Aluminum-Activated Malate Transporters Can Facilitate GABA Transport\[OPEN\]

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Plant aluminum-activated malate transporters (ALMTs) are currently classified as anion channels; they are also known to be regulated by diverse signals, leading to a range of physiological responses. Gamma-aminobutyric acid (GABA) regulation of anion flux through ALMT proteins requires a specific amino acid motif in ALMTs that shares similarity with a GABA binding site in mammalian GABA\(_A\) receptors. Here, we explore why TaALMT1 activation leads to a negative correlation between malate efflux and endogenous GABA concentrations ([GABA]) in both wheat (Triticum aestivum) root tips and in heterologous expression systems. We show that TaALMT1 activation reduces [GABA], because TaALMT1 facilitates GABA efflux but GABA does not complex Al\(^{3+}\). TaALMT1 also leads to GABA transport into cells, demonstrated by a yeast complementation assay and via \(^{14}\)C-GABA uptake into Xenopus laevis oocytes; this was found to be a general feature of all ALMTs we examined. Mutation of the GABA motif (TaALMT1\(^{F213C}\)) prevented both GABA influx and efflux, and resulted in no correlation between malate efflux and [GABA]. We conclude that ALMTs are likely to act as both GABA and anion transporters in planta. GABA and malate appear to interact with ALMTs in a complex manner to regulate each other's transport, suggestive of a role for ALMTs in communicating metabolic status.

INTRODUCTION

Gamma-aminobutyric acid (GABA) is a four-carbon nonproteinogenic amino acid that was first discovered in potato tubers (Steward et al., 1949) but has mainly been studied in mammals as an inhibitory neurotransmitter (Sigel and Steinmann, 2012). When plants encounter stress—whether abiotic (e.g., hypoxia, heat, cold, salt, drought) or biotic (e.g., herbivory, pathogen infection)—they rapidly accumulate GABA (Shelp et al., 2012). This accumulation has been shown to play an important role in the regulation of C:N balance (Fait et al., 2008, 2011), cytosolic pH (Carroll et al., 1994; Shelp et al., 1999), salt tolerance (Renault et al., 2010), and oxidative stress tolerance (Bouché et al., 2003b; Bouché and Fromm, 2004). GABA has also been proposed to be an endogenous plant signaling molecule (Kinnersley and Turano, 2000; Palanivelu et al., 2003; Roberts, 2007). More recently, it was shown that GABA at low micromolar concentrations regulates anion currents through aluminum-activated malate transporter (ALMT) proteins from various species. While the wheat (Triticum aestivum) TaALMT1 can be activated by aluminum (Al\(^{3+}\)), this is not a general feature of ALMTs, despite their name. Some ALMTs can facilitate anion efflux when activated by external anions such as sulfate (SO\(_4^{2-}\)) or malate\(^{2-}\) in alkaline solutions (Ramesh et al., 2015). Such activation by an ion from the same side as the direction of current is referred to in the literature as transactivation. A putative GABA binding motif was discovered in ALMTs with homology to the one found in mammalian GABA\(_A\) receptors, and treatment with micromolar concentrations of muscimol (an analog of GABA) resulted in inhibition of anion flux (Ramesh et al., 2015). Addition of bicuculline (a GABA receptor antagonist) attenuated the effect of both muscimol and GABA (Ramesh et al., 2015). These results suggest that there are distinct classes of anion channels in plant and animal cells that have comparable modes of GABA regulation (Żárský, 2015; Gilliham and Tyerman, 2016; Ramesh et al., 2017).

It is well established that in acidic soils TaALMT1 confers Al\(^{3+}\) tolerance in wheat through exuding malate from the root tips and chelating toxic Al\(^{3+}\) (Delhaize and Ryan, 1995; Ma et al., 2001; Sasaki et al., 2004). Exogenous application of GABA or muscimol to the roots of wheat seedlings with high TaALMT1 expression inhibited malate efflux and impaired root growth in the presence of Al\(^{3+}\), which phenocopied a near isogenic line with less exudation (Zárský, 2015; Gilliham and Tyerman, 2016; Ramesh et al., 2015). This reciprocal relationship remained unexplained and may indicate either TaALMT1 activation caused...
changes in [GABA], or that [GABA], is altered in some way that then regulates TaALMT1.

Identification of a putative GABA binding motif in ALMTs provided a possible mechanism by which plant GABA may act as a signal (Ramesh et al., 2015; Žárský, 2015), but we are yet to fully understand the molecular and physiological basis of how this occurs and the relationship between anion flux and GABA regulation in plant cells. A number of pharmacological agents have been used to characterize animal GABA receptors, some of which are plant or fungal derived, either as agonists, i.e., muscimol, or as regulators of GABA synthesis or catabolism i.e., amino-oxyacetate (AOA) and vigabatrin, respectively (Wood and Peesker, 1973; Jackson et al., 1982; Grant and Heel, 1991). Using these inhibitors to manipulate [GABA] in plant cells may increase our understanding of how ALMT-mediated anion efflux is regulated.

It was previously hypothesized that ALMT might sense and signal metabolic status via regulation by GABA and malate, which alters membrane voltage and transduces the signal into a physiological response (Gilliham and Tyerman, 2016). Cellular efflux of GABA has been well documented (Bown and Shelp, 1989; Chung et al., 1992). Micromolar concentrations of GABA are found in root exudates and the apoplast, and among all amino acids exuded from wheat roots, GABA shows the highest efflux (Warren, 2015). It was envisaged that such carbon and nitrogen loss might only be justified energetically if GABA was involved in signaling (Gilliham and Tyerman, 2016). While a high affinity GABA influx transporter (AtGAT1) has been characterized and is expressed in Arabidopsis thaliana roots (Meyer et al., 2006), no transporter that can efflux GABA from the cytoplasm into the apoplast has been identified. This raises an interesting question as to how GABA exits the cytoplasm and enters the apoplast.

In this study, we used two GABA analogs to manipulate [GABA] in cells expressing TaALMT1: vigabatrin, a GABA transaminase (GABA-T) inhibitor used as an antiepileptic in humans (Livingston et al., 1989; Nanavati and Silverman, 1991), and AOA, an inhibitor of both GABA-T and glutamate decarboxylase (GAD) (John and Charteris, 1978; Miller et al., 1991; Snedden et al., 1992). We demonstrate that exogenous application of AOA and vigabatrin had an effect on both anion efflux via TaALMT1 and [GABA], resulting in negative correlations between [GABA] and anion efflux, which were also evident following Al3+ application. Interestingly, these negative correlations were abolished when the site-directed mutant TaALMT1F213C—impaired in its GABA regulation of malate transport—was expressed instead of TaALMT1. Although vigabatrin and AOA can affect [GABA] via inhibition of GABA-T and glutamate decarboxylase, respectively, their primary effect appeared to be via TaALMT1 (vigabatrin blocks and AOA activates). We propose that the reduction in [GABA] upon Al3+ treatment at low pH is due to efflux of GABA via TaALMT1. ALMT activity strongly affects [GABA], likely resulting in changes in metabolic flux through the GABA shunt. In the case of the wheat root, this may provide a signaling mechanism by which TaALMT1 can regulate root growth in acidic and alkaline conditions.

RESULTS

Validating Measurement of Intracellular GABA Concentration

GABA concentrations are routinely measured using the GABase enzyme assay. GABase is a commercially available enzyme mixture composed of γ-aminobutyric acid aminotransferase and succinic semialdehyde dehydrogenase from Pseudomonas fluorescens. The coupled enzyme reaction results in GABA conversion to succinate with the stoichiometric reduction of NADP+.
(Zhang and Bown, 1997). While examining the effects of inhibitors on cell and tissue GABA concentrations ([GABA]), we found that this assay was inhibited by AOA when added directly to the enzyme mix (Supplemental Figure 1C). We therefore tested if the GABAase assay was compromised when it was used on extracts of tissues that had been treated with AOA, Al^{3+}, or vigabatrin by performing GABA spike and recovery experiments (Supplemental Figures 1A to 1L). After 22-h treatment with 1 mM AOA, the recovery of spiked GABA from wheat seedling root extract was 97% (Supplemental Figures 1A to 1C); Al^{3+} treatment also did not compromise measurement of [GABA]. (Supplemental Figures 1D to 1F). Vigabatrin-treated wheat root tips also yielded full recovery of spiked GABA (Supplemental Figures 1G to 1I). Xenopus laevis oocytes treated with these compounds were also examined and similarly full recovery of GABA was obtained (Supplemental Figures 1J to 1L). We further used ultra-high-performance liquid chromatography (UPLC) to measure [GABA], on the same wheat root tip tissue extracts, after treatment with Al^{3+} and AOA, validating the results obtained by the enzyme assay (Supplemental Figure 1M). Thus, we expect the GABase assay to faithfully measure [GABA], following Al^{3+}, AOA, or vigabatrin treatments.

**Wheat Root Malate Efflux and [GABA]_{i}**

External Al^{3+} at low pH was previously shown to reduce [GABA] while stimulating malate efflux from roots of wheat seedlings (Ramesh et al., 2015). The Al^{3+}-tolerant wheat line ET8 has higher expression of TaALMT1 compared with ES8, its near isogenic line (NIL) (Yamaguchi et al., 2005); we found that TaALMT1 expression was further increased in ET8 by external Al^{3+} treatment (Supplemental Figure 2). We confirmed that ET8 exhibited Al^{3+} (100 μM) induced malate efflux from roots of 3-d-old wheat seedlings and that this coincided with a reduction in root tip [GABA] (Figure 1A). We also confirmed that line ET8 had higher [GABA] than ES8 in pH 4.5 and that ES8 [GABA] did not respond to Al^{3+} treatment (Supplemental Figure 1N) (Ramesh et al., 2015). When the corresponding values of Al^{3+}-stimulated malate efflux and root tip [GABA] from the same plants were plotted against each other, we observed that they were negatively correlated (Figure 1A). Delhaize et al. (1993) and others have shown through the use of excised root tips that they are the major site for malate efflux via TaALMT1. Both in the presence and absence of external SO_{4}^{2-}, AOA significantly stimulated malate efflux at pH 4.5 (Figure 2A), confirming the results we observed for excised root tips (Figure 1). The corresponding [GABA] of root tips was lower in the presence of AOA (Figure 2B). The presence of SO_{4}^{2-} at pH 4.5 made no difference to root tip [GABA], and vigabatrin did not elevate [GABA]. A significant negative correlation was observed between malate efflux and root tip [GABA] at pH 4.5 (Figure 2C) when all the individual replicates were plotted for two independent experiments. At pH 7.5, malate efflux was significantly higher in the presence of SO_{4}^{2-}, and vigabatrin significantly decreased [GABA] by the addition of SO_{4}^{2-}—stimulated efflux (Figure 2D). Root tip [GABA] at pH 7.5 was slightly lower but not significantly so than at pH 4.5 (Figures 2B and 2E); however, it was significantly decreased by the addition of SO_{4}^{2-}, contrasting with the lack of effect seen at pH 4.5. At pH 7.5, treatment with SO_{4}^{2-} plus vigabatrin significantly increased [GABA] compared with SO_{4}^{2-} treatment alone (Figure 2E). At pH 4.5, vigabatrin elevated [GABA], when applied on its own, but this did not occur at pH 7.5 (Figures 2B and 2E); furthermore, AOA did not significantly affect malate efflux or [GABA] at pH 7.5 (Figure 2B,E). Again, there was a significant negative correlation between root tip [GABA], and
malate efflux (Figure 2F), though the slope was lower than that observed at pH 4.5 (P < 0.001). Taken together, these results show that in roots of intact wheat seedlings, there exists a consistent negative correlation between malate efflux and [GABA] in root tips that is common across the treatments that were used to alter malate flux.

Malate Efflux and [GABA] in Tobacco BY2 Expressing TaALMT1

Tobacco BY2 (Nicotiana tabacum cv Bright Yellow-2) cells were used to test whether heterologous expression of TaALMT1 was sufficient to induce the negative linear relationship between malate efflux and [GABA] that was observed in wheat roots. Both Al³⁺ and AOA stimulated malate efflux in TaALMT1-expressing BY2 cells (Figure 3A); the effect of AOA was greatest at pH 4.5 compared with higher pHs (Supplemental Figure 3). At pH 4.5, the stimulated malate efflux corresponded to a lowered [GABA] at the end of the efflux period (Figure 3B). There was no effect of SO₄²⁻ by itself on either parameter at pH 4.5 (Supplemental Figures 4A and 4B). Neither malate efflux nor [GABA] varied at pH 4.5 in BY2 cells transformed with the empty vector when treated with AOA or Al³⁺ (Figures 3A and 3B). As observed for wheat roots, BY2 cells expressing TaALMT1 exhibited a significant negative linear correlation between malate efflux and [GABA], across individual replicates and treatments at pH 4.5 (Figure 3C; Supplemental Figure 4).

In contrast to its effect at pH 4.5, SO₄²⁻ at pH 7.5 significantly stimulated malate efflux from BY2 cells expressing TaALMT1 (Figure 3E), as it did in ET8 wheat roots. The addition of vigabatrin inhibited SO₄²⁻ stimulated malate efflux in a dose-dependent manner, with complete block observed at 100 μM (Supplemental Figure 5), but had little effect on controls expressing the empty vector (Figure 3E). In BY2 cells expressing TaALMT1, the reduction of malate efflux correlated with an increase in [GABA]. (Figure 3E), [GABA] in BY2 cells was elevated by vigabatrin as expected from its proposed action on GABA-T in both TaALMT1 and empty vector-expressing cells. Following both AOA and vigabatrin treatment, the significant relationships between malate efflux and [GABA] (Figures 3C and 3G) were dependent upon the expression of TaALMT1 since empty vector controls did not show a significant correlation (Supplemental Figure 6).

The F213C Mutation in TaALMT1 Abolishes the Relationship between [GABA] and Malate Efflux

A phenylalanine residue (F) has been shown to be important for GABA sensitivity in GABAₐ receptors and TaALMT1 (Boileau et al., 1999; Ramesh et al., 2015). Substitution of this residue by cysteine (C) impairs GABA sensitivity of TaALMT1 but did not abolish activation of the malate efflux by Al³⁺ or external anions (Ramesh et al., 2015). Therefore, we tested whether the exogenous application of Al³⁺ or AOA affected the relationship between malate efflux and [GABA], in tobacco BY2 cells expressing site-directed mutant TaALMT₁F213C. In the presence of Al³⁺ or AOA at low pH, malate efflux and [GABA] in the TaALMT₁-expressing cells were significantly negatively correlated (Figure 3C), but these were not correlated in cells expressing the TaALMT₁F213C mutant at pH 4.5.
Malate efflux increased with exogenous application of Al³⁺ or AOA in cells expressing TaALMT1F213C similarly to cells expressing TaALMT1 (Figure 3A), but unlike for TaALMT1-expressing cells, [GABA]ᵢ was not significantly reduced by these treatments (Figure 3B). At pH 7.5, malate efflux from TaALMT1F213C-expressing cells was stimulated by SO₄²⁻ to a similar degree as for those expressing TaALMT1 (Figure 3E). However, vigabatrin did not inhibit efflux from cells expressing TaALMT1F213C, and this contrasts with cells expressing TaALMT1, even though [GABA]ᵢ was elevated in both (Figure 3F). Thus, the relationship between malate efflux and [GABA]ᵢ was also abolished in cells expressing the TaALMT1F213C mutant at pH 7.5 (Figure 3H).

**Activation of TaALMT1 Results in GABA Efflux**

To determine if the reduction in [GABA]ᵢ was due to transport of GABA through TaALMT1, we tested if Al³⁺ not only activated malate efflux but also GABA efflux from the wheat NILs ET8 and ES8 (Figure 4). We observed that ET8 showed not only significantly higher malate efflux (Figure 4A) but also higher GABA efflux from intact seedling roots over 22 h when compared with ES8 (Figure 4C). This higher efflux could be detected from excised ET8 root tips even after 2 h (Figure 4D), corresponding to a stimulated malate efflux (Figure 2B). Similarly, transgenic barley (Hordeum vulgare) expressing TaALMT1 (OE) (Delhaize et al., 2004; Ramesh et al., 2015) when exposed to Al³⁺ at low pH showed significantly increased malate efflux (Figure 4E) as well as higher GABA efflux (Figure 4F) when compared with the Golden Promise background alone. It is also interesting to note that the efflux of GABA was reduced by vigabatrin (100 μM) in cells expressing TaALMT1 (OE) (Figure 4F).

![Figure 3. Tobacco BY2 Cells Expressing TaALMT1 Show the Same Negative Correlation between Malate Efflux and Endogenous GABA Concentration as Intact ET8 Roots in Response to Al³⁺ and AOA at pH 4.5 and SO₄²⁻ and Vigabatrin at pH 7.5.](image)

(A) Al³⁺ (100 μM) and AOA (1 mM) activate malate efflux from BY2 cells expressing TaALMT1 and TaALMT1F213C at pH 4.5 in standard (basal) solution over 22 h.
(B) [GABA]ᵢ in BY2 cells expressing TaALMT1 and TaALMT1F213C at the end of the efflux period.
(C) Wild-type TaALMT1 malate efflux versus [GABA]ᵢ in the cells at the end of the efflux period (22 h) at pH 4.5 after treatment with 100 μM Al³⁺ or 1 mM AOA addition to basal solution. Linear regression (Y = −1.47X + 3.90), R² = 0.43, P = 0.008.
(D) Site-directed mutant TaALMT1F213C at pH 4.5; treatments as in (C). ns, regression not significant.
(E) SO₄²⁻ (10 mM Na₂SO₄) activates malate efflux from BY2 cells expressing TaALMT1 and TaALMT1F213C at pH 7.5 in standard (basal) solution over 22 h but vigabatrin (100 μM) only inhibits TaALMT1 malate efflux.
(F) [GABA]ᵢ in BY2 cells expressing TaALMT1 and TaALMT1F213C at the end of the efflux period.
(G) Wild-type TaALMT1 malate efflux versus [GABA]ᵢ in the cells at the end of the efflux period (22 h) at pH 7.5 after treatment with SO₄²⁻ or SO₄²⁻ + vigabatrin addition to basal solution. Linear regression (Y = −0.64X + 2.75), R² = 0.39, P = 0.01.
(H) Site-directed mutant TaALMT1F213C at pH 7.5; treatments as in (G). Different lowercase letters indicate significant differences within a treatment group between genotypes; different uppercase letters indicate significant differences between treatments at a particular pH for each genotype (P < 0.05). For (A), (B), (D), and (F), n = 5 replicates each consisting of subculture of BY2 suspension culture. For (C), (D), (G), and (H), each point is a single biological replicate. No significant relationship exists between malate efflux and endogenous GABA concentrations in cells expressing empty vector at pH 4.5 or 7.5 (Supplemental Figure 6).
higher than that of malate on a molar basis by 3-fold in ET8 wheat and over 500-fold in TaALMT1-expressing barley roots. GABA efflux was also examined in tobacco BY2 cells expressing TaALMT1 and the TaALMT1F213C mutant, where at low pH we observed very large GABA efflux with Al\textsuperscript{3+} treatment only for cells expressing TaALMT1 (Figure 4G). GABA efflux in response to Al\textsuperscript{3+} from BY2 cells expressing TaALMT1 was also higher than malate efflux (compare Figures 3A and 4G).

Given that Al\textsuperscript{3+} activates GABA efflux through TaALMT1 in addition to malate, we tested the possibility that GABA may also complex Al\textsuperscript{3+}, since there was no data in the literature on possible interactions between GABA and Al\textsuperscript{3+}. Using a fluoride competitive ligand method and comparing GABA with the organic anions citrate, oxalate, malate, and salicylate, we found that GABA had very low affinity for Al\textsuperscript{3+} compared with these other compounds, with the strength of complexation following the order: citrate>oxalate>malate>salicylate>GABA (detailed in Supplemental Methods). We also found no synergistic or antagonistic interaction between GABA and malate in Al\textsuperscript{3+} complexation.

Al\textsuperscript{3+}, AOA, and Vigabatrin Have Direct Effects on TaALMT1 Correlating with Rapid Changes in [GABA] in X. laevis Oocytes

Since AOA had similar effects to Al\textsuperscript{3+} in activation of malate efflux and on reducing [GABA], it prompted us to examine if the GABA
analogues AOA and vigabatrin may have direct effects on TaALMT1 that may then alter [GABA], (Figure 5). Using two-electrode voltage clamp electrophysiology (TEVC) on X. laevis oocytes expressing TaALMT1 and TaALMT1F213C, we perfused the bath with 1 mM AOA or 100 μM Al3+ to compare the inward current activation corresponding to activation of malate efflux (Figures 5A and 5B). AOA activates TaALMT1 and the TaALMT1F213C mutant rapidly and with similar kinetics to Al3+. Vigabatrin was also examined for its effect on anion-activated currents at pH 7.5 (Figure 5C; Supplemental Figure 7). Vigabatrin acted rapidly on the SO42− and malate-activated inward current giving close to 100% inhibition at the external concentration of 100 μM. Furthermore, this inhibition was consistent for repeated applications indicating no enduring affect that would be expected if [GABA], were increased by inhibition of GABA-T. Water injected control oocytes showed no responses to these treatments (Figure 5D).

Oocyte [GABA], and GABA efflux were examined in separate batches of oocytes after 10-min treatment with Al3+, AOA, and vigabatrin and compared with water-injected controls (Figures 5E to 5G). Both Al3+ and AOA resulted in reduced [GABA], but only in TaALMT1-expressing oocytes. The reduction in [GABA], was almost fully accounted for by the amount of GABA effluxed from the oocytes in 10 min (Figures 5E and 5F). The effect of vigabatrin was examined at pH 7.5. Vigabatrin increased [GABA] compared with Na2SO4-treated oocytes only in TaALMT1-expressing oocytes. All these data are consistent with rapid effects of Al3+ and vigabatrin on TaALMT1, which appears to alter [GABA], by activation of GABA efflux through TaALMT1 (Al3+) or inhibition (vigabatrin). In the case of AOA, although AOA rapidly activated the anion currents and rapidly reduced [GABA], within 10 min (Figure 5G), we could not confirm that this occurred via GABA efflux due to the GABA enzyme assay being inhibited by 1 mM AOA in the external medium.

TaALMT1 and Other ALMTs Also Facilitate GABA Influx

To test if TaALMT1 also facilitated GABA influx, we expressed TaALMT1 and TaALMT1F213C in X. laevis oocytes and tested their ability to influx 14C-GABA. The GABA transporter AtGAT1 from Arabidopsis was used as a positive control in these uptake experiments (Figure 6). At pH 4.5, TaALMT1-expressing oocytes showed significantly higher GABA uptake from an external concentration of 1 mM compared with both control and mutant TaALMT1F213C-expressing oocytes and similar to that facilitated by AtGAT1 (Figure 6A). At pH 7.5, TaALMT1-expressing oocytes (without an external activating anion) did not show significant GABA influx compared with control oocytes, with the rate being significantly lower than AtGAT1-expressing oocytes (Figure 6B). AtGAT1-expressing oocytes had significantly reduced influx at pH 7.5 compared with that at pH 4.5, consistent with the hypothesis that this transporter uses the proton motive force to drive transport (Meyer et al., 2006). Activation of TaALMT1 at pH 7.5 with 10 mM Na2SO4 increased GABA uptake significantly into the TaALMT1-expressing oocytes when compared with either the controls or TaALMT1F213C-expressing oocytes (Figure 6B).

Wheat ALMT1 is the founding member of the ALMT family (Sasaki et al., 2004). A large number of these genes are present in Arabidopsis, barley, and rice (Oryza sativa) as well as other plants. Not all ALMTs are activated by Al3+ but can be (trans-)activated by millimolar concentrations of external anions (Ramesh et al., 2015) and play diverse roles in plant physiology (Roelfsema et al., 2012; Sharma et al., 2016). Thus, we tested ALMTs from Arabidopsis, barley, and rice that had previously been shown to exhibit GABA-inhibited malate currents, for their ability to transport GABA into X. laevis oocytes. All the ALMTs tested (HvALMT1, AtALMT1, OsALMT5, and OsALMT9) facilitated transport of GABA into the respective cRNA-injected oocytes at pH 4.5 (Figure 6C). In all cases, 100 μM Al3+ reduced this uptake to control levels. However, the presence of Al3+ did not affect the uptake of GABA by AtGAT1.

These results demonstrate that transport of GABA is a general feature of the ALMT family and that extracellular Al3+ is a common inhibitor for GABA uptake despite differences between the family in Al3+-activated malate currents.

TaALMT1 Complements Growth of a Yeast Mutant Deficient in the Transport of GABA

To further explore GABA transport by TaALMT1, we tested if GABA could be used as a nitrogen source for yeast growth, where TaALMT1 and site-directed mutant TaALMT1F213C were transformed into a yeast triple mutant strain 22754d (MATa ura3-1, gap1-1, put4-1, uga4-1) (Figure 7). This yeast strain carries mutations in general amino acid permease (gap1), proline (put4), and GABA (uga4) and is unable to grow on proline, citrulline, or GABA as the sole nitrogen source: it was used to characterize high affinity GABA transport (Meyer et al., 2006). The efflux of malate was observed to be higher in each of the transformants expressing TaALMT1 and the mutant when grown on galactose, which induces expression of the transgene via the GAL1 promoter (Figure 7A). This is consistent with TaALMT1 and its mutant being located on the plasma membrane and able to efflux malate, as previously shown in X. laevis and tobacco BY2 cells (Ramesh et al., 2015). All the yeast strains were capable of growth on selective drop out medium (SC-ura) supplemented with 2% glucose or galactose as carbon source and ammonium sulfate as nitrogen source (Supplemental Figures 8A and 8B). When the yeast strains were starved of nitrogen by growth in nitrogen-free medium and transferred to medium with no GABA, there were no significant differences in growth of the yeast strains (Supplemental Figure 8C). However, in medium supplemented with 1 mM GABA as the sole nitrogen source, yeast cells expressing TaALMT1 showed significantly higher relative growth rate compared with control (empty vector) or mutant TaALMT1F213C (Figure 7B; Supplemental Figure 8E). On medium supplemented with GABA at a concentration of 20 or 37.83 mM, which corresponds to SC medium with GABA at the concentration equivalent to that of ammonium sulfate, all the yeast strains showed similar growth (Supplemental Figure 8D), indicating that the stimulation of growth by 1 mM GABA was already saturating. Therefore, we examined the GABA dose-response of yeast growth (Figure 7C). The apparent Km for growth stimulation by GABA was 0.56 mM, indicating at least moderate affinity for GABA transport by TaALMT1. This growth stimulation was completely inhibited by the addition of 2 mM malate to the medium for TaALMT1 (Figure 7D).
Figure 5. Rapid Activation of Inward Currents (Anion Efflux) by Al^{3+}, AOA, Sulfate, and Inhibition by Vigabatrin for TaALMT1 Expressed in X. laevis Oocytes and Changes in [GABA] and GABA Efflux over 10 min.

(A) TaALMT1-expressing oocytes under TEVC showing inward current activation (at ~80mV) in response to 100 µM Al^{3+} and 1 mM AOA addition to the bath (pH 4.5) (continuous perfusion). Rates of activation of the inward current by Al^{3+} and AOA were similar.

(B) Current responses of TaALMT1^{F213C} mutant-expressing oocytes under TEVC as in (A). Rates of activation of the inward current by Al^{3+} and AOA were similar. Currents were consistently larger for TaALMT1^{F213C} and less cRNA was injected for these oocytes (16 ng compared with 32 ng in [A]).

(C) Vigabatrin (100 µM) effect on sulfate-activated inward currents at pH 7.5.

(D) Water-injected control oocytes treated with Al^{3+}, AOA, or vigabatrin.

(E) Effect of Al^{3+} (100 µM) treatment over 10 min on [GABA] (volume basis) and GABA efflux into the external medium (right plots, external GABA normalized to oocyte volume).

(F) Effect of vigabatrin (100 µM) in the presence of SO_{4}^{2-} (as 10 mM Na_{2}SO_{4}) at pH 7.5 on [GABA] (volume basis) and GABA efflux into the external medium (right plots, external GABA normalized to oocyte volume).

(G) Effect of AOA (1 mM) treatment (10 min) on [GABA] (volume basis). Different letter indicates significance (P < 0.05).

For (E) to (G), data are n = 4 to 5 replicates each consisting of four oocytes. For (A) to (D), individual oocyte examples are shown from repeated experiments on at least two harvests of oocytes.
DISCUSSION

GABA Transport by TaALMT1 Is Demonstrated across a Wide Range of Expression Systems and May Be a General Feature of ALMTs

Anion transport via ALMTs is an important and well-accepted mechanism for signaling (Bouché et al., 2003a; Roberts, 2007; Piñeros et al., 2008; Dreyer et al., 2012; Ligaba et al., 2013; Ramesh et al., 2015), stomatal pore regulation (Meyer et al., 2010; Roelfsema et al., 2012; De Angeli et al., 2013; Kollist et al., 2014; Palmer et al., 2016), phosphorous nutrition (Liang et al., 2013; Balzerque et al., 2017), and for providing tolerance to Al³⁺ at low pH (Delhaize and Ryan, 1995; Sasaki et al., 2004; Zhang et al., 2008; Ryan et al., 2011). We have demonstrated that TaALMT1 is also able to facilitate GABA efflux (Figures 4 and 5) and influx (Figure 6) at very high rates. This mostly accounts for the negative linear relationship observed between malate influx and endogenous GABA concentrations in cells expressing TaALMT1.

We have shown GABA transport by TaALMT1 using NILs of wheat that differ in the expression of TaALMT1 (Figure 4C), transgenic barley expressing TaALMT1 (Figure 4F), and complementation by Figure 6.

Figure 6. ALMTs but Not TaALMT1F213C Facilitate pH-Dependent GABA Influx from 1 mM External GABA When Expressed in X. laevis Oocytes and Are Blocked by 100 μM Al³⁺.

(A) Comparison of ¹⁴C-GABA uptake into X. laevis oocytes expressing TaALMT1 and AtGAT1 high-affinity GABA transporter at pH 4.5 (Exp. 1). Exp. 2: Comparison of ¹⁴C-GABA uptake between control, TaALMT1, and TaALMT1F213C -expressing oocytes and the effect of 100 μM Al³⁺.

(B) Comparison of ¹⁴C-GABA uptake for TaALMT1 and AtGAT1 at pH 7.5 (Exp. 1). Exp. 2: Effect of 10 mM Na₂SO₄ on ¹⁴C-GABA uptake comparing control, TaALMT1, and TaALMT1F213C -expressing oocytes.

(C) Comparison of ¹⁴C-GABA uptake and the effect of 100 μM Al³⁺ for oocytes expressing ALMTs from wheat (TaALMT1), barley (HvALMT1), Arabidopsis (AtALMT1), rice (OsALMT5 and OsALMT9), and Arabidopsis GABA transporter (AtGAT1) at pH 4.5. Fluxes were normalized to the median of AtGAT1. Different letters denote significant difference (P < 0.05) within each experiment. For all data, n = 7 to 10 biological replicates each consisting of eight oocytes from three oocyte harvests.
TaALMT1 of a yeast mutant deficient in GABA transport (Figure 7). The yeast mutant 22574d that we used to characterize GABA transport and nitrogen utilization in yeast cells (Grenson et al., 1970; Breitkreuz et al., 1999; Meyer et al., 2006) will likely be a very useful system in which to characterize other ALMTs for interactions with GABA and to further explore the pharmacology of the transporter.

Uptake studies with *X. laevis* oocytes showed that GABA uptake via ALMTs is not unique to TaALMT1, but ALMTs from barley, rice, and Arabidopsis also transported significantly more GABA into the cells than controls (Figure 6). Interestingly, the addition of Al3+ reduced the uptake in each case. We applied Al3+ since this activates TaALMT1 to efflux both malate and GABA at low pH. The block of GABA influx by Al3+ indicates that influx is mutually exclusive of efflux. In contrast, the uptake of GABA by AtGAT1 was not reduced in response to Al3+ (Figure 6C). It is interesting to note that there is evidence for interactions of Al3+ with GABAA receptors (Trombley, 1998) and GABA transporters in animals (Albrecht and Norenberg, 1991; Cordeiro et al., 2003).

**Reconciling GABA Efflux via TaALMT1 and GABA Block of Malate Efflux**

External GABA inhibits malate efflux and inward membrane currents through ALMTs with high affinity (IC50 1 to 7 µM) dependent...
on the presence of a motif containing phenylalanine (F213 in TaALMT1), which has similarities to a region in GABA<sub>a</sub> receptors (Smith and Olsen, 1995; Boileau et al., 1999; Ramesh et al., 2015). This external GABA block highlights the question of how GABA is exported from the cell to the apoplast to regulate TaALMT1. As far as we are aware, no transport system has been identified that is able to account for GABA efflux across the plasma membrane into the apoplast (Shelp and Zarei, 2017). This is despite numerous examples of relatively high extracellular concentrations of GABA being measured (Chung et al., 1992; Snedden et al., 1992; Crawford et al., 1994) and particularly the case for wheat roots where GABA is by far the most exported amino acid from roots (Warren, 2015). We propose that GABA efflux through TaALMT1 could account for this pathway and that the resulting GABA accumulation in the apoplast then inhibits malate transport via TaALMT1.

We note that there is some controversy regarding the membrane topology of ALMT proteins (Sharma et al., 2016), and this is important in the context of the location of F213 within the GABA motif of TaALMT1 and the rapidity of effect of externally applied GABA (Ramesh et al., 2015). Most recent algorithms (e.g., MEMSAT-SVM, TOPCONS; Nugent and Jones, 2012; Tsirigos et al., 2015) predict six to seven transmembrane domains for the more hydrophobic N terminus half of the protein and with the hydrophilic C terminus located in the cytosol. This is consistent with the model proposed by Zhang et al. (2013) in their study of the putative pore-forming domains of the vacuolar anion channel, AATLMT9. A similar model was also presented by Ramesh et al. (2017), who computed from evolutionary sequence variation over 3688 alignments using the EvFold web portal (Marks et al., 2012).

Notably, the GABA motif previously characterized is located toward the end of TMD 6 (or 7) just before the long C terminus, which in most predictions is oriented toward the cytoplasm. It was suggested that this was oriented toward the apoplast to account for the rapid effect of GABA (Ramesh et al., 2015) and corresponding with immunocytochemical evidence for the C terminus to be located on the apoplastic side (Motoda et al., 2007). Another model formulated from extensive sequence alignments across the family and secondary structure predictions also indicates that the GABA motif may be oriented toward the apoplast, but with a further two TMDs within the C terminus (Dreyer et al., 2012). If the GABA motif (beginning at F213 in TaALMT1) does orient toward the cytosolic side, the transport of GABA that we demonstrate here may reconcile the rapid action of GABA on malate currents, which presumably also applies to some GABA analogs that block depending on F213, such as muscimol and vigabatrin. Equally, the apoplastic localization of the motif is still consistent with our data. Clearly, a crystal structure will resolve this issue.

**Inhibitors That Target GABA Metabolism and Catabolism Directly Interact with TaALMT1**

Manipulating [GABA]<sub>i</sub> in cells and studying the effects of this perturbation on anion transport may provide information on the role of GABA in ALMT regulation for signaling and growth. Arabidopsis mutants deficient in GABA metabolism and catabolism have been previously used to demonstrate the role of altered [GABA]<sub>i</sub> in directing pollen tube growth, guard cell closure under drought, and systemic signaling upon wounding (Palanivelu et al., 2003; Mekonnen et al., 2016; Scholz et al., 2017). In this study, we used inhibitors assumed to disturb key steps in the GABA shunt pathway because of the rapidity of their effects, albeit compromised by possible lack of target specificity. Inhibition of GAD enzyme activity by AOA has been indicated for isolated mesophyll cells of asparagus (Asparagus officinalis; Snedden et al., 1992). Vigabatrin, by contrast, is a known inhibitor of GABA-T in animal cells that catalyzes the breakdown of GABA into succinate (Ben-Menachem, 2011). Both of these agents superficially appeared to have the desired affects in that AOA reduced [GABA]<sub>i</sub> and activated malate efflux when TaALMT1 was expressed, while vigabatrin increased [GABA]<sub>i</sub> and inhibited malate efflux.

Apart from the expected associations with the inhibitors described above, we observed that activation of the TaALMT1-mediated malate efflux by external Al<sup>3+</sup> at low pH (Figures 1C and 3B) and by SO<sub>4</sub><sup>2-</sup> at pH 7.5 (Figure 2E) also reduced [GABA]<sub>i</sub>. Based on the prevailing view that both these treatments activate malate efflux through TaALMT1 directly, these observations could be explained by [GABA]<sub>i</sub>, being depleted in order to support malate synthesis, since internal malate concentrations remain stable when malate efflux is stimulated by Al<sup>3+</sup> in wheat root apices (Delhaize et al., 1993). If GABA is required for malate synthesis and the demand from increased malate efflux through TaALMT1 results in depletion of [GABA]<sub>i</sub>, we would have expected to observe a reduction in [GABA]<sub>i</sub> when the TaALMT1<sup>F213C</sup> mutant channel was activated. This was not observed in BY2 cells, where [GABA]<sub>i</sub> remained at similar levels to controls when malate efflux was activated resulting in no relationship between [GABA]<sub>i</sub> and malate efflux (Figure 3). Therefore, we conclude that the reduction in [GABA]<sub>i</sub> in response to Al<sup>3+</sup> at low pH, and anions at high pH, is due to the large GABA efflux through the activated TaALMT1.

A direct effect of Al<sup>3+</sup> and anions on TaALMT1 and some other ALMTs has been demonstrated by the rapid activation observed through continuous current recording of voltage-clamped X. laevis oocytes (Figure 5A) (Sasaki et al., 2004; Hoekenga et al., 2006; Kobayashi et al., 2007; Kovermann et al., 2007; Piñeros et al., 2008; Meyer et al., 2011; De Angeli et al., 2013; Ramesh et al., 2015). Similarly, GABA and muscimol inhibition of the currents was as rapid as could be expected for solution change kinetics (Ramesh et al., 2015). AOA at pH 4.5 rapidly activated inward current in TaALMT1-expressing X. laevis oocytes consistent with malate anion efflux. The activation occurs with the same velocity (initial current rise) as that induced by Al<sup>3+</sup> (Figure 5A). Within 10 min, [GABA]<sub>i</sub> in the oocytes was significantly reduced by AOA, exactly as was observed when TaALMT1 was activated by Al<sup>3+</sup> (Figures 5E and 5G). The fact that neither of these treatments changed [GABA]<sub>i</sub>, in control water-injected oocytes indicates that the change in [GABA]<sub>i</sub> was due to activation of TaALMT1. The mutant TaALMT1<sup>F213C</sup> that is not responsive to GABA was also activated by AOA both in X. laevis oocytes (Figure 5B) and tobacco BY2 cells (Figure 3A). Thus, AOA is interacting with a site/s on TaALMT1 independently from the GABA binding motif.

Vigabatrin also inhibits the TaALMT1 inward current carried by anions rather rapidly (Figure 5C), which is not consistent with increasing [GABA]<sub>i</sub>. Though vigabatrin did increase [GABA]<sub>i</sub> in tobacco BY2 cells not expressing TaALMT1 (Figure 3E), the effect of vigabatrin was greater when TaALMT1 or TaALMT1<sup>F213C</sup> was
expressed in BY2 (Figure 3F) and when activated by SO₄ at pH 7.5 in wheat root (Figure 2E) and in X. laevis oocytes (Figure 5F). Thus, we conclude that both AOA and vigabatrin not only alter [GABA] by their known target enzymes, but also directly target TaALMT1 to change GABA efflux. The negative correlations observed between malate efflux and [GABA] in both wheat roots and tobacco BY2 cells is mainly indicative of altered GABA efflux through TaALMT1. Notwithstanding this conclusion, higher expression of TaALMT1 appeared to elevate [GABA], independently of any treatments as was initially observed when comparing ET8 with ES8 wheat NILs (Figure 1 in Ramesh et al., 2015; Supplemental Figure 1N). This was observed with tobacco BY2 cells expressing TaALMT1 (Figure 3B) at pH 4.5 and TaALMT1F213C at pH 7.5 (Figure 3F), but not in X. laevis oocytes (Figure 5E). Interestingly TaALMT1F213C does not transport GABA (Figures 4G, 6A, and 6B), yet its expression is able to influence [GABA], depending on pH. Currently we have no explanation for these observations, and further research is needed on the interaction between TaALMT1 and GABA metabolism related to the GABA interaction motif.

It may not be surprising that both AOA and vigabatrin interact with TaALMT1 since both are structural analogs of GABA. In the case of AOA, it inhibits GAD and GABA-T via interaction with the pyridoxal phosphate cofactor binding site (Wallach, 1961; John and Charteris, 1978; Löscher et al., 1989; Miller et al., 1991). It is interesting that AOA strongly activates TaALMT1, so far the only known external organic compound besides transported anions that has this effect. AOA is one atom shorter than GABA and is also a zwitterion that has no net charge at neutral pH. The possibility that ALMTs also have a pyridoxal phosphate binding site needs to be explored, though there is no clear pyridoxal phosphate (vitamin B₆) binding motif (InterPro IPR021115, Prosite entry Ps00392) evident in TaALMT1. GABA-T is also a pyridoxal phosphate-dependent enzyme and is reported to be inhibited by AOA (Storici et al., 2004). Vigabatrin has a similar structure to GABA; therefore, it may inhibit TaALMT1 as does the GABA analog muscimol. We conclude that the effect of vigabatrin and AOA on [GABA] is mainly via interactions with TaALMT1.

Potential Roles for GABA Transport via ALMTs

Until now, the identity of a GABA efflux transporter across the plasma membrane was not known. Our studies show that TaALMT1 in addition to mediating efflux of organic anions also mediates GABA efflux from cells when activated (Figures 4 and 5). Its transport capacity for GABA is very high as indicated by its activation being able to significantly reduce [GABA] in root tips and other cells expressing TaALMT1. In fact, we have shown higher capacity for GABA efflux relative to malate efflux on a molar basis (Figure 4). This is quite novel as changes in [GABA], are likely to have broad effects on carbon metabolism and signaling. We have shown that GABA does not significantly complex Al³⁺ (Supplemental Methods); therefore, we can exclude the hypothesis that GABA efflux with malate may provide additional protection against Al³⁺. Although it is not clear why high GABA efflux may be an advantage when TaALMT1 is activated by Al³⁺ at low pH, or activated at high pH, we may speculate along the following lines.

First, GABA may act as an extracellular pH buffer. GABA addition to solutions at both low and high pH tends to bring the pH...
toward neutrality. Thus, at low pH where GABA synthesis by GAD acts to regulate pH (Snedden et al., 1992; Crawford et al., 1994; Shelp et al., 1999; Snedden and Fromm, 1999), its efflux to the external medium will also tend to increase the external pH. It has been reported that exogenously supplied GABA (10 μM) significantly improved root growth of barley seedlings at pH 4.5 and when exposed to 20 μM Al3+ at pH 5.0 (Song et al., 2010). This was considered to be via alleviation of oxidative damage.

Second, AtALMT1 has been implicated as the key regulator in the attraction of the beneficial Bacillus subtilis strain FB17 to the Arabidopsis root (Lakshmanan et al., 2013), in addition to its role in Al3+ tolerance. Pseudomonads are known for their specific GABA receptors and positive chemotactic response to GABA (Reyes-Darias et al., 2015) and B. subtilis has positive chemotaxis to Arabidopsis roots with chemoreceptors that recognize a range of amino acids.

Third, GABA transported by TaALMT1 also provides feedback regulation on the cotransport of organic anions. As GABA builds up in the apoplast, this will tend to reduce malate efflux. Apoplastic GABA may also signal to adjacent cells via its effect on TaALMT1. Malate has the opposite effect since it activates TaALMT1 from the trans side of TaALMT1 particularly at higher pH. Thus, the two transported substrates have opposite effects on the trans (apoplastic) side of the transporter (Figure 8). ALMTs in general have rather complex regulation via anions, for example, the activation of AtALMT9 on the tonoplast membrane by cytosolic malate to efflux Cl− into the vacuole of guard cells during stomatal opening (De Angeli et al., 2013).

The Transport Mechanism for GABA and Its Interaction with Malate

Considering the transport of GABA via TaALMT1, it is necessary to account for the likely ionic charge on GABA. GABA is a zwitterion at neutral pH and is thus uncharged in the cytosol. Only at very high pH (above pKå 10.43) or at low pH (below pKå 4.23) does either a negative or positive charge become >50% of the total concentration. At pH 4.5, we calculate that there would be 35% of GABA in the external medium that has a net positive charge due to a proportion of molecules not deprotonated at the carboxyl end, while the amino terminal will remain positively charged. Efflux from the cytoplasm (slightly alkaline pH) would not be detectable as an electrical current, but influx at pH 4.5 could be detected as an inward current if the cationic form of GABA is transported, particularly if there is a high affinity of transport as suggested by the IC50 of block by GABA of the anion current, and from the high influx measured relative to that of AtGAT1 at pH 4.5. It is also possible that GABA fluxes may be coupled to the movement of protons in the same direction, similar to GABA transport via AtGAT1 (Meyer et al., 2006), which would be detectable as an electrogenic current. GABA influx into X. laevis oocytes was substantially higher at pH 4.5 than pH 7.5 (10-fold), and the flux at pH 7.5 was similar to that of control water-injected oocytes. However, GABA influx was activated at pH 7.5 by the addition of Na2SO4 to the bathing medium, which also stimulates malate efflux.

The ALMTs we tested that facilitated GABA influx (Figure 6) also showed external GABA inhibition of the malate efflux current at pH 7.5 (Ramesh et al., 2015). However, GABA on the inside of the cell clearly allows efflux of malate and GABA together, since in the heterologous expression systems used here and in the wheat NIL lines both GABA and malate efflux occur simultaneously. In the case of the X. laevis oocyte, the [GABA], is well above the concentrations that block malate efflux from the external (trans) side. The F213C mutation in TaALMT1 greatly reduces the external GABA sensitivity of anion efflux currents (Ramesh et al., 2015). We have shown here that this mutation also effectively abolishes GABA influx and GABA efflux via TaALMT1, but still allows activation by external anions and external Al3+, further confirming the importance of this site in GABA interactions. The F213C mutation also shows extremely high malate efflux when expressed in X. laevis oocytes compared with the unmutated TaALMT1, suggesting that “cotransport” of malate and GABA has been uncoupled. A summary of the observed interactions between GABA and malate transport via TaALMT1 and how this is expected to affect internal and external GABA concentrations is presented in Figure 8. Although Figure 8 depicts the ALMT as a channel, as this is the consensus in the literature, it is possible that the mechanism of transport may also be carrier-like.

To fully understand the transport mechanism in ALMTs, more detailed kinetic studies will be required wherein GABA and malate concentrations are varied on both cis and trans faces, most probably best achieved via patch-clamp studies. Our work reported here indicates that ALMTs are more complicated than a relatively simple ligand-gated anion channel, and we suggest that at least some (e.g., TaALMT1) could be considered as GABA transporters with anion channel activity. Why this has not been revealed from the many previous studies is probably related to several factors including the focus upon Al3+ tolerance resulting from the malate transport through TaALMT1 and the probable lack of or low electrogenic activity associated with GABA transport.

The dual function of TaALMT1 and other ALMTs is analogous to some transporters that display channel activity such as the excitatory amino acid transporters from animals that function as both glutamate transporters and chloride channels (Cater et al., 2016). There is also a precedent for GABA transport in that the mammalian GAT1 transporter displays sodium channel activity (Risso et al., 1996). In the context of the biological link between GABA and anion transport and particularly that of malate, the regulation of efflux of both substrates is titrated finely by apoplastic concentrations of both substrates, making them uniquely positioned to provide intercellular and intracellular communication of metabolic status (Figure 8).

METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich. 14C-GABA was obtained from American Radiolabeled Chemicals.

cRNA Synthesis

Plasmid DNA from ALMT and site-directed mutants (Nugent and Jones, 2012; Ramesh et al., 2015) was extracted using the Mini Prep kit from Sigma-Aldrich, and 1 μg of plasmid DNA was linearized with the restriction enzyme NheI. Capped cRNA was synthesized using the MESSAGE Mmachine T7 Kit (Ambion) as per the manufacturer’s instructions.
Voltage-Clamp Electrophysiology

Electrophysiology was performed on Xenopus laevis oocytes 2 d after injection with water/cRNA (Preuss et al., 2010; Ramesh et al., 2015). Oocytes were injected with 46 nL of RNase-free water using a microinjector (Nanoject II, automatic nanoliter injector; Drummond Scientific) ± 16 to 32 ng cRNA. Sodium malate (10 mM, pH 7.5) was injected into oocytes 1 to 4 h before measurement. Aluminum activation was performed in ND88 solution at pH 4.5 (Sasaki et al., 2004; Høkenga et al., 2006) ± aluminum chloride (AlCl₃ 100 mM). Basal external solutions for anion activation contained 0.5 mM CaCl₂ (pH 4.5) or 0.7 mM CaCl₂ (pH 7.5) and mannitol to 220 mM osm kg⁻¹ ± 10 mM sodium sulfate or 10 mM malate buffered with 5 mM BTP/MES from pH 4.5 to 7.5. Vigabatrin and AOA were added at 100 μM and 1 mM, respectively, in the experiments. In all X. laevis oocyte experiments, solutions were applied to gene-injected oocytes in the same order as controls (water injected). Randomly selected oocytes were alternated between control and gene injected to limit any bias caused by time-dependent changes after gene injection or malate injection. The University of Adelaide Animal Ethics Committee approved the X. laevis oocyte experiments (project number S-2014-192).

Root Assays

NILs of wheat (Triticum aestivum) ET8 and ES8 (Sasaki et al., 2004) and barley (Hordeum vulgare) transgenic line overexpressing TaALMT1 (a gift from Peter Ryan, CSIRO, Canberra; Delhaize et al., 2004) were surface sterilized in 1% bleach for 1 min, rinsed three times in water, and germinated on moist filter paper in the dark on the bench. The 3-d-old seedlings were placed in a microcentrifuge tube with roots immersed for 22 h in 3 mM CaCl₂ and 5 mM MES/BTP to pH 4.5 ± treatments. For root flux assays, experiments were performed wherein the identity of the treatment solutions was unknown to the person performing the experiments to remove any bias. Malate concentrations were measured on an OMEGA plate-reading spectrophotometer (BMG) following the K-LMALR/K-LMALL assay11 kit (Megazyme) as per the manufacturer’s instructions. The change in absorbance at 340 nm was used to calculate the concentration of malate in the samples.

Endogenous GABA Concentrations and GABA Efflux

GABA concentrations were measured on an OMEGA plate-reading spectrophotometer following the GABase enzyme assay (Zhang and Bown, 1997). Briefly, 5 mm of root tips was excised and snap frozen in liquid nitrogen after seedlings were subjected to treatment solutions for 22 h and further growth. The transformed calli were used to extract DNA to confirm the presence of transgenes by PCR and maintained by subculturing every 3 weeks. The transient BY2 cell lines were used in further experiments.

Tobacco BY2 Transgenic Lines

Tobacco suspension cells (Nicotiana tabacum cv Bright Yellow-2) were transformed with TaALMT1, site-directed mutants, or the empty vector pTOOL37 (Supplemental Table 1) using a slightly modified protocol (An, 1985). Briefly, fresh suspension cells in Murashige and Skoog (MS) medium were cocultivated with agrobacterium strains carrying the binary vectors with genes of interest for 48 h with 20 μM acetosyringone at 25°C. The cells were washed four times in MS medium, with a final wash in MS medium with 1.018 mM GABA for 2 h. The oocytes were washed twice with ice-cold uptake buffer and placed in 0.1N nitric acid before addition of scintillation mobile solvents AccQ Tag Ultra Eluents A and B (Waters). Calibration range for GABA was 0 to 500 μM. The results were analyzed with Empower 3 chromatography software by Waters.

Tobacco BY2 Malate Efflux and GABA Concentrations

Tobacco suspension cells were grown in MS medium on a rotary shaker (~100 rpm) until the logarithmic phase. Aliquots of suspension cells containing ~1 g of cells were centrifuged and gently resuspended in a basal BY2 solution (Zhang et al., 2008). TaALMT1 and site-directed mutants expressing or vector control tobacco BY2 suspension cells (0.15 g) were placed in 5 mL of 3 mM CaCl₂, 3 mM sucrose, and 5 mM MES/BTP to pH 4.5 to 7.5 ± treatments as shown in the figure legends, in 50−mL tubes on a rotary shaker for 22 h, unless otherwise stated. Malate fluxes were measured as described above. Cells were harvested after treatments by centrifugation at 5000g in a desktop microcentrifuge and snap frozen in liquid nitrogen. Frozen cells were submitted to extraction and GABA concentrations measured as described above using GABase enzyme (Sigma-Aldrich).

³¹C-GABA Tracer Flux Experiments

X. laevis oocytes expressing ALMTs, site-directed mutant, controls, and AtGAT1 (At1g08230) were incubated in uptake buffer containing 0.5 mM CaCl₂ (pH 4.5) or 0.7 mM CaCl₂ (pH 7.5), buffered with 5 mM MES/BTP ± AlCl₃ (100 mM)/sodium sulfate (10 mM), mannitol to 220 mM osm kg⁻¹ and 1.018 mM GABA for 2 h. The oocytes were washed twice with ice-cold uptake buffer and placed in 0.1 n nitric acid before addition of scintillation fluid (4 mL) and radioactivity measured in a Beckman and Coulter multi-purpose scintillation counter (LS6500). The AtGAT1 used as a positive control was a gift from Doris Rentsch, University of Bern, Switzerland.

Yeast Experiments

Saccharomyces cerevisiae strain 22574d (MATα ura3-1, gap1-1, ptt4-1, uga4-1) (Jauniaux et al., 1987) was transformed with TaALMT1 and
site-directed mutants cloned (Supplemental Table 1) into yeast transformation vector pDEST52, which carries the promoter and enhancer sequences from the GAL1
gene for galactose-regulated expression according to Schiestl and Gietz (1989), and the transformants were selected on minimal medium supplemented with 2% glucose. Selected trans- formants were grown in Grenson’s medium (Grenson et al., 1970) lacking nitrogen supplemented with 2% galactose for 16 to 18 h before transfer to medium supplemented with either 1 mM GABA as the sole nitrogen source for growth studies or different concentrations of GABA (0–1 mM) for dose- response curves. The yeast strain used was a gift from Doris Rentsch.

Ligand Complexation Method for Testing Al3+-GABA Complexation

A competitive ligand method was developed (Supplemental Methods) and tested wherein the ligand of interest, L (e.g., GABA), is added to a solution containing Al3+ and fluoride (F). Depending on the strength of Al3+ complexation with the ligand of interest, F will be displaced from the AlF2+ complex, resulting in higher free F concentration, which can be detected with a fluoride ion selective electrode (ISE).

$$\text{AlF}_{2+} + L^{−} \rightarrow \text{AlF}_{2−} + F^{−}$$

Five organic ligands were used in the measurements: citric acid, oxalic acid, malic acid, salicylic acid, and GABA. Stock solutions of 0.05 and 0.005 M were made and adjusted to pH 4.5 using NaOH. A solution containing 10 mM NaCl, 60 μM Al3+ (as AlCl3), 60 μM F (as NaF), and 10 mM MES, adjusted to pH 4.5 was made. Ten milliliters of this solution was titrated with organic ligand, and the free F concentration was determined using a fluoride ion selective electrode (Orion 9609BNWP), allowing several minutes between each titration step until the reading stabilized. The ISE was calibrated using solutions with 10 mM NaCl, 10 mM MES (pH 4.5), and 1 to 100 μM F. All ISE measurements were made in aluminum foil-covered beakers while stirring the solution. The pH of the solution was checked at the end of each titration run and was always between 4.45 and 4.55.

Statistics

All graphs and data analysis were performed in GraphPad Prism 7 (version 7.02). All data shown are mean ± se. Where appropriate, one-way and two-way ANOVA were performed with post tests to determine significance between individual treatments (Supplemental Table 2).

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: TaALMT1 (DQ072260), HvALMT1 (EF424084), OsALMT5 (Os04g0417000), OsALMT9 (Os10g0572100), AtALMT1 (AT1G08430), AtALMT7 (AT1G08230), Actin (KC775782), Tubb4 (U76895.1), Cyclophilin (EU035525.1), and GAPDH (EF592190.1).

Supplemental Data

Supplemental Figure 1. GABA spike and recovery experiments with root tips of 3-d-old wheat seedlings (ET8) and X. laevis oocytes (control and TaALMT1 injected) to test the impact of AOA, Al3+, and vigabatrin on the enzyme assay of GABA and UPLC versus GABase measurements.

Supplemental Figure 2. Expression of TaALMT1 relative to three housekeeping genes (GAPDH, Cyclophilin, and Actin) in seedling roots of plants treated with 100 μM AlCl3, pH 4.5 or basal solution for 22 h.

Supplemental Figure 3. Malate efflux via TaALMT1 expressed in tobacco BY2 cells as a function of external pH and the effect of AOA.

Supplemental Figure 4. Further evidence of a negative linear correlation between malate efflux and [GABA] in tobacco BY2 cells expressing TaALMT1 showing that sulfate does not activate malate efflux at low pH.

Supplemental Figure 5. Vigabatrin, an inhibitor of GABA transaminase, inhibits malate efflux activated by 10 mM Na2SO4 while elevating endogenous GABA concentrations in BY2 cells expressing TaALMT1 at pH 7.5.

Supplemental Figure 6. Tobacco BY2 empty vector controls show no significant correlation (F > 0.05) between malate efflux and endogenous GABA concentrations at pH 4.5 when treated with Al3+, AOA, or at pH 7.5 when treated with Na2SO4 or vigabatrin.

Supplemental Figure 7. Malate-activated inward currents in TaALMT1-expressing X. laevis oocytes at pH 7.5 (−80 mV) are also rapidly inhibited by 100 μM vigabatrin.

Supplemental Figure 8. Details of complementation of yeast strain 22754d (MATa ura3-1, gap1-1, put4-1, uga4-1) expressing TaALMT, TaALMT1318.

Supplemental Table 1. Primers used for cloning and site directed mutagenesis.

Supplemental Table 2. ANOVA tables.

Supplemental Methods. Details of measurement of possible complexation of Al3+ by GABA.

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AUTHOR CONTRIBUTIONS

S.D.T. supervised the research. S.A.R., S.D.T., and M.G. planned and designed experiments. S.A.R. performed experiments with oocytes and tobacco BY2 cells, as well as yeast and plant experiments. M.K. performed additional plant experiments. W.S. assisted in plant, oocyte, and tobacco cell experiments. M.O. and L.C. optimized and carried out UPLC measurements. M.M. and F.D. optimized and carried out GABA-Al3+ binding assays. S.A.R. and S.D.T. analyzed the data and wrote the article with edits from M.G. All authors had intellectual input into the project and commented on the manuscript.

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