Tonoplast intrinsic proteins and vacuolar identity

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Abstract

TIPs (tonoplast intrinsic proteins) have been traditionally used as markers for vacuolar identity in a variety of plant species and tissues. In the present article, we review recent attempts to compile a detailed map of TIP expression in *Arabidopsis*, in order to understand vacuolar identity and distribution in this model species. We discuss the general applicability of these findings. We also review the issue of the intracellular targeting of TIPs and propose key emerging questions relative to the cell biology of this protein family.

Introduction

Aquaporins are membrane proteins that play a major role in regulating the plant water balance by acting as channels for water and small uncharged molecules. Plant aquaporins are part of the large family of MIPs (major intrinsic proteins), which is subdivided according to subcellular localization. Thus the MIP family is subdivided into plasma membrane (PIP), tonoplast (TIP), nodulin-like (NIP), small basic (SIP) intrinsic proteins and the newly identified, as yet unlocalized XIPs [1,2].

A large research effort has been spent over the last 15 years into understanding the function of plant aquaporins, in particular with relation to their structural features, solute specificity and role in water balance regulation (for recent comprehensive reviews of plant aquaporin functions see [3,4]). Recently, studies have also been initiated to understand the intracellular targeting of aquaporins, in particular TIPs and PIPs [2,5]. Beside their biological roles, TIPs have a relatively long history as vacuolar markers [6]. The discovery that different TIP isoforms localized to separate tonoplasts within individual cells indicated that multiple vacuoles may be present within the same cell [7]. Therefore the localization of different TIPs to vacuolar membranes was instrumental to the definition of a working model for vacuole biogenesis and identity over the past decade. In general, the model predicts the existence of multiple vacuoles in plant cells, with PSVs (protein storage vacuoles) having α-TIP (TIP3;1) and δ-TIP (TIP2;1) on their tonoplast, and LVs (lytic vacuoles), functionally equivalent to mammalian lysosomes, having γ-TIP (TIP1;1) [8]. The existence of separate vacuoles implies the presence of at least two distinct sorting routes to the PSV or the LV. Such separate routes, and the putative vacuolar sorting signals that assign cargo proteins to a given route, have been described [9–12].

Research into the subcellular localization of TIPs – and subsequent vacuolar-type identification – has so far been performed by immunofluorescence [8,13] and by transient or stable expression of fluorescent protein fusions [14–17]. Results have been gathered from a variety of plant species, tissues and cell lines. The resulting maps of TIP-based vacuolar distribution have been useful conceptual frameworks for research into vacuolar sorting and biogenesis, but conveyed the optimistic notion that findings in a particular experimental system could be extrapolated to many plant species, which would then be expected to share a similar vacuolar system architecture and sorting mechanisms. In fact it now appears that, although the basic features are conserved, there is a degree of variability among different species and possibly even among different tissues of the same plant [6,18].

As mentioned above, the basic model for TIP distribution, based on immunofluorescence studies, posited that plant cells may contain PSVs with TIP3;1 and TIP2;1, and LVs with TIP1;1 [7,8,13]. More recently, the expression of different TIP family members was also mapped by tagging TIPs with fluorescent proteins [16,19–21]. In general, emphasis was on the three above-mentioned TIP isoforms, which had been the subject of immunofluorescence studies [1]. When TIP–XFP (any fluorescent protein) fusions are expressed under control of the 35S promoter in whole plants, their expression pattern is virtually ubiquitous [5,15,16,21,22]. In mature tissues, all TIP fusions reported to date seem to localize to the tonoplast of the central vacuole (reviewed in [6]). It is, however, possible that overexpression of these membrane proteins may lead to them reaching the tonoplast regardless of whether this is their actual destination.

Developmental regulation of TIP expression

In an effort to focus on the distribution of vacuoles labelled by TIPs in a model plant species, and to reduce the risk of localization artefacts due to overexpression, we mapped the expression of the three isoforms which were initially used to discriminate between LVs and PSVs: TIP1;1, TIP2;1 and TIP3;1 [21]. YFP (yellow fluorescent protein) fusions to these TIPs were expressed under their native genomic control sequences in transgenic plants (which, however, still express the corresponding endogenous TIPs). The chimaeric

Key words: aquaporin, fluorescent protein, intrinsic protein, tonoplast, tonoplast intrinsic protein (TIP), vacuole.

Abbreviations used: endo H, endoglycosidase H; ER, endoplasmic reticulum; LV, lytic vacuole; MIP, major intrinsic protein; PIP, plasma membrane intrinsic protein; PSV, protein storage vacuole; TIP, tonoplast intrinsic protein; YFP, yellow fluorescent protein.

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TIP–YFP fusions localized to the tonoplast of the central vacuole in leaves (TIP1;1 and TIP2;1) and embryos (TIP3;1). This localization was independent of the position of the YFP tag [21]. TIP1;1 and TIP3;1 appeared to be developmentally separated, with TIP3;1 being abundant in seeds but sharply declining after germination, to be effectively replaced by TIP1;1 [21]. This confirms that storage vacuoles (in seeds) are enriched in TIP3;1 and that LVs (in vegetative organs) are defined by TIP1;1. These two isoforms, however, barely overlapped during germination, but when they did they appeared to localize to the same tonoplast [21]. This hints at a developmental, rather than spatial, regulation of the TIPs and, more importantly, at the existence of a single vacuole which, from being a storage organelle in seeds, develops into a lytic compartment during germination. A similar developmental transition between TIP3;1 and TIP1;1 was also observed in pea and barley root tips by immunofluorescence [23].

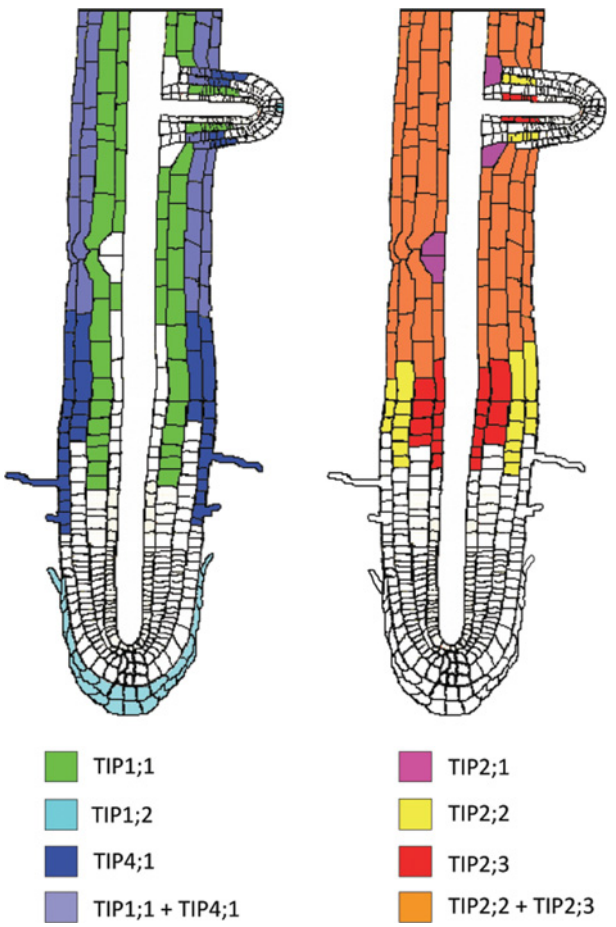
Because the TIP isoforms examined in the above studies have very close relatives in the *Arabidopsis* genome (see Figure 3) [6], it is possible that these relatives may not be distinguished by isoform-specific antisera and therefore be responsible for the separate pattern observed by immunofluorescence. This led us to extend our analysis to the systematic localization study of every member of the *Arabidopsis* TIP family, with the exception of two isoforms (TIP1;3 and TIP5;1) which are predicted to be exclusively expressed in pollen [6]. We recently generated fusions to the complete genomic sequences of all *Arabidopsis* TIPs whose transcripts are detected in root tissues [20]. Our findings (summarized in Figure 1) reveal an unexpected degree of tissue specificity for different TIP isoforms. For example, a YFP fusion with TIP1;2, whose transcript is abundant and distributed throughout the root axis [19], is only detected at the root cap, whereas TIP1;1, whose transcriptional profile is very similar to TIP1;2, is found throughout the root axis but not in the root cap (Figure 1) [16,24]. TIP2;1, which is widespread in leaves, is confined to the lateral root primordium and subsequently to a small set of cells at the base of the lateral root (Figure 1). Other previously unmapped isoforms, such as TIP2;2 and TIP2;3, have an overlapping pattern of expression but are apparently absent in the cells where TIP2;1 is expressed [20]. Another isoform with a strictly root-specific expression is TIP4;1, which is found exclusively in the epidermal and cortical layers (Figure 2). TIP4;1 is expressed very early during germination, as the radicle emerges from the seed (Figure 2A), then its expression remains confined to the differentiation and elongation zones (Figure 2B) and declines as the root matures (Figure 2C).

Apart from the very localized pattern observed for TIP2;1 and TIP1;2, expression of several isoforms overlaps, especially in the mature root axis (Figure 1). There all these TIP isoforms are mainly detected at the tonoplast of the central vacuole, with no other vacuole-like structures being evident [20].

It is important to point out that none of the TIP isoforms analysed are found in the meristematic region of the root tip [20]. Root tips of pea and barley were the source of cells for the initial TIP immunolabelling experiments which revealed multiple vacuoles [7] and whole sections of pea root, including the meristem, were subsequently confirmed to have detectable TIPs [23]. The absence of TIPs from the root tip/meristem region in *Arabidopsis* indicates that it may be difficult to generalize the findings obtained in this particular species to a universally applicable model for vacuolar identity. The expression mapping results in *Arabidopsis* point towards the presence of a single vacuolar compartment which differentiates either into a PSV or a LV during plant development. As TIPs too are developmentally regulated, the association of a particular isoform with a specific type of vacuole at any given developmental stage remains a useful indicator. For example, the association of TIP3;1 with PSVs and of TIP1;1 with LVs still holds true and is fully compatible with earlier findings [8].

Figure 1 | A map of TIP expression in *Arabidopsis* root tissues

The expression map is based on TIP–YFP localization data [20]. The root anatomy diagram was taken and modified from the eFP Browser interface [32].

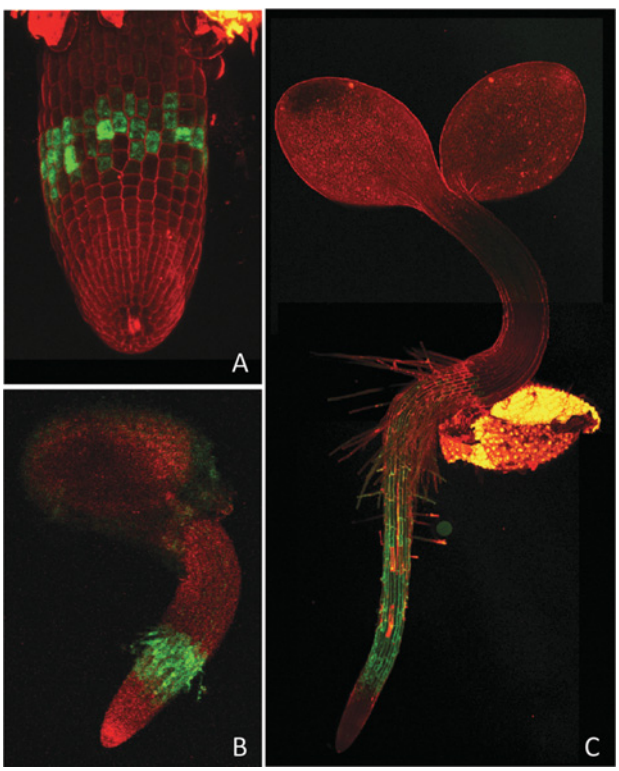


TIP targeting

Despite recent advances in TIP localization and function, remarkably little is known about the route(s) that sort

Figure 2 | **Root-specific expression of TIP4;1**

Germinating seeds and seedlings of transgenic plants expressing TIP4;1–YFP (green) were stained with propidium iodide (red) and visualized by confocal microscopy. The images are the maximal projection of 16 optical *z* sections (4 μm step-size). The image in (**C**) was assembled from three adjacent *z*-stacks.



TIPs to the tonoplast, or about specific tonoplast sorting signals on these proteins. The most abundant experimental information is available for TIP3;1. Early pulse–chase experiments on mesophyll protoplasts from transgenic tobacco overexpressing TIP3;1 showed that TIP3;1 can reach the tonoplast in a route that is insensitive to brefeldin A treatment, indicating that it may not involve trafficking through the Golgi complex [25]. These findings were confirmed by transient expression of HA (haemagglutinin)-tagged TIP3;1 [26]. Moreover, addition of an N-glycosylation sequon to a luminal loop of TIP3;1 resulted in the protein being glycosylated, but the N-linked glycan remained sensitive to endo H (endoglycosidase H) digestion. This indicates further that the protein had not visited the Golgi complex, where endo H resistance is acquired [26]. These findings were extended further by the observation that TIP3;1 (and TIP3;2) contains a C-terminal extension which is responsible for its Golgi-independent trafficking [17]. In a yeast two-hybrid assay, this C-terminal sequence was found to interact with two novel proteins, AtSRC2 (*Arabidopsis thaliana* soya bean gene regulated by cold 2), a type II membrane protein that moves from the ER (endoplasmic reticulum) together with TIP3;1, and AtVAP (*Arabidopsis thaliana* vesicle-associated protein), that localizes to the vacuole. It appears that deletion of the C-terminal tail causes TIP3;1 to follow a different route to the vacuole [17]. These experiments were performed by transient expression in a heterologous system (tobacco suspension culture cell protoplasts), where TIP3;1 is unlikely to be present [6,21].

Very little is known about the targeting of other TIP isoforms. The availability of transgenic plants with tagged TIPs expressed at quasi-native levels may provide a starting point for this study. It should, however, be possible to further improve reliability by expressing native TIP fusions in their corresponding knockout backgrounds and by producing fusions both at the N- and the C-terminus to rule out mistargeting resulting from the masking of otherwise exposed sorting signals [27]. For the fine dissection of the TIP-targeting process, however, heterologous expression may still represent a necessary alternative. Whenever a TIP has been fused to a fluorescent protein, the result has been delivery to the tonoplast of a single vacuolar type [15,16,19–22,28,29]. Be it a mistargeting artefact or true localization, there do not seem to be multiple vacuole-like compartments in plant cells that can be differentially highlighted using TIPs. Small structures other than the central vacuole and the ER have been occasionally highlighted [20], but their nature still awaits characterization.

Localization versus function?

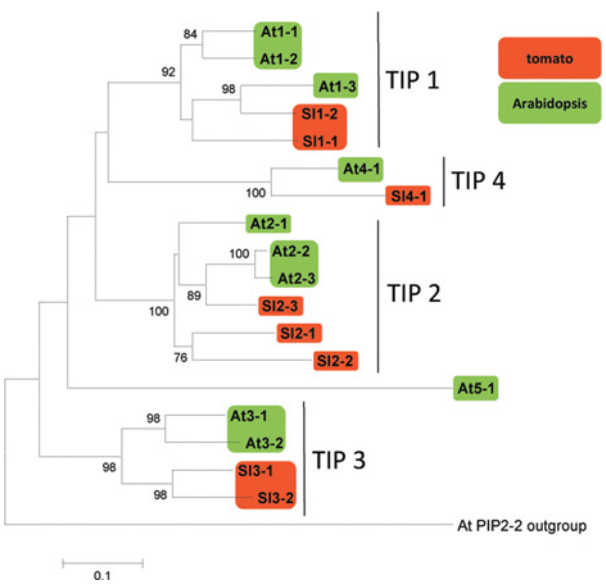
So far, the genetic knockout of TIPs has yielded no obvious phenotype. Insertional inactivation of TIP1;1 [16] and of both TIP1;1 and TIP1;2 resulted in apparently normal plants [30]. T-DNA (transferred DNA) mutant lines for TIP2;1, TIP2;2, TIP2;3, TIP4;1 and TIP3;2 also show normal growth and development under standard growth conditions (S. Gattolin and L. Frigerio, unpublished work). In the light of the expression map shown in Figure 1, this lack of phenotype can be explained by redundancy. Even when the abundant TIP1;1 is missing, both roots and leaves have at least two other remaining TIP isoforms. Given their different tissue localization, it is unlikely that the closely related isoforms TIP1;1 and TIP1;2 would be redundant (Figure 1). Although the lack of TIP1;1 could be compensated by other isoforms, one would predict a phenotype for the TIP1;2 knockout, although this could be subtle or not evident under laboratory growth conditions.

Beside the use of TIPs as vacuolar markers, which as we have seen is potentially flawed, or limited to closely related plant species, what is the benefit of knowing where *Arabidopsis* TIPs are located? The TIP family appears to be highly conserved in higher plants. As far as a major water-demanding crop plant such as tomato is concerned, the structure of the gene family is strikingly close to that of *Arabidopsis* (Figure 3). It is therefore likely that functional information obtained in *Arabidopsis* may be rapidly translatable into commercially relevant Brassicaceae and Solanaceae. As discussed above, however, this may not extend to other crops such as legumes.

A recent report shows that simply up-regulating SlTIP2;2 (*Solanum lycopersicum* TIP2;2) has a beneficial effect on water

Figure 3 | High conservation of the TIP family between *Arabidopsis* and tomato

The amino acid sequences of the *Arabidopsis thaliana* and *Solanum lycopersicum* TIP family members were aligned with ClustalW. The tree was produced with MEGA4.1 using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analysed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. Bootstrap test results are shown where higher than 50. *Arabidopsis* PIP2;2 was used as an outgroup.



usage by this plant and also results in larger fruits [31]. Modulating the levels of other root-specific isoforms may contribute further to improving these characteristics.

Outstanding questions

The description of the sites of expression of TIPs is only the starting point for a thorough functional investigation of both TIP targeting and function. Several questions are currently unanswered: what are the signals that sort different TIPs to the tonoplast and what are their sorting routes? Do some TIPs visit the Golgi complex? What proteins do TIPs interact with en route to the tonoplast? Can we use TIPs to study vacuolar biogenesis? For example, where do the TIPs localize when cells regenerate vacuoles after evacuolation? Would they accumulate in the early secretory pathway or be redirected to the plasma membrane? Several questions are also outstanding on TIP function: what is the solute specificity of each TIP? What is the role of each isoforms? Is there a separate role for individual isoforms/complexes or are they just providing sufficient redundancy to ensure that cellular water homoeostasis remains under control? It is likely that several of these exciting questions will be addressed in the near future.

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