ORIGINAL ARTICLE

BAT1, a bidirectional amino acid transporter in *Arabidopsis*

Ekrem Dündar · Daniel R. Bush

Abstract The *Arabidopsis thaliana* At2g01170 gene is annotated as a putative gamma amino butyric acid (GABA) permease based on its sequence similarity to a yeast GABA transporting gene (*UGA4*). A cDNA of At2g01170 was expressed in yeast and analyzed for amino acid transport activity. Both direct measurement of amino acid transport and yeast growth experiments demonstrated that the At2g01170 encoded-protein exhibits transport activity for alanine, arginine, glutamate and lysine, but not for GABA or proline. Significantly, unlike other amino acid transporters described in plants to date, At2g01170 displayed both export and import activity. Based on that observation, it was named bidirectional amino acid transporter 1 (*BAT1*). Sequence comparisons show BAT1 is not a member of any previously defined amino acid transporter family. It does share, however, several conserved protein domains found in a variety of prokaryotic and eukaryotic amino acid transporters, suggesting membership in an ancient family of transporters. *BAT1* is a single copy gene in the *Arabidopsis* genome, and its mRNA is ubiquitously expressed in all organs. A transposon—GUS gene-trap insert in the *BAT1* gene displays GUS localization in the vascular tissues (Dundar in Ann Appl Biol, 2009) suggesting BAT1 may function in amino acid export from the phloem into sink tissues.

Keywords Amino acid transport · Nitrogen metabolism · Assimilate partitioning

E. Dündar
Biyoloji Bölümü, Fen Edebiyat Fakültesi,
Balıkesir Üniversitesi, Balıkesir, Turkey
e-mail: ekremdundar@yahoo.com

E. Dündar
Program in Physiological and Molecular Plant Biology,
University of Illinois at Urbana-Champaign,
Urbana, IL 61801, USA

D. R. Bush (✉)
Department of Biology, Colorado State University,
Fort Collins, CO 80523, USA
e-mail: dbush@colostate.edu

Introduction

Amino acids are the currency for nitrogen exchange in plants as multicellular organisms (Bush 1999). Amino acid transporters are the principle mediators of nitrogen distribution in plants and hence, they are important regulators of resource allocation. Physiological and molecular analysis of all classes of amino acid transporters is essential to establishing a complete understanding of resource allocation in plants (Bush 1993; Fischer et al. 1998; Ortiz-Lopez et al. 2000). Moreover, determining the molecular regulation and transport mechanisms of plant amino acid transporters holds great promise for manipulating the amino acid content and nutritional value of important crops (Patrick 1988; Fischer et al. 1998; Bush 1999; Liu and Bush 2006).

γ-Aminobutyric acid or 4-aminobutyric acid (GABA) is a four-carbon nonprotein amino acid that is found ubiquitously in many organisms (Narayan and Nair 1990). In humans and animals it functions as an essential inhibitory neurotransmitter (McCormick 1989). In plants, it has been widely reported to accumulate under various stress conditions and many functions have been attributed to GABA accumulation including pH regulation, nitrogen storage, developmental regulation, and plant defense (Shelp et al. 1999). Because of the variety of functions proposed for GABA, several groups have looked for GABA transporters. One study identified two previously described amino acid transporters, AAP3 and ProT2, as GABA transporters

based on their GABA transport capacity (Breitkreuz et al. 1999). More recently, a high affinity GABA transporter was described in *Arabidopsis thaliana* (Meyer et al. 2006).

A single copy *Arabidopsis* gene, At2g01170, has been annotated in the *Arabidopsis* genome as a putative GABA permease based on its sequence similarity to known transporters. It has an overall amino acid identity of 34% with the GABA permease of *Aspergillus nidulans* (Hutchings et al. 1999), 29% with the yeast GABA permease *UGA4* (Andre et al. 1993), and 27% with the yeast choline transport protein (Nikawa et al. 1990). Sequence comparisons of At2g01170 at the amino acid level against the NCBI plant protein database identified orthologous permeases in rice with sequence identity as high as 73%. CAT1 (Frommer et al. 1995), a cationic amino acid transporter with 23% identity was the highest level of similarity in the *Arabidopsis* genome.

Amino acid transporters characterized in plants to date are unidirectional, proton-coupled amino acid symporters that import amino acids into the cell using the proton motive force generated across the plasma membrane by the H-pumping ATPase (Bush 1993). In contrast, uptake assays with ^{14}C-labeled amino acids and growth experiments on amino acid containing plates of defined media suggested a bidirectional, facilitated transport activity for At2g01170. Therefore this gene was named *BAT1* for Bidirectional Amino Acid Transporter 1.

Transport of solutes in both directions across the plasma membrane can be mediated by a variety of independent importers and exporters (Bush 1993). Alternatively, bidirectional movement through a single facilitated transporter is another mechanism that has been well described in various organisms (Bush 1993; Solsbacher et al. 2000; Kottra and Daniel 2001; Boulland et al. 2003). Bidirectional amino acid transporters in plants have not been reported to date and thus, BAT1 appears to be the first example of a transporter capable of amino acid export across the plasma membrane.

Materials and methods

Escherichia coli strains and growth conditions

Escherichia coli (*E. coli*) strains used for transformation and cloning were DH5-α and Top10 (Invitrogen Corp., Carlsbad, CA, USA), and they were maintained in Luria-Bertani (LB) medium (2% glucose, 2% Bacto Tryptone, 1% yeast extract, 0.1% NaCl, pH 7, 2% Bacto agar for solid media). For selecting transformants, ampicillin (100 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) were added to the media. Cells were transformed using electroporation as described

by the manufacturer's manual for the BioRad Pulser (Bio-Rad, Hercules, CA, USA).

Subcloning *BAT1* into NEVE

The EST clone M41C3STM was obtained from *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University in a Blue Script SK plasmid. The purified pBSBAT1 was used as a template to amplify the *BAT1* cDNA with T3 and T7 primers. The PCR-amplified cDNA was then cloned into pCR4.0 (TOPO TA cloning, Invitrogen Corp., Carlsbad, CA, USA), and then subcloned into NEVE (Sauer et al. 1990). *E. coli* strain DH5-α was transformed with NEVEBAT1. Colonies carrying 5′ to 3′ oriented *BAT1* cDNA in front of the *S. cerevisiae PMA1* promoter in NEVE were identified using restriction digestion (Sauer et al. 1990).

Yeast media and transformation

Saccharomyces cerevisiae strain JT16 [*MATa hip1-614 his4-401 ura3-52 ino1 can1*] (Tanaka and Fink 1985) was transformed with NEVEBAT1 using the polyethylene glycol method in pESC Yeast Epitope Tagging Vectors Instruction Manual (Stratagene, La Jolla, CA, USA). Transformed yeast cells were selected on a modified version of S1 medium (Hsu et al. 1993) containing 0.17% yeast nitrogen base (without NH$_4$SO$_4$, without amino acids), 2% Bacto™ Agar (BD Biosciences, Boston, MA, USA), 2% glucose, 0.5% NH$_4$SO$_4$, 0.002% inosine, 0.01% arginine, and 0.002% histidine (Sigma-Aldrich, St Louis, MO, USA). *AAP1* (Hsu et al. 1993) was used as a control.

Amino acid transport in yeast

Amino acid transport in yeast was quantified using ^{14}C-labeled amino acids as adapted from Hsu et al. (1993). The ^{14}C-labeled amino acids were purchased from Perkin Elmer (Boston, MA, USA). Three-day-old yeast colonies were inoculated into the modified S1 media and grown until mid-logarithmic phase. An aliquot of this culture was used to inoculate 50 mL of fresh media, and grown overnight. The cells from BAT1 expressing and control (insert-free NEVE cells) flasks were harvested at the desired optical density, washed once with amino acid free growth media and collected in the same media. Transport experiments were initiated by adding an aliquot of suspended yeast cells (5–10 mg of fresh weight) into the transport solution (400 μL, pH 5) that contained 0.4 μCi of ^{14}C-labeled amino acid and unlabelled amino acid to a final concentration of 200 μM. An aliquot of the suspended yeast cells were collected at 4 and 8 min on a micropore filter under vacuum. Transport was linear over this time frame and rates were calculated based on the slope of the line. The cells were

washed twice with 2 mL of cold transport buffer (growth media minus required amino acids plus 200 μM of the amino acid being assayed) and accumulated radioactivity was measured with liquid scintillation spectroscopy. Transport assays were repeated at least three times with a standard error that was less than 10%.

Arabidopsis growth conditions

Arabidopsis seeds were soaked in 95% alcohol for 10 min, 20% Clorox plus 0.1% tween 20 for 5 min, washed twice with sterile water, and suspended in 400–800 μL 0.1% agar. The sterilized seeds were transferred onto MS plates containing 1% sucrose, and 4.5 g L^{-1} Agargel (Sigma-Aldrich, St Louis, MO, USA) or 6 g L^{-1} Bacto™ Agar (BD Biosciences, Boston, MA, USA) and kanamycin (50 μg mL^{-1}). Plated seeds were stratified in a cold room for 1–3 days, and then they were transferred into a controlled growth chamber that was set to 21°C with a 10 h light (150 μE m^{-2} s^{-1}) and 14 h dark cycle. For soil grown plants, seedlings were transferred into well-watered Sunshine Mix soil (Wetsel Seed Co., Harrisonburg, VA, USA), and placed in chambers set to 21°C with a 8 h light (200 μE m^{-2} s^{-1}), 16 h dark cycle.

RNA extraction

Total RNA was isolated based on Logemann et al. (1987). Half a gram of *Arabidopsis* tissue was ground in a mortar with liquid nitrogen. 1 mL extraction buffer [25 mM Tris HCL pH 8, 25 mM ethylene diamine tetra acetic acid (EDTA), 75 mM NaCl, 1% sodium dodecyl sulfate, and 1 M β-mercaptoethanol] was added followed by additional grinding. The homogenate was transferred to a microfuge tube and mixed with an equal volume (1 mL) of phenol:chloroform:isoamyl alcohol (25:24:1). The solution was then centrifuged for 10 min at 12,000 rpm at 4°C. The aqueous phase was extracted with phenol–chloroform and then with chloroform. The aqueous phase was then incubated at 4°C in 2 M LiCl overnight, and centrifuged 20 min at 4°C at 12,000 rpm. The pellet was rinsed with 2 M LiCl, resuspended in 500 μL TE (Tris-EDTA pH 8) after air drying, and precipitated by adding 3 M sodium acetate pH 5.2 (0.1 volume) and 100% ice cold ethanol (2.5 volume). The solution was then centrifuged 10 min at 12,000 rpm at 4°C, after incubating 15–45 min at −80°C. The pellet was washed with ice-cold 70% ethanol (centrifuged 1 min at 14,000 rpm), and resuspended in 30–50 μL DEPC water after air or vacuum drying. All solutions used were prepared with DEPC-treated water and autoclaved. The mortar and pestle were treated with RNaseZap (Ambion, Inc., Austin, TX, USA) before use.

RNA gel-blot analysis

RNA gel-blot analysis was as described in Sambrook et al. (1989). Briefly, 20 μg total RNA from each sample was separated on a formaldehyde gel at 50 V for 3 h (3–4 V cm^{-1}). The gel was composed of 1% agarose, 1× formaldehyde gel-running buffer (0.1 M MOPS pH 7, 40 mM sodium acetate pH 5.2, and 5 mM EDTA pH 8, FGB), and 2.2 M formaldehyde. RNA samples were prepared in 2 μL 5× FGB, 3.5 μL formaldehyde, 4 μL of gel loading buffer (50% glycerol, 1 mM EDTA pH 8, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 1 μg mL^{-1} ethidium bromide), and brought to a total volume of 25 μL with DEPC treated-autoclaved water. The samples were heated at 65°C for 15 min and then chilled on ice.

RNA was transferred onto Hybond N+ membranes following the manufacturer's instructions (Amersham-Pharmacia, Piscataway, NJ, USA). The probe was a 1 kb fragment of *BAT1* cDNA amplified via PCR using primers 1 kb-L (5′-TTC TCC AAT TTC GCC ATC TC-3′) and 1 kb-R (5′-ATC GCT CCA TCT CTC GAA AA-3′). Probes were prepared using a Megaprime DNA labeling system (Amersham-Pharmacia, Piscataway, NJ, USA) and α-^{32}P-dCTP (Perkin Elmer Boston, MA, USA), and cleaned with Bio-Spin columns (Bio-Rad, Hercules, CA, USA). The specific activity of each probe was measured using a liquid scintillation spectroscopy.

Pre-hybridization and hybridization were performed in Ultrahyb™ (Ambion, Inc., Austin, TX, USA) for 16 h at 42°C. Blots were washed twice in 50 mL 2× SSC/0.1% SDS for 5 min each time, and twice in 50 mL 0.1× SSC/0.1% SDS for 15 min each time. Both washes were in 42°C. The blots were then exposed to an X-ray film for 1–7 days in an X-ray cassette at −80°C.

PCR and sequencing

RT-PCR was performed using the Retroscript RT-PCR kit (Ambion, Inc., Austin, TX, USA) following the manufacturer's instructions. 2 μg total RNA template was used for reverse transcription and PCR was performed in a DNA-Engine PTC-200 (MJ Research, Inc., Watertown, MA, USA). The PCR primers for *BAT1* cDNA were 1 kb-L and 1 kb-R as given above, and they were designed to amplify a 1 kb region of *BAT1* cDNA and a 1.2 kb region of *BAT1* genomic DNA, to easily detect any genomic DNA contamination.

Templates (1 μL) from transcriptase reactions were used for real-time PCR using Qiagen's Sybergreen PCR kit (Qiagen Inc., Valencia, CA, USA) and a Smart Cycler System (Cepheid, Sunnyvale, CA, USA). The primers used for real-time PCR were as follows: UQ10-L (5′-CGA CTT GTC ATT AGA AAG AAA GAG ATA ACA GG-3′),

UQ10-R (5′CTT GGT CCT GCG TCT TCG TGG TGG TTT C-3′), BAT1-L (5′-GGG TAT GCC ATT GCT GAG AT-3′), BAT1-R (5′-ATC GCT CCA TCT CTC GAA AA-3′). All real-time PCR primers were designed to amplify a 170–200 bp long product, and had a melting temperature of 59–60°C. UBQ-10 (At4g05320, Acc no: NM_178970) was used as a normalizer.

For sequencing, the PCR products were gel extracted using a Gel Extraction kit (Qiagen Inc., Valencia, CA, USA), and sequenced by the Keck Center at the University of Illinois at Urbana-Champaign. Align and EditSeq (DNA-STAR, Inc., Madison, WI, USA) were used for sequence analysis. Hydropathy plots were generated with the Kyte and Doolittle (1982) algorithm using DNA Strider 1.1 (Marck 1988) while membrane topology predictions was generated using TopPred II (Claros and von Heijne 1994). Phylogenetic analysis was conducted using Clustal W algorithm (Thompson et al. 1994) and Phylip program (Felsenstein 1989).
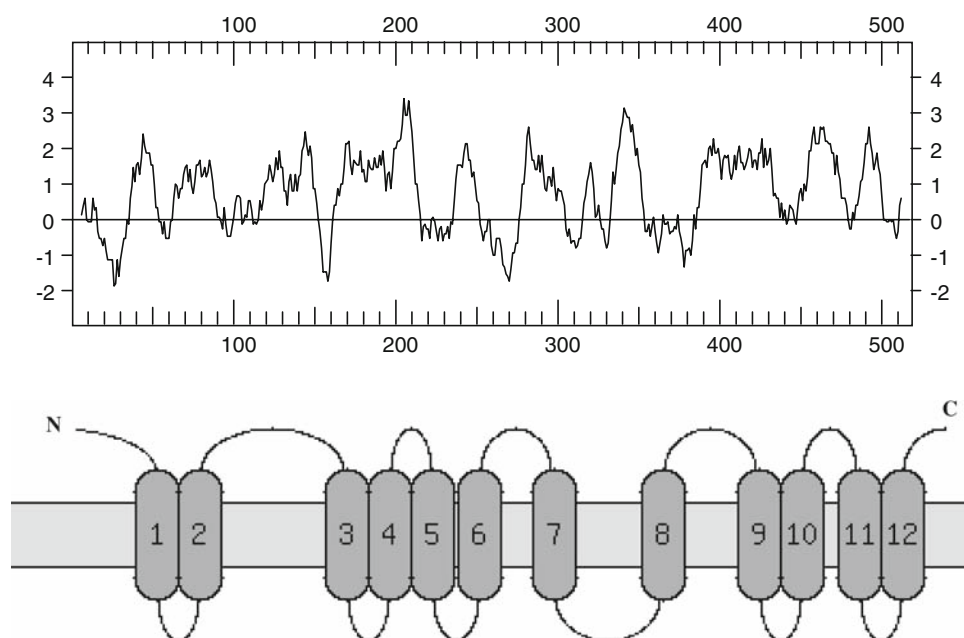
Results

Amino acid transport in BAT1 expressing yeast cells

We undertook an investigation of the amino acid transport activity of At2g01170 (BAT1) because of its sequence similarity with previously described amino acid transporters. Moreover, hydropathy analysis and membrane topology predictions suggested BAT1 is a polytopic, integral membrane protein with 12 transmembrane domains (Fig. 1). Although it exhibits a maximum of 34% identity to any pre-

viously characterized transporters, it does match multiple conserved domains in several amino acid transporters when searched against the conserved domain database (Marchler-Bauer et al. 2005, 2007). Those amino acid transporters belong to the AA permease (pfam00324: amino acid permease), LysP (COG0833: amino acid transporters), AnsP (COG1113: GABA permease and related permeases), and PotE (COG0531: amino acid transporters) families (Fig. 2). Among the known plant amino acid transporters, only BAT1 and CAT1 matched more than two conserved protein domains. In contrast, the other plant transporters matched only two protein domains. Likewise, BAT1 and CAT1 are classified as members of the amino acid–polyamine–organocation (APC) Superfamily (Fischer et al. 1998; Jack et al. 2000) (see also Aramemnon Database at http://aramemnon.botanik.uni-koeln.de). A phylogenetic analysis of BAT1 and other plant amino acid transporters using Clustal W algorithm (Thompson et al. 1994) and Protdist function of BioEdit program (Hall 1999) confirmed Fischer et al.'s (1998) suggestion of placing BAT1 as the only member of a separate family (GABA permease related family: note that this name originates from the annotation in the public database) under the APC superfamily, while also placing the BAT family closer to the multi-member CAT family (Su et al. 2004) than to other plant amino acid transporter families (Fig. 3).

BAT1 cDNA was obtained from the Ohio State University *Arabidopsis* Biological Resource Center (ABRC) and sequenced to confirm it was a full length clone that it matches the sequence in the NCBI databank (Altschul et al. 1990). The *BAT1* gene has been predicted to exist in two splice variants (At2g01170.1 with 516 amino acids and

Fig. 1 Hydropathy analysis and membrane topology of BAT1. The Hydropathy plot (*upper panel*) was generated with Kyte and Doolittle (1982) algorithm using DNA Strider 1.1. The membrane topology prediction (*lower panel*) was generated using TopPred II (Claros and von Heijne 1994)

LysP (COG0833: Amino acid transporters)

BAT1	TG**ITTTY**NTGLRF**GGTVT**LVYG**WF**LA**G**S**FTMCVGLSMAE**IC**SSY**P	96
gi 15896412	Lys-specific permease [*Clostridium*]	TG**LFFTS**GNTIST**AG**P**GG**AIAA**YA**IM**G**V**LVYF**LMTSLGE**MATMI**P	79
gi 15832302	Lys-specific permease [*E. coli*]	TG**LFVAS**GATISQ**AG**P**GG**ALLS**YM**LI**G**LMVYF**LMTSLGE**LAAYM**P	79
gi 15674175	Lys-specific permease [*Lactococcus*]	TG**LFLTS**GDVIHT**AG**P**FG**ALTA**YV**LI**G**A**MVYF**LMTSLGE**MATYL**P	97
gi 15599824	Lys-specific permease [*Pseudomonas*]	TG**LFVAS**GATVAT**AG**P**GG**ALLS**YA**LI**G**LMVYF**LMTSLGE**MAAYM**P	78
gi 15927260	Lys-specific permease [*Staphylococcus*]	TG**LFVTS**GGAIHD**AG**A**LG**ALIG**YA**II**G**IMVFF**LMTSLGE**MATYL**P	78
gi 16765530	Lys-specific permease [*Staphylococcus*]	TG**LFVAS**GATISQ**AG**P**GG**ALFS**YI**LI**G**LMVYF**LMTSLGE**LAAYM**P	78
gi 1708894	Lys-specific permease [*S. cerevisiae*]	TG**LFVG**ISTPLSN**AG**P**VG**SLIA**YI**FM**G**TIVYF**VTQSLGE**MATFI**P	172
gi 15802712	Lys-specific permease [*E. coli*]	TG**LFVAS**GATISQ**AG**P**GG**ALLS**YM**XI**G**LMVYF**LMTSLGE**LAAYM**P	79
gi 16130094	Lys transporter [*E. coli*]	TG**LFVAS**GATISQ**AG**P**GG**ALLS**YM**LI**G**LMVYF**LMTSLGE**LAAYM**P	79

AnsP (COG1113: GABA permease and related permeases)

BAT1	LA**G**SFT**MC**VGL**SMAE**ICSSYPTSG**GL**YY**W**SAMLA**G**P**RWAP**LAS**W**M**TGWF**	122
gi 15841162	Amino acid permease [*Mycobacterium*]	II**G**FFV**FF**VLRA**MGE**LLLSNLNYK**SF**VDFAADLL**G**P**AAGF**FVG**W**SYWFA	108
gi 3025135	Putative transporter [*Bacillus*]	IT**G**IIC**FL**IMR**SLGE**LLLSNLNYH**SF**VDFVQDYL**GD**MAAF**ITG**W**TYWFC	106
gi 19551713	GABA permease [*Corynebacterium*]	II**G**FML**FF**VMRA**MGE**LLLANLNYK**SL**RDAVSDIL**G**P**GAGF**VTG**W**TYWFC	88
gi 15829868	Phe transport system [*E. coli*]	VA**G**IIA**FL**IMRQ**LGE**MVVEEPVSG**SF**AHFAYKYW**G**P**FAGF**LSG**W**NYWVM	110
gi 15600290	Probable aap [*Pseudomonas* PA01]	VA**G**AAV**YM**VMRA**LGE**MAVHEPVSG**SF**GHYASSYL**G**P**LAGF**VTG**W**TYAFE	98
gi 15800289	Probable aap [*Pseudomonas* PA01]	VA**G**IIA**FL**IMRQ**LGE**MVVEEPVSG**SF**AHFAYKYW**G**P**FAGF**LSG**W**NYWVM	110
gi 16799854	Hypothetical protein [*Listeria*]	II**G**AIL**FF**VMRA**LGE**LLIHNPTTG**SF**TEFAEQFI**G**P**WAGF**ITG**W**TYWFC	99
gi 130068	Phe specific permease [*E. coli*]	VA**G**IIA**FL**IMRQ**LGE**MVVEEPVSG**SF**AHFAYKYW**G**P**FAGF**LSG**W**NYWVM	110
gi 15608842	Probable Ser/Ala/Gly transporter [*Mycobacterium*]	II**G**FFV**FF**VLRA**MGE**LLLSNLNYK**SF**VDFAADLR**G**P**AAGF**FVG**W**SYWFA	108

PotE (COG0531: Amino acid transporters)

BAT1	**W**FLAGSFTMCVGLS**MAE**ICS**SY**PT**S**GGLY**YW**SAMLA	107
gi 15965967	Putative aap [*Sinorhizobium*]	**W**PLGCFISFVFAVA**MAQ**ISS**AY**PT**A**GGLY**HW**GSILG	103
gi 15214395	Uncharacterized aap [*S. pombe*]	**W**LIAMFFLICVSMS**MAE**ICS**AM**PT**S**GGLY**YA**AAVFA	139
gi 1351705	Uncharacterized aap [*S. pombe*]	**W**IIGCICLIPVSIS**LGE**LAS**SM**PT**S**GGLY**FW**IFTLA	121
gi 19112716	Hypothetical protein [*S. pombe*]	**W**LIAGAGCMCIALS**VAE**LVS**AY**PT**S**GGLY**FT**CKDLV	135
gi 1351645	Uncharacterized aap [*S. pombe*]	**W**LIAMVFVQCVANG**MAE**LCS**SM**PT**S**GGLY**YA**AAVLA	115
gi 15214364	Uncharacterized aap [*S. pombe*]	**W**LIAMVFIICIALS**MAE**LCS**AM**PT**S**GGLY**YA**AAVLA	134
gi 15214362	Uncharacterized aap [*S. pombe*]	**Y**IIAIPLQLISAAT**MAE**VCS**AL**PT**A**GSLY**FW**ASAAG	119
gi 418150	GABA permease [*S. cerevisiae*]	**W**FVAAFFILLVGIT**MAF**HAS**SI**PT**A**GGLY**YW**TYYYA	147
gi 13472699	Amino acid permease [*Mesorhizobium*]	**W**LVGCFVSLTFAVA**MSQ**ISS**AY**PT**A**GGLY**HW**GSILG	105

AA_permease (pfam00324: Amino acid permease)

BAT1	**F**VLGLLMSQ**Y**TIT**G**YDASAHMTE**E**TVD**A**D**K**NG**P**RGIISAIGIS	283
gi 75058334	Na-K-Cl cotransporter [*Bos taurus*]	**F**FSVFAIFF**P**AAT**G**ILAGANISG**D**LAD**P**Q**SA**I**P**KGTPLAILIT	521
gi 34395946	Lys-specific permease [*E. coli*]	**M**IGVAMIVG**F**SFQ**G**TELIGIAAG**E**SED**P**AK**N**I**P**RAVRQVFWRI	249
gi 77416678	Ser/Ala/Gly transporter [*E. coli*]	**F**FAGFQIAV**F**AFV**G**IELVGTTAA**E**TKD**P**EK**S**L**P**RAINSIPIRI	256
gi 728862	L-Asn permease [*Salmonella*]	**A**LVLIQGVV**F**AFA**S**IELVGTAAG**E**CKD**P**QK**M**V**P**KAINSVIWRI	264
gi 462414	Aap [*Trichoderma*]	**F**CSVFVTAA**F**SFS**G**TELVGLAAA**E**STN**P**TK**N**M**P**GAIKQVFWRI	300
gi 1703255	Basic aap [*S. cerevisiae*]	**W**VSSLINAA**F**TYQ**G**TELVGITAG**E**AAN**P**RK**A**L**P**RAIKKVVVRI	311
gi 586542	General aap AGP2 [*S. cerevisiae*]	**F**LACLIQAS**F**TIA**G**GEYISMLAG**E**VKR**P**RK**V**L**P**KAFKQVFVRL	331
gi 549738	Polyamine transporter [*S. cerevisiae*]	**F**IIGFQQSN**F**TLQ**G**FSMLPALAD**E**VKV**P**EK**D**I**P**RGMSNAVLLS	302
gi 74752434	Cation-chloride cotransporter-interacting protein 1 isoform b. [*H. sapiens*]	**F**ASVFAVLF**N**GCT**G**IMAGANMSG**E**LKD**P**SR**A**I**P**LGTIVAVAYT	309

Fig. 2 Partial alignments of BAT1 and other transport proteins *highlighting* conserved protein domains. BAT1 aligned with nine proteins exhibiting the highest level of conserved protein domains from the Conserved Domain Databank (Marchler-Bauer et al. 2005, 2007). Identical residues are *boxed*. Significant positives are labeled with *bold face*. *Numbers* at the end of each domain sequence are the residue position of the respective proteins

At2g01170.2 with 437 amino acids) based on genomic annotation (NCBI Databank). We decided to work with the longer version which is consistent with the EST sequences reported (GenBank ID numbers: 86080462, 125317171, 49161850, 5845280, 8697411, 19834984, 19804998, 42530307, 85924219, 37428902, 2596680, 85924222, 124788928, 42534435, 85924220, 125128551, 86082258, 42535234, 19860171, 125152801, 8720353, 85924218, 23302765, 85924221, 42533747). The *BAT1* cDNA was subcloned into yeast expression vector NEVE (Sauer et al. 1994) and transformed into *Saccharomyces cerevisiae* (JT16) for transport and growth experiments.

Initial transport experiments with radiolabeled amino acids yielded contradictory results suggesting substrate import for one amino acid, no activity for another, and evidence of amino acid export activity for other amino acids
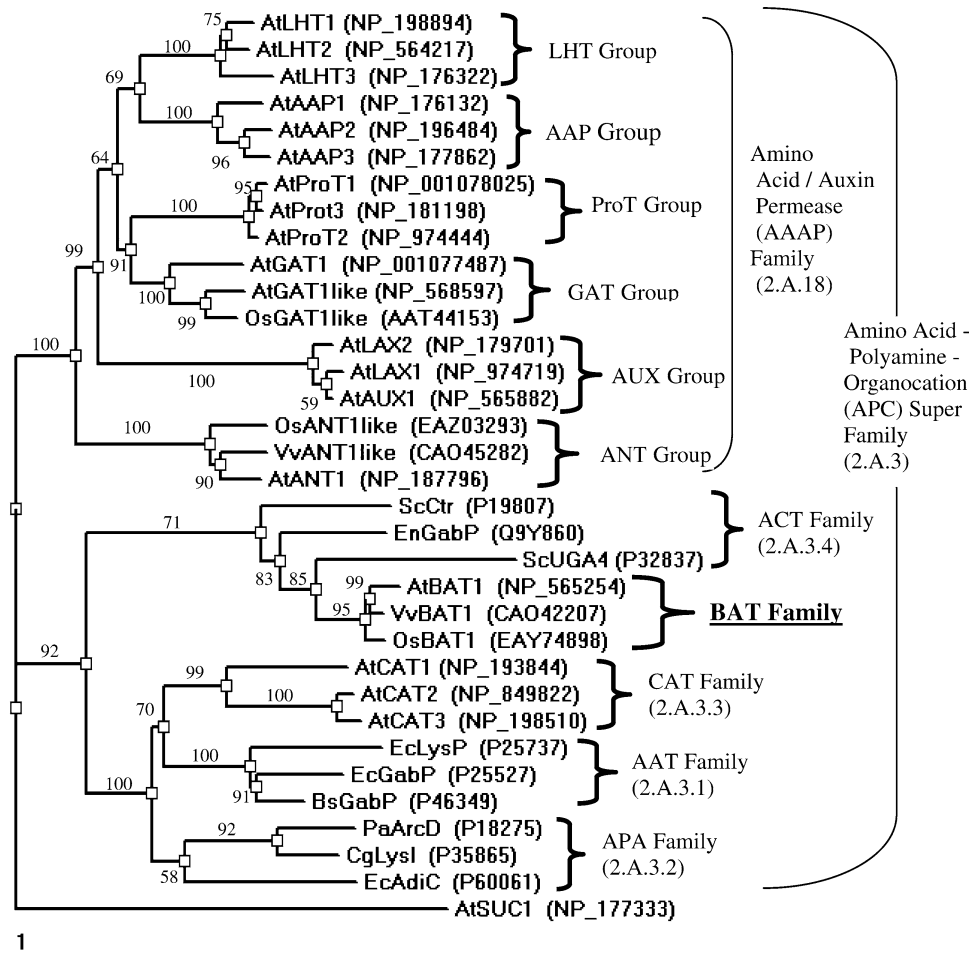
Fig. 3 An unrooted cladogram displaying the phylogenetic position of BAT1 among other transporters. SUC1 was used as an outgroup. The tree was generated using Clustal W algorithm (Thompson et al. 1994) and Protdist function of BioEdit program (Hall 1999). Percentage bootstrap values (of 1,000 replicates) shown on branches of the tree were calculated by PAUP program (Swofford 2001). The accession numbers of each transporter sequence and the Transport Classification Databank (TCDB, http://www.tcdb.org, (Saier 2000) number of each family were indicated in parenthesis. Note that groups (or subfamilies) according to TCDB can be regarded as families, and AAAP family members are classified under ATF (amino acid transporter) super family elsewhere in the literature (Fischer et al. 1998; Grallath et al. 2005; Meyer et al. 2006). Three transporters to represent each family/group were chosen to construct the tree. Organism names were added before each transporter; At: *Arabidopsis thaliana*, Os: *Oryza sativa*, Vv: *Vitis vinifera*, Sc: *Saccharomyces cerevisiae*, En: *Emericella nidulans*, Ec: *Escherichia coli*, Bs: *Bacillus subtilis*, Pa: *Pseudomonas aeroginosa*, Cg: *Corynobacterium glutamicum*. Branch lengths are proportional to phylogenetic distance

(data not shown). Analysis of these results showed the growth stage of the yeast cultures, as measured with OD_{600}, varied slightly in these initial experiments and, therefore, we examined amino acid transport activity of BAT1 at different growth stages. The results in Fig. 4 show that BAT1-expressing cells imported Ala and Arg and exported Glu and Lys. Significantly, we did not observe any transport activity for GABA and proline. Interestingly, we noted maximum transport activity for both controls and BAT1 expressing cells at OD_{600} around 1.0. Surprisingly, BAT1 appears to be an efflux pathway for glutamate during early stages of the growth curve, OD_{600} 0.5–0.8, but then becomes an import pathway at higher cell densities. No evidence for histidine, valine, serine, phenylalanine, tyrosine, tryptophan, and glutamine transport was observed (data not shown).

To explore further BAT1 transport activity, we compared cell growth of BAT1 expressing yeast to controls grown on defined media containing select amino acids. Growth inhibition in response to excess amino acid accumulation often results from feed-back inhibition of biosynthetic pathways associated with that amino acid that negatively impacts the synthesis of other amino acids (Proteau and Silver 1991; Galili 1995; Lee and Duggleby 2001). We observed that almost all amino acids tested inhibit JT16 growth on agar plates that contained more than 3 mM of a given amino acid. Growth inhibition was observed for BAT1-expressing cells

Fig. 4 Amino acid transport activity in BAT1-expressing yeast and controls as a function of OD_{600}. Cells were harvested at the indicated OD_{600}. 200 μM of non-labeled amino acid was present in each transport reaction. Controls represent uptake by yeast cells transformed with insert-free NEVE. Arg and Ala were imported into cells while Lys and Glu were exported. No difference of transport was observed in any phase of cells for GABA and proline between BAT1 expressing cells and the control. Transport assays were repeated at least three times and the standard error for all experiments was less than 10%
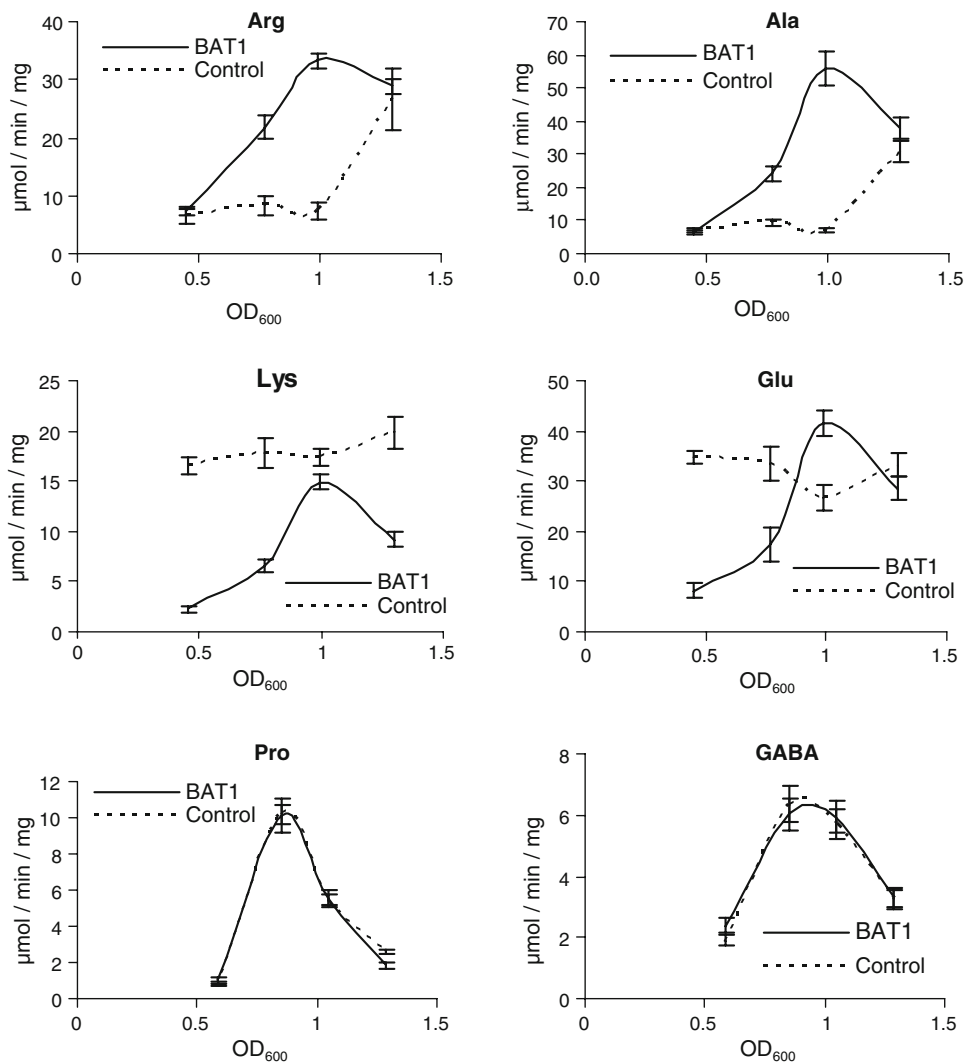
grown on plates containing 5 mM arginine or alanine (Fig. 5), the two amino acids that BAT1 serves as an import pathway. Likewise, growth was inhibited in control cells grown on lysine and glutamate, presumably because of native transporters that accumulated high concentrations of these amino acids resulting in feedback inhibition as noted above. In contrast, BAT1-expressing cells grew well on lysine and glutamate plates, suggesting BAT1 exported excess lysine and glutamate. That observation is consistent with an export function of BAT1 for these two amino acids. Taken together with direct measurements of lysine and glutamate transport, these results are evidence of a bidirectional transport function for BAT1.

Expression pattern of *BAT1* in *Arabidopsis*

RNA gel-blot analysis suggested *BAT1* is widely expressed across early stages of seedling development and in the primary organ systems (Fig. 6a). Those results were confirmed

and extended to cauline leaves in real-time PCR experiment (Fig. 6b).

Discussion

BAT1 is a facilitated transporter that mediates bidirectional transport of amino acids

Both direct quantification of amino acid uptake and growth rates of BAT1-expressing yeast cells suggest BAT1 has bidirectional transport activity. Plant amino acid transporters previously characterized in heterologous expression systems are proton coupled symporters that mediated amino acid uptake (Bush 1999; Ortiz-Lopez et al. 2000; Fischer et al. 2002; Okumoto et al. 2002; Bush et al. 1996; Chen et al. 2001). In contrast, the observations reported here show BAT1 moves amino acids in both directions. Alanine and arginine transport reported in Fig. 4 demonstrates net uptake when compared to non-expressing yeast
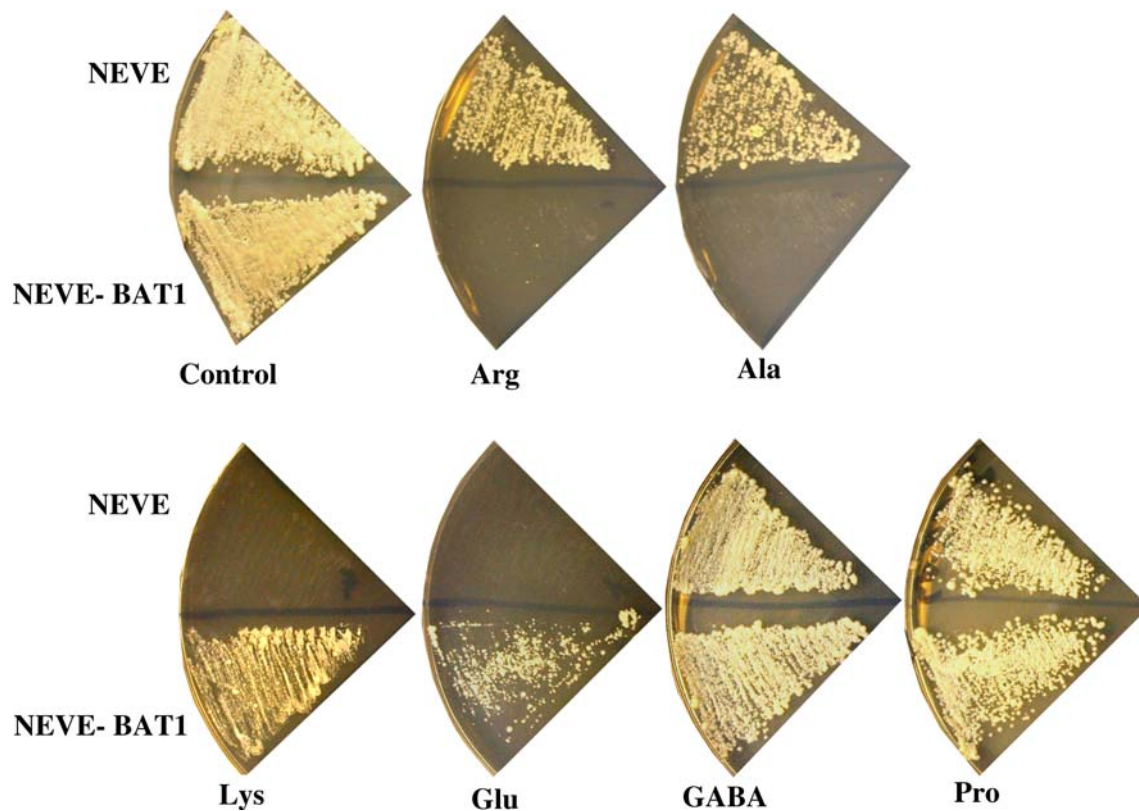
Fig. 5 Growth of BAT1 cDNA expressing yeast cells on 5 mM (pH 6) amino acids. *Top* sectors in each picture contain yeast cells that have the insert free vector NEVE, and the *bottom* ones were streaked with BAT1-expressing yeast cells containing NEVEBAT1. The control plate does not contain any additional amino acids. Pictures were taken at 48 h

Fig. 6 Spatial and temporal expression of *BAT1*. **a** RNA gel-blot analysis. Whole seedlings were used to extract total RNA at weekly time points. *Rt* Roots, *RL* rosette leaves, *St* stems, *F* flowers, *S* siliques, *Cont* control. 20 µg total RNA was loaded for RNA gel-blot analysis. 2 µg total RNA was first run on a 1% agarose gel containing ethidium bromide, and used for the adjustment for the subsequent equal loading. **b** *BAT1* mRNA levels revealed by real-time PCR. *BAT1* expression in organs from 6-week-old, soil grown plants. *UBQ-10* (Polyubiquitin 10, At4g05320, Acc no: NM_178970) was used to normalize *BAT1* transcript numbers (calculated *BAT1* transcript numbers were divided by the calibration factor that was obtained by dividing each UBQ-10 transcript number by the largest one)
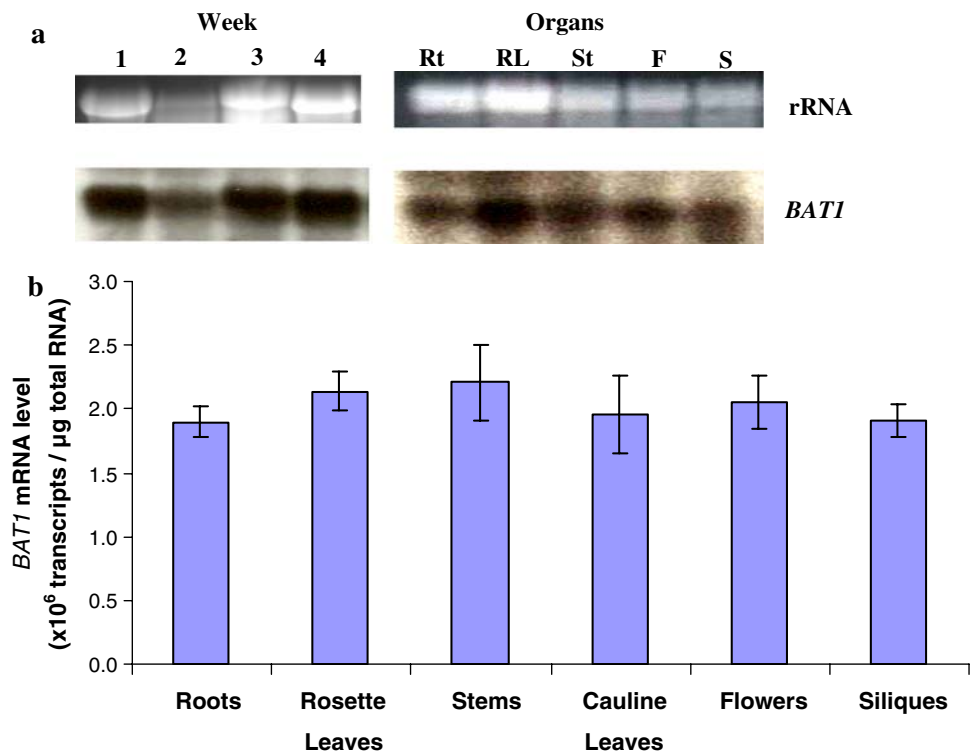
controls at comparable growth stages. Lysine accumulation is much lower in BAT1-expressing cells than in non-expressing controls. This suggests lysine taken up by native yeast transporters is exported by BAT1. Given this evidence for bidirectional movement, we conclude BAT1 mediates facilitated diffusion in which the substrate moves across the membrane down its potential energy gradient (Bush 1993). For alanine and arginine, we observed net uptake, suggesting higher electrochemical potential outside the cells than inside. The lysine data, on the other hand, suggest active uptake by native transport systems accounts for lysine accumulation against its electrochemical gradient in the control cells. In BAT1-expressing cells active accumulation by the yeast transporters is short-circuited by BAT1-mediated efflux of lysine back down its electrochemical potential gradient. Bidirectional transport of amino acids in plants is needed for exchange of amino acids between xylem and phloem (Sharkey and Pate 1975; Gessler et al. 2003), in addition to phloem loading and unloading (Lalonde et al. 2003). A transposon—GUS gene-trap insert in the *BAT1* gene displays GUS localization in the vascular tissues (Dundar 2009). This localization is consistent with BAT1 playing a key role in phloem physiology, representing the first example of an export pathway for phloem unloading to sink tissues.

The growth stage of the yeast cells (OD_{600} level) had a significant effect on amino acid transport in both control and BAT1 expressing yeast cells (Fig. 4). Cimprich et al. (1995) and Pena et al. (1995) reported that the average cytoplasmic pH of yeast cells can change depending on the carbon source they grow on. Imai and Ohno (1995) reported changes in yeast intracellular pH as a function of the growth curve. Cytoplasmic pH was 5.7 during the lag phase of growth, pH 6.8 for log phase and pH 5.5 for stationary phase. A more recent study (Karagiannis and Young 2001) also confirmed the higher pH of log phase than that of stationary phase. Our transport results also varied as a function of the growth curve. That variation may be linked to the changes in cytoplasmic pH reported above. However, it is also possible this variation reflected global changes in yeast gene expression during growth that impacted background activity and/or directly influenced the activity of BAT1.

BAT1 belongs to a new family of amino acid transporters

Bioinformatic analysis of conserved protein domains (Fig. 2) and phylogenetic relationships (Fig. 3) show BAT1 is not a member of previously described plant amino acid transporter families. Results from Conserved Domain Databank (Marchler-Bauer et al. 2005; Marchler-Bauer et al. 2007) blast search revealed that only BAT1 and CAT1 (result for CAT1 is not shown) match four different conserved protein domains (Fig. 2) while the other amino acid transporters match 2 domains at most. Furthermore, the previously described amino acid transporters match eukaryotic protein domains while BAT1 and CAT1 have matches from both prokaryotes and eukaryotes. Likewise, BAT1 and CAT1 are reported to be the members of the same super family named APC superfamily (Fischer et al. 1998; Jack et al. 2000, http://aramemnon.botanik.uni-koeln.de). BAT1 is not confidently placed into any previously described transporter family. Based on our analysis of BAT1 transport activity, phylogenetic relationships and conserved protein domains, we suggest BAT1 is a distinct amino acid transporter in plants.

In conclusion, the data presented here show BAT1 mediates facilitated diffusion of alanine, arginine, glutamate, and lysine. *BAT1* is expressed in all major organ systems, and it appears to be the first example of a bidirectional transporter capable of amino acid export from the cell.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andre B, Hein C, Grenson M, Jauniaux JC (1993) Cloning and expression of the *UGA4* gene coding for the Inducible GABA-specific transport protein of *Saccharomyces cerevisiae*. Mol Gen Genet 237:17–25

Boulland JL, Rafiki A, Levy LM, Storm-Mathisen J, Chaudhry FA (2003) Highly differential expression of SN1, a bidirectional glutamine transporter, in astroglia and endothelium in the developing rat brain. Glia 41:260–275

Breitkreuz KE, Shelp BJ, Fischer WN, Schwacke R, Rentsch D (1999) Identification and characterization of GABA, proline and quaternary ammonium compound transporters from *Arabidopsis thaliana*. FEBS Lett 450:280–284

Bush DR (1993) Proton-coupled sugar and amino acid transporters in plants. Ann Rev Plant Physiol Plant Mol Biol 44:513–542

Bush DR (1999) Amino acid transport. In: Singh BK (ed) Plant amino acids. Marcel Dekker Inc., New York, pp 305–318

Bush DR, Chiou TJ, Chen LS (1996) Molecular analysis of plant sugar and amino acid transporters. J Exp Bot 47:1205–1210

Chen LS, Ortiz-Lopez A, Jung A, Bush DR (2001) ANT1, an aromatic and neutral amino acid transporter in *Arabidopsis*. Plant Physiol 125:1813–1820

Cimprich P, Slavik J, Kotyk A (1995) Distribution of individual cytoplasmic pH values in a population of the yeast *Saccharomyces cerevisiae*. FEMS Microb Lett 130:245–251

Claros MG, von Heijne G (1994) TopPred II: an improved software for membrane protein structure predictions. Comp Appl Biosci 10:685–686

Dundar E (2009) Multiple GUS expression patterns of a single *Arabidopsis* gene. Ann Appl Biol 154:33–41

Felsenstein J (1989) PHYLIP—phylogeny inference package (Version 3.2). Cladistics 5:164–166

Fischer WN, Andre B, Rentsch D, Krolkiewicz S, Tegeder M, Breitkreuz K, Frommer WB (1998) Amino acid transport in plants. Trends Plant Sci 3:188–195

Fischer WN, Loo DDF, Koch W, Ludewig U, Boorer KJ, Tegeder M, Rentsch D, Wright EM, Frommer WB (2002) Low and high

affinity amino acid H+ -cotransporters for cellular import of neutral and charged amino acids. Plant J 29:717–731

Frommer WB, Hummel S, Unseld M, Ninnemann O (1995) Seed and vascular expression of a high-affinity transporter for cationic amino acids in *Arabidopsis*. Proc Natl Acad Sci 92:12036–12040

Galili G (1995) Regulation of lysine and threonine synthesis. Plant Cell 7:899–906

Gessler A, Weber P, Schneider S, Rennenberg H (2003) Bidirectional exchange of amino compounds between phloem and xylem during long-distance transport in Norway spruce trees (*Picea abies* [L.] Karst). J Exp Bot 54:1389–1397

Grallath S, Weimar T, Meyer A, Gumy C, Suter-Grotemeyer M, Neuhaus J-M, Rentsch D (2005) The AtProT family. Compatible solute transporters with similar substrate specificity but differential expression patterns. Plant Physiol 137:117–126

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucl Acids Symp Ser 41:95–98

Hsu LC, Chiou TJ, Chen LS, Bush DR (1993) Cloning a plant amino acid transporter by functional complementation of a yeast amino acid transport mutant. Proc Natl Acad Sci 90:7441–7445

Hutchings H, Stahmann KP, Roels S, Espeso EA, Timberlake WE, Arst HN, Tilburn J (1999) The multiply-regulated gabA gene encoding the GABA permease of *Aspergillus nidulans*: a score of exons. Mol Microb 32:557–568

Imai T, Ohno T (1995) Measurement of yeast intracellular pH by image processing and the change it undergoes during growth phase. J Biotech 38:165–172

Jack DL, Paulsen IT, JrMH Saier (2000) The amino acid/polyamine/organocation (APC) superfamily of transporters specific for amino acids, polyamines and organocations. Microb 146:1797–1814

Karagiannis J, Young PG (2001) Intracellular pH homeostasis during cell-cycle progression and growth state transition in *Schizosaccharomyces pombe*. J Cell Sci 114:2929–2941

Kottra G, Daniel H (2001) Bidirectional electrogenic transport of peptides by the proton-coupled carrier PEPT1 in *Xenopus laevis* oocytes: its asymmetry and symmetry. J Phsiol London 536:495–503

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157:105–132

Lalonde S, Tegeder M, Throne-Holst M, Frommer WB, Patrick JW (2003) Phloem loading and unloading of sugars and amino acids. Plant Cell Environ 26:37–56

Lee YT, Duggleby RG (2001) Identification of the regulatory subunit of *Arabidopsis thaliana* acetohydroxyacid synthase and reconstitution with its catalytic subunit. Biochemistry 40:6836–6844

Liu X, Bush DR (2006) Expression and transcriptional regulation of amino acid transporters in plants. Amino Acids 30:113–120

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163:16–20

Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Bryant SH (2005) CDD: a conserved domain database for protein classification. Nucleic Acids Res 33:D192–D196

Marchler-Bauer A, Anderson JB, Derbyshire MK, DeWeese-Scott C, Gonzales NR, Gwadz M, Hao L, He S, Hurwitz DI, Jackson JD, Ke Z, Krylov D, Lanczycki CJ, Liebert CA, Liu C, Lu F, Lu S, Marchler GH, Mullokandov M, Song JS, Thanki N, Yamashita RA, Yin JJ, Zhang D, Bryant SH (2007) CDD: a conserved domain database for interactive domain family analysis. Nucleic Acids Res 35:237–240

Marck C (1988) "DNA Strider": a "C" program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res 16:1829–1836

McCormick DA (1989) GABA as an inhibitory neurotransmitter in human cerebral cortex. J Neurophysiol 62:1018–1027

Meyer A, Eskandari S, Grallath S, Rentsch D (2006) AtGAT1, a high affinity transporter for γ-aminobutyric acid in *Arabidopsis thaliana*. J Biol Chem 281:7197–7204

Narayan VS, Nair PM (1990) Metabolism, enzymology and possible roles of 4-aminobutyrate in higher-plants. Phytochemistry 29:367–375

Nikawa J, Hosaka K, Tsukagoshi Y, Yamashita S (1990) Primary structure of the yeast choline transport gene and regulation of its expression. J Biol Chem 265:15996–16003

Okumoto S, Schmidt R, Tegeder M, Fischer WN, Rentsch D, Frommer WB, Koch W (2002) High affinity amino acid transporters specifically expressed in xylem parenchyma and developing seeds of *Arabidopsis*. J Biol Chem 277:45338–45346

Ortiz-Lopez A, Chang HC, Bush DR (2000) Amino acid transporters in plants. Biochim Biophys Acta 1465:275–280

Patrick JW (1988) Assimilate partitioning in relation to crop productivity. Hortscience 23:33–40

Pena A, Ramirez J, Rosas G, Calahorra M (1995) Proton pumping and the internal pH of yeast cells, measured with pyranine introduced by electroporation. J Bacteriol 177:1017–1022

Proteau G, Silver M (1991) Acetohydroxy acid synthetase of *Thiobacillus acidophilus*. Biomed Lett 46:121–128

Saier MHJ (2000) Families of transmembrane transporters selective for amino acids and their derivatives. Microbiology 146:1775–1795

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sauer N, Caspari T, Klebl F, Tanner W (1990) Functional expression of the *Chlorella* hexose transporter in *Schizosaccharomyces pombe*. Proc Natl Acad Sci 87:7949–7952

Sauer N, Baier K, Gahrtz M, Stadler R, Stolz J, Truernit E (1994) Sugar transport across the plasma membranes of higher plants. Plant Mol Biol 26:1671–1679

Sharkey PJ, Pate JS (1975) Selectivity in xylem to phloem transfer of amino acids in fruiting shoots of white lupin (*Lupinus albus* L.). Planta 127:251–262

Shelp BJ, Bown AW, McLean MD (1999) Metabolism and functions of gamma-aminobutyric acid. Trend Plant Sci 4:446–452

Solsbacher J, Maurer P, Vogel F, Schlenstedt G (2000) Nup2p, a yeast nucleoporin, functions in bidirectional transport of importin alpha. Mol Cell Biol 20:8468–8479

Su HY, Frommer WB, Ludewig U (2004) Molecular and functional characterization of a family of amino acid transporters from *Arabidopsis*. Plant Physiol 136:3104–3113

Swofford DL (2001) PAUP* 4.0b10: phylogenetic analysis using parsimony (*and other methods). In Sinauer Associates, Sunderland

Tanaka J, Fink GR (1985) The histidine permease gene (HIP1) of *Saccharomyces cerevisiae*. Gene 38:205–214

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680