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1 Heterologous expression reveals that GABA does not directly inhibit the 2 vacuolar anion channel *AtALMT9*

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5 One-Sentence Summary:

6 GABA, a molecule involved in the regulation of the stomata aperture and drought tolerance,
7 does not modify vacuolar anion fluxes mediated by the anion channel *AtALMT9*.

8
9
10 Dear Editor,

11 GABA (gamma-aminobutyric acid) is a well-known neurotransmitter activating Cl⁻ channels
12 in synapses and having an inhibitory effect on neural activity. In plants, GABA was proposed
13 to have different functions, for example, in the regulation of the carbon/nitrogen balance and
14 resistance/tolerance to different biotic and abiotic stresses (Steward, 1949; Bouché et al.,
15 2003; Bouché and Fromm, 2004; Fait et al., 2008; Renault et al., 2010; Fait et al., 2011; Sigel
16 and Steinmann, 2012). Plants unable to synthesize GABA present a modified leaf
17 transpiration suggesting a role of GABA in the regulation of stomatal movements (Mekonnen
18 et al., 2016). More recently, it was proposed that GABA regulates vacuolar ion transport in
19 guard cells directly targeting the anion channel *AtALMT9* (*Arabidopsis thaliana* Aluminum
20 Activated Malate Transporter 9; Xu et al., 2021a). Indeed, Xu and colleagues proposed that
21 GABA might affect stomata aperture regulation via direct inhibition of *AtALMT9*. However,
22 although crucial, straightforward evidence showing a direct effect of GABA on *AtALMT9* ion
23 transport activity is missing (Xu et al., 2021b). Therefore, we designed patch-clamp
24 experiments to detect possible direct effects of GABA on *AtALMT9* mediated ionic currents.
25 In the following *Letter*, we demonstrate that, in our experimental system, GABA has no direct
26 influence on *AtALMT9* ion transport activity across the vacuolar membrane.

27 *AtALMT9* is a vacuolar anion channel mediating Cl⁻ influx into the vacuole during stomatal
28 opening (De Angeli et al., 2013). Notably, GABA inhibits *TaALMT1* ionic currents in
29 *Xenopus* oocytes from the cytosolic side (Long et al., 2020). Based on these findings, we
30 decided to test whether the application of GABA from the cytosolic side (GABA_{cyt}) impacts

31 *AtALMT9* mediated Cl^- currents. Since *AtALMT9-GFP* complements *almt9* knock-out
32 phenotypes (Xu et al., 2021a), indicating that GFP tagging does not modify ion channel
33 properties, we transiently expressed *AtALMT9-GFP* into *Nicotiana benthamiana* for
34 electrophysiological experiments. We performed patch-clamp experiments in cytosolic-side-
35 out configuration from isolated vacuoles (De Angeli et al., 2013; Zhang et al., 2013) as this
36 experimental design allows us to directly access the cytosolic face of the vacuolar membrane
37 and to apply different cytosolic conditions to the same patch, *i.e.* to the same population of ion
38 channels (Zhang et al., 2014). In planta GABA concentrations are in the range of $60\ \mu\text{M} - 1$
39 mM (Renault et al., 2011; Xu et al., 2021a). Therefore, we spanned a concentration range
40 applying $100\ \mu\text{M}$, $2\ \text{mM}$ and $10\ \text{mM}$ GABA at the cytosolic side of *AtALMT9* (Fig 1A). We
41 measured *AtALMT9* mediated Cl^- currents in presence of $100\ \text{mM}$ Cl^- in the cytosol (black
42 triangles; Figure 1B), and subsequently upon addition of different GABA_{cyt} concentrations
43 (Figure 1A and B; open triangles). We could observe typical *AtALMT9* mediated inward
44 currents, with no rundown over 20 minutes (Figure 1D). However, we could not measure any
45 significant effect after the application of the three different concentrations of GABA_{cyt} on
46 *AtALMT9* mediated Cl^- currents (Figure 1A). Notably, even a concentration as high as 10
47 mM GABA_{cyt} was unable to inhibit *AtALMT9* ion transport activity (Figure 1A).

48 Cytosolic malate is a known activator of *AtALMT9* (De Angeli et al., 2013), thus we tested
49 whether application of GABA_{cyt} modifies the activation of *AtALMT9* mediated Cl^- currents
50 by $1\ \text{mM}$ malate $^{2-}_{\text{cyt}}$. Therefore, we quantified the activation by $1\ \text{mM}$ malate $^{2-}_{\text{cyt}}$ of *AtALMT9*
51 in the presence and in the absence of $2\ \text{mM}$ GABA_{cyt} sequentially applied to the same patch
52 (Figure 1A, B; black squares and C). Additionally, we tested the effect of $100\ \mu\text{M}$ or $10\ \text{mM}$
53 GABA_{cyt} on the activation by $1\ \text{mM}$ malate $^{2-}_{\text{cyt}}$. Also under these conditions we did not
54 observe any reduction of the ionic currents over time (*i.e.* no rundown; Figure 1E).
55 Importantly, our data show that the application of GABA_{cyt} does not influence the activation
56 of *AtALMT9* mediated ionic currents by malate $^{2-}_{\text{cyt}}$ (Figure 1A and B). Since the regulation of
57 the vacuolar ion channels can also occur from the vacuolar lumen side, in a last set of
58 experiments, we tested this possibility for GABA. To check whether GABA_{lum} modifies
59 *AtALMT9* mediated Cl^- currents and also malate $^{2-}_{\text{cyt}}$ activation of *AtALMT9* we used a
60 vacuolar side buffer (*i.e.* pipette solution) containing, in addition to all the components used in
61 the previous experiments, $2\ \text{mM}$ GABA (full description in the legend of the Figure 1). We
62 could not detect any effect of GABA_{lum} (Figure 2A).

63 Overall, the results we have obtained answer the question whether GABA directly regulates
64 *AtALMT9* ion transport activity (Xu et al., 2021b). Indeed, the present findings show that
65 GABA, applied from either the cytosolic or the vacuolar side, has no effect on *AtALMT9*
66 mediated Cl^- currents. Consequently, GABA does not have a direct influence on the ion
67 transport activity of *AtALMT9* across the vacuolar membrane. Interestingly, by analogy with
68 *TaALMT1*, it was proposed that *AtALMT9* harbors a putative “GABA binding motif”
69 (Ramesh et al., 2015; Xu et al., 2021a). However, the lack of a direct effect of GABA on
70 *AtALMT9* ionic currents shows that direct inhibition by GABA is not a feature of all
71 ALMT’s. Notably, *AtALMT9* and *AtALMT1* belong to different clades of the ALMT family
72 (Kovermann et al., 2007), therefore the different behavior possibly reflects structural
73 differences between the two ion channels.

74 Overall, evidence suggests that GABA influences stomata aperture (Mekonnen et al., 2016;
75 Xu et al., 2021a) but our data suggest that this does not occur through a direct inhibition of
76 *AtALMT9*. In this context different scenarios are possible. Indeed, we can speculate that *in*
77 *vivo* GABA could indirectly modify *AtALMT9* transport activity via a so far unknown
78 regulatory factor (e.g. a GABA activated protein, Figure 2B). Alternatively, GABA could
79 regulate other vacuolar ion channels and transporters residing in the vacuolar membrane
80 (Figure 2B). Finally, GABA metabolism modifies organic acid homeostasis in *Arabidopsis*
81 (*Arabidopsis thaliana*) leaves (Mekonnen et al., 2016) and therefore we cannot exclude that
82 this indirectly modifies guard cell responses. In summary, our data show that more research is
83 needed to identify the molecular mechanism mediating the effects of GABA *in vivo*.

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98 The author responsible for distribution of materials integral to the findings presented in this
99 article in accordance with the policy described in the Instructions for Authors
100 (<https://academic.oup.com/plphys/pages/General-Instructions>) is Alexis De Angeli.

101

102 Author contributions:

103 J.J. performed patch-clamp experiments; J.J. and A.DA. designed research; J.J. and A.DA.
104 wrote the manuscript.

105

106 **Figure Legends**

107 **Figure 1. Cytosolic GABA does not directly inhibit AtALMT9-mediated Cl⁻ currents.**

108 (A) AtALMT9 mediated chloride currents without and after activation by 1mM malate at the cytosolic
109 side in presence or absence of GABA (currents are normalized to their initial 100mM cytosolic Cl⁻).
110 Data represent means ± SE (n = 3-5). No statistical differences were found between presence and
111 absence of GABA (paired or un-paired two-sample Student's *t*-test). (B) Mean current-voltage
112 relationship from vacuolar patches expressing AtALMT9 (black symbols and lines) or mock controls
113 (blue symbols and lines) exposed to different cytosolic conditions. Symbols represent means ± SE (n =
114 3-10). (C) Representative current traces recorded in cytosolic-side-out excised patch from *N.*
115 *benthamiana* mesophyll vacuole expressing AtALMT9 under different cytosolic conditions. (D) and
116 (E) Time courses of representative currents upon perfusion with different cytosolic conditions. Each
117 graph displays a representative patch-clamp experiment. Cytosolic side solutions were sequentially
118 exchanged by a perfusion system. Data presented in A, B, C, D and E were recorded from patches of
119 *N. benthamiana* mesophyll vacuoles from transiently transformed leaves as previously described (De
120 Angeli et al. 2013). For consistency with study performed by Xu et al.(2021a) AtALMT9-GFP fusion
121 was used. Currents were elicited from a holding potential of 0 mV with 3s pulses from +40 mV to -
122 120 mV with -20 mV steps (Figure 1B and C) or from 0 mV to -160 mV (Figure 1A, D and E).
123 Recordings were performed in cytosolic-side-out configuration using as cytosolic side solution: 100
124 mM BTP-Cl⁻, 0,1 mM CaCl₂, pH = 7,5 adjusted with BTP and π = 500 mOsm adjusted with D-sorbitol
125 with or without 1 mM malate²⁻_{cyt} (as indicated). Pipette solution (i.e. vacuolar side) contained 11,2 mM

126 malate²⁻, 100 mM HCl, pH = 6 adjusted with BTP and π = 550 mOsm adjusted with D-sorbitol. To
127 study GABA effect 100 μ M, 2 mM or 10 mM GABA was added to the cytosolic side (GABA_{cyt})
128 solution or 2 mM GABA was added to the pipette solution (GABA_{lum}; Figure 2).

129

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131

132 **Figure 2. Vacuolar GABA does not directly influence AtALMT9 currents.**

133 (A) Mean current-voltage relationship obtained from vacuolar patches expressing AtALMT9 in
134 absence (grey symbols) or presence (black symbols) of the GABA in the vacuole (GABA_{lum}) under
135 different cytosolic conditions. Solutions and applied voltage protocols are described in Figure 1.
136 Symbols represents means \pm SE (n = 3-10). (B) Graphical summary showing that GABA has no direct
137 impact on the transport capacities of AtALMT9. Indirect effects of GABA on vacuolar ion transport
138 mediated by AtALMT9 might involve currently unknown regulatory factor (RC). Direct effect of
139 GABA could be mediated by regulation of other vacuolar ion carriers (channel or transporter, IC).

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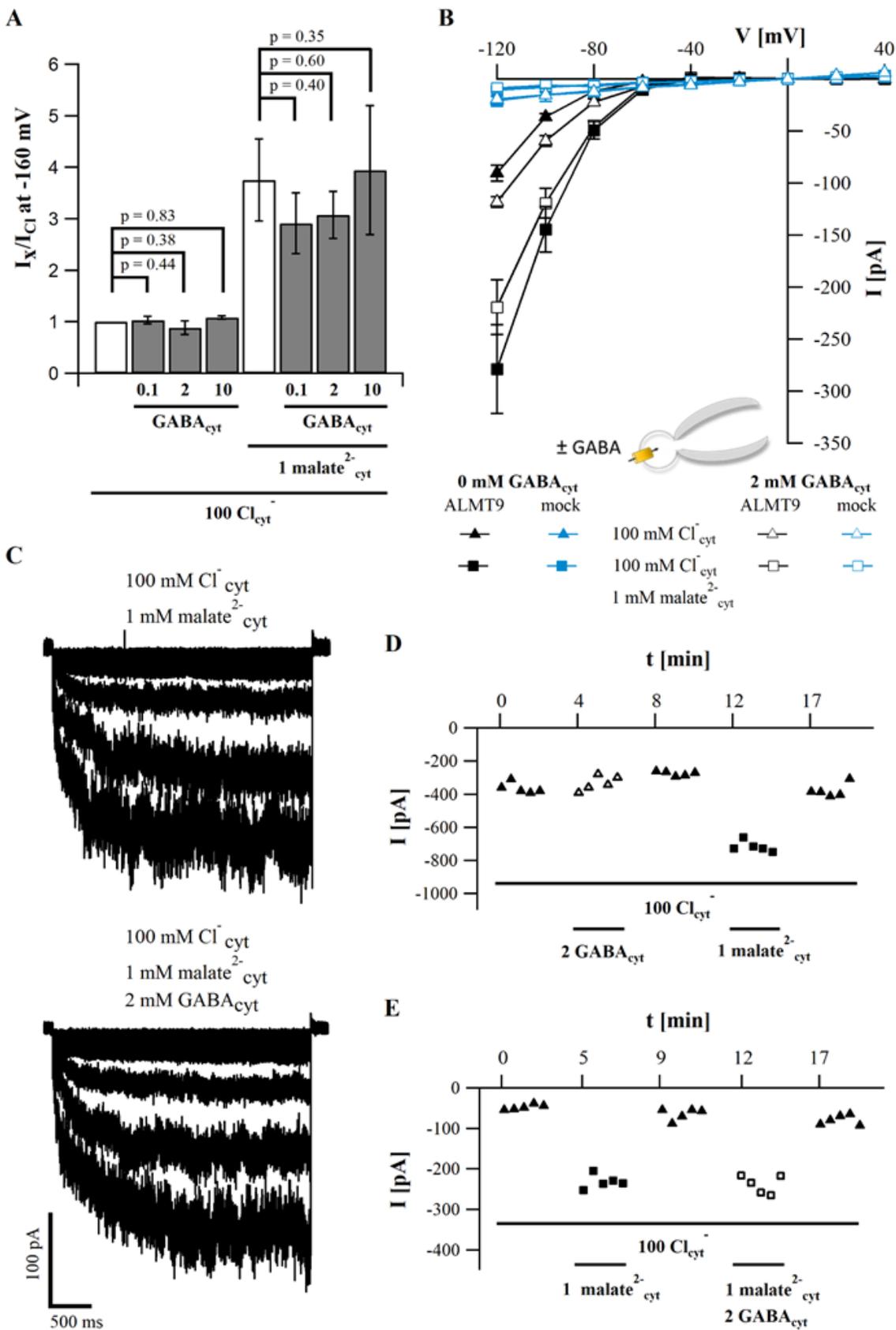
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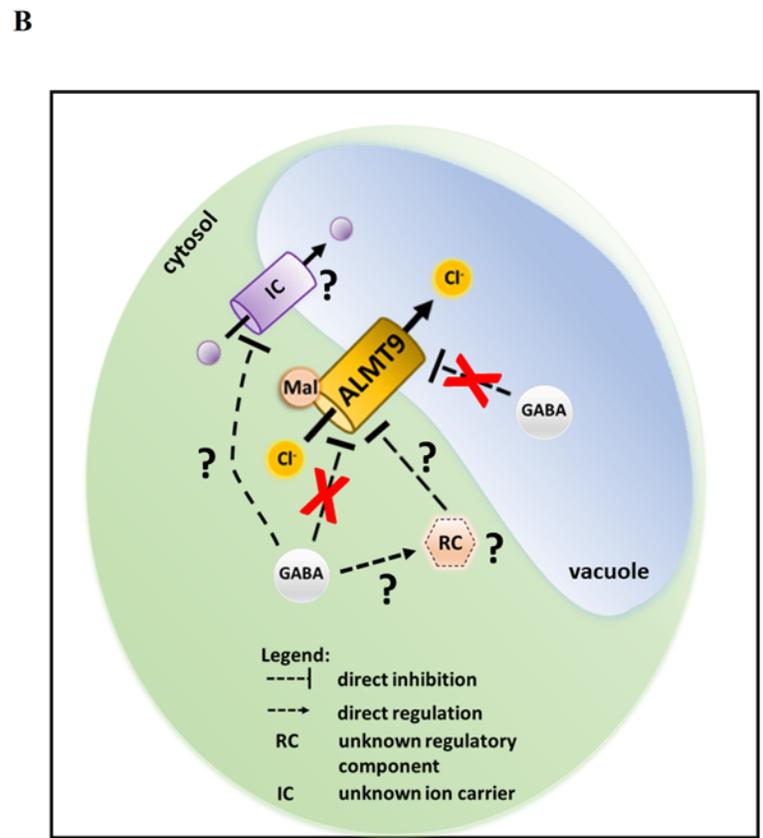
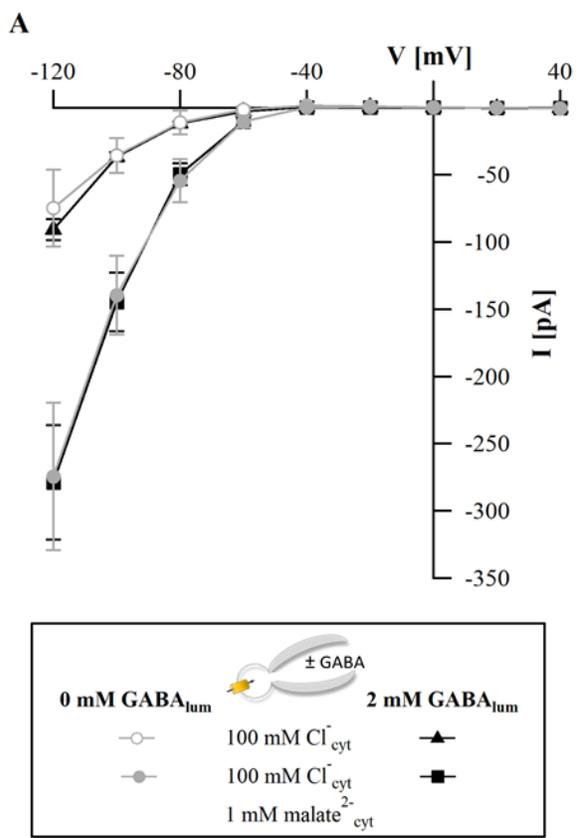
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