Multiple ALMT subunits combine to form functional anion channels: A case study for rice ALMT7

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The Aluminum Activated Malate Transporter (ALMT) family members are anion channels that play important roles in organic acid transport, stress resistance, growth, development, fertilization and GABA responses. The rice malate permeable OsALMT7 influences panicle development and grain yield. A truncated OsALMT7 mutant, panicle apical abortion1 (paab1) lacking at least 2 transmembrane helices, mediates reduced malate efflux resulting in yield reducing. Here, we further investigated the contribution of OsALMT7 transmembrane helices to channel activity, using heterologous expression in Xenopus laevis oocytes. We further found that OsALMT7 formed as a homomer by co-expressing OsALMT7 and paab1 proteins in oocytes and detecting the physical interaction between two OsALMT7, and between OsALMT7 and paab1 mutant protein. Further study proved that not just OsALMT7, mutants of TaALMT1 inhibit wild-type TaALMT1 channel, indicating that ALMTs might perform channel function as homomers. Our discovery brings a light for ion channel structure and homomultimer regulation understanding for ALMT anion channels and potential for crop grain yield and stress response improvement in the context of the essential role of ALMTs in these plant processes.

KEYWORDS
ALMT7, malate flux, transmembrane helices, homomer, channel activity

Introduction

Aluminum Activated Malate Transporters (ALMTs) are important plant anion channels by playing roles in organic acid transport, stress resistance, growth and development, nutrient absorption, and the GABA response (Hedrich 2012; Ramesh et al., 2015; Sharma et al., 2016; Balzergue et al., 2017; Medeiros et al., 2018; Long et al.,
development, and grain yield (Heng et al., 2018). Furthermore, OsALMT7 plays an important role in panicle anion transport, panicle abortive and Serbia (through the use of gene mapping, a mutation in paab1 was found to underpin a panicle apical abortion phenotype (Heng et al., 2018). Compare with other ALMTs, OsALMT7 has special molecular characteristics that contribute to anion channel regulation. This strengthens our understanding of ALMT function. By defining the mechanism by which subunit modification has a dominant effect on channel function, this provides a new avenue by which genetic modification or gene editing can have important effects without first creating knockout mutants, enabling crop stress resistance and grain yield improvements.

Materials and methods

Electrophysiological measurements in X. laevis oocytes

All chemicals were sourced from Sigma Aldrich. Capped complementary RNA (cRNA) production, X. laevis oocytes preparation, and whole oocyte two-electrode voltage clamping studies. We proposed and tested the hypothesis that OsALMT7 transports malate as a multimer and that paab1 interaction with OsALMT7 inhibits transport capacity. Furthermore, we extended this to examine whether this is a feature of other ALMTs, specifically TaALMT1.

A number of plant channels are already known to function as homo- or hetero-multimers, and heteromerization is acknowledged to be an important mechanism of channel regulation (Hedrich, 2012). For example, aquaporins of both the PM and tonoplast assemble as homo- or heterotetramers, such as PIP1s with no or weak water permeability with itself, combine with PIP2s and increase their activity in different plant species (Harvengt et al., 2000; Bienert et al., 2012; Fetter et al., 2004; Heinen et al., 2014; Berny et al., 2016). Shaker family potassium (K) channels share a similar structure with ALMTs, 6 transmembrane α-helices with both N-terminal and C-terminal cytosolic domains, and function as homo- or heterotetramers; notably, KC1, the silent channel subunit, forms a heterotetramer with other shaker K channels to change their voltage dependence (Baizabal-Aguirre et al., 1999; Pilot et al., 2001; Very and Sentenac, 2003; Xicluna et al., 2007; Duby et al., 2008; Lebudy et al., 2008; Geiger et al., 2009; Lebudy et al., 2010; Wang et al., 2010; Leanguenin et al., 2011). Cyclic nucleotide-gated channel 2 (CNGC2) and CNGC4 have been proposed to form a heteromeric channel to mediate Ca2+ currents (Tian et al., 2019). For plant channel subunits, the S-type channel SLAC1 has been proposed to combine with the shaker K’ channels KAT1 and KAT2 and inhibit their channel activity (Zhang et al., 2016). Zhang et al. (2013) showed that tonoplast-localized AtALMT9 formed homomultimeric complex by coexpressing the mutant channel and wild-type channel in tobacco leaves; however, it has not been determined whether PM-localized ALMTs, including OsALMT7, and R-type anion channels, such as ALMT12/13/14, function as multimers or monomers.

Here, we determined that OsALMT7 function as multimeric proteins and that combinations of ALMT subunits can contribute to anion channel regulation. This strengthens our understanding of ALMT function. By defining the mechanism by which subunit modification has a dominant effect on channel function, this provides a new avenue by which genetic modification or gene editing can have important effects without first creating knockout mutants, enabling crop stress resistance and grain yield improvements.

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(TEVC) recording were performed as our previous work (Heng et al., 2018; Long et al., 2020). 46 nL of 200 mM Na2-malate (pH 7.2) was pre-loaded before TEVC recording. The bath solution for malate current recording consisted of 80 mM Na-glucuronate, 1 mM Ca-glucuronate, 1 mM K-glucuronate, 1 mM Mg-glucuronate, 25 mM malic acid, 0.1 mM LaCl3, and 10 mM MES/Tris (pH 5.8). And the bath solution for different anion permeability contained 25 mM NaNO3, 25 mM Na2-malate, 25 mM NaCl, and 25 mM Na2SO4 respectively. The method for creating inside-out patch clamp in X. laevis oocytes followed our previous protocols (Long et al., 2020) with the solution modified, 20 mM Na2-malate in the bath solution (cytosolic side) and 10 mM Na2-malate in the pipette solution (outside) and the pH of both was adjusted to 7.2 (Hepes/Tris).

**BiFC assay**

The coding regions of OsALMT7, paab1-t1, paab1-t2, and TaALMT1 were cloned into pSPYCE and pSPYNE vectors (Waadt et al., 2008; Waadt and Kudla, 2008). The BiFC assays were performed as described previously (Walter et al., 2004; Waadt and Kudla, 2008). The tested construct pairs were expressed in leaves of Nicotiana benthamiana for 3 d before microscopy observation. The YFP fluorescence in the transformed leaves was imaged using a confocal laser scanning microscope (ZEISS LSM710).

**Results**

**Paab1 mutant proteins lacking the last 2-3 transmembrane helices mediate malate efflux**

Heng et al. (2018) found two transcripts in a panicle apical abortion (paab1) mutant rice, paab1-t1 and paab1-t2. Figure 1A depicts the full-length OsALMT7 protein and the paab1-t1 and paab1-t2 variants in which the last 2-3 transmembrane α-helices (depending on topology predictions) and the cytosolic C-terminus are absent. Both paab1 truncations encode malate-transport competent proteins but with a much-reduced capacity compared to full-length OsALMT7 when expressed in X. laevis oocytes (Heng et al., 2018). Here, we carried out an in-depth analysis of the behaviour of paab1 proteins in X. laevis oocytes. Our results confirmed that the paab1-t1 and paab1-t2 proteins conducted significantly greater inward currents than water-injected oocytes at the polarization range of membrane potential (more negative than -100 mV), but these currents were lower in magnitude than those of wild-type OsALMT7 (malate efflux; Figure 1B; Supporting Figure 1). Channel conductance (G) analysis of the paab1 mutant truncated proteins found that the maximal conductance was lower, and voltage-dependent activation occurred at a more negative potentials than that of the wild-type channel (OsALMT7). The Gmax values of OsALMT7, paab1-t1, and paab1-t2 were 112.2 ± 13.1 µS, 20.7 ± 5.8 µS, and 16.7 ± 5.2 µS, and the V1/2 values of OsALMT7, paab1-t1, and paab1-t2 were -118.4 ± 11.9 mV, -136.9 ± 16.0 mV, and -138.1 ± 18.0 mV, respectively (Figure 1C). Isolated inside-out membrane patches (cytosolic side facing the bath solution) from X. laevis oocytes injected with OsALMT7, paab1-t1, or paab1-t2 cRNA showed, compared to water-injected controls, weak activated currents with short open times reminiscent of TaALMT1 (=1 pA; Long et al., 2020). paab1 mutants showed both a smaller magnitude of activated currents and less open probability (Popen), with single channel currents at -160 mV of 1.03 ± 0.03 pA, 0.42 ± 0.05, and 0.40 ± 0.04 pA and Popen of 16.9 ± 3.8%, 9.3 ± 1.3%, and 10.5 ± 1.2% for OsALMT7, paab1-t1, and paab1-t2, respectively (Figures 1D, E).

**Paab1 mutant channels inhibit OsALMT7**

Heng et al., 2018 showed that transgenic plants expressing both OsALMT7 and paab1 had a similar phenotype to paab1-1 plants; we propose that this may be the result of paab1 mutant channels inhibiting OsALMT7 channel function. To investigate the impact of paab1 on OsALMT7 channel activity, we coexpressed OsALMT7 and either of the two paab1 transcripts. The TEVC recording showed that co-expression of either of the paab1-t1 and paab1-t2 proteins inhibited the channel activity of OsALMT7. Current magnitudes mediated by OsALMT7 and paab1 co-injection with identical amounts of cRNA fell between those obtained with sole injection of OsALMT7 or paab1 alone (Figures 2A, B). Interestingly, we found that OsALMT7 and paab1 mutant channels showed different time dependence, with OsALMT7 exhibiting instantaneous currents while paab1 currents had time-dependent activation at negative membrane potentials (Figure 2A). What about the current curve of paab1 and OsALMT7 coexpressing situation? As shown in Figures 2A, C, OsALMT7 and paab1 co-injection resulted in currents possessing both instantaneous and time-dependent components, taking on hybrid characteristics of both parent channels.

To confirm that paab1 inhibited OsALMT7, we injected different proportions of cRNA, increasing the cRNA amount of the paab1 mutant to the same amount of OsALMT7 cRNA. As paab1 cRNA was increased, the inhibition of OsALMT7 increased (Figures 2D, E). These TEVC results in X. laevis oocytes are consistent with the inhibition of OsALMT7 by paab1 mutant channels.

In Heng et al, we reported that OsALMT7 had a high permeability to NO3− and malate, and a low permeability to Cl− and SO42−. In this study, we examined the anion permeability following paab1 and OsALMT7 co-injection by substitution of the anions in the bath solution. TEVC showed that paab1
OsALMT7 truncate mutant proteins (paab1-t1 and paab1-t2) showed channel activity in X. laevis oocytes. (A) Schematic of OsALMT7, paab1-t1, and paab1-t2. (B) Current-Voltage relationship from TEVC recordings of whole X. laevis oocytes expressing OsALMT7, paab1-t1, paab1-t2, and water injected control with 46 nM 200 mM Na 2-malate preloaded. The data are derived from the recordings shown in (A) and presented as mean ± SE. Student’s t test was used to analyze statistical significance from water injected control (*P<0.1 and **P<0.01). (C) G/Gmax-Voltage relationship from OsALMT7, paab1-t1, and paab1-t2 expressed oocytes. The data are derived from the recordings shown in (A). (D) Channel activity of representative inside-out membrane patch from OsALMT7, paab1-t1, and paab1-t2 expressed oocytes with 20 mM malate in the bath (equivalent to the cytosol) and 10 mM malate in the pipette (equivalent to the cell exterior) both at pH 7.2, at a holding voltage equivalent to -160 mV in the whole cell configuration. Downward current deflections are indicative of anion efflux from the cell. The numbering, 0 (and blue line) indicates channel closure and 1, 2 indicate number of channels simultaneously open (red lines). (E) The single channel current (left panel) and open probability (right panel) on OsALMT7, paab1-t1, and paab1-t2 expression oocytes. The data are derived from the recordings shown in (D) and additional data and presented as means± SE (n>10 for each data). Student’s t test was used to analyze statistical significance from control conditions (**P<0.01). (F) Histogram analysis of OsALMT7, paab1-t1, and paab1-t2 induced currents from inside-out recording blue and red lines indicate Gaussian fits using Clampfit. This and additional data were used to generate (D).
mutant channels had no effect on anion permeability to OsALMT7; the currents following paab1-OsALMT7 co-injection shared the same anion selectivity as OsALMT7 (Supplemental Figure 1). We also investigated the effect of pH on OsALMT7, paab1-t1, paab1-t2, paab1-t1-OsALMT7, and paab1-t2-OsALMT7 channels, with the external bath pH setting to 7.2, 5.8, and 4.2, representing alkali, neutral, and acidic soil conditions. The TEVC recording showed that all these channels shared no dependence on external pH (Supplemental Figure 2), unlike wheat ALMT1 (Delhaize et al., 2004; Sasaki et al., 2004). In summary, paab1 did not affect the anion selectivity or pH dependence of OsALMT7.

OsALMT7 functions as a multimer

We hypothesized that paab1 mutant channels inhibited OsALMT7 by combining into heteromers. To test our hypothesis, we first examined the physical interactions between OsALMT7 itself, OsALMT7 and paab1 mutant channels and the two paab1 mutant channels. BIFC experimental results were consistent with that OsALMT7 interacting with itself, paab1-t1, and paab1-t2 proteins in tobacco leaves and the two paab1 channels interacting with each other as well (Figure 3). To confirm that paab1 mutant channels and OsALMT7 assemble to form homo- and heteromultimers.

The paab1 mutant terminates transcription in the middle of the 5th transmembrane α-helices, causing a lack of the last 2 transmembrane α-helices and C-terminal cytosolic domains (Figure 1A, Heng et al., 2018). Truncated mutants of OsALMT7 with different numbers of transmembrane α-helices were constructed to examine their contribution to channel activity in the context of the whole protein (Figure 5A). These truncations were named OsALMT7-M1 to OsALMT7-M6 and contained 2 to 7 transmembrane α-helices respectively. Surprisingly, we found that OsALMT7-M2 with just 3 transmembrane α-helices mediated malate efflux, while OsALMT7-M6 with all 7 helices showed no channel activity (Figures 5B, C and Supplemental Figure 3).

The TEVC recordings showed that, in contrast to the wild-type TaALMT1, all the truncations showed no channel activity at pH 4.5 with Al3+ or pH 7.2 in the bath, the truncated mutants TaALMT1-M1 and TaALMT1-M3 inhibited the channel activity of TaALMT1, indicating that TaALMT1 functions as a homomultimer like OsALMT7 (Supplemental Figures 4D, E).

To investigate the broader implications of our observations in other ALMTs, we examined the effect of combinations between TaALMT1 and its truncated mutants. When exposed to either pH 4.5 with Al3+ or pH 7.2 in the bath, the truncated mutants TaALMT1-M1 and TaALMT1-M3 inhibited the channel activity of TaALMT1, indicating that TaALMT1 functions as a homomultimer like OsALMT7 (Supplemental Figures 4D, E).

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According to the studies in OsALMT7 and TaALMT1, we summarized that mediating anion flux with lacking of transmembrane helices was special for OsALMT7, and the dominant deactive proformance of its truncations to wild-type channels was common to the ALMT family as they function as dimer.
Discussion

ALMTs lacking their complete transmembrane α-helices can still retain the ability to transport anions

Ligaba et al. (2013) removed the C-terminal domain of TaALMT1 to various degrees and retained the full complement of transmembrane domains (TaALMT1D219-459) and found amino acid residues in C-terminal important for Al³⁺ sensitivity and the retention of malate transport ability, they also truncated transmembrane domains and found no activity of the protein. Zhang et al. (2013) found that key sites in the transmembrane helices of AtALMT9 affected channel activity. Recently, Li et al. (2020) found that truncating apple ALMT9 at the C-terminus affected channel activity. Our work is the first to show that truncating the transmembrane domains of ALMTs can still result in a transport competent protein and that such truncation results in an in planta phenotype (Heng et al., 2018).

FIGURE 2
paab1-t1 and paab1-t2 inhibits channel activity of OsALMT7 in X. laeviss oocytes. (A) TEVC current recording in X. laeviss oocytes. Whole-cell currents were recorded in oocytes injected with different cRNAs and cRNA combines: OsALMT7, paab1-t1, paab1-t2, OsALMT7+paab1-t1, OsALMT7+paab1-t2, and water as control. Voltage protocols and time and current scale bars for the recordings are shown. (B) 1-V relationship of the currents recordings of oocytes expressing OsALMT7, paab1-t1, paab1-t2, OsALMT7+paab1-t1, OsALMT7+paab1-t2, and water injected control. The data are derived from the current recordings as shown in (A) and presented as mean ± SE. (C) Current of whole oocytes expression OsALMT7, paab1-t1, paab1-t2, OsALMT7+paab1-t1, and OsALMT7+paab1-t2 at -120 mV. (D) 1-V relationship of the currents recordings of oocytes injected with different amount of paab1 mutant cRNA coinjected with OsALMT7. The data are presented as mean ± SE in: • 12 for each datal. (E) Rel. conductance of different amount of paab1 mutant cRNAs injected oocytes. Student’s t-test (**P<0.01) was used to analyze statistical significance.
FIGURE 3
BiFC analysis between OsALMT7 and OsALMT7 (upper pannel, left image), OsALMT7 and paab1-t1 (upper pannel, middle image), OsALMT7 and paab1-t1 (upper pannel, right image), paab1-t1 and paab1-t2 (lower pannel left image), and TaALMT1 and TaALMT1 (lower pannel middle image). YN-TaALMT1 and YC-OsALMT7 was co-expressed as a negative control.

FIGURE 4
Properties of currents recorded in oocytes injected with different cRNAs encoding tandem subunits. (A) Diagram illustrating the construction of the tandems, stop code of the 5' terminal eDNA was removed and a linker encoding [GGGG]h was involved between the two subunits. The ATG of 3' terminal eDNA was arrayed after the linker and the TAA was set at the end of the tandems. (B) TEVC current recording in X. laevis oocytes. Whole-cell currents were recorded in oocytes injected with different tandem cRNAs: paab1-t1-paab1-t1, OsALMT7-OsALMT7, paab1-t1-OsALMT7, and OsALMT7-paab1-t1. Voltage protocols and time and current scale bars for the recordings are shown. (C) 1-V relationship of the currents recordings of oocytes expressing paab1-t1-paab1-t1, OsALMT7-OsALMT7, paab1-t1-OsALMT7, and OsALMT7-paab1-t1. The data are derived from the current recordings as shown in (A) and presented as mean ± SE.
We truncated different transmembrane helices of OsALMT7 and investigated the activity of them. However, only OsALMT7-M2, with 3 transmembrane helices, and paab1 showed channel activity, suggesting that the first 3 transmembrane helices are important for the pore formation on PM for malate permeability, and OsALMT7-M2 and paab1 could form a pore but other truncations with more transmembrane helices could not. Previous studies showed that the last 2 transmembrane helices were essential for pore formation and channel activity (Ligaba et al., 2013; Zhang et al., 2013), yet we found that OsALMT7-M2 and paab1 were functional for transport. These results are novel and will inform ALMT structural studies.

For TaALMT1, we got none channel activity for all the truncations at both pH 4.5 with Al³⁺ or pH 7.2. However, Ligaba et al. (2013) found that TaALMT1-M3 (TaALMT1D219-459) showed channel activity but lost Al sensitivity at pH 4.5, while our study failed to obtain TaALMT1-M3 channel activity for at least 5 times TEVC experiments. We think that this was caused by the different conditions of the X. laevis oocytes in the two labs.

**ALMTs function as multimers**

In this study, we have shown that the PM-localized channels OsALMT7 and TaALMT1 function as multimers by TEVC.
recording in X. laevis oocytes and physical interaction analysis in tobacco leaves. Although structural biology evidence is lacking, in light of the cases of OsALMT7, we propose that it functions as multimers. Zhang et al., 2013 proposed that the vacuolar ALMT channel AtALMT9 functions as a multimer. Similar to our study, they coexpressed point mutations and wild-type AtALMT9 channels in tobacco mesophyll protoplasts, detecting the inhibition of channel activity, and they further showed the multimer formation by immunoblot analysis. Recently, Wang et al., 2022 and Qin et al., 2022 reported that ALMT1 and ALMT12 functions as a dimer. Our study started from the clew in Heng et al. (2018). When wild-type plants were transformed with a genomic fragment containing the paab1 base substitution causing both OsALMT7 and paab1 expressing and resulting in a panicle abortion phenotype. That dominant-negative phenotype implied that paab1 might inhibit OsALMT7 channel activity and the following experiment approved that. Although we cannot predict the OsALMT7 forming a dimer or a trimer, it is credible that the geometric symmetry structure formed with monomer unit is necessary for the ALMT anion channels. Furthermore, we propose that introduction of mutant ALMT channels to wildtype plants would be a method to alter ALMT function as a tool to manipulate plant phenotype.

In the BiFC experiment, we detected interaction fluorescence only when YFP truncations were fused at the N-terminus of OsALMT7 or paab1 (Figure 3) but no fluorescence when YFP truncations were fused at the C-terminus (data not shown). One reason is that unlike TaALMT1, OsALMT7 was predicted to have 7 transmembrane helices, and the C-termini of OsALMT7 and paab1 might face different side of the PM respectively. Another is that Mumm et al. (2013) showed that fusing a YFP at the N-terminus had no effect on channel characteristics and PM localization for ALMT12, while the C-terminus fused YFP affected function and PM localization of ALMT12. So, we proposed that YFP truncations fusing in the C-terminus might inhibit the interaction between OsALMT7 and paab1. These might be the reason that the tandem construction causes the malate conductance reducing comparing to WT OsALMT7 channel (Figure 4). Furthermore, for the case of the tandems of paab1-t1-OsALMT7 and OsALMT7-paab1-t1, which protein was designed at the N-terminal did affect the malate permeability of the tandem. According to the TEVC data, we proposed that the paab1-t1-OsALMT7 provided more complete pore than OsALMT7-paab1-t1. Moreover, the tandem with paab1 in the N-terminus had stronger channel activity than in C-terminus (Figure 4) suggesting that paab1 mutants and OsALMT7 channel need a especial combination to mediate malate transporting.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

HZ and ZH (co-first author) performed the electrophysiology and BiFC experiments. YXL made the vector constructions. YL and CF(corresponding author) designed the project and wrote the
manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1012578/full#supplementary-material
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