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Characterization of a New Vacuolar Membrane Aquaporin Sensitive to Mercury at a Unique Site

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The membranes of plant and animal cells contain aquaporins, proteins that facilitate the transport of water. In plants, aquaporins are found in the vacuolar membrane (tonoplast) and the plasma membrane. Many aquaporins are mercury sensitive, and in AQP1, a mercury-sensitive cysteine residue (Cys-189) is present adjacent to a conserved Asn-Pro-Ala motif. Here, we report the molecular analysis of a new Arabidopsis aquaporin, δ-TIP (for tonoplast intrinsic protein), and show that it is located in the tonoplast. The water channel activity of δ-TIP is sensitive to mercury. However, the mercury-sensitive cysteine residue found in mammalian aquaporins is not present in δ-TIP or in γ-TIP, a previously characterized mercury-sensitive tonoplast aquaporin. Site-directed mutagenesis was used to identify the mercury-sensitive site in these two aquaporins as Cys-116 and Cys-118 for δ-TIP and γ-TIP, respectively. These mutations are at a conserved position in a presumed membrane-spanning domain not previously known to have a role in aquaporin mercury sensitivity. Comparing the tissue expression patterns of δ-TIP with γ-TIP and α-TIP showed that the TIPs are differentially expressed.

INTRODUCTION

The membranes of plant and animal cells contain aquaporins, proteins that form water-selective channels (reviewed in Chrispeels and Agre, 1994; Chrispeels and Maurel, 1994; Engel et al., 1994) that belong to the ancient major intrinsic protein (MIP) family (Reizer et al., 1993). These 23- to 29-kD proteins have six putative membrane-spanning α-helical domains and a number of absolutely conserved amino acids, including the signature sequence SerGlyxHisxAsnProAla or SGxHxNPA (where x is any amino acid), which is partially repeated as NPA in the second half of the protein.

The existence of water channel proteins has long been suspected, because a number of membranes and tissues have been found to have a permeability to water that is much greater than the predicted intrinsic membrane permeability. In addition, water movement can be inhibited by protein-modifying sulfhydryl reagents (Macey, 1984; Pratz et al., 1986; Wayne and Tazawa, 1990). Xenopus oocytes provide a convenient system to determine the water transport activity of a single protein expressed as the plasma membrane of these cells. When oocytes injected with in vitro–synthesized complementary RNA (cRNA) are moved to a hypotonic solution, an increase in volume has been observed; oocytes expressing aquaporin cRNA rapidly swell and burst. This method first was used to characterize the mammalian protein AQP1 as an aquaporin (Preston et al., 1992), and aquaporin function has since been found in plant proteins, including γ-TIP (for tonoplast intrinsic protein; Maurel et al., 1993), the plasma membrane intrinsic proteins (PIPs; Kammerloher et al., 1994), and RD28-PIP (Daniels et al., 1994) from Arabidopsis and α-TIP from bean (Maurel et al., 1995). Water transport activity has been determined for several other plant MIP proteins, including TobRB7 from tobacco (Opperman et al., 1994).

Determining the structure of the aquaporin protein is currently the focus of a number of studies (for an overview, see Agre et al., 1995). Work is progressing by analyzing site-directed and insertional mutations (Preston et al., 1993, 1994; Daniels et al., 1994; Jung et al., 1994b), intergenic and intragenic complementation of mutants (Jung et al., 1994b), and chimeric aquaporins (Mulders et al., 1995) to detect functionally important domains. Spectroscopy (Farinas et al., 1993; van Hoek et al., 1993), two-dimensional x-ray crystallography (Walz et al., 1994), and electron diffraction (Jap and Li, 1995) are being used to establish the tertiary structure of aquaporins. The ability of sulfhydryl reagents, such as mercuric chloride and *p*-chloromercuribenzenesulfonate, to inhibit aquaporin function has been a useful tool in marking regions of the protein that form, or are proximal to, the water pore. It is thought that these reagents bind the sulfhydryl moiety of cysteine(s) present in the aquaporin protein and sterically occlude the pore (Preston et al., 1993), which is predicted to be a narrow structure created by the two conserved NPA motifs (Jung et al., 1994b). To date, only residues flanking the NPA motifs have been shown to be involved in aquaporin mercury sensitivity (Preston et al., 1993; Zhang et al., 1993; Daniels et al., 1994;

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Jung et al., 1994b). In this study, we found that a cysteine in a predicted transmembrane sequence distant from the putative pore-forming motifs also is involved in mercury sensitivity. This may indicate a previously unidentified region of the protein involved in the pore structure.

RESULTS

δ-TIP Is an Arabidopsis MIP Family Homolog

A large number of unique expressed sequence tags (ESTs) in Arabidopsis show homology with members of the MIP gene family (A. Weig and M.J. Chrispeels, unpublished results), but only a few of the proteins encoded by these cDNAs have been functionally characterized. To further our understanding of MIPs and aquaporins in Arabidopsis, we screened a λZAP library of Arabidopsis cDNAs for phage with inserts homologous to β-*tip* (Höfte et al., 1992). One insert had a partial open reading frame (ORF) with greatest sequence identity to γ-TIP (63.7% sequence identity over 525 bp), and a cDNA with an identical full-length ORF was obtained from the French CNRS Arabidopsis EST project (Höfte et al., 1993) for further characterization. This cDNA was sequenced, and the ORF was found to encode a polypeptide of 251 amino acids with a calculated molecular weight of 25,025, which is closer in homology with Arabidopsis TIP than it is with PIP aquaporins. Consequently, this putative protein was named δ-TIP.

δ-TIP contains the MIP family signature sequence SGxHxNPAVT, and the membrane topology is predicted to be similar to other MIP family proteins, with six transmembrane domains and cytoplasmically oriented N and C termini. A comparison of the amino acid sequences for a number of known Arabidopsis aquaporins—the mammalian aquaporins AQP1 (Preston et al., 1992) and AQP4 (Jung et al., 1994a)—and δ-TIP is shown in Figure 1. The genomic sequence of δ-*tip* was isolated by screening a genomic Arabidopsis library with the δ-*tip*

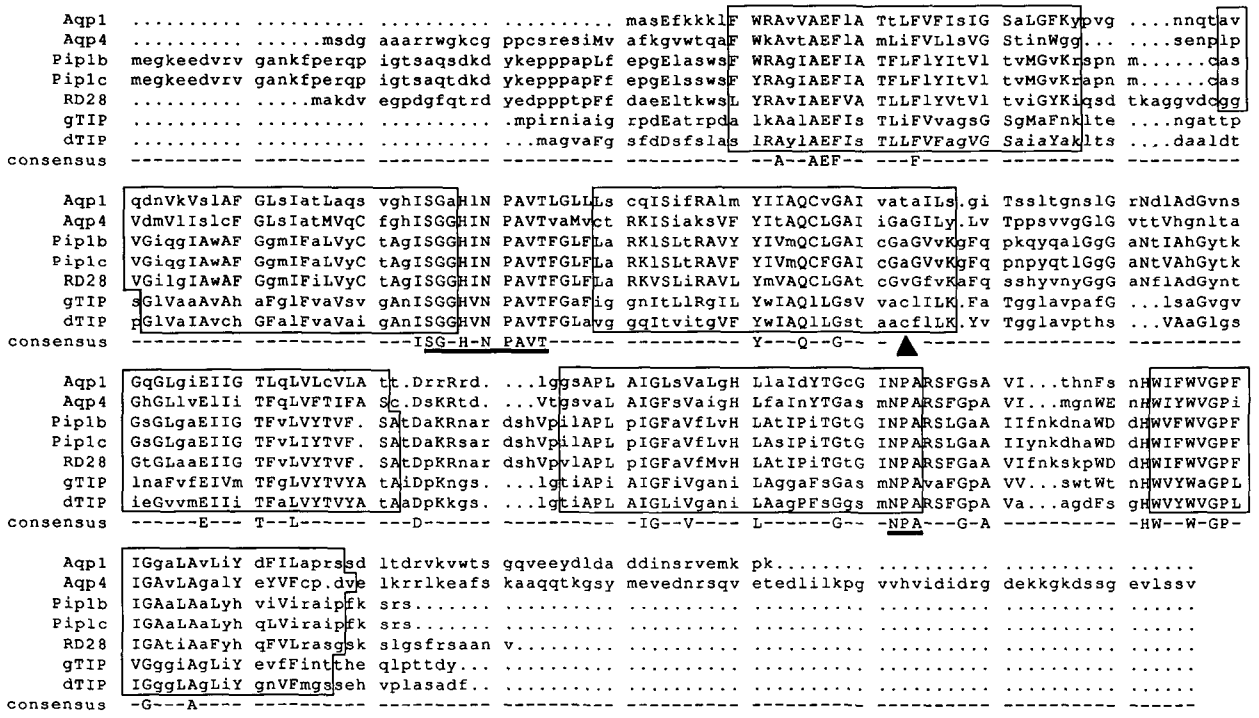

Figure 1. Amino Acid Comparisons of Aquaporins for Which Mercury Sensitivity Has Been Characterized.

The amino acid sequences were obtained from the following sources: Aqp1 (Preston et al., 1992), Aqp4 (Hasegawa et al., 1994; Jung et al., 1994a), Pip1b and Pip1c (Kammerloher et al., 1994), RD28 (Daniels et al., 1994), γ-TIP (gTIP) (Maurel et al., 1993), and δ-TIP (dTIP) (this article). The single-letter amino acid code is used. Gaps added for aligning sequences are noted with dots. Similar and conserved amino acids are shown in uppercase letters; others are in lowercase letters. The consensus line shows those residues conserved in all sequences. The position of the cysteine residue mutated in δ-TIP C116S and γ-TIP C118T is noted by an arrowhead in the consensus line. The MIP family signature sequences SGxHxNPA and NPA residues (Reizer et al., 1993) are underlined in the consensus sequence line. Boxed regions are predicted to form transmembrane α-helices.

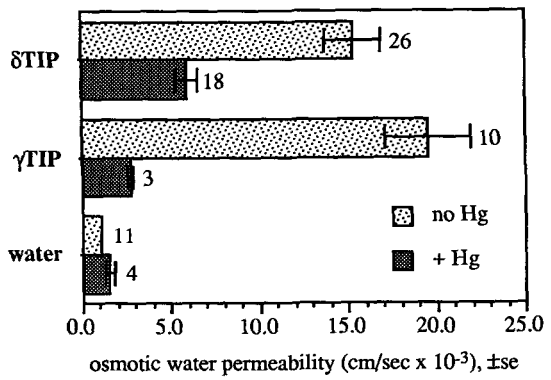

Figure 2. P_f Values of Individual δ-*tip* and γ-*tip* cRNA-Injected Oocytes Derived from Volume Change Measurements Made over Four (γ-*tip*) or Five (δ-*tip*) Independent Preparations of Oocytes.

Where indicated, the assay was performed in the presence (+) of mercury ions (Hg in 1 mM $HgCl_2$ with a 10-min preincubation). Data are expressed as the mean ± SE, with the number of replicates indicated next to each bar.

cDNA sequence. The 2287-bp genomic fragment includes 1.2 kb of promoter sequence and 602 bp of coding sequence interrupted by two small introns of 103 and 398 bp.

δ-TIP Forms Water Channels in Xenopus Oocytes

In vitro–transcribed cRNA encoding δ-TIP or γ-TIP, each with the 5′ and 3′ untranslated regions of a β-globin gene from Xenopus, was injected into 1-day-old Xenopus oocytes, and osmotically driven water transport was investigated 3 days after injection. Oocytes were exposed to hypo-osmotic conditions (160-mosmol gradient), and the initial changes in cell volume were measured. Water-injected control oocytes swelled slowly after the change in osmotic strength and burst only after 40 to 45 min (data not shown). In contrast, oocytes injected with either δ-*tip* or γ-*tip* cRNA rapidly increased their volume and ruptured in 2 to 5 min, indicating the appearance of a new pathway for facilitated transport of water into the hypertonic cell. With δ-TIP and γ-TIP, the osmotic water permeability (P_f) increased 12- to 16-fold and 15- to 20-fold, respectively, over the control value (Figure 2). Recent evidence shows that γ-TIP is an aquaporin, a proteinaceous pore highly permeable to water (Maurel et al., 1993; reviewed in Chrispeels and Agre, 1994), similar to the mammalian aquaporins AQP1 (Preston et al., 1992) and AQP4 (Hasegawa et al., 1994). Mercury sulfhydryl reagents, such as mercuric chloride, are characteristic inhibitors of water channel proteins (Macey, 1984; Preston et al., 1993). Water channeling through the δ-TIP aquaporin is inhibited 69% by 1 mM mercuric chloride (Figure 2), whereas the γ-TIP aquaporin was inhibited by 95% in this experiment (see also Maurel et al., 1993).

GlpF is a member of the MIP protein family in yeast and has been described recently as a glycerol channel that does not permit water transport (Maurel et al., 1994). The mammalian MIP protein AQP3, on the other hand, permits water, urea, and glycerol transport (Echevarria et al., 1994; Ishibashi et al., 1994; Ma et al., 1994). δ-TIP is nearly impermeable to glycerol; oocytes injected with δ-*tip* cRNA showed a 1.8-fold increase in uptake of radiolabeled glycerol over water-injected oocytes. *GlpF* cRNA–injected oocytes showed a 17-fold increase (data not shown).

δ-TIP and γ-TIP Mutants Form Mercury-Insensitive Water Channels

A model of plant tonoplast aquaporins showing the six membrane-spanning domains and the NPA signature sequences of these MIP family proteins in the first and second halves of the polypeptide is shown in Figure 3. These NPA sequences are present in all members of the Arabidopsis MIP family (A. Weig and M.J. Chrispeels, unpublished results). Work with the mammalian aquaporin AQP1 and the plant aquaporin RD28-PIP has revealed that the presence or absence of a cysteine in the amino acid residues just before either of the two NPA signature sequences generally correlates with the ability of mercury sulfhydryl reagents to inhibit aquaporin activity (Preston et al., 1993; Zhang et al., 1993; Daniels et al., 1994; Jung et al., 1994b). These results indicate that this is a functionally important domain of the aquaporin, and it is thought that the binding of mercury to a cysteine at this region sterically blocks the flow of water through the water pore (Preston

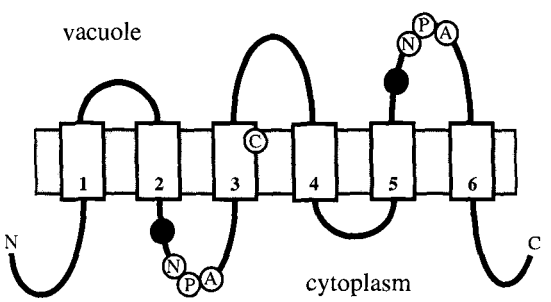

Figure 3. Model of the δ-TIP Protein and Its Topography in the Vacuolar Membrane.

The six putative transmembrane domains are numbered from the N-terminal end. The two conserved Asn-Pro-Ala (NPA) sequences are noted, as is Cys-116 (C) in its predicted transmembrane domain. The dark circles indicate cysteine residues previously implicated in aquaporin mercury sensitivity.

et al., 1993). However, δ-TIP and γ-TIP have no cysteine residues near either NPA sequence, yet both are sensitive to inhibition by mercury. γ-TIP has only one cysteine at position 118. At this location, the aquaporin δ-TIP also has a cysteine residue (see Figure 1). This residue is within a predicted transmembrane sequence (Höfte et al., 1992), which may form an amphipathic helix with cysteine in the hydrophilic side, as shown in Figure 4.

To examine the effects of replacing the cysteine residue in γ-TIP and the homologous cysteine residue at position 116 in δ-TIP with an amino acid of similar size and hydrophilicity, polymerase chain reaction (PCR) site-directed mutagenesis (Higuchi, 1990) was used to make the mutants γ-TIP C118T (cysteine replaced by threonine) and δ-TIP C116S (cysteine replaced by serine). The osmotic water permeability permitted by these mutant aquaporins was investigated using the Xenopus oocyte expression system. As seen in Figure 5A, the

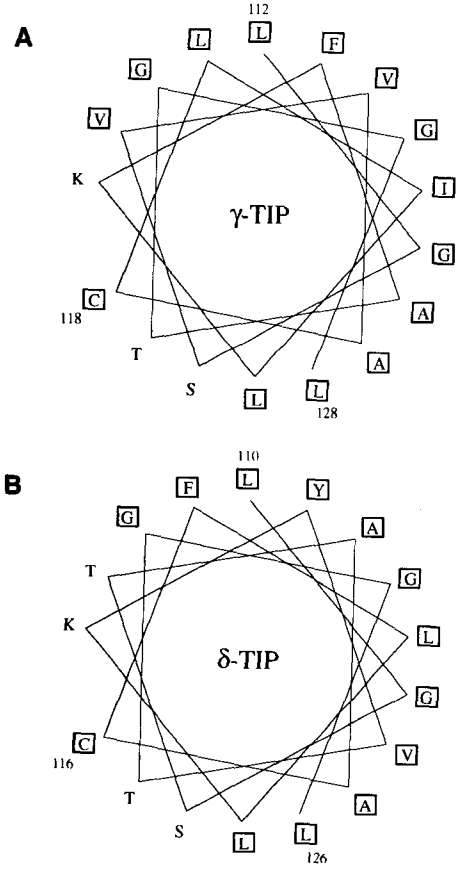

Figure 4. Helical Wheel Projections of Amino Acid Residues 112 to 128 from γ-TIP and 110 to 126 from δ-TIP.

The single-letter amino acid code is used, with the residue number following. Hydrophobic residues are indicated where the amino acid is boxed; hydrophilic residues are not boxed. The Cys-118 of γ-TIP and Cys-116 of δ-TIP are numbered as such.

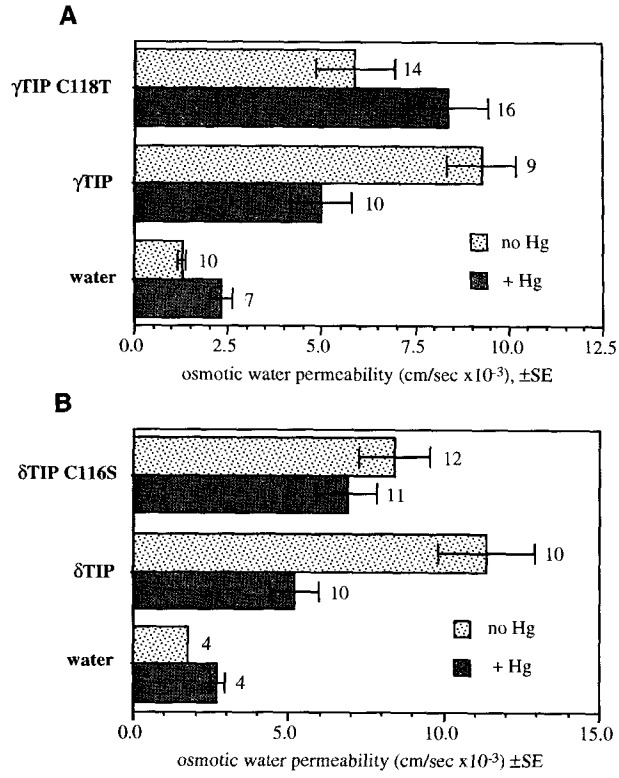

Figure 5. Water Transport Activity of Mutant and Wild-Type *tip* cRNA–Injected Oocytes.

(A) *P_f values of γ-tip and γ-tip C118T cRNA–injected oocytes derived from volume change measurements made over four independent preparations of oocytes.*

(B) *P_f values of δ-tip and δ-tip C116S cRNA–injected oocytes derived from volume change measurements made over three independent preparations of oocytes.*

Where indicated, the assays were performed in the presence (+) of mercury ions (Hg in 1 mM $HgCl_2$ for 10 min of preincubation). Data are expressed as the mean ± SE, with the number of replicates indicated.

mutant γ-TIP C118T has only 60% of the water channel activity of control γ-TIP, indicating that this conservative amino acid substitution may have sufficiently disturbed the structure of the molecule to slow water flow. A substitution of cysteine by serine or alanine produced inactive γ-TIP channels (data not shown). The δ-TIP C116S mutant (Figure 5B) was functional but only 70% as active as the control δ-TIP. Mercury sensitivity was assessed for both the wild-type channels (see also Figure 2) and the mutants. Mercuric chloride inhibited the activity of γ-TIP by 67% in this experiment (but 95% inhibition is indicated in Figure 2) and inhibited the activity of δ-TIP by 75%.

In the presence of mercuric chloride, the γ-TIP C118T mutant was stimulated to 125% of the wild-type activity and δ-TIP

C116S was inhibited by 33%, considerably less than the 75% value found for control δ-TIP. Thus, the mutants in which cysteine is replaced are either not inhibited by mercury (γ-TIP) or are much less sensitive to mercury (δ-TIP). Variability in the baseline osmotic water permeability of oocytes expressing mutant aquaporins also was observed with mammalian AQP1 by Preston et al. (1993) and Jung et al. (1994b).

δ-TIP Is Localized to the Tonoplast

To be able to perform immunocytochemical studies on δ-TIP, we raised an antiserum against a peptide consisting of the C-terminal 13 amino acids of δ-TIP coupled to BSA. The C terminus of the protein has no amino acid identity with other known proteins or putative proteins derived from known ESTs in Arabidopsis (GenBank release 90.0, EMBL release 43.0, and dbEST release 081795). Fractionation of isolated microsomal, tonoplast, intracellular, and plasma membranes, followed by immunoblotting, showed that the serum detects bands of ~21, 36.5, and 60 kD, which probably correspond to monomeric and multimeric forms of δ-TIP, because MIP proteins easily form nondissociable aggregates, even in the presence of SDS. As seen in Figure 6A, the tonoplast fraction is enriched greatly in δ-TIP compared with the plasma membrane fraction and the intracellular membrane fraction. An immunoblot using antibodies against the 80.8-kD tonoplast pyrophosphatase (Sarafian et al., 1992) showed that the plasma membrane fraction is essentially free of tonoplast contamination (Figure 6B). δ-TIP appears as a mixture of multimers, even when denaturation is carried out at 55°C with 1% β-mercaptoethanol and 1% SDS. This is a common observation with the highly hydrophobic MIP proteins (Daniels et al., 1994; Kammerloher et al., 1994; Qi et al., 1995), and even in the presence of strongly denaturing buffers, such as used by Qi et al. (1995), δ-TIP appears as a dimer or a mixture of dimeric and monomeric forms (data not shown). Preincubation of the δ-TIP antiserum with the δ-TIP peptide antigen resulted in an antiserum that would not bind to Arabidopsis total protein extracts (data not shown), indicating that the antiserum is specific for the amino acid sequence at the δ-TIP C terminus.

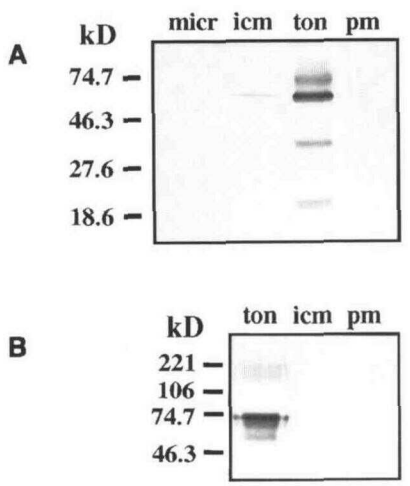

Figure 6. Immunodetection of Membranes from Arabidopsis.

Plasma membrane (pm) and intracellular fractions (icm) were taken from fractions U4 and L4, respectively, of a two-phase separation purification of plasma membranes. These fractions, a tonoplast (ton) sample, and a microsome (micr) sample were fractionated by SDS-PAGE and transferred to nitrocellulose. The positions of marker proteins are indicated at left, with molecular mass in kilodaltons.

(A) The blot was developed with anti–δ-TIP.

(B) The blot was developed with anti–tonoplast pyrophosphatase.

Expression of δ-TIP in Tissues and during Development

To characterize the distribution of δ-*tip* expression and differences in tissue specificity with the γ-TIP protein, the pattern of β-glucuronidase (GUS) expression driven by the δ-*tip* promoter was examined. The 1.1 kb of the δ-*tip* promoter sequence was fused with the coding sequence of GUS (*uidA*), and this construct was introduced into tobacco by Agrobacterium-mediated transformation. Results of the histochemical staining of intact and sectioned δ-*tip*::*uidA* transgenic tobacco tissues are shown in Figure 7. The staining of young seedlings (Figure 7A) shows GUS activity only in the shoot, especially in the cotyledons and hypocotyl, with some staining in the developing true leaves. GUS signal in older leaves was observed to be restricted generally to vascular tissues (Figure 7B). Staining was not affected by sectioning the leaf perpendicular to the leaf axis (see Figure 7B), indicating that the GUS substrate can penetrate intact leaf tissue fully. Strong GUS staining was observed also in stem sections, especially in the phloem (Figures 7C and 7D) and in the flower receptacle, gynoecium, and filament (Figure 7E). No expression was observed in the anther except in the tissue surrounding the vascular bundle at the junction with the filament (Figure 7F). GUS activity was seen in developing seed pods only in the upper portion of the capsule endocarp (Figure 7G). No δ-*tip* promoter–driven gene expression could be detected in primary meristematic tissues (data not shown).

RNA gel blot analysis was performed on total RNA isolated from a number of distinct tissues in Arabidopsis plants during several points in its developmental cycle. Antisense RNA was generated from 3′ untranslated region sequences of δ-*tip*, γ-*tip*, and α-*tip* to obtain gene-specific probes and from the coding sequence of the Arabidopsis 18S rRNA gene, which was used as a gel-loading control. The 1.15-kb δ-*tip* transcript was seen in all tissues, primarily in flower, bolt, and young shoot tissue (Figure 8). The γ-*tip* gene also is expressed in all tissues but is most heavily expressed in flowers, bolt and cauline leaves, and root tissues (Figure 8). The longer 2.0-kb RNA species observed with the δ-*tip*–probed RNA gel blot is likely to be unprocessed or misprimed RNA, because genomic δ-*tip* has at

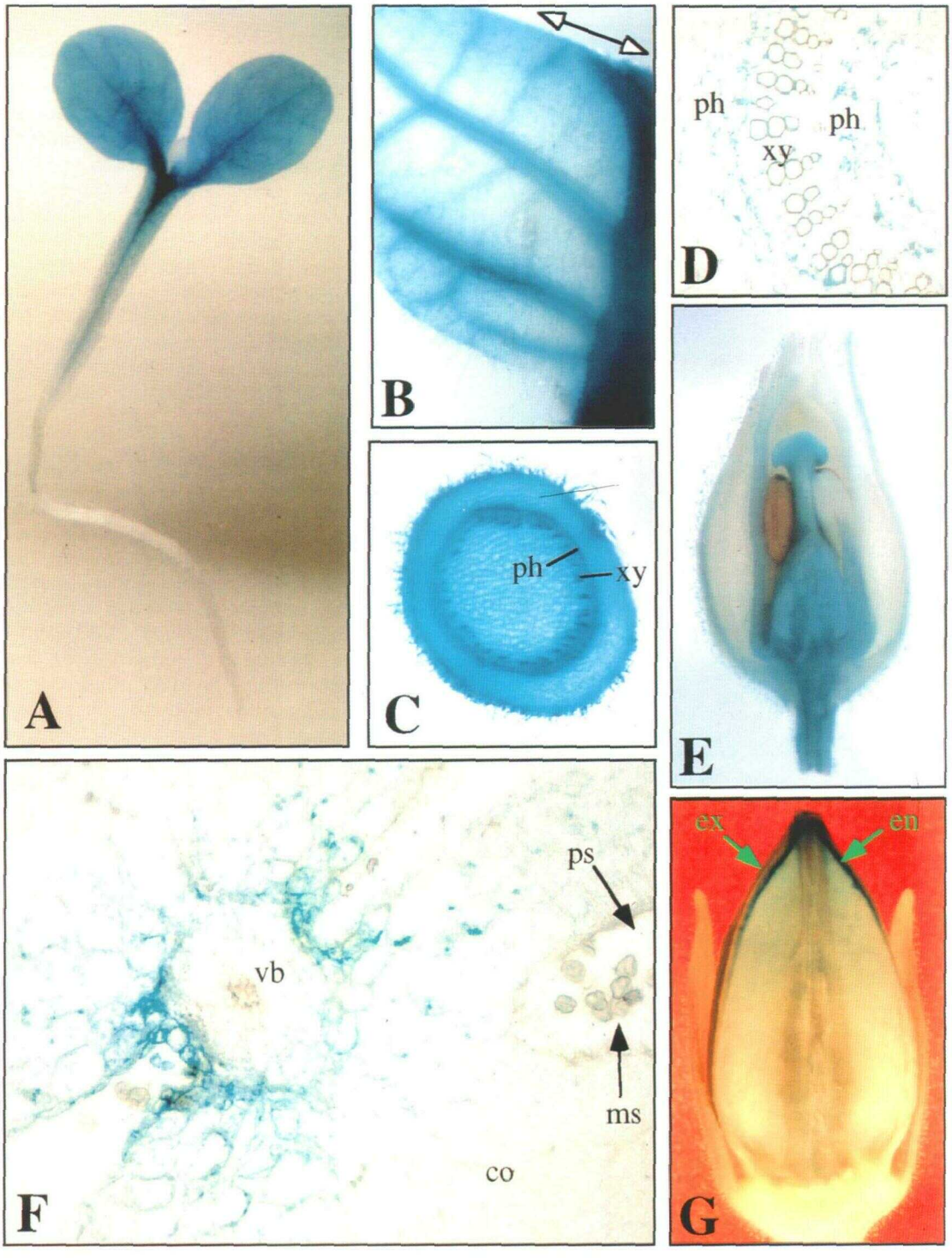

Figure 7. GUS Histochemical Staining of δ-*tip*::*uidA*–Transformed Tobacco Plants.

(A) Young seedling.
(B) Portion of a leaf from a 3-month-old plant, with cut indicated by open arrows.
(C) Hand-cut section of a mature stem. ph, phloem; xy, xylem.
(D) Microtome cross-section of a mature leaf petiole.
(E) Hand-cut section of a flower.
(F) Microtome cross-section of an anther. co, connective; ms, microspores; ps, pollen sac; vb, vascular bundle.
(G) Hand-cut section of an immature seed pod. en, capsule endocarp; ex, capsule exocarp.

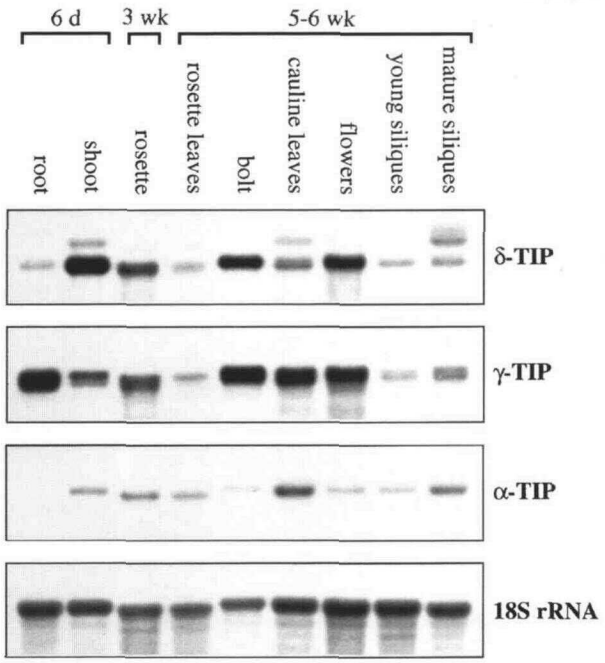

Figure 8. RNA Gel Blot Analysis of Arabidopsis Total RNA Hybridized with Sequence-Specific RNA Probes Synthesized from δ-*tip*, γ-*tip*, α-*tip*, and 18S rRNA.

Each lane contains 10 μg of RNA from the indicated organs. The age of the harvested tissue is noted above. Electrophoresis was carried out such that the direction of RNA migration was toward the bottom. d, day; wk, weeks.

least two small introns and several potential transcriptional start sites. Except for the roots, α-*tip* is expressed in all other tissues. The greatest expression of α-*tip* is in cauline leaves and *mature siliques* (*Figure 8*).

DISCUSSION

δ-TIP Is a New Tonoplast Aquaporin

Our study identifies δ-TIP as an aquaporin of the Arabidopsis tonoplast that is highly expressed in green tissues. Together with the aquaporin γ-TIP (Maurel et al., 1993), δ-TIP was used to define a new domain of the aquaporin polypeptide involved in mercury sensitivity and possibly in the water pore structure itself.

The MIP family of proteins includes members found in a wide variety of organisms, from bacteria to animals and higher plants (Chrispeels and Agre, 1994). They are predicted to form transmembrane proteins with channel-like properties, and many are involved in the movement of water and small solutes across a membrane (Chepelinsky, 1994). The physiological role of the MIP proteins and aquaporins in higher plants has yet to be resolved, but there are indications that they may have functions in cell expansion (Ludevid et al., 1992), solute transport (Opperman et al., 1994), water uptake (Maggio and Joly, 1995), drought tolerance (Yamaguchi-Shinozaki et al., 1992), and salt tolerance (Yamada et al., 1995).

From hydropathy analysis, MIP proteins are predicted to have six α-helical membrane-spanning domains (Pao et al., 1991; Reizer et al., 1993), and these conclusions appear to be confirmed by proteolytic analysis of AQP1 and mutant AQP1s (Preston et al., 1994). Animal MIP proteins are known to form homotetrameric structures in the membrane, as determined by size-exclusion chromatography (Smith and Agre, 1991), freeze–fracture electron microscopy of proteoliposomes (Verbavatz et al., 1993; Zeidel et al., 1994), scanning transmission electron microscopy (Walz et al., 1994), and electron diffraction (Jap and Li, 1995). Radiation inactivation has shown that the AQP1 functional unit has a molecular mass of ~30 kD (van Hoek et al., 1992), which corresponds to the size of the AQP1 monomer, and coexpression in Xenopus oocytes of wild-type and mercury-insensitive AQP1 produces oocytes with partially mercury-sensitive water channel activity (Preston et al., 1993). These results indicate that in the aquaporin tetramer, each subunit exists as an independently functioning aquaporin. In addition, work with dimeric AQP1 mutant constructs suggests that the aquaporin subunits need to form multimers to function (Jung et al., 1994b). Based on these results, Jung et al. (1994b) have proposed an hourglass model in which the aquaporin protein takes an hourglass-like form in the membrane, with the hairpin turns of both conserved Asn-Pro-Ala motifs in each aquaporin coming together to form a nonpolar size-exclusion pore surrounded by the six α-helices.

MIP proteins have been characterized as aquaporins either through the incorporation of purified or in vitro–translated protein into artificial lipid bilayers, or by their expression in Xenopus oocytes (reviewed in Chrispeels and Agre, 1994). It should not be concluded summarily that the water channel activity observed in these artificial systems is a true reflection of MIP protein function in plants. However, recent work validates these systems for use in the characterization of plant aquaporins. Maggio and Joly (1995) have shown that in tomato roots exposed to mercuric chloride, water flow quickly decreases without any effect on the movement of potassium ions. This result is presented as evidence for the existence of a water-selective, proteinaceous route of transcellular water flow in plants. Also, in transgenic Arabidopsis plants expressing antisense *AthH2*, which encodes a MIP protein family member and possible plasma membrane aquaporin, Kaldenhoff et al. (1995) have found that leaf protoplasts swell under hypoosmotic conditions at a much slower rate than do protoplasts from wild-type plants. Expression of the antisense construct is most likely preventing or reducing the production of AthH2 protein from sense mRNA and, if *AthH2* encodes an aquaporin, therefore reduces the protoplast transmembrane water flow.

Analysis of the water transport activity resulting from δ-TIP in Xenopus oocytes shows it to be an aquaporin comparable to γ-TIP, which is also a TIP (Maurel et al., 1993). δ-TIP is specific for water molecules and does not transport glycerol at a significantly greater rate than water-injected oocytes. Both aquaporins are inhibited by mercury sulfhydryl reagents, but the site responsible for this sensitivity is not known, because near the putative pore-forming NPA residues there is no cysteine residue. Mammalian aquaporins also lack this residue.

δ-TIP Has a Unique Mercury-Sensitive Site Not Found in Other Aquaporins

To determine the mercury-sensitive residue, mutation of each cysteine in the TIPs was unnecessary because the γ-TIP protein has only one cysteine at a position conserved in δ-TIP. Replacement of the amino acid at this location with a non–mercury-reactive substitute resulted in a mutant γ-TIP or δ-TIP aquaporin that retained its water channel activity and had a changed mercury sensitivity. The baseline value and the percentage of inhibition obtained with 1 mM $HgCl_2$ with the γ-TIP C118T mutant were variable, even though many oocytes were used for each experiment. Similar results were obtained by Preston et al. (1993) and Jung et al. (1994b), who found that the baseline water channeling activity of an AQP1 C189S mutant fluctuated between 17% stimulation and 40% inhibition when compared with control AQP1. We found that γ-TIP C118T was stimulated to 125% of the γ-TIP wild-type activity.

The mode of action of mercury sensitivity is thought to be a blockage of the aquaporin pore by the large mercury ion bound to a cysteine proximal to the pore opening (Preston et al., 1993). Our results therefore indicate that Cys-118 of γ-TIP (Figure 4B) and Cys-116 of δ-TIP (Figure 4A) either are involved in forming the pore or are very near, even though their location is some distance from either NPA sequence in the conserved motifs of the amino acid chain. The aquaporin may be folded so that these residues are positioned around the pore. A helical wheel plot of this region in both δ-TIP and γ-TIP shows that it is capable of forming an amphipathic helical structure, with cysteine in the hydrophilic face. This plot indicates that the cysteine faces the aqueous environment, and being within a transmembrane sequence, this residue could be part of the pore-forming structure.

An illustration of our prediction, in the context of the hourglass model of Jung et al. (1994b), is presented in Figure 9. This model predicts that cysteine residues in the amphipathic transmembrane helix 3 that do not face the pore would have no effect on mercury sensitivity. The mercury-insensitive RD28 aquaporin has a cysteine two amino acids to the N-terminal side of δ-TIP C-116 (see Figure 1), but this residue would be situated in a hydrophobic, nonaqueous region of the amphipathic helix. That RD28 is a mercury-insensitive water channel (Daniels et al., 1994) is then additional support for our model.

We predict that the region of δ-TIP and γ-TIP forming an amphipathic helix (Figure 9, helix 3) does not coincide entirely

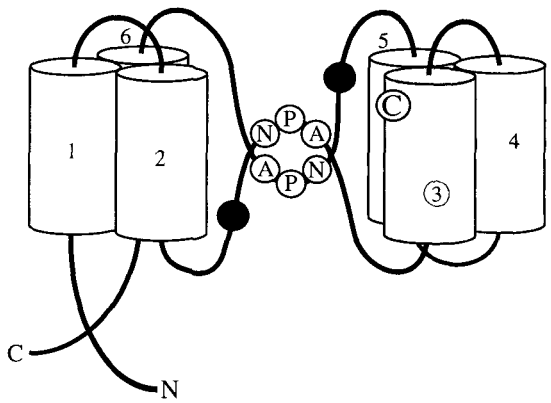

Figure 9. Predicted Location of δ-TIP Cys-116 and γ-TIP Cys-118 in the Hourglass Model.

The six putative transmembrane domains are numbered from the N-terminal end. The two Asn-Pro-Ala signature sequences are indicated in single-letter amino acid code, as is the cysteine residue conserved in δ-TIP and γ-TIP. The darkened circles indicate residues previously implicated in aquaporin mercury sensitivity.

with the transmembrane domain previously predicted for γ-TIP or other MIP proteins (Höfte et al., 1992; Reizer et al., 1993). As discussed by Agre et al. (1995), algorithm-based predictions of MIP aquaporin topology do not agree well with experimental evidence. The membrane structure of these proteins still is unresolved, and we hope our results will help to clarify this matter. Recently, Jap and Li (1995) have predicted from their electron diffraction analysis of AQP1 that the aquaporin protein is made up of seven transmembrane α-helices. This prediction contradicts the hourglass model of Jung et al. (1994b) but provides an interesting alternative model to test.

In protein gel electrophoresis, the δ-TIP protein forms multimers that we could not dissociate with reducing agents. Plant MIP proteins are known to form aggregates (Höfte et al., 1992; Daniels et al., 1994; Kammerloher et al., 1994), and it is not clear whether they aggregate only with themselves or with other hydrophobic proteins. Qi et al. (1995) obtained mostly monomers of a beet plasma membrane MIP by the use of 100 mM DTT, but we did not obtain the same result with δ-TIP (data not shown). The aggregates may form in vivo or as an artifact of the isolation procedure. Generally speaking, a large proportion of γ-TIP formed monomers, whereas most of the δ-TIP formed aggregates. Nevertheless, control experiments in which the serum was depleted of its specific IgG molecules with the C-terminal portion of δ-TIP used as the immunogen allowed the conclusion that all of these bands represent δ-TIP cross-reactivity.

The propensity of plant MIP proteins to migrate as multimeric forms during gel electrophoresis may be an indication that they exist as multimers in the plant membrane. Mammalian MIP family aquaporins are known to form homotetramers (re-

viewed in Engel et al., 1994); therefore, it is likely that plant MIP aquaporins also will form multimeric structures in vivo. It will be difficult to gain a better understanding of plant aquaporin structure and function until we know its arrangement in the membrane.

δ-TIP Is Expressed Primarily in the Shoot and Reproductive Organs

The pattern of δ-*tip* expression observed in promoter–GUS gene fusion-transformed plants matches that determined by RNA gel blot analysis. Therefore, it appears we have isolated a functionally complete promoter. Expression of δ-*tip* seems to correlate with regions of developing vascular tissue and is limited to aboveground organs. The γ-TIP aquaporin may be involved in cell expansion in regions of rapid cell growth (Ludevid et al., 1992). From our results, we propose that δ-TIP may function to generate water flow in developing vascular cells and maintain cellular water permeability in mature vascular tissues. δ-TIP is primarily a shoot aquaporin, whereas γ-TIP is primarily but not exclusively a root aquaporin.

In some tissues, δ-TIP and γ-TIP are present together, although it is unknown whether both proteins are expressed in the same cells or whether both are present simultaneously in the same cell. However, the possibility arises that the two aquaporins could interact to form heteromultimers. What role, if any, this may serve in plants or other organisms coexpressing MIP proteins in the same cell is a matter of some interest. The formation of heteromeric structures could modulate the activity or specificity of MIP proteins or result in a novel function.

METHODS

Growing Conditions for Arabidopsis Plants

Plants (*Arabidopsis thaliana* ecotype Columbia) were grown from seed under continuous bright illumination at 22 to 24°C after 2 days of vernalization at 4°C. Plants were grown in 4-inch pots filled with Terra-Lite Redi-Earth (W.R. Grace and Co., Ajax, Ontario, Canada) and subirrigated with 0.05% Peter's Peat-Lite fertilizer (J.M. McConkey, Sumner, WA).

Isolation and Sequencing of the δ-*tip* cDNA Clone

An unamplified λYES (Elledge et al., 1991) library of Arabidopsis seedling cDNA was screened by using the tonoplast intrinsic protein β-*tip* cDNA fragment (Höfte et al., 1992) as a probe. λYES plaque fields were grown and screened according to Sambrook et al. (1989). Three positive plaques were isolated after two screenings and automatically subcloned into plasmid form as described by Elledge et al. (1991). Following subcloning, both DNA strands of the insert cDNA were sequenced using the Sequenase 2.0 system (United States Biochemical Corp.) and a primer walking strategy. One partial cDNA clone was found to have homology with the major intrinsic protein (MIP) family. A full-length clone was obtained from the French CNRS Arabidopsis expressed sequence tag (EST) project (Höfte et al., 1993) and sequenced as given above. The GenBank accession number for the δ-*tip* cDNA is U39485.

Genomic Library Construction and Sequencing

Genomic DNA was isolated from seedling tissue of Arabidopsis by the procedure of Rogers and Bendich (1988) and partially digested with Sau3AI. The fragments were ligated into λFIXII (Stratagene Cloning Systems, La Jolla, CA) and packaged according to the manufacturer's instructions. The library was screened with a random primer–generated ^{32}P-labeled probe prepared from a fragment of the full-length δ-*tip* cDNA, from the 5′ end multiple cloning site BamHI to the δ-*tip* internal BamHI. Three hybridizing plaques were identified and plaque purified (Sambrook et al., 1989). The clones were digested with BamHI, and the restriction fragments were electrophoretically separated on an agarose gel and transferred to nitrocellulose. The blot was hybridized with the previously mentioned δ-*tip* BamHI-BamHI fragment probe, and a hybridizing 4.5-kb BamHI fragment was identified and subcloned. This clone was sequenced using a primer walking strategy starting with oligonucleotides within the δ-*tip* coding region. We determined 1.2 kb of sequence upstream of the δ-*tip* ATG initiation codon and 1 kb of sequence downstream of the initiation codon. The GenBank accession number for the genomic δ-*tip* fragment is U39486.

DNA Sequence Analysis

Sequence data were compiled and analyzed using MacVector (IBI, New Haven, CT). Aquaporin protein sequence alignment, amino acid similarities, and consensus sequences were determined by the Lineup and Pretty programs of the Genetics Computer Group (Madison, WI) sequence analysis package (Smith, 1988), with adjustments made by eye when appropriate. Membrane topology was predicted using the PepPlot, PlotStructure, and HelicalWheel programs of the Genetics Computer Group sequence analysis package.

β-Glucuronidase Fusion Construction

Sequences in the promoter region of the δ-*tip* gene between 4 and 1125 bp upstream of the initiator ATG were amplified by the polymerase chain reaction (PCR), using the following oligonucleotide primers: forward primer, 5′-GGATTCATCAGTAAGCTTTAAAATCGTAC-3′, which created a HindIII 1131 bp upstream of the initiator ATG; and reverse primer, 5′-CCAGCCATTGTTCTAGATGAAGAAGG-3′, which created an XbaI site 4 bp upstream of the initiator ATG. PCR was performed with Vent DNA polymerase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Then the 1.1-kb PCR product was transformed into *Agrobacterium tumefaciens* LBA4404 and used to transform *Nicotiana tabacum* cv Xanthi.

Plasmid Constructions and in Vitro RNA Synthesis

cDNA encoding δ-TIP was cloned into the BglII site of a pSP64T-derived BlueScript vector carrying 5′ and 3′ untranslated sequences of a β-globin gene from Xenopus (Preston et al., 1992). Cloning of γ-*tip* into the pSP64T-derived BlueScriptII vector was described previously (Maurel

et al., 1993). The cloned δ-*tip* was a NotI-NotI fragment containing the entire δ-*tip* coding sequence plus 42 and 178 bp at the 5′ and 3′ termini, respectively, with the NotI ends filled in and ligated to the filled-in ends of the BglII cut vector. Clones with insert in the correct orientation were determined by restriction mapping and subsequent sequencing of portions of the insert. Capped complementary RNA (cRNA) encoding δ-TIP and γ-TIP were synthesized in vitro by using T3 RNA polymerase and purified as described by Preston et al. (1992).

Site-Directed Mutagenesis to Create a Mercury-Insensitive δ-TIP and γ-TIP

The pSP64T-derived BlueScriptII vector containing either the δ-*tip* or γ-*tip* coding sequence was used for site-directed mutagenesis by recombinant PCR as described by Higuchi (1990). To create the δ-TIP mutant C116S, the sense strand mutagenic oligonucleotide 5′-CACCGCCGCTAGTTTCCTCCTT-3′ was used to change the cysteine codon TGT to the serine codon AGT. To create the γ-TIP mutant C118T, the sense strand mutagenic oligonucleotide 5′-CGTCGTCGCTACCCTCATCCTTAAAT-3′ was used; it changed the cysteine codon TGC to the threonine codon ACC. The sense oligonucleotide was used in combination with an oligonucleotide complementary to the T7 promoter in the pSP64T-derived BlueScript vector, whereas the antisense oligonucleotide was used in combination with an oligonucleotide complementary to the T3 promoter in the pSP64T-derived BlueScript vector. The products of these two separate PCRs were gel purified and used as templates for a secondary PCR by using only the T7 and T3 primers. The resulting product was digested with HindIII and BamHI and cloned into the corresponding sites of pBS (Stratagene). The resulting clones were partially sequenced to verify that the desired mutation had been introduced.

Oocyte Preparation and Injection

Fully grown oocytes (stage V and VI) were isolated from *Xenopus laevis* and incubated in Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes-NaOH, 0.33 mM $Ca[NO_3]_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, pH 7.4) supplemented with gentamycin sulfate to 50 μg/mL at 18°C as described by Cao et al. (1992). In vitro transcripts (1 mg/mL) or nuclease-free water were injected in a volume of 50 nL.

Osmotic Water Permeability Assay

Oocytes were transferred 3 days after cRNA injection from Barth's solution (200 mosmol) at room temperature to the same solution diluted to 40 mosmol with distilled water. Changes in cell volume were followed with a microscope by taking photographs at 20- to 120-sec intervals. Oocyte diameters were measured four times along two sets of perpendicular axes. The volume (V) was estimated as the mean of two ellipsoid volumes. The osmotic permeability coefficient (P_f) was calculated from $P_f = V_o[d(V/V_o)/dt]/[S \times V_w(Osm_{in} - Osm_{out})]$, where the initial oocyte volume $V_o = 9 \times 10^{-4}$ cm^3, initial oocyte surface area $S = 0.045$ cm^2, and molar volume of water $V_w = 18$ cm^3/mol (Zhang and Verkman, 1991).

Antisera

A peptide representing the C terminus of the predicted amino acid sequence of δ-*tip* was chemically synthesized and coupled to BSA with glutaraldehyde, following the method of Harlow and Lane (1988). The sequence of the synthetic peptide is H_2N-KSSEHVPLASADF-COOH. The coupled peptide was injected into a New Zealand White rabbit, and the peptide was boosted at 4-week intervals. After five boosts, we obtained a highly specific serum that was used at a 1:500 dilution for the preparation of immunoblots. The antiserum to vacuolar pyrophosphatase was provided by P.A. Rea (University of Pennsylvania, Philadelphia, PA).

Preparation of Protein and Membrane Fractions

For determining membrane localization, plasma membrane, intracellular membrane, and microsome fractions were prepared as described by Daniels et al. (1994) by using the two-phase partition method of Kjellbom and Larsson (1984). Tonoplast membranes were isolated following the methods of Höfte et al. (1992) by purifying protoplasts, followed by osmotic shock lysis to release and isolate intact vacuoles.

Immunodetection

For immunoblotting, appropriate quantities of protein were fractionated by SDS-PAGE and transferred to nitrocellulose, and the proteins were detected using the rabbit anti-δ-TIP antiserum. Goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad) was used as the secondary antibody. Extracts were incubated in denaturing buffer (with 1% β-mercaptoethanol) for 10 min at 55°C before loading the gel.

RNA Isolation and RNA Gel Blot Analysis

Arabidopsis total RNA was isolated either from fresh plant tissue by using the Quiagen tips method (protocols QGVP 4113, 1992; Quiagen Inc., Chatsworth, CA) or from frozen tissue, as described previously (de Vries et al., 1988). Total RNA was separated on Hepes–formaldehyde, 1.5% agarose gels, following the protocol of Tsang et al. (1993), and transferred to Hybond-N nylon membranes (Amersham), according to the methods of Sambrook et al. (1989). The RNA was bound to the membrane with UV illumination and hybridized to δ-*tip*, γ-*tip*, α-*tip*, or 18S rRNA antisense RNA probes in hybridization buffer with 50% formamide at 45°C. Hybridized membranes were washed with high stringency (0.2 × SSPE [1 × SSPE is 150 mM NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.4], 0.5% SDS at 65 to 75°C for 45 min). The membranes were then exposed to x-ray film with intensifying screens at −70°C. Between hybridizations, the membranes were stripped of probe as recommended by Sambrook et al. (1989) and exposed to x-ray film to verify that the probe was removed entirely.

Probes were generated by RNA polymerase run-off transcription of the desired gene fragment in the presence of ^{32}P-radiolabeled UTP. The δ-*tip* probe was generated by linearizing the cDNA clone with XmnI and transcribing with T3 RNA polymerase, resulting in a probe hybridizing to 25 and 163 bp of coding sequence and 3′ untranslated region sequence, respectively. The γ-*tip* probe was generated by linearizing the plasmid pgt-1 (Höfte et al., 1992) with AseI and transcribing with T7 RNA polymerase, which resulted in a probe hybridizing to 188 bp of 3′ untranslated region sequence. The α-*tip* probe was generated by linearizing an α-*tip* (Höfte et al., 1992) cDNA clone with BstXI and transcribing with T7 RNA polymerase, which generated a probe hybridizing to 47 bp of coding sequence and 181 bp of 3′ untranslated region sequence. The 18S rRNA control probe was generated from the Arabidopsis Biological Resource Stock Center (Ohio State University, Columbus, OH) DNA stock 88K22T7 by linearizing the EST clone

with KpnI and transcribing with SP6 RNA polymerase, producing a probe hybridizing to ~1200 bp of the coding sequence (Unfried et al., 1989).

Histochemical β-GUS Staining and Image Processing

Histochemical staining of β-glucuronidase (GUS) activity was performed on seedlings or hand-cut sections at 37°C in 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid, 50 mM $NaPO_4$, pH 7.0, 0.1% β-mercaptoethanol, 0.1% Triton X-100, 0.02% NaN_3 after 5 min of vacuum infiltration. To eliminate background GUS activity in anthers, the staining solution of flower organs was adjusted to pH 8.0 (van der Meer et al., 1992). Untransformed tobacco tissue was used as a negative control. After 6 to 16 hr of incubation, the GUS substrate solution was removed and chlorophyll was solubilized in ethanol. For sectioning, stained tissue was fixed and dehydrated by freeze substitution with ethanol (Meyerowitz, 1987), cleared with xylene, and embedded in paraffin (Paraplast; Oxford Labware, St. Louis, MO).

Slide photos taken of the GUS-stained tissue were digitized using a slide scanner (model CoolScan; Nikon Inc., Melville, NY). Brightness, contrast, and color balance were adjusted using Adobe Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA). Blots were digitized on a flatbed scanner (model Reli 4830T; Relisys, Milpitas, CA), and background was subtracted using Photoshop 3.0 (Adobe). Composite figures were prepared in Canvas 3.5 (Deneba Software, Miami, FL) and printed using a dye-sublimation color printer (model Phaser IIsdx; Tektronix Inc., Wilsonville, OR).

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REFERENCES

Agre, P., Brown, D., and Nielsen, S. (1995). Aquaporin water channels: Unanswered questions and unresolved controversies. Curr. Opin. Cell Biol. **7**, 472–483.

Cao, Y., Anderova, M., Crawford, N.M., and Schroeder, J.I. (1992). Expression of an outward-rectifying potassium channel from maize mRNA and complementary RNA in Xenopus oocytes. Plant Cell **4**, 961–969.

Chepelinsky, A.B. (1994). The MIP transmembrane channel gene family. In Handbook of Membrane Channels, C. Peracchia, ed (San Diego, CA: Academic Press), pp. 413–432.

Chrispeels, M.J., and Agre, P. (1994). Aquaporins: Water channel proteins of plant and animal cells. Trends Biol. Sci. **19**, 421–425.

Chrispeels, M.J., and Maurel, C. (1994). Aquaporins: The molecular basis of facilitated water movement through living plant cells. Plant Physiol. **105**, 9–15.

Daniels, M.J., Mirkov, T.E., and Chrispeels, M.J. (1994). The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is a homolog of the tonoplast water channel protein TIP. Plant Physiol. **106**, 1325–1333.

de Vries, S., Hoge, H., and Bisseling, T. (1988). Isolation of total and polysomal RNA from plant tissues. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 1–13.

Echevarria, M., Windhager, E., Tate, S., and Frindt, G. (1994). Cloning and expression of AQP3, a range water channel from the medullary collecting duct of rat kidney. Proc. Natl. Acad. Sci. USA **91**, 10997–11001.

Elledge, S., Mulligan, J.T., Ramer, S.W., Spottswood, M., and Davis, R.W. (1991). λYES: A multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc. Natl. Acad. Sci. USA **88**, 1731–1735.

Engel, A., Walz, T., and Agre, P. (1994). The aquaporin family of membrane water channels. Curr. Opin. Struct. Biol. **4**, 545–553.

Farinas, J., van Hoek, A., Shi, L.-B., Erickson, C., and Verkman, A. (1993). Nonpolar environment of tryptophans in erythrocyte water channel CHIP28 determined by fluorescence quenching. Biochemistry **32**, 11857–11864.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Hasegawa, H., Ma, T., Skach, W., Matthay, M.A., and Verkman, A.S. (1994). Molecular cloning of a mercurial-insensitive water channel expressed in selected water-transporting tissues. J. Biol. Chem. **269**, 5497–5500.

Higuchi, R. (1990). Recombinant PCR. In PCR Protocols: A Guide to Methods and Applications, M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds (San Diego, CA: Academic Press), pp. 177–183.

Höfte, H., Hubbard, L., Reizer, J., Ludevid, D., Herman, E.M., and Chrispeels, M.J. (1992). Vegetative and seed-specific isoforms of a putative solute transporter in the tonoplast of *Arabidopsis thaliana*. Plant Physiol. **99**, 561–570.

Höfte, H., Desprez, T., Amselem, J., Chiapello, H., Caboche, M., Moisan, A., Jourjon, M.-F., Charpenteau, J.-L., Berthomieu, P., Guerrier, D., Giraudat, J., Quigley, F., Thomas, F., Fu, D.Y., Mache, R., Raynal, M., Cooke, R., Grellet, F., Delseny, M., Parmentier, Y., Demarcillac, G., Gigot, C., Fleck, J., Philipps, G., Axelos, M., Bardet, C., Tremousaygue, D., and Lescure, B. (1993). An inventory of 1152 expressed sequence tags obtained by partial sequencing of cDNAs from *Arabidopsis thaliana*. Plant J. **4**, 1051–1061.

Ishibashi, K., Sasaki, S., Fushimi, K., Uchida, S., Kuwahara, M., Saito, H., Furukawa, T., Nakajima, K., Yamaguchi, Y., Gojobori, T., and Marumo, F. (1994). Molecular cloning and expression of a member of the aquaporin family with permeability to glycerol and urea in addition to water expressed at the basolateral membrane of kidney collecting duct cells. Proc. Natl. Acad. Sci. USA **91**, 6269–6273.

Jap, B., and Li, H. (1995). Structure of the osmo-regulated H_2O-channel, AQP-CHIP, in projection at 3.5Å resolution. J. Mol. Biol. **251,** 413–420.

Jung, J.S., Bhat, R.V., Preston, G.M., Guggino, W.B., Baraban, J., and Agre, P. (1994a). Molecular characterization of an aquaporin cDNA from brain—candidate osmoreceptor and regulator of water balance. Proc. Natl. Acad. Sci. USA **91,** 13052–13056.

Jung, J.S., Preston, G.M., Smith, B., Guggino, W.B., and Agre, P. (1994b). Molecular structure of the water channel through aquaporin CHIP: The tetrameric-hourglass model. J. Biol. Chem. **269,** 14648–14654.

Kaldenhoff, R., Kölling, A., Meyers, J., Karmann, U., Ruppel, G., and Richter, G. (1995). The blue light–responsive *AthH2* gene of *Arabidopsis thaliana* is primarily expressed in expanding as well as in differentiating cells and encodes a putative channel protein of the plasmalemma. Plant J. **7,** 87–95.

Kammerloher, W., Fischer, U., Piechottka, G.P., and Schaffner, A.R. (1994). Water channels in the plant plasma membrane cloned by immunoselection from a mammalian expression system. Plant J. **6,** 187–199.

Kjellbom, P., and Larsson, C. (1984). Preparation and polypeptide composition of chlorophyll-free plasma membranes from leaves of light-grown spinach and barley. Physiol. Plant. **62,** 501–509.

Ludevid, D., Höfte, H., Himelblau, E., and Chrispeels, M.J. (1992). The expression pattern of the tonoplast intrinsic protein γ-TIP in *Arabidopsis thaliana* is correlated with cell enlargement. Plant Physiol. **100,** 1633–1639.

Ma, T., Frigeri, A., Hasegawa, H., and Verkman, A. (1994). Cloning of a water channel homolog expressed in brain meningeal cells and kidney collecting duct that functions as a stilbene-sensitive glycerol transporter. J. Biol. Chem. **269,** 21845–21849.

Macey, R.I. (1984). Transport of water and urea in red blood cells. Am. J. Physiol. **246,** C195–C203.

Maggio, A., and Joly, R.J. (1995). Effects of mercuric chloride on the hydraulic conductivity of tomato root systems: Evidence for a channel-mediated water pathway. Plant Physiol. **109,** 331–335.

Maurel, C., Reizer, J., Schroeder, J.I., and Chrispeels, M.J. (1993). The vacuolar membrane protein γ-TIP creates water specific channels in *Xenopus* oocytes. EMBO J. **12,** 2241–2247.

Maurel, C., Reizer, J., Schroeder, J.I., Chrispeels, M.J., and Saier, M.H., Jr. (1994). Functional characterization of the *Escherichia coli* glycerol facilitator GlpF in *Xenopus* oocytes. J. Biol. Chem. **269,** 11869–11872.

Maurel, C., Kado, R.T., Guern, J., and Chrispeels, M.J. (1995). Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. EMBO J. **14,** 3028–3035.

Meyerowitz, E. (1987). Experimental protocols: *In situ* hybridization to RNA in plant tissue. Plant Mol. Biol. Rep. **5,** 242–250.

Mulders, S.M., Preston, G.M., Deen, P.M.T., Guggino, W.B., Van Os, C.H., and Agre, P. (1995). Water channel properties of major intrinsic protein of lens. J. Biol. Chem. **270,** 9010–9016.

Opperman, C.H., Taylor, C.G., and Conkling, M.A. (1994). Root-knot nematode-directed expression of a plant root-specific gene. Science **263,** 221–223.

Pao, G.M., Wu, L.-F., Johnson, K.D., Höfte, H., Chrispeels, M.J., Sweet, G., Sandal, N.N., and Saier, M.H., Jr. (1991). Evolution of the MIP family of integral membrane transport proteins. Mol. Microbiol. **5,** 33–37.

Pratz, J., Ripoche, P., and Corman, B. (1986). Evidence for proteic water pathways in the luminal membrane of kidney proximal tubule. Biochem. Biophys. Acta **856,** 259–266.

Preston, G.M., Carroll, T.P., Guggino, W.B., and Agre, P. (1992). Appearance of water channels in *Xenopus* oöcytes expressing red cell CHIP28 protein. Science **256,** 385–387.

Preston, G.M., Jung, J.S., Guggino, W.B., and Agre, P. (1993). The mercury-sensitive residue at cysteine 189 in the CHIP28 water channel. J. Biol. Chem. **268,** 17–20.

Preston, G.M., Jung, J.S., Guggino, W.B., and Agre, P. (1994). Membrane topology of Aquaporin CHIP. J. Biol. Chem. **269,** 1668–1673.

Qi, X., Tai, C.-Y., and Wasserman, B. (1995). Plasma membrane intrinsic proteins of *Beta vulgaris* L. Plant Physiol. **108,** 387–392.

Reizer, J., Reizer, A., and Saier, M.H., Jr. (1993). The MIP family of integral membrane channel proteins: Sequence comparisons, evolutionary relationships, reconstructed pathway of evolution, and proposed functional differentiation of the two repeated halves of the proteins. Crit. Rev. Biochem. Mol. Biol. **28,** 235–257.

Rogers, S., and Bendich, A. (1988). Extraction of DNA from plant tissues. In Plant Molecular Biology Manual, S. Gelvin, R. Schilperoort, and D.P.S. Verma, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. A6/1–A6/10.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sarafian, V., Kim, Y.C., Poole, R.J., and Rea, P.A. (1992). Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **89,** 1775–1779.

Smith, B.L., and Agre, P. (1991). Erythrocyte Mr 28,000 transmembrane protein exists as multisubunit oligomer similar to channel proteins. J. Biol. Chem. **266,** 6407–6415.

Smith, D.W. (1988). A complete, yet flexible system for DNA/protein sequence analysis using VAX/VMS computers. Comput. Appl. Biosci. **4,** 212.

Tsang, S., Yin, X., Guzzo-Arkuran, C., Jones, V., and Davidson, A. (1993). Loss of resolution of gel electrophoresis of RNA: A problem associated with the presence of formaldehyde gradients. BioTechniques **14,** 380–381.

Unfried, I., Stocker, U., and Gruendler, P. (1989). Nucleotide sequence of the 18 rRNA gene from *Arabidopsis thaliana*. Nucleic Acids Res. **17,** 7513.

van der Meer, I.M., Stam, M.E., van Tunen, A.J., Mol, J.N.M., and Stuitje, A.R. (1992). Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell **4,** 253–262.

van Hoek, A.N., Luthjens, L., Hom, M., van Os, C., and Dempster, J. (1992). A 30 kDa functional size for the erythrocyte water channel determined *in situ* by radiation inactivation. Biochem. Biophys. Res. Commun. **184,** 1331–1338.

van Hoek, A.N., Wiener, M., Bicknese, S., Miercke, L., Biwersi, J., and Verkman, A.S. (1993). Secondary structure analysis of purified, functional CHIP28 water channels by CD and FTIR spectroscopy. Biochemistry **32,** 11847–11856.

Verbavatz, J.M., Brown, D., Sabolic, I., Valenti, G., Ausiello, D.A., Van Hoek, A.N., Ma, T., and Verkman, A.S. (1993). Tetrameric assembly of CHIP28 water channels in liposomes and cell membranes: A freeze-fracture study. J. Cell Biol. **123,** 605–618.

Walz, T., Smith, B., Agre, P., and Engel, A. (1994). The three-dimensional structure of human erythrocyte aquaporin CHIP. EMBO J. **13,** 2985–2993.

Wayne, R., and Tazawa, M. (1990). Nature of the water channels in the internodal cells of *Nitellopsis*. J. Membr. Biol. **116,** 31–39.

Yamada, S., Katsuhara, M., Kelly, W., Michalowski, C., and Bohnert, H. (1995). A family of transcripts encoding the water channel proteins: Tissue-specific expression in the common ice plant. Plant Cell **7,** 1129–1142.

Yamaguchi-Shinozaki, K., Koizumi, M., Urao, S., and Shinozaki, K. (1992). Molecular cloning and characterization of 9 cDNAs for genes that are responsive to desiccation in *Arabidopsis thaliana*: Sequence analysis of one cDNA clone that encodes a putative transmembrane channel protein. Plant Cell Physiol. **33,** 217–224.

Zeidel, M., Nielsen, S., Smith, B., Ambudkar, S., Maunsbach, A., and Agre, P. (1994). Ultrastructure, pharmacologic inhibition, and transport selectivity of aquaporin channel-forming integral protein in proteoliposomes. Biochemistry **33,** 1606–1615.

Zhang, R., and Verkman, A.S. (1991). Water and urea permeability properties of *Xenopus* oocytes: Expression of mRNA from toad urinary bladder. Am. J. Physiol. **260,** C26–C34.

Zhang, R., van Hoek, A.N., Biwersi, J., and Verkman, A.S. (1993). A point mutation at cysteine 189 blocks the water permeability of rat kidney water channel CHIP28k. Biochemistry **32,** 2938–2941.