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Heterologous expression reveals that GABA does not directly inhibit the vacuolar anion channel *At*ALMT9

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Letter

Dear Editor,

GABA (gamma-aminobutyric acid) is a well-known neurotransmitter activating Cl^- channels in synapses and having an inhibitory effect on neural activity. In plants, GABA was proposed to have different functions, for example, in the regulation of the carbon/nitrogen balance and resistance/tolerance to different biotic and abiotic stresses (Steward, 1949; Bouché et al., 2003; Bouché and Fromm, 2004; Fait et al., 2008; Renault et al., 2010; Fait et al., 2011; Sigel and Steinmann, 2012). Plants unable to synthesize GABA present a modified leaf transpiration suggesting a role of GABA in the regulation of stomatal movements (Mekonnen et al., 2016). More recently, it was proposed that GABA regulates vacuolar ion transport in guard cells directly targeting the anion channel *At*ALMT9 (*Arabidopsis thaliana* aluminum activated malate transporter 9; Xu et al., 2021a). Indeed, Xu and colleagues proposed that GABA might affect stomata aperture regulation via direct inhibition of *At*ALMT9. However, although crucial, straightforward evidence showing a direct effect of GABA on *At*ALMT9 ion transport activity is missing (Xu et al., 2021b). Therefore, we designed patch-clamp experiments to detect possible direct effects of GABA on *At*ALMT9 mediated ionic currents. In the following *Letter*, we demonstrate that, in our experimental system, GABA has no direct influence on *At*ALMT9 ion transport activity across the vacuolar membrane.

*At*ALMT9 is a vacuolar anion channel mediating Cl^- influx into the vacuole during stomatal opening (De Angeli et al., 2013). Notably, GABA inhibits *Ta*ALMT1 ionic currents in *Xenopus* oocytes from the cytosolic side (Long et al., 2020). Based on these findings, we decided to test whether the application of GABA from the cytosolic side ($GABA_{cyt}$) impacts *At*ALMT9-mediated Cl^- currents. Since *AtALMT9-GFP* complements *almt9* knock-out phenotypes (Xu et al., 2021a), indicating that GFP tagging does not modify ion channel properties, we transiently expressed *At*ALMT9-GFP into *Nicotiana benthamiana* for electrophysiological experiments. We performed patch-clamp experiments in cytosolic-side-out configuration from isolated vacuoles (De Angeli et al., 2013; Zhang et al., 2013) as this experimental design allows us to directly access the cytosolic face of the vacuolar membrane and to apply different cytosolic conditions to the same patch, that is to the same population of ion channels (Zhang et al., 2014). In planta GABA concentrations are in the range of 60 μM–1 mM (Renault et al., 2011; Xu et al., 2021a). Therefore, we spanned a concentration range applying 100 μM, 2 mM, and 10 mM GABA at the cytosolic side of *At*ALMT9 (Figure 1A). We measured *At*ALMT9-mediated Cl^- currents in presence of 100 mM Cl^- in the cytosol (black triangles; Figure 1B), and subsequently upon addition of different $GABA_{cyt}$ concentrations (Figure 1, A and B; open triangles). We could observe typical *At*ALMT9-mediated inward currents, with no rundown over 20 min (Figure 1D). However, we could not measure any significant effect after the application of the three different concentrations of $GABA_{cyt}$ on *At*ALMT9-mediated Cl^- currents (Figure 1A).

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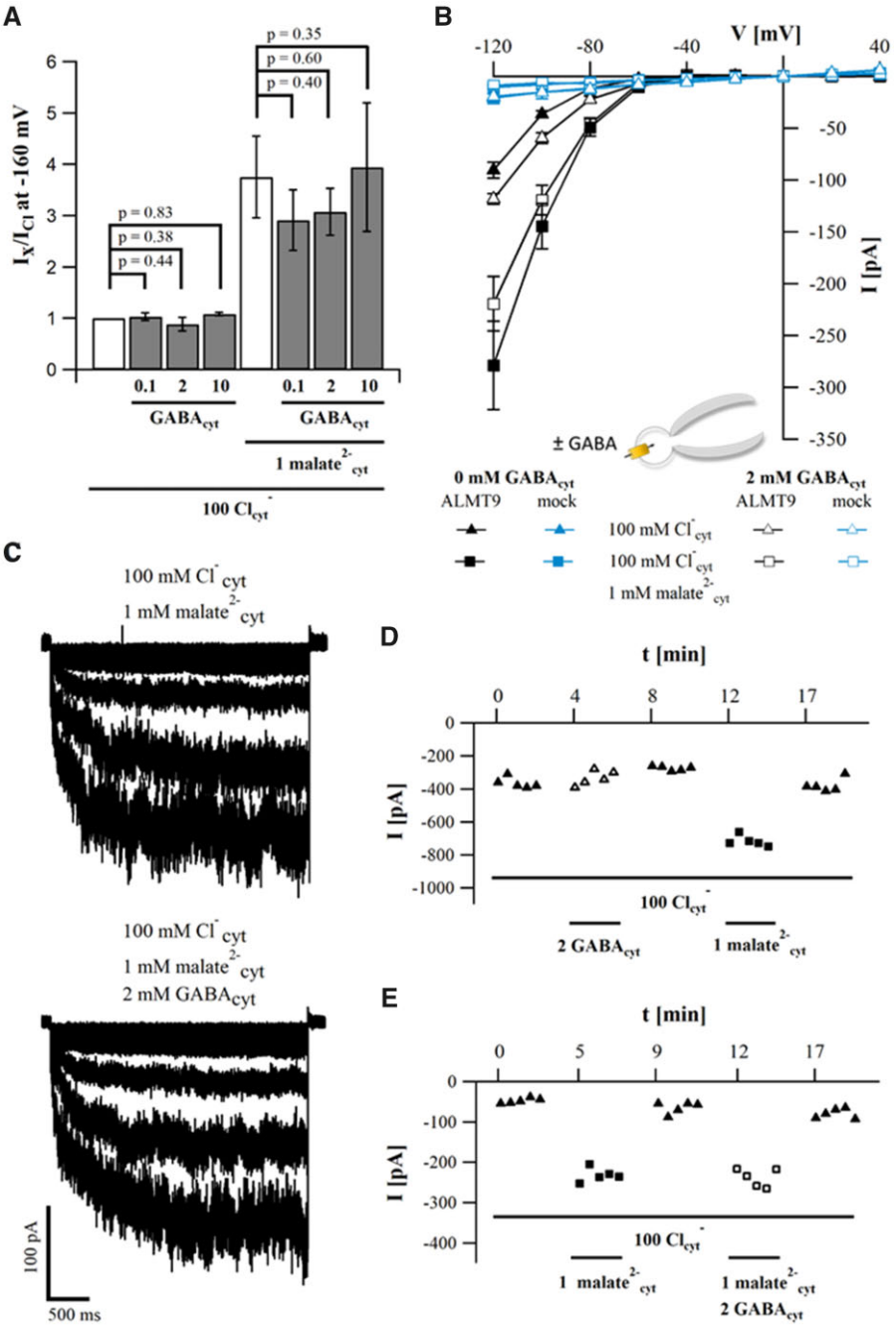

Figure 1 Cytosolic GABA does not directly inhibit AtALMT9-mediated Cl^- currents. A, AtALMT9-mediated chloride currents without and after activation by 1 mM malate at the cytosolic side in presence or absence of GABA (currents are normalized to their initial 100 mM cytosolic Cl^-). Data represent means ± SE (n = 3–5). No statistical differences were found between presence and absence of GABA (paired or un-paired two-sample Student's t test). B, Mean current–voltage relationship from vacuolar patches expressing AtALMT9 (black symbols and lines) or mock controls (blue symbols and lines) exposed to different cytosolic conditions. Symbols represent means ± SE (n = 3–10). C, Representative current traces recorded in cytosolic-side-out excised patch from *N. benthamiana* mesophyll vacuole expressing AtALMT9 under different cytosolic conditions. D and E, Time courses of representative currents upon perfusion with different cytosolic conditions. Each graph displays a representative patch-clamp experiment. Cytosolic side solutions were sequentially exchanged by a perfusion system. Data presented in A–E were recorded from patches of *N. benthamiana* mesophyll vacuoles from transiently transformed leaves as previously described (De Angeli et al., 2013). For consistency with study performed by Xu et al. (2021a), AtALMT9-GFP fusion was used. Currents were elicited from a holding potential of 0 mV with 3 s pulses from + 40 to −120 mV with −20 mV steps (Figure 1, B and C) or from 0 to −160 mV (Figure 1, A, D, and E). Recordings were performed in cytosolic-side-out configuration using as cytosolic side solution: 100 mM BTP-Cl^-, 0.1 mM $CaCl_2$, pH = 7.5 adjusted with BTP, and π = 500 mOsm adjusted with D-sorbitol with or without 1 mM $malate^{2-}_{cyt}$ (as indicated). Pipette solution (i.e. vacuolar side) contained 11.2 mM $malate^{2-}$, 100 mM HCl, pH = 6 adjusted with BTP, and π = 550 mOsm adjusted with D-sorbitol. To study GABA effect, 100 µM, 2 mM, or 10 mM GABA was added to the cytosolic side ($GABA_{cyt}$) solution or 2 mM GABA was added to the pipette solution ($GABA_{lum}$; Figure 2).

Notably, even a concentration as high as 10 mM $GABA_{cyt}$ was unable to inhibit *At*ALMT9 ion transport activity (Figure 1A).

Cytosolic malate is a known activator of *At*ALMT9 (De Angeli et al., 2013), thus we tested whether application of $GABA_{cyt}$ modifies the activation of *At*ALMT9-mediated Cl^- currents by 1 mM $malate^{2-}_{cyt}$. Therefore, we quantified the activation by 1 mM $malate^{2-}_{cyt}$ of *At*ALMT9 in the presence and in the absence of 2 mM $GABA_{cyt}$ sequentially applied to the same patch (Figure 1, A and B; black squares and C). Additionally, we tested the effect of 100 μM or 10 mM $GABA_{cyt}$ on the activation by 1 mM $malate^{2-}_{cyt}$. Also under these conditions we did not observe any reduction of the ionic currents over time (i.e. no rundown; Figure 1E). Importantly, our data show that the application of $GABA_{cyt}$ does not influence the activation of *At*ALMT9-mediated ionic currents by $malate^{2-}_{cyt}$ (Figure 1, A and B). Since the regulation of the vacuolar ion channels can also occur from the vacuolar lumen side, in a last set of experiments, we tested this possibility for GABA. To check whether $GABA_{lum}$ modifies *At*ALMT9-mediated Cl^- currents and also $malate^{2-}_{cyt}$ activation of *At*ALMT9, we used a vacuolar side buffer (i.e. pipette solution) containing, in addition to all the components used in the previous experiments, 2 mM GABA (full description in the legend of the Figure 1). We could not detect any effect of $GABA_{lum}$ (Figure 2A).

Overall, the results we have obtained answer the question whether GABA directly regulates *At*ALMT9 ion transport activity (Xu et al., 2021b). Indeed, the present findings show that GABA, applied from either the cytosolic or the vacuolar side, has no effect on *At*ALMT9-mediated Cl^- currents. Consequently, GABA does not have a direct influence on the ion transport activity of *At*ALMT9 across the vacuolar membrane. Interestingly, by analogy with *Ta*ALMT1, it was proposed that *At*ALMT9 harbors a putative "GABA binding motif" (Ramesh et al., 2015; Xu et al., 2021a). However, the lack of a direct effect of GABA on *At*ALMT9 ionic currents shows that direct inhibition by GABA is not a feature of all ALMT's. Notably, *At*ALMT9 and *At*ALMT1 belong to different clades of the ALMT family (Kovermann et al., 2007), therefore the different behavior possibly reflects structural differences between the two ion channels.

Overall, evidence suggests that GABA influences stomata aperture (Mekonnen et al., 2016; Xu et al., 2021a) but our data suggest that this does not occur through a direct inhibition of *At*ALMT9. In this context, different scenarios are possible. Indeed, we can speculate that in vivo GABA could indirectly modify *At*ALMT9 transport activity via a so far unknown regulatory factor (e.g. a GABA activated protein, Figure 2B). Alternatively, GABA could regulate other vacuolar ion channels and transporters residing in the vacuolar membrane (Figure 2B). Finally, GABA metabolism modifies organic acid homeostasis in Arabidopsis (*A. thaliana*) leaves (Mekonnen et al., 2016) and therefore we cannot exclude that this indirectly modifies guard cell responses. In summary, our data show that more research is needed to

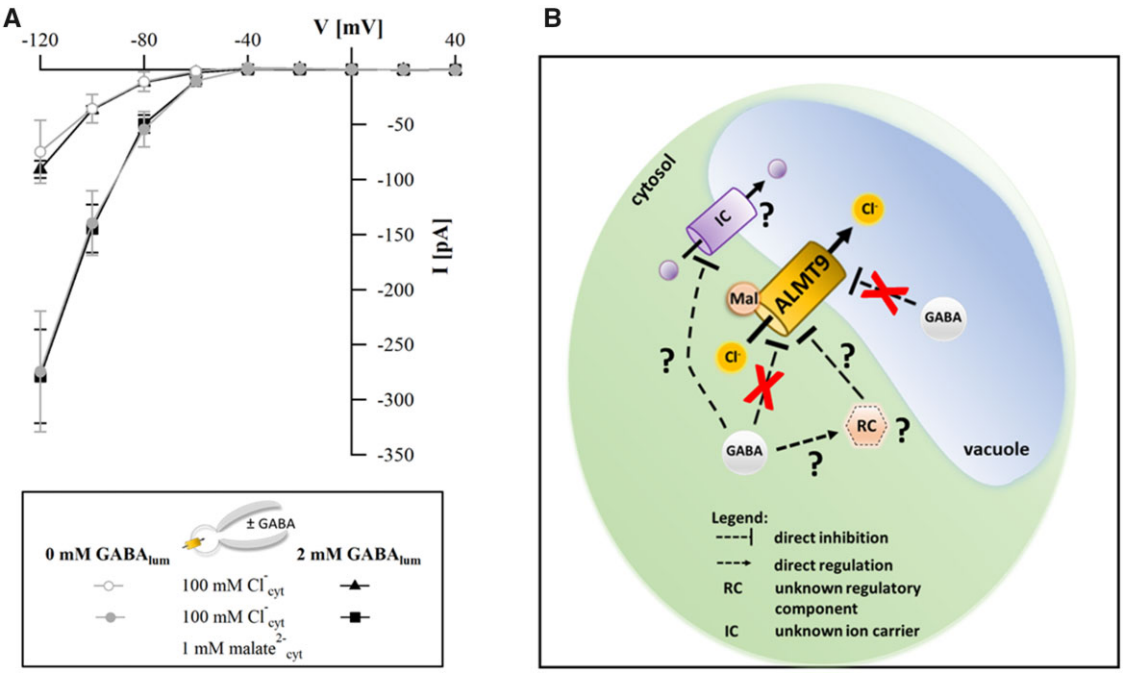

Figure 2 Vacuolar GABA does not directly influence AtALMT9 currents. A, Mean current–voltage relationship obtained from vacuolar patches expressing AtALMT9 in absence (gray symbols) or presence (black symbols) of the GABA in the vacuole ($GABA_{lum}$) under different cytosolic conditions. Solutions and applied voltage protocols are described in Figure 1. Symbols represents means ± SE (n = 3–10). B, Graphical summary showing that GABA has no direct impact on the transport capacities of AtALMT9. Indirect effects of GABA on vacuolar ion transport mediated by AtALMT9 might involve currently unknown regulatory factor (RC). Direct effect of GABA could be mediated by regulation of other vacuolar ion carriers (channel or transporter, IC).

identify the molecular mechanism mediating the effects of GABA in vivo.

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Conflict of interest statement. None declared.

References

Bouché N, Fait A, Bouchez D, Moller SG, Fromm H (2003) Mitochondrial succinic-semialdehyde dehydrogenase of the -aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. Proc Natl Acad Sci USA **100**: 6843–6848

Bouché N, Fromm H (2004) GABA in plants: just a metabolite? Trends Plant Sci **9**: 110–115

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

Fait A, Fromm H, Walter D, Galili G, Fernie AR (2008) Highway or byway: the metabolic role of the GABA shunt in plants. Trends Plant Sci **13**: 14–19

Fait A, Nesi AN, Angelovici R, Lehmann M, Pham PA, Song L, Haslam RP, Napier JA, Galili G, Fernie AR (2011) Targeted enhancement of glutamate-to-γ-aminobutyrate conversion in Arabidopsis seeds affects carbon–nitrogen balance and storage reserves in a development-dependent manner. Plant Physiol **157**: 1026–1042

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

Long Y, Tyerman SD, Gilliham M (2020) Cytosolic GABA inhibits anion transport by wheat ALMT1. New Phytol **225**: 671–678

Mekonnen DW, Flügge U-I, Ludewig F (2016) Gamma-aminobutyric acid depletion affects stomata closure and drought tolerance of *Arabidopsis thaliana*. Plant Sci **245**: 25–34

Ramesh SA, Tyerman SD, Xu B, Bose J, Kaur S, Conn V, Domingos P, Ullah S, Wege S, Shabala S, et al. (2015) GABA signalling modulates plant growth by directly regulating the activity of plant-specific anion transporters. Nat Commun **6**: 7879

Renault H, El Amrani A, Palanivelu R, Updegraff EP, Yu A, Renou J-P, Preuss D, Bouchereau A, Deleu C (2011) GABA accumulation causes cell elongation defects and a decrease in expression of genes encoding secreted and cell wall-related proteins in *Arabidopsis thaliana*. Plant Cell Physiol **52**: 894–908

Renault H, Roussel V, El Amrani A, Arzel M, Renault D, Bouchereau A, Deleu C (2010) The Arabidopsis pop2-1 mutant reveals the involvement of GABA transaminase in salt stress tolerance. BMC Plant Biol **10**: 20

Sigel E, Steinmann ME (2012) Structure, function, and modulation of GABAA receptors. J Biol Chem **287**: 40224–40231

Steward FC (1949) γ-Aminobutyric acid: a constituent of the potato tuber? Science **110**: 439–440

Xu B, Long Y, Feng X, Zhu X, Sai N, Chirkova L, Betts A, Herrmann J, Edwards EJ, Okamoto M, et al. (2021a) GABA signalling modulates stomatal opening to enhance plant water use efficiency and drought resilience. Nat Commun **12**: 1952

Xu B, Sai N, Gilliham M (2021b) The emerging role of GABA as a transport regulator and physiological signal. Plant Physiol. kiab347

Zhang J, Baetz U, Krügel U, Martinoia E, De Angeli A (2013) Identification of a probable pore-forming domain in the multimeric vacuolar anion channel AtALMT9. Plant Physiol **163**: 830–843

Zhang J, Martinoia E, De Angeli A (2014) Cytosolic nucleotides block and regulate the Arabidopsis vacuolar anion channel AtALMT9. J Biol Chem **289**: 25581–25589