Heterologous expression reveals that GABA does not directly inhibit the vacuolar anion channel AtALMT9

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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 AtALMT9 (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 AtALMT9. However, although crucial, straightforward evidence showing a direct effect of GABA on AtALMT9 ion transport activity is missing (Xu et al., 2021b). Therefore, we designed patch-clamp experiments to detect possible direct effects of GABA on AtALMT9 mediated ionic currents. In the following Letter, we demonstrate that, in our experimental system, GABA has no direct influence on AtALMT9 ion transport activity across the vacuolar membrane.

AtALMT9 is a vacuolar anion channel mediating Cl⁻ influx into the vacuole during stomatal opening (De Angeli et al., 2013). Notably, GABA inhibits TaALMT1 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 AtALMT9-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 AtALMT9-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$_{cyt}$ on AtALMT9 (Figure 1A). We measured AtALMT9-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 1A, A and B; open triangles). We could observe typical AtALMT9-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 AtALMT9-mediated Cl⁻ currents (Figure 1A).
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 p = 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 p = 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 GABAcyt was unable to inhibit AtALMT9 ion transport activity (Figure 1A).

Cytosolic malate is a known activator of AtALMT9 (De Angeli et al., 2013), thus we tested whether application of GABAcyt modifies the activation of AtALMT9-mediated Cl− currents by 1 mM malate2−cyt. Therefore, we quantified the activation by 1 mM malate2−cyt of AtALMT9 in the presence and in the absence of 2 mM GABAcyt sequentially applied to the same patch (Figure 1A and B; black squares and C). Additionally, we tested the effect of 100 μM or 10 mM GABAcyt on the activation by 1 mM malate2−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 GABAcyt does not influence the activation of AtALMT9-mediated ionic currents by malate2−cyt (Figure 1A 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 AtALMT9-mediated Cl− currents and also malate2−cyt activation of AtALMT9, 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 AtALMT9 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 AtALMT9-mediated Cl− currents. Consequently, GABA does not have a direct influence on the ion transport activity of AtALMT9 across the vacuolar membrane. Interestingly, by analogy with TaALMT1, it was proposed that AtALMT9 harbors a putative "GABA binding motif" (Ramesh et al., 2015; Xu et al., 2021a). However, the lack of a direct effect of GABA on AtALMT9 ionic currents shows that direct inhibition by GABA is not a feature of all ALMT’s. Notably, AtALMT9 and AtALMT1 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 AtALMT9. In this context, different scenarios are possible. Indeed, we can speculate that in vivo GABA could indirectly modify AtALMT9 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.

Acknowledgments

We acknowledge the Heterologous Expression and Plant Electrophysiology (EHEV) platform from BPMP lab in Montpellier for their advices and assistance.

Funding

A.D.A. and J.J. were funded by CNRS ATIP-AVENIR grant 2018 and ANR PARASOL (2021-2024).

Conflict of interest statement. None declared.

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