Fluorescent Reporter Proteins for the Tonoplast and the Vacuolar Lumen Identify a Single Vacuolar Compartment in Arabidopsis Cells¹[W]

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We generated fusions between three Arabidopsis (Arabidopsis thaliana) tonoplast intrinsic proteins (TIPs; α-, γ-, and δ-TIP) and yellow fluorescent protein (YFP). We also produced soluble reporters consisting of the monomeric red fluorescent protein (RFP) and either the C-terminal vacuolar sorting signal of phaseolin or the sequence-specific sorting signal of proricin. In transgenic Arabidopsis leaves, mature roots, and root tips, all TIP fusions localized to the tonoplast of the central vacuole and both of the luminal RFP reporters were found within TIP-delimited vacuoles. In embryos from developing, mature, and germinating seeds, all three TIPs localized to the tonoplast of protein storage vacuoles. To determine the temporal TIP expression patterns and to rule out mistargeting due to overexpression, we generated plants expressing YFP fused to the complete genomic sequences of the three TIP isoforms. In transgenic Arabidopsis, γ-TIP expression was limited to vegetative tissues, but specifically excluded from root tips, whereas α-TIP was exclusively expressed during seed maturation. δ-TIP was expressed in vegetative tissues, but not root tips, at a later stage than γ-TIP. Our findings indicate that, in the Arabidopsis tissues analyzed, two different vacuolar sorting signals target soluble proteins to a single vacuolar location. Moreover, TIP isoform distribution is tissue and development specific, rather than organelle specific.

Vacuoles are the intracellular endpoint of the plant secretory pathway. This model for vacuolar biogenesis and transport posits that plant cells can contain separate, functionally distinct vacuoles (Paris et al., 1996; Neuhaus and Rogers, 1998; Epimashko et al., 2004; Surpin and Raikhel, 2004). This model results from two major sets of observations. First, proteins can be targeted to vacuoles by different types of vacuolar sorting signals (VSSs), which appear to recruit cargo to separate sorting pathways (Neuhaus and Rogers, 1998; Jolliffe et al., 2005; Vitale and Hinz, 2005). Thus, proteins destined to lytic vacuoles (LVs) are thought to carry sequence-specific VSSs (ssVSSs), which interact with receptors of the vacuolar sorting receptor (VSR) family (Kirsch et al., 1994, 1996; Ahmed et al., 2000; Laval et al., 2003). Proteins destined to protein storage vacuoles (PSVs) are instead thought to bear hydrophobic C-terminal VSSs (ctVSSs), which may interact with a different receptor, RMR (Jiang and Rogers, 1998; Park et al., 2005). Second, different isoforms of the tonoplast intrinsic protein (TIP) have been localized to the tonoplast of separate vacuolar compartments in a variety of species and cell types and are therefore used as specific markers for different vacuolar types, with α-TIP identifying PSVs and γ-TIP labeling LVs (Jauh et al., 1998, 1999). This model for vacuolar biogenesis and transport therefore arises from the amalgamation of a large number of biochemical and microscopic observations in different plant species and tissues.

Recently, evidence has shown that the separate vacuole-separate transport pathways model may not be as clean-cut as initially postulated. Proteins whose precursors carry ssVSSs can ultimately be found in the PSV (Brown et al., 2003; Jolliffe et al., 2004; Maruyama et al., 2006). Genetic knockout of one isoform of VSR, AvVSR1, leads to secretion of PSV-destined proteins (Shimada et al., 2003; Fuji et al., 2007). Moreover, indirect evidence indicates that Arabidopsis (Arabidopsis thaliana) embryos may contain only one type of vacuole, the PSV (Otegui et al., 2006).

Remarkably, the available information about vacuolar systems in the most popular model plant, Arabidopsis, is rather incomplete. GFP fusions to chitinase or aleurain and prototype cargoes for PSVs and LVs, respectively, were both found in the lumen of the central vacuole in leaves or roots (Flückiger et al., 2003). This is in apparent contrast with the behavior of the same markers in tobacco (Nicotiana tabacum) cells (Di Sansebastiano et al., 1998, 2001). GFP-chitinase gave rise to punctate structures reminiscent of PSVs (Di Sansebastiano et al., 1998; Flückiger et al., 2003). However, these were not characterized further. More detailed, in vivo observation has been hampered by the fact that currently available GFP-based markers are somewhat unreliable.

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due the propensity of GFP itself to travel to vacuoles (Frigerio et al., 2001a; Zheng et al., 2004) and its lack of fluorescence in the acidic vacuolar lumen (Fluckiger et al., 2003; Tamura et al., 2003; Samalova et al., 2006).

Regarding the distribution of TIPs in Arabidopsis, the available evidence is equally fragmentary. Park et al. (2004) identified a compartment in Arabidopsis leaf protoplasts, which seems to exist as a separate entity to the central vacuole when α-TIP is overexpressed. However, no direct evidence for an endogenous α-TIP protein within leaf protoplasts was provided and no concomitant localization of γ-TIP was performed.

In Arabidopsis seeds, α-TIP was very clearly localized to the rim of the PSV by immunofluorescence and immuno-electron microscopy (EM; Oufattole et al., 2005; Poxleitner et al., 2006). However, no concurrent detection of γ-TIP was performed. When the latter was attempted by others, no labeling was observed (Otegui et al., 2006).

Thus, definitive evidence for the presence of multiple vacuoles in Arabidopsis cells, with particular respect to leaves, root tips, and seeds, is so far lacking. The idea that Arabidopsis cells contain separate vacuolar compartments should be supported by the following, testable hypotheses: (1) Soluble secretory proteins carrying a C-terminal VSS should be targeted to the PSV lumen; (2) soluble secretory proteins carrying a ssVSS should accumulate in the LV lumen; (3) LV tonoplasts should contain γ-TIP; and (4) PSV tonoplasts should contain α- and/or δ-TIP (Jauh et al., 1998, 1999).

In this work, we tested these hypotheses systematically. We studied the localization of a novel set of fluorescent reporters for the vacuolar lumen. They are based on the monomeric red fluorescent protein (RFP), which has recently been shown to be both transport neutral (Zheng et al., 2005) and fluorescent in the vacuolar lumen (Samalova et al., 2006). An additional feature of these reporters is that they only contain well-characterized, minimal VSSs, thus minimizing the possibility of mistargeting induced by the presence of additional, unnecessary sequences. We also fused yellow fluorescent protein (YFP) to the three TIP isoforms of Arabidopsis whose sequences have previously been used to generate peptide antibodies (Jauh et al., 1998, 1999). These reporter proteins were either constitutively expressed or driven by their native control sequences. Our results demonstrate that, in the Arabidopsis tissues we analyzed, all reporters localize to a common vacuolar location. We also show that the three TIP isoforms are differentially expressed during development, which calls into question their use as markers to identify coexisting vacuoles in single cell types.

RESULTS

Soluble RFP-VSS Reporters Localize in the Lumen of Central Vacuoles in Arabidopsis Leaves and Roots

We generated soluble vacuolar reporters based on the monomeric RFP1 (Campbell et al., 2002). As a control, RFP was preceded by a signal peptide (sp) and no VSS (spRFP). We then produced two RFP fusions: one with the ctVSS of phaseolin, the tetrapeptide AFVY (Frigerio et al., 1998), appended to the C terminus of
RFP (spRFP-AFVY; Fig. 1); and one in which the ssVSS of proricin, the linker peptide (Frigerio et al., 2001b), was appended to the N terminus of RFP (spL-RFP). As a control for the sequence specificity of the ricin linker-based reporter, we mutated the crucial Ile residue within its VSS to Gly (spL<sub>I</sub>-RFP). This mutation completely abolishes capacity for targeting proteins to the vacuole when the linker peptide is located anywhere in the protein sequence, apart from the C terminus, where it acts as a cVSS (Jolliffe et al., 2003).

**Figure 2.** Two vacuole-targeted RFP reporters localize to the same vacuole in Arabidopsis leaves and embryos. Leaves (A–H) and mature embryos (I–P) from transgenic plants expressing the indicated constructs or transformed with empty vector alone as a control (Co; Q–T) were analyzed by confocal microscopy. RFP was excited at 543 nm. All images shown were acquired using the same photomultiplier gain and offset settings. For each sample, two magnifications are shown. Scale bars: 40 μm (A, C, E, G, and Q, and I, K, M, O, and S) and 10 μm (B, D, F, H, and R, and J, L, N, P, and T).
We produced and analyzed transgenic Arabidopsis plants expressing the RFP-based reporters (Fig. 1). In mature leaves, spRFP was exclusively detected in the apoplast (Fig. 2, A and B), as previously observed in tobacco epidermal cells (Zheng et al., 2005). In contrast, spRFP-AFVY and spL-RFP were both localized exclusively in the lumina of the large, central vegetative vacuoles (Fig. 2, C, D, E, and F). Mutation of the critical Ile to Gly in the ricin VSS promoted complete secretion of spLIG-RFP into the apoplast (Fig. 2, compare G and H with A and B). This indicates that the ricin VSS is acting as a true sequence-specific sorting signal.

We then analyzed embryos dissected from dry seeds. In mature embryos, the common destination for spRFP-AFVY and spL-RFP was the lumen of the PSV (Fig. 2, K–N). To confirm this, we took advantage of the fact that mature or near-mature PSVs are autofluorescent (Shimada et al., 2003; Li et al., 2006). The fluorescence emission spectrum of embryos excited at 405 nm reveals a peak of autofluorescence at 500 nm (Supplemental Fig. S1A). This is useful for identifying the lumen of the PSV (pseudocolored in blue in Supplemental Fig. S1B), while permitting the simultaneous detection of reporters emitting at longer wavelengths, such as YFP and RFP. Autofluorescence and spRFP-AFVY signals clearly colocalized (Supplemental Fig. S1B). Mutation of the linker or absence of VSSs once again resulted in complete secretion of RFP (Fig. 2, compare O and P with I and J). With the same confocal microscope settings, no background signal in the RFP channel was detectable in corresponding tissues from plants transformed with empty vector as a control (Fig. 2, Q–T).

We then analyzed root axes and root tips (Fig. 3). In root axes, VSS-bearing RFP localized uniformly in the lumen of the central vacuole (Fig. 3, A and D). This was also the predominant localization pattern in root tips (Fig. 3, B, C, E, and F). Again, the absence of VSSs or the mutated ricin VSS led to clear secretion of RFP (Fig. 3, G–J). In some cells expressing the vacuolar reporters, in addition to the central vacuole, other smaller, punctate structures could be observed (Fig. 3, C and F). The nature of these structures is unknown. However, they are always detectable regardless of what type of VSS is appended to RFP. Therefore, our results indicate that both VSSs target RFP to the same destination in all cell types analyzed.

**TIP-YFP Reporters Localize to the Tonoplast of the Central Vacuole**

We generated translational fusions between the cDNAs for three well-studied Arabidopsis TIP isoforms (Jauh et al., 1998), α-TIP (At1g73190), γ-TIP (At2g36830), and δ-TIP (At5g16240), and YFP (Fig. 1). To rule out mistargeting due to the presence of the reporter, we appended GFP/YFP either at the N or C terminus of the TIPs. In both cases, the fluorescent protein is exposed to the cytosol. We initially assayed expression of these constructs by *Agrobacterium* infiltration of tobacco leaf epidermal cells. All YFP-tagged TIPs localized to the tonoplast of the large central vacuole, with tonoplast localization being confirmed by coexpression with the plasma membrane marker EGFP-LTI6b (Kurup et al., 2005; Supplemental Fig. S2). We therefore employed the same constructs to generate stable Arabidopsis transgenic lines and analyzed the localization of the reporter proteins. Figure 4 shows that all three TIP-YFP fusions localize to the tonoplast of the central vacuole in cotyledons and young and mature leaves. In cotyledonary leaves (Fig. 4, A–C), or young rosette leaves (Fig. 4, D–F), the fluorescent TIPs also label globular structures, previously named bulbs (Saito et al., 2002). As leaves age, the number of bulbs declines (Fig. 4, compare A–C with G–I), as previously observed. Appending GFP to the N terminus of the
TIPs resulted in an identical pattern of localization (Supplemental Fig. S3), thus ruling out missorting due to the position of the fluorescent protein.

In protoplasts prepared from rosette leaves, distribution of the three TIPs remained unchanged and was strictly limited to the tonoplast of the large central vacuole (Fig. 4, J–L). This is in contrast with previous immunofluorescence studies that localized exogenously expressed hemagglutinin (HA)-tagged α-TIP to small, separate structures, identified as PSVs, within the same cells (Park et al., 2004).

In mature Arabidopsis root axes, again all the TIPs present a comparable pattern and label the tonoplast of the central vacuole and occasionally smaller, punctate structures (Fig. 5, A, D, and G). In root tips, all three TIPs label very bright, punctate structures (Fig. 5, B, E, and H). However, the tonoplast of the central vacuole was also labeled (Fig. 5, C, F, and I). Once again, and crucially, all isoforms produce the same fluorescence pattern. As described above, these structures are likely to be bulbs, in which the fluorescent markers are concentrated, resulting in saturating levels of signal (Saito et al., 2002). If, however, the brightly fluorescent structures are indeed separate compartments, they cannot be distinguished from the central vacuole by specific localization of any of the TIP isoforms studied here.

**TIP-YFP Labels the PSV in Embryos**

Immunofluorescence data indicate that TIP localization is complex in storage tissues. In Arabidopsis seed sections, α- and δ-, but not γ-TIP, were found to decorate the PSV tonoplast (Poxleitner et al., 2006). We studied the distribution of our TIP-YFP reporters in embryos from mature and germinating seeds by confocal microscopy. We isolated embryos from dry seeds or at different stages of germination (Fig. 6). As de-
scribed above (Supplemental Fig. S1), we used a 405-nm laser to excite the autofluorescent compounds of the lumen of the PSV (pseudocolored in blue). In dry seeds, all three TIP-YFP fusions clearly outlined the rim of the PSV, thus defining their tonoplasts (Fig. 6, A, E, and I). During the progression of germination, the tonoplast underwent extensive remodeling (Fig. 6, B, F, and J) and large, bulb-like structures became visible (Fig. 6, C, D, G, H, K, and L). This phenomenon affected every observable cell in the germinating seed (Supplemental Fig. S4). The development of the bulbous structures was paralleled by a decline in auto-fluorescence (Fig. 6, compare C, G, and K with B, F, and J), probably reflecting the degradation of storage material within the PSV. With the emergence of the cotyledons from the germinating seed (day 3.5), the TIPs localized to the tonoplast of what now resembled a large, central, lytic vacuole (Fig. 6, D, H, and L). We conclude that, in embryos, there is only one type of vacuole—the PSV—which, upon germination, degrades its storage material and eventually reconverts into a vegetative-type LV.

Expression of TIP Isoforms Is Developmentally Separated

Our findings imply that all vacuole-directed RFP fusions and all three TIP-YFP fusions share the same intracellular localization. It is possible that 35S-driven expression of the reporters may lead to mislocalization due to overexpression. We resolved this by studying the expression and localization of the TIPs under the control of their native genomic sequences. Some reports indicate that α- and γ-TIP distribution is developmentally regulated (Hofte et al., 1992; Ludevid et al., 1992; Wang et al., 2007). This is also shown by their gene expression patterns as revealed by Genevestigator analysis (Zimmermann et al., 2004; Supplemental Fig S5). Whereas δ- and γ-TIP show abundant expression in vegetative tissue, their transcript levels become undetectable in developing and mature seeds. In contrast, α-TIP expression seems to be confined to seed maturation and germination (Supplemental Fig S5A). These patterns mirror earlier observations using immunoblot (Hofte et al., 1992) and GUS promoter fusions (Ludevid et al., 1992). To extend this analysis to the subcellular localization of these proteins in vivo, we fused YFP to the complete genomic sequences of the three TIPs (Fig. 1) and generated transgenic plants. Native α-TIP-YFP expression was strictly confined to seeds (Fig. 7, I and J). During embryo development, native α-TIP-YFP became detectable only at the late torpedo stage (Supplemental Fig. S5). In dry seeds, the activity of the native α-TIP promoter was much stronger than that of the 35S promoter (Fig. 7, compare E and F with I and J). This confirms that the phenotypes...
described in Figure 6 are unlikely to represent overexpression artifacts. In contrast, native γ-TIP-YFP was not expressed in seeds, but was turned on immediately after germination, as α-TIP expression began to decline (Fig. 7, compare I–L with M–P). Native δ-TIP-YFP was also absent in seeds and its expression in vegetative tissues initiated approximately 24 h later than γ-TIP, with fluorescence becoming visible in the vascular tissue (Fig. 7, Q–T).

We analyzed the transition between TIP isoforms in embryos at the subcellular level by confocal microscopy (Fig. 8). As already shown for constitutively expressed α-TIP-YFP (Fig. 6), native α-TIP-YFP initially decorated the tonoplast of the PSV and, as germination progressed, its expression extended to the autophagic, bulbous structures within the PSV lumen (Fig. 8, A and B). After 2.5 d, when most autofluorescent material in the PSV had disappeared, native α-TIP-YFP was less strongly expressed (Fig. 8C), whereas native γ-TIP began to be detectable (Fig. 8G). During this brief overlap, both TIP-YFP fusions appeared to localize to the same membrane (Fig. 8, compare C and G). One day later, native α-TIP-YFP was no longer detectable and γ-TIP was joined by δ-TIP at the tonoplast of the central vacuole (Fig. 8, compare D, H, and L). This transition between isoforms was equally well observable by immunoblot with anti-GFP antiserum (Supplemental Fig. S5B). In seedlings, native γ-TIP-YFP was expressed in developing roots, but remarkably excluded from root cap, meristem, and elongation zone (Fig. 9A). Native δ-TIP-YFP was only detectable in mature, but not developing, roots or root tips (Fig. 9E). In mature root cells, both γ- and δ-TIP were found on the tonoplast of the central vacuole (Fig. 9, B and F). Likewise, both TIPs localized to the central vacuole in leaf cells (Fig. 9, C, D, G, and H).

DISCUSSION

Different VSSs Target RFP to the Same Vacuole

We generated a set of RFP-based soluble vacuolar reporters. These consist of a signal peptide, RFP— which, unlike GFP, is transport neutral in plant cells and fluorescent in both the apoplast (Zheng et al., 2005) and the vacuolar lumen (Samalova et al., 2006)— and one of two minimal, but classically distinct, VSSs. Localization in the lumen of the vacuole was unequivocally observed in mature leaves, roots, and embryos, with vacuolar targeting of the RFP being entirely reliant on the VSS. The secretion of RFP in the absence of sorting signal or with a mutant ricin ssVSS was also unambiguous. The mutant ricin VSS confirmed the robustness of our reporter set, with a single amino acid substitution being sufficient to completely abolish vacuolar targeting.

We expected different VSSs to target the same fluorescent protein to different vacuolar compartments, as previously suggested (Di Sansebastiano et al., 1998, 2001). RFP fluorescence instead invariably highlighted
the same vacuole. Indeed, the available, albeit limited, literature on Arabidopsis tends to be in agreement with our findings. When GFP was fused to the C-terminal sorting signal of pumpkin 2S albumin, it was visualized in the central vacuoles of vegetative tissues incubated in the dark (Tamura et al., 2003). GFP fusions to the chitinase VSS (considered to be a PSV marker) and GFP barley (*Hordeum vulgare*) lectin VSS were recently either seen in the lumen of the central vacuole or in the endoplasmic reticulum (ER) of Arabidopsis leaves (Sanmartín et al., 2007). Flückiger et al. (2003) observed that aleurain GFP (containing a region of aleurain including the ssVSS) was targeted to the central vacuole. GFP-chitinase was found in the central vacuole, but it also labeled the ER and smaller, uncharacterized structures, depending on the cell types studied (Flückiger et al., 2003). For both reporters, the observation was complicated by the use of GFP, which presented erratic patterns of fluorescence and consistently showed relatively strong background fluorescence in the ER. In fact, when we substituted GFP for RFP in our constructs, the GFP-based reporter constructs generated comparable results (data not shown). We believe this reflects the well-documented inadequacy of GFP as a marker for the vacuolar lumen (Tamura et al., 2003; Samalova et al., 2006).

A Common Tonoplast Destination for Different TIPs

We also generated a comprehensive set of reporters for the tonoplast. Although some TIP-GFP fusions have been studied previously in Arabidopsis (Tian et al., 2007), YFP fusions to the TIP isoform genomic sequences highlight their temporal and tissue-specific regulation. Germinating seeds from the indicated Arabidopsis transgenic lines were imaged with a stereomicroscope at the indicated times. YFP was excited with a xenon lamp equipped with a GFP filter and detected with a digital camera. Chlorophyll autofluorescence appears red. All images were acquired using the same camera sensitivity and shutter speed. Insets show white-light images of the same samples. DAG, Days after germination.
et al., 2004), we performed a systematic analysis of the localization of the three TIP isoforms widely used as markers for different vacuolar compartments (Jauh et al., 1999).

Although work in tobacco BY-2 cells has previously shown that constitutively expressed α- and γ-TIP fused to GFP localize to the tonoplast of the large central vacuole (Mitsuhashi et al., 2000), we expected the TIP-YFP fusions to localize to different vacuolar membranes, in particular α-TIP to be found on PSV in non-seed Arabidopsis tissues (Park et al., 2004). However, looking at the distribution of our TIP-YFPs, we invariably detected only one type of vacuole in the Arabidopsis tissues analyzed. In particular, there seems to be little doubt that this is the case in developing and mature embryos, where all three constitutively expressed TIPs localized to the tonoplast of the PSV. We conclude that only PSVs are present in mature and germinating seeds, with no evidence for separate, possibly lytic vacuoles. A similar conclusion has recently been reached by Otegui et al. (2006), who found no evidence for a lytic vacuole in embryos, based on immunogold labeling with anti-γ-TIP. This is perhaps not surprising: Transcriptomic data (Supplemental Fig. S5), GUS-promoter fusion data (Ludevid et al., 1992), and our own results with native TIP genomic sequences strongly indicate that, of the three isoforms under study, only α-TIP is present during embryo maturation and germination. This is in apparent contrast with results obtained by immunofluorescence in Arabidopsis seeds, using peptide antibodies against the δ-TIP (Poxleitner et al., 2006). The Arabidopsis genome contains two additional δ-TIP isoforms (Johanson et al., 2001). It is possible that the antibody specificity extends to these isoforms, but it should be noted that their mRNAs are absent or decline steadily during seed maturation (Supplemental Fig. S6).

Developmental Specificity of TIPs

Our results indicate that the expression of native α-TIP-YFP is firmly restricted to storage tissues, being turned on at the late torpedo stage (Supplemental Fig. S5) and becoming barely detectable 3 d after germination (Fig. 8D). No α-TIP expression was detected in leaves. Only when it was constitutively expressed did α-TIP-YFP colocalize to the central vacuole with γ- and δ-TIP-YFP (Fig. 4). Recent reports have identified a PSV in Arabidopsis leaf protoplasts, using overexpressed, HA-tagged α-TIP as a membrane reporter and transiently expressed phaseolin as a soluble vacuolar marker (Park et al., 2004). It is difficult to reconcile these findings with our observation that native α-TIP-YFP is normally not expressed in such cells. In addition, both immunofluorescence and EM have previously shown that phaseolin forms aggregates within the lumen of the central (presumably lytic) vacuole in tobacco protoplasts (Frigerio et al., 1998, 2001c), rather than in separate structures. It is possible that, upon protoplast fixation and immunofluorescence, the phaseolin aggregates become so brightly fluorescent as to require a low detector gain on the confocal microscope. This, in turn, would result in fluorescence at the tonoplast of the central vacuole.
falling below detection within the same image, thus conveying the impression of two separate tonoplasts with a different TIP composition.

Our findings raise the question as to why proteins that are ultimately targeted to the same organelle can carry different types of sorting signals. In a recent analysis of the soluble and membrane proteome of vegetative vacuoles (the sum of LV + PSV in green tissues), 402 proteins were identified (Carter et al., 2004). Within a subset of about 130 bona fide vacuolar proteins bearing a signal peptide, 42 were found to contain sequence-specific-type sorting signals. In contrast, only 10 proteins, mostly peroxidases, were unequivocally assigned a putative ctVSS. Because this type of signal has no conserved sequence, its detection relied on the comparison between cytosolic and vacuolar members of the peroxidase family, with the latter bearing a C-terminal hydrophobic propeptide (Carter et al., 2004). Besides these known examples, almost three-fourths of the soluble vacuolar proteome are unaccounted for in terms of sorting signal. It is probable that several proteins will carry ctVSSs because these cannot easily be detected by direct sequence analysis. In addition, ctVSS deduction by coalignment is impossible if the proteins are not annotated and/or lack relatives of similar functions located in different compartments. Even so, it is very likely that the number of soluble vacuolar proteins not carrying known types of sorting signals exceeds that of proteins bearing canonical VSSs. This potential diversity of signals is likely to reflect a multiplicity of vacuolar sorting pathways. Attempting to invoke the existence of different vacuolar types to accommodate different signals and their related pathways may therefore prove a daunting task.

Our data show that, in the Arabidopsis tissues we analyzed, either there is a single type of vacuole or the panel of reporters we developed is not capable of discriminating between different, coexisting vacuolar types. We have also shown that three TIP isoforms localize to the same tonoplast in leaves, roots, and seeds, with α-TIP being confined to seeds. In addition, δ- and γ-TIP are not expressed in root tips—historically a choice location for the detection of separate vacuoles (Paris et al., 1996; Jauh et al., 1998, 1999). Whereas it is very likely that one or more TIP isoforms are indeed expressed within root tips (Supplemental Fig. S6), the absence of the variants we studied raises concerns about their use as markers for subcellular organelles in these tissues. Finally, the data shown here, while convincing for the tissues studied, do not exclude the possibility that two vacuolar types may indeed coexist in other cell types. For example, in cells of the maturing embryo it is possible that PSVs may arise de novo at the expense of a shrinking lytic vacuole, as previously described in other species (Hoh et al., 1995). We suspect it will be difficult to document this transition by light microscopy. The approach most suitable to unravel this level of complexity would require EM and is beyond the scope of this study.

Figure 9. Native δ- and γ-TIP are expressed in vegetative tissues but not root tips. Roots (A, B, E, and F) and leaves (C, D, G, and H) of transgenic Arabidopsis plants expressing the indicated constructs were analyzed by confocal microscopy. In A and E, YFP fluorescence is superimposed to the transmitted light image. Note that γ-TIP expression is restricted to the mature part of the root. Scale bars: 20 μm (A and E); 10 μm (B–D, F–H).
MATERIALS AND METHODS

Recombinant DNA

A schematic representation of the constructs used in this study is shown in Figure 1. All primer sequences are shown in Supplemental Figure S8.

**RFP Reporters**

To generate spRFP and related constructs, the monomeric RFP1 coding region was amplified by PCR with Pwo DNA polymerase (Roche) from pcdNA1-mRFP (Campbell et al., 2002), using primers 3 and 4. The signal peptide of phaseolin was amplified from pDH4A-d418 (Frigierio et al., 1998) with primers 1 and 2. The amplified RFP and signal peptides were spliced together by fusion PCR with primers 1 and 4 to yield spRFP. This was used as a template for subsequent constructs. To generate spRFP-AFVY, primer 1 was used in combination with primer 5. For spl-RFP, the signal peptide of phaseolin was amplified with primers 1 and 7 and fused to RFP (amplified with primers 4 and 6) using primers 1 and 4. To generate the point mutation in splI-RFP, spl-RFP was subject to Quickchange mutagenesis (Stratagene) using primers 8 and 9. All PCR products were cloned into the SmalI-NotI sites of pGreenII-0029 (Hellens et al., 2000).

**TIP Reporters**

The coding regions of the three TIP isoforms were amplified by reverse transcription-PCR from total leaf RNA for γ and δ-TIP or seed RNA for α-TIP using primers 10 to 15 (Supplemental Fig. S8; SmalI and NotI sites are underlined). The products were cloned into the NotI site of pGreenII-0029 between the 33S promoter and the YFP coding sequence followed by the nopaline synthase terminator.

For N-terminal GFP-TIP fusions, primers 16 to 19 were used (Supplemental Fig. S8; SmalI and SacI sites are underlined). PCR products were cloned into the SmalI-SacI sites of pVKH18-ErcGFP (Batonko et al., 2000).

**Native TIP-YFP Cloning Strategy and Primers**

The open reading frame of YFP was amplified using the following primers 20 and 21 (Supplemental Fig. S8; Xhol and SacI sites are underlined). The resultant product was ligated into the cloning vector pBlueScript KS (Stratagene).

DNA was extracted from mature leaves of Arabidopsis (Arabidopsis thaliana ecotype Columbia-0) using GenElute Plant Genomic DNA Miniprep Kit (Sigma) and used as template for PCR amplification of genomic regions of the TIPs. The 5' promoter and gene (2.5-kb region or to next upstream gene) were amplified using primers 22 to 27 (Supplemental Fig. S8; Kpn I and Xhol sites are underlined). From the same DNA preparation, the 3' terminators were amplified using primers 28 to 33 (Supplemental Fig. S8; SacI and NotI sites are underlined). Promoter and gene regions were cloned into the KpnI-Xhol sites of pBlueScript KS, followed by YFP in Xhol-SmalI sites, followed by 3' untranslated regions in SmalI-NotI sites. The whole cassette was then inserted into the KpnI-NotI sites of pGreenII-0029.

All the resulting constructs were introduced into strain CS8 of Agrobacterium tumefaciens harboring the pSoup vector (Hellens et al., 2000).

**Transgenic Plants**

Transgenic Arabidopsis plants expressing the above-described constructs were generated by Agrobacterium-mediated transformation (Clough and Bent, 1998). Suspensions of Agrobacterium from 5-mL overnight cultures containing 0.25% Silwet were pipetted directly onto floral buds or opening flowers and plants covered in a plastic sheet overnight before resuming growth in greenhouse conditions.

**Microscopy**

All samples were mounted in water and imaged with a Leica TCS SP2 confocal laser-scanning microscope, using a 10× (NA 0.3) air, a 40× (NA 1.25), or a 63× (NA 1.4) oil immersion objective. YFP was excited at 514 nm and detected in the 525- to 583-nm range. RFP was excited at 543 nm and detected in the 553- to 638-nm range. Chlorophyll autofluorescence was detected in the 660- to 700-nm range. When imaging mature embryos, PSV autofluorescence was excited at 405 nm and detected in the 450- to 510-nm range. Simultaneous detection of PSV autofluorescence and YFP or RFP was performed by combining the settings indicated above in the sequential scanning facility of the microscope, as instructed by the manufacturer.

For observation of TIP expression during seed germination, germinating seeds were imaged using a Leica MZFL3 stereomicroscope equipped with a Nikon DXM1200 digital camera. YFP was excited with a mercury UV lamp and detected with a 525- to 550-nm bandpass filter. After acquisition, images were resized and, where appropriate, converted to grayscale with Adobe Photoshop CS. No image enhancement was performed.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** PSV identification by autofluorescence.

**Supplemental Figure S2.** Three TIP-YFP fusions localize to the tonoplast in tobacco epidermal cells.

**Supplemental Figure S3.** The position of the fluorescent protein does not affect TIP targeting to the tonoplast.

**Supplemental Figure S4.** TIP-YFP label large autophagic structures during Arabidopsis seed germination.

**Supplemental Figure S5.** mRNAs and protein expression of three Arabidopsis TIP isoforms is developmentally regulated.

**Supplemental Figure S6.** Expression of native α-TIP-YFP during seed development and maturation.

**Supplemental Figure S7.** Developmental regulation of the three δ-TIP isoforms of Arabidopsis.

**Supplemental Figure S8.** List of all oligonucleotide primers used in this study.

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**LITERATURE CITED**


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