT12 cDNA was then cloned into pIREs2-eGFP vectors using T4 DNA Ligase (New England Biolab) and transformed the construct into DH5α E. coli cells. The pIREs-eGFP construct allows expression of both the channel and eGFP from the same promoter, but unattached in protein construction. This allows for selection of channel expressing cells during patch clamp expression via florescence. In order to detect and determine the expression of BdALMT12 in HEK293 cells, a Myc-tag was fused to the C-terminus of QUAC1 in the pIREs-eGFP-QUAC1 plasmid. The plasmid was PCR amplified with primers F-myc and R-myc (Table 4) containing the Myc-tag sequence. The PCR products were then treated with DpnI (New England Biolabs), to digest all parental plasmids, and transformed into DH5α E. coli cells. Human Embryonic Kidney 293 (HEK293) cells were grown in DMEM with 10 % Fetal Bovine Serum, 1 % Penicillin Streptomycin, and 1 % L-Glutamine at 37 °C in 95 % air / 5 % CO2. Cells were transfected using Fugene HD transfection reagent (Promega), according to the manufacturer’s protocol. Transfected cells were used in patch clamp experiments the following day.

Electrophysiology - Whole cell patch clamp was performed with HEKA EPC 10 amplifier; and flowing cells were chosen for recording. After capacitance compensation, membrane voltage was clamped from +60 mV to -195 mV with 15 mV decrements. The holding potential was -20 mV. Voltage pulse protocol is shown in Figure 2I. The standard external solution contained (in mM): 150 NaCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 mannitol, 10 Hepes, pH 7.3, adjusted with N-methyl-d-glucamine. The external solution with malate contained (in mM): 120 NaCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 mannitol, 10 Hepes, 30 malic acid at pH 7.3, was added by perfusion. The pipette solution contained (in mM): 130 CsCl, 10 EGTA, 1 MgCl2, 10 Hepes, pH 7.3, and various free Ca2+ concentrations; free Ca2+ concentration was calculated with WEBMAXC software. 60 μM staurosporine (a kinase inhibitor) or 1, 5 or 10 μM W-7 (a CaM inhibitor (Caymanchem)) was added to the pipette solution in some experiments.

Peak current density of each cell was used to determine Δ current density and conductance. The Δ current density was calculated by subtracting current density recorded before adding malate to the current density recorded after adding malate to the bath solution. Conductance was calculated by division of peak current (after addition of malate) to the driving force and fit with a Boltzmann equation. For clarity, the driving force was the difference of clamped voltages and the chloride equilibrium potential. Chloride equilibrium potential was calculated based on the concentrations of chloride in the pipette and bath solutions (as described in the manuscript), with the following equation VEq = RT/(zF) ln(Cbath/Cpipette) (where R is the universal gas constant, T was set to 296.15 Kelvin, which is equivalent to 23 Celsius, z is the valence of chloride, F is the Faraday’s constant, Cbath is the concentration of chloride in the bath solution and Cpipette is the concentration of chloride in the pipette solution). The fit was a standard 4 parameter Boltzmann equation with free running parameters. Specifically the equation (f= y0+a/(1+exp(-(x-x0)/b))) was used; a = max(y)-min(y); b = xwtr(x,y-min(y).5)/4; x0 = x50(x,y-min(y).5); y0 = min(y)). For clarity, while there is no explicit variable of V1/2, one can calculate V1/2 from the x50 value, which is the potential at which conductance is halfway between max(y) and min(y). The x50 value was calculated using Sigma Plot from the given sets of data. V1/2 is the value when x = x50. z is the valance of the ion moving across, which can be back calculated by the slope of the Boltzmann equation. Slope = RT/(zF), therefore z = RT/(F*slope) (R is the universal gas constant, T is the temperature in Kelvin, F is the Faraday’s constant.

Only Δ current densities below -100 mV were used in statistical analyses. Overall Δ current densities were analyzed by 2-way repeated measure ANOVA, where voltage points were the repeated factor and either the concentrations of Ca2+, the presence of inhibitors or the type of QUAC1 (wildtype, variants) expressed were the second factor. The significance of overall Δ
current densities were presented with alphabet letters. Matching notations indicate no significant difference. Conductance was statistical analyzed and presented in the same way as Δ current density.

Time constant (tau) was calculated with the following equation: \( \tau = C_mR_s - R_o \) where \( \tau \) (ms) is the time constant, \( C_m \) (pF) is the membrane capacitance, \( R_s \) (mV/pA) is the resistance at steady state, and \( R_o \) is the resistance at peak.

**Stomatal aperture measurements** – Stomatal assays were performed according to previously reported methods (51, 52). Detached leaves from 4 to 8 week old plants, or at others times as indicated in the results, were soaked in opening buffer (50 mM KCl, 10 mM Mes, pH 6.1) for 2 hours. Leaves were soaked for another 3 hours in control buffer (10 mM Mes, 25 μM CaCl₂, pH 6.1) or control buffer with addition of either 150 mM malic acid (pH 6.1), 20 μM of a Ca²⁺ ionophore A23187 or 5, 10 and 20 μM W-7, or two of the three, or all three. Leaves were removed from the buffer and immediately dried by gentle blotting with kim-wipe and then painted with a layer of clear nail polish, a process taking less than 30 seconds. The nail polish peal was used for microscopy using a Zeiss Axiocam 135 microscope and obtained images visualized using Image J (53), and ensuring a blind analysis by relabelling of the image files by a third party. At least 100 stomata were measured, arising from 4 leaves obtained from 4 plants for each condition tested. Significance of differences was determined by 1-way ANOVA with Fisher’s LSD post-hoc.

**BdALMT12 Site Directed Mutagenesis** - The three basic residues R335, R338, and K342 were changed to alanine using the QuikChange site-directed mutagenesis kit according to the manufacturer’s protocol. pIRESE-GFP vector carrying BdALMT12 was used as template with primers F-R338A and R-R338A (Table 4). The plasmid-encoding variant R338A was then used as a template to make the double variant R335A/R338A and triple variant R335A/R338A/K342A with pairs of primers F-DM1 with R-DM1 and F-TM with R-TM respectively (Table 4). The plasmid encoding the triple variant was used as template to make the double variants R335A/K342A and R338A/K342A with the 2 pairs of primers F-DM2 with R-DM2 and F-DM3 with R-DM3 respectively (Table 4). Variants were confirmed by sequencing and expressed in HEK293 cells for patch clamp experiments. Myc-tag was also fused to the C-terminus of variants, in the same manner as wildtype, for determining of expression in HEK293 cells by western blot. The variant plasmids were PCR with primers F-myc and R-myc (Table 4) containing the myc sequence. The PCR products were then treated with DpnI (New England Biolab) and transformed into DH5α E.coli cells.

**BdALMT12 protein extraction and western blotting** - Wildtype and variant BdALMT12 fused with Myc-tag were expressed in HEK293 cells. All proteins present in the cell membranes of HEK293 cells were extracted with biotinylation using the Pierce Cell Surface Protein Isolation kit (Thermo Fisher), according to the manufacturer’s protocol. The extracts were fractionated by 10 % SDS-PAGE and transferred to PVDF membranes using a transfer apparatus (Bio-Rad). The membranes were blocked with RapidBlock solution 10x (Amresco), according to the manufacturer’s protocol. After blocking, the membranes were incubated with HRP-conjugated anti-Myc (1:5000 dilution) and anti-sodium potassium ATPase (1:5000 dilution) antibodies for 90 minutes at room temperature. Blots were washed with TBST buffer (50 mM Tris-Cl, 150 mM NaCl, and 0.1% Tween) three times for 5 minutes each time, incubated in Chemiluminescent substrate (Amersham) for 5 minutes and developed with the ChemiDoc Imaging System (Bio-Rad). Western bands were quantified with Image Lab software (Bio-Rad).

**Isothermal titration calorimetry** - The ITC experiments were performed on a Nano-ITC (TA-instrument). BdALMT12 peptides (AA334 – AA355) representing the wildtype CaM binding domain helix, as well as double variants R335D/R338D, R335D/K342D, R338D/K342D, and triple variants R335D/R338D/K342D with >90% purity were synthesized by Genscript. The peptides and bovine CaM (BioOcean) were dialyzed with 0.1-0.5 kD MWCO tubing overnight in buffer containing 20 mM HEPES, 100 mM KCl, and 5 mM CaCl₂ at pH 7.4. The peptide concentrations were adjusted to 400 μM and titrated into the Nano-ITC cell containing either 60 μM CaM, 60 μM CaM with 60 μM W-7.
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or just dialysis buffer. Since W-7 inhibits by competing with the peptides and binds to CaM, W-7 was mixed with CaM 30 minutes prior to the ITC experiment to ensure CaM was fully inhibited. The ‘no Ca2+’ sample was prepared exactly as described above, except that peptide and CaM were dialyzed overnight with 3 buffer changes, into buffer containing 20mM HEPES, 100mM KCl, and 1.5 mM EGTA at pH 7.4. The data was processed using the NanoAnalyze software by subtracting the background heat obtained from titrating the peptides into dialysis buffer to the heats obtained from titrating the peptides into the CaM-containing solutions.

CaM agarose affinity pull-down - Wildtype BdALMT12 and variant 335/342 fused with Myc-tag were expressed in HEK293 cells. Cells were rinsed with PBS, pH 7.4 (Thermo Fisher) and gently scraped off the bottom of the flask into PBS. Cell pellets were collected by centrifuge at 500 x g for 3 minutes then re-suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150mM NaCl, 1% Triton X-100, 10% glycerol and 1 x Halt protease inhibitor cocktail (Thermo Fisher)) and incubated at 4°C for 30 minutes. Total cell lysate was clarified by centrifuge at 10,000 x g for 2 minutes. Ca2+ was added to the cell lysate to make a final concentration of 8 mM Ca2+. CaM-Sepharose beads (Biovision) were transferred to mini spin columns (VWR) and equilibrated with wash buffer (50mM Tris-HCl pH 7.4, 150 mM NaCl, 0.25% Triton X-100, 10% glycerol, 1 x Halt protease inhibitor cocktail) by centrifuge at 1000 x g for 1 minute, repeated two times. The cell lysate was added to the CaM beads and incubated for 8 hours at 4°C. All unbound proteins were removed by centrifuge at 1000 x g for 1 minute and the columns were rinsed four times with wash buffer at 1000 x g for 1 minute each time. SDS-PAGE sample buffer containing 20 mM EGTA (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 20 mM EGTA) was then added to the columns and incubated for 2 hours at room temperature. Bound proteins were eluted by centrifuge at 1000 x g for 2 minutes and loaded onto SDS-page gel for QUAC1 detection by western blotting as described above. Western bands were quantified by normalizing against total (eluted) protein stain with Image Lab software (Bio-Rad).

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Conflict of Interest: The authors declare that they have no conflicts of interest with the contents of this article.
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References

1. Ward, J. M., Mäser, P., and Schroeder, J. I. (2009) Plant Ion Channels: Gene Families, Physiology, and Functional Genomics Analyses. *Annu. Rev. Physiol.* 71, 59–82
2. Hedrich, R. (2012) Ion Channels in Plants. *Physiol. Rev.* 92, 1777–1811
3. Marten, H., Konrad, K. R., Dietrich, P., Roelfsema, M. R. G., and Hedrich, R. (2007) Ca2+-Dependent and -Independent Abscisic Acid Activation of Plasma Membrane Anion Channels in Guard Cells of Nicotiana tabacum 1. *Plant Physiol.* 143, 28–37
4. Lee, S. C., Lan, W., Buchanan, B. B., and Luan, S. (2009) A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proc. Natl. Acad. Sci.* 106, 21419–21424
5. Dreyer, I., Gomez-porras, J. L., Riaño-pachón, D. M., and Hedrich, R. (2012) Molecular evolution of slow and quick anion channels (SLACs and QUACs/ALMTs). *Front. Plant Sci.* 3, 1–12
6. Imes, D., Mumm, P., Bohm, J., Al-Rasheid, K. A. S., Marten, I., Geiger, D., and Hedrich, R. (2013) Open stomata 1 (OST1) kinase controls R-type anion channel QUAC1 in Arabidopsis guard cells. *Plant J.* 74, 372–382
7. Kwak, J. M., Mori, I. C., Pei, Z., Leonhardt, N., Torres, M. A., Dangl, J. L., Bloom, R. E., Bodde, S., Jones, J. D. G., and Schroeder, J. I. (2003) NADPH oxidase AtbboH and AtbboF genes function in ROS-dependent ABA signaling in Arabidopsis. *Eur. Mol. Biol. Organ. J.* 22, 2623–2633
8. Mori, I. C., Murata, Y., Yang, Y., Munemasa, S., Wang, Y., Andreoli, S., Alonso, J. M., Harper, J. F., Ecker, J. R., Kwak, J. M., and Schroeder, J. I. (2006) CDPKs CPK6 and CPK3 Function in ABA Regulation of Guard Cell S-Type Anion- and Ca2+-Permeable Channels and Stomatal Closure. *PLOS Biol.* 4, 1749–1762
9. Brandt, B., Brodsky, D. E., Xue, S., Negi, J., Iba, K., Kangasjarvi, J., Ghassemian, M., Stephan, A. B., Hu, H., and Schroeder, J. I. (2012) Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. *Proc. Natl. Acad. Sci.* 109, 10593–10598
10. Scherzer, S., Maierhofer, T., Al-rasheid, K. A. S., Geiger, D., and Hedrich, R. (2012) Multiple Calcium-Dependent Kinases Modulate ABA-Activated Guard Cell Anion Channels. *Mol. Plant.* 5, 1409–1412
11. Schroeder, J. I., and Keller, B. U. (1992) Two types of anion channel currents in guard cells with distinct voltage regulation. *Proc. Natl. Acad. Sci. U. S. A.* 89, 5025–9
12. Linder, B., and Raschke, K. (1992) A slow anion channel in guard cells, activating at large hyperpolarization, may be principal for stomatal closing. *FEBS Lett.* 313, 27–30
13. Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., Uchimiya, H., Hashimoto, M., and Iba, K. (2008) CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature.* 452, 483–486
14. Vahisalu, T., Kollist, H., Wang, Y. F., Nishimura, N., Chan, W. Y., Valerio, G., Lamminmäki, A., Brosché, M., Moldau, H., Desikan, R., Schroeder, J. I., and Kangasjärvi, J. (2008) SLAC1 is required for plant guard cell S-type anion channel function in stomatal signalling. *Nature.* 452, 487–491
15. Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., Ache, P., Matschi, S., Liese, A., Al-Rasheid, K. A. S., Romeis, T., and Hedrich, R. (2009) Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proc. Natl. Acad. Sci.* 106, 21425–21430
16. Maierhofer, T., Diekmann, M., Offenborn, J. N., Lind, C., Bauer, H., Hashimoto, K., Al-Rasheid, K. A. S., Luan, S., Kudla, J., Geiger, D., and Hedrich, R. (2014) Site-and kinase-specific phosphorylation-mediated activation of SLAC1, a guard cell anion channel stimulated by abscisic
BdALMT12 activation requires calcium/calmodulin

17. Geiger, D., Maierhofer, T., AL-Rasheid, K. A. S., Scherer, S., Mumm, P., Liese, A., Ache, P., Wellmann, C., Marten, I., Grill, E., Romeis, T., and Hedrich, R. (2011) Stomatal Closure by Fast Abscisic Acid Signaling Is Mediated by the Guard Cell Anion Channel SLAH3 and the Receptor RCAR1. Sci. Signal. 4, ra32-ra32

18. Meyer, S., Mumm, P., Imes, D., Endler, A., Weder, B., Al-Rasheid, K. A. S., Geiger, D., Marten, I., Martinova, E., and Hedrich, R. (2010) AtALMT12 represents an R-type anion channel required for stomatal movement in Arabidopsis guard cells. Plant J. 63, 1054–1062

19. Sasaki, T., Yamamoto, Y., Ezaki, B., Katsuahara, M., Ahn, S. J., Ryan, P. R., Delhaize, E., and Matsumoto, H. (2004) A wheat gene encoding an aluminum-activated malate transporter. Plant J. 37, 645–653

20. Palmer, A. J., Baker, A., and Muench, S. P. (2016) The varied functions of aluminium-activated malate transporters—much more than aluminium resistance. Biochem. Soc. Trans. 44, 856–862

21. Medeiros, D. B., Martins, S. C. V, Cavalcanti, J. H. F., Daloso, D. M., Martinova, E., Nunes-Nesi, A., DaMattia, F. M., Fernie, A. R., and Araújo, W. L. (2016) Enhanced Photosynthesis and Growth in ataquac1 Knockout Variants Are Due to Altered Organic Acid Accumulation and an Increase in Both Stomatal and Mesophyll Conductance. Plant Physiol. 170, 86–101

22. Sasaki, T., Mori, I. C., Furuichi, T., Munemasa, S., Toyooka, K., Matsuoka, K., Murata, Y., and Yamamoto, Y. (2010) Closing Plant Stomata Requires a Homolog of an Aluminum-Activated Malate Transporter. Plant Cell Physiol. 51, 354–365

23. Schachtman, D. P., Schroeder, J. I., Lucas, W. J., Anderson, J. A., and Gaber, R. F. (1992) Expression of an inward-rectifying potassium channel by the Arabidopsis KAT1 cDNA. Science. 258, 1654–1658

24. Cao, Y. W., Ward, J. M., Kelly, W. B., Ichida, A. M., Gaber, R. F., Anderson, J. A., Uozumi, N., Schroeder, J. I., and Crawford, N. M. (1995) Multiple genes, Tissue-specificity, and Expression-dependent Modulation Contribute to the Functional Diversity of Potassium Channels in Arabidopsis thaliana. Plant Physiol. 109, 1093–1106

25. Leng, Q., Mercier, R. W., Yao, W., and Berkowitz, G. A. (1999) Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiol. 121, 753–761

26. Szabó, I., Negro, A., Downey, P. M., Zoratti, M., Lo Schiavo, F., and Giacometti, G. M. (2000) Temperature-dependent functional expression of a plant K(+) channel in mammalian cells. Biochem. Biophys. Res. Commun. 274, 130–5

27. Leng, Q., Mercier, R. W., Hua, B.-G., Fromm, H., and Berkowitz, G. A. (2002) Electrophysiological analysis of cloned cyclic nucleotide-gated ion channels. Plant Physiol. 128, 400–410

28. Pilot, G., Lacombe, B., Gaynard, F., Chérel, I., Boucherez, J., Thibaud, J. B., and Sentenac, H. (2001) Guard Cell Inward K+ Channel Activity in Arabidopsis Involves Expression of the Twin Channel Subunits KAT1 and KAT2. J. Biol. Chem. 276, 3215–3221

29. Ivashikina, N., Deeken, R., Fischer, S., Ache, P., and Hedrich, R. (2005) AKT2/3 subunits render guard cell K+ channels Ca2+ sensitive. J. Gen. Physiol. 125, 483–492

30. Ligaba, A., Kochian, L., and Piñeros, M. (2009) Phosphorylation at S384 regulates the activity of the TaALMT1 malate transporter that underlies aluminum resistance in wheat. Plant J. 60, 411–423

31. Mumm, P., Imes, D., Martinova, E., Al-Rasheid, K. A. S., Geiger, D., Marten, I., and Hedrich, R. (2013) C-terminus-mediated voltage gating of arabidopsis guard cell anion channel QUAC1. Mol. Plant. 6, 1550–1563

32. Karaman, M. W., Herrgard, S., Treiber, D. K., Gallant, P., Atteridge, C. E., Campbell, B. T., Chan, K. W., Ciceri, P., Davis, M. I., Edeen, P. T., Faraoni, R., Floyd, M., Hunt, J. P., Lockhart, D. J., Milanov, Z. V, Morrison, M. J., Pallares, G., Patel, H. K., Pritchard, S., Wodicka, L. M., and Zarrinkar, P. P. (2008) A quantitative analysis of kinase inhibitor selectivity. Nat. Biotechnol. 26, 127–132
33. Hidaka, H., and Tanaka, T. (1983) Naphthalenesulfonamides as Calmodulin Antagonists. *Methods Enzymol.* 102, 185–194
34. Minhas, F. U. A. A., and Ben-Hur, A. (2012) Multiple instance learning of Calmodulin binding sites. *Bioinformatics.* 28, i416–i422
35. Mruk, K., Farley, B. M., Ritacco, A. W., and Korbetz, W. R. (2014) Calmodulation meta-analysis: Predicting calmodulin binding via canonical motif clustering. *J. Gen. Physiol.* 144, 1–10
36. Cole, C., Barber, J. D., and Barton, G. J. (2008) The Jpred 3 secondary structure prediction server. *Nucleic Acids Res.* 36, W197-201
37. Edlund, M., and Blikstad, I. (1996) Calmodulin Binds to Specific Sequences in the Cytoplasmic Domain of C-CAM and Down-regulates C-CAM Self-association. *J. Biol. Chem.* 271, 1393–1399
38. Ben-Johny, M., and Yue, D. T. (2014) Calmodulin regulation (calmodulation) of voltage-gated calcium channels. *J. Gen. Physiol.* 143, 679–692
39. Donovan, N., Martin, S., and Donkin, M. E. (1985) Calmodulin Binding Drugs Trifluoperazine and Compound 48/80 Modify Stomatal Responses of Commelina communis L. *J. Plant Physiol.* 118, 177–187
40. Cousson, A., Cotelle, V., and Vavasseur, A. (1995) Induction of Stomatal Closure by Vanadate or a Light/Dark Transition Involves Ca2+-Calmodulin-Dependent Protein Phosphorylations. *Plant Physiol.* 109, 491–497
41. Shimazaki, K., Kinoshita, T., and Nishimura, M. (1992) Involvement of Calmodulin and Calmodulin-Dependent Myosin Light Chain Kinase in Blue Light-Dependent H Pumping by Guard Cell Protoplasts from Vicia faba L. *Plant Physiol.* 99, 1416–1421
42. De Silva, D. L. R., Cox, R. C., Hetherington, A. M., and Mansfield, T. A. (1985) Suggested Involvement of Calcium and Calmodulin in the Responses of Stomata to Abscisic Acid. *New Phytol.* 101, 555–563
43. Erdahl, W. L., Chapman, C. J., Taylor, R. W., and Pfeiffer, D. R. (1994) Ca2+ Transport Properties of Ionophores A23187, ionomycin, and 4-BrA23187 in a Well Defined Model System. *Biophys. Journal.* 66, 1678–1693
44. Peterson, F. C., Burgie, E. S., Park, S., Jensen, D. R., Joshua, J., Bingman, C. A., Chang, C., Cutler, S. R., Jr, G. N. P., and Volkman, B. F. (2010) Structural basis for selective activation of ABA receptors. *Nat Struct Mol Biol.* 17, 1109–1113
45. Weiner, S., Geffre, A., and Toth, A. L. (2017) Functional genomics in the wild: A case study with paper wasps shows challenges and prospects for RNA-interference in ecological systems. *Genome.* 61, 266–272
46. Reichow, S. L., Clemens, D. M., Freites, J. A., Németh-cahalan, K. L., Heyden, M., Tobias, D. J., Hall, J. E., and Gonen, T. (2013) Allosteric mechanism of water-channel gating by Ca 2+ -calmodulin. *Nat. Struct. Mol. Biol.* 20, 1085–1092
47. Alaimo, A., Nuñez, E., Aivar, P., Fernández-Orth, J., Gomis-Perez, C., Bernardo-Seisdedos, G., Malo, C., and Villarroel, A. (2017) Calmodulin confers calcium sensitivity to the stability of the distal intracellular assembly domain of Kv7.2 channels. *Sci. Rep.* 7, 13425
48. Zhou, Y. P., Duan, J., Fujibe, T., Yamamoto, K. T., and Tian, C. E. (2012) AtIQM1, a novel calmodulin-binding protein, is involved in stomatal movement in Arabidopsis. *Plant Mol. Biol.* 79, 333–346
49. Reisser, S., Strandberg, E., Steinbrecher, T., and Ulrich, A. S. (2014) 3D hydrophobic moment vectors as a tool to characterize the surface polarity of amphiphilic peptides. *Biophys. J.* 106, 2385–2394
50. Mann, D. G. J., LaFayette, P. R., Abercrombie, L. L., King, Z. R., Mazarei, M., Halter, M. C., Poovaiah, C. R., Baxter, H., Shen, H., Dixon, R. A., Parrott, W. A., and Neal Stewart Jr, C. (2012) Gateway-compatible vectors for high-throughput gene functional analysis in switchgrass (Panicum virgatum L.) and other monocot species. *Plant Biotechnol. J.* 10, 226–236
51. Hilu, K. W., and Randall, J. L. (1984) Convenient Method for Studying Grass Leaf Epidermis. *Taxon.* 33, 413–415
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52. Berger, D., and Altmann, T. (2000) A subtilisin-like serine protease involved in the regulation of stomatal density and distribution in Arabidopsis thaliana. Genes Dev. 14, 1119–1131
53. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. Nat. Methods. 9, 671–675
54. Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D., and Higgins, D. G. (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539
55. Nicholas, K. B., Nicholas, H. B., and Deerfield, D. W. (1997) GeneDoc: analysis and visualization of genetic variation. EMBnet News. 4, 14
56. Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425
57. Zuckerkandl, E., and Pauling, L. (1965) Evolutionary divergence and convergence in proteins. in Evolving Genes and Proteins (V. Bryson and H.J. Vogel ed), pp. 97–166, Academic Press, New York, 10.1209/epl/i1998-00224-x
58. Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874
### Table 1: Changes in average stomatal widths induced by BdALMT12 over-expression or exogenous application of a calcium ionophore + malate. Values representing the averages from 100 stomata for each treatment are shown. Significance was determined by a lower-tailed Z test comparing each treatment against the opening control.

|                     | WT Stomata width (µm) | KD2 Stomata width (µm) | Δ (WT – KD2) |
|---------------------|------------------------|------------------------|--------------|
| Young – untreated   | 1.52 ± 0.02            | 1.62 ± 0.02            | -0.1 ± 0.005 |
| Adult – untreated   | 1.55 ± 0.02            | 1.48 ± 0.02            | 0.06 ± 0.003  (p < 0.001) |
| Adult – treated with malate and A23187 | 1.47 ± 0.02 | 1.32 ± 0.02 | 0.15 ± 0.004 (2 sample T-test) |
| Adult – Δ (untreated – treated) | 0.08 ± 0.005 | 0.16 ± 0.005 | 0.08 ± 0.005 (2 sample T-test) |

### Table 2: Binding parameters of wildtype and variant CaM binding peptide. Unless otherwise indicated, all measurements were made in the presence of 5 mM Ca²⁺. The ‘no Ca²⁺’ condition included 1.5 mM EGTA. W-7 concentration was equimolar to CaM concentration.

| Peptide                  | Kd1 (M) | ΔH1 (kJ/mol) | AS1 (J/mol.K) | Kd2 (M) | ΔH2 (kJ/mol) | AS2 (J/mol.K) |
|--------------------------|---------|--------------|---------------|---------|--------------|---------------|
| Wildtype peptide         | 3.452E⁻⁵ | 105.4        | 4.962E⁻⁸      | 3.925E⁻⁶ | -170.4       | -4.8E⁻⁷       |
| Wildtype peptide, no Ca²⁺ | 9.165E⁻⁷ | 69.16        | 3.437E⁻⁶      | 1.003E⁻⁶ | -199.9       | -5.636E⁻⁷     |
| Variant 338/342 peptide  | 4.101E⁻⁶ | -88.78       | -2.217E⁻¹     | 6.366E⁻⁶ | -199.2       | -5.685E⁻⁷     |
| Variant 335/338 peptide  | 3.721E⁻⁷ | -75.77       | -131.1        | ND      | ND           | ND            |
| Variant 335/342 peptide  | 5.5E⁻⁷   | -470.8       | -1478         | ND      | ND           | ND            |
| Wildtype peptide + W-7   | ND      | ND           | ND            | ND      | ND           | ND            |

### Table 3: CaM binding site prediction comparison for the region homologous to B. distachyon amino acids 334 - 351. Sequences were obtained from the NCBI database and aligned using Clustal Omega (54). Regions homologous to the predicted BdALMT12 CBD were evaluated using the Calmodulation Database and Meta-analysis Predictor Software to analyze the full protein sequence (35). The higher the positional prediction score numbers, the higher the likelihood for a binding site, with scoring letters being a continuation of the numbers with A=10, B=11, C=12 etc. Amphipathicity of the helices are expressed as the absolute value of the 3D hydrophobic moment of the helix, calculated using the 3D-HM prediction software and is expressed as Å²kT/e, where kT/e is the electrostatic potential and Å is the length of the vector (49).

| Species                  | Annotation | NCBI Accession # | Motif Starting Residue # | Putative CBD Motif | CalM Motif Positional Prediction Scores | Hydrophobic Moment Vector | Å²kT/e |
|--------------------------|------------|------------------|--------------------------|--------------------|----------------------------------------|----------------------------|--------|
| Brachypodium distachyon  | BdALMT12   | XP_003574370     | 334                      | TRVAREAVKVLQELAVSI | 55A999CAAA98898946                 | 12.479                      |        |
| Zea mays                 | ZmALMT12   | XP_008648985     | 334                      | TRVAREAVKVLQELAVSI | 557666755543343335                 | 12.512                      |        |
| Glycine max              | GmALMT12   | XP_01564622      | 334                      | TRVAREAVKVLQELAVSI | 557666755543343335                 | 12.405                      |        |
| Sorghum bicolor          | SBdALMT12  | XP_002437417     | 337                      | TRVAREAVKVLQELAVSI | 557666755543343335                 | 12.479                      |        |
| Aeolopsis balsaei         | AgtALMT1X  | XP_020161007     | 334                      | TRVAREAVKVLQELAVSI | 557666755543343335                 | 10.084                      |        |
| Ananas comosus           | AcaALMT12  | OAY77120         | 309                      | IRYVEGSVVLKELGSI   | 55A999CAAA98898946                 | 8.570                       |        |
| Asparagus officinalis     | AoaALMT1X  | XP_020261302     | 325                      | TRVATESVKVLLELOSI  | 559998977787565557                 | 8.206                       |        |
| Vitis vinifera           | VaALMT1X   | XP_002278594     | 345                      | FQVAAEVSVKLRELNC   | 55A999888854453315                 | 3.002                       |        |
| Spinacia oleracea         | SoALMT1X   | XP_012815304     | 340                      | IRLAAEVSVVLKELGSI  | 55A999888854453315                 | 3.040                       |        |
| Nicotiana tabacum         | NtALMT1X   | XP_016493112     | 340                      | IRLAEVTKVLKELGSI   | 557666755544453315                 | 8.815                       |        |
| Glycine max              | GmALMT1X   | XP_003516635     | 344                      | IRLAEVSVVLKELGSI   | 55A999888854453315                 | 6.646                       |        |
| Solanum lycopersicum      | SlaALMT1X  | XP_004244701     | 339                      | IRLGVAYKVLKELGSI   | 96877866665653315                  | 8.329                       |        |
| Arabidopsis thaliana      | AegALMT1X  | O49696          | 331                      | VNRAGEVCKALIELGSI  | 857666755544453315                 | 8.872                       |        |
| Triticum aestivum         | TaALMT1X   | Q764L1          | 324                      | GEMSLHSSVLRDLAMAT   | 533342222554476666                 | 4.941                       |        |

### Table 4: Primer Sequences
**FIGURE LEGENDS**

**FIGURE 1.** Primary structure elements of BdALMT12 and its evolutionary relationships. A) Alignment of AtQUAC1 (A) and BdALMT12 (B) amino acid sequences. The alignment was made using Clustal Omega (54) and visualized using GeneDoc (55). Regions highlighted in black are conserved. The location of the six predicted transmembrane helices are highlighted in pink and a predicted CaM binding domain is shaded according to Figure 4G. B) Evolutionary relationships of ALMT family members from *A. thaliana* and *B. distachyon*. The evolutionary history was inferred using the Neighbor-Joining method (56). The optimal tree with the sum branch length = 5.50656346 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (57) and are in the units of the number of amino acid substitutions per site. The analysis involved 21 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 105 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (58). Sequence sources (NCBI accession#s): *A. thaliana* AtALMT1 (AAE28289.1), AtALMT2 (Q9SJ8.2), AtALMT3 (Q9LPQ8.1), AtALMT4 (Q9C6L8.1), AtALMT5 (Q9Z29.1), AtALMT6 (Q9SHM1.1), AtALMT7 (Q9XIN1.1), AtALMT8 (Q9SRM9.1), AtALMT9 (AEE76098.1), AtALMT10 (O23086.2), AtALMT11 (Q3E9Z9.1), AtALMT12 (Q49696.1), AtALMT13 (Q9LS23.1), AtALMT14 (Q9LS22.1); *B. distachyon* sequence sources are indicated in the figure.

**FIGURE 2.** Effect of Ca\(^{2+}\) and pharmacological inhibitors on activity of BdALMT12 after malate activation. Statistical tests and notations are described under Experimental procedures. A) Ca\(^{2+}\)-dependent Δ current density of BdALMT12. HEK cells transfected with BdQUAC1 were patched using different Ca\(^{2+}\) concentrations in the pipette solution including 0 μM (black circle), 0.05 μM (white circle), 0.1 μM (black triangle), 0.5 μM (white triangle) and 5 μM (black square) free Ca\(^{2+}\), n ≥ 8 for each concentration. B) Conductance of BdALMT12 channels at different Ca\(^{2+}\) concentrations including 0.1 μM (solid line), 0.5 μM (broken line), 5 μM (dotted line) free Ca\(^{2+}\). No statistical differences were detected (p > 0.05). C) Representative traces of BdALMT12 current, presented in current density (pA/pF), at -180 mV with 0 and 5 μM free Ca\(^{2+}\) in the pipette solution. D) Effect of staurosporine. Pipette solutions contained 0.1 μM free Ca\(^{2+}\) (white circle) or 0.1 μM free Ca\(^{2+}\) and 60 nM staurosporine (black circle) with n = 9 for each treatment. E) Conductance of BdQUAC1 channels with addition of staurosporin in the pipette solution. Pipette solutions contained 0.1 μM free Ca\(^{2+}\) (solid line) or 60 nM staurosporine (dotted line). F) Representative traces of BdQUAC1 current, presented in current density (pA/pF), at -180 mV with 0.5 μM free Ca\(^{2+}\) and the presence or absence of W-7. G) Effect

**TABLE 1.** Primer sequences.

| Name | Primer Sequence |
|------|-----------------|
| F-P1 | 5'-CAGGCCGAGCCACGTTTGATAAAAAGCAAGCTGCTTCAAGCTTGGCAAGGCCACCATGGCTTGCACTCTACATCC-3' |
| R-P1 | 5'-CCACAGCAGTTGATACAAAGGCAAGGTCGCTTGATATTATTACCATCAGCTAGCATAGAAAATCTG-3' |
| F-R338A | 5'-CTATGACTAGTAAAGGCAAGGTCGCTTGATATTATTACCATCAGCTAGCATAGAAAATCTG-3' |
| R-R338A | 5'-GGAAACTTCTGGCCACTCCGCCTGCTACTCTAGTGGACTAGCATAGAAAATCTG-3' |
| F-DM1 | 5'-CGGAGATCATGCACTGAGCAGCAGCAGGGAAGTGGCC-3' |
| R-DM1 | 5'-GGCACTTCCCCTGCTACTGAGCATGGAATTTAC-3' |
| F-TM | 5'-GGAATCTGATCGTACGAGGGAATTTAC-3' |
| R-TM | 5'-AGCTCGTCTGAGGGAATTTAC-3' |
| F-DM2 | 5'-CCATGCTCTTCATGCACGAAAGTGGCCGGCTTCTA-3' |
| R-DM2 | 5'-TGACACGGCGACTTCTCCTGAGCATGGAATTTAC-3' |
| F-DM3 | 5'-CGGACCTTCCCCTGCTACTCTAGTGGACTAGCATGGAATTTAC-3' |
| R-DM3 | 5'-GGCACTTCCCCTGCTACTTCTAGTGGACTAGCATGGAATTTAC-3' |
| F-BdUBC18 | 5'-GGAGGCCACCTCAAGTCTATTT-3' |
| R-BdUBC18 | 5'-ATAGCGGTTCATGCTTCTGAG-3' |
| F-BdALMT12 | 5'-ACTGTTGGCTGACTTCTGAG-3' |
| R-BdALMT12 | 5'-CTAGGCTTCTGGTGTTGTA-3' |
| F-myc | 5'-GATATACCGCGGCGGATCCGCTTCTCC-3' |
| R-myc | 5'-GATATACCGCGGCGGATCCGCTTCTCC-3' |
of W-7. Pipette solutions contained 0.5 μM free Ca\(^{2+}\) (black triangle), 0.5 μM free Ca\(^{2+}\) and 1 μM W-7 (white triangle), 0.5 μM free Ca\(^{2+}\) and 5 μM W-7 (black circle), 0.5 μM free Ca\(^{2+}\) and 10 μM W-7 (black circle), and 0 μM free Ca\(^{2+}\) (black square) with n ≥ 6 for each treatment. H) Conductance of BdQUAC1 channels with addition of W-7 in the pipette solution. Pipette solutions contained 0.5 μM free Ca\(^{2+}\) (broken line), 0.5 μM free Ca\(^{2+}\) and 1 μM W-7 (dotted line), or 0.5 μM free Ca\(^{2+}\) and 5 μM W-7 (solid line). I) Voltage protocol used for the experiments.

**FIGURE 3.** Effect of malate, a Ca\(^{2+}\) ionophore and a CaM inhibitor on stomatal aperture in *B. dystachyon* wildtype and knockdown plants. Statistical symbols *, # indicates p≤0.05, and **, ## indicates p ≤ 0.01. A) Plot of the averages from 100 stomata for each treatment are shown. Significance was determined by a lower-tailed Z test comparing each treatment against the opening control. Representative stomatal images for each treatment are labeled with lower case Roman numeral. B) PCR quantification of RNAi knockdown of QUAC1 in *B. distachyon* T0 generation plants. C) PCR quantification of QUAC1 expression and stomata width of the T1 generation KD2 adult plants (at 8-10 weeks). QUAC1 has rebounded and been overly expressed (by a 1-sample T-test, n= 100 stomata). D) PCR quantification of QUAC1 expression and stomata width of the T1 generation KD2 adult plants (at 8-10 weeks). QUAC1 has rebounded and been overly expressed (by a 1-sample T-test, n = 19, with wildtype normalized to 1). Stomata width of KD2 plants is significantly smaller both in the absence (empty pattern) and presence (crosshatched lines) of malate and A23187 (by a 2-sample T-test, n = 100 stomata). All error bars represent standard error.

**FIGURE 4.** Effect of mutations in the putative BdALMT12 CaM binding domain on BdALMT12 channel activity. Statistical tests and notations of patch clamp data are described under Experimental procedures - Electrophysiology. A) Δ current density of BdALMT12 and BdALMT12 variants. Transfected HEK cells were patched with 0.5 μM Ca\(^{2+}\) in the pipette solution. Wildtype (black circle), variant 335/338 (white circle), variant 335/342 (white triangle), variant 338/342 (black square), variant 335/338/342 (black triangle). Each variant was compared to wildtype only, with n ≥ 8 for each variant or wildtype. Same letters indicate no significant difference. B) Channel conductance of BdALMT12 and BdALMT12 variants including wildtype (solid line), variant 335/338 (long broken line), variant 335/342 (dotted line) and variant 338/342 (short broken line). C) Representative traces of variant and wildtype BdALMT12 currents, presented in current density (pA/pF), at -180 mV. D) Inactivation time constants of each double variant compared to wild type BdALMT12. E) Protein expression of variants and wildtype Myc-tagged BdALMT12 in HEK293 cell membranes. Total isolated cell membrane proteins were analysed by Western Blot using an anti-Myc-tag antibody, with n ≥ 6 for each variant and wildtype. Wildtype expression was normalized to 1. Significance was determined by 1 sample T test, * p = 0.014, *** p < 0.001. F) Δ current density of BdALMT12 and BdALMT12 variants after normalization to protein expression. Normalization was calculated by subtracting the Δ current density to the GFP Δ current density then dividing by protein expression for wildtype (black circle), variant 335/338 (white circle), variant 335/342 (black triangle), variant 338/342 (white triangle), and variant 335/338/342 (black square). Significance is denoted as described for panel A. G) Helical wheel depiction of the putative CaM binding domain in BdALMT12 (amino acids 334 to 351), highlighting the amphiphilic nature of the helix. Non-polar, hydrophobic residues are yellow, polar uncharged residues are green, polar acidic residues are red and polar basic residues are blue. Residues selected for mutagenesis are indicated with an * in both panels. All error bars represent standard error.

**FIGURE 5.** Binding of wildtype and variant CaM binding peptides to CaM. A) Isothermal titration calorimetry analyses of the binding of wildtype to CaM in the presence of 5 mM Ca\(^{2+}\), 5 mM Ca\(^{2+}\) with 60 μM W-7, or no added Ca\(^{2+}\) with 1.5 mM EGTA for the 0 μM free Ca\(^{2+}\) effect. The amount of W-7 used in
the above mentioned experiment is equal to the amount of CaM presence (60 µM). The 5 mM Ca²⁺ and no Ca²⁺ data were fit using the ‘multiple sites’ model and the 5 mM Ca²⁺ with W-7 data was fit using the ‘independent’ data. B) Isothermal titration calorimetry analyses of the binding of variant CBD peptides to CaM. The binding of all peptides was carried out in the presence of 5mM Ca²⁺. Variant 335/342 data were fit using the ‘multiple sites’ model yielding a two-state binding result. The others were fit using the ‘independent’ model and yielded either a single binding event (variant 335/338 and 335/342) or no binding (variant 335/338/342). C) Representative Western Blot and reversible protein stain image of full-length wildtype QUAC1 and variant 335/342 binding to CaM. D) Quantification of full-length QUAC1 protein bound to CaM. Wiltype BdALMT12 and variant 335/342 binding to CaM were analyzed by Western Blot using an anti-Myc-tag antibody, n = 4. Wiltype and variant QUAC1 were quantified against total proteins eluted from the CaM affinity pull-down, and wild type QUAC1 was normalized to 1. Error bars represent standard error. Significance was determined by 1 sample T test, ** indicates p ≤ 0.01).
BdALMT12 activation requires calcium/calmodulin

Figure 1
BdALMT12 activation requires calcium/calmodulin

Figure 3
Figure 5

A

B

C

D

BdALMT12 activation requires calcium/calmodulin
The malate-activated ALMT12 anion channel in the grass Brachypodium distachyon is co-activated by Ca^{2+}/calmodulin

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