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Vegetative and Seed-Specific Forms of Tonoplast Intrinsic Protein in the Vacuolar Membrane of *Arabidopsis thaliana*[1]

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ABSTRACT

Reports from a number of laboratories describe the presence of a family of proteins (the major intrinsic protein family) in a variety of organisms. These proteins are postulated to form channels that function in metabolite transport. In plants, this family is represented by the product of NOD26, a nodulation gene in soybean that encodes a protein of the peribacteroid membrane, and tonoplast intrinsic protein (TIP), an abundant protein in the tonoplast of protein storage vacuoles of bean seeds (KD Johnson, H Höfte, MJ Chrispeels [1990] Plant Cell 2: 525–532). Other homologs that are induced by water stress in pea and in *Arabidopsis thaliana* and that are expressed in the roots of tobacco have been reported, but the location of the proteins they encode is not known. We now report the presence and derived amino acid sequences of two different TIP proteins in *A. thaliana*. α-TIP is a seed-specific protein that has 68% amino acid sequence identity with bean seed TIP; γ-TIP is expressed in the entire vegetative body of *A. thaliana* and has 58% amino acid identity with bean seed TIP. Both proteins are associated with the tonoplast. Comparisons of the derived amino acid sequences of the seven known plant proteins in the major intrinsic protein family show that genes with similar expression patterns (*e.g.* water stress-induced or seed specific) are more closely related to each other than the three *A. thaliana* homologs are related. We propose that the nonoverlapping gene expression patterns reported here, and the evolutionary relationships indicated by the phylogenetic tree, suggest a functional specialization of these proteins.

The vacuoles of plant cells play an important role in the sequestration and temporary storage of inorganic ions and organic metabolites (for a review, see ref. 2). The exchange of ions and metabolites between the cytoplasm and the vacuole is driven by the pH gradient across the tonoplast, generated by H^+/ATPase and H^+/pyrophosphatase. Transport is mediated by carriers, allowing fluxes of solutes against the electrochemical gradient and channels that permit passive transport, *i.e.* down the electrochemical gradient. Because the vacuoles of different cell types can show dramatic differences in the nature and magnitude of the ion and metabolite exchange with the cytoplasm (*e.g.* transport of potassium in guard cells or of malate in the mesophyll of CAM plants), the tonoplasts most likely have different channels and carriers with specialized functions. Recently, published reports point to a small, but growing, family of homologous intrinsic membrane proteins from quite different organisms (22, 28). This family has been termed the MIP[4] family after its best characterized member, MIP from bovine lens fiber junctional membranes. MIP has been shown to form tetrameric structures (1) with channel activity in artificial lipid membranes (7). One of the MIP homologs, GLPF, in the inner membrane of *Escherichia coli* plays a role in the facilitated transport of glycerol. By analogy, it seems reasonable to postulate that the other proteins in this superfamily also form channels that are involved in passive transmembrane transport of ions and/or metabolites. The first member of this family to be identified in plants was NOD26 (24), a plant-encoded protein in the peribacteroid membrane of soybean root nodules infected with *Rhizobium* bacteroids. Recently, three other members of the MIP family were identified in plants: a water stress-induced cDNA in pea (*Pisum sativum*) (11), a root-specific gene in tobacco (*Nicotiana tabacum*) (31), and a seed-specific gene in bean (*Phaseolus vulgaris*), which is widely conserved in the plant kingdom (15). In bean seeds, the protein is associated with the tonoplast (protein body membranes), and for this reason we called it TIP.

The evidence presented in this paper shows that *Arabidopsis thaliana* contains two quite different TIP homologs in the tonoplasts of different cells. One, α-TIP, is expressed in the developing embryos, and the other one, γ-TIP, is expressed in all the vegetative organs but not in seeds. The amino acid sequences of these two tonoplast proteins show 59% identity. We determined the evolutionary distances of the seven known plant proteins in the MIP family and found that proteins with similar expression patterns in different plant species are more closely related to each other than the three proteins of *A.*

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[4] Abbreviations: MIP, major intrinsic protein; kb, kilobase; TIP, tonoplast intrinsic protein; bp, base pair; ORF, open reading frame; SSC, standard sodium citrate; poly(A^+) RNA, polyadenylated RNA.

thaliana. We suggest that these data support the idea that the genes encode functionally specialized proteins with specific expression patterns. Whether they are all located in the tonoplast and regulate the passage of the same or different ions or metabolites remain to be investigated.

MATERIALS AND METHODS

Arabidopsis Growth Conditions

Arabidopsis thaliana, ecotype Columbia, seeds were a gift from Dr. Nigel Crawford, University of California, San Diego. Plants were grown under continuous illumination at 25 to 27°C in a soil-peat mixture (J.M. McConkey and Co., Sumner, WA) and subirrigated at 3- to 4-d intervals with 0.125% (w/v) Peter's peat-lite fertilizer (J.M. McConkey). Tissues were harvested, directly frozen in liquid nitrogen, and stored at −80°C.

Isolation and Sequencing of *Arabidopsis* Genomic Clones

A genomic library in phage EMBL3, made from total DNA from *A. thaliana*, ecotype Columbia, was kindly provided by Dr. Nigel Crawford. Plaque screening with a ^{32}P-labeled probe was done using standard procedures (21). Hybridizations were done in 6× SSC at 65°C with a final wash was in 0.2× SSC at 60°C. The bean TIP cDNA probe consisted of a 580-bp fragment containing 307 bp of the coding sequence and 257 bp of the 3′-untranslated sequence, followed by a short polyadenylate tail. The *A. thaliana* α-TIP probe used in the second library screen was a 577-bp fragment containing bp 1264 to 1841 of the sequence shown in Figure 1A (downward arrowheads). Hybridizations with this probe were in 6× SSC at 60°C with a final wash in 1× SSC at 60°C. Sequencing was done on both strands using the dideoxy chain termination method (25). DNA sequence analyses were performed using the intelligenetics programs GEL, SEQ, SEARCH, and GENALIGN (18).

Chimeric Gene Constructs and *in Vitro* RNA Synthesis

For *in vitro* synthesis of α-TIP, sense RNA, plasmid pa t-1, containing the entire α-TIP gene (from bp 411–1841, Fig. 1A) cloned behind the T7 promoter in the *Sph*I site of pBS$^-$ (Promega, Madison, WI), was linearized with *Hin*dIII and transcribed *in vitro* using T7 RNA polymerase as described previously (Stratagene, La Jolla, CA, RNA transcription instruction manual catalog No. 600111, 1989). Antisense RNA was synthesized from *Sty*I-linearized pa t-2, using T7 RNA polymerase, giving rise to a 280-bp transcript complementary to the region between bp 1557 and 1841 (Fig. 1A, upward arrowheads). pa t-2 is pBS$^-$ that contains a 3.5-kb *Hin*dIII fragment containing the α-TIP gene in an opposite orientation with respect to the T7 promoter. γ-TIP sense and antisense RNAs were synthesized from plasmid pg t-1 using T3 RNA polymerase after linearizing the plasmid with *Bgl*II and T7 RNA polymerase after linearizing with *Hin*dIII, respectively. pg t-1 is pBS$^-$ containing a 300-bp *Acc*I to *Bgl*II fragment from γ-TIP (from bp 1104–1398, Fig. 1B, downward arrowheads) downstream from the T3 promoter.

For synthesis in *E. coli*, we introduced a 259-bp *Acc*I to *Bal*I fragment (bp 1103–1362, Fig. 1B) into the *Bam*HI site of pGEX2-T (27). In this way, an in-frame fusion was created between glutathione *S*-transferase and the 33 C-terminal amino acids of γ-TIP.

DNA and RNA Blot Analysis

A. thaliana genomic DNA was isolated as described by Wilkinson and Crawford (29). DNA blotting onto Hybond-N (Amersham, Arlington Heights, IL) membranes was as described by Maniatis *et al.* (21), and hybridization was carried out at 42°C for 30 h according to the method of Klessig and Berry (17) with a final wash at 60°C in 1× SSC. The probes were made with the same 577-bp DNA fragments used in the plaque screen (see above).

Total RNA was isolated from frozen tissue as described before (5). Total RNA samples were applied onto Nytran membranes (Schleicher and Schuell) with a minifold II (Schleicher and Schuell) slot blot appliance using the procedure described in the manual. To quantify the α-TIP and γ-TIP mRNAs, a dilution series of *in vitro* synthesized sense RNA was applied on the same blots. The blots were probed with *in vitro* synthesized [^{32}P]UTP-labeled antisense RNAs. Hybridization was carried out at 65°C with 50% formamide in 5× SSC using the conditions described in the Stratagene RNA transcription instruction manual. Final washes were at 65°C with 0.2× SSC.

Expression in *E. coli*, Protein Purification, and Generation of a Rabbit Antisera

A saturated overnight culture of *E. coli* (DH5α) containing the glutathione *S*-transferase-γ-TIP fusion construct (see above) was inoculated 1:50 in Luria broth (21) and grown at 37°C until an absorbance (600 nm) of 0.2 to 0.4 was reached. Isopropyl thiogalactoside (0.1 mM) was added to induce the tac promoter, and the culture was grown for 4 to 6 h. Cells were collected by centrifugation (10 min, 2500*g*), and the cell pellet was stored at −80°C. After thawing on ice, the cells were resuspended in 1/50th volume (50 mM Tris HCl, pH7.5; 10 mM EDTA; 12% (w/w) sucrose; 0.5 mM PMSF; 1 mg/mL lysozyme) and incubated for 30 min at room temperature. The spheroplasts were lysed by passing them three times through a French press. The lysate was centrifuged for 30 min, 20,000*g*. More than 90% of the fusion protein, as estimated from Coomassie-stained SDS-PAGE, was in the pellet fraction. The pellet was washed three times with 1 M NaCl, 1% Triton X-100 in 20 mM Tris-HCl (pH 7.5) and solubilized in 1/100th of the culture volume 1% SDS, 20 mM Tris-HCl, and 1 mM DTT. The protein concentration was determined according to the method of Lowry *et al.* (20), with BSA as a protein standard. The fusion protein was purified from the pellet fraction through preparative gel electrophoresis and electroelution as described previously (12). Typically, we obtained approximately 1 mg of purified fusion protein from 100 mL culture. An antiserum was raised against the SDS-denatured protein in a rabbit using standard procedures (12). The antiserum raised against bean seed TIP has been described previously (15).

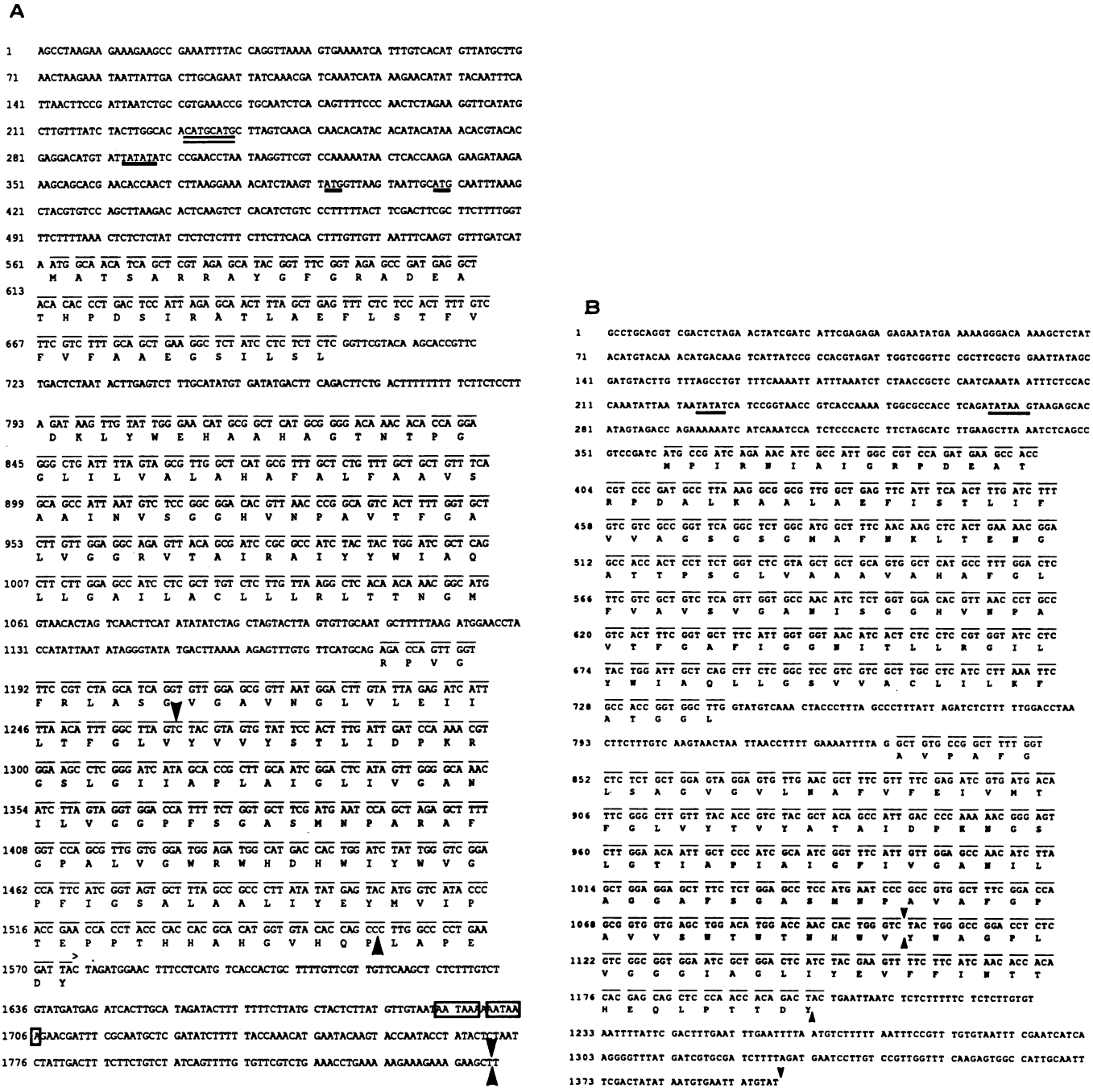

Figure 1. Nucleotide and deduced amino acid sequences of *A. thaliana* TIP homologs. Intron positions were derived from the comparison with the cDNA sequences. A, α-TIP: A consensus TATA box (position bp 293) upstream of the ATG initiator codon is underlined as well as two ATG codons located between the TATA sequence and the initiator codon. The seed-specific expression box CATGCAT (19) is underlined twice. Two consensus polyadenylate-addition recognition sequences downstream of the stop codon are boxed. Downward arrowheads indicate the fragment that was used to probe the genomic library and the Southern blot (Fig. 2). Upward arrowheads delineate the fragment used to generate a sequence specific probe for the northern analysis and the RNA slot blots (Fig. 3). B, γ-TIP: Two TATA consensus sequences upstream of the initiator codon are underlined. Downward arrowheads delimit the fragment used to synthesize a sequence specific probe for the northern analysis and the RNA slot blots (Fig. 3). Upward arrowheads indicate the peptide fragment used to generate a γ-TIP-specific antiserum.

Immunoblot Analysis

For immunoblotting, protein samples were fractionated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose sheets were blocked with 3% BSA before reaction with a rabbit antiserum. A standard procedure with horseradish-coupled second antibody was used for visualization (12).

Protoplast and Vacuole Isolation

Protoplasts were isolated from rosette leaves of young *A. thaliana* plants grown sterilely in magenta boxes. The protoplast isolation procedure was according to that of Damm and Willmitzer (4). Vacuoles were isolated using an osmotic lysis procedure adapted from that of Boller and Kende (3), which was described previously (13), with minor modifications. Briefly, 5×10^6 protoplasts were pelleted by centrifugation for 5 min at 50*g*. After removal of the supernatant, the protoplasts were resuspended in 5 mL prewarmed (42°C) lysis medium by pipetting twice up and down with a wide-bore 10-mL pipet. The lysis medium consisted of 0.2 M mannitol, 20 mM EDTA, 2 mM DTT, 5 mM Hepes, 10% Ficoll type 400 (Sigma), and 0.5% BSA adjusted to pH 8.0. The vacuoles were purified by flotation using a three-step gradient. The gradient was made by layering 3 mL of a 4% Ficoll solution on top of the lysed protoplasts followed by 1 mL of vacuole buffer. The 4% Ficoll solution was made by mixing 1 volume of lysis medium with 1.5 volumes of 0.45 M mannitol solution supplied with 10 mM Hepes (pH 7.5), 1 mM L-cysteine, and 1 mM PMSF. The vacuole buffer had 0.6 M betaine, 10 mM Hepes (pH 7.5), 1 mM PMSF, and 1 mM L-cysteine. The gradient was centrifuged for 30 min at 50,000*g* in a swing out rotor at 10°C, and the purified vacuoles were isolated from the interphase between the middle and upper layer and stored at −80°C. To separate the tonoplast from the vacuolar sap, the vacuoles were thawed and vortexed and then centrifuged at 40,000 rpm for 60 min in a TI-50 rotor (Beckman).

Preparation of Microsomes

Frozen tissue was homogenized on ice in (10 mL/mg tissue) extraction buffer (12% sucrose [w/w], 1 mM EDTA, 100 mM Tris/HCl [pH 7.8], 0.5 mM PMSF, 0.1 mg/mL leupeptin, and 10^{-7} M pepstatin) using a mortar and pestle or a tissue grinder (Fisher Scientific, Tustin, CA) in an Eppendorf tube. Cell debris was removed by centrifugation at 9000*g* for 10 min. The microsomal fraction was isolated from the supernatant through centrifugation at 40,000 rpm for 60 min in a TI-50 rotor. The microsomal pellet was dissolved in (3 mL/mg tissue) twofold denaturing buffer (40 mM Tris/HCl [pH 8.6], 2% SDS, 34% glycerol, and 0.6% 2-mercaptoethanol) before SDS-PAGE.

Electron Microscopic Immunocytochemical Localization

The immunocytochemical localization of α-TIP(At) in the embryo and silique tissues of near-mature siliques of *A. thaliana* was done with antiserum against bean seed TIP exactly as previously described (13).

Sequence Alignment and Phylogenetic Tree

Protein sequence alignment and relative evolutionary distances for construction of the phylogenetic trees were determined for the seven fully sequenced plant proteins in the MIP family using the progressive sequence alignment programs of Feng and Doolittle (8) and Doolittle and Feng (6) modified for the execution of these programs on the University of California, San Diego, VAX/VMS DNA system. The seven sequences utilized are α-TIP(At), γ-TIP(At) (sequences reported in this paper), and TIP(16), called α-TIP(Pv), NOD26(24), TobR7(31) referred to as γ-TIP(Nt), clone 7A(11) referred to as WsiTIP(Ps), and RD28(26) referred to as WsiTIP(At). The sequences were named in the following way: α for the seed-specific sequences, γ for the vegetative organ sequences, and wsi for the water stress-induced sequences. The species is recorded in parentheses with a two-letter abbreviation, one letter for the genus and one for the species.

The FASTA and RDF2 programs were used to calculate percentage identity and the comparison score, respectively (23). Comparison scores are recorded in SD values higher than those obtained with 100 comparisons of randomized sequences of the protein segments analyzed.

RESULTS

Isolation and Sequence of Genomic Clones Encoding *Arabidopsis* Homologs of Bean TIP

Proteins that cross-react with a bean TIP antiserum and with similar M_r values have been found in the seeds of all angiosperms and one pine species tested (15). This antiserum against bean TIP also identified a 26-kD protein in extracts from *A. thaliana* seeds (see below). The presence of at least one TIP-related gene was further confirmed through southern blotting of total DNA with the bean TIP cDNA as a probe under low stringency conditions (not shown). Using the same probe, we isolated four phages carrying identical inserts from an *A. thaliana* genomic library (50,000 plaques).

A 3.5-kb *Hin*dIII fragment hybridizing to the probe was subcloned and sequenced on both strands. The sequence confirmed the presence of three ORFs with significant sequence identity to the bean TIP cDNA (68% on 264 amino acids) interspersed by two short sequences with all the characteristics of introns in genes from plants (10). This nucleotide sequence is hereafter referred to as α-TIP(At) (Fig. 1A). Southern blotting with a DNA fragment containing the third exon (Fig. 1A, downward arrowheads) as a probe demonstrated the presence of at least one and perhaps two additional related genes in the *A. thaliana* genome (Fig. 2). To isolate these other potential members of the TIP gene family, we rescreened 30,000 plaques of the same library with the probe derived from the *A. thaliana* clone. This screen yielded 19 phages carrying inserts hybridizing to the DNA probe.

Three classes of overlapping clones hybridizing with decreasing intensity could be distinguished. One class of strongly hybridizing clones contained inserts, which on the basis of their restriction map, seemed to overlap with the previously isolated clone of α-TIP(At). The second class of clones has not yet been characterized in detail, but preliminary sequence

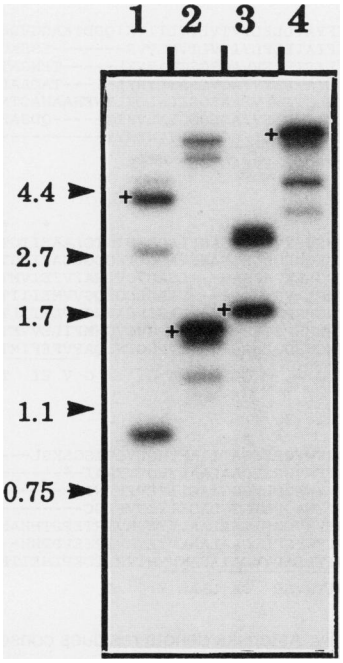

Figure 2. Southern blot of *A. thaliana* genomic DNA, hybridized with a ^{32}P-labeled DNA fragment from α-TIP (shown in Fig. 1A). Lanes 1 to 4 contain DNA digested with *Hin*dIII, *Pvu*II, *Eco*RV, and *Eco*RI, respectively. Arrows indicate the position of M_r markers (in kb). Bands corresponding to the α-TIP gene are indicated with a "+" on the left side; other hybridizing bands are fragments from other TIP-genes.

data from a clone of this class revealed an ORF highly similar to the first gene (86% identity on 49 amino acids). The third group of clones hybridized very weakly. We subcloned a 3.2-kb *Eco*RI fragment from this class and sequenced it on both strands (Fig. 1B). The sequence contained two large ORFs, the predicted amino acid sequence of which showed 58% identity (235 amino acids) with bean TIP and 59% identity (243 amino acids) with the α-TIP(At) sequence. The sequence comparison indicates the presence of only one intron, at exactly the same position as the second intron in the α-TIP(Ara) sequence (Fig. 2). The position of this intron was further confirmed through sequencing of a cDNA obtained with the polymerase chain reaction (data not shown).

The alignment of the predicted amino acid sequence of the α-TIP(At) gene with the sequence derived from the bean cDNA suggests that the translation starts at position 562 (Fig. 1A), thus encoding a polypeptide of 268 amino acids with a calculated M_r value of 28,306. At a distance of 269 bp upstream of this initiation codon is a TATA consensus sequence characteristic for polymerase II promoters. Downstream of this TATA box (93 and 110 bp) are two ATG codons (underlined in Fig. 1A) followed by in-frame stop codons four and 10 codons downstream, respectively. We do not know whether these codons are on the mature mRNA. Interestingly, the initiation codon is preceded by a region rich in T strands characteristic of plant introns (10), suggesting that another intron might be present in the untranslated leader. Two consensus polyadenylate addition sequences are present downstream from the coding sequence (positions 1694 and 1701 in Fig. 1A, boxed sequences). Based on the amino acid sequence similarity and the seed-specific expression (see below), this gene was considered the *A. thaliana* homolog of the bean seed TIP gene.

The second and the third genes were called β-TIP(At) and γ-TIP(At), respectively. γ-TIP encodes a polypeptide of 251 amino acids with a calculated M_r value of 25,618. The initiation codon for this gene was identified at bp position 359 based on the amino acid sequence alignment with the other TIP genes (Fig. 2) and based on the fact that this is the first ATG codon downstream (27 bp) of an in-frame stop codon. Upstream of the initiation codon, the nucleotide sequence shows two TATA boxes that are underlined in Figure 1B. The 5′-upstream sequence of α-TIP(At), but not of γ-TIP(At), contains a typical CATGCAT box present in the 5′-upstream sequences of genes that are expressed in dicot seeds. This box has recently been shown to be necessary for seed-specific expression of a glycinin gene (19). No consensus polyadenylation signals could be identified downstream from the coding sequence within the sequenced fragment.

The membrane topology of both the α-TIP and γ-TIP polypeptides is predicted to be similar to that of the TIP protein from bean: six membrane-spanning domains with cytoplasmically oriented N- and C-termini (Fig. 3). Both predicted proteins lack glycosylation consensus sites on their luminal loops. Both proteins have an internal sequence identity and appear to have arisen through a duplication of a protein with three membrane-spanning domains (16, 22).

In addition to the alignment with bean TIP, Figure 2 also shows the alignment of the other four plant sequences in the MIP family. These include NOD26(24); TobRB7(31), here called γ-TIP(Nt); clone 7A, here called WsiTIP(Ps) (11); and RD28(26), here called WsiTIP(At). The sequence AtRB7(30) is almost identical with γ-TIP(At) and is not included here (see "Discussion").

Residues common to at least four of the seven sequences are shown as a consensus sequence in Figure 3, whereas asterisks denote residues conserved in all plant MIP proteins. The sequence SGGHXNPAV appears to be the signature sequence for these proteins and should help to identify new proteins in this family and distinguish them from other proteins with six membrane-spanning domains. This signature sequence is found in the MIP sequences of all organisms (data not shown).

Analysis of Evolutionary Relationship of Plant MIP Proteins

The statistical analyses of the protein sequences are summarized in Figure 4, which shows the percentage identity between the sequences followed by the length of the segments that were compared. In most cases, nearly the entire amino acid sequence was used for the statistical analyses. The percentage identity and comparison scores SD establish that all these proteins are homologous, *i.e.* derived from a common ancestral protein. The proteins with similar expression patterns (*e.g.* seed specific or water stress induced) are more closely related to each other than the proteins of one organism (*e.g. A. thaliana*).

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                                                                            **   *                                    *
WsiTIP(At)    1   M-AKDVE---GPDGFQTR--------------DYEDPPPTPFFDAEELTKWSLYRAVIAEFVATLLFLYVTVLTVLIIYKIQSDTKAGGVDCGGVGILG-AWAF
WsiTIP(Ps)    1   MEAKEQDVSLGANKFPERQPLGIAAQSQDEPKDYQEPPPAPLFEPSELTSWSFYRAGIAEFIATFLFLYITVLTVMGVVR-------ESSKCKTVGIQGIAWAF
γTIP(At)      1   MP------------------------------I---RNIAIGRPDEATRPDALKAALAEFISTLIFVVAGSGSGMAFNKL-----TENGATTPSGLVAAAVAH
RtTIP(Nt)     1   M-------------------------------V---R-IAFGSIGDSFSVGSLKAYVAEFIATLLFVFAGVGSAIAYNKL-----TADAALDPAGLVAVAVAH
αTIP(At)      1   MA------------------------------TSARRAYGFGRADEATHPDSIRATLAEFLSTFVFVFAAEGSILSLDKLYWEHAAHAGTNTPGGLILVALAH
αTIP(Pv)      1   MA------------------------------T---RRYSFGRTDEATHPDSMRASLAEFASTFIFVFAGEGSGLALVKIY-----QDSAFSAGELLALALAH
NOD26(Gm)     1   MA----------DYSA------GTESQEVVVNVTKNTSETIQRSDSLVSVPFLQKLVAEAVGTYFLIFAGCASLVVNENYY----------NMITFPGIAIVW
Consensus         M                                    R   FGR DE T     RA  AEF  T  FVFAG GS      K              GL   A AH

                         *      **** ****                 *   * **                                       *   ** *
WsiTIP(At)   86   GGMIFILVYCTAGISGGHINPAVTFGLFLTRKVSLIRAVLYMVAQCLGAICGVGFVKAFQSSH-YVNYGGGANFLADGYNTGTGLAAEIIGTFVLVYTVFS-AT
WsiTIP(Ps)   98   GGMIFALVYCTAGISGGHINPAVTFGLFLARKXSLTRAIFYMVMQVLGAICGAGVVKGFEGKQRFGDLNGGANFVAPGYTKGDGLGAEIVGTFILVYTVFS-AT
γTIP(At)     66   AFGLFVAVSVGANISGGHVNPAVTFGAFIGGNITLLRGILYWIAQLLGSVVACLILKFATGGLAVPAFG-----LSAGVGVLNAFVFEIVMTFGLVYTVYATAI
RtTIP(Nt)    64   AFALFVGVSIAANISGGHLNPAVTLGLAVGGNITILTGFFYWIAQLLGSTVACLLLKYVTNGLAVPTHG-----VAAGLNGLQGVVMEIIITFALVYTVYATAA
αTIP(At)     74   AFALFAAVSAAINVSGGHVNPAVTFGALVGGRVTAIRAIYYWIAQLLGAILACLLLRLTTNGMRPVGFR-----LASGVGAVNGLVLEIILTFGLVYVVYSTLI
αTIP(Pv)     66   AFALFAAVSASMHVSGGHVNPAVSFGALIGGRISVIRAVYYWIAQLLGSIVAALVLRLVTNNMRPSGFH-----VSPGVGVGHMFILEVVMTFGLMYTVYGTAI
NOD26(Gm)    78   GLVLTVLVYTVGHISGGHFNPAVTIAFASTRRFPLIQVPAYVVAQLLGSILASGTLRLLFMGNHDQFSG-----TVPNGTNLQAFVFEFIMTFFLMFVICGVAT
Consensus         AF LF  VS  A ISGGH NPAVTFG   GG   LIRA  YWIAQLLGSI A L LK  T G      G      A G     G V EI  TF LVYTVY TA

                  *             *   **              *   ***   *              *     *  *
WsiTIP(At)  198   DPKRNARDSHVPVLAPLPIGFAVFMVHLATIPITGTGINPARSFGAAVIFNKSKPWDDHWIFWVGPFIGATIAAFYHQFVLRASGSKSL------GSFRSAANV-
WsiTIP(Ps)  201   DAKRSARDSHVPILAPLPIGFAVFLVHLATIPITGTGINPARSLGAAIVFNKKIGWNDHWIFWVGPFIGAALAALYHQVVIRAI------------PFKSK----
γTIP(At)    165   DPKNGS----LGTIAPIAIGFIVGANILAGGAFSGASMNPAVAFGPAVV---SWTWTNHWVYWAGPLVGGGIAGLIYEVFFINTT---------HEQLPTT-DY-
RtTIP(Nt)   163   DPKKGS----LGTIAPIAIGFIVGANILAAGPFSGGSMNPARSFGPAVV---AGDFSQNWIYWAGPLIGGGLAGFIYGDVFIGC----------HTPLPTSEDYA
αTIP(At)    173   DPKRGS----LGIIAPLAIGLIVGANILVGGPFSGASMNPARAFGPALV---GWRWHDHWIYWVGPFIGSALAALIYEYMVIPTEPPTHHAHGVHQPLAP-EDY-
αTIP(Pv)    165   DPKRGA----VSYIAPLAIGLIVGANILVGGPFDGACMNPALAFGPSLV---GWQWHQHWIFWVGPLLGAALAALVYEYAVIPIEPPPHH----HQPLAT-EDY-
NOD26(Gm)   177   DNR--A----VGELAGIAIGSTLLLNVIIGGPVTGASMNPARSLGPAFV---HGEYEGIWIYLLAPVVGAIAGAWVYNIVRYTDKPLSEITKSASF-LKGRAASK
Consensus         DPKRGA    VG IAPLAIGFIVGANILAGGPF GASMNPARSFGPA V      W  HWIYWVGP  GA LAAL Y    I            H PL    DY
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Figure 3. Multiple alignment of the seven sequenced plant members of the MIP protein family. Asterisks denote residues conserved in all seven plant proteins of this family. Residues common to at least four of the seven sequenced proteins are shown in the consensus sequence (Consensus). Numbers to the left of the sequences shown refer to the first amino acid in the row. The putative transmembrane segments (22) are indicated with lines below the aligned sequences. The gene nomenclature is explained in the text.

Tissue-Specific and Developmentally Regulated Expression of α- and γ-TIP mRNA

To study the expression patterns of the two TIP isoforms, we used sequence-specific antisense RNA probes to analyze slot blots with total RNA from various tissues of *A. thaliana* (Fig. 5). α-TIP was expressed exclusively in siliques beginning some time between 6 and 9 d after flowering, reaching a peak at about 15 d. Densitometer scanning of the slot blots, with a dilution series of *in vitro* synthesized antisense RNA as a reference (not shown), allowed us to estimate the concentration of α-TIP messenger at this stage at 100 pg/mg total RNA or approximately 1% of poly(A^+) RNA. The γ-TIP expression pattern was complementary to that of α-TIP: γ-TIP was expressed in all plant organs except mature siliques. Its expression in all tissues was quantitatively similar (between 10 and 40 pg/mg total RNA or 0.1 to 0.4% of poly(A^+)RNA) but declined in the siliques at the time when α-TIP expression increased. The decline in γ-TIP expression in siliques coincided with the senescence of the fruit wall, suggesting that γ-TIP expression in siliques is contributed by the fruit tissues

	γTIP(At) (251)	αTIP(At) (268)	RtTIP(Nt) (250)	αTIP(Pv) (256)	NOD26(Gm) (271)	WsiTIP(Ps) (289)
WsiTIP(At) (285)	33(246) [29]	37(253) [35]	35(226) [28]	34(247) [28]	31(190) [19]	69(265) [103]
γTIP(At) (251)		59(243) [62]	65(245) [63]	58(235) [61]	32(230) [20]	34(229) [29]
αTIP(At) (268)			52(243) [60]	68(264) [100]	32(239) [22]	37(240) [31]
RtTIPT(Nt) (250)				48(240) [56]	34(222) [25]	34(227) [29]
αTIP(Pv) (256)					33(229) [23]	37(235) [30]
NOD26(Gm) (271)						28(265) [21]

Figure 4. Binary comparisons of the alignments of the seven sequenced plant members of the MIP protein family. Values in parentheses below the source of the protein indicate the number of amino acids in the protein. Values reported in the table correspond to the percentage identity in the other segments compared. The number of amino acids in the aligned segment is provided in parentheses. Values in brackets denote the comparison score in SD values higher than those obtained with 100 comparison of randomized sequences of these protein segments. The FASTA and RDF2 programs were used to calculate the percentage identity and the comparison score, respectively (23).

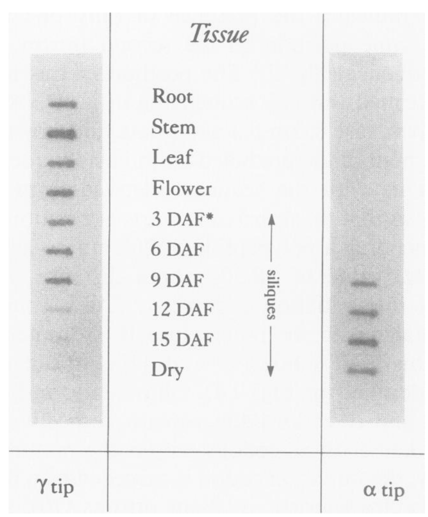

Figure 5. Slot blot analysis of *A. thaliana* total RNAs hybridized with sequence-specific RNA probes synthesized from α-TIP and γ-TIP. Each slot contains 2 μg of total RNA from different organs.

and not the seeds. This idea was confirmed by the analysis of transgenic *A. thaliana* plants containing the β-glucuronidase gene fused to the α-TIP and γ-TIP promoters, respectively (D. Ludevid, H. Höfte, and M.J. Chrispeels, unpublished). The size of both the α-TIP and γ-TIP transcripts was approximately 1200 bp, as determined using northern blotting on total RNA (not shown). No differences in mobility were observed for the γ-TIP transcripts in the different organs. In conclusion, these data show that α-TIP and γ-TIP isoforms clearly have nonoverlapping expression patterns in seeds and vegetative tissues, respectively.

α-TIP Is a Seed-Specific Protein in the Tonoplast of the Protein Storage Vacuoles of the Embryo and Endosperm

The α-TIP homolog in bean is located specifically in the vacuolar membrane (tonoplast) of the protein storage vacuoles in seeds, and antibodies against bean seed TIP cross-react with a similarly sized protein in the seeds of all species tested. Nevertheless, because the sequence identity between bean α-TIP and *A. thaliana* α-TIP is only 68%, we decided to determine the subcellular location of α-TIP in *A. thaliana* seeds by immunocytochemistry.

The results (Fig. 6) show abundant colloidal gold labeling of the membrane (tonoplast) surrounding the protein storage vacuoles in the embryo (Fig. 6A), whereas the tonoplasts of the cells of the silique (Fig. 6B) were not labeled. In Figure 6A about one-third of the protein storage vacuole area represents a grazing cut through the tonoplast. The colloidal gold particles look scattered, but on the inside face of the membrane there is a clear row of particles (triangles), whereas on the cytoplasmic face, the particles are less numerous (arrows). That this is a grazing cut can be seen by the faint appearance of several oil bodies that lie underneath. Our polyclonal serum has more antibodies directed to epitopes on the vacuolar side of the TIP polypeptide than to epitopes on the cytoplasmic side. We generally found somewhat more nonspecific labeling with *A. thaliana* seeds than with bean or soybean seeds. Some nonspecific labeling is also evident in Figure 6B, but the tonoplast is completely devoid of label. In contrast, immunolocalization of TIP with the same antiserum in leaves of tobacco plants transformed with a bean TIP gene driven by the cauliflower mosaic virus 35S promoter showed very heavy labeling of the tonoplast in leaf cells, using the same serum (13). Together, these results support our conclusion that α-TIP(At) is a seed-specific protein that is uniquely associated with the tonoplast, as is its homolog in beans.

γ-TIP Is a Tonoplast Protein Present in Vegetative Tissues

To investigate the subcellular location of γ-TIP, we first raised a γ-TIP-specific antiserum. A fusion protein was produced in *E. coli* consisting of glutathione *S*-transferase fused to the C-terminal 33 amino acids of γ-TIP (see Fig. 1B, upward arrowheads). Antibodies raised against the fusion protein purified by SDS-PAGE recognized a 26-kD protein in total extracts and in microsomes prepared from leaves of *A. thaliana*. No such polypeptide was found in the supernatant fraction of the homogenate (data not shown). To determine

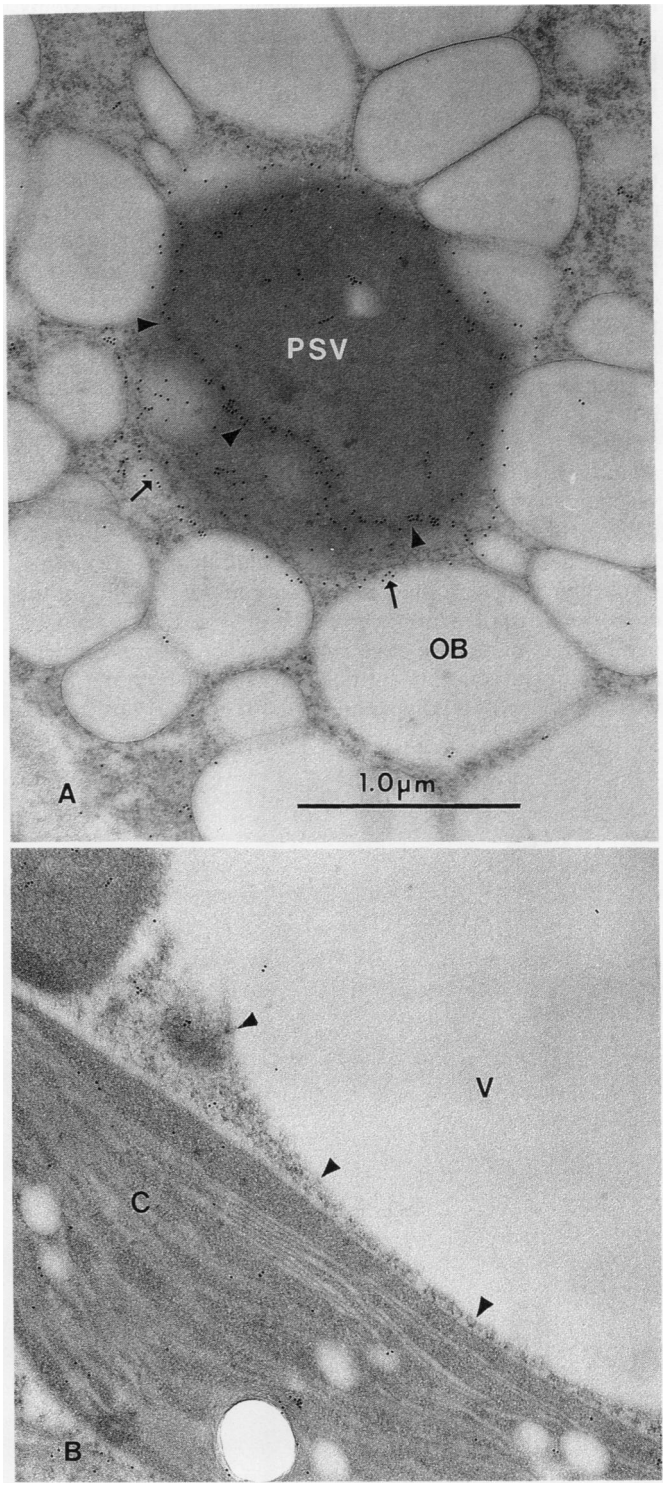

Figure 6. Immunocytochemical localization of α-TIP(At). A, Near-mature embryo cell. PSV, Protein storage vacuole; OB, oil bodies; arrowheads, vacuolar face of the tonoplast; arrows, cytoplasmic face of the tonoplast. Magnification, ×30,000. B, Mesocarp parenchyma cell of the silique. v, Vacuole; c, chloroplast; arrowheads, tonoplast. There is some background labeling, but no labeling of the tonoplast.

whether the accumulation of γ-TIP is organ specific or occurs in all organs, we prepared microsomes from leaves, stems, roots, and flowers, as well as from seeds, and analyzed them by immunoblotting with α-TIP and γ-TIP antibodies. The results (Fig. 7) show that all vegetative organs contained a γ-TIP cross-reactive polypeptide of 26 kD, and only seeds contained α-TIP. In addition to the polypeptide of 26 kD, there is in some lanes an additional band of lower mobility (higher molecular mass) visible. This band is most likely an aggregate and is often present in immunoblots of proteins in the MIP family (for example, see ref. 9). Although the abundance of this band is diminished by heating the samples only to 70°C instead of to 100°C, we could not completely eliminate its presence. The higher molecular mass aggregate is seldom observed when total cell extracts are immunoblotted but is nearly always present when microsomes or purified tonoplasts are analyzed (see also Fig. 8 for example). The abundance of the γ-TIP protein was comparable in different vegetative tissues and paralleled the steady-state mRNA levels detected with the γ-TIP-specific probe. These data indicate the absence of posttranscriptional regulation of γ-TIP expression. Interestingly, some heterogeneity in the mobility of the cross-reacting polypeptides could be observed; a single polypeptide was present in root extracts, whereas in stem, leaf, and flower extracts, TIP formed a doublet. We do not know whether the second polypeptide is the result of a posttranslational modification such as phosphorylation or whether it represents the product of another gene.

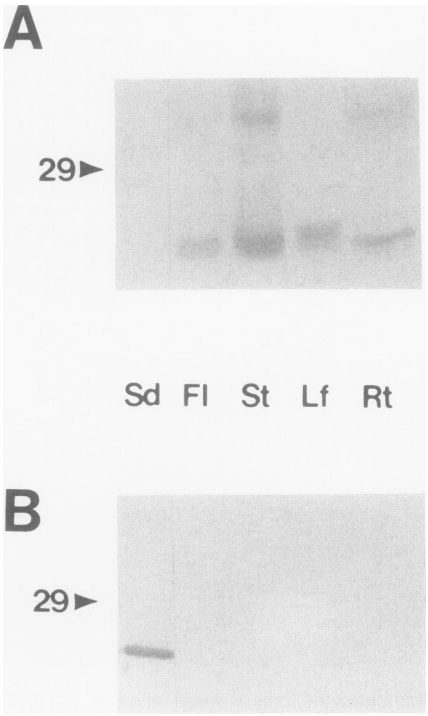

Figure 7. Immunoblot analysis of *A. thaliana* TIP isoforms in different tissues. A, With serum against γ-TIP; B, with serum against bean seed-TIP. Ten micrograms of microsomal proteins were loaded in each lane. Sd, Seed; Fl, flower; St, stem; Lf, leaf; and Rt, root. Arrows indicate the position of the 29-kD marker. Higher M_r immunoreactive bands presumably are TIP dimers (see text).

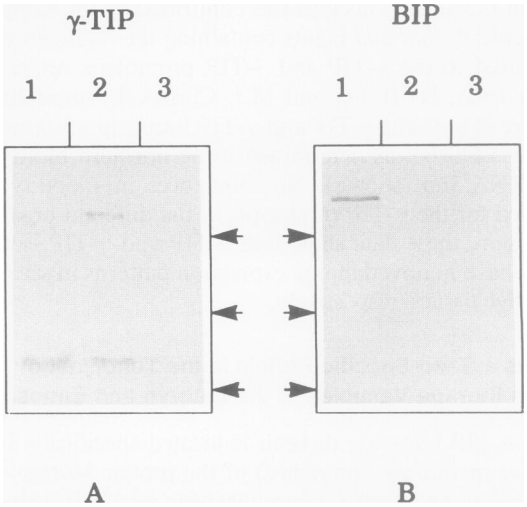

Figure 8. Immunoblot analysis of protoplasts (lane 1), purified tonoplasts (lane 2), and vacuolar content (lane 3) from *A. thaliana* leaves with a γ-TIP antiserum and a binding protein (BIP) antiserum. Samples in lanes 1 and 2 were equalized on the basis of acid phosphatase activity. Lanes 2 and 3 contain the same amount of vacuolar material Arrows indicate the position of the 18- (lower) and the 29- and 45-kDa (upper) markers. The higher M_r immunoreactive band present in the tonoplast lane corresponds to γ-TIP dimer. Note the absence of BIP in the vacuolar fractions (lane 2 and 3).

To determine the subcellular location of γ-TIP, we purified vacuoles from *A. thaliana* leaf protoplasts, separated the vacuolar contents from the tonoplast by centrifugation after lysis, and analyzed the proteins using immunoblotting with the γ-TIP-specific antiserum. To equalize the loading of the lanes in Figure 8, we determined acid phosphatase activity in the protoplast and vacuole fractions and loaded aliquots corresponding to equal acid phosphatase activity in lanes 1 and 3. Lane 2 (tonoplast) contains an amount of tonoplast protein corresponding to the vacuolar contents shown in lane 3. The γ-TIP antiserum detected the same amount of cross-reacting protein in the protoplast and tonoplast fractions, confirming the presence of γ-TIP in the tonoplast (Fig. 8). A parallel immunoblot was probed with an antiserum against the ER marker protein BiP (binding protein), and this protein was found in the protoplasts, but not in the tonoplast or vacuolar content fractions, demonstrating that the isolated vacuoles were not contaminated with ER.

DISCUSSION

In this paper, we describe and extend (26, 30) the identification of a small gene family encoding tonoplast-associated proteins in *A. thaliana*. We isolated three different homologous genes. One gene (α-TIP) is seed specific, whereas another gene (γ-TIP) is expressed only in vegetative tissues. For the third gene (β-TIP), we do not have expression data yet. However, this gene is likely to encode a seed-specific protein for the following reason. Preliminary sequence data indicate that the putative protein that is encoded by β-TIP is highly

similar to α-TIP (86% over 49 amino acids). Therefore, it is likely that this protein, like α-TIP, will also be recognized by the antiserum raised against bean seed TIP. The antiserum against bean seed TIP detected a cross-reacting protein only in seeds of *A. thaliana* and not in other organs; it is, therefore, not unreasonable to assume that β-TIP will also be specifically expressed in seeds. Interestingly, bean seeds also contain two highly conserved (75% amino acid identity) TIP isoforms (13) with similar temporal expression patterns (H Höfte, unpublished data).

Evolutionary Relationships between the Different MIP Proteins of Plants

The cloning and Southern blotting data presented in this paper indicate that *A. thaliana* contains three TIP genes. Other genes, such as the water stress-induced gene of *A. thaliana* (26) escaped detection with the DNA probes used. In addition to the genes mentioned above, three other members of the TIP family have been isolated: NOD26 expressed in soybean nodules (24), the water stress-induced clone 7A of pea roots (11), and the tobacco root-specific protein Tob RB7 (31). The screening of an *A. thaliana* cDNA library with a DNA probe from this tobacco gene yielded a gene that is almost identical with our γ-TIP sequence (30). Except for a few base changes throughout the sequence, the surprising difference between the two sequences is a frameshift near the 3′ end of the coding sequence, resulting in a predicted polypeptide with a different carboxyterminus (10 amino acids). In addition, northern blot data indicate that this gene, in contrast to γ-TIP, is specifically expressed in *A. thaliana* roots and not in other vegetative tissues (MA Conckling, personal communication). We did not find any evidence for the presence of another gene highly similar but different from γ-TIP in *A. thaliana*. However, we cannot exclude the possibility that the sequence differences reflect allelic differences between the *A. thaliana* ecotypes used in the two studies (Colombia *versus* Landsberg).

The phylogenetic tree for the seven fully sequenced plant proteins in the MIP family is shown in Figure 9. The results show a clustering of the two seed-specific proteins (α-TIP(Pv) and α-TIP(At)), the two water stress-induced proteins (WsiTIP(Nt) and WsiTIP(At)), and the two vegetatively expressed proteins (γ-TIP(At) and γ-TIP(Nt)). The water stress-induced proteins are more distantly related from the seed-specific and vegetatively expressed proteins, which form a cluster in the phylogenetic tree.

Both α- and γ-TIP are integral tonoplast proteins. We do not know whether all members of the TIP superfamily in plants are tonoplast associated. NOD26 is present in the peribacteroid membrane of soybean nodules (24); however, it is not established whether this membrane is derived from the plasma membrane, the tonoplast, or both. The intracellular location of the root-specific tobacco protein (31) remains to be determined. The clustering of the seed-specific and the vegetatively expressed homologs in the phylogenetic tree leads us to suggest that they all are probably tonoplast proteins. However, the water stress-induced protein could well have a different location in the cell.

Recently, another TIP homolog was described in the yeast *Saccharomyces cerevisiae* (28). The gene, FPS1, complements

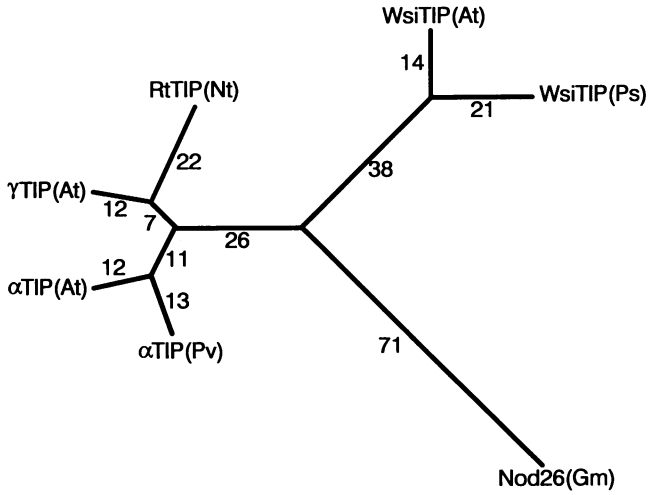

Figure 9. Parsimonious phylogenetic tree of the seven fully sequenced members of the MIP protein family in plants. The relative lengths of the branches indicating evolutionary distance are shown as numbers next to the segments.

the growth defect on fermentable sugars of a mutant (*fdp1*) but does not complement the defect in glucose-induced *ras*-mediated cAMP signaling, the initial cause for the observed growth defect. It will be interesting to see whether the FPS1 protein is also a tonoplast-associated protein in yeast cells and whether TIP is also able to complement the *fdp1*-associated growth defect. These experiments are now in progress.

Nonoverlapping Tissue-Specific Expression of α-TIP and γ-TIP May Reflect Functional Specialization

Our current working hypothesis is that TIP is a solute channel in the vacuolar membrane (15). In this study, we show that different TIP isoforms have nonoverlapping expression patterns in vegetative and in seed tissues. In addition, a more detailed analysis of the expression pattern of the reporter gene β-glucuronidase fused to the γ-TIP promoter region in transgenic *A. thaliana* showed that the γ-TIP promoter is developmentally regulated and preferentially expressed in certain vegetative cell types and absent in others. For instance, transcription is completely turned off in meristematic cells (D. Ludevid, H. Höfte, M. J. Chrispeels, unpublished). We suggest that different TIP isoforms may have different transport characteristics adapted to the specific physiological environment of vacuoles in different cell types. Indeed, it is well established that the vacuole in storage parenchyma cells of seeds has a number of specialized functions associated with the storage, breakdown, and mobilization of proteins and metabolites, whereas vacuoles in vegetative cells have other specialized functions depending on the cell type. These different functions may require a different set of active solute transporters. The tentative conclusion that TIP proteins may be functionally specialized is supported by the evolutionary data showing that proteins from different organisms with similar expression patterns are more closely related to one another than to other proteins in the same organism.

Different isoforms of channel proteins can vary in selectiv-

ity, voltage dependence, and/or regulation by other factors such as phosphorylation. In this respect it is interesting to note that bean seed TIP is phosphorylated by a tonoplast-associated Ca^{2+}-dependent serine protein kinase (14). We are currently investigating whether this phosphorylation has any functional significance. How could we go about finding out whether different isoforms of TIP have different functions? Future experiments will address the physiological role of the variation in TIP isoforms in the tonoplast of different cell types. To this end, we are currently studying in more detail the relationship between the presence of different TIP isoforms and the morphology and the physiology of the vacuole. In addition, we are investigating the electrophysiological properties of *Xenopus laevis* oocytes injected with different TIP mRNAs. In this way we hope to confirm the channel function of TIP and to determine whether different isoforms indeed have different functional characteristics.

Note Added in Proof

In a recent paper, Maeshima describes the characteristics of the abundant protein present in the tonoplast of radish roots. The aminotermino amino acid sequence of this protein has sequence identity with γ-TIP, indicating that it is another member of the TIP family. (Maeshima M [1991] Characterization of the major integral protein of vacuolar membrane. Plant Physiol **98:** 1248–1254).

The GenBank accession number for α-TIP(At) is M84343 and for γ-TIP(At) is M84344. The EMBL accession numbers are X63551 and X63552, respectively.

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