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The Expression Pattern of the Tonoplast Intrinsic Protein γ-TIP in *Arabidopsis thaliana* Is Correlated with Cell Enlargement[1]

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ABSTRACT

The vacuolar membrane (tonoplast) contains an abundant intrinsic protein with six membrane-spanning domains that is encoded by a small gene family. Different isoforms of tonoplast intrinsic protein (TIP) are expressed in different tissues or as a result of specific signals. Using promoter-β-glucuronidase (GUS) fusions and in situ hybridization, we have examined the expression of γ-TIP in *Arabidopsis thaliana*. GUS staining of plants transformed with promoter-GUS fusions showed that γ-TIP gene expression is high in recently formed tissues of young roots. In the shoot, γ-TIP gene expression was highest in the vascular bundles of stems and petioles, as well as in the stipules and in the receptacle of the flower. No GUS activity was detected in root or shoot meristems or in older tissues, suggesting temporal control of γ-TIP gene expression associated with cell elongation and/or differentiation. In situ hybridization carried out with whole seedlings confirmed that in root tips, γ-TIP mRNA was present only in the zone of cell elongation just behind the apical meristem. In seedling shoots, mRNA abundance was also found to be correlated with cell expansion. These results indicate that γ-TIP may be expressed primarily at the time when the large central vacuoles are being formed during cell enlargement.

The vacuolar membrane or tonoplast contains an intrinsic protein with six membrane-spanning domains that is encoded by a small gene family. Different members of this family of TIPs[4] are expressed in different organs or as a result of specific signals. For example, α-TIP is specifically expressed in seeds (9, 10), γ-TIP is expressed in vegetative tissues (7, 22, 23), NOD26 is a peribacteroid membrane protein in the root nodules of soybean plants (17), and a water stress-induced TIP appears when plants are allowed to dry out (5). The TIP proteins belong to a superfamily called the MIPs that has members in many other organisms, including bacteria, yeast (20), *Drosophila*, and mammals (see refs. 14 and 15). The function of these membrane proteins has not yet been elucidated, but circumstantial evidence indicates that they are capable of forming channels in the membrane (4) and could be involved in the transport of ions, metabolites, or water. Incorporation of MIP into artificial bilayers results in the formation of voltage-gated channels that are highly selective for anions (4). Ouyang et al. (13) recently suggested that NOD26 mediates malate transport across the peribacteroid membrane of soybean nodules, and that the activity of the protein is mediated by its phosphorylation state. A number of proteins in the MIP family, including seed-specific α-TIP (8), can be phosphorylated, and such phosphorylation could regulate their activity. Of particular interest is a recent report that CHIP 28, an abundant protein in the erythrocyte plasma membrane, is able to greatly increase the osmotic water permeability in the plasma membrane of *Xenopus* oocytes and may constitute the functional unit of a membrane water channel (16).

Starting from the premise that knowing more precisely where a gene is expressed can lead to a better understanding of the function of the gene product, we studied the expression of γ-TIP in greater detail. Transcriptional fusions between the promoters of *Arabidopsis thaliana* γ-TIP and the gene encoding GUS were transformed into *A. thaliana*. GUS activity was assayed histochemically to indicate spatial patterns of γ-TIP gene expression. In addition, we adapted an in situ hybridization method previously used with *Drosophila* embryos for use with intact seedlings, and found that in roots, γ-TIP mRNA is present only in a small zone just behind the apical meristem. We suggest on the basis of our results that in elongating tissues, γ-TIP mRNA accumulates at the time of vacuolation or shortly thereafter and subsides as the cells reach their full size.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis thaliana, ecotype Columbia, was grown with continuous illumination at 25 to 27°C in a soil-peat mixture (J.M. McConkey, Sumner, WA) and subirrigated at 3-d intervals with 0.125% Peter's peat-lite fertilizer (J.M. McConkey). *Raphanus sativus* seeds were obtained from a local supplier and germinated in the light for 2 d on moist filter paper.

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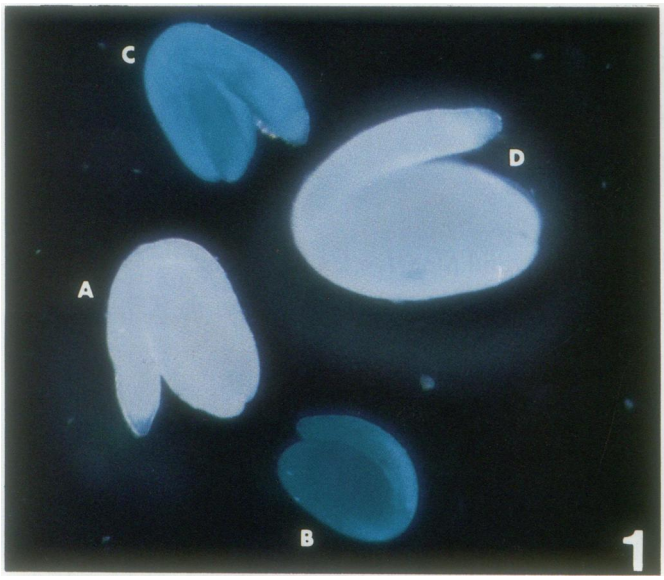
[4] Abbreviations: TIP, tonoplast intrinsic protein; MIP, major intrinsic protein; CaMV, cauliflower mosaic virus; GUS, β-glucuronidase.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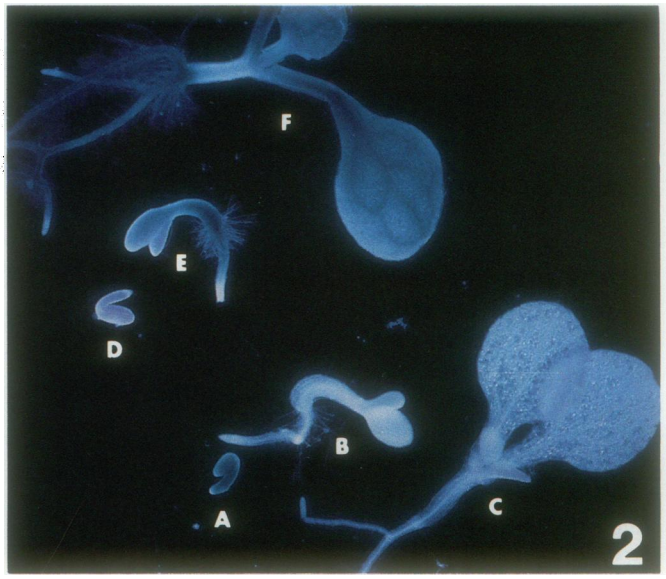
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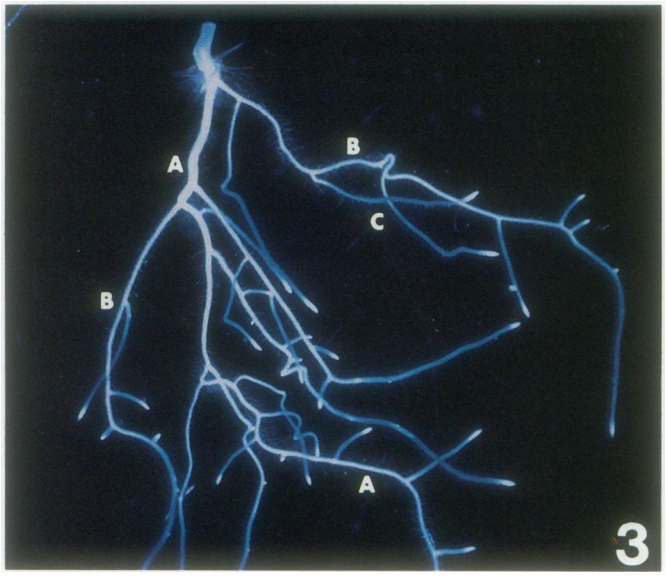
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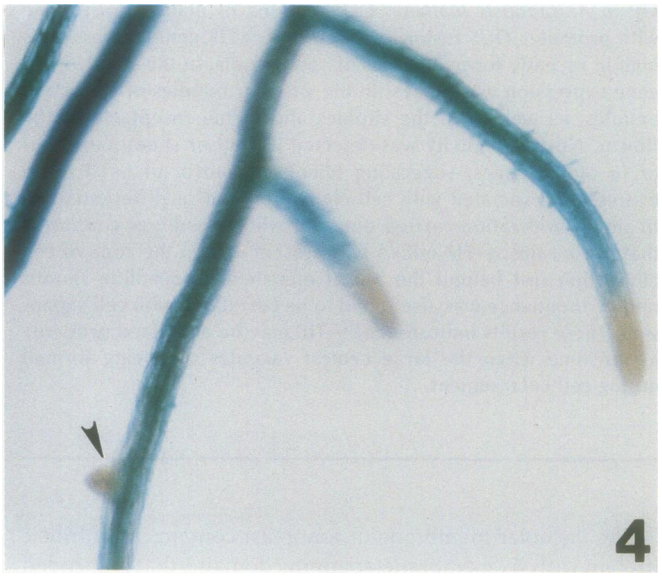
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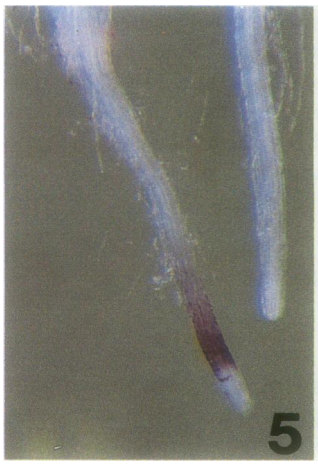
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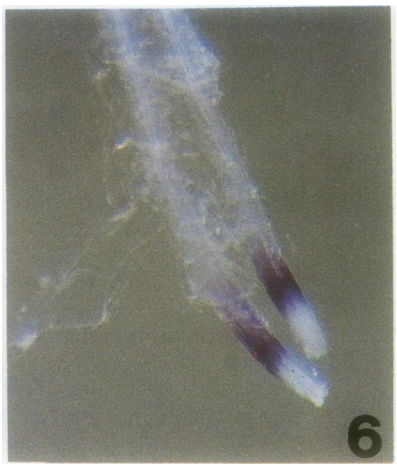
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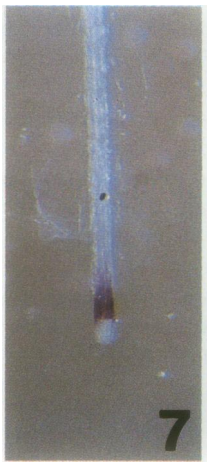
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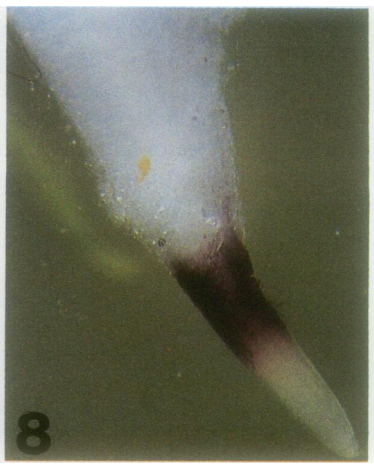
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Plant Transformation

A. thaliana var Columbia root explants were transformed using the recombinant LBA4404 strain by the method of Valvekens et al. (19) and regenerated on medium containing 50 mg/L kanamycin. *A. thaliana* var Columbia seeds transformed with the CaMV 35S-GUS construct were kindly provided by Dr. Nigel Crawford (University of California, San Diego).

Constructs

To construct α-TIP-GUS, the following elements were inserted between the *Eco*RI and the *Hin*dIII site of the polylinker of pDE1001 (3): (a) a 2-kb *Hin*dIII-*Sph*I fragment covering the region upstream of the *A. thaliana* α-TIP gene (7) 5′ to −152 bp from the ATG initiation codon (position 410 in the α-TIP sequence); (b) a 1.87-kb *Sal*I-*Eco*RI fragment from pRAJ275 (Clontech Labs, Inc., Palo Alto, CA) containing the entire GUS coding sequence with modifications around the initiation codon to optimize the translation initiation; (c) a 400-bp fragment containing the 3′ untranslated region of transcript 7 of the right portion (TR) of the T-DNA of an octopine-type Ti-plasmid of *Agrobacterium tumefaciens* (2).

γ-TIP-GUS is identical to α-TIP-GUS except that the α-TIP promoter region was replaced by a 1.8-kb *Hin*dIII fragment of γ-TIP (7) covering the region upstream of −20 bp from the ATG initiation codon (position 339 in the sequence, ref. 7).

The chimeric binary plasmids were transformed into *A. tumefaciens* strain LBA4404 using the procedure described by Höfgen and Wilmitzer (6) and with a selection on Luria Broth medium containing 100 mg/L spectinomycin and 300 mg/L streptomycin.

Histochemical Localization of GUS

Histochemical localization of GUS was performed as follows. Aseptically grown seedlings were submersed in staining solution (1 mM 5-bromo-4-chloro-3-indolyl glucuronide [X-gluc] in 100 mM sodium phosphate buffer [pH 7.0], 10 mM EDTA, 0.5 mM potassium ferrocyanide, and 0.5 M potassium ferricyanide) in an uncapped microfuge tube, placed under vacuum for 2 to 3 min, and incubated at 37°C for 12 to 16 h. The stained tissue was fixed by incubation for 10 min in 5% formaldehyde, 5% acetic acid, and 20% ethanol and cleared of Chl by incubation for 10 min in 10% commercial bleach followed by a rinse in distilled water.

In Situ Hybridization

For whole-mount in situ hybridization, *A. thaliana* and *R. sativus* seedlings were fixed in PBS, 67 mM EGTA, and 6% formaldehyde (Fisher) for 25 min at room temperature. In situ hybridization was performed essentially as described by Tautz and Pfeifle (18) with the following modifications: heptane washes were eliminated, fixed seedlings were stored 2 to 4 d in ethanol, and hybridization was carried out at 55°C using a digoxygenin-labeled RNA probe. Anti-digoxygenin antibody coupled to alkaline phosphatase (Boehringer Mannheim) was diluted 1:10 in PBS containing 0.1% Tween 20 and 2% BSA and preabsorbed to *Arabidopsis* seedlings overnight at room temperature. Seedlings used in the preabsorption were fixed in PBS, 67 mM EGTA, and 3% formaldehyde for 25 min. The final dilution of antidigoxygenin antibody in the posthybridization mixture was 1:2000. Seedlings were incubated with the antibody overnight at 4°C. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt was carried out for 20 to 50 min. A sense probe was used as a control.

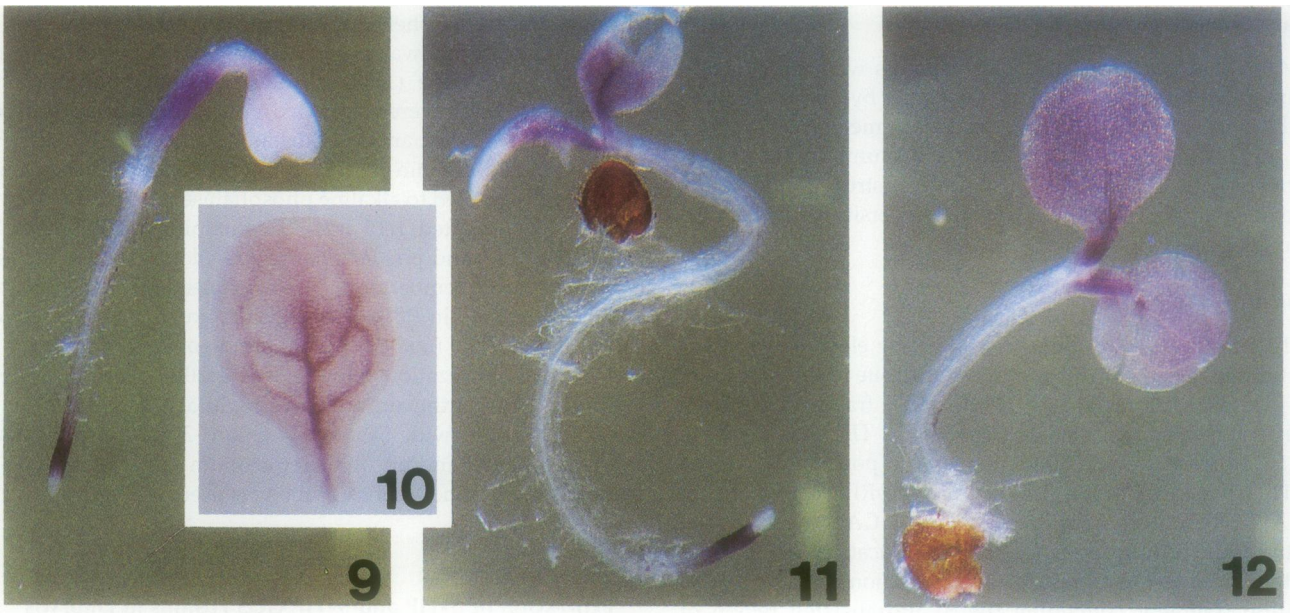
RESULTS

Expression of α-TIP and γ-TIP GUS Fusions in Embryos and Seedlings

Embryos obtained from imbibed mature seeds in which the expression of GUS is driven by different promoters are shown in Figure 1. There was no detectable GUS activity in a control (untransformed) embryo (D) or in an embryo obtained from a plant transformed with the γ-TIP-GUS construct (A). A light blue coloring was obtained with a CaMV 35S-GUS construct (C) and intense blue staining in an embryo obtained from a plant transformed with the α-TIP-GUS construct (B). These results confirm our earlier work showing that α-TIP is expressed during seed development (7).

The expression of α-TIP-GUS and γ-TIP-GUS during the first week of seedling growth is shown in Figure 2. In this figure, A, B, and C are seedlings of 0, 2, and 8 d of age and are transformed with the α-TIP-GUS construct. The GUS activity present initially in a mature embryo (A), as a result of the expression of the α-TIP promoter during embryogeny, rapidly decays (B), and there is no accumulation of GUS activity later (C). In Figure 2, D, E, and F are seedlings of 0, 2, and 8 d of age, respectively, in which GUS expression is driven by the γ-TIP promoter. At 2 d, GUS activity is highest in the hypocotyl, with low activity in the cotyledons and none in the root tip. By 8 d, there is no longer any expression of γ-TIP-GUS in the young stem (hypocotyl), with intense staining in the roots and the expanded cotyledons. In cotyledons, GUS activity appears highest in the vascular bundles, with much lower levels in the mesophyll. These staining patterns suggest that the γ-TIP-GUS construct reflects the expression pattern of the γ-TIP gene, as our previous work

Figures 1–8. Expression of promoter-GUS fusions in transformed *A. thaliana* using α-TIP and γ-TIP promoters. Figure 1, Imbibed embryos obtained from seeds of plants transformed with γ-TIP-GUS (A), α-TIP-GUS (B), or CaMV 35S-GUS (C) and from the untransformed control (D). Figure 2, Embryos and seedlings of plants transformed with α-TIP-GUS (A, B, C) and γ-TIP-GUS (D, E, F) at 0 d (A, D), 2 d (B, E), and 8 d (C, F) of seedling growth; Figures 3 and 4, Root system of a 2-week-old plant transformed with a γ-TIP-GUS fusion; A, primary root; B, GUS-negative portion of secondary root; C, GUS-positive portion of secondary root; arrowhead, unstained lateral root meristem. Figures 5–8, Localization of γTIP mRNA in roots by in situ hybridization with antisense mRNA. Figure 5, 2-d-old *A. thaliana*; an unstained control treated with sense mRNA is shown to the right. Figure 6, 4-d-old *A. thaliana* plant. Figure 7, 8-d-old *A. thaliana* plant. Figure 8, Root from a 2-d-old radish seedling.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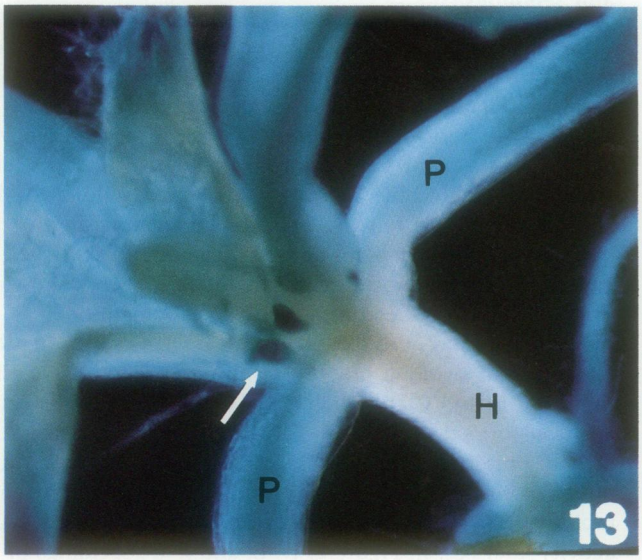
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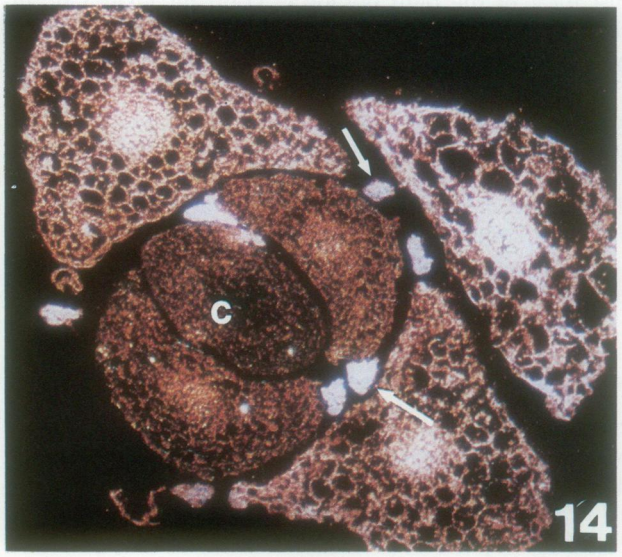
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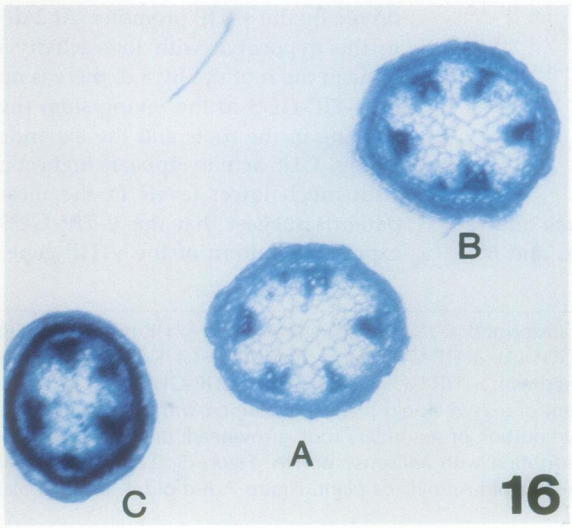
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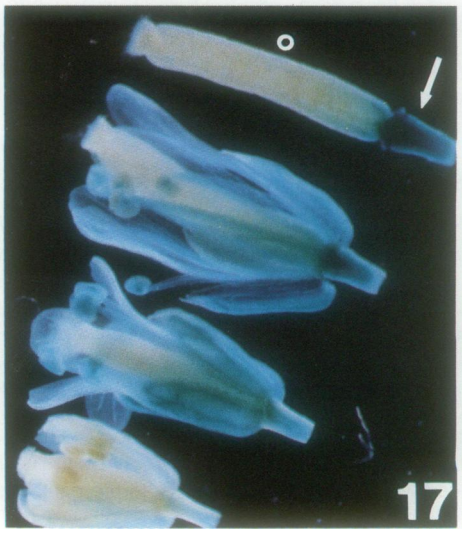
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had shown that α-TIP is expressed specifically in developing embryos and γ-TIP in the body of the plant (7). Spatial patterns of GUS activity in the root system of a 3-week-old plant transformed with the γ-TIP-GUS construct are shown in Figures 3 and 4. In Figure 3, the primary root (A) and the old portions of the secondary roots (B) are not stained, whereas younger portions of secondary roots (C) are stained. The apical root meristems and the root caps do not stain for GUS activity. A newly formed lateral root (arrowhead in Fig. 4) is unstained. This pattern is consistent with a wave of TIP expression in the roots: expression is absent from meristematic cells, and the gene is activated when the cells begin to elongate and is inactivated sometime later.

Localization of γ-TIP mRNA in Roots Using in Situ Hybridization

The use of promoter-GUS gene fusions has two major drawbacks. First, one can never be sure that the entire regulatory region has been included in the promoter fragment; second, GUS appears in the cells when the gene is activated, but does not necessarily disappear from the cells as soon as the mRNA decays. Localization of mRNA by in situ hybridization circumvents both problems. To localize γ-TIP mRNA, we adapted a whole-mount method developed for *Drosophila melanogaster* embryos, and the results showing γ-TIP mRNA in seedling roots are presented in Figures 5 to 8. The purple reaction product indicates the presence of γ-TIP mRNA in the roots of 2-, 4-, and 8-d-old *A. thaliana* plants, as shown in Figures 5, 6, and 7, respectively. In the primary root, γ-TIP mRNA is always in a narrow band just behind the root tip. A control with sense mRNA synthesized with digoxygenin UTP is also shown (Fig. 5) and is completely unstained. This method for in situ hybridization is also applicable to other plant species containing mRNA that cross-hybridizes with *A. thaliana* γ-TIP mRNA, and the location of γ-TIP mRNA in a root tip of a 2-d-old radish seedling is shown in Figure 8. These in situ hybridization results confirm the results obtained with GUS fusion constructs. The γ-TIP mRNA is absent from the apical meristem of the root and occurs in the zone of cell elongation. GUS activity in root segments that have recently elongated is probably caused by the persistence of GUS enzyme rather than by continued expression of the γ-TIP-GUS gene fusion.

Localization of γ-TIP mRNA in the Shoots of Young Seedlings

Localization of γ-TIP mRNA in an entire 2-d-old seedling is shown in Figure 9. At this time, there is a high level of γ-TIP mRNA in the zone of root elongation and less in the hypocotyl. Slightly later (days 3–4), γ-TIP mRNA in the petioles and cotyledons appears while it disappears from the hypocotyl (Figs. 10–12). At day 4, γ-TIP mRNA is evident in the vascular bundles (Fig. 10), but much less so in the mesophyll. Later, mRNA levels decline, first in the vascular bundles (day 5, Fig. 12), and 1 or 2 d later in the other tissues of the shoot (data not shown). These data support the notion of a transient pattern of gene expression that is associated with cell expansion: at the time of cell elongation of the hypocotyl or petioles or cell expansion (mesophyll), the gene is expressed, and this expression subsides soon thereafter.

Expression of γ-TIP in the Rosette and the Stipules

Expression of γ-TIP-GUS gene fusion in the rosette of a 2-week-old plant is shown in Figures 13 and 14. In this rosette, the hypocotyl (H) is completely unstained, whereas the petioles (P) show low levels of GUS activity with the vascular bundles being clearly visible. The most intense staining (arrow) is in the stipules, small structures that are present in pairs at the base of each petiole. Stipules develop from the same vegetative meristem as the leaf primordium that gives rise to the mature leaf, but the development of the stipules is restricted. A different view of the stipules is shown in Figure 14. A cross-section through the rosette of a young plant photographed in dark field shows the stipules as very bright spots (white arrows). Under the conditions of dark-field photography, the highest GUS activity is in the whitest areas. Thus, the vascular bundles at the center of each petiole cross-section are more brightly stained than the surrounding cortex tissues. In addition, the older petioles (outer ring of three) are more brightly stained than the youngest petioles/leaves, which have not yet developed, at the center (C) of the rosette (inner group of three). Particularly marked is the contrast between the brightly stained stipules and the youngest leaves, which do not yet express GUS activity. This contrast is also shown in Figure 15, in which a group of blue cells—a longitudinal section through a stipule—stand out against a completely unstained meristem region of the rosette. Like the root meristem, the shoot meristem does not express the γ-TIP-GUS fusion gene. Expression occurs after cell enlargement and cell differentiation when the leaf primordia have grown into young leaves.

Expression of γ-TIP in the Inflorescence and Flowers

GUS staining of plants transformed with the γ-TIP-GUS fusion is shown in Figures 16 and 17. Cross-sections through the inflorescence stem (Fig. 16, A and B) and the flower stem

Figures 9–17. Localization of γ-TIP mRNA in shoots of *A. thaliana* seedlings. Figure 9, 2-d-old plant showing staining in the zone of cell elongation of the root and in the hypocotyl. Figure 10, Cotyledon of a 4-d-old plant. Figure 11, Shoot of a 3-d-old plant; staining has disappeared from the hypocotyl but is present in the cotyledons. Figure 12, Shoot of a 5-d-old plant; staining in the petioles and cotyledons. Figures 13–17, Expression of γ-TIP-GUS fusions in *A. thaliana* plants. Figure 13, Rosette of a 2-week-old plant; H, hypocotyl; P, petiole of cotyledon; arrow indicates stipule. Figure 14, Dark-field photography of a section through the rosette; bright areas have the highest GUS activity. C, Center of the rosette; all profiles are cross-sections through petioles; arrows, brightly stained stipules. Figure 15, A single stipule is shown against the unstained cells of the meristem. Figure 16, Cross-sections through the stem. A, Inflorescence stem about 1 cm above the rosette; B, inflorescence stem near the top; C, flower stem. Figure 17, Floral organs of 4- to 5-week-old plants shown in a developmental sequence from left to right. O, Ovary. Note high GUS activity in the receptacle.

(Fig. 16C) show very intense staining of the vascular bundles and the cortex. The staining is most intense in the flower stems (most recently elongated), least intense at the base of the inflorescence stem (Fig. 16A), and intermediate at the top of the inflorescence stem (Fig. 16B). GUS activity is very low at the earliest stage of flower development examined here (Fig. 17, lower left), but gradually increases, especially in the vascular bundles with the most activity in the receptacle, whereas the ovary (O) has no activity. GUS activity is detected in the ovary later as it develops into a silique (data not shown). As the silique develops, the seedcoats are stained, but the embryos are not (data not shown).

DISCUSSION

The techniques of molecular cloning make it possible to identify with relative ease proteins with interesting structures whose functions remain unknown. We did so in the case of TIP when we cloned this member of an ancient family of channel proteins and showed that it is abundantly present in the tonoplasts of protein storage vacuoles (9, 11). Several other members of the superfamily have now been cloned from diverse organisms, and we are somewhat closer to understanding the functions of these proteins. One way to gain insight into the function of a gene product is to use molecular techniques to define the cell types, the period during development, or the signals that trigger gene expression. For example, the demonstration that CHIP28, a mammalian member of this family, is abundantly expressed in renal tubules led to the suggestion, later confirmed, that it may be a water channel. We previously reported that γ-TIP mRNA is present in roots, shoots, and reproductive organs, whereas α-TIP mRNA is present only in developing embryos. Here we extend the observations on γ-TIP, using both in situ hybridization and promoter GUS fusions.

Our results show that GUS activity from the γ-TIP-GUS fusion is present in considerably more (older) tissue than γ-TIP mRNA. This is especially evident in the roots. This discrepancy could be the result of the persistence of GUS activity in these tissues after the GUS mRNA has already decayed, or to a lower sensitivity of the whole-mount in situ hybridization method compared to the detection of GUS activity. The tissues that stain positively for GUS but not for mRNA may contain low levels of γ-TIP mRNA that are not detected by this method. Low abundance mRNAs are more readily detected by isotopic in situ hybridization methods.

We found, as did others (see ref. 12), that GUS activity preferentially occurred in the vascular system of some organs. It is not known whether this is caused by specific characteristics of the cytoplasm of these cells, leading to greater GUS stability or to higher levels of γ-TIP gene expression. Higher levels of γ-TIP gene expression might be expected in the vascular system because the level of expression appears to correlate with the time of cell elongation. Cells of the vascular system elongate much more than cells of surrounding tissues, such as the cortex or mesophyll.

Relationship of γ-TIP Expression to Cell Elongation

Of particular interest is the relationship of γ-TIP gene expression to cell elongation, as demonstrated by the expression pattern in the roots. There is no expression in the root meristem, and the complete absence of GUS activity in older parts of the root system indicates that the expression of the γ-TIP gene is completely shut down. This interpretation is confirmed by the results of in situ hybridization. In root tips, γ-TIP mRNA is confined to a narrow zone just behind the apical meristem. These results indicate that γ-TIP gene expression is under developmental (or temporal) control and that the gene is activated coincidentally with vacuolation and cell differentiation. A similar expression pattern is found in the stem. Young stems show γ-TIP expression (Figs. 2E and 9), but there is no expression in slightly older stems (Figs. 2F, 12, and 13) and no expression in the shoot apical meristem (Fig. 14). As in the root, expression appears to be associated with cell elongation/differentiation. These results are consistent with the findings of Maeshima (12), who analyzed the abundance of a 25-kD tonoplast protein in the hypocotyls of soybean seedlings. It is likely that this abundant tonoplast protein is a TIP homolog. He observed that the protein was present at very low levels in the hypocotyl hook and appeared in the zone of elongation below the hypocotyl hook. Meristematic cells are characterized by numerous small vacuoles, and our results imply that γ-TIP is absent or present in low amounts from these small vacuoles, but starts being synthesized as the cells expand.

The expression pattern of the γ-TIP homolog TobRB7 in tobacco plants is quite different from that of γ-TIP in *A. thaliana*. First, the mRNA for TobRB7 is found only in roots and not in other organs. Second, when a promoter-GUS fusion was expressed in tobacco, GUS activity was present in the root meristem and the central cylinder (23). There appears to be no correlation between the expression of TobRB7 and cell enlargement.

It seems unlikely to us that the dramatic difference in the level of γ-TIP expression between the apical meristem and the zone of elongation is merely a reflection of the amount of tonoplast per cell. Indeed, stain intensity is expressed on a per volume basis, and it is doubtful that there is such an increase in the amount of tonoplast per volume of tissue. Rather, we see this increase as an indication that the tonoplast differentiates and acquires a new function. One possibility is that the tonoplasts of central vacuoles are active in the transport of ions or metabolites that are not transported by the tonoplasts of small meristematic cells, and that γ-TIP functions in the transport of these substances. A second possibility, supported by preliminary observations from our laboratory (1), is that γ-TIP acts as a water channel. CHIP28, a mammalian homolog of TIP that is abundantly present in erythrocyte membranes and renal tubules, has recently been shown to act as a water channel in *Xenopus* oocytes (15). It is noteworthy in this context that another member of the TIP family is induced when plants are subjected to water stress. This may be an adaptation of the plant to allow water to flow more rapidly into or out of certain cell types. The high level of α-TIP in the membranes of protein storage vacuoles (9, 10) is also consistent with this interpretation. The drying and rehydration of embryos requires a rapid flux of water in and out of the vacuoles. α-TIP and γ-TIP are both abundant proteins, and water channels in membranes are known to be abundant (21). We do not wish to suggest that there is a

causal relationship between γ-TIP expression and cell elongation, but only that the formation of large central vacuoles occurs at the same time as the accumulation of new proteins and the acquisition of new functions. Antisense experiments are now underway to determine if failure to express γ-TIP results in a discernable phenotype.

Expression in the Shoot

The γ-TIP gene is expressed strongly in the roots but is also expressed in the shoots: in elongating hypocotyls, elongating petioles, expanding leaves, the elongating influorescence stem, and flower stems. GUS staining is greater in the vascular tissues than in the parenchyma tissues, suggesting that the level of expression is higher there, or that the enzyme is more stable in the vascular system. Higher expression in the vascular tissues may be related to the greater elongation that these cells undergo prior to their differentiation into functional vascular cells. In these organs and cell types, as in the roots, expression correlates with cell elongation or expansion.

Of particular interest is the high GUS activity in the stipules and the receptacle. The high GUS activity in the receptacle persists during development of the silique, and high expression of the gene in this tissue was confirmed by in situ hybridization (data not shown). However, we could not ascertain if the high mRNA levels persist later in silique development. Similarly, we do not know if the high GUS levels in the stipules are caused by the continued expression of the gene or by the stability of GUS in these organs. The clearest correlation between γ-TIP expression and another cellular activity is with cell elongation, but we cannot rule out other cellular conditions in which there is high γ-TIP expression.

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