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Research article

Expression pattern of a nuclear encoded mitochondrial arginine-ornithine translocator gene from Arabidopsis

Elisabetta Catoni[1], Marcelo Desimone[1], Melanie Hilpert[1], Daniel Wipf[1], Reinhard Kunze[2], Anja Schneider[2], Ulf-Ingo Flügge[2], Karin Schumacher[1] and Wolf B Frommer*[1]

Address: [1]Plant Physiology, ZMBP, Auf der Morgenstelle 1, D-72076 Tübingen, Germany and [2]Botany Institute II, University of Cologne, Gyrhofstr. 15, D-50931 Cologne, Germany

Email: Elisabetta Catoni - elisabetta.catoni@zmbp.uni-tuebingen.de; Marcelo Desimone - marcelo.desimone@zmbp.uni-tuebingen.de; Melanie Hilpert - melanie.hilpert@zmbp.uni-tuebingen.de; Daniel Wipf - daniel.wipf@zmbp.uni-tuebingen.de; Reinhard Kunze - reinhard.kunze@uni-koeln.de; Anja Schneider - anja.schneider@uni-koeln.de; Ulf-Ingo Flügge - ui.fluegge@uni.koeln.de; Karin Schumacher - karin.schumacher@zmbp.uni-tuebingen.de; Wolf B Frommer* - frommer@zmbp.uni-tuebingen.de

* Corresponding author   †Equal contributors

Abstract

Background: Arginine and citrulline serve as nitrogen storage forms, but are also involved in biosynthetic and catabolic pathways. Metabolism of arginine, citrulline and ornithine is distributed between mitochondria and cytosol. For the shuttle of intermediates between cytosol and mitochondria transporters present on the inner mitochondrial membrane are required. Yeast contains a mitochondrial translocator for ornithine and arginine, Ort1p/Arg11p. Ort1p/Arg11p is a member of the mitochondrial carrier family (MCF) essential for ornithine export from mitochondria. The yeast *arg11* mutant, which is deficient in Ort1p/Arg11p grows poorly on media lacking arginine.

Results: High-level expression of a nuclear encoded *Arabidopsis thaliana* homolog (AtmBAC2) of Ort1p/Arg11p was able to suppress the growth deficiency of *arg11*. RT-PCR analysis demonstrated expression of *AtmBAC2* in all tissues with highest levels in flowers. Promoter-GUS fusions showed preferential expression in flowers, i.e. pollen, in the vasculature of siliques and in aborted seeds. Variable expression was observed in leaf vasculature. Induction of the promoter was not observed during the first two weeks in seedlings grown on media containing NH_4NO_3, arginine or ornithine as sole nitrogen sources.

Conclusion: AtmBAC2 was isolated as a mitochondrial transporter for arginine in Arabidopsis. The absence of expression in developing seeds and in cotyledons of seedlings indicates that other transporters are responsible for storage and mobilization of arginine in seeds.

Background

In most organisms, amino acid metabolism is distributed between at least two compartments: cytosol and mitochondria. In addition, plastids contribute significantly to amino acid metabolism of plants [1]. For instance in plants, biosynthesis, conversion and degradation of arginine, ornithine and citrulline are distributed across mitochondria, plastids and cytosol [2].

In many plants, arginine is stored in seeds as a major nitrogen source, e.g. in *Arabidopsis thaliana* arginine accounts for 11% of total seed nitrogen [2]. During seed germination, stored arginine is degraded for conversion into transport forms (e.g. glutamine or glutamate). The enzyme arginase, which catalyzes degradation of arginine to ornithine and urea is localized in mitochondria [3]. Thus arginine needs to be imported into mitochondria to allow mobilization. Transport steps across the mitochondrial membranes are also required during biosynthesis. In yeast, ornithine, a precursor of arginine, is synthesized in mitochondria from glutamate, which is imported from the cytosol. Ornithine is transferred to the cytosol before conversion to arginine. Additionally, arginine needs to be imported into mitochondria to allow mitochondrial protein synthesis. Ornithine serves as an intermediate of arginine and proline metabolism, and of the ureide citrulline. In plants, ornithine serves also a precursor for polyamine and alkaloid biosynthesis [4]. Thus also in various other metabolic pathways, transport of arginine, ornithine and also citrulline is required. Although mitochondrial in- and efflux of citrulline was originally described in rat liver and heart [5], it has not been described in plants.

Thus for metabolite exchange and coordination of all these pathways, multiple carriers must be present on the inner mitochondrial membrane of the different organisms. In the yeast genome, 35 members of the mitochondrial carrier family (MCF) were identified; 12 of them were characterized in terms of substrate specificity [6]. MCF proteins have similar structural features: a relatively low molecular mass of around 30 kDa, a tripartite structure (three repeats of about 100 amino acids), each consisting of two transmembrane-helices separated by a hydrophilic loop. Furthermore, a consensus sequence [P-X-(DE)-X-(LIVAT)-(RK)-X-(LRH)-(LIVMFY)], called the "mitochondrial energy signature", is present in all carriers in one to three copies at the C-terminal end of the first helix of each repeat [7]. Most transporters belonging to this family function as antiporters. A combination of transport studies in mitochondria [8,9] and reconstitution of carriers expressed in *E. coli* lead to the identification of the first mammalian mitochondrial carrier gene belonging to the MCF family [10]. Since then, mitochondrial transporter genes encoding various predicted transport systems have been identified in yeast and human genomes [11–14].

The Arabidopsis genome contains at least 48 members of the MCF family, 7 with 3 copies of the mitochondrial energy transfer signature, 21 with two copies and 20 with one copy. Until now, only few plant MCF carriers have been characterized [11]: the adenine nucleotide translocators [15,16], uncoupling proteins [17,18] and recently also a dicarboxylate-tricarboxylate carrier named DTC

[19] and a succinate transporter [20]. However, despite their importance none of the postulated plant mitochondrial transporters for amino acids has been identified, such as proline transporters for the compartmentalized proline metabolism [21], GABA transporters for the GABA shunt [22], glutamate/aspartate transporters for photorespiratory exchange with peroxisomes or ornithine/arginine carriers for arginine metabolism that were biochemically characterized in isolated soybean mitochondria [3].

A yeast *arg11* mutant unable to synthesize arginine was identified [23]. The corresponding gene was cloned by complementation of the *arg 11* mutant, revealing that Arg11p is a member of MCF. On the basis of amino acids content of the mutant, it was suggested that ARG11p would function as an mitochondrial ornithine translocator of yeast [24]. The properties of Ort1p were characterized after expression in *E. coli* and subsequent reconstitution. The major function of Ort1p/Arg11p is ornithine export from mitochondria. Ort1p/Arg11p mediates electroneutral antiport of ornithine, arginine and lysine, and with lower efficiency also ornithine/H^+ antiport [25]. Since mitochondria of an *arg11* deletion mutant retained arginine/ornithine carrier activity, a second transporter for basic amino acids named Bac1 was postulated [26]. Sequence homologies to the yeast gene were used to identify the human ORNT1 transporter and to demonstrate that the HHH syndrome (Hyperornithinaemia-Hyperammoniaemia-Homocitrullinuria) is due to defects in the respective gene [27]. In the case of citrullinaemia, a different member of the MCF family was defective [28], namely the aspartate/glutamate carrier [29]. Yet another amino acid carrier identified by reconstitution in liposomes was the human glutamate transporter [14]. However in yeast, and despite extensive attempts, none of the crucial carriers for amino acids or other metabolites was identified in genetic studies apart from *arg11*, *acr*1 and *dic1*, i.e. mutants have been found for all metabolic steps in many of the amino acid pathways but not the respective mutations in mitochondrial carriers. Since the yeast genome contains a large number of MCF proteins one may hypothesize that transporters have overlapping substrate specificities and yeast may thus be able to compensate loss of single MCF genes.

To date, the most efficient approach to identify plant plasma membrane transporters has been suppression cloning in yeast mutants. Since in most cases mitochondrial transporters do not contain identifiable targeting sequences, it remained open whether yeast complementation may be a suitable tool to identify the plant homologs. Searches in the Arabidopsis genome identified a gene 32% identical to the yeast Ort1p/Arg11p. The Arabidopsis gene *AtmBAC2*, when expressed in yeast, was able to restore growth of the yeast *arg11* mutant on media lacking ar-
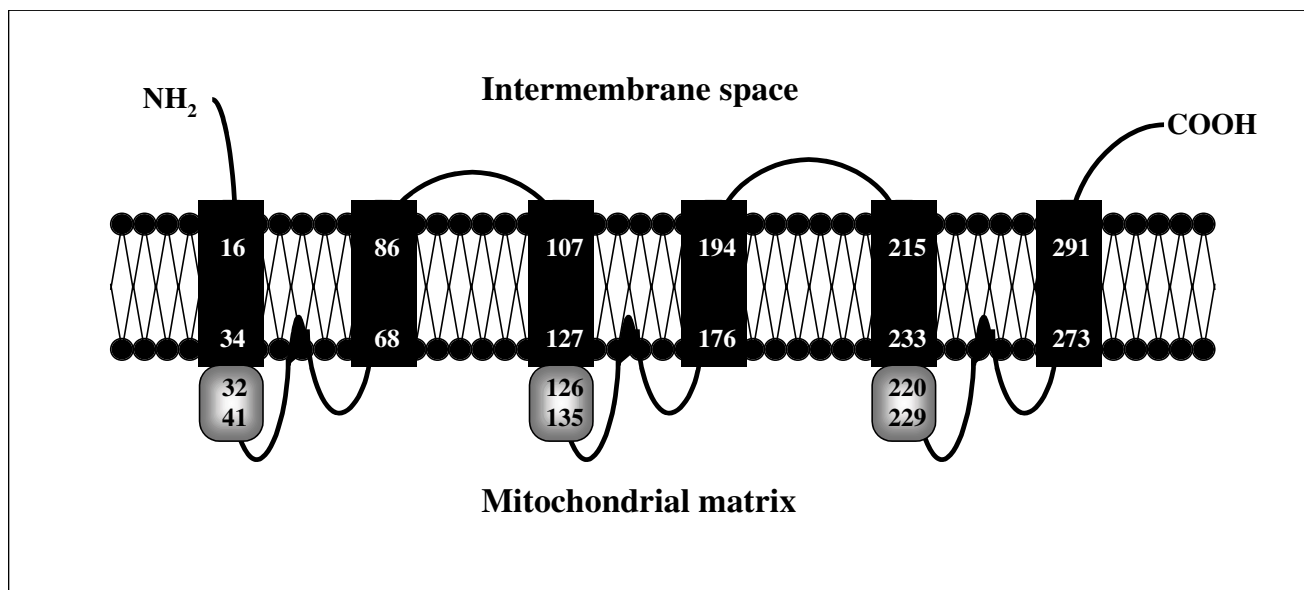
Figure 1
Structural model of AtmBAC2. The prediction was performed using the TMHMM program http://www.cbs.dtu.dk/serv-ices/TMHMM-2.0. Six hydrophobic domains are depicted as black boxes and the three mitochondrial energy transfer signatures as grey boxes (amino acid positions are given).

ginine, indicating that AtmBAC2 catalyzes the export of ornithine from mitochondria to the cytosol. RT-PCR analysis and promoter-GUS fusions were used to study expression and regulation of *AtmBAC2*. Absence of expression in developing seeds and in cotyledons of seedlings indicated that, in addition to AtmBAC2, another transporter, supposedly *AtmBAC1* [30], is involved in storage and mobilization of arginine.

Results and Discussion
Complementation of the yeast arg11 mutant by a plant cDNA
The yeast mutant *arg11* is bradytrophic for arginine, due to deletion of the mitochondrial transporter for ornithine, an intermediate in arginine metabolism [24]. To identify a corresponding Arabidopsis transporter gene, the *arg11* yeast mutant was transformed with an Arabidopsis cDNA library in the yeast expression vector pFL61 [31]. Around 10^6 transformants were screened for growth on a medium lacking arginine. Potentially due to the background growth of the bradytroph mutant, no genes able to complement the yeast *arg11* mutant were found. Therefore, a homology search was carried out with the ORT1p/Arg11p sequence from *Saccharomyces cerevisiae* against the predicted protein sequences encoded by the Arabidopsis genome and in the Aramemnon [32] integral membrane protein database http://aramemnon.botanik.uni-koeln.de. Two

putative proteins AtmBAC1 and AtmBAC2 encoded by the loci At5g46800 and At1g79900 respectively scored highest similarity values. By RT-PCR, the *AtmBAC2* cDNA corresponding to the locus At1g79900 was cloned. *AtmBAC2* encodes a protein of 296 amino acids with a calculated molecular mass of 32 kDa that shares 32% identity and 52% similarity with the yeast ORT1p/Arg11p protein. Hydrophobicity analysis of the polypeptide (HMMTOP 2.0; http://www.enzim.hu/hmmtop/) predicted six putative membrane spanning domains, consistent with the known structures of MCF carriers [33]. Six other programs predict between zero (soluble) and five transmembrane spans [32]. In general, the prediction of transmembrane spans in case of MCF carriers is problematic since hydrophobic domains are present, however, confidence levels are below the assumed critical value. Thus, such proteins would be missing from databases, which rely solely on membrane protein predictions. The structural prediction is supported by the presence of "mitochondrial energy transfer signatures" after the first, third, and fifth transmembrane domains. The combination of homology searches, hydrophobicity analysis and the presence of the signature motif indicate that AtmBAC2 comprises all features of the mitochondrial carrier family (Fig. 1). AtmBAC2 does not contain a predicted targeting sequence for import into mitochondria, similar to many

Δ*arg11* + pDR195 Δ*arg11* + pDR195-AtmBAC2
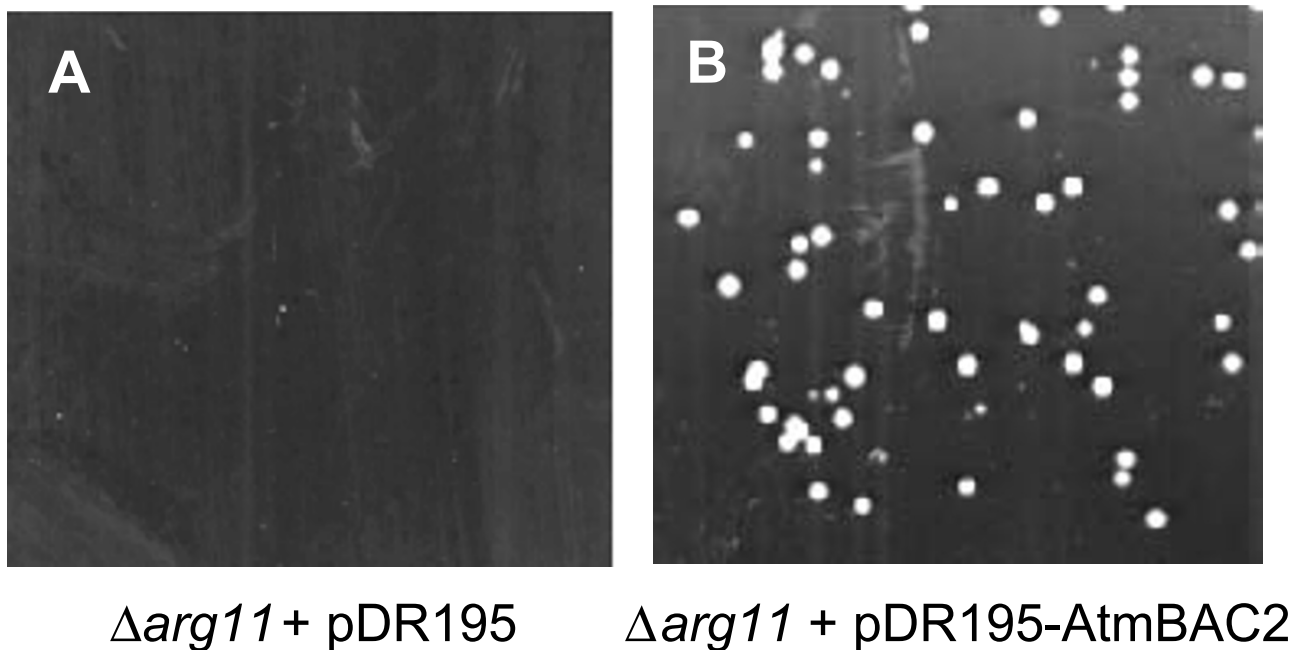
Figure 2
Functional complementation of yeast. The yeast strain Y02386 (*arg11*) was transformed with the empty vector pDR195 (A), or with AtmBAC2 cloned in pDR195 (B).

other mitochondrial proteins belonging to the MCF family.

The *AtmBAC2* cDNA was cloned in the yeast vectors pFL61, containing the *PGK* promoter, and pDR195, with the stronger *PMA1* promoter fragment. When expressed from the *PMA1* promoter, *AtmBAC2* was able to efficiently complement the yeast arginine bradytrophy (Fig. 2). Similar experiments using the comparatively weaker *PGK* promoter showed only weak complementation, i.e. slower growth as compared to cells expressing *AtmBAC2* under control of the PMA1 promoter (data not shown). To study the substrate specificity, AtmBAC2 was fused to a his-tag and the fusion protein was expressed under the strong PMA1 promoter fragment in yeast. Mitochondria were isolated and AtmBAC2 was purified as a 32 kDa polypeptide (data not shown). Functional reconstitution can be used to characterize the properties of AtmBAC2 in detail [12]. In a parallel study, Hoyos *et al.* [30] identified AtmBAC1, a closely related ornithine transporter. AtmBAC1 was successfully reconstituted into artificial proteoliposomes and was able to carry out ornithine/ornithine exchange as well as exchange of various amino acids against arginine. AtmBAC1 showed a preference for

basic amino acids with highest affinity for arginine and lysine, but transported also histidine. Thus AtmBAC1, similar to many other characterized MCF transporters, is promiscuous. This may reflect that transporters do not have a binding pocket, but filtering and recognition probably take place along the translocation pathway within the pore. From an evolutionary perspective, the low selectivity may be advantageous since mutations would allow rapid adaptation to changes in compartmentation of metabolism. However, the modulation of substrate specificity makes it difficult to define the physiological function based only on the sequence. The promiscuity may also explain why so far only few mutants in mitochondrial carrier functions have been identified, e.g. *arg11* in yeast or the disease related HHH syndrome.

So far, the most efficient methodology to characterize mitochondrial transporters was a candidate gene approach. Candidates were expressed in *E. coli* [19], purified proteins were reconstituted in lipid vesicles and transport properties were determined [34]. The approach is difficult and laborious and no standard protocols applicable for all candidates are available [12]. Therefore, an alternative method was developed allowing identification of mito-

chondrial carriers via suppression of auxo- or bradytrophic phenotypes of yeast mutants. Although there is no evidence for a mitochondrial targeting sequence in the *AtmBAC2* sequence, and little is known about signals for import of nuclear encoded proteins in the mitochondria of yeast and plants, the yeast import mechanisms are able to recognize also plant mitochondrial proteins and target them to mitochondria. In this way, the identification of *AtmBAC2* and of *AtmBAC1* [30] from Arabidopsis as ornithine carriers confirms the validity of yeast complementation for the functional assignment of candidate genes.

Phylogenetic tree of MCF translocators
A detailed analysis of transporter databases indicates that the Arabidopsis MCF family is highly divergent and a full alignment showed only eight perfectly conserved residues. Since a similarity tree branches into two major clades plus a few single and very distantly related members, the phylogenetic analysis was carried out with the subgroup containing the amino acid transporters, several other Arabidopsis MCF members and selected fungal and mammalian members falling into the same branch (Fig. 3). These selected polypeptides can be classified into six major clades: (i) amino acid carriers, (ii) two clades of di- and tricarboxylate carriers, (iii) uncoupling proteins (UCPs), and (iv) at least two groups of carriers for which no function has been assigned yet (Fig. 3). Within the amino acid carriers, the *Saccharomyces* Ort1p/Arg11p and related transporters from *Neurospora* and *Aspergillus* are most closely related to the Arabidopsis AtmBAC2 and AtmBAC1, whereas glutamate and glutamate/asparate carriers are more distantly related [14,29]. No other Arabidopsis proteins fall into this clade. Interestingly, the mammalian carnitine transporter [35] also belongs to the amino acid carrier clade, being more closely related to the basic amino acid transporters. Since so far no evidence exists for carnitine transport in plants, one may predict that the other members of this clade and those belonging to the groups of unknown transporters may be candidates for the missing carrier involved in amino acid transport functions in mitochondria.

Expression pattern of **AtmBAC2** in Arabidopsis
To study the expression pattern of *AtmBAC2*, total RNA was extracted from different tissues of *Arabidopsis thaliana* plants grown in soil. As the expression level was too low to be detected by RNA gel blot analysis, RT-PCR was carried out. *AtmBAC2* expression was found in all tissues analyzed, with highest transcript levels in flowers (Fig. 4a). To investigate a potential influence of the nitrogen source on the expression of *AtmBAC2*, RT-PCR was carried out on mRNA extracted from Arabidopsis seedlings grown on nitrogen-free MS medium supplemented with different inorganic and organic nitrogen sources NH_4NO_3, arginine or ornithine. Material was harvested at different develop-

mental stages. *AtmBAC2* was expressed at higher levels in a medium supplemented with arginine or ornithine as compared to NH_4NO_3 (Fig. 4b). A slight increase in expression was observed also under conditions of nitrogen starvation (Fig. 4b). The regulation and expression pattern are compatible with a role of AtmBAC2 in arginine and ornithine catabolism of mitochondria.

To study the tissue specificity in more detail, the *AtmBAC2* promoter region was isolated. The promoter was cloned as a longer 2 kb and a shorter 1.5 kb fragment as transcriptional fusions with GUS. In both cases, the expression pattern of independent transformants was comparable. While expression was undetectable in seedlings, first GUS activity was detectable in three-weeks-old plants (Fig. 5A,5B,5C,5D,5E). The discrepancy between promoter-GUS analysis and RT-PCR in seedlings may be due to higher sensitivity of the RT-PCR or due to missing regulatory elements present e.g. in the intron of *AtmBAC2*. Leaf veins showed patchy staining, in some cases at the leaf rims (Fig. 5F,5G). Consistent with RT-PCR, histochemical GUS analysis showed elevated expression levels in flowers, specifically in stamens and petals (Fig. 5H,5I), and both developing and germinating pollen (Fig. 5J, data not shown). Furthermore, expression was observed in siliques in the main vasculature and in aborted seeds, but not in developing seeds (Fig. 5K,5L,5M).

Basic amino acid transporters in the inner mitochondrial membrane are supposed to play roles in arginine metabolism in developing seedlings and during the mobilization of proteins, e.g. during seed germination. Unexpectedly, no expression was found in developing seeds or in young seedlings during germination, suggesting that a different carrier, potentially *AtmBAC1*, might be responsible for these processes [30]. Proline represents by far the most abundant amino acid in pollen [36]. Thus the expression of AtmBAC2 in pollen may be taken as a hint that arginine can serve as a precursor for proline biosynthesis, since arginase is localized in mitochondria, and thus arginine has to be taken up into mitochondria. Alternatively, the expression pattern may also be relevant for other metabolic pathways since ornithine can also serve as a precursor for polyamine and alkaloid biosynthesis [4].

Conclusions
Functional expression of a candidate gene under control of a strong promoter allowed identification of a plant mitochondrial transporter for basic amino acids. This carrier seems to have overlapping, but different functions as AtmBAC1 in the plant. The study also pinpoints candidates for the missing amino acid transport systems in plant mitochondria. RNAi studies together with *knock-out* mutants will enable a detailed physiological characterization of the mitochondrial compartmentation of amino
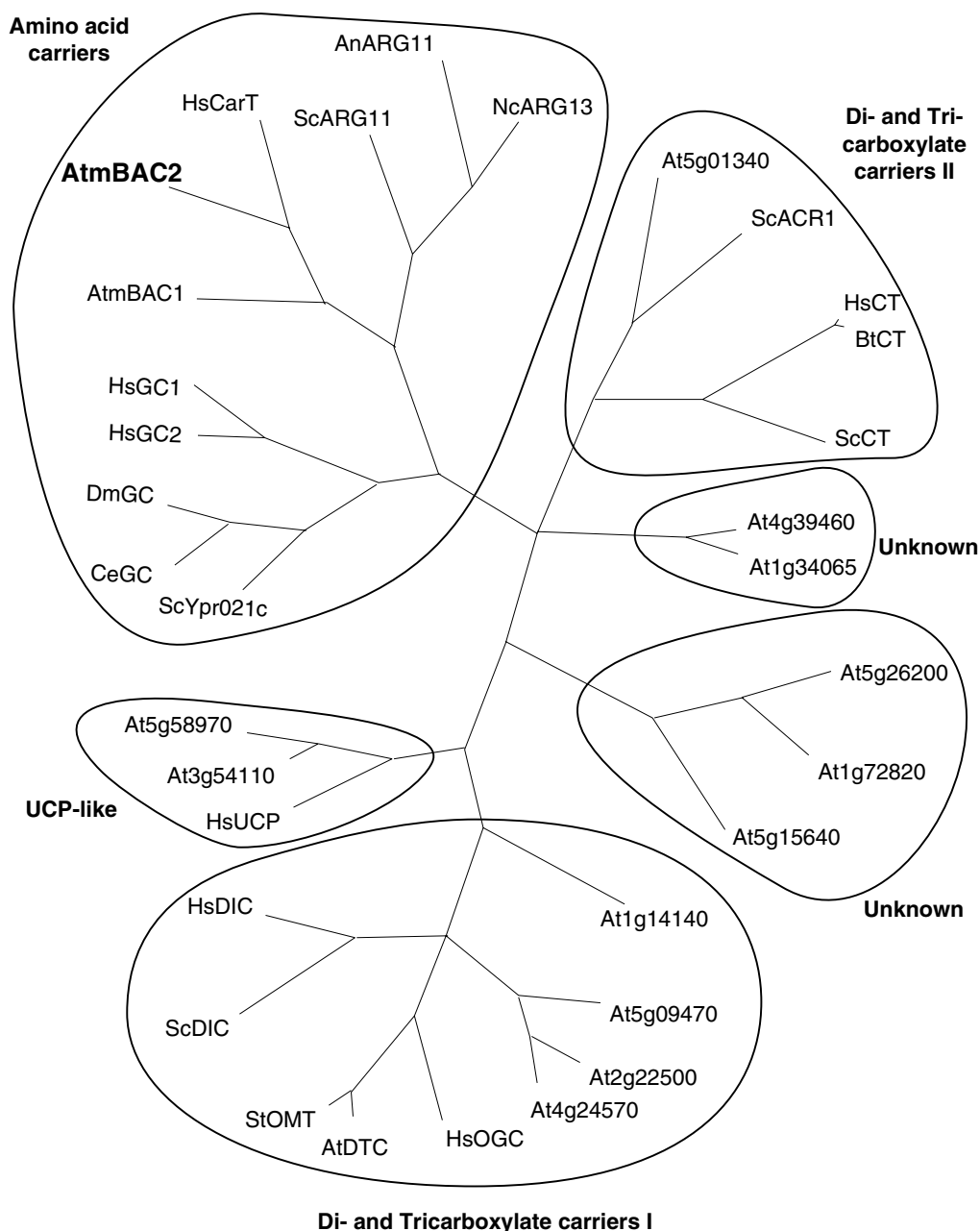
Figure 3
Phylogenetic analyses of AtmBAC2 and other mitochondrial carriers. The alignment was restricted to the conserved domains (between pos. 11 and 259 in AtmBAC2 amino acid sequence). Maximum parsimony analyses were performed using PAUP 4b10 with all amino acid characters unweighted and gaps scored as missing characters. Heuristic tree searches were executed using 1000 random sequence additions and the tree bisection-reconnection branch-swapping algorithm with random sequence analysis. The complete alignment was based on 342 sites; 306 were phylogenetically informative. At5g01340, NP195754; ScACR1, CAA80973; HsCT, NP005975; BtCT, P79110; ScCT, NP009850; At4g39460, NP568060; At1g34065, NP564436; At5g26200, NP197992; At1g72820, NP565048; At5g15640, NP568317; At1g14140, NP172866; At5g09470, NP196509; At2g22500, NP179836; At4g24570, NP194188; HsOGC, NP003553; AtDTC, CAC84549; StOMT, X99853; ScDIC, AAB71336; HsDIC, NP036272; HsUCP, NP003346; At3g54110, NP190979; At5g58970, NP568894; ScYpr021c, NP015346; CeGC, NP497274; DmGC, AAF57048; HsGC1, CAD21007; HsGC2, CAD21008; AtmBAC1, NP568670; AtmBAC2, NP178108; HsCarT, O43772; ScARG11, CAA60862; AnARG11, AAD44763; NcARG13, AAF87777.
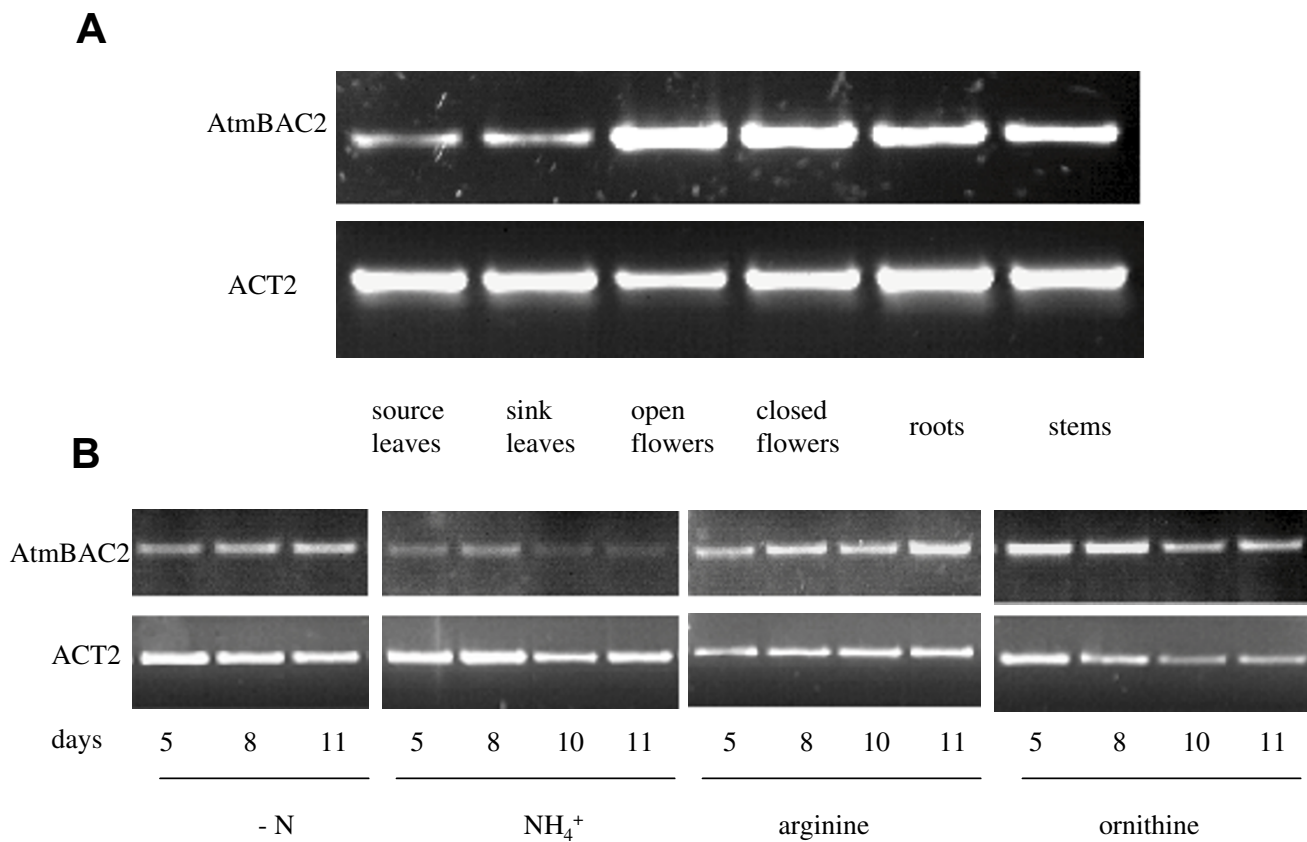
A

B

Figure 4
Expression of *AtmBAC2* mRNA expression in different Arabidopsis organs (A) and in whole seedlings grown on different nitrogen sources (B). RNAs were extracted and converted to cDNA by reverse transcription, and a 267 bp *AtmBAC2* fragment was amplified by 25 PCR cycles. As control, a 377 bp actin2 fragment was amplified simultaneously by 20 PCR cycles (ACT2).

acid biosynthesis. The studies also demonstrate that in addition to the 53 potential amino acid transporters for plasma membrane and tonoplast [37], plants contain additional carriers for organellar compartmentation.

Methods
Yeast strains
The yeast strain Y02386 [Matα; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0; YOR130c::kanMX4] from Euroscarf (Frankfurt, Germany), carrying a deletion of the yeast gene *ARG11*, was used for yeast complementation. Selective conditions for Y02386 were established by using yeast nitrogen base without amino acids and ammonium sulfate (Difco, Heidelberg, Germany) supplemented with (mg L^{-1}): 5000 $(NH_4)_2SO_4$, 20 histidine, 100 leucine, 20 methionine with 2% glucose and eventually 50 uracil in case of untransformed yeast. As negative control Y02386 was transformed with the empty vector pDR195 [38].

Cloning of the **AtmBAC2** *gene and yeast transformation*
AtmBAC2 cDNA was amplified by PCR on an Arabidopsis cDNA library (ecotype Landsberg; [31]) using primers spanning the predicted ORF from ATG to stop: 5'-agcctc-gagatggatttctggccggagtttatg, containing a *Xho*I restriction site and 5'-ttcggatcctcaatctcctgtgacaatatc, containing a *Bam*HI restriction site. The PCR product was restricted with *Xho*I and *Bam*HI and ligated into the yeast expression vector pDR195. The resulting plasmid was amplified in *E. coli* DH5 and transferred into Y02386, according to Gietz *et al.* [39]. cDNA and protein sequence were deposited in Genbank under the accession number AC011717_10.

Plant material and mRNA expression
Arabidopsis thaliana L. Heynh. ecotype Col-0 was used for plant studies. For organ specificity studies, all plant organs were harvested from plants grown in soil in the greenhouse with the exception of roots, which were obtained from plants cultured on MS agar plates. For expression
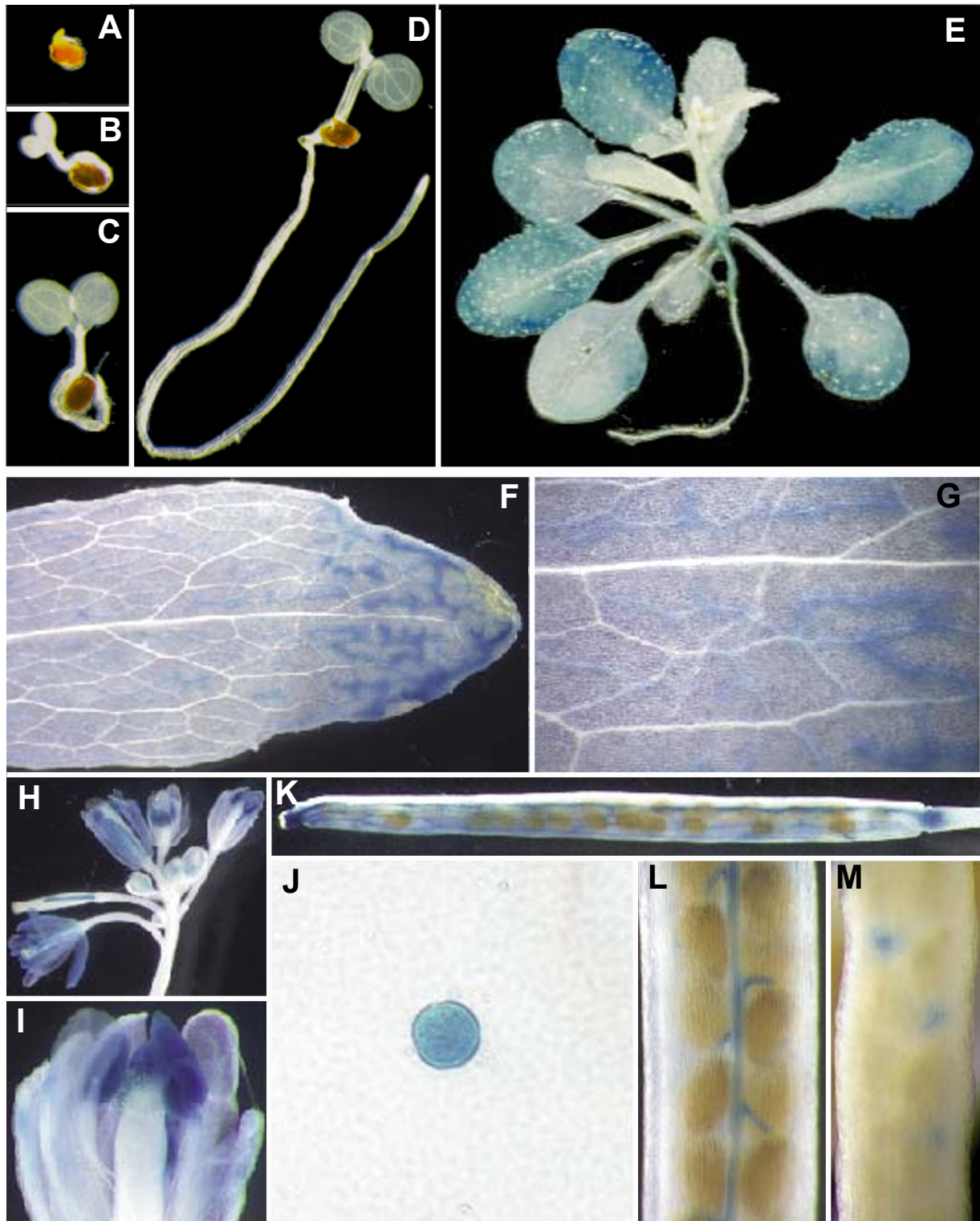
Figure 5
GUS staining of Arabidopsis plants transformed with the *AtmBAC2* promoter-GUS fusion. Histochemical analysis revealed promoter activity in 1, 2, 4, 7 and 21 days old plants (**A** to **E**). Details of expression in mature leaves (**F** and **G**); activity in inflorescences and flower (**H** and **I**), individual pollen grains (**J**) and siliques (**K** to **M**).

studies with different nitrogen sources, *Arabidopsis* seeds were placed on sterile N-free MS-medium supplied with 10 mM nitrogen either as NH_4NO_3, arginine or ornithine. After 5, 8, 10 and 11 days, plants were harvested and total RNA extracted. For RT-PCR analysis, RNAs were converted to cDNA using Retroscript (Ambion) following the manufacturer's instructions. A 267 bp cDNA fragment was amplified by 25 PCR cycles using specific primers for *AtmBAC2* (5'-GGACAAGAAAACCTCAGGACCAT, 5'-GAATAGACACCGCAAGGCTACCT). As a control, a 377 bp cDNA fragment of the constitutively expressed actin 2 gene (*AtACT2*) was simultaneously amplified by 20 PCR cycles [40]. Identity of the amplicons was confirmed by sequencing.

Promoter-GUS fusion

For promoter-GUS fusions two different lengths (2020 and 1480 bp) of the *AtmBAC2* promoter region including the start codon were amplified by PCR on *Arabidopsis thaliana* Heynh., ecotype Col0 genomic DNA using the primers 5'-CTGACCTCTAGACCAGCTGGTGACAGCATCAG; 5'-CTGACCTCTAGAAAACTTGGGTTTGAACAGCC and 5'-CTGACCACCCGGGACCATACCAAGGATGGGATTG-TAC. Forward primers contained a *Xba*I restriction site, the reverse primer a *Sma*I site. The PCR product was restricted with *Sma*I and *Xba*I and ligated in frame to the GUS gene in binary vector pCB308 [41].*Agrobacterium tumefaciens* GV2260 was transformed with the resulting plasmids. *Arabidopsis thaliana* L. Heynh., ecotype Col0, grown in soil in the greenhouse, was transformed by floral dipping [42]. Transgenic Arabidopsis plants were selected for BASTA resistance (Aventis Crop Science, Frankfurt).

Histochemical analysis of GUS activity

Transformed T2 Arabidopsis plants were grown in soil. At different developmental stages, plants or organs were harvested and stained for GUS activity. Seedlings were grown on solid MS plates. Histochemical assays for β-glucuronidase activity in transgenic plants were performed as described in Martin *et al.* [43]. Tissues were incubated for ~18 h at 37°C in staining solution containing (mM) 100 sodium phosphate (pH 7), 10 EDTA, 3 K_4 [Fe(CN)$_6$], 0.5 K_3 [Fe(CN)$_6$], 1 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) and 0.2% (v/v) Triton X-100. Slight vacuum was applied to facilitate substrate infiltration. Chlorophyll containing tissues was cleared in 70% EtOH. Photographs were taken on a CCD camera (Visitron).

Authors' contributions

EC, MD, RK, AS, and MH carried out the molecular cloning and the promoter GUS study. DW performed the phylogenetic analysis. KS and RK participated in the design of the study. WBF and UIF conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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