



RESEARCH PAPER

Root phloem-specific expression of the plasma membrane amino acid proton co-transporter AAP3

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Abstract

Amino acids are regarded as the nitrogen 'currency' of plants. Amino acids can be taken up from the soil directly or synthesized from inorganic nitrogen, and then circulated in the plant via phloem and xylem. AtAAP3, a member of the Amino Acid Permease (AAP) family, is mainly expressed in root tissue, suggesting a potential role in the uptake and distribution of amino acids. To determine the spatial expression pattern of AAP3, promoter–reporter gene fusions were introduced into *Arabidopsis*. Histochemical analysis of AAP3 promoter–GUS expressing plants revealed that AAP3 is preferentially expressed in root phloem. Expression was also detected in stamens, in cotyledons, and in major veins of some mature leaves. GFP–AAP3 fusions and epitope-tagged AAP3 were used to confirm the tissue specificity and to determine the subcellular localization of AtAAP3. When overexpressed in yeast or plant protoplasts, the functional GFP–AAP3 fusion was localized in subcellular organelle-like structures, nuclear membrane, and plasma membrane. Epitope-tagged AAP3 confirmed its localization to the plasma membrane and nuclear membrane of the phloem, consistent with the promoter–GUS study. In addition, epitope-tagged AAP3 protein was localized in endodermal cells in root tips. The intracellular localization suggests trafficking or cycling of the transporter, similar to many metabolite transporters in yeast or mammals, for example, yeast amino acid permease

GAP1. Despite the specific expression pattern, *knock-out* mutants did not show altered phenotypes under various conditions including N-starvation. Microarray analyses revealed that the expression profile of genes involved in amino acid metabolism did not change drastically, indicating potential compensation by other amino acid transporters.

Key words: Amino acid transport, *Arabidopsis thaliana*, long-distance transport, phloem, plasma membrane, root.

Introduction

Plants take up nitrogen nutrient via roots, mainly in the form of inorganic nitrogen such as NO_3^- and NH_4^+ . Once taken up into the plant body, NH_4^+ is reduced to organic nitrogen, mainly amino acids, and translocated to the other tissues via phloem and xylem. However, plants may also take up amino acids directly from the soil (Näsholm and Persson, 2001). Since relatively high concentrations of amino acids are present in both phloem ($\sim 10^{-1}$ M) and xylem sap ($\sim 10^{-2}$ M), they are thought to be the major transported forms of organic nitrogen in most plant species. Indeed, partitioning of amino acids plays a key role in the delivery of reduced nitrogen to the heterotrophic tissues (Pate and Sharkey, 1975), and the amino acid content of phloem and xylem sap seems to be tightly regulated under various conditions to meet the nitrogen requirements of different tissues (Lam *et al.*, 1995).

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Amino acid distribution requires a number of membrane transport steps along the translocation pathway. Amino acids have to cross the plasma membrane when taken up from the soil into root cells. Amino acids synthesized in root tissue have to be exported to the shoot via xylem. Since mature xylem tracheary elements are dead cells, amino acids need to cross the plasma membrane to enter the xylem, potentially via facilitators, exchangers, or antiporters. The concentration of amino acids in the phloem and in the mesophyll cytoplasm is significantly higher than in the apoplast, suggesting an active transport of amino acids into the phloem (Lohaus *et al.*, 1995). Furthermore, large parts of amino acids fed directly to the xylem sap appear unchanged in the phloem sap, indicating that amino acids can be exchanged between xylem and phloem (Atkins, 2000; Pate and Sharkey, 1975). Besides intercellular amino acid transport, translocation processes also occur between intracellular compartments such as chloroplasts, mitochondria, cytosol, and vacuoles during the processes of synthesis and storage (Catoni *et al.*, 2003; Hoyos *et al.*, 2003). In fact, vacuolar/lysosomal amino acid transporters have been identified (Rusnak *et al.*, 2001).

Early physiological studies of amino acid transport in plants suggested the existence of several amino acid carriers exhibiting broad substrate specificity and being energized by cotransport with protons (Williams *et al.*, 1992; Wyse and Komor, 1984). Amino acid uptake into plasma membrane vesicles showed complex kinetics, indicating the existence of multiple transport systems (Li and Bush, 1990a, b).

Arabidopsis amino acid transporters were identified by complementation of yeast mutants defective in the uptake of amino acids (Chen and Bush, 1997; Chen *et al.*, 2001; Fischer *et al.*, 1995; Frommer *et al.*, 1993, 1995; Hsu *et al.*, 1993; Kwart *et al.*, 1993; Rentsch *et al.*, 1996). Amino acid transporters from other plant species (Marvier *et al.*, 1998; Neelam *et al.*, 1999; Miranda *et al.*, 2003; Tegeder *et al.*, 2000) were also isolated and characterized in yeasts.

Recent analysis of the *Arabidopsis* genome revealed that at least 53 putative amino acid carriers are present at the plasma membrane and tonoplast (Wipf *et al.*, 2002).

On the basis of sequence homologies, these transporters were subdivided into two major clades: the APC and the ATF1 superfamilies (Fischer *et al.*, 1998; Wipf *et al.*, 2002). The APC superfamily consists of two families: the CAT family and the GABA-permease-related family. The ATF1 superfamily can be divided into five subfamilies: AUX1-related proteins, proline transporters (ProTs), the lysine-histidine-transporters (LHTs), a new branch comprising vacuolar and vesicular amino acid carriers from yeast and animals, and amino acid permeases (AAPs).

Among the plant amino acid carriers, the AAPs (amino acid permeases) are the best characterized so far. All the members of the AAP family that have been characterized have overlapping spectra of transported substrates. Inter-

estingly, each member of the AAP family shows a distinct expression pattern, indicating non-redundant roles for each AAP in plants. Previous promoter-GUS studies of *AAP1* and *AAP2* revealed that *AAP1* is expressed in developing embryos, whereas *AAP2* is expressed in the vascular tissue of siliques (Hirner *et al.*, 1998). The two high affinity systems *AAP6* and *AAP8* were expressed in the xylem and in the seeds, respectively (Okumoto *et al.*, 2002). However, the cellular expression pattern of other AAPs has not been investigated so far. In this paper, the spatial localization pattern of *AAP3*, the only AAP expressed to high levels preferentially in roots and which transports neutral and basic amino acids (Fischer *et al.*, 1995, 2002) is presented. Histochemical analysis of transgenic *Arabidopsis* expressing *AAP3* promoter-GUS fusions or c-Myc epitope-tagged *AAP3* showed expression of *AAP3* in the phloem of roots. The analysis of GFP-AAP3 fusions expressed in tobacco protoplasts, and transgenic *Arabidopsis* expressing c-Myc epitope-tagged *AAP3* localized, *AAP3* to the plasma membrane, the nuclear membrane, ER, Golgi bodies, and endosomal vesicles, showing that *AAP3* is a plasma membrane transporter that is also present on internal membranes along the trafficking pathway. Despite the specific expression pattern, *knock-out* mutants failed to reveal a specific function of *AAP3* in amino acid uptake.

Materials and methods

Plant material, growth, transformation, and analysis

Arabidopsis thaliana L. ecotype Col-0 and *Nicotiana tabacum* cv. Samsun NN were grown either in axenic culture on MS medium supplemented with 2% sucrose or in soil culture in the greenhouse. *Arabidopsis* plants were transformed using *Agrobacterium tumefaciens* pGV2260 via vacuum infiltration (Bechtold and Pelletier, 1998). Tobacco plants were transformed as described by Martin *et al.* (1993). The suspension culture of the Tobacco Bright Yellow-2 (BY-2) cell line was grown as described by Merkle *et al.* (1996).

DNA constructs

A genomic clone carrying the promoter region of *AAP3* was isolated from a genomic cosmid library from *Arabidopsis thaliana* C24 (Martin *et al.*, 1993). A 500 bp promoter fragment including a native *HindIII* site at the 5'-end was amplified by PCR to introduce *Bam*HI sites before the first ATG of the *AtAAP3* gene using primers 5'-GAGGGAAAATCCTTTTGTGTGCTCT and 5'-AATACGACTCACTATAG. The PCR product was fused with the flanking upstream 1 kb *SacII/HindIII* fragment excised from the cosmid and cloned together into pBluescript. The resulting 1.5 kb promoter fragment was cloned into the *SacII/Bam*HI sites of pBlueGUS3 (pBlueGUS3 carries a 1809 bp *uidA* fragment followed by a 203 bp octopine synthase terminator sequence cloned as a *SmaI* fragment into the *SmaI/HindIII* (blunted) site of pBluescript SK-; T Martin and WB Frommer, unpublished result), creating a 1.5 kb promoter-GUS transcriptional fusion, which was then digested with *SacII/XbaI* and cloned into pGPTV-HPT (Becker *et al.*, 1992). For the longer 3.8 kb *AAP3* promoter-GUS fusion, a 2.3 kb *HindIII/SacII* fragment further upstream was partially digested from the cosmid clone and inserted in front of the 1.5 kb promoter. For constructing the GFP-AAP3 fusion protein, a 300 bp fragment corresponding to the 5'-end of the *AAP3* open reading frame and carrying a *Bgl*II site and 15 bp of linker

sequence in front of the start ATG of *AtAAP3* was amplified by PCR using primers 5'-ACCAAAAGATCTGGGGGAGGGGAGGGA-TGGTTCAAAACCACCAACAGTTCTCGC-3' and 5'-GTGAC-GGCAGAGAAGAGCAACATCACCACC-3'. The PCR product was exchanged with the original sequence of the *AAP3* cDNA in pBluescript using a native *RsrII* restriction site, and then cloned into the *BglIII* site of pAVA321 (von Arnim *et al.*, 1998), carrying the *GFP* gene under the control of the CaMV 35S promoter. For yeast transformation, the *GFP-AAP3* fusion gene was excised with *XhoI/NotI* and cloned into pDR195 (Rentsch *et al.*, 1996). For the construction of *c-MycAAP3*, the *EcoRI/HindIII* *AAP3* genomic fragment was cloned in pBluescript. Two different PCR fragments (a fragment corresponding to the 680 bp upstream sequence of the starting codon of *AAP3* and carrying the *SalI* site at the 3'-end, and a fragment corresponding to 370 bp behind the start codon of *AAP3* and the carrying *SalI* and *Eco47III* sites at the 5'-end) were generated by PCR using primers 5'-GCTGTTTTCTAAATAAATTTAGTTTGTCCA-CGG-3', 5'-TCCGTCGAGCATCTTTTGTGTTTGTCTGCTG-3', 5'-GATGGTCGACGGAAGCGCTGGAAGTGGAGATATGGTT-CAAAACCACCAACAG-3' and 5'-GTGACGGCAGAGAAGA-GCAACATCACCACC-3'. The resulting fragments were fused at the *SalI* site and exchanged with the original sequence of the *AAP3* genomic clone using native *SnaBI* and *RsrII* sites, resulting in the *AAP3* genomic clone carrying *SalI* and *Eco47III* site after the starting codon (*SalI-Eco47III-AAP3*). Three repeats of the *c-Myc* epitope motif were generated by annealing complementary 100 bp primers containing overhangs for *SalI* and *Eco47III* (5'-TCGACGGAGAGCAAAAGCTGATCTCAGAGGAGGACCT-GCTTGAGAACAGAAGTTAATTTCTGAGGAAGACCTCCTG-GGAGAGCAAAAGCTGATTAGCGAGGAGGATCTGCTCAGC and 5'-GCTGAGCAGATCCTCCTCGCTAATCAGCTTTTGCTC-TCCCAGGAGGTCTTCTCAGAAATTAACCTTCTGTTCTCCA-AGCAGGTCCTCCTCTGAGATCAGCTTTTGCTCTCCG). The annealed primers were inserted into the *SalI* and *Eco47III* sites of *SalI-Eco47III-AAP3*, resulting in a *c-Myc* sequence fused to the *AAP3* genomic clone (*c-MycAAP3*). The *c-MycAAP3* sequence was cloned into two plant binary vectors pJH212 and pPZP312, both pPZP212 (U10462) derived vectors carrying kanamycin and basta resistance genes, respectively.

Yeast growth

Yeast 22Δ8AA [MATα, *ura3-1*, *gap-1*, *put4-1*, *uga4-1*, *can1::HisG*, *lyp/alp::HisG*, *hip1::HisG*, and *dip5::HisG*] (Fischer *et al.*, 2002) was transformed, selected on NAAG-ammonium medium (1.7 g l⁻¹ Yeast Nitrogen Base, Difco, 10 g l⁻¹ glucose, 20 g l⁻¹ Oxoid Agar, Difco, supplemented with 5 g l⁻¹ NH₄SO₄), and used for growth tests on NAAG medium supplemented with 3 mM L-proline as the sole nitrogen source. For microscopy, yeast cells were grown overnight in NAAG at 28 °C, fixed in 4% paraformaldehyde and 3.4% sucrose, stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI 2.5 μg ml⁻¹) and observed with Leica DM RE microscopy (Leica, Germany).

Histochemical localization of GUS activity

Histochemical assays for β-glucuronidase activity were performed as described (Martin *et al.*, 1992). Tissues were cut into 2×5 mm pieces, incubated in GUS staining solution containing 100 mM sodium phosphate (pH 7), 10 mM EDTA, 3 mM K₄[Fe(CN)₆], 0.5 mM K₃[Fe(CN)₆], 0.1% (v/v) Triton X-100, 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) for 3–24 h at 37 °C. Slight vacuum was applied before incubation to facilitate substrate infiltration. For resin sections, X-Gluc-stained tissues were fixed in 4% glutaraldehyde, and 50 mM sodium phosphate (pH 7) overnight at 4 °C. Fixed tissues were dehydrated in ethanol and embedded in LR White resin (London Resin Company Ltd). Embedded material was

cut into 1.5–5 μm sections with glass knives using an ultramicrotome and observed by bright field and phase contrast microscopy. Root sections were counterstained with periodic acid and Schiff's reagent.

Transient expression of GFP-AAP3 in BY-2 protoplasts

Protoplasts from tobacco BY-2 cultures were prepared as described by Merkle *et al.* (1996). Transient transformation of the protoplasts with PEG was performed according to the protocol of Negrutiu *et al.* (1987). For confocal laser scanning microscopy, protoplasts were incubated overnight at 24 °C in the dark after transformation and observed with a Leica DM RE microscope (Leica, Germany).

Immunodetection of epitope-tagged AAP3

Immunolabelling was performed as described by Otto and Kaldenhoff (2000) with slight modifications. Roots of *c-MycAAP3*-expressing tobacco plants were embedded in 5% low melting temperature agarose. Blocks were sectioned (~100 μm) on a Leica VT1000S microtome (Leica, Germany). Sections were fixed immediately in 3% formaldehyde in PBS for 30 min on ice, washed three times with PBS (PBS; 58 mM Na₂HPO₄, 15 mM NaH₂PO₄, 68 mM NaCl, pH 7.4, and 6.7 mM EGTA), and labelled with mouse monoclonal anti-c-Myc antibody (Santa Cruz Biotechnology, Inc., USA) overnight at 4 °C. The first antibody was visualized by either FITC-conjugated goat anti-mouse antibody (Sigma, USA) or Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Inc., USA). Sections were observed with a Leica DM RE confocal microscope (Leica, Bensheim, Germany). Whole mount immunodetection on *Arabidopsis* roots was performed essentially as described in Lauber *et al.* (1997) using PBS (137 mM NaCl, 8.1 mM Na₂HPO₄, 2.68 mM KCl, and 1.47 mM KH₂PO₄) instead of microtubule-stabilizing buffer (MSB; 50 mM PIPES, 5 mM EGTA, and 5 mM MgSO₄ pH 7.0). For thin cryosection labelling, root tips were fixed with 4% formaldehyde in MSB for 30 min, followed by fixation with 8% formaldehyde in MSB for 45 min on ice. Fixed root tips were embedded in 10% gelatine; gelatine blocks containing root tips were infiltrated in 2.1 M sucrose in PBS, pH 7.2, and frozen in liquid nitrogen. Ultrathin (100 nm) and semithick cryosections (300 nm) were obtained using a Leica Ultracut UCT/EM FCS cryo-ultramicrotome at -100 °C and -80 °C, respectively. Thawed cryosections were incubated with monoclonal anti-c-Myc antibody (1:100) in blocking buffer (1% milk powder, 0.5% BSA, in PBS) for 60 min. After washing with blocking buffer, bound antibodies were detected with Cy3-conjugated goat anti-mouse antibody or goat anti-mouse IgG-Nanogold (Nanoprobes, USA). The silver enhancement was performed as described by Danscher (1981) and Stierhof *et al.* (1991). Final embedding was done in 2% methyl cellulose (Sigma, M-6385) for transmission electron microscopy or in Mowiol 4.88 (Hoechst, Germany) for immunofluorescence microscopy. Semithick sections were viewed in a Zeiss Axiophot light microscope, ultrathin sections in a LEO 906 transmission electron microscope.

Identification of T-DNA insertion lines

Arabidopsis T-DNA insertion lines generated at the University of Wisconsin Knockout *Arabidopsis* facility (<http://www.biotech.wisc.edu/Arabidopsis/>) were screened as described by Krysan *et al.* (1996). Insertions were detected with PCR primers specific to the *AAP3* gene (5'-CAGTTCTCGCCGTCGATATGCCACAAACC-3'; 5'-GATCAAGAAGTACTCCAGCTATGGACCC-3') and to the left border T-DNA insertion sequence (5'-CATTTTATAATAACGC-TGCCGACATCTAC-3'). Homozygosity was determined by PCR with *AAP3* primers spanning the coding region of *AAP3*.

RT-PCR

Total RNA was isolated from 18-d-old whole *Arabidopsis* plants grown on MS media containing 58 mM sucrose, under 14/10 h light/

dark at 21.5 °C. First strand cDNA synthesis was performed using M-MLV reverse transcriptase (MBI Fermentas, Germany) and oligo-dT₁₈, according to the manufacturer's protocol. RT-PCR reactions were performed using *AAP3* gene-specific primers (5'-CTTGCTGCTTGTACCGCTCC; 5'-CTACAATTCTTGCCTTCAGGG).

Array analyses

Total RNA from 18-d-old whole *Arabidopsis* plants were isolated as described above. The hybridization and analysis of the nuclear chloroplast gene array containing 3292 *Arabidopsis* gene-sequence tags (GSTs) were performed as described in Richly *et al.* (2003).

Results

RNA gel blot analyses indicated that *AAP3* is specifically expressed in roots. To study the potential role of *AAP3* in uptake or distribution of organic nitrogen in *Arabidopsis*, a genomic clone was isolated from a cosmid library from *Arabidopsis thaliana* ecotype C24 and the promoter region was isolated and analysed. 2.2 kb of this fragment were sequenced (Genbank acc. AX300470). The sequence is 96% identical to that of ecotype Col-0.

Expression of *AAP3* in the phloem of root tissue

To analyse the expression pattern in more detail, promoter-GUS studies were performed. A 1.5 kb promoter fragment was fused to the GUS gene (*AAP3-GUS_s*) and *Arabidopsis* plants were transformed (Fig. 1). As opposed to previous RNA gel blot analyses showing high expression mainly in roots (Fischer *et al.*, 1995), in 3-week-old transgenic plants, the promoter was active mainly in cotyledons and in major veins of some leaves, but only weakly active in roots (Fig. 2A). Twelve out of 14 lines showed weak and patchy staining or no staining (three lines out of 14) in roots. To test

whether additional elements upstream are required for high level expression in roots, a 3.8 kb fragment of the *AAP3* promoter (*AAP3-GUS_L*) was used to include additional elements (Fig. 1). All 19 lines carrying the 3.8 kb promoter showed strong staining in root vascular tissue in addition to expression in cotyledons and major veins of some leaves (Fig. 2B). Considering that *AAP3* expression is also strongly detected in root tissue at the RNA level (Fischer *et al.*, 1995), the GUS expression pattern of the 3.8 kb promoter construct is in agreement with the native expression in wild-type plants. Therefore, further studies were focused on *AAP3-GUS_L* plants. In mature roots, expression of GUS is localized to two vascular strands in the central cylinder (Fig. 2C). The *Arabidopsis* root is typically diarch, containing two phloem strands. Normally, cells between two protoxylems differentiate into metaxylem, generating a continuous file of xylem elements. Therefore, the staining pattern indicates expression in phloem tissue. Cross-sections of mature, secondary-thickened roots verified expression in the phloem (Fig. 2D). GUS staining in root vascular tissue was stronger in the side roots (Fig. 2E), and was detected all along the root down to ~1.5–2 cm from the root tip. No staining was detected in the tip of the root (Fig. 2F). *AAP3* promoter-GUS expression was also observed in flowers, i.e. at the tip of filaments (Fig. 2G). Interestingly, GUS activity in stamens was not found in earlier developmental stages (data not shown). In *Arabidopsis*, dehiscence usually takes place just before anthesis. Cross-sections of flowers showed GUS activity in the filament of the stamen before dehiscence (Fig. 2H, I), indicating that the expression is induced just before dehiscence. To test whether the promoter is also functional in other species, tobacco plants were transformed with both constructs. Similar to *Arabidopsis*, *AAP3-GUS_s* plants showed almost no GUS expression in the vascular system of roots (data not shown), whereas *AAP3-GUS_L* plants showed strong staining (Fig. 2K). No GUS activity was detected in leaves of *AAP3-GUS_L* tobacco plants (data not shown). These results confirm the finding that the larger fragment contains putative enhancer motifs.

Analysis of the *AAP3* promoter sequence

Sequence analysis of the complete 5'-upstream region of *AAP3* from Col-0 revealed several features in the putative promoter (Fig. 1). A potential TATA-box was located at position –78 bp relative to the first base of the *AAP3* cDNA (Fischer *et al.*, 1995). Since transcriptional initiation was not confirmed experimentally, it is unknown whether this corresponds to the native TATA-box. The sequence between –1076 bp to –1675 bp is AT-rich and repetitive in the *Arabidopsis* genome; more than 50 homologous repeats (>85%) were found. This region consists of four parts, an AT-rich *AtREP2* sequence, a pair of internal repeats (named *repeat1* and *repeat2* sharing 88% homology; Fig. 1) and an *AtREP3* sequence. Bioinformatic analysis using the PLACE database (Higo *et al.*, 1999) revealed multiple

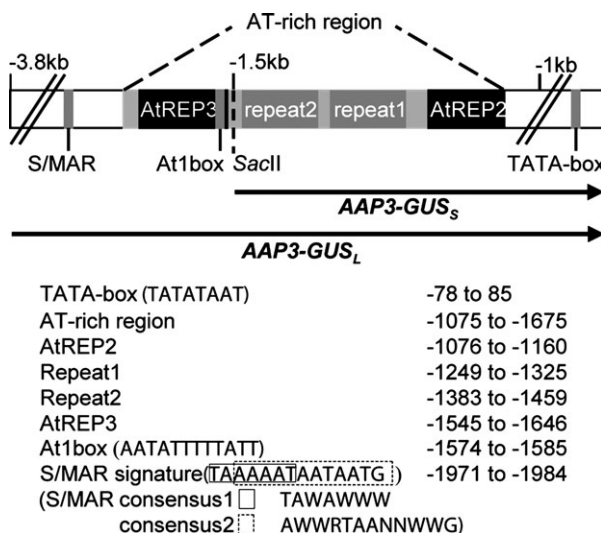


Fig. 1. Structure of the *AAP3* promoter. The promoter regions used for *AAP3-GUS_s* (1.5 kb) and *AAP3-GUS_L* (3.8 kb) constructs are indicated by arrows. Positions and sequences of the motifs found in the promoter are indicated.

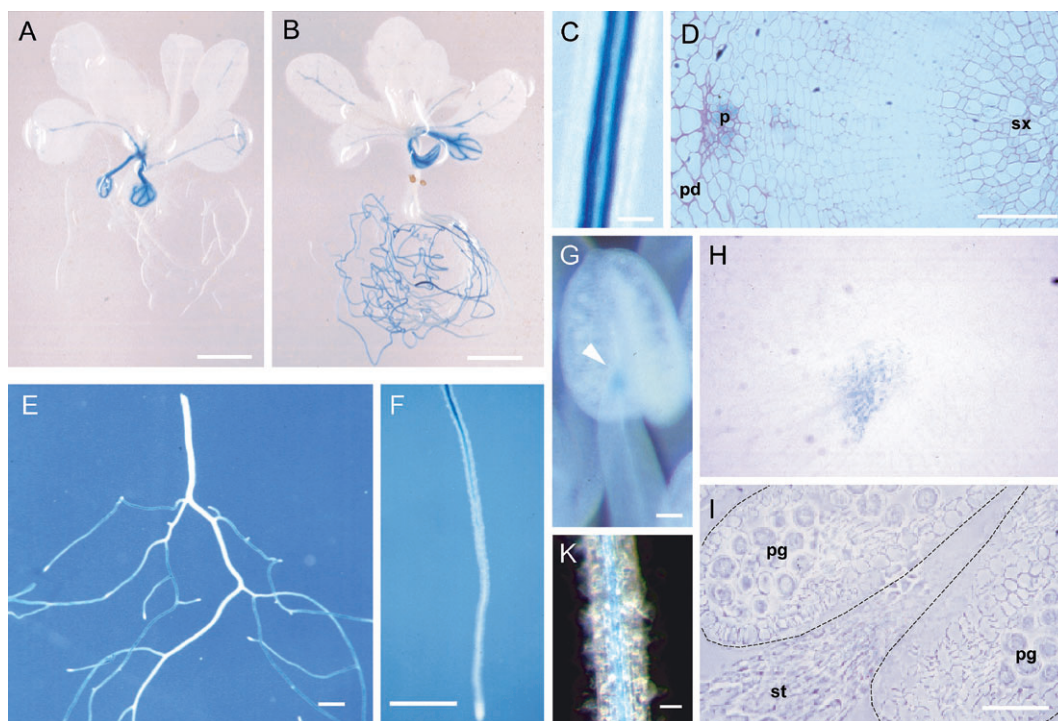


Fig. 2. *Arabidopsis* plants expressing AAP3 promoter-GUS constructs. (A, B) GUS-stained *Arabidopsis* seedlings expressing either AAP3-GUS_S (A) or AAP3-GUS_L (B). The staining is mainly observed in the root tissue of AAP3-GUS_L expressing plants. Bar=5 mm. (C) Root of AAP3-GUS_L plant. Two strands are visible, presumably phloem tissue. Bar=50 µm. (D) Cross-section of mature root stained with Schiff's reagent. Phloem tissue is stained. p, Phloem; pd, peridermis; sx, secondary xylem. Bar=20 µm. (E) Root of GUS-stained *Arabidopsis* seedlings. The staining is hardly visible in the main root whereas the side roots are stained. Bar=5 mm. (F) Root tip of GUS-stained root. Staining stops ~1.5 cm from the root tip. Bar=5 mm. (G) Stamen of AAP3-GUS_L plant. The tip of the filament is stained (arrowhead). Bar=20 µm. (H, I). Cross-section of an undehiscent anther. Bright field (H); phase contrast (I). The anther tissues are indicated by dashed lines. pg, Pollen grain; st, stamen. Bar=20 µm. (K) Root of AAP3-GUS_L expressing tobacco. The vascular tissue is stained. Bar=500 µm.

putative regulatory boxes. Considering that the longer promoter is required for strong root expression, AAP3-GUS_L may contain additional enhancer elements, i.e. an At1box enhancer found in *rbc-3A* (Ueda *et al.*, 1989), or a scaffold/matrix attachment region (S/MAR) signature sequence found ~2 kb upstream of ATG. This sequence occurs rarely and is conserved in seven individual S/MAR from *Arabidopsis* (van Drunen *et al.*, 1997).

Intracellular localization using GFP fusions

To investigate the intracellular localization of AAP3, a GFP-AAP3 fusion was constructed. As GFP is quenched by acidic pH, the fusion protein was constructed in such a way as to allow GFP to face the cytosol. Experimental analysis of the topology of AAP1 (NAT2) suggested that the N-terminus of AAP1 is located in the cytoplasm whereas the C-terminus is directed towards the apoplast (Chang and Bush, 1997). Bioinformatic analyses (Schwacke *et al.*, 2003) suggest the same topology for AAP3. Therefore the coding sequence of AAP3 was fused at the 3'-end of the GFP gene (Fig. 3B). Yeast complementation showed that the fusion encodes a functional transporter (Fig. 4A-C). The GFP-AAP3 fusion protein was detected in the yeast plasma membrane, and also in

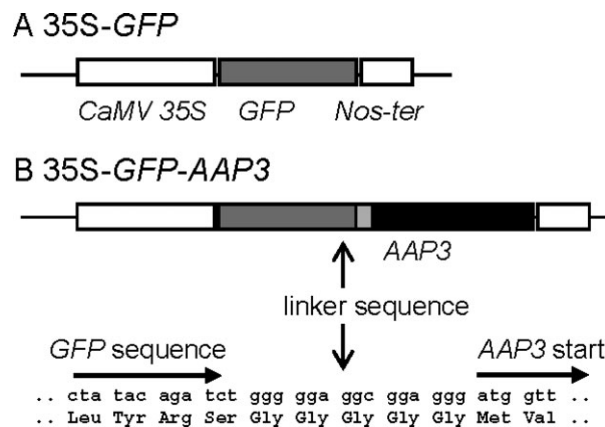


Fig. 3. GFP constructs used for plant transformation. (A) Control vector, GFP coding sequence under control of the CaMV 35S promoter. (B) AAP3 coding sequence was fused to the 3'-end of GFP after linker sequence. The amino acid sequence of the fusion region is described.

internal structures, especially as a ring around the nucleus, potentially indicating accumulation in karmellae (Fig. 4H-L). In BY-2 cells, fluorescence from GFP alone was found in the cytosol and the nucleus (Fig. 5A). GFP-AAP3 was found preferentially in punctate structures in the cytosol

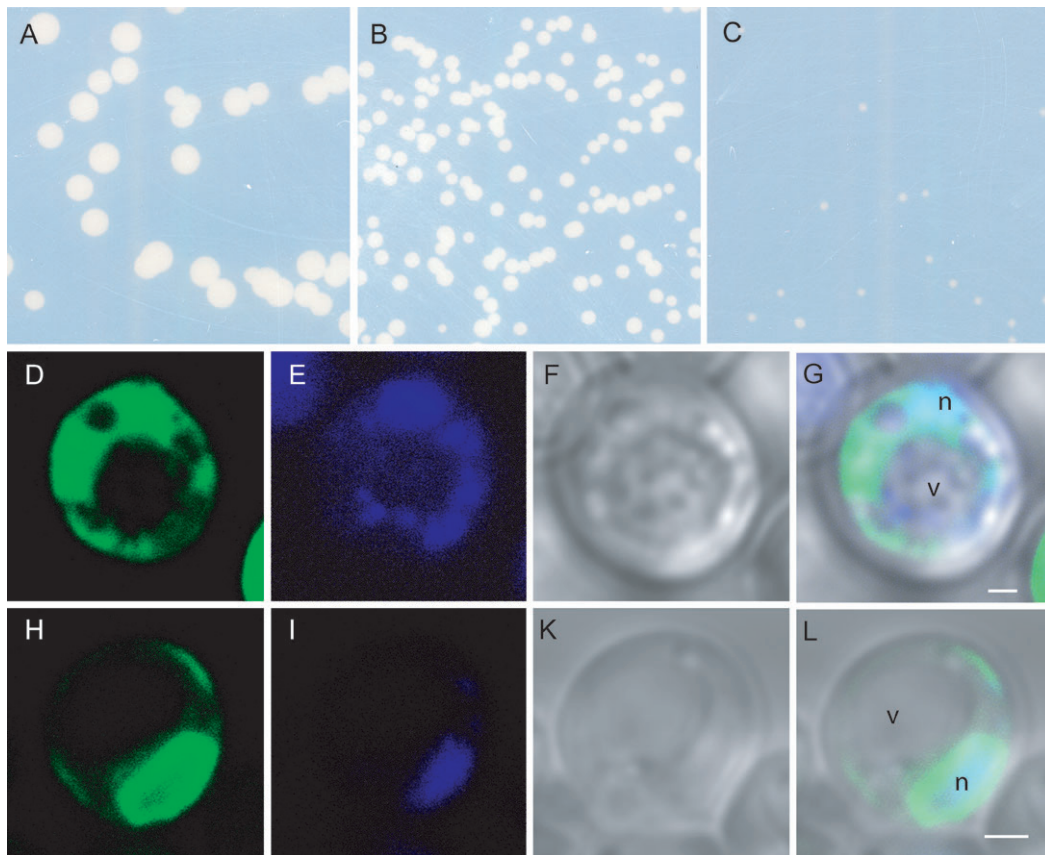


Fig. 4. Yeast strain 22Δ8AA cells expressing GFP and GFP-AAP3 fusion protein. (A–C) Growth of yeast colonies expressing GFP–AAP3 fusion protein (A), AAP3 (B) and the vector pDR195 (C) on minimal media containing 3 mM proline as the sole nitrogen source. Each panel represents 4 cm² area of a plate. (D–L) Images of single yeast cells expressing either GFP (D–G) or GFP–AAP3 (H–L). GFP (D, H), DAPI (E, I) and transmission (F, K) images were captured by confocal microscopy and merged (G, L). In cells expressing GFP (D–G), GFP fluorescence is observed throughout the cytosol and nucleus, whereas in cells expressing GFP–AAP3 (H–L), the fluorescence is observed at the plasma membrane and the nuclear membrane. n, Nucleus; v; vacuole. Bar=1 μm.

(Fig. 5B). Localization of GFP–AAP3 protein to the plasma membrane was also visible, whereas the tonoplast was never associated with GFP fluorescence. To test whether the accumulation in intracellular structures is due to accumulation of the membrane protein in the late endosome, Brefeldin A (BFA), a vesicle trafficking inhibitor, was added (Ritzenthaler *et al.*, 2002). BFA treatment led to the disappearance of the dot-like structure and a more diffuse pattern (Fig. 5C), indicating that the punctate structures might correspond to Golgi bodies.

Tagged AAP3 protein localizes in the phloem

Since the studies using the GFP–AAP3 fusion protein did not lead to a clear elucidation of the subcellular localization, and to confirm the AAP3 promoter–GUS studies, protein localization was investigated using epitope-tagged AAP3. Three copies of the *c-Myc* motif were fused to the 5'-end of the AAP3 translational start within the genomic clone (Fig. 6A). The construct, which contains ~2.5 kb of its native promoter region, the whole coding region including all introns, and 400 bp of the 3'-UTR, was used to transform

tobacco and *Arabidopsis*. In tobacco plants, *c-Myc*AAP3 was detected mainly in the phloem, consistent with the results of promoter–GUS experiments (Fig. 6C). Nine independent transgenic tobacco lines were analysed and six of them gave comparable patterns of localization, whereas no signals were detected in the roots of the other three lines (data not shown). In several cases, nuclei were observed in the cells expressing *c-Myc*AAP3, suggesting that these were companion cells (Fig. 6D). The protein was present both on the plasma membrane and the nuclear membrane. In *Arabidopsis*, the protein was also found in the cells in the phloem (Fig. 6E). The immunoreaction was not uniform, but was in patches as in BY-2 cells. In addition, endodermal cells at the primary and secondary root tips also expressed *c-Myc*AAP3, again not only at the plasma membrane but also in the internal membranes, i.e. nuclear envelopes (Fig. 6F, G, I). The presence of *c-Myc*AAP3 in the endodermis was not seen in the AAP3–GUS_L plants, indicating that sequences downstream of the promoter are responsible for expression in the endodermis. When roots were treated with Brefeldin A, an inhibitor of

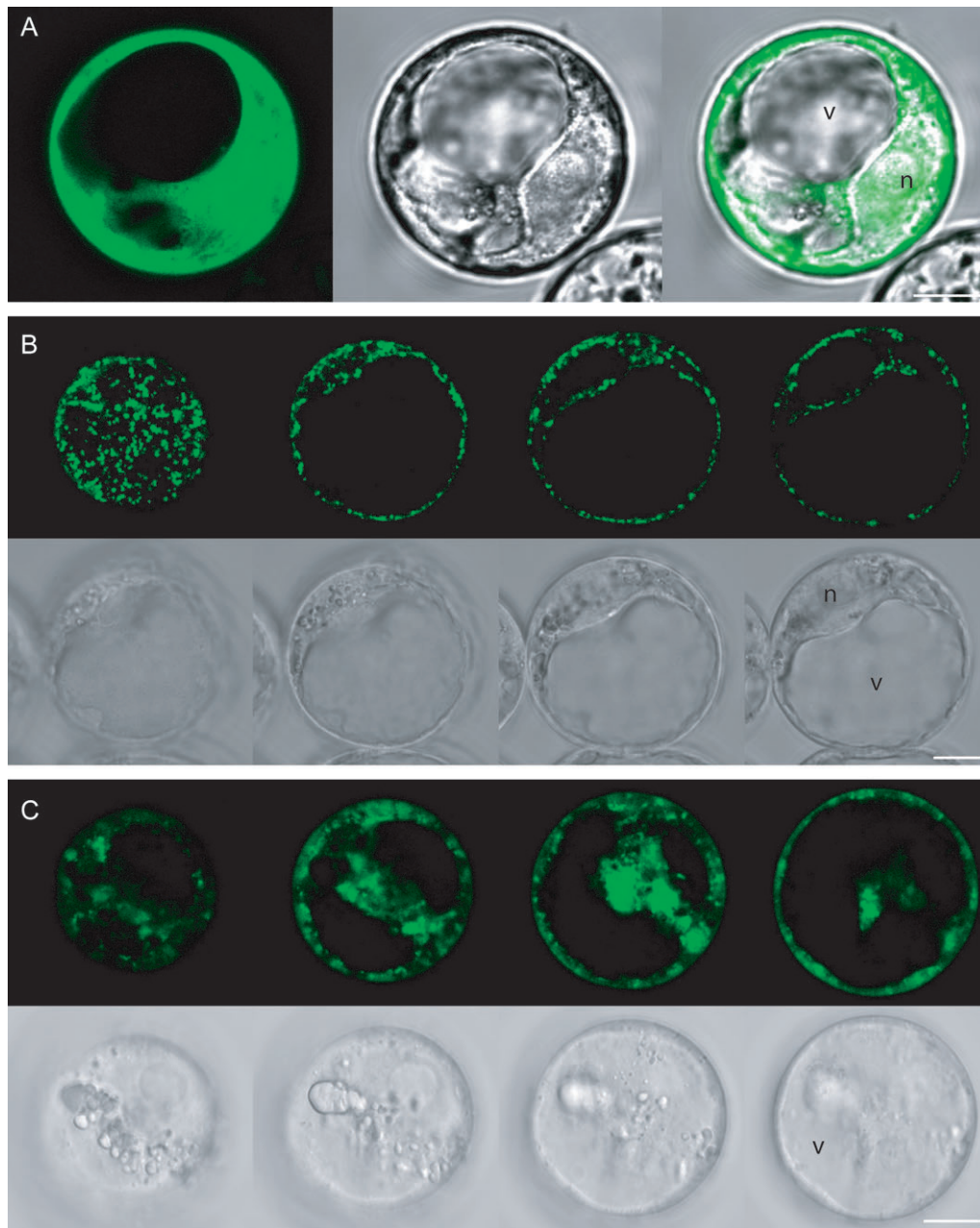


Fig. 5. Subcellular localization studies of GFP–AAP3 fusion protein. AAP3 protein was tagged with GFP and expressed transiently in tobacco BY-2 cells. (A) Protoplast expressing GFP protein under the control of the CaMV 35S promoter. GFP fluorescence is detected throughout the cytosol. The GFP fluorescence (left panel) and the bright field image (middle panel) are overlayed in the right panel. n, Nucleus; v; vacuole. Bar=10 μ m. (B) Serial sections of a protoplast which expresses GFP–AAP3 fusion protein. The GFP fluorescence (upper panels) is detected around the nucleus, dot-like structure in the cytosol and plasma membrane. Note that the tonoplast is signal free. The lower panels represent the bright field images of the upper panels. n, Nucleus; v; vacuole. Bar=10 μ m. (C) Serial sections of GFP–AAP3 expressing protoplast which was treated with 100 μ M of Brefeldin A. The pictures were taken 30 min after BFA treatment. v; Vacuole. Bar=10 μ m.

vesicular transport, the fusion protein accumulated in intracellular particles, supporting the idea that $c\text{-Myc}$ -AAP3 is on its way to the plasma membrane (Fig. 6G). Immuno-gold localization on ultrathin cryosections at the transmission electron microscope (TEM) level shows plasma membrane localization (Fig. 6K, M) as well as intracellular accumulation at the nuclear membrane (Fig. 6L), at multi-

vesicular body-like vesicles (Fig. 6M), at Golgi bodies (Fig. 6N), at the ER, and the cell plate (not shown).

AAP3 T-DNA insertion lines

To investigate the function of AAP3, two independent *Arabidopsis* lines carrying T-DNA insertions in the AAP3 gene were isolated by reverse genetic screens of *Arabidopsis*

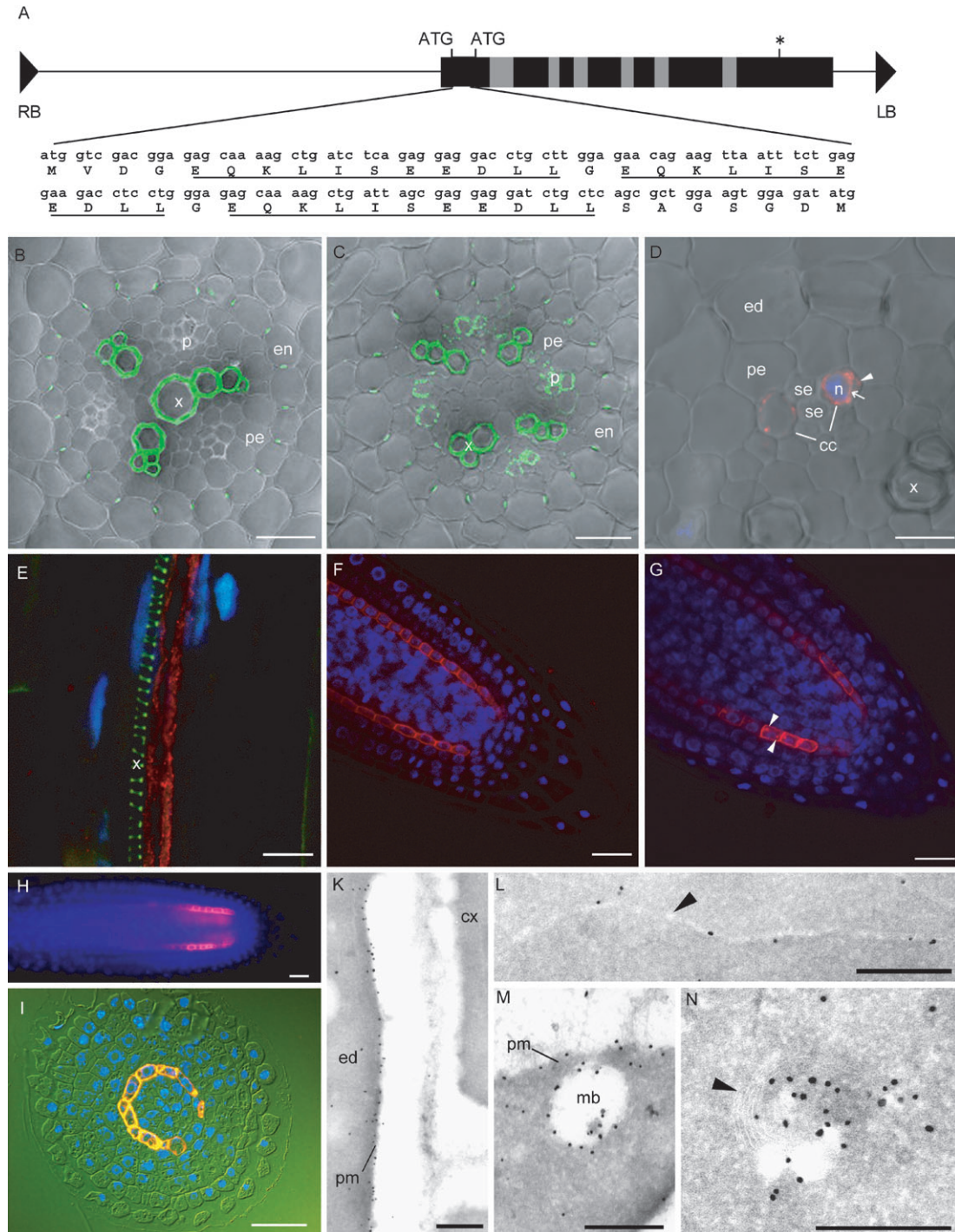


Fig. 6. Cellular and subcellular localization studies using *c-MycAAP3* protein. (A) *c-MycAAP3* construct. The three repeats of the *c-Myc* motif (indicated by an underline in the amino acid sequence) are fused to the 5'-end of *AAP3* translational start. Black boxes indicate exons. The termination codon is indicated by an asterisk. (B, C) Immunolocalization on fresh sections from tobacco roots. The *c-MycAAP3* protein localization (indicated as green fluorescence) is overlaid on the bright-field image. The antibody did not react on the wild-type root section (B) (the signals in the xylem cell wall and casparian strips are autofluorescence) whereas the phloem cells of *c-MycAAP3* line root section (C) were recognized by anti *c-Myc* antibody. p, Phloem; pd, peridermis; x, xylem. Bar=20 μm. (D) Magnified image of a root section from *c-MycAAP3* tobacco line. The nucleus in a companion cell is visualized by DAPI (indicated by a blue colour). Note that *c-MycAAP3* protein (indicated by red fluorescence) is associated with both plasma membrane (arrowhead) and nuclear membrane (arrow). pd, Peridermis; x, xylem; se, sieve element; cc, companion cell. Bar=10 μm. (E–H). Whole-mount immunodetection on roots of the *c-MycAAP3 Arabidopsis* line. Nuclei are visualized by DAPI (indicated by the blue colour). (E) Image of the central cylinder. Two cell files, most probably phloem cells, are recognized by the anti *c-Myc* antibody (red). Green autofluorescence from the xylem is also shown. x, Xylem. Bar=10 μm. (F) In the root tips, *c-MycAAP3* protein is localized to the plasma membrane of the endodermal cells. Bar=10 μm. (G) When the root is treated with Brefeldin A (50 μM, 4 h), *c-MycAAP3* protein accumulates in vesicle-like structures (indicated by arrowheads). Bar=10 μm. (H) The expression of

mutants generated at the University of Wisconsin Knockout *Arabidopsis* facility. Sequence analysis confirmed that *aap3-1* and *aap3-2* contain T-DNA insertions in the sixth and second exon of *AAP3*, respectively (Fig. 7A). RT-PCR revealed that both alleles do not express functional transcripts. Primer pairs specifically amplifying a unique fragment of *AAP3* downstream (*aap3-2*) or on the both sides (*aap3-1*) of the insertions from seedling cDNA confirmed that the insertions destroyed the locus (Fig. 7B).

After isolating homozygous *aap3-1* and *aap3-2* and after backcrossing, plants were grown under various conditions (soil, on agar medium with ammonium, nitrate or amino acids as the sole nitrogen source, toxic amino acid analogues, nitrogen starvation) and compared to the Wassilewskija wild type. However, under the conditions tested, no obvious difference in growth was observed between wild-type and *aap3* plants (data not shown).

To investigate whether *aap3* mutants are affected in amino acid uptake, the transport of ^{14}C -labelled amino acids was measured. Since *AAP3* mediates the uptake of neutral and basic amino acids when expressed in yeast and *Xenopus* oocyte cells, glutamine, proline, aspartate, histidine, and lysine were chosen for the assay. In all three cases, uptake was sensitive to the protonophore CCCP (Fig. 7C, D), however, no significant differences in uptake rates relative to the wild type were observed.

Even though *aap3* mutants did not show visible phenotypes under the conditions tested, the insertion might have effects on gene regulation. To investigate whether *aap3* mutants show a molecular phenotype, DNA microarray analysis was performed. Since many reactions of amino acid synthesis pathways are located in chloroplasts (Singh, 1999), custom-made GST arrays representing 3292 genes, most of which code known or predicted chloroplast proteins, were used. The arrays were hybridized with labelled cDNA probes from 18-d-old wild-type or *aap3-1* *Arabidopsis* seedlings. Among 3292 genes, 239 genes were significantly up-regulated in *aap3-1*, whereas 581 genes were down-regulated (Supplementary data: see Supplementary Table 1 at *Journal of Experimental Botany* online). Overall, the observed change in the transcript profile was relatively small, the highest overrepresentation and lowest underrepresentation in *aap3-1* was 2.1-fold (observed for the *Lhcb1.2* gene, At1g29910) and 0.46-fold (observed for a putative protein, At4g22650) compared with the wild type, respectively. Among the genes involved in amino acid metabolism, the largest increase was observed for the putative 2-isopropylmalate synthase gene

(At1g18500, 1.9-fold) and the largest decrease was observed for a plastid-localized aspartate aminotransferase (At4g31990, 0.59-fold). The relatively small change in the gene expression profile, together with the fact that significant differences could not be detected in amino acid levels in *aap3-1* and *aap3-2* plants compared with the wild type (data not shown), indicates that the defect in *AAP3* function does not lead to a prominent change in metabolism, at least under the conditions tested, possibly due to compensation.

Discussion

The broad specificity amino acid transporter *AAP3* is expressed in roots (Fischer *et al.*, 1998, 1995). Coupling of amino acid with proton transport indicates that *AAP3* serves as a cellular uptake system or a vesicular release system. Contrary to the expectation that *AAPs* might be involved in direct uptake from the soil (Näsholm and Persson, 2001), *AAP3* is not expressed in the rhizodermis or in roots hairs, but was found to be expressed specifically in the phloem of roots. Promoter-GUS analyses were confirmed by immunolocalization of the tagged transporter. *AAP3* is mainly present at the plasma membrane, although a certain amount of the protein was also found in intracellular compartments. In addition to the phloem tissue, *AAP3* seems to serve other functions in a cell file at the root tip and in the connective tissue of the anthers.

Root-specific expression of *AAP3*

AAP3 is expressed in the vascular tissues of *Arabidopsis* roots, mainly in the phloem of the root and in a cell file corresponding to the endodermis in the root tip. Phloem is the continuous tissue throughout the plant, but there is a longitudinal differentiation along the path. While the sucrose transporter *SUT1* is present all along the phloem from major veins to the minor veins in the sink tissues, a galactinol synthase gene and the sucrose transporter *SUT4* are preferentially expressed in minor vein phloem, indicating different roles of major/minor vein phloem (Weise *et al.*, 2000). Thus the expression of *AAP3* in root phloem might suggest a specific role required in root phloem.

Based on knowledge of the physiology of amino acid partitioning, several hypotheses for a function of *AAP3* at the plasma membrane may be considered. Firstly, *AAP3* might play a role in the retrieval of amino acids leaked from the phloem. For sucrose transport in the phloem, retrieval of

c-Myc-*AAP3* protein in the endodermal cells stops after the elongation zone. Bar=10 μm (I). Cross-section of a root tip. The *c-Myc*-*AAP3* protein (indicated by the red fluorescence) is localized at the plasma membrane. *c-Myc*-*AAP3* protein is also localized to the nuclear membrane (DAPI-stained nuclei are blue) although the strength of the signal is much weaker. Bar=20 μm . (K) Electron microscopy of an endodermal cell. Note that the plasma membrane of the neighbouring cortex cell is not labelled. n, Nucleus; cx, cortex; ed, endodermis; pm, plasma membrane. Bar=0.2 μm . (L) *c-Myc*-*AAP3* protein localizing in nuclear membrane (arrowhead). Bar=0.1 μm . (M) *c-Myc*-*AAP3* protein localizing at a multivesicular body-like vesicle and plasma membrane. mb, Multivesicular body; pm, plasma membrane. Bar=0.1 μm . (N) *c-Myc*-*AAP3* protein localizing at a Golgi body (arrowhead). Bar=0.1 μm .

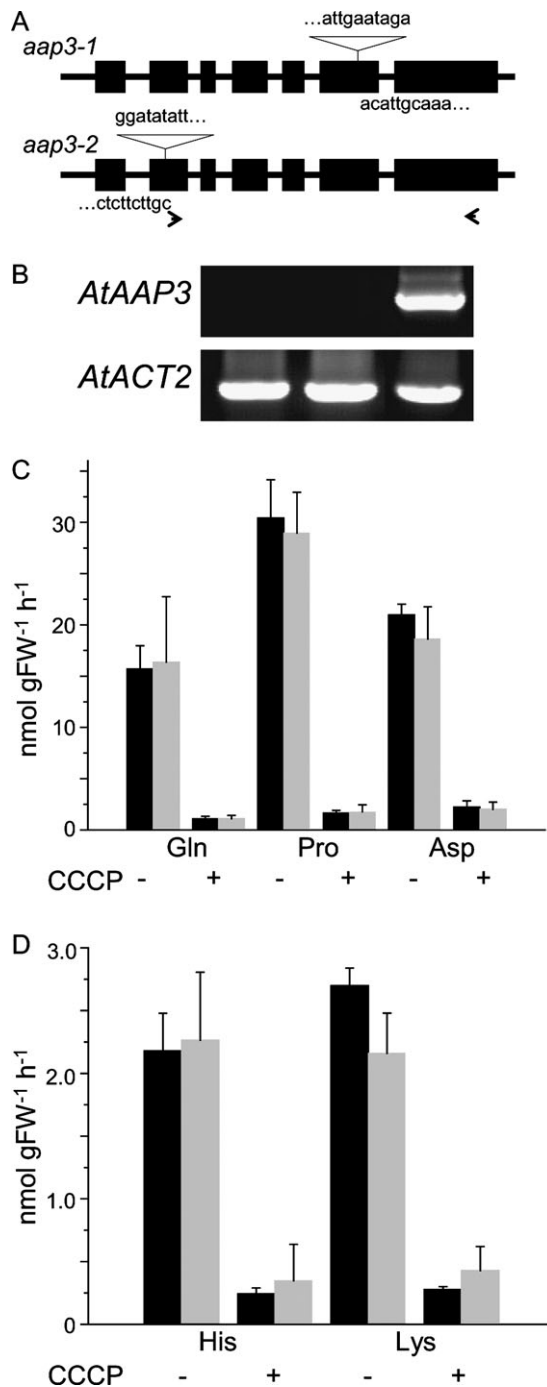


Fig. 7. Analysis of T-DNA insertion lines of *AAP3*. (A) Locations of the T-DNA insertions within the sequence of the *AAP3* gene. The sequences of the gene-T-DNA insertion junction are indicated. (B) RT-PCR performed on the cDNA from *aap3-1* (left lane), *aap3-2* (middle lane), and *Wassilewskija* wild-type (right lane) plants. The RT-PCR using *AAP3* gene-specific primers (indicated by arrows in (A)) did not detect the transcription of the *AAP3* in *aap3-1* and *aap3-2* lines (upper panel). Lower panel, the control reaction performed with the gene-specific primer of *AtACT2* gene. (C, D) Uptake studies of neutral (C) and basic (D) amino acids using whole seedlings from *Wassilewskija* wild-type (solid bar) and *aap3-1* (grey bar) plants. Seedlings were incubated in ^{14}C -labelled amino acids in the absence/presence of 0.1 mM CCCP.

sugars leaking out of the phloem is thought to play an important role for maintaining the pressure gradient (Maynard and Lucas, 1982). Consistently, the sucrose transporters SUT1 and SUC2 are not only present in leaf phloem, but also along the path and in sink phloem, i.e. roots, siliques, and tubers (Kühn *et al.*, 2003; Truernit and Sauer, 1995). *AAP3* may serve a similar function, i.e. retrieval of amino acids specifically along the root phloem of *Arabidopsis*.

Secondly, *AAP3* may load certain amino acids synthesized in root tissue and transported into the root phloem, from which they are either unloaded symplasmically in root tips or trans-loaded into the xylem for translocation into shoots. The *Arabidopsis* mutant *dhdps2*, deficient in one of the lysine biosynthesis enzymes, accumulates aspartate and threonine in root and shoot tissue, whereas most of the aspartate- and pyruvate-derived amino acids accumulate only in root tissue, indicating that the synthesis of these amino acids occurs mainly in the roots (Sarrobot *et al.*, 2000). *AAP3*, one of two *AAP* transporters mediating the uptake of basic amino acids, might specialize in the uptake of root-synthesized amino acids (Fischer *et al.*, 2002).

Thirdly, *AAP3* might be involved in the unloading of amino acids into the cells surrounding the phloem. Since the phloem tissue seems to be symplasmically isolated from the parenchyma cells, except at the root tip (Wright *et al.*, 1996), amino acids imported into the parenchyma cells must cross the plasma membrane of the phloem and parenchyma cells. The amino acids taken up in parenchyma cells can either circulate round the plant body via the xylem, or supply the cells in the root tissue, and *AAP3* might mediate these steps. A similar hypothesis has been put forward in the case of the tuber localization of SUT1, since a block of sucrose transport activity by antisense inhibition leads to retarded tuber development (Kühn *et al.*, 2003).

The potential role of AAP3 in tips of filaments

AAP3 is also expressed in flowers, i.e. in the connective tissue of stamens, where it is induced shortly before dehiscence. Dehiscence is linked to desiccation and is controlled by jasmonic acid (Sanders *et al.*, 2000). It is assumed that the accumulation of osmotically active compounds, such as sugars, proline and betaine is accelerated, leading to water efflux from the anther wall and thus triggering dehiscence. The sucrose transporter *AtSUC1* is co-expressed in connective tissue at the same stage of the anther development as *AAP3*, indicating its role in triggering dehiscence (Stadler *et al.*, 1999). *AAP3* transports a variety of amino acids including proline, a major osmolyte, therefore it may contribute to the accumulation of osmotically active compounds in the connective tissue. Alternatively, *AAP3* could function in the uptake of amino acids from anther tissue. Although the extracellular concentration of amino acids in the tapetum is unknown, it was

speculated to be in the millimolar range considering the rapid degradation of the tapetum (Schwacke *et al.*, 1999). AAP3 might serve in the recovery of organic nitrogen from dehiscid anthers, which no longer require amino acids.

Subcellular localization of AAP3

Recently, as many as 53 putative amino acid carriers were identified in the *Arabidopsis* genome by homology searches (Wipf *et al.*, 2002). The amino acid carriers were divided into three superfamilies; APC, ATF1, and MFS superfamilies. Each of these has related animal and yeast members and contains a wide spectrum of amino acid carriers, differing in the coupling mechanism (e.g. H^+ / Na^+ -coupled symporters, uniporters, and pH-gradient driven), substrate specificity, and localization. Some members of the ATF1 superfamily are localized at the plasma membrane (Swarup *et al.*, 2001), whereas other members are known to be involved in the import or export of amino acids at the membrane of intracellular vesicles and vacuoles (Rusnak *et al.*, 2001). AAPs, a subfamily within the ATF1 superfamily, mediate amino acid uptake in yeast and oocytes, which indicates the plasma membrane localization of these proteins. However, the localization in heterologous expression systems is not necessarily an indicator of the native localization of the protein, for example, AHA1 and AHA2, the plant plasma membrane H^+ -ATPases, are not targeted to the plasma membrane, but rather stay 'trapped' in the ER when expressed in yeast (Palmgren and Christensen, 1993). Therefore, the subcellular localization was investigated by using GFP–AAP3 protein and $c\text{-Myc}$ –AAP3 protein.

The GFP–AAP3 fusion proteins localized in punctate structures, nuclear membrane, and plasma membrane when transiently expressed in BY-2 protoplasts. Brefeldin A (BFA), a fungal toxin, has various effects on plant cells including the accumulation of Golgi membranes in clusters, the fusion of the Golgi with the ER, and the loss of distinct Golgi stacks (Ritzenthaler *et al.*, 2002). The treatment with BFA led to the loss of the punctate structures in protoplasts, suggesting identity to Golgi bodies or prevacuolar compartments.

The immunolocalization using *Arabidopsis* and tobacco plants expressing $c\text{-Myc}$ –AAP3 revealed that AAP3 is mainly localized at the plasma membrane. This finding was of particular importance considering the existence of vesicular and vacuolar carriers in the ATF1 superfamily (Rusnak *et al.*, 2001). The $c\text{-Myc}$ –AAP3 protein was also localized in intracellular organelles and immunoelectron microscopy showed gold labelling on nuclear membrane, ER, Golgi, and multivesicular body-like vesicles. The distribution ratio of the protein in plasma membrane/intracellular membrane was different between protoplasts overexpressing GFP–AAP3 and *Arabidopsis* plants expressing $c\text{-Myc}$ –AAP3. This might reflect the difference in the expression level in both experiments. Considering that $c\text{-Myc}$ –AAP3 was expressed under its own promoter in *Arabidopsis* plants, it is believed

that native AAP3 protein is mainly localized at the plasma membrane, as the experiment using $c\text{-Myc}$ –AAP3 indicated.

The localization of GFP–AP3 and $c\text{-Myc}$ –AAP3 in Golgi bodies and ER may reflect protein in the sorting pathway, since membrane proteins are first integrated into intracellular vesicles before they are sorted to their 'final destination' (Söllner *et al.*, 1993). The localization in Golgi bodies and ER may also represent the proteins that are endocytosed and recycled. Other plant membrane proteins such as plasma membrane H^+ -ATPase and PIN1, a putative auxin efflux carrier, are turned over rapidly, and plasma membrane localization is rapidly abolished by various membrane trafficking inhibitors (Geldner *et al.*, 2001). Alternatively, AAP3 might be targeted to these organelles as well as to the plasma membrane, although the AAP3 protein sequence lacks a known signal peptide. A dual localization and function has been described for the metal transporter DMT1, which functions in the intestinal import at the plasma membrane and in the release of iron from intracellular vesicles (Roth *et al.*, 2000). Furthermore, many transport proteins are known to be targeted to, or removed from the plasma membrane, for example, in the basal state, the glucose transporter GLUT4 is localized in subapical vesicles and is released to the plasma membrane upon stimulation by insulin (Al-Hasani *et al.*, 2002). The yeast general amino acid permease GAP1 is subject to nitrogen catabolite inhibition, which involves ubiquitination and endocytosis (Springael *et al.*, 2002). Similarly, the yeast plasma membrane siderophore-iron transporter Arn1p are localized to the plasma membrane and undergo rapid endocytosis at higher substrate concentration (Kim *et al.*, 2002). Currently, little is known about the regulation of subcellular localization of plant transporters, but AAP3 might undergo such dynamic intracellular relocation as GLUT4, GAP1, and Arn1p. The tagged AAP3 may thus serve as a tool to study the conditional localization of AAP3 by analysing its subcellular distribution in response to different nitrogen nutrition regimes, temperatures etc.

Physiological function of AAP3

Despite the complexity of amino acid transport at the molecular level, antisense inhibition of an amino acid transporter StAAP1 leads to a reduction of amino acid levels in potato tubers, demonstrating its importance for long-distance transport of amino acids (Koch *et al.*, 2003). However, the results do not exclude that antisense affects more than one member of the family. The potential role of AtAAP3 in root phloem was analysed using insertion mutants. The homozygous *aap3-1* and *aap3-2* mutants showed no effects in response to alterations in nitrogen nutrition. Under none of the conditions did the mutants behave differently from the wild type. Analyses of the nuclear transcriptome in the *aap3-1* mutant also revealed a minor change in the genes involved in amino acid metabolism.

Within the AAP family, AAP2, AAP5, and AAP6 were also found to be expressed in roots (Fischer *et al.*, 1995), and they may be compensating for the loss of AAP3 activity in the mutants. Therefore, studies using double- or multi- *knock-out* mutants or RNAi approaches, combined with high-throughput transcriptome analyses to monitor the expression level of all amino acid carriers, might be required to deduce the function of AAPs.

Moreover, functional compensation could be achieved by one or more of the 54 putative amino acid carriers (Wipf *et al.*, 2002). Due to the complexity of the gene families involved in amino acid transport, it may be expected that only the combination of mutants, together with new analytical tools such as nanosensors, will enable this complexity to be resolved.

Supplementary material

The complete result of GST array is available as Supplementary material (Supplementary Table 1) at *Journal of Experimental Botany* online.

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