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Characterization of a protein of the plastid inner envelope having homology to animal inorganic phosphate, chloride and organic-anion transporters

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Abstract A protein from *Arabidopsis thaliana* (L.) Heynh. showing homology to animal proteins of the NaPi-1 family, involved in the transport of inorganic phosphate, chloride, glutamate and sialic acid, has been characterized. This protein, named ANTR2 (for *anion transporters*) was shown by chloroplast subfractionation to be localized to the plastid inner envelope in both *A. thaliana* and *Spinacia oleracea* (L.). Immunolocalization revealed that ANTR2 was expressed in the leaf mesophyll cells as well as in the developing embryo at the upturned-U stage. Five additional homologues of ANTR2 are found in the *Arabidopsis* genome, of which one was shown by green fluorescent protein (GFP) fusion to be also located in the chloroplast. All ANTR proteins share homology to the animal NaPi-1 family, as well as to other organic-anion transporters that are members of the Anion:Cation Symporter (ACS) family, and share the main features of transporters from this family, including the presence of 12 putative transmembrane domains and of a 7-amino acid motif in the fourth putative transmembrane domain. ANTR2 thus represent a novel protein of the plastid inner envelope that is likely to be involved in anion transport.

Keywords Anion · *Arabidopsis* · Chloroplast · Inner envelope · Transporter

Abbreviations ACS: Anion:Cation Symporter · GFP: green fluorescent protein · Pi: inorganic phosphate

Introduction

The plastid is the site for a number of biosynthetic functions essential to plants, including photosynthesis, nitrogen assimilation and the synthesis of amino acids, as well as the synthesis of starch, fatty acids and numerous secondary products. These diverse biochemical functions rely on the controlled exchange of metabolites between the plastid and the surrounding cytosol. Plastids are double-membrane organelles. Although the outer envelope membrane contains a number of proteins that have been shown to have channel-like activities (Pohlmeier et al. 1997), and therefore may act as a selectivity filter, the major site of the controlled exchange of metabolites across the plastid is the inner envelope (Fischer and Weber 2002). Numerous proteins located in the inner envelope membrane are needed for the transport of a variety of metabolites, including inorganic anions such as phosphate, sulfate, nitrite and chloride, organic anions such as amino acids and pyruvic acid, as well as adenylates and phosphorylated intermediates (Fischer and Weber 2002; Neuhaus and Wagner 2000).

To date, only a small subset of transporters of the plastid inner envelope has been biochemically characterized and the corresponding gene identified. These include the triose phosphate/phosphate exchanger, phosphoenolpyruvate/phosphate exchanger, 2-oxoglutarate/malate and glutamate/malate translocators, ADP/ATP translocator, glucose-6-phosphate/phosphate translocator, pentose phosphate/phosphate translocator and the glucose translocator (Flügge et al. 1989; Weber et al. 1995, 2000; Fischer et al. 1997; Neuhaus et al.

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1997; Kammerer et al. 1998; Eicks et al. 2002; Taniguchi et al. 2002; Renné et al. 2003). It is thus clear that numerous additional transporters of the plastid inner envelope remain to be identified and characterized.

Several approaches can be used to identify and characterize transporters present in the plastid inner envelope. These range from the classical approach of biochemical purification of proteins followed by reconstitution into artificial membranes, to approaches using genomics, including the *in silico* analysis of the genome of *Arabidopsis thaliana* for the presence of genes encoding putative plastid membrane proteins as well as the analysis of the proteome of the plastid inner envelope using organic solvent extraction and MALDI-TOF analysis (Ferro et al. 2002, 2003; Koo and Ohlogge 2002).

In this work, we demonstrate the identification of a novel protein present in the plastid inner envelope of *A. thaliana* and *Spinacia oleracea* that shows homology to animal transporters involved in inorganic phosphate (Pi), chloride and glutamate transport.

Materials and methods

Plant material

Wild-type *Arabidopsis thaliana* (L.) Heynh., accession Columbia (*Arabidopsis* Biological Resources Center, Ohio), was grown under continuous light on a mixture of 2/3 soil and 1/3 perlite at 19 °C and 60% relative humidity. Spinach (*Spinacia oleracea* L.) leaves were purchased at the local supermarket.

DNA constructs and expression in *Escherichia coli*

The full-length *ANTR1* cDNA was isolated by screening the PRL-2 cDNA library (Newman et al. 1994) with a probe derived from a partial expressed sequence tag (EST). A similar screen for *ANTR2* gave rise only to an incomplete cDNA. The full-length *ANTR2* cDNA was isolated by a 5'-RACE-PCR approach (Frohman et al. 1988) on the *Arabidopsis* PRL-2 cDNA library. The PCR product corresponding to the missing 5' end was inserted at the *Bgl*II site located near the 5' end of the incomplete cDNA using standard molecular biology procedures.

The plasmid psmRS-GFP was used for the construction of the ANTR1-GFP fusion (Davis and Vierstra 1998). A PCR fragment including the whole coding region of *ANTR1* was inserted in the *Xba*I-*Bam*HI sites of psmRS-GFP in order to create an in-frame fusion protein with the ANTR1 protein sequence at the N-terminus and green fluorescent protein (GFP) at the C-terminus. The cassette containing the CaMV 35S promoter-ANTR1-GFP fusion-Nos terminator was excised by *Hind*III-*Eco*RI and inserted in the binary vector pBI121 (Clontech, Palo Alto, CA, USA). As a control the GFP from the plasmid psmRS-GFP was also subcloned in the binary vector pBI121.

Polypeptides representing the N-terminal hydrophilic portion of the mature ANTR1 and ANTR2 proteins were expressed in *E. coli* using the pET expression system (Novagen, Madison, WI, USA). Briefly, DNA fragments encoding portions of ANTR1 and ANTR2 were amplified by PCR using oligonucleotides creating *Nde*I and *Bam*HI restriction sites at the 5' and 3' ends, respectively, and cloned in the same sites in the pET14b vector. The proteins were expressed in the *E. coli* strain BL21(DE3)LysE growing in media containing isopropyl β -D-thiogalactopyranoside (IPTG). Total protein extracts were analyzed by Western blotting using ANTR2 antibodies.

Plant transformation

Arabidopsis was transformed with the GFP and ANTR1-GFP constructs by the floral-dip method using *Agrobacterium tumefaciens* pGV3101 (Clough and Bent 1998). Transgenic plants were selected on Murashige and Skoog media containing 1% sucrose and 50 μ g/ml kanamycin. Epidermal peels of the abaxial surface of leaves of transgenic plants were analyzed by epifluorescence microscopy.

Chloroplast isolation

Chloroplasts were isolated according to Douce and Joyard (1982) on a Percoll gradient. Leaves were cut from young plants (less than 4 weeks old) and stored in air at 4 °C overnight to reduce the starch content of the chloroplasts. For spinach leaves, the mid-rib of each leaf was removed before storing overnight at 4 °C. All further steps were performed at 4 °C. Leaves were homogenised with two short bursts (3–5 s each) in a Waring Blender in 3 vol. of Buffer H [0.33 M sucrose, 30 mM Na-pyrophosphate (pH 7.8), 0.1% BSA]. The homogenate was filtered through one layer of Miracloth and centrifuged for 10 min at 1,200 g. The pellet was gently resuspended in Buffer J [0.33 M sucrose, 10 mM MOPS-KOH (pH 7.8)] with a paintbrush. The suspension was filtered through one layer of Miracloth, layered on a 40/80% (v/v) Percoll step gradient in Buffer J and centrifuged at 3,000 g for 20 min. The intact chloroplasts were collected from the 40/80% interphase, diluted at least 5 times with Buffer J and centrifuged at 3,000 g for 5 min. Finally the chloroplasts were resuspended with a paintbrush in an appropriate volume of Buffer J. The amount of chlorophyll in the chloroplast preparation was determined by mixing 10 μ l of purified chloroplasts with 10 ml of 80% (v/v) acetone. Aliquots of 1 ml were centrifuged for 30 s. The absorbance at 652 nm was determined and the amount of chlorophyll calculated with the formula: chlorophyll (mg/ml) = OD₆₅₂ × 28.99.

Isolation of chloroplast envelopes

Envelopes were separated by methods adapted from Joyard et al. (1982). Intact chloroplasts were resuspended in a hypertonic medium [0.66 M sucrose, 10 mM MOPS-KOH (pH 7.8), 4 mM MgCl₂], frozen at -20 °C for at least 1 h and thawed at room temperature for 1 h. The chloroplast envelopes were then stripped-off by homogenisation in a Dounce (Sigma, St. Louis, MO, USA) homogeniser (20–40 strokes). The freezing and homogenising were repeated once more. The homogenate was supplemented with sucrose to a final concentration of 1.1 M, overlaid with hypertonic medium (0.66 M sucrose) and Buffer J [0.33 M sucrose, 10 mM MOPS-KOH (pH 7.8)]. This gradient was centrifuged at 113,000 g at 4 °C for at least 3 h. Highly purified outer envelopes were isolated from the 0.33/0.66 M interphase, diluted at least 3 times with Buffer J and centrifuged at 208,000 g. Enriched inner envelopes were collected from the 0.66/1.1 M interphase, diluted at least 3 times with Buffer J and centrifuged for 60 min at 208,000 g. The envelope pellets were resuspended in an appropriate volume of Buffer J.

Total protein extraction and quantification

For small-scale extractions, 100 mg of plant material was ground with 100 μ l of extraction buffer [80 mM Tris-HCl (pH 8.0), 5 mM DTT, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100] in an Eppendorf tube. For larger amounts the plant material was ground in liquid nitrogen, and the powder extracted with the same amount (w/v) of extraction buffer. Cell debris was separated by centrifugation at 10,000 g in a table-top centrifuge. The supernatant was stored at -80 °C. The amount of protein in the different organelle fractions and from the total

protein extractions was determined with the Bradford assay from BioRad (Richmond, CA, USA). In the Western analysis of crude extracts from various tissues, care was taken to load equal amounts of proteins on the gel, and the quality of the extracts and of the transfer was controlled by Ponceau Red staining.

Analysis of organelle and membrane fractions

The purity of organelles and membrane fractions was tested by Western blot analysis. Proteins were fractionated by SDS-PAGE. The sample denaturation was done in Laemmli buffer on ice for 1 h. The proteins were transferred onto nitrocellulose in a Trans-Blot electrophoretic cell (BioRad). Free binding sites were blocked with blocking buffer (8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na₂HPO₄, 0.24 g/l KH₂PO₄, 0.1% Tween-20, 5% milk powder). The nitrocellulose membranes were incubated with the following organelle-specific antibodies: aquaporin RD28 for plasma membranes (Daniels et al. 1994), pyrophosphatase VPPase326 for tonoplast (Kim et al. 1994), porin for mitochondria (Heins et al. 1994), SEC12 for the ER (Bar-Peled and Raikhel 1997), TOC75 for outer-envelope proteins (Tranel et al. 1995), TIC110 for inner-envelope proteins (Lübeck et al. 1996) and LHCI for thylakoids. The antibody dilutions used were 1:500 for RD28, TIC110 and At-SEC12, 1:800 for porin, 1:1,000 for VPPase326 and TOC75, and 1:2,000 for LHCI. AtSEC12 antibody was purchased from Rose Biotechnology (Palo Alto, CA, USA). The ANTR2 rabbit antibody was made against the peptide NSIKINRSRAYKSEESDIA that was coupled to keyhole-limpet protein (Eurogentec, Belgium). Antibodies were visualized with horseradish peroxidase conjugated to protein A and the Enhanced Chemiluminescence Kit (ECL; Amersham, UK) as recommended by the manufacturer.

Immunolocalization

Tissues were fixed in a solution containing 3.7% (w/v) formaldehyde, 5% (v/v) acetic acid, 50% (v/v) ethanol, for 4 h at room temperature, followed by 3 days at 4 °C. Tissues were then dehydrated in an ethanol gradient, cleared in xylol and embedded in paraffin wax. Section of 10 µm were dewaxed, hydrated and washed in water, and incubated in blocking buffer containing 10% (w/v) BioRad blotting-grade blocker in 30 mM Tris-HCl (pH 7.5), 37 mM NaCl and 0.5% (v/v) Tween-80. Sections were incubated with immune or pre-immune sera in blocking buffer for 2 h at room temperature followed by washes in TBS-T buffer containing 30 mM Tris-HCl (pH 7.5), 37 mM NaCl and 0.5% (v/v) Tween-80. The secondary antibody was a goat anti-rabbit immunoglobulin linked to alkaline phosphatase (Dako, Switzerland). The secondary antibody was diluted in blocking buffer and incubated with the sections for 1 h at room temperature. After washing (first washing with TBS-T buffer with Tween-80 followed by buffer without Tween), colour was developed using the Dako NBT/BCIP system. Sections were counterstained with 0.1% (w/v) alcian green for 20 s, dehydrated in an ethanol series, cleared in xylene and mounted with Eukitt (Kindler, Germany).

Results

Identification of two genes homologous to animal anion transporters

NaPi-1 was initially characterized as a gene expressed in rabbit kidney cells that confers sodium-dependant phosphate transport when expressed in *Xenopus* oocytes (Werner et al. 1991). Further studies have shown that in *Xenopus* oocytes, NaPi-1 acts as anion channel permeable to chloride and organic anions (Busch et al. 1996).

Fig. 1 Alignment of the *Arabidopsis thaliana* ANTR protein family. The consensus sequence for ACS family members is indicated by *asterisks*. The putative transit-peptide cleavage sites for the proteins ANTR1, ANTR2 and ANTR3 are indicated by the digits 1, 2 and 3, respectively, placed over the sequences. Alignment was made with the ClustalW1.74 program

More recently, the *Caenorhabditis elegans* EAT4 homologue was shown to be involved in glutamatergic neurotransmission (Lee et al. 1999), while the human homologue VGLUT1 has been shown to be a neuronal glutamate transporter that also exhibits a conductance for chloride (Bellocchio et al. 2000; Takamori et al. 2000). Another human homologue of NaPi-1, named AST, has been implicated in the transport of sialic acid (Verheijen et al. 1999).

We initially selected two expressed sequence tags from *Arabidopsis* that showed homology to the rabbit NaPi-1 gene. Two full-length cDNAs were subsequently isolated and named *ANTR1* and *ANTR2* for *anion transporters*. *ANTR1* corresponds to the gene At2g29650, while *ANTR2* corresponds to the gene At4g00370. ANTR1 and ANTR2 share 70% amino acid identity and 80% amino acid similarity (Fig. 1). The homology is high throughout the proteins except at the N-termini where the sequences are highly divergent.

Analysis of ANTR1 and ANTR2 with the BlastP program for homology with proteins having a characterized function reveals highest homology with the human VGLUT1 and AST, the *C. elegans* EAT4 and the rabbit NaPi-1. Alignment of ANTR2 protein with these animal proteins, as well as with a rice homologue, is shown in Fig. 2. The level of similarity between ANTR2 and the animal homologues ranges from 47% for NaPi-1 to 53% for VGLUT1 or AST, with the N-terminal hydrophilic domain being poorly conserved for all proteins. All these proteins belong to the Anion:Cation Symporter (ACS) family which itself is included in the major facilitator superfamily (MFS; Pao et al. 1998). Analysis of the hydropathy profiles of ANTR1 and ANTR2 revealed the presence of 12 putative transmembrane-spanning domains separated in two clusters of six domains by a larger hydrophilic stretch (Fig. 3), a feature common to members of the MFS superfamily. Both ANTR1 and ANTR2 also contain the seven residues that form a characteristic motif present in the members of the ACS family (Fig. 2; Pao et al. 1998).

Analysis of the *Arabidopsis* proteins with the ChloroP (Emanuelsson et al. 1999) and TargetP (Emanuelsson et al. 2000) programs revealed a putative chloroplast transit peptide for ANTR1 (ChloroP score 0.58, TargetP score 0.99), with a cleavage site between amino acid 59 (Arg) and 60 (Ser; Fig. 1). Use of the same programs for the analysis of ANTR2 gave ambiguous and non-conclusive results (ChloroP score 0.49, TargetP score 0.68).

AtANTR1 1 ---MNARALLCSSNIHSLYTSNRPPEKTSS²-----RSLRNLKPSPKSLR-----VW
AtANTR2 1 ---MALGGLISNRNFGSFIGSGNGCQRLGKSGAEVSKLFPNALLCRNHQPLOASLHHESGHMRRSFGCF
AtANTR3 1 ---MATVGSIKPLHHSSCSSSFFPRNPVNRK-----ALLG---FVFD⁴SARKN-----QI
AtANTR4 1 MCYSLSIQSSIDFHNRNALKIHGDRAILTSNL-----PTLRRIPLFLPERDRRRK-----LV
AtANTR5 1 -----
AtANTR6 1 -----MARLTLRPHNHFFSS--PIYAHKQ-----PFLSVYTIFPHHHQN-----PL

AtANTR1 45 IYPRNRSSVFRVLRSSDKSESSNSY--VEGDKVSGNNDVVSDSPSSIVL-----PWEE--FPR
AtANTR2 67 LQPR-MDSVIR--FRNSIKINRSRAYYKSEBESDITEGVVPSADGSAEAILVEGNLQNASPWVQ--FPRR
AtANTR3 44 RCENLRYSE---SDGKRRNAAAKRNPSPERCAAEVLTGGGSEAEIAE-----VRT---MMPER
AtANTR4 52 LCTGRVVNSLK--FTGNTSVDLCGIPRHRLRVSCSDARRTPREETAAELTAQ-----PNFSEFITSER
AtANTR5 1 -----MKLSN--IPQR
AtANTR6 40 IKSRVKCSASG--TERVRESKKLPKDFIEDPKPQLPIPEVLSTETGFEQN-----WPPWKN--IPQR

AtANTR1 103 WVIVLLCFSAFLCNMDRVNMSIALPMSAEYGNPATVGLIQSSFFWGYLLTQIAGGIWADTVGGKRVL
AtANTR2 132 WVIVLLCFSSFLCNMDRVNMSIALPMSQEYNWSSATVGLIQSSFFWGYLLTQILGGIWADKFGGKVVL
AtANTR3 99 IKVVI¹L²TACMMCLCNADRVVMSVAVPLADKLGWSSFLG²VVQSSFLWGYLFSSVIGGALVD¹RYGGKRVL
AtANTR4 112 VKVVAMLAALALCNADRVVMSVAIVPLSLSRGWSKFSFGIVQSSFLWGYLISPIAGGTLVDRYGGKVV¹M
AtANTR5 10 YVIV¹FL²TL²ST²CV¹YIERVGFSTAYTVAADAAGINO¹SSKGTIL²ST²FFVGY¹AC¹SQV¹PGG¹WAAQ¹KI¹GG¹KRVL
AtANTR6 99 YKLIGATSLAFVICNMDKVNLSIALPMSHQFGWSSVAGLVQSSFFWGYALSOLPGGWLSKIFGGKRVL

AtANTR1 173 GFGVIWWSIATILTPVAAKLGLEPYLLV^{*}RAF^{*}MGV^{*}EGVAMPAMNNILSKWV^{*}PVQ^{*}ERSRSLALVYSGMYL^{**}
AtANTR2 202 GFGVWWSFATIMTPAARLGLPEYLLV^{*}RAF^{*}MGV^{*}EGVAMPAMNNMLSKWIP^{*}V^{*}SERSRSLALVYSGMYL^{**}
AtANTR3 169 AWGVALW¹SLATILTPWAAAHSTLALLC²VRA²FFGLA²EGVAMP²MTTL²LSR²WFPMD²ERASAVG²ISMAG²FHMG
AtANTR4 182 AWGVALW¹SLATFLTPWAA²SSLWALLA²ARAMV²GVA²EGVAL²PC²MNMVARW²FPT²TERSRAV²GIAMAG²FQLG
AtANTR5 80 LLSFV¹LW²ST²CF¹L¹V¹LP¹DPNR-VGL¹L¹V¹ARL¹LV¹GA¹O¹G¹F¹PS¹IHT¹VLA¹Q¹W¹PP¹H¹ERSR¹LV¹S¹IT¹SGMYL¹
AtANTR6 169 EIGVFTWSFATALVPLLAF-MEGLIFSRILVGI^{*}EGV^{*}SPSA^{*}ATDLI^{*}ARTI^{*}IPVKERSRAVGFVFGGLSLG

AtANTR1 243 SVTGLAFSPFLIHQFGWPSVFY¹SF¹SG¹SLGT¹VW¹LTLWL¹TKA¹ESS¹PL-EDPTLLPEERKLIADNC--ASKE--
AtANTR2 272 SVTGLAFSPMLITKFGWPSVFY¹SF¹SG¹SLGT¹W¹FLLWL¹KFA¹VSS¹PK-DDPDLSEEEKVILGGS--KPRE--
AtANTR3 239 NVVGLLLTPLMLSSITG¹ISG¹P¹FIL¹FA¹SLGLLW¹STW¹SSG¹V¹TNNPQ-DSPFITRSELRLIQAGKPVQPS---
AtANTR4 252 NVVGLMLSPILMSQGGIYGF¹FV¹IF¹GLSG¹FLW¹LVLS¹ATS¹SSAPD-RHPQITKSELEYIKQKKQISTMENK
AtANTR5 149 AALGMWLLPALVELRGPESVFLA¹EALAGVI¹WSL¹LW¹RYATDP¹PRSEH¹PKAAAAG¹FGGALLPTNVNH--
AtANTR6 238 SVMGLLLAPPIIETFNWESVFY¹L¹FL¹LE¹GV¹W¹FG¹V¹GF¹QFLNEEVSYKGNEISTSHKSE¹NATKEELGSS---

AtANTR1 308 --PVKSIP--WRLILSKPPVWALISCHF¹CHNWGT¹FILLTW¹MPTY¹YHOVLK¹FNLMESGLLSVFPWMTMAISA
AtANTR2 337 --PVTVIP--WKLILSKPPVWALISCHF¹CHNWGT¹FILLTW¹MPTY¹YNOVLK¹FNLTESGLLCVLPWLTMAVFA
AtANTR3 305 TISPKNP¹SLRLILSKLPT¹WALIFANVTN¹NGYFVLLSWMPVY¹FQ¹TFVFNVLKQAAWF¹SALP¹WATMAISG
AtANTR4 321 RISTSGT¹PFGRLLSKMPTWAVIVANSMSHWG¹FFVLLSWMP¹LYFN¹SVYHVLKQAAW¹FAV¹PWSMMAFTG
AtANTR5 216 --KVTHIP--WKKIMLSL¹PVWALV¹VNNET¹FHYALV¹LMNW¹LPT¹YFELGLQISLQ¹GMDSSK¹MPY¹L¹NMFV¹FS
AtANTR6 305 ---LKEIP--WKSFFQSPAVWAMYTHFCGS¹WGHY¹TCLSW¹LPT¹YFSEALS¹NL¹TEAAW¹VSILP¹PLASIVVT

AtANTR1 375 NAGGWIADTLVS-RGFSVTNVRKIMOTIG¹FLGPAFFL¹TOLKHIDS---PTMAVLCMACSQG¹TDAFSQSGL
AtANTR2 404 NIGGWIADTLVS-RGLSITNVRKIMOSIG¹FLGPAFFLSQLSHVKT---PAMAVLCMACSQGS¹DAFSQSGL
AtANTR3 375 YYAGAASDELIR-TGHSVTSVRKIMOSIG¹FMG¹PGLSLLCLN¹FAKS---PSCAAVFMTIALSLSSFSQAG¹F
AtANTR4 391 YIAGFWSDLLIR-RGTSITLTRKIMOSIG¹FG¹PGIALIGL¹TAKQ---PLVASAWLSLAVGLK¹SF¹SHLGF
AtANTR5 283 IVGGFIADYLI¹TKRILSVTRTRKFLNTVGLTASAA¹LMVLP¹MPFRT---ENGVI¹L¹CSSVALG¹FLALGRAGF
AtANTR6 371 SLASQFADYLI¹T-NGVDTT¹TVR¹KICQ¹TIA¹FA¹VAPAI¹CMT-LSSVDIG¹LPWEIVGIL¹TAGLALSSFALSGL

AtANTR1 441 YSNHODIAPRYSGVLLGLSNTAGVL¹AGV¹LGTAA¹TGHILO-----HG---SWDDVFTIS-VGLYL¹VGT
AtANTR2 470 YSNHODI¹GPYAGVLLG¹LSNTAGVL¹AGV¹FGTAA¹TGYILO-----RG---SWDDVFKVA-VALYL¹IGT
AtANTR3 441 LLNMODIAPQYAGLEHGISNCAGT¹LAATV¹STIGTGYFVQW-----LG---SQAF¹LTVT-AFLYFATT
AtANTR4 457 LINLOEIAPEYSGVLHGMC¹LTA¹GLAA¹IVGT¹VGAGFFVEL-----LG---SFQGFILLT-AILYL¹LSA
AtANTR5 350 AVNHMDIAPRYAGIVM¹CVS¹NTAGTLAGIT¹GVDLT¹GRKLEASKLVYS¹DL¹SHPE¹SWRVVF¹FIP-GL¹LCIFSS
AtANTR6 439 YCTHODISPEYASILLGITNTVGAV¹PGIV¹GV¹ALTGFL¹LD¹S-----TH---SWTMSLFVPSIFFY¹LTGT

AtANTR1 499 VIWNL¹FSTGE-KIID-----
AtANTR2 528 LVWNL¹FATGE-KILD-----
AtANTR3 500 VFWLL¹FATGE-RVF-----
AtANTR4 516 LFYNI¹YATGE-RVDFDTTAA
AtANTR5 419 VVFL¹LSTGE-RIF-----
AtANTR6 499 VVWLAFASSE¹PQTE¹RKEDS-

AtANTR2 1 MALGGLISNRNFGSFIGSNGCQRLGKSGAEVSKLFPNALLCRNHQPLQASLHHESGHMRRSF-GCFLQP
 OsANTR 1 -----MLYLLPLSVSCRVPGSPAPRRSRRLDPPGGRR---GVGDGLGGVRVFRRLRGRGTDVRS
 HsVGLUT1 1 -----MEFRQEEFRKLAGRALGKLRHLLLEKR-----QEGAETLELSADGR--PVTTQT
 HsAST 1 -----MRSPVRDLARNDGEESTDRTPLLPGAP-----RAEAAFPVCCSARYN---LAILA
 CeEAT4 1 -----MVGEPLAKMTAAAASA-----TGAAPPQQMQEEGNENPMQMHS
 OcNaPi-1 1 -----MDNQFFPSR-----KG-PCFCSFRYV---LALFM

AtANTR2 70 RMDSVIRFRNSIKINRSRAYYKSE-ESDITEGVVP--SADGSAEAILVEGNLQNASPWQQPFRRWVIVL
 OsANTR 57 NTSSSSSRKGRHDDARHDGGYDGDGAGALLASVRRLLLSGSAQDDAAEGEAEEDE--QGQFPKRWAIVF
 HsVGLUT1 47 -RDPPVVDCTCFGLPR-RYIIAIMSGLGFCISFGIR-CNLGVAIVSMVNNSTTHRGG-----
 HsAST 47 FFGFFIVYALRVNLSV-ALVDMVDSNTTLEDNRTSKACPEHSAPIKVHNNQT--G-----
 CeEAT4 39 NKVLQVMEQWTWIGKCRKRWLLAILANMGFMISFGIR-CNFGAAKTHMYKNYTDPYG-----
 OcNaPi-1 25 HFCNIVIIAQRMCLSL--TMVAMVNNTNLHGS-----PNTSAEKRLDNTKN-----

AtANTR2 137 LCFSSFLLCNMDRVNMSIAILPMSQEYNWSSATVGLIQSSFFWGYLLTQILGGIWAADKFGGKVVLGFGVV
 OsANTR 125 LCFSAFLLCNMDRVNMSIAILPMSAEFGWNPQTVGLIQSSFFWGYLLTQIAGGIWAADTVGGKTVLGFGVI
 HsVGLUT1 101 -----HVVVQKAQFSWDPETVGLIHGSSFFWGYIVTQIPGGFICQKFAANRVFGFAIV
 HsAST 99 -----KKYQWDAETQGWILGSFFYGYIITQIPGGYVASKIGGKMLLGFGIL
 CeEAT4 94 -----KVHMHFNWITIDELSVMESSYFYGYLVTQIPAGFLAAKFPNKLFGFGIG
 OcNaPi-1 69 -----PVYNWSPDVOGIIIFSSIFYGAFLIQIPVGYISGIYSIKKLI GFALF

AtANTR2 207 WWSFATIMTPIAARLGLP-FLLVVRAFMGIGEGVAMPAMNNMLSKWIPVSERSRSLALVYSGMYLGSVTG
 OsANTR 195 WWSIATALTTPFAAKLGLP-FLLVTRAFMGVGEVAMPAMNNILSKWVPVSERSRSLALVYSGMYLGSVTG
 HsVGLUT1 153 ATSTLNMLIPSAARVHYG-CVIFVRILQGLVEGVTPACHGIWSKWAPPLERSRLATTAFCGSYAGAVVA
 HsAST 145 GTAVLTLFTPIAADLGVG-PLIVLRALGLEGEGVTFPAMHAMWSSWAPPLERSKLLSISVYAGAQLGTVIS
 CeEAT4 144 VGAFNLNLLPYGFKVSDYLVAFIQITQGLVQGVCPAMHGVWRYWAPPMERSKLATTAFTGSYAGAVLG
 OcNaPi-1 115 LSSLVSLIFIPQAAAVGET-WIIVCRVVOGITQGTVTTAQHEIIVKWAPPLERGLTSMLSLGFLLGPFIV

AtANTR2 276 LAFSPMLITKFGWPSVFYSFGSLGSIWFLWLKFAYS SPKDDPDLSEEEKVILGGSKPREPVT-----
 OsANTR 264 LAFSPLLIHNFGWPSVFYSFGSLGVFWFSTWASKAYSSPLEDPGISAEKKLITISQTTGGEPVK-----
 HsVGLUT1 222 MPLAGVLVQVSGWSSVFYVYGSFGIFWYLFWLLVSYESPALHPSISEEERKYIEDAIGESAKLMNPLTKF
 HsAST 214 LPLSGIICYMNTYVYFFGTIGIFWFLWLWLVSDTPQKHKRISHYEKEYILSSLRNQLSSQ---K--
 CeEAT4 214 LPLSAFLVSYVSWAAPFYLYGVCGVIAILWFCVTFEKPAPFHTISQEEKIFIEDAIGHVSNTH-PTIR-
 OcNaPi-1 184 LLVLTGIICESLWPMVFIYFACGCAVCLLWFLVLYDDPKDHPCVSLHEKEYITSSLIQQGSST---RQ

AtANTR2 340 VIPWKLILSKPPVWALIIISHFCHNWGTFILLTWMPTYYNQVLKFNLTESGLL CVLPWLTMAVFANIGGWI
 OsANTR 328 EIPWGLILSKPPVWALIVSHFCHNWGTFILLTWMPTYYNQVLKFNLTESGLFCVLPWLTMAV SANFGGWI
 HsVGLUT1 292 STPWRRFFTSMPPVYAIIVANFCRSWTFYLLLSQPAYFEEVFGFEISKVGLV SALPHLVTIIVPIGGQI
 HsAST 279 SVPWVPIKSLPLWAIIVVAHFSYNWTFYLLTLLPTYMKEILRFNVQENGFLSSLPYLGSWLCMILGQQA
 CeEAT4 282 SIPWKAIIVTSKPPVWAIIVANFARSWTFYLLLQNLTYMKEALGMKIADSGLLAAIPHLV MGCVVLMMGGQL
 OcNaPi-1 250 SLPIKAMIKSLPLWAIISFCCFAYLWYTSRLIVYTPTLINSMLHVDIRENGLLSSLPYLF AWICGVIAGHT

AtANTR2 410 ADTLVS-RGLSITNVRKIMQSIGFLGPAFFLSQLSHVKTP-AMAVLCMACSQGSDAFSOSGLYSNHQDIG
 OsANTR 398 ADTLVS-RGLSVTTVRKIMQSIGFLGPAFFLTQLSHIDSP-AMAVLCMACSQGTDAFSOSGLYSNHQDIG
 HsVGLUT1 362 ADFLRSRRIMSTTNVRKLMNCGFGMEATLLLVVGYSHSK-GVAISFLVLAVGFSGF AISGFNVNHL DIA
 HsAST 349 ADNLRKWNFSTLCVRRIFSLIGMIGPAVFLVAAGFIGDYSLAVAFLTISTTLGGFCSSGFSINHL DIA
 CeEAT4 352 ADYLRSNKILSTTAVRKIFNCGFGGEEAFMLIVAYTTSD-TTAIMALIAAVGMSGF AISGFNVNHL DIA
 OcNaPi-1 320 ADFLMSRNMLS LTAIRKLF TAI GLLLP I V F S M C L L Y L S S G F Y S T I T F L I L A N A S S F C L G G A L I N A L D L A

AtANTR2 478 PRYAGVLLGLSNTAGVLAGVFGTAATGYILQR---GSWDDVFKVAVALYLI GTLVWNLFATGEKILD---
 OsANTR 466 PRYAGVLLGLSNTAGVLAGVFGTAATGYILQH---GSWDDVFKVSUVLYLVGLTLVWNLFSTGEKIIDZ--
 HsVGLUT1 431 PRYASILMGISNGVGTLSGMVCPPIIVGAMTKHKTREEWQYVFLIASLVHYGGVIFYGVFASGEKQPWAE
 HsAST 419 PSYAGILLGITNTFATIPGMVGPVIAKSLTPDNTVGEWQTVFYIAAAINVF GAIFFTLFAKGEVQNWALN
 CeEAT4 421 PRYAAILMGFSNGIGTLAGLTCPFVTEAFTAH-SKHGWTSVFLLASLIHFTGVTFYAVYASGELQEWAE
 OcNaPi-1 390 PRYVVFIKGVTTLIGMTGGMTSSTVAGLFLSQDPSSWFKIFLLMSIINVISVIFYLIFAKAEIQDWAKE

AtANTR2 -----
 OsANTR -----
 HsVGLUT1 501 ---EEMSEE---KCGFVG-----HDQLAGSDDSEMEDEAEPPGAPPAPPPSYGATHSTFQPPRP
 HsAST 489 ---DHHGHRH-----
 CeEAT4 490 KEEEWSNKELVNKTGINGTGYGAAETTFTQLPAGVDSSYQAQAAP--APGTPNFASAWDEHGSSGVVEN
 OcNaPi-1 460 ---KQHTRL-----

AtANTR2 -----
 OsANTR -----
 HsVGLUT1 555 PPVRDY
 HsAST -----
 CeEAT4 558 PHYQQW
 OcNaPi-1 -----

Fig. 2 Alignment of the *Arabidopsis thaliana* ANTR2 with a rice (*Oryza sativa*) homologue (OsANTR), and with the human (*Homo sapiens*) VGLUT1 and AST, the *Caenorhabditis elegans* EAT-4 and the rabbit (*Oryctolagus cuniculus*) NaPi-1 proteins. The consensus sequence for ACS family members is indicated by asterisks. Alignment was made with the ClustalW 1.74 program

ANTR2 is a chloroplast inner-envelope protein

The intracellular localization of the ANTR2 protein was examined by organelle and membrane fractionation. Analysis of the purity of the fractions was tested using antibodies recognizing proteins specific to various membranes. Antibodies against ANTR2 were raised against a unique 19-amino-acid peptide present in the N-terminal hydrophilic domain (see Materials and methods).

Preliminary analysis, by Western blotting, of fractions derived from *Arabidopsis* leaves and enriched in either plasma membrane, tonoplast, chloroplast or mitochondria, indicated that ANTR2 was mainly a chloroplast protein (data not shown). Subsequent analysis was thus focused on purification and fractionation of the chloroplast. Figure 4a shows that ANTR2 protein is enriched in the chloroplast fraction relative to a crude extract from whole shoots. ANTR2 antibodies recognize a single protein of 45 kDa. Analysis of the chloroplast

fractions by Western blotting with antibodies raised against the endoplasmic reticulum protein Sec12, the tonoplast pyrophosphatase, the plasma membrane aquaporin RD28 and the mitochondrial porin, revealed that the chloroplast fraction was depleted of proteins derived from the membranes of other organelles (data not shown).

Chloroplasts were then fractionated to separate the inner and outer envelopes. The extent of enrichment and contamination of the two fractions were analyzed by Western blotting with antibodies against the inner-envelope protein TIC110, the outer-envelope protein TOC75 and the thylakoid protein LHCII. Figure 4b (lanes 1–3) shows that ANTR2 is found enriched in the inner-envelope fraction and depleted in the outer-envelope fraction of *Arabidopsis* chloroplasts. Both envelope fractions had undetectable contamination from thylakoids, excluding the localization of the ANTR2 protein in these membranes. Similar results were obtained in experiments with envelopes isolated from spinach chloroplasts (Fig. 4b, lanes 4–6).

The localization of the ANTR2 protein to the plastid inner envelope indicated that the N-terminal

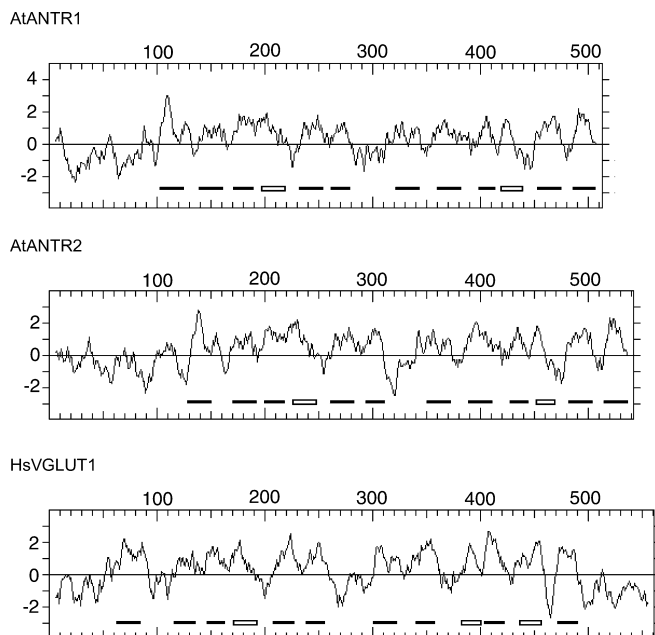


Fig. 3 Hydropathy profiles of ANTR1, ANTR2 and the human VGLUT1 homologue. Evaluation of the potential transmembrane-spanning domains was performed using the programs HMMTOP (<http://www.enzim.hu/hmmtop>), TMHMM (<http://www.cbs.dtu.dk/services/TMHMM>) and Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html). The results were transposed on a Kyte–Doolittle plot using a window of 11 amino acids. Transmembrane segments predicted by at least two programs are indicated by black boxes while segments predicted by only one program are indicated by open boxes

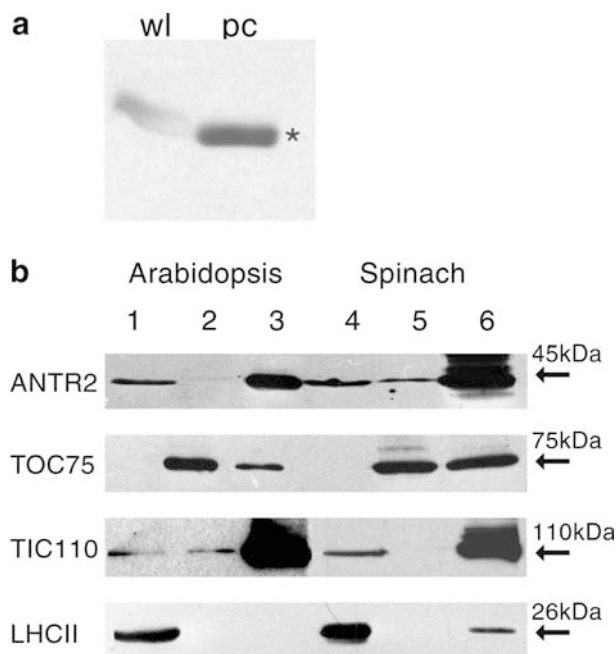


Fig. 4a, b ANTR2 is localized in the chloroplast inner envelope. **a** Proteins (25 μ g) from whole-leaf extracts (*wl*) and purified chloroplasts (*pc*) were separated on an SDS–PAGE gel and analyzed by Western blotting using polyclonal rabbit antibodies against ANTR2. The asterisk denotes 45 kDa. **b** Proteins were isolated from purified chloroplasts as well as inner and outer chloroplast envelopes and analyzed by Western blotting with antibodies against ANTR2, the outer envelope protein TOC75, the inner envelope protein TIC110 and the thylakoid protein LHCII. Lane 1, *Arabidopsis* purified chloroplasts (10 μ g); lane 2, *Arabidopsis* outer envelopes (3 μ g); lane 3, *Arabidopsis* inner envelopes (3 μ g); lane 4, spinach (*Spinacia oleracea*) purified chloroplasts (30 μ g); lane 5, spinach outer envelopes (16 μ g); lane 6, spinach inner envelopes (16 μ g)

hydrophilic domain may contain a cleavable transit peptide. Analysis of the protein with ChloroP and TargetP, combined with manual inspection of the N-terminal hydrophilic domain, revealed the presence of two potential cleavage sites, one between amino acids 28 (Ser) and 29 (Gly) and a second site between 87 (Arg) and 88 (Ala; Fig. 1). Since the second potential cleavage site is within the peptide that was used for production of antibodies, the ability of the antibodies to detect the hydrophilic peptide included between amino acids 88 and 139 of ANTR2 was checked. Figure 5 shows that the ANTR2 antibodies can detect the ANTR2 peptide expressed in *E. coli* while they failed to recognize a peptide representing the mature hydrophilic portion of ANTR1 comprising the region between amino acids 60 and 110.

Expression pattern of ANTR2

Western analysis of crude extracts from different tissues of *Arabidopsis* was performed with antibodies against ANTR2. Figure 6 shows that ANTR2 is predominantly expressed in leaves, stems and developing siliques, while expression in roots and flowers was below detection.

Expression of ANTR2 was further examined in leaves and developing seeds by immunolocalization. Cross-sections through leaves reveal expression of ANTR2 in both palisade and spongy mesophyll cells (Fig. 7a, b). No expression of ANTR2 protein is evident in cells of the leaf epidermis. In 7- to 9-day-old siliques containing embryos at the upturned-U stages, expression of ANTR2 is strongest in the embryo and weak but detectable in the cells of the outer integument, endosperm and cells of the silique walls (Fig. 7c, d).

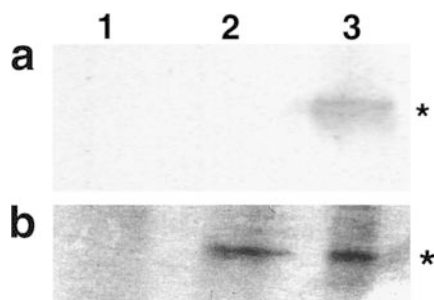


Fig. 5a, b Specificity of the ANTR2 antibodies. Proteins (5 μ g) from whole *Escherichia coli* transformed with the pET14b vector (lanes 1) or with constructs leading to the expression of peptides representing the N-terminal hydrophilic portion of the mature ANTR1 (amino acids 60–110; lanes 2) or ANTR2 (amino acids 88–139; lanes 3) were separated on an SDS-PAGE gel. **a** Western blot analysis using polyclonal rabbit antibodies against ANTR2. **b** Coomassie blue staining of the SDS-PAGE gel showing the overexpressed ANTR1 and ANTR2 peptides. The asterisk denotes 6 kDa

ANTR1 is a chloroplast protein

Rabbit antibodies raised against the entire N-terminal hydrophilic domain (110 amino acids) of ANTR1 were found to significantly cross-react with the ANTR2 N-terminal hydrophilic domain of the mature peptide (amino acids 88–139), making its use inappropriate for localization of ANTR1 (data not shown). A hybrid protein was thus created whereby GFP was translationally fused to the C-terminal end of the full-length ANTR1. GFP and the ANTR1–GFP constructs were expressed under the control of the CaMV 35S promoter and stably transformed into *Arabidopsis*. Epidermal peels for the abaxial surface of leaves were examined by epifluorescence microscopy (Fig. 8). While GFP was found in the cytoplasm and nucleus but not in plastids of epidermal cells of transgenic plants expressing the unfused GFP, the green fluorescence was clearly localized in plastids in epidermal cells of transgenic plants expressing the ANTR1–GFP fusion. These data indicated that ANTR1 was localized to the plastids.

The ANTR gene family in *Arabidopsis*

Analysis of the genome of *Arabidopsis* for homologues of ANTR1 and ANTR2 revealed the presence of four additional genes homologous to ANTR1 and ANTR2, namely At2g38060 (ANTR3), At3g46980 (ANTR4), At5g44370 (ANTR5) and At5g20380 (ANTR6). No complete cDNAs were available for ANTR3 and ANTR6. The protein sequences of ANTR3 and ANTR6 were thus deduced using a combination of approaches, including the annotation provided in TAIR (<http://www.arabidopsis.org>) or TIGR (<http://www.tigr.org>), alignment with ESTs from *Arabidopsis* and other plants, as well as alignments with the ANTR members for which the annotation has been confirmed with full-length cDNAs (ANTR1, ANTR2, ANTR4 and ANTR5). Analysis of the gene structure of ANTR6 and comparison with the exon/intron structure of ANTR1, ANTR2 and ANTR4 led to a predicted protein sequence slightly different from the TAIR annotation. The difference is mainly due to a selection of splice acceptor sites. Although a full-length cDNA was reported for ANTR6 (accession AY095993), its structure appeared aberrant since only a short open reading frame is generated. Analysis of this sequence indicates an insertion

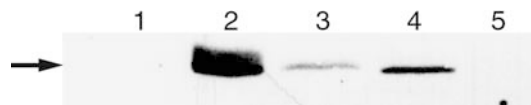
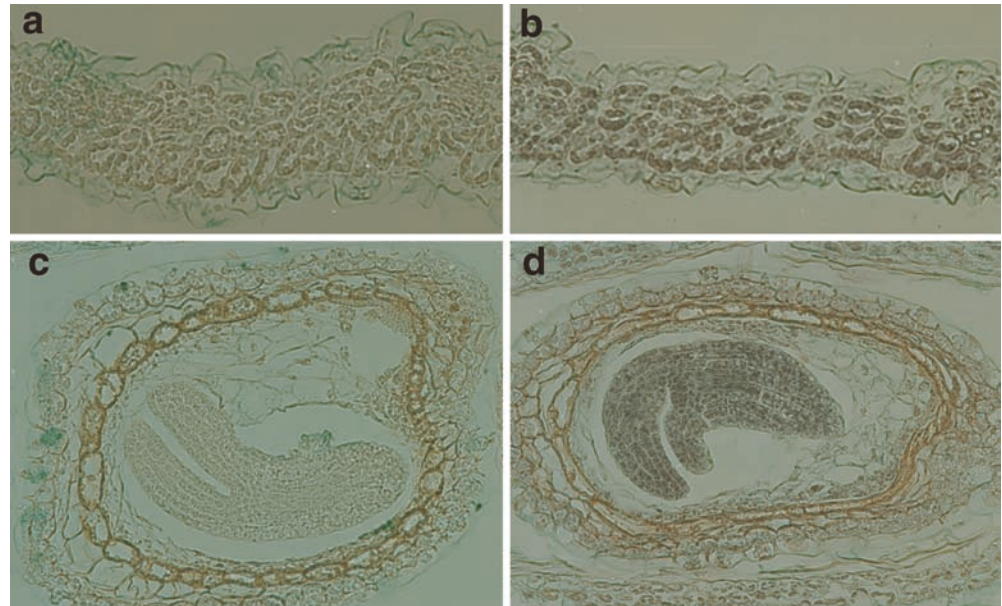


Fig. 6 Expression pattern of ANTR2 in *Arabidopsis*. Western blotting was performed with equal loading (50 μ g) of protein isolated from roots (lane 1), leaves (lane 2), inflorescence stems (lane 3), green siliques (lane 4), and flowers (lane 5). The arrow indicates 45 kDa

Fig. 7a–d Immunolocalization of ANTR2 in leaves and seeds of *Arabidopsis*. Paraffin sections of leaves (**a, b**) and 7- to 9-day-old siliques (**c, d**) were analyzed with antibodies against ANTR2 (**b, d**), as well as the corresponding pre-immune serum (**a, c**)



of 16 bases, which leads to a change in reading frame and a premature stop codon. The prediction of the ANTR6 protein is thus based on the exon/intron structure of the cDNA with the removal of the 16 bases.

The alignment of all ANTR protein sequences is shown in Fig. 1. The level of homology between the various members of the family ranges from 29% amino acid identity and 49% similarity between ANTR4 and ANTR5 to 70% identity and 80% similarity between ANTR1 and ANTR2. The homology between ANTR members is lowest at the N-terminal hydrophilic region. The programs ChloroP and TargetP both predict a chloroplast targeting sequence for ANTR3 (ChloroP score 0.543, TargetP score 0.905; Fig. 1). The predictions with the same programs for ANTR4 and ANTR6 are more ambiguous. The structure of the ANTR5 protein appears unusual in that it lacks an extensive N-terminal hydrophilic domain common to all other ANTR proteins. This indicates that the protein may be located in a different sub-cellular compartment compared to other members of the family. The SignalP program (Nielsen et al. 1997) predicts an anchoring signal peptide at the N-terminal end of ANTR5. The gene structure of *ANTR5* is also unusual in that it is composed of a single exon while all other *ANTR* genes are composed of at least eight exons. The various members of the ANTR family contain at least five of the seven residues that form the characteristic motif present in the members of the ACS family (Fig. 1).

Discussion

Chloroplast purification and membrane subfractionation have shown that ANTR2 is a protein located in the inner envelope of chloroplasts in both *Arabidopsis* and spinach. ANTR2 has not been previously identified as a

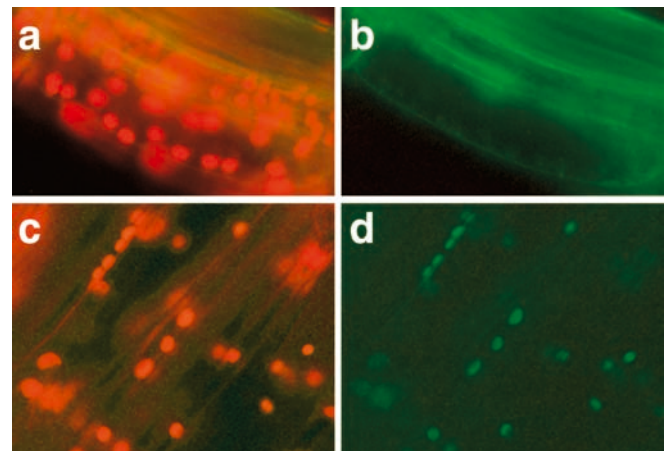


Fig. 8a–d Localization of an ANTR1–GFP fusion in chloroplasts. Epidermal pavement cells from the abaxial surface of transgenic *Arabidopsis* leaves expressing GFP (**a, b**) and the fusion protein ANTR1–GFP (**c, d**) were examined by epifluorescence microscopy. Autofluorescence from the plastids (**a, c**) was visualized with a Leitz I2 filter (excitation filter 450–490 nm and long-pass emission filter 515 nm) while GFP (**b, d**) was visualized with a Leitz L3 filter (excitation filter 450–490 nm, band-pass emission filter 520–525 nm)

plastid protein either through proteomic approaches combining membrane protein extraction with organic solvents and analysis of peptides with MALDI–TOF (Ferro et al. 2002, 2003), or *in silico* searches of the *Arabidopsis* genome using programs such as ChloroP or TargetP (Ferro et al. 2002; Koo and Ohlrogge 2002). This may be due, in part, to the failure of the ChloroP and TargetP programs to recognize the chloroplast targeting sequence.

Western analysis of crude protein extracts revealed that ANTR2 is mainly expressed in leaves, stems and developing siliques, while expression in roots and

flowers is below detection. Immunolocalization revealed that ANTR2 is expressed in the leaf spongy and palisade mesophyll cells while expression in the leaf epidermal cells is undetectable. In developing seeds, ANTR2 expression is mainly located in the embryo with some weak expression in the integument, endosperm and cells of the silique wall. Although the developing embryo of *Arabidopsis* at the upturned-U stage is green and could be competent at photosynthesis, the integument and endosperm are non-photosynthetic. ANTR2 is thus expressed in both autotrophic and heterotrophic tissues. Although the expression pattern of the ANTR1 protein could not be determined, the localization of an ANTR1–GFP fusion protein to the chloroplast shows that ANTR1 is also a chloroplast protein.

Four additional homologues of ANTR1 and ANTR2 are found in the *Arabidopsis* genome. Although the localization of these additional proteins within the cell is unknown, the apparent lack of a chloroplast-targeting signal for ANTR5 suggests that not all ANTR proteins may be chloroplastic. All ANTR proteins are homologous to the animal NaPi-1 protein family as well as to other organic-anion transporters found in the ACS family of transporters, albeit at a lower level, including bacterial hexuronate and galactonate transporters. ANTR proteins share the main features of members of the ACS family, including the presence of 12 putative membrane-spanning domains clustered in 2 groups of 6, as well as the presence of a 7-amino-acid motif in the fourth putative transmembrane domain. To our knowledge, the ANTR genes from *Arabidopsis* are the first characterized plant genes that are members of the ACS family of permeases (Pao et al. 1998).

The *ANTR1* and *ANTR2* genes of *Arabidopsis* were isolated based on the high homology of the encoded proteins to the rabbit and human NaPi-1. Although NaPi-1 was initially identified as a protein expressed in rabbit kidney that induced a sodium-dependant Pi transport when expressed in *Xenopus* oocytes (Werner et al. 1991), the role of this protein in vivo still remains unclear. In contrast to NaPi-2, a protein unrelated to NaPi-1 but also expressed in rabbit kidney and mediating a strong sodium-dependant Pi transport in *Xenopus* oocytes, the rabbit NaPi-1 shows only low affinity to Pi and the gene is poorly regulated by Pi supply (Werner et al. 1998). Expression of NaPi-1 in *Xenopus* has been shown to induce a chloride conductance that was inhibited by chloride-channel blockers, as well as a conductance for organic anions, such as benzylpenicillin (Busch et al. 1996). Detailed studies of the Pi transport induced by NaPi-1 expression in *Xenopus* oocytes indicated that NaPi-1 might be up-regulating a Pi transporter that is endogenous to *Xenopus* oocytes (Bröer et al. 1998). Mutation in a homologue of NaPi-1 in humans results in a deficiency in the transport of the organic anion sialic acid, reinforcing the concept of NaPi-1 as an organic-anion transporter (Verheijen et al. 1999). More recently, the human brain-specific NaPi-1 homologue (BNPI) has been shown to be responsible for

the transport of glutamate into synaptic vesicles (Bellocchio et al. 2000; Takamori et al. 2000). In addition, this protein, which has been renamed VGLUT1, was shown to exhibit a conductance for chloride that is blocked by glutamate. VGLUT1 is expressed specifically in glutamatergic neurons (Bellocchio et al. 1998), and mutation in the NaPi-1 homologue EAT4 in *C. elegans* results in a defect in neurotransmission from these neurons (Lee et al. 1999). Together, these results indicate that NaPi-1 homologues in mammalian cells are primarily involved in the transport of glutamate (in neurons) and of other organic acids, as well as acting as a chloride channel. Although the evidence for a role of mammalian NaPi-1 homologues in Pi transport is unclear, it remains possible that these proteins may have Pi transport activity under some conditions. It is also possible that NaPi-1 may have multiple activities, similar to the animal EAAT5/ASCT1 protein, which exhibits both glutamate/amino acid transport activity and a chloride conductance (Zerangue and Kavanaugh 1996), and the chloroplast triose phosphate translocator, which also exhibits channel activity towards anions (Schwarz et al. 1994).

In view of the homology between the *Arabidopsis* ANTR proteins and the mammalian NaPi-1, VGLUT and AST proteins, three putative functions may be assigned to the plant chloroplast proteins, namely Pi transport, glutamate/organic-anion transport or chloride channel.

Several proteins have been identified in the plastid inner envelope that mediate the 1:1 counter-exchange of Pi with phosphorylated compounds. These include the triose phosphate/phosphate translocator (Flügge et al. 1989), the phosphoenolpyruvate/phosphate translocator (Fischer et al. 1997), the glucose-6-phosphate/phosphate translocator (Kammerer et al. 1998) and the pentose phosphate/phosphate translocator (Eicks et al. 2002). A proton-dependant phosphate transporter has also been recently identified in chloroplasts (Versaw and Harrison 2002). There is evidence for the presence of additional Pi transporters in the plastid envelope. For example, a transporter exchanging malate for Pi has been identified at the biochemical level in leukoplast envelopes from castor seed endosperm (Eastmond et al. 1997) while sulfate transport across the envelope of spinach chloroplast was linked to counter-transport of Pi (Mouriaux and Douce 1979). In non-green plastids, synthesis of starch from glucose-6-phosphate and ATP leads to a 2:1 imbalance between phosphate import and phosphate export. A Pi transporter would be required for the exit of Pi out of the plastid since a high level of Pi would inhibit starch biosynthesis. Unidirectional transport of Pi across the envelope membrane of cauliflower-bud amyloplasts has been measured, although the corresponding protein has not been identified (Neuhaus and Maass 1996).

The inner envelope of the plastid contains transport systems for the entry and exit of numerous non-phosphorylated organic anions. These include the

translocators for the exchange of glutamine and glutamate (Yu and Woo 1988) or glycerate and glycolate (Howitz and McCarty 1985), for which no proteins have been identified. Furthermore, unidentified transporters are also thought to be involved in the translocation of numerous amino acids synthesized in the plastids.

Chloride channels have been characterized in the envelopes of the chloroplast, either after reconstitution into artificial membranes (Heiber et al. 1995) or in native membranes (van den Wijngaard and Vredenberg 1997). One of these channels was implicated in chloroplast protein import (van den Wijngaard and Vredenberg 1997). Chloride and anion channels may be involved in the maintenance of charge balance and pH across the inner envelope. Besides the inner envelope triose phosphate/phosphate translocator, which has been shown to have anion channel activity in reconstituted membranes (Schwarz et al. 1994), the proteins involved in the various chloride channel activities of the plastid inner envelope have not been identified.

It is difficult at this point to define which of the multiple potential candidate solutes is most likely transported by the ANTR1 and ANTR2 proteins. Functional characterization of transport activity of a chloroplast protein through heterologous expression is made difficult by the inappropriate targeting of the proteins to endomembranes. Expression of chloroplast membrane transporters in yeast followed by protein purification and/or reconstitution into artificial liposomes has been a method of choice used to characterize a number of plastid transporters. Use of this reconstitution system, as well as analysis of transport activity in whole yeast expressing the ANTR 1 and ANTR2 proteins without the transit peptide, has however failed to detect any significant transport activity of ANTR1 and ANTR2 towards either Pi, chloride, glutamate or glutamine (data not shown). Reverse genetics and the inactivation of the *ANTR* genes may give some clues as to the role of the proteins in the plastids. The potential expression of several ANTR homologues in the plastids may complicate the analysis by reverse genetics and require the simultaneous inactivation or down-regulation of several members of the *ANTR* gene family. Although challenging, the study of the *ANTR* genes promises to bring novel information on the biochemistry of metabolites across the envelope of the plastids, and perhaps also of other membranes.

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References

- Bar-Peled M, Raikhel NV (1997) Characterization of AtSEC12 and AtSAR1. proteins likely involved in endoplasmic reticulum and golgi transport. *Plant Physiol* 114:315–324
- Bellocchio EE, Hu H, Pohorille A, Chan J, Pickel VM, Edwards RH (1998) The localization of the brain-specific inorganic phosphate transporter suggests a specific presynaptic role in glutamatergic transmission. *J Neurosci* 18:8648–8659
- Bellocchio EE, Reimer RJ, Freneau RT, Edwards RH (2000) Uptake of glutamate into synaptic vesicles by an inorganic phosphate transporter. *Science* 289:957–960
- Bröer S, Schuster A, Wagner CA, Bröer A, Forster I, Biber J, Murer H, Werner A, Lang F, Busch AE (1998) Chloride conductance and Pi transport are separate functions induced by the expression of NaPi-1 in *Xenopus* oocytes. *J Membr Biol* 164:71–77
- Busch AE, Schuster A, Waldegger S, Wagner CA, Zempel G, Broer S, Biber J, Murer H, Lang F (1996) Expression of a renal type I sodium/phosphate transporter (NaPi-1) induces a conductance in *Xenopus* oocytes permeable for organic and inorganic anions. *Proc Natl Acad Sci USA* 93:5347–5351
- Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 6:735–43
- Daniels MJ, Mirkov TE, Chrispeels MJ (1994) The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homologue of the tonoplast water channel protein TIP. *Plant Physiol* 106:1325–1333
- Davis SJ, Vierstra RD (1998) Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Mol Biol* 36:521–52
- Douce R, Joyard J (1982) Purification of the chloroplast. In: Edelman M, Hallick RB, Chua NH (eds) *Methods in chloroplast molecular biology*. Elsevier, Amsterdam, pp 239–256
- Eicks M, Maurino V, Knappe S, Flügge U-I, Fischer K (2002) The plastidic pentose phosphate translocator represents a link between the cytosolic and the plastidic pentose phosphate pathways in plants. *Plant Physiol* 128:512–522
- Eastmond PJ, Dennis DT, Rawsthorne S (1997) Evidence that a malate/inorganic phosphate exchange translocator imports carbon across the leucoplast envelope for fatty acid synthesis in developing castor seed endosperm. *Plant Physiol* 114:851–856
- Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* 8:978–984
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* 300:1005–1016
- Ferro M, Salvi D, Rivière-Rolland H, Vermaat T, Seigneuri-Berny D, Grunwald D, Garin J, Joyard J, Rolland N (2002) Integral membrane proteins of the chloroplast envelope: identification and subcellular localization of new transporters. *Proc Natl Acad Sci USA* 99:11487–11492
- Ferro M, Salvi D, Brugière S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, Rolland N (2003) Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana*. *Mol Cell Proteomics* 2:325–345
- Fischer K, Weber A (2002) Transport of carbon in non-green plastids. *Trends Plant Sci* 7:345–351
- Fischer K, Kammerer B, Gutensohn M, Arbinger B, Weber A, Häusler RE, Flügge UI (1997) A new class of plastidic phosphate translocators: a putative link between primary and secondary metabolism by the phosphoenolpyruvate/phosphate antiporter. *Plant Cell* 9:453–462
- Flügge UI, Fischer K, Gross A, Sebald W, Lottspeich F, Eckerskorn C (1989) The triose phosphate-3-phosphoglycerate-phosphate translocator from spinach chloroplasts: nucleotide sequence of a full-length cDNA clone and import of the in vitro synthesized precursor protein into chloroplasts. *EMBO J* 8:39–46

- Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 85:8998–9002
- Heiber T, Steinkamp T, Hinnah S, Schwarz M, Flügge UI, Weber A, Wagner R (1995) Ion channels in the chloroplast envelope membrane. *Biochemistry* 34:15906–15917
- Heins L, Mentzel H, Schmid A, Benz R, Schmitz UK (1994) Biochemical, molecular, and functional characterization of porin isoforms from potato mitochondria. *J Biol Chem* 269:26402–26410
- Howitz KT, McCarty RE (1985) Substrate specificity of the pea chloroplast glycolate transporter. *Biochemistry* 24:3645–3650
- Joyard J, Grossman A, Bartlett SG, Douce R, Chua NH (1982) Characterization of envelope membrane polypeptides from spinach chloroplasts. *J Biol Chem* 257:1095–1101
- Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber A, Flügge UI (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: the glucose 6-phosphate/phosphate antiporter. *Plant Cell* 10:105–117
- Kim EJ, Zhen RG, Rea PA (1994) Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. *Proc Natl Acad Sci USA* 91:6128–6132
- Koo AJK, Ohlrogge JB (2002) The predicted candidates of *Arabidopsis* plastid inner envelope membrane proteins and their expression profiles. *Plant Physiol* 130:823–836
- Lee RY, Sawin ER, Chalfie M, Horvitz HR, Avery L (1999) EAT-4, a homolog of a mammalian sodium-dependent inorganic phosphate cotransporter, is necessary for glutamatergic neurotransmission in *Caenorhabditis elegans*. *J Neurosci* 19:159–167
- Lübeck J, Soll J, Akita M, Nielsen E, Keegstra K (1996) Topology of IEP110, a component of the chloroplastic protein import machinery present in the inner envelope membrane. *EMBO J* 15:4230–4238
- Mourioux G, Douce R (1979) Transport du sulfate à travers la double membrane limitante, ou enveloppe, des chloroplastes d'épinard. *Biochimie* 61:1283–1292
- Neilsen H, Engelbrecht J, Brunak S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 10:1–6
- Neuhaus HE, Maass U (1996) Unidirectional transport of orthophosphate across the envelope of isolated cauliflower-bud amyloplasts. *Planta* 198:542–548
- Neuhaus HE, Wagner R (2000) Solute pores, ion channels, and metabolite transporters in the outer and inner envelope membranes of higher plant plastids. *Biochim Biophys Acta* 1465:307–323
- Neuhaus HE, Thom E, Möhlmann T, Steup M, Kampfenkel K (1997) Characterization of a novel ATP/ADP translocator located in the plastid envelope of *Arabidopsis thaliana* L. *Plant J* 11:73–82
- Newman T, de Bruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikhel N, Somerville SC, Thomashow M, Retzel E, Somerville CR (1994). Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. *Plant Physiol* 106:1241–1255
- Pao SS, Paulsen IT, Saier MH (1998). Major facilitator superfamily. *Microbiol Mol Biol Rev* 62:1–34
- Pohlmeier K, Soll J, Steinkamp T, Hinnah S, Wagner R (1997) Isolation and characterization of an amino acid-selective channel protein present in the chloroplastic outer envelope membrane. *Proc Natl Acad Sci USA* 94:9504–9509
- Renné P, Dressen U, Hebbeker U, Hille D, Flügge UI, Westhoff P, Weber APM (2003) The *Arabidopsis* mutant *det* is deficient in the plastidic glutamate/malate translocator DiT2. *Plant J* 35:316–331
- Schwarz M, Gross A, Steinkamp T, Flügge UI, Wagner R (1994) Ion channel properties of the reconstituted chloroplast triose phosphate/phosphate translocator. *J Biol Chem* 269:29481–29489
- Takamori S, Rhee JS, Rosenmund C, Jahn R (2000) Identification of a vesicular glutamate transporter that defines a glutamatergic phenotype in neurons. *Nature* 407:189–194
- Taniguchi M, Taniguchi Y, Kawasaki M, Takeda S, Kato T, Sato S, Tabata S, Miyake H, Sugiyama T (2002) Identifying and characterizing plastidic 2-oxoglutarate/malate and dicarboxylate transporters in *Arabidopsis thaliana*. *Plant Cell Physiol* 43:706–717
- Tranel PJ, Froehlich J, Goyal A, Keegstra K (1995) A component of the chloroplastic protein import apparatus is targeted to the outer envelope membrane via a novel pathway. *EMBO J* 14:2436–2446
- van den Wijngaard PWJ, Vredenberg WJ (1997) A 50-picosiemens anion channel of the chloroplast envelope is involved in chloroplast protein import. *J Biol Chem* 272:29430–29433
- Verheijen FW, Verbeek E, Aula N, Beerens CEMT, Havelaar AC, Joose M, Peltonen L, Aula P, Galjaard H, van der Spek PJ, Mancini GMS (1999) A new gene, encoding an anion transporter, is mutated in sialic acid storage diseases. *Nature Biotechnol* 23:462–465
- Versaw WK, Harrison MJ (2002) A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. *Plant Cell* 14:1751–1766
- Weber A, Menzclaff E, Arbinger B, Gutensohn M, Eckerskorn C, Flügge UI (1995) The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: molecular cloning of a transporter containing a 12-helix motif and expression of the functional protein in yeast cells. *Biochemistry* 34:2621–2627
- Weber A, Servaites JC, Geiger DR, Kofler H, Hille D, Gröner F, Hebbeker U, Flügge UI (2000) Identification, purification and molecular cloning of a putative plastidic glucose translocator. *Plant Cell* 12:787–801
- Werner A, Moore ML, Mantei N, Biber J, Semenza G, Murer H (1991) Cloning and expression of cDNA for a Na/P_i cotransport system of kidney cortex. *Proc Natl Acad Sci USA* 88:9608–9612
- Werner A, Dehmelt L, Nalbant P (1998) Na⁺-dependent phosphate cotransporters: the NaPi protein families. *J Exp Biol* 201:3135–3142
- Yu J, Woo KC (1988) Glutamine transport and the role of the glutamine translocator in chloroplasts. *Plant Physiol* 88:1048–1054
- Zerangue N, Kavanaugh MP (1996) Flux coupling in a neuronal glutamate transporter. *Nature* 383:634–637