Identification and characterization of GABA, proline and quaternary ammonium compound transporters from *Arabidopsis thaliana*

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Abstract *Arabidopsis thaliana* grows efficiently on GABA as the sole nitrogen source, thereby providing evidence for the existence of GABA transporters in plants. Heterologous complementation of a GABA uptake-deficient yeast mutant identified two previously known plant amino acid transporters, AAP3 and ProT2, as GABA transporters with Michaelis constants of 12.9 ± 1.7 and 1.7 ± 0.3 mM at pH 4, respectively. The simultaneous transport of [1-14C]GABA and [2,3-3H]proline by ProT2 as a function of pH, provided evidence that the zwitterionic state of GABA is an important parameter in substrate recognition. ProT2-mediated [1-14C]GABA transport was inhibited by proline and quaternary ammonium compounds.
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Key words: *Arabidopsis thaliana*; Amino acid transport; GABA; Proline; Zwitterionic; Quaternary ammonium compound

1. Introduction

In plants, the concentration of 4-aminobutyrate (GABA), a ubiquitous four-carbon non-protein amino acid, is markedly stimulated by a variety of stress conditions [1,2]. GABA accumulation is likely mediated via increased synthesis from glutamate [2] and possibly by decreased catabolism [1,3] or changes in intracellular or intercellular transport. Cellular efflux [4] and vascular transport [1] of GABA have been documented. Like other amino acids, GABA import into the cell, across the plasma membrane, likely requires the presence of H^+-coupled transporters [5,6], however, the existence of GABA transporters in plants has not been reported. GABA transport proteins, which vary in sequence homology, localization and substrate specificities, have been isolated and well characterized in representative species from animal, fungal and bacterial systems [7–10]. Most use GABA, but also transport other compounds such as proline, 3-aminopropionate, taurine and glycine betaine, a quaternary ammonium compound.

In the present study, heterologous complementation of a GABA transport-deficient yeast mutant and analysis of previously isolated amino acid transporters [11–13] were used to identify GABA transporters in *Arabidopsis thaliana*. Two known amino acid transporters, amino acid permease 3 (AAP3) [12] and the proline transporters 2 (ProT2) [13], effectively mediated GABA transport. Like the animal, fungal and bacterial GABA transport systems, GABA transport by ProT2 was inhibited by other related compounds.

2. Materials and methods

2.1. Plant growth

A. thaliana (L.) Heynh (C24 ecotype) were germinated and grown to maturity on AM medium according to Schmidt and Willmitzer [14]. Plates either contained no nitrogen, or were supplemented with 20 mM GABA or 10 mM N in the form of $(NH_4)_2SO_4$ and KNO_3 (66% NH_4^+ and 33% NO_3^-).

2.2. Yeast growth, transformation and selection

The *S. cerevisiae* strain 22574d (*ura3-1, gap1-1, put4-1, uga4-1*), deficient in GABA transport [15], was transformed with a cDNA library from *A. thaliana* seedlings [16]. Transformants were selected on SD medium [17], washed from the plates in nitrogen-free liquid SD medium and reselected on nitrogen-free SD medium supplemented with 20 mM GABA as the sole nitrogen source. Colonies able to grow were reselected in liquid SD medium. Plasmid DNA was isolated and reintroduced into the mutant 22574d. Resulting strains were tested for reversion by using 5-fluoroorotic acid [18]. Further characterization of the plasmids was performed by restriction digest analysis and full or partial sequencing of up to 500 bp at both 3′- and 5′-ends.

2.3. DNA sequencing

The cDNAs isolated by complementation were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) on the ABI PRISM Sequencer Model 310 or 377 (Perkin Elmer).

2.4. Yeast growth assay

The mutant strain 22574d was transformed with the amino acid permease cDNAs (*AAP1-6* and *ProT1-2*) in the yeast expression vector pFL61 [12,13]. The transformed yeast strains and the clones found by complementation were grown in liquid SD medium to the same optical density (OD_{600}) in the logarithmic phase, washed in nitrogen-free medium, and equal amounts streaked onto plates containing varying concentrations of proline and GABA. The plates were incubated for 4 days at 28°C.

2.5. Transport measurements

Yeast cells were grown in liquid SD medium at 28°C and harvested at an OD_{600} of 0.5. Cells were washed twice and resuspended in 0.3 M sorbitol to a calculated OD_{600} of 20. Prior to the transport measurements, 100 μl aliquots of cells were supplemented with 100 mM glucose and incubated for 10 min at 28°C. To start the reaction, each aliquot was added to an equal volume of 100 mM potassium phosphate buffer containing 0.3 M sorbitol, the appropriate radiolabeled substrate (either 18.5 kBq of [1-14C]GABA or [U-14C]proline; simultaneous transport experiments utilized both 18.5 kBq [1-14C]GABA and 37 kBq of [2,3-3H]proline; Sigma), and 1 mM of the respective unlabeled substrate. The pH of the transport buffer was 4.0 [6], unless stated otherwise. Where the pH was altered, the relative proportion of zwitterionic GABA and proline was calculated using the Henderson–Hasselbalch equation ($pH = pK + \log([A^-]/[HA])$) and pK_1 values of 4.03 for GABA and 1.95 for proline [19]. Samples (45 μl) were taken after 1, 2, 3 and 4 min, filtered on GF/C glass fiber filters (Whatman),

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and washed immediately with 8 ml of ice-cold water containing 5 mM GABA or proline to reduce non-specific binding. Radioactivity ([14]C- and ^{3}H-labeled substrates) accumulated in the cells on the filter was measured by liquid scintillation spectrometry (Beckman LS 6800). Competition for GABA transport was performed in the presence of a 5-fold molar excess of competitors. All kinetic data represent the mean of three independent experiments ± S.D. performed on separate days. Kinetic constants were calculated using non-linear regression analysis.

3. Results

3.1. Growth of Arabidopsis on GABA

Arabidopsis seeds germinated and grew on nitrogen-free medium supplemented with nitrogen in the form of NH_4^+ and NO_3^- or GABA; seeds germinated on nitrogen-free medium did not grow (Fig. 1A). These observations indicate that external GABA, when supplied as the sole nitrogen source, can support the growth of *Arabidopsis*, and suggest that plants contain GABA transporters, at least in roots.

3.2. Heterologous complementation of a GABA transport mutant

The *S. cerevisiae* strain 22574d, which is deficient in proline and GABA transport, grows poorly on minimal media supplemented with proline or GABA as the sole nitrogen source [9]. Transformation of the mutant with an *Arabidopsis* expression library resulted in several clones which mediated growth on selective GABA concentrations. Restriction digests and sequence analysis revealed that two of the cDNAs (named *ZFP1* for zinc finger protein, differing in length of ∼0.9 and 1.1 kb, respectively), encode proteins that show homology (>50% identity) to RMA1 [20] and RZF [21], two zinc finger proteins from *Arabidopsis*; these were not pursued because they represent putative transcription factors that probably activate an endogenous yeast GABA transporter. Two cDNAs encode the previously characterized amino acid transporter AAP3 [12]; however, one cDNA designated *AAP3′*, has a 5′-untranslated region 61 bp shorter than *AAP3*.

Table 1
Influence of pH upon the catalytic efficiency of [1-^{14}C]GABA or [2,3-^{3}H]proline transport into yeast cells expressing *ProT2*

Substrate	Condition	V_{max}/K_m[a]	
		Total	Zwitterionic[b]
Proline	pH 4.0	4.19 ± 0.94	-
	pH 4.5	3.14 ± 0.86	–
	pH 5.0	2.48 ± 0.81	–
	pH 5.5	0.94 ± 0.16	–
GABA	pH 4.0	1.39 ± 0.32	2.83
	pH 4.5	1.22 ± 0.22	1.67
	pH 5.0	1.39 ± 0.08	1.55
	pH 5.5	0.57 ± 0.05	0.61

[a]Criterion of catalytic efficiency ($\times 10^6$); values are mean ± S.D., $n = 3$.
[b]The concentration of zwitterionic GABA was calculated using the Henderson–Hasselbalch equation ($pK_{GABA} = 4.03$ [19]).

3.3. Growth assay of yeast transformants

The growth of the mutant yeast strain 22574d expressing previously cloned amino acid permeases (*AAP1-6* and *ProT1-2*) and the cDNAs found by complementation in this study were compared on various concentrations of GABA or 1 mM proline. Growth media supplemented with 10 mM $(NH_4)_2SO_4$ served as a control; wild-type yeast and the mutant 22574d transformed with the different cDNAs or *pFL61* grew equally well. All cDNAs mediated growth on 1 mM proline, however, growth on GABA was variable. With the exception of *AAP1*, all *AAP*s and *ProT*s mediated growth on 20 mM GABA (Fig. 1B; of the *AAP*s, only 22574d-*AAP3* is shown). However, 22574d expressing *AAP3′* and *ProT2* grew more vigorously on lower GABA concentrations than cells expressing *AAP3* and *ProT1*, respectively.

3.4. Characterization of AAP3 and ProT2 as GABA transporters

GABA or proline transport by AAP3′ and ProT2 was linear for at least 5 min and exhibited saturable, concentration-dependent uptake [22]. At pH 4.0, the apparent K_m
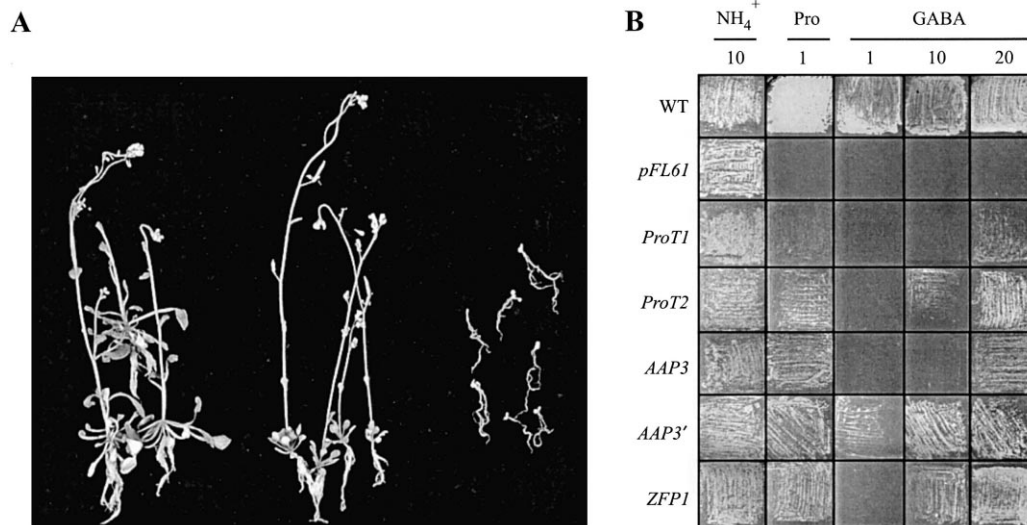

Fig. 1. Growth of *Arabidopsis* (A) and yeast transformants (B) on media supplemented with GABA as the sole nitrogen source. *A. thaliana* ecotype C24 seeds were sown on media containing either 10 mM N in the form of $(NH_4)_2SO_4$ and KNO_3 (66% NH_4^+ and 33% NO_3^-) (left), 20 mM GABA (middle) or no nitrogen (right; panel A). The *S. cerevisiae* mutant 22574d transformed with *pFL61* (control), or with the same vector carrying *ProT1*, *ProT2*, *AAP3*, *AAP3′* and *ZFP1* ([12,13], this study). Transformants were streaked on medium containing either 10 mM $(NH_4)_2SO_4$, 1 mM proline, or 1, 10 and 20 mM GABA (panel B).

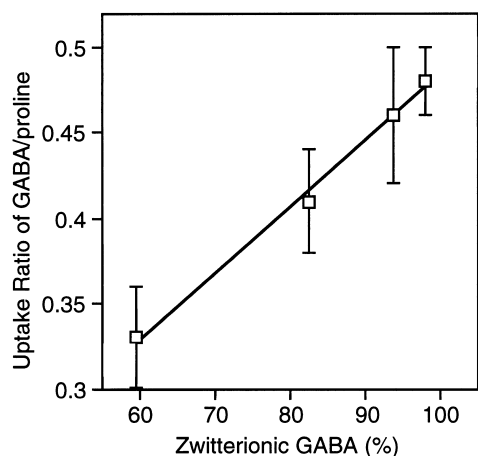Fig. 2. Ratio of [1-^{14}C]GABA/[2,3-^{3}H]proline transport into yeast cells expressing *ProT2* as a function of relative zwitterionic GABA. Simultaneous uptakes of 1 mM GABA and proline were performed at various pHs ranging from 4.2 to 5.7. Data represent the mean of three independent experiments ± S.D.

(mean ± S.D.) of AAP3′ for GABA was estimated to be 12.9 ± 1.7 mM with an apparent V_{max} of 3.22 ± 0.25 μmol min^{-1} g^{-1} yeast dry weight (DW). In contrast, the affinities of ProT2 for GABA and proline were higher, with apparent K_m of 1.70 ± 0.34 mM and 0.42 ± 0.06 mM, respectively. The apparent V_{max} of ProT2 for GABA (2.31 ± 0.39 μmol min^{-1}
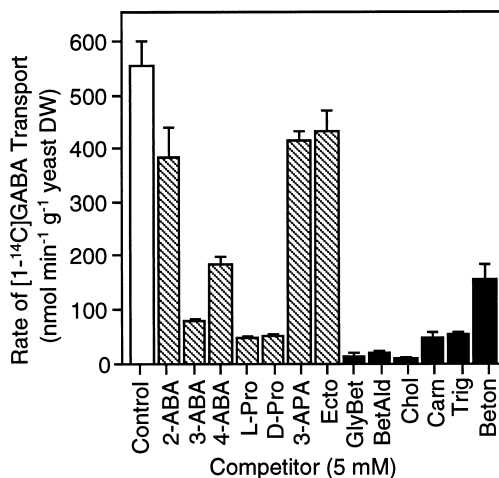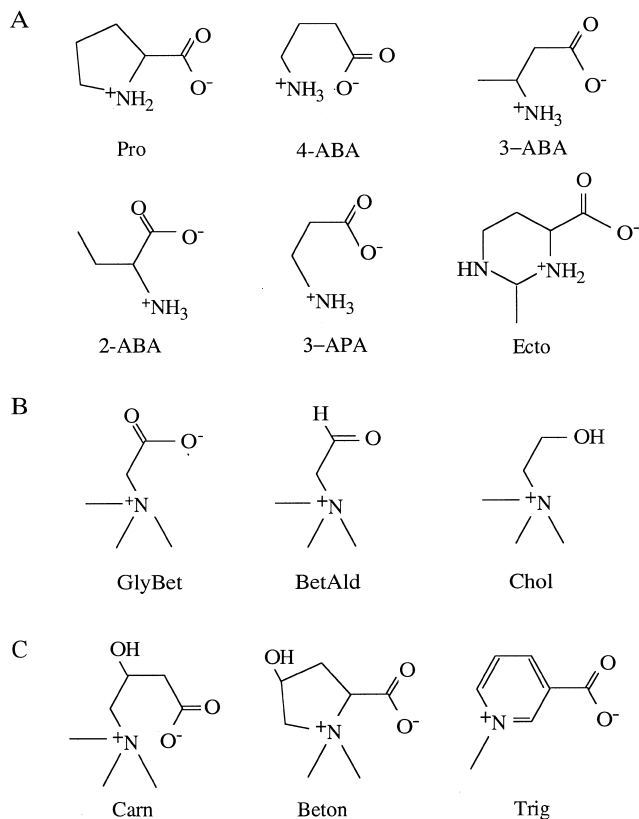

Fig. 3. Chemical structures of various amino acids (A), glycine betaine and precursors (B), and other betaines (C). 2-ABA, 2-aminobutyric acid; 3-ABA, 3-aminobutyric acid; 4-ABA, 4-aminobutyric acid (GABA); 3-APA, 3-aminopropionic acid; GlyBet, glycine betaine; BetAld, glycine betaine aldehyde; Beton, betonicine; Carn, carnitine; Chol, choline; Ecto, ectoine; Pro, proline; Trig, trigonelline.

Fig. 4. Biochemical characterization of ProT2. Competition of 1 mM [1-^{14}C]GABA uptake by the *S. cerevisiae* mutant 22574d expressing *ProT2* in the presence of the respective competitors at a concentration of 5 mM. The uncompeted uptake rate of GABA served as the control (open bar); competition with amino acids (cross-hatched bars) or quaternary ammonium compounds (solid bars). Data represent the mean of three independent experiments ± S.D. performed at pH 4.0. 2-ABA, DL-2-aminobutyrate; 3-ABA, DL-3-aminobutyrate; 4-ABA, 4-aminobutyrate (GABA); 3-APA, 3-aminopropionate; Gly-Bet, glycine betaine; BetAld, glycine betaine aldehyde; Beton, betonicine; Carn, carnitine; Chol, choline; Ecto, ectoine; Pro, proline; Trig, trigonelline.

g^{-1} yeast DW) and proline (1.74 ± 0.21 μmol min^{-1} g^{-1} yeast DW) were not significantly different. When V_{max}/K_m, a criterion of catalytic efficiency, was determined for either proline or GABA uptake in response to increasing pH, the ratio decreased 3-fold for proline, but remained relatively unchanged for GABA until pH 5.5 (Table 1). These different trends are attributable to pH effects on the carrier and differences in the ionization of substrates. Throughout the pH range investigated, proline is almost exclusively in the zwitterionic form, whereas the percentage of zwitterionic GABA doubles over the same range from 48.2% to 96.7%, respectively. When the percentage of zwitterionic GABA is considered, the catalytic efficiency of ProT2 for GABA was about half that for proline and decreased in a similar manner.

To investigate the importance of the zwitterionic or neutral substrate for ProT2, the transport of [1-^{14}C]GABA and [2,3-^{3}H]proline was simultaneously measured in response to increasing pH, and the ratio of ^{14}C/^{3}H plotted against the predicted percentage of zwitterionic GABA (Fig. 2). The ratio of GABA/proline never exceeded 0.48 and increased linearly ($y = 0.004x+0.096$; $r^2 = 0.996$) with the predicted increase in pH-dependent zwitterionic GABA concentration indicating that this species is preferentially transported.

Of the compounds tested which are structurally similar to GABA (Fig. 3), glycine betaine, choline and betaine aldehyde inhibited [1-^{14}C]GABA transport most effectively (between 96 to 99%) (Fig. 4). The betaines, carnitine and trigonelline, in addition to L- and D-proline, were also very effective inhibitors ($\sim$91%), followed by DL-3-aminobutyrate, betonicine, and 4-aminobutyrate (70–86%). Ectoine, 3-aminopropionate and DL-2-aminobutyrate inhibited GABA transport slightly (20–30%), whereas, taurine, and the polyamines, putrescine and spermine, had no inhibitory effects (data not shown).

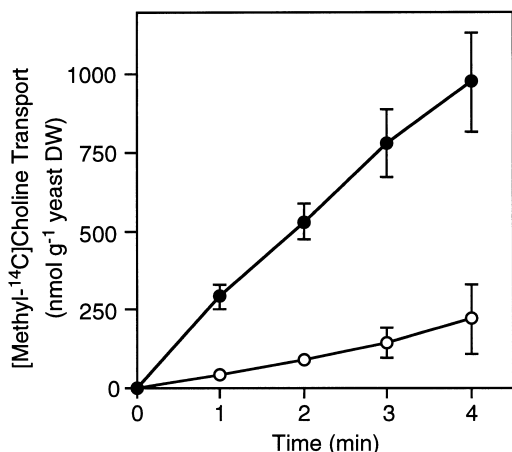Since choline was one of the most effective inhibitors, a

Fig. 5. Time course of [methyl-^{14}C]choline uptake into yeast cells expressing *ProT2*. Uptakes were measured with the *S. cerevisiae* mutant 22574d transformed with *pFL61* (open symbol) or with the same vector carrying *ProT2* (closed symbol). Yeast was grown in standard medium supplemented with 1 mM choline to suppress the endogenous choline transporter. Data represent the mean of three independent experiments ± S.D. performed at pH 4.0.

time course of [methyl-^{14}C]choline transport into the mutant 22574d transformed with *pFL61* or with the same vector carrying *ProT2* was performed (Fig. 5). Expression of the endogenous yeast choline transporter (CTR1) was partially repressed by the addition of 1 mM choline to standard yeast growth media [23]. With corrections for [methyl-^{14}C]choline transport by the control, the mutant expressing *ProT2* had a net transport rate of 180 nmol min^{-1} g^{-1} yeast DW, a rate 3-fold higher than the control and in a same order of magnitude as that for [1-^{14}C]GABA transport in the absence of inhibitor (Fig. 4). Choline did not affect yeast vitality or the electrochemical gradient, since the transport of [U-^{14}C]adenine by endogenous yeast H$^+$-cotransport systems [24] was uninhibited by the presence of 0, 5 or 25 mM choline (783 ± 97, 778 ± 63 and 800 ± 34 nmol adenine min^{-1} g^{-1} yeast DW).

4. Discussion

Growth of *Arabidopsis* on medium supplemented with GABA as the sole nitrogen source, provided preliminary evidence that plants possess proteins capable of transporting GABA. Expression of *Arabidopsis* amino acid transporters (*AAP2-6* and *ProT 1-2*) [11–13] allowed the growth of a GABA transport-deficient yeast mutant on high concentrations of GABA. Previously, ProT1 and ProT2 were identified as H$^+$-coupled transporters that exhibit considerable selectivity for proline and are able to discriminate against many of the common α-amino acids [13]; GABA as a potential substrate was never tested. In the present study, uptake experiments with [1-^{14}C]GABA demonstrated that ProT2 mediates GABA transport. However, calculation of the catalytic efficiency (V_{max}/K_m), simultaneous studies of [1-^{14}C]GABA and [2,3-^{3}H]proline transport, and competition of [1-^{14}C]GABA uptake by D- or L-proline versus GABA, indicated that ProT2 preferred proline over GABA. A similar preference has been shown for the proline transporter (PUT4) from *S. cerevisiae* [15]. Furthermore, the ratio of GABA/proline transport increased with the pH-dependent availability of zwitterionic GABA. Therefore, GABA as well as proline (which was

present in the fully ionized state under the experimental conditions used), seem to be preferentially transported in the zwitterionic or neutral form. GABA in this form may adopt a cyclic structure, which according to Christensen et al. [25], is structurally similar to proline.

Since ProT2 had a preference for zwitterionic GABA, the apparent K_m for neutral GABA at pH 4.0 can be recalculated as 0.82 mM, one-half of the value for total GABA and double that for proline. With respect to other non-plant GABA transporters, the affinity of ProT2 for GABA is between those of the low affinity vesicular GABA transporters [26] and the high affinity animal, fungal and bacterial GABA transporters [7–10]. The GABA transporter from *E. coli* (GabP) prefers to transport zwitterionic GABA [27,28] and analogs that mimic a cyclic conformation of GABA [28].

The most effective inhibitors of GABA transport by ProT2 were betaines, which also accumulate and are probably transported in plants in response to stress [29,30]. Glycine betaine and its immediate metabolic precursors, betaine aldehyde and choline, were strong inhibitors; other betaines, including carnitine, trigonelline and betonicine, also inhibited GABA transport, but were not as effective. As some of these compounds are achiral, and both D- and L-proline inhibited GABA uptake, it is suggested that ProT2 cannot discriminate between stereoisomers [31,32]. Despite the structural dissimilarity between these compounds, the separation distance between polar functional groups may be the main factor determining substrate recognition. For example, the extra methyl group in 3-aminobutyrate prevents the charge separation in comparison to 3-aminopropionate, thereby improving the efficacy of its transport by *E. coli* GabP [28]. The ability of ProT2 to mediate significant [methyl-^{14}C]choline transport suggests that the inhibition of GABA transport by choline reflects competition at the binding site, rather than inhibition at an allosteric site. Glycine betaine transport and inhibition of proline uptake has recently been shown for LeProT1, a member of the ProT family from tomato [33]. Thus, the ProTs may represent general carriers that facilitate the transport of a variety of stress-related compounds which could act as osmolytes, free radical scavengers and protein stabilizers.

Heterologous complementation of a GABA transport-deficient yeast strain identified the previously reported AAP3 (AAP3′) as a putative GABA transporter. Even though the affinity of AAP3′ was about one order of magnitude lower than ProT2 and several orders of magnitude lower than non-plant GABA transporters [7–10], yeast cells expressing *AAP3′* grew better than those expressing other plant amino acid transporter genes. Growth of *S. cerevisiae* using glucose results in acidification of unbuffered medium through an activation of the plasma membrane H$^+$-ATPase [35], thereby decreasing the availability of zwitterionic GABA, the form recognized by ProT2. In addition to zwitterionic GABA, yeast cells expressing the general amino acid permease *AAP3* may also transport dibasic GABA, thereby increasing the nitrogen availability to these cells. Dibasic GABA has a charge symmetry similar to lysine, which is a good transportable substrate of AAP3 [12].

In planta, the apoplasmic pH is about 5.5 [36], implying that 95% of GABA is in the zwitterionic form. Furthermore, in unstressed plants, the GABA levels in xylem sap are about 0.1 mM [36,37] and are dramatically increased (230%) during drought conditions [37]; the level of this amino acid in the

phloem during severe plant stress conditions has never been determined. GABA concentrations in the range of 6–39 mM in cell suspension cells adapted to water stress have been documented [38–40]. In *Arabidopsis*, *AAP3* is expressed in roots [12], a result consistent with the observed growth of *Arabidopsis* on GABA as the sole N source and *ProT2* is induced under stress conditions. These data suggest that AAP3 and ProT2 are physiologically significant for the transport of GABA within the plant.

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