Mapping of tonoplast intrinsic proteins in maturing and germinating Arabidopsis seeds reveals dual localisation of embryonic TIPs to the tonoplast and plasma membrane

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Running title: seed- specific tonoplast intrinsic proteins

Abstract

We have mapped the expression of the tonoplast intrinsic protein (TIP) gene family members in Arabidopsis seeds by fluorescent protein tagging of their genomic sequences and confocal microscopy. Three isoforms (TIP1;1, TIP2;1 and TIP2;2) have distinct patterns of expression in maternal tissues (outer integuments and placento-chalazal region). Two isoforms, TIP3;1 and the previously uncharacterised TIP3;2, are the only detectable TIPs in embryos during seed maturation and the early stages of seed germination. Throughout these developmental stages, both isoforms co-locate to the tonoplast of the protein storage vacuoles, but also appear to label the plasma membrane. Plasma membrane labelling is specific to TIP3;1 and TIP3;2, is independent of the position of the fluorescent protein and appears to be specific to early seed maturation and early germination stages. We discuss these results in the context of the predicted distribution of aquaporins in Arabidopsis seeds.

Keywords: Aquaporin, vacuole, tonoplast, intrinsic protein, Arabidopsis, seed, embryo, protein storage, endosperm, endoplasmic reticulum, fluorescent protein.

Introduction

Tonoplast intrinsic proteins (TIPs) are tonoplast-located aquaporins which function as transmembrane channels for water and small, uncharged solutes. Research in the last 15 years has led to insights on the structure, function and regulation of TIPs (for recent reviews see Heinen et al., 2009; Maurel et al., 2009; Wudick et al., 2009). Expression of several TIP isoforms has been mapped by a variety of approaches (reviewed in Wudick et al., 2009). Some TIP isoforms have been used extensively as vacuolar markers (reviewed in Frigerio et al., 2008; Gattolin et al., 2010), and antibodies against TIP isoforms were used to discriminate between different vacuolar types (Paris et al., 1996; Jauh et al., 1998; Jauh et al., 1999). Immunofluorescence experiments performed in root tips of different species revealed that alpha-TIP (now renamed TIP3;1; Johanson et al., 2001) and gamma-TIP (TIP1;1) preferentially label protein storage vacuoles (PSV) and lytic vacuoles, respectively. TIP3;1 is indeed found in seeds and is a useful marker for the PSV tonoplast (Hunter et al., 2007). The association of given TIP isoforms with a specific vacuolar type contributed to the formulation of the 'multivacuole hypothesis' (Jauh et al., 1999).

The Arabidopsis genome encodes 10 TIP isoforms. The functions, or even the localisation of some of these isoforms, in particular at the tissue/organ level, have not yet been elucidated in detail. We have recently undertaken a systematic analysis of TIP expression and distribution in the vacuolar system of *Arabidopsis thaliana*. By using fluorescent protein fusions to the genomic sequences of several TIP family members, we showed that, in roots and leaves, different TIPs have distinct patterns of tissue and organ-specific expression, but tend to coexist on the same tonoplast (Hunter et al., 2007; Gattolin et al., 2009).

In the present work we have focussed on the TIP isoforms which are predicted to be expressed in seed tissues, both during maturation and germination. We show that TIPs 1;1, 2;1 and 2;2, normally regarded as being confined to vegetative tissues, are also expressed in

seed maternal tissues (seed coat integuments and placento-chalazal region). In contrast, TIP3;1, and the previously uncharacterised TIP3;2, are the only genuinely endosperm- and embryo-specific TIP isoforms in Arabidopsis. We report that the PSV-specific TIP3;1 and the 'vegetative' TIP1;1 isoforms coexist in seedlings for up to 7 days from imbibition, and co-localise to the same tonoplasts throughout. By performing detailed developmental time courses, we also show that both the embryo-specific TIP3;1 and TIP3;2 appear to localise to both the plasma membrane (PM) and the PSV tonoplast during seed development and germination.

Results and discussion

'Vegetative' TIPs are expressed in maternal seed tissues

We have previously generated transgenic Arabidopsis lines expressing the genomic sequences (including promoters) of several 'vegetative' TIPs fused to YFP or RFP (Gattolin et al., 2009). After analysis of these lines we were able to identify three TIP isoforms (TIP1;1, TIP2;1 and TIP2;2) which are also expressed in seeds.

We have shown that TIP2;1 is restricted to the base of the lateral root primordium in hypogean organs (Gattolin et al., 2009). In seeds, however, TIP2;1 is expressed in the outer integument of the seed coat (Fig. 1a). Within this tissue, TIP2;1 localises to the tonoplast of seed coat cells (Suppl. Fig. S1a). In contrast, TIP2;2 is detected in the cell layer immediately beneath the layer labelled by TIP2;1-YFP, which we interpret as being the second layer of the outer integument (Windsor et al., 2000; Fig. 1b, arrowhead; compare with panel a: note the gap between the seed exterior, as highlighted by propidium iodide, and the YFP signal. See also higher magnification in Suppl. Fig. S1b). These expression patterns confirm the detection of TIP2;1 and TIP2;2 transcripts in seeds (Schmid et al., 2005; Frigerio et al., 2008) but clearly indicate that these isoforms are restricted to the seed coat. While TIP localisation

in the seed coat has not been observed previously, it is possible to speculate that their presence may help facilitate mucilage production and water exchanges during seed maturation (Windsor et al., 2000).

We and others have previously shown that TIP1;1 is the most widespread TIP isoform in Arabidopsis, being present both in roots and aerial parts (Ludevid et al., 1992; Hunter et al., 2007; Beebo et al., 2009; Gattolin et al., 2009). Analysis of seeds from transgenic plants expressing TIP1;1-YFP under its native genomic sequences shows that TIP1;1 expression is limited to the placento-chalazal region, where vascular tissues terminate (Fig 1c).

We previously generated transgenic plants co-expressing TIP2;2-YFP and TIP1;1-RFP (Gattolin et al., 2009). While these two isoforms coexist in root tissues, their expression patterns appear to be mutually exclusive in seeds (Fig. 1d). These results provide tissue specific information on TIP isoform distribution in seeds and indicate that three of the vegetative TIP isoforms (TIP2;1, TIP2;2 and TIP1;1) are expressed in distinct maternal seed tissues, but not in the embryos.

TIP3;1 and 3;2 are embryo and endosperm specific

Developmental microarray datasets indicate that transcripts of TIP3;1 and TIP3;2 (henceforth referred to collectively as TIP3) are only detected in maturing and mature seeds (Schmid et al., 2005; Winter et al., 2007; Le et al., 2010). This has been confirmed experimentally for TIP3;1 (Ludevid et al., 1992; Hunter et al., 2007). We produced translational fusions between the genomic sequence of TIP3;1 and YFP, and between TIP3;2 and mCherry. We then analysed seeds from transgenic plants co-expressing both chimeric proteins. Expression of both TIP3 was indeed only detected in the endosperm and embryo: in embryos from mature seeds, TIP3;1 and TIP3;2 colocalised on the tonoplast of PSV (Fig. 2). TIP3;1-YFP and, to a lesser extent, TIP3;2-mCherry also highlighted a network-like structure, which most likely

represents the endoplasmic reticulum (ER). This ER location is confirmed by the labelling of the nuclear envelopes (Fig 2 a-c, asterisks). In the endosperm, TIP3;1 and 3;2 also colocalised to the PSV tonoplast (Fig. 2, g-i).

According to transcriptomic data, the synthesis of TIP3 transcripts is confined to seed maturation (Schmid et al., 2005; Vander Willigen et al., 2006; Frigerio et al., 2008). To measure how long TIP3 protein expression persists during germination, we produced double transgenic plants expressing TIP3;1-YFP and TIP1;1-RFP and performed a germination time course (Fig. 3, panels a-h). TIP3;1-YFP is abundant and fluoresces very brightly in dry seeds (Hunter et al., 2007) and during imbibition (Fig. 3a). Fluorescence persists during radicle emergence (Fig. 3,b-c). As the radicle breaks through the seed coat, the part of the endosperm that eases radicle emergence lies outside the seed after it has been shed, and is still strongly labelled by TIP3;1 (Fig. 3d). TIP3;1 fluorescence remains detectable for several days after radicle emergence (Fig. 3, e-g). TIP1;1 is present in all seedling tissues apart from the root tip (Gattolin et al., 2009) but not in embryos (Hunter et al., 2007). While TIP3;1 is clearly dominant during the first 72 h from imbibition (Fig. 3, a-f), TIP1;1 expression begins around 3-4 days and is initially detectable in the root elongation zone (Fig. 3f), whence it gradually extends to the cotyledons (Fig. 3g), eventually replacing TIP3;1 around a week after germination (Fig. 3h). During this developmental transition, the two TIPs happen to co-express in some seedling tissues for about 2 days (Fig 3, f-g). At higher magnification, it appears that during their co-expression, TIP3;1-YFP and TIP1;1-RFP mostly label the same tonoplast of the central vacuoles (fig. 3, panels i-k and l-n). This result confirms and extends our previous finding that TIP3;1 and TIP1;1 overlapped only briefly (Hunter et al., 2007) and indicates that in cells coexpressing TIP3;1 and TIP1;1, only a single vacuole is labelled by these isoforms.

Another conclusion arising from our developmental analysis is that TIP3;1 and 3;2 are the only TIPs detectable during the first two phases of seed water uptake during germination (Bewley, 1997; Vander Willigen et al., 2006; see further below). TIP1;1 expression commences only after during phase III, when tissue expansion is well under way.

TIP3;1 and 3;2 localise to both PSV and the PM during seed maturation and germination

TIP3;1 and TIP3;2 colocalise to the PSV tonoplast in dry seeds (Fig. 2). In order to assess whether expression of the two isosorms differs in any way during seed development, we performed a time course of seed maturation by imaging embryos taken from seeds belonging to siliques at different stages of maturation within the same plant (see Fig. S2 for a graphic representation of the sampling procedure). Figure 4 shows that TIP3;2-mCherry is detectable throughout the embryo at the early torpedo stage (Fig. 4a), while TIP3;1-YFP expression is initially only detectable in the cotyledons (Fig. 4, a-b), then spreading through the cotyledons towards the radicle (Fig. 4c). As soon as TIP3;1-YFP appears in patches of cotyledonary cells, the protein colocalises with TIP3;2-mCherry on structures that resemble both the tonoplast and the plasma membrane (Fig. 4, d-f).

In order to further characterise the intracellular localisation of TIP3;2 during maturation, and because of its earlier expression compared to TIP3;1, we imaged maturing embryos from plants expressing TIP3;2-YFP (Fig. 5). To minimise potential artefacts due to overexpression (Moore and Murphy, 2009), we inserted the genomic TIP3;2-YFP construct into a *tip3;2* T-DNA insertion mutant, in which the endogenous TIP3;2 transcripts are undetectable (Suppl. Fig. S3). These knockout mutant plants have no obvious phenotype. Given the overlap and common location of TIP3;1 and TIP3;2, it is likely that this results from redundancy between the two TIP3 isoforms.

As shown in fig. 5, TIP3;2-YFP appears at early torpedo stage and highlights both the vacuolar membranes and an outer membrane which is clearly distinct from the tonoplast (Fig. 5, b-c). We confirmed that this is the plasma membrane by co-localisation of the TIP3;2-YFP signal with the styryl dye FM4-64 (Fig. 7, a-c). The signal at the PM persists throughout seed maturation (Fig. 5, panels a through to l) but fades significantly, to become barely detectable in mature, drying seeds (Panels m-o). This is in agreement with our previous observations of mature seeds (Fig. 2; Hunter et al., 2007). A very similar dual localisation was also observed during a maturation time course of seeds expressing TIP3;1-YFP (Suppl. Fig. S2). In terms of subcellular localisation, therefore, TIP3;1 and TIP3;2 appear to be very similar. The fact that both TIP3 isoforms only appear to label the tonoplast in mature seeds may explain why this dual localisation was not observed in previous studies performed in mature or dry seeds (Gillespie et al., 2005; Hunter et al., 2007). In some cases, however, anti-alpha TIP (TIP3;1) was reported to label both PSV tonoplasts and a membrane surrounding clusters of PSV which is likely to be PM (Poxleitner et al., 2006).

We asked whether dual localisation of TIP3 also occurred during seed germination. Both TIP3;1-YFP and TIP3;2-YFP still presented labelling of both the PM and the tonoplast in germinating seeds (data not shown). It is possible that C-terminal fusion of YFP masks potential targeting signals in the cytosolic tail of TIP3;1 (Oufattole et al., 2005). Therefore we generated plants expressing N-terminally YFP tagged TIP3;1 (YFP-TIP3;1, in Columbia ecotype background) or TIP3;2 (YFP-TIP3;2, in *tip3;2* background) and performed a detailed time course of germination (Fig. 6). We took care to use exactly the same CLSM settings throughout the experiment. During the first 24 h no major changes were observed. At 24 h TIP3 are mostly localised to the tonoplast but both ER and plasma membrane are detectable (Fig. 6, a-d). The PM localisation persists through the next 24 h (Fig. 6, e-h). However, as the radicle emerges from the seed (60h), plasma membrane labelling is greatly reduced, while

remaining detectable in the still-seed enveloped cotyledons (Fig. 6, panels m and o). Once the seedling has fully emerged, TIP3 only label the tonoplast of the expanding central vacuoles but no residual PM labelling is visible. This situation persists until TIP3 expression ceases (data not shown). These results indicate that dual tonoplast/plasma membrane localisation is independent of the position of the YFP tag, as both C-terminal and N-terminal YFP fusions to TIP3 showed the same localisation pattern. This rules out the possibility that C-terminally appended YFP may be masking potential targeting signals in the cytosolic tail of the TIP3. Moreover, dual labelling is specific to seed maturation and the early phases of germination/tissue expansion as it seems to cease when the seed dries and when the seedling has fully emerged from the seed, respectively.

Dual PM-tonoplast localisation is specific to TIP3

The fact that PM labelling appears to be both developmentally regulated and independent of YFP position in the chimeric proteins seems to indicate that it is not artefactual. It is however possible that it may still represent a mislocalisation artefact attributable to the presence of the YFP tag. If this were the case, we would expect other YFP-labelled TIP isoforms to label the PM in maturing/germinating seeds. Therefore we placed YFP-TIP1;1 under the control of the TIP3;2 promoter and generated transgenic plants in *tip3;2* background. When embryos from maturing seeds were analysed by CLSM, tip3;2pro::YFP-TIP1;1 was exclusively detected at the tonoplast of the PSV, but never at the plasma membrane (Fig. 7, panels g-i). This result strengthens our hypothesis that the observed developmentally-specific, dual intracellular localisation is not a generic TIP mislocalisation artefact due to the presence of YFP. Therefore our data indicate that the seed-specific TIP3 are unique in their ability to reach the plasma membrane as well as the tonoplast.

In this report we have mapped the expression of all the TIPs which are predicted to be present in Arabidopsis seeds. Our results identify two subsets, one of embryo-specific TIPs (TIP3) and one of TIPs which reside in maternal tissues. In particular, isoforms TIP2;1 (delta-TIP) and TIP1;1 (gamma-TIP), appear to be confined to specific maternal structures (the outer integument and the placento-chalazal region) and excluded from embryos. Previous immunofluorescence experiments showed that anti delta-TIP antibodies label the PSV membranes in cotyledons of germinating Arabidopsis (Poxleitner et al., 2006) and tobacco (Jiang et al., 2000) seeds. As we have now mapped the expression of all three TIP2 isoforms (Gattolin et al., 2009 and this study) and found that none is present in embryos, it is therefore unlikely that the delta-TIP antibody cross-reacts with a different TIP2 isoform, as previously hypothesised (Hunter et al., 2007). Immunohistochemistry also found that anti gamma-TIP antibodies label structures internal to the PSV in *Brassica napus* (Gillespie et al., 2005) and globoids in tobacco, tomato and snapdragon seed PSV (Jiang et al., 2001). Again, having now analysed the pattern of expression of TIP1;1 and TIP1;2 in Arabidopsis (Gattolin et al., 2009), we can rule out crossreactivity, as TIP1;2 was not detected in embryos (data not shown). It is possible that our TIP1s and TIP2s chimeric YFP fusions are expressed in embryos at levels which are below detection. The expression patterns we observed, however, match very closely the developmental transcriptomic data collected for each TIP isoform (Hunter et al., 2007; Frigerio et al., 2008).

We have also studied the expression of TIP3;2, which was not previously described. TIP3;1 and 3;2 have very similar expression patterns (with a delay in TIP3;1 appearance: Fig. 4) and, as far as their subcellular localisation is concerned, can be considered to be overlapping. The fact that the insertional knockout of TIP3;2 has no apparent phenotype likely indicates that these isoforms, whose sequence identity is very high (82%), are functionally redundant.

Apart from the TIP3 isoforms, TIP isoform overlap in seeds is negligible. When the TIP3 are present at the same time as TIP1;1 during germination, their intracellular location seems to coincide. The co-localisation of TIP3;1 and TIP1;1 mirrors previous data obtained in BY-2 cells (Mitsuhashi et al., 2000) and suggests that, if Arabidopsis cells do contain multiple vacuolar types, TIP3 and TIP1;1 - when fused to XFP and expressed at near-native levels - may not be useful markers to distinguish them.

In addition to their expected tonoplast localisation, XFP fusions to TIP3 also appear to label the plasma membrane. Our control experiments seem to rule out artefactual mislocalisation. We do not yet know whether this is the result of dual targeting or of vectorial transport between the plasma membrane and the tonoplast. While our data show that when synthesis of the TIP3 ceases - in drying seeds or after germination - PM labelling fades well before tonoplast labelling, suggesting a vectoriality of transport, it is also possible that TIP3 are turned over more rapidly at the plasma membrane, therefore conveying the impression of directional trafficking between PM and tonoplast. Clearly much work is needed to clarify this issue. In any case, while the elucidation of the targeting routes and signals of the TIP3 is beyond the scope of this initial report, it is reasonable to ask what the physiological role of the dual localisation of TIP3;1 and TIP3;2 may be.

Water uptake during seed germination is a triphasic process, divided in: phase I, rapid initial uptake by dry seeds (1-3 h in Arabidopsis); phase II, plateau, and phase III, further increase of water uptake during tissue expansion (Bewley, 1997; Manz et al., 2005). As plasma membrane intrinsic proteins (PIP), but not TIP, are generally found at the PM, one would expect extensive involvement of PIP in seed water uptake. Intriguingly, however, both microarray data (Schmid et al., 2005) and macroarray experiments (Vander Willigen et al., 2006) indicate that, out of 13 PIPs encoded by the Arabidopsis genome (Johanson et al.,

2001), only 3 isoforms (PIP1;2, PIP1;4, PIP1;5) have detectable transcripts in seeds (Suppl. Figure S5). Moreover their mRNA levels decrease dramatically after torpedo stage, concurrently with the sharp rise in TIP3 expression. Vander Willigen et al (2006) observe that, during germination, TIP3 protein levels are very high but there is no new gene transcription, indicating that TIP3 are translated from extant mRNA. Concurrently, PIP1;2 and PIP2,1 are being highly transcribed but, as is the case for TIP1;1 (Fig. 3), their relative polypeptides do not become detectable until about 60 hours after germination, i.e. well into phase III of water uptake. As pointed out by these authors, it is puzzling how such low concentrations of PIP protein during the early phases of germination can achieve basic transcellular water transport in the seed (Vander Willigen et al., 2006).

In the light of our results, we speculate that TIP3 may be the only aquaporins mediating seed water intake, and that the presence of TIP3 at the plasma membrane may compensate for the absence (or low concentration) of PIP. It should be noted, however, that gene expression analysis of the MIP superfamily based on microarray data in Arabidopsis seeds (Schmid et al., 2005; Le et al., 2010) shows that the nodulin 26-like aquaporin NIP1;2 is also expressed in maturing seeds (albeit at a significantly lower levels than TIP3), with a developmental pattern that closely resembles TIP3 (Schmid et al., 2005; Le et al., 2010; Suppl. Fig. S5). Nothing is known about NIP1;2 function or localisation, but its closest homologue, NIP1;1, is localised to the plasma membrane (Kamiya et al., 2009). It is therefore possible that NIP1;2 also participates with TIP3 in a hypothetical, developmentally controlled compensation/complementation of PIPs.

In conclusion, we have characterised the entire complement of TIPs present in Arabidopsis seeds and found remarkable tissue-specificity of different isoforms. We also showed that none of the seed TIP combinations allow us to identify distinct vacuolar types in maturing or germinating seeds. Finally, the observation that the embryo-specific TIP3 isoforms also

localise to the plasma membrane paves the way to a detailed study of their intracellular targeting.

Methods

Recombinant DNA and transgenic plants

The construction of TIP1;1-YFP, , TIP2;1-YFP, TIP2;2-YFP, TIP2;2-YFP/TIP1;1-RFP and TIP3;1-YFP has been described previously (Hunter et al., 2007; Gattolin et al., 2009). For the C-terminal TIP3;2 YFP and mCherry fusions, the genomic sequence of TIP3;2 (At1g17810), including 817bp of the promoter (the whole intergenic region), was amplified using Phusion proofreading Taq polymerase (Finnzymes, Espoo, Finland), sequenced, and inserted into the KpnI and XhoI sites of pGREENII0029 (Hellens et al., 2000) containing either a YFP-OCS (Gattolin et al 2009) or a mCherry-OCS fragment (amplified from pKS391 pFA6a-mCherry-natMX6, http://www-rcf.usc.edu/~forsburg/vectors.html). A SacI site in the forward primer allowed for the generation of TIP3;1-YFP/TIP3;2-mCherry co-expression constructs, as described in Gattolin et al (2009). For N-terminal YFP fusions, pGREENII0029 was also used for the cloning of the various fragments. The whole promoter regions of TIP3;1 (703bp, EcoRI/BamHI) and TIP3;2 (817bp, EcoRI/BglII) were amplified and fused to YFP (BamHI/XbaI), subsequently the genomic coding regions of TIP3;1, TIP3;2 and TIP 1;1 were amplified (XbaI/SacI) and translationally fused to the YFP coding sequence. These included the whole 279bp (TIP3;1), 308bp (TIP3;2) and 431bp (TIP1;1) 3'UTRs regions.

All constructs were introduced into *Arabidopsis thaliana* ecotype Columbia by the floral dip method (Clough and Bent, 1998). Transgenic plants were either selected on kanamycin or directly identified by YFP fluorescence of seeds under a stereomiscroscope.

Time courses

The seed maturation time course was performed by isolating embryo from seeds taken from siliques from individual plants, sampled at different distances from the first mature (shattering) silique (for a graphic representation see Suppl. Fig. 2).

For the germination time course, seeds were sterilised and plated on 0.5X MS medium, 0.8% agar and incubated at 4°C for 24 hours before being incubated at 25°C until complete seedling establishment. Starting from the 24 h point, embryos were dissected or seedlings separated from the ruptured seeds and imaged every 12 hours.

Confocal microscopy

Whole dry seeds or embryos dissected from maturing, dry or germinating seeds were mounted in sterile 0.5X MS medium (Murashige and Skoog, 1962) and visualised with a Leica TCS SP5 using a 10X air objective (NA 0,3), or 63X oil immersion objective (NA 1.4). In some cases seeds were preincubated for 2 min in 10 µg/ml propidium iodide or with FM4-64 (8 nM), diluted in 0.5X MS medium. YFP was excited at 514 nm and detected in the 525 to 550 nm range. RFP and mCherry were excited at 561 nm and detected in the 563 to 640 nm range. Propidium iodide was excited at 561 nm and detected in the 650 to 720 nm range. FM4-64 was excited at 514 nm and detected in the range 616 nm – 645 nm. Autofluorescence of the seed coat was visualised by exciting at 514 nm and detecting in the 680-750 nm range. Simultaneous detection of YFP and RFP, YFP and propidium iodide or YFP and mCherry was performed by combining the settings indicated above in the sequential scanning facility of the microscope, as instructed by the manufacturer. 3D reconstruction of z-stacks of optical sections was performed with the Leica LAS-AF Lite free software (Leica Microsystems, Germany).

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Figure legends

Figure 1. Expression of fluorescent TIP fusions in maternal tissues of *Arabidopsis* seeds.

Seeds from transgenic *Arabidopsis* expressing the indicated constructs were visualised by confocal microscopy and, where indicated, after staining with propidium iodide (PI). Each panel shows a single XY focal plane (central section), and the XZ (bottom section) or YZ (right section) projection of a stack of 40 optical sections. Green, YFP fluorescence. Red, propidium iodide fluorescence (a-c) or RFP fluorescence (d). Scale bars, 50 μm

Figure 2. Expression of TIP3 in Arabidopsis embryos and endosperm.

Embryos and endosperm from transgenic Arabidopsis co-expressing TIP3;1-YFP (green) and TIP3;2-mCherry (red) were visualised by confocal microscopy. Asterisks indicate the nuclei. Scale bars, 10 μm.

Figure 3. Developmental transition between TIP3;1 ans TIP1;1 during germination.

Seeds from transgenic Arabidopsis co-expressing TIP3;1-YFP (green) and TIP1;1-RFP (red) were plated, incubated for the indicated amounts of time and visualised by confocal microscopy. The blue colour in panels (a) to (d) indicates autofluorescence from the seed coat. Panels (i-k) and (l-n) show subcellular co-localisation of TIP3;1-YFP and TIP1;1-RFP at 96 and 120 h after plating, respectively.

Scale bars: a-d: 100 μm; e-h: 500 μm; i-n, 20 μm.

Figure 4. Co-expression of TIP3;1-YFP and TIP3;2-mCherry in Arabidopsis embryos

Mature embryos and endosperms were extracted from seeds taken from siliques at progressive stages of maturation of transgenic plants co-expressing TIP3;1-YFP (green) and TIP3;2-mCherry (red) and visualised by confocal microscopy. (a-c) projections of stacks of 17 optical sections. (d-f) single optical sections of regions of the cotyledon where TIP3;1 and TIP3;2 are coexpressed. Scale bars: a-c: 100 μm; d-f: 10 μm.

Figure 5. Dual localisation of TIP3;2-YFP during seed maturation.

Embryos were extracted from seeds taken from siliques at progressive stages of maturation of transgenic Arabidopsis *tip3;2* mutants expressing TIP3;2-YFP and visualised by confocal microscopy. Note that TIP3;2-YFP is visible both at the plasma membrane and tonoplast very early during maturation but is absent from the PM in drying seeds. Scale bars: a,d,g,j,m: 100 μm; b-c, e-f, h-i, k-l, n-o: 10 μm.

Figure 6. Dual localisation of YFP-TIP3;2 and YFP-TIP3;1 during seed germination.

Embryos were extracted from germinating seeds at the indicated times from plating and visualised by confocal microscopy. Note that YFP-TIP3 fluorescence is visible both at the plasma membraneand PSV tonoplast throughout germination but it fades from the plasma membrane after seedling emergence from the seed (60h). Scale bar, 5 μm.

Figure 7. Dual PM-tonoplast localisation is specific to TIP3.

Embryos were extracted from seeds taken from siliques at progressive stages of maturation of transgenic Arabidopsis expressing the indicated constructs and visualised by confocal microscopy. (a-c) co-localisation of TIP3;2-YFP with FM4-64 at the plasma membrane in early torpedo embryo (compare with Fig. 5, panels d-f).

(d-f), N-terminal fusion of YFP to TIP3;2 does not affect its dual localisation. (g-i) When TIP1;1-YFP is expressed in embryos under the control of the TIP3;2 promoter, it only localises to the tonoplast of the PSV, but not the plasma membrane. Scale bars, 10 μm.

Supplementary material

Supplementary Figure S1. Localisation of TIP2;1 at the outer seed coat.

Seeds from transgenic *Arabidopsis* expressing the indicated constructs were visualised by confocal microscopy. Note that TIP2;2 labels the cell layer immediately beneath the outermost layer of the outer integument (b). The image in (c) is a maximal fluorescence projection of 17 z-stacks. Red, propidium iodide. Scale bars, 20 μm. (d) Diagrammatic TIP expression map in maturing seed tissues. The image was copied and modified from http://seedgenenetwork.net/. While we have also detected TIP3 expression in the endosperm

during germination, we do not know whether this occurs at the developmental stage depicted in the diagram. This is reflected in the paler orange colour coding.

Supplementary Figure S2. Dual localisation of TIP3;1-YFP during seed maturation.

Embryos were extracted from seeds taken from siliques at progressive stages of maturation of transgenic Arabidopsis expressing TIP3;1-YFP (green) and visualised by confocal microscopy. Note that TIP3;1-YFP is visible both at the plasma membrane and tonoplast very early during maturation but it fades from the PM as seeds dry. Scale bars: a,d,g,j: 100μm; b-c, e-f, h-i, k-l: 10 μm.

Supplementary Figure S3. Characterisation of *tip3;2* knockout line.

(a) diagram (not drawn to scale) indicating the position of the T-DNA insertions within the *TIP3;2* gene. The mutant line has two t-DNAs inserted back-to-back within the second exon (blue). The insertion resulted in a 22-bp deletion between the two T-DNAs. the arrows indicate the positions of the T-DNA left borders as determined by DNA sequencing.

(b) RT-PCR analysis on total RNA extracted from mature seeds of wild-type, *tip3;2* knockout, and *tip3;2* complemented with YFP-TIP3;2. Note that while the TIP3;2 transcript has become undetectable in the *tip3;2* mutant, the transcripts of TIP3;1 are still present. The sequences of the primers used to detect specific target transcripts are also shown.

Supplementary Fig. S4. eFP browser and AtGenexpress expression predictions for PIP1;1-PIP1;5.

Anatomical expression maps were generated using the Arabidopsis eFP browser (Winter et al., 2007) (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Supplementary Fig. S5. eFP browser and AtGenexpress expression predictions for TIP3;1, TIP3;2 and NIP1;2.

Anatomical expression maps were generated using the Arabidopsis eFP browser (Winter et al., 2007) (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Note that maximal mRNA expression levels for NIP1;2 are at least 5- fold lower than those reported for the TIP3 .

Figure 1

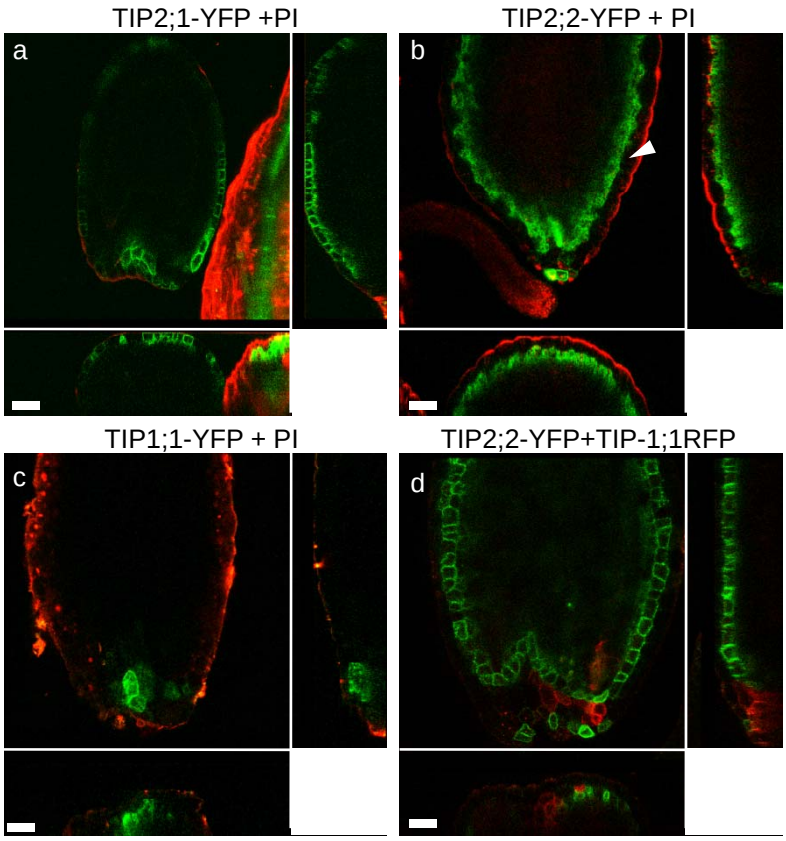
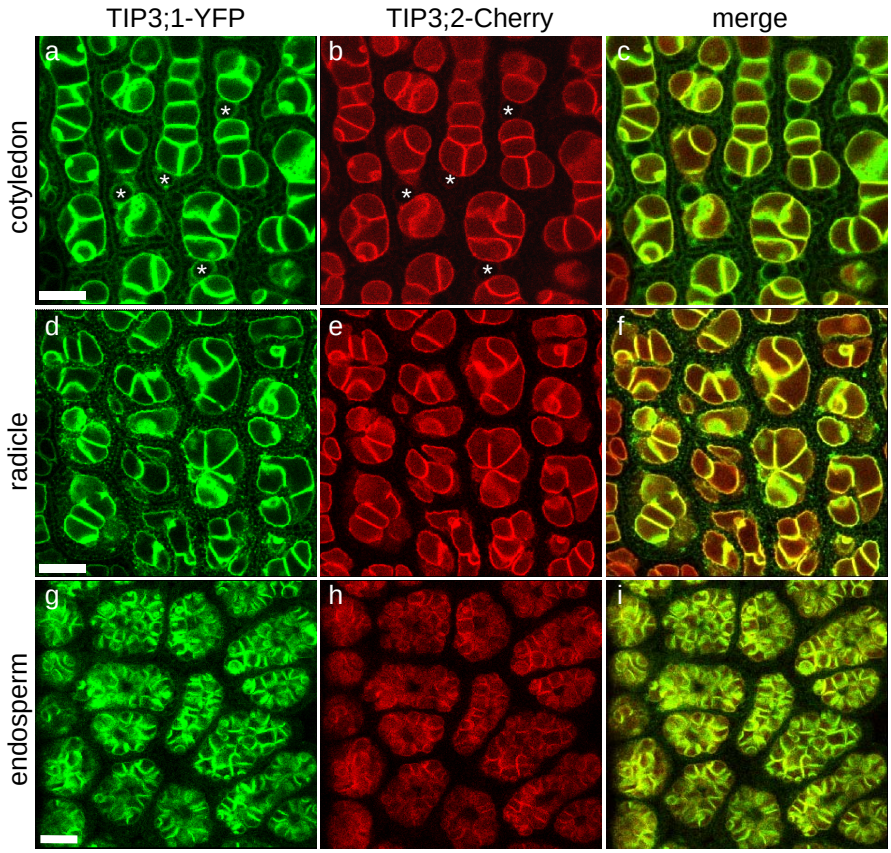


Figure 2



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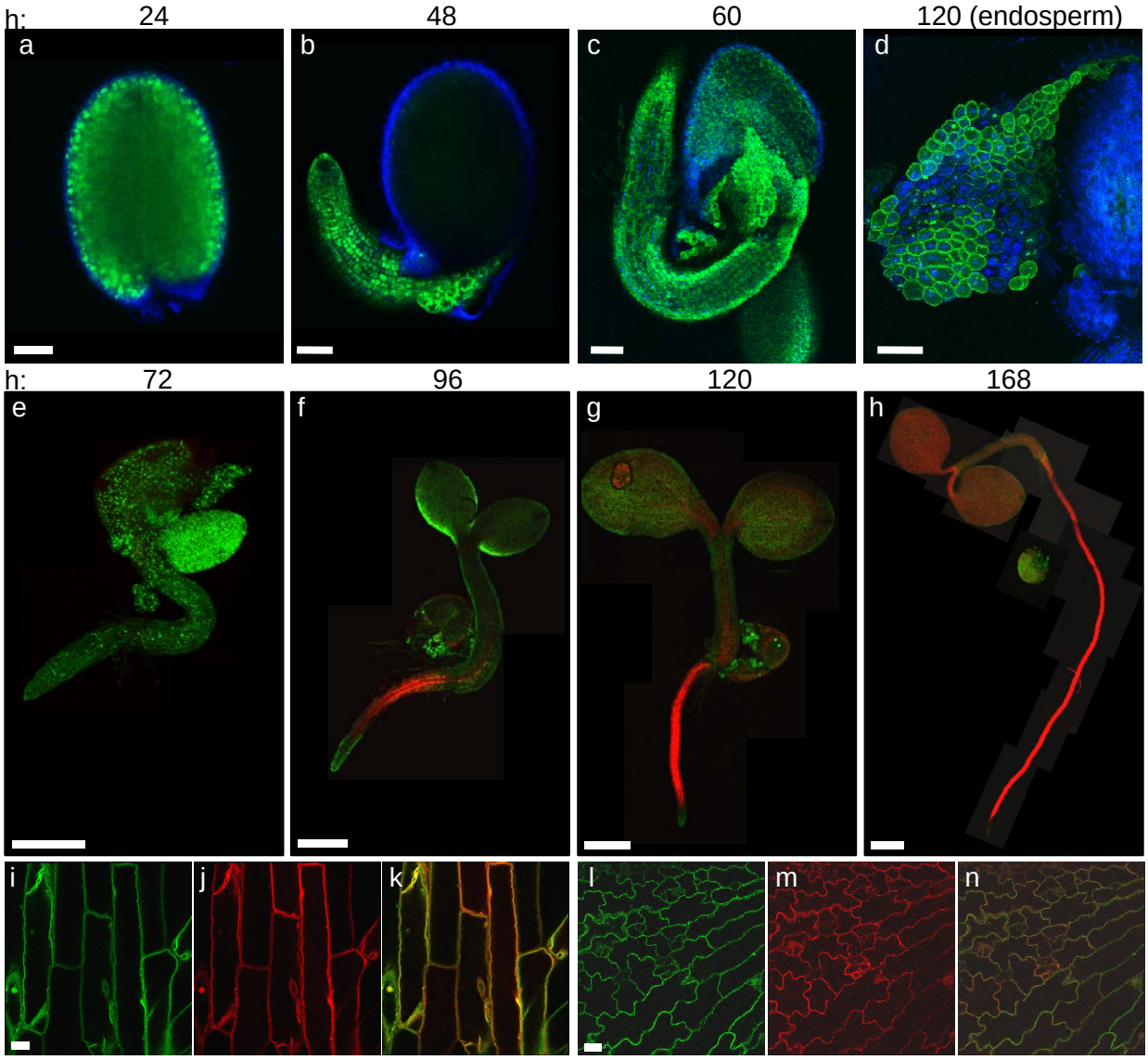

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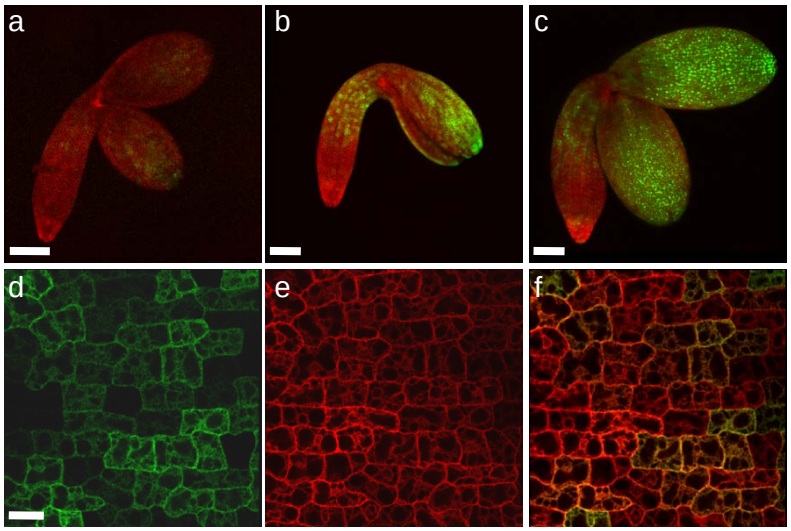

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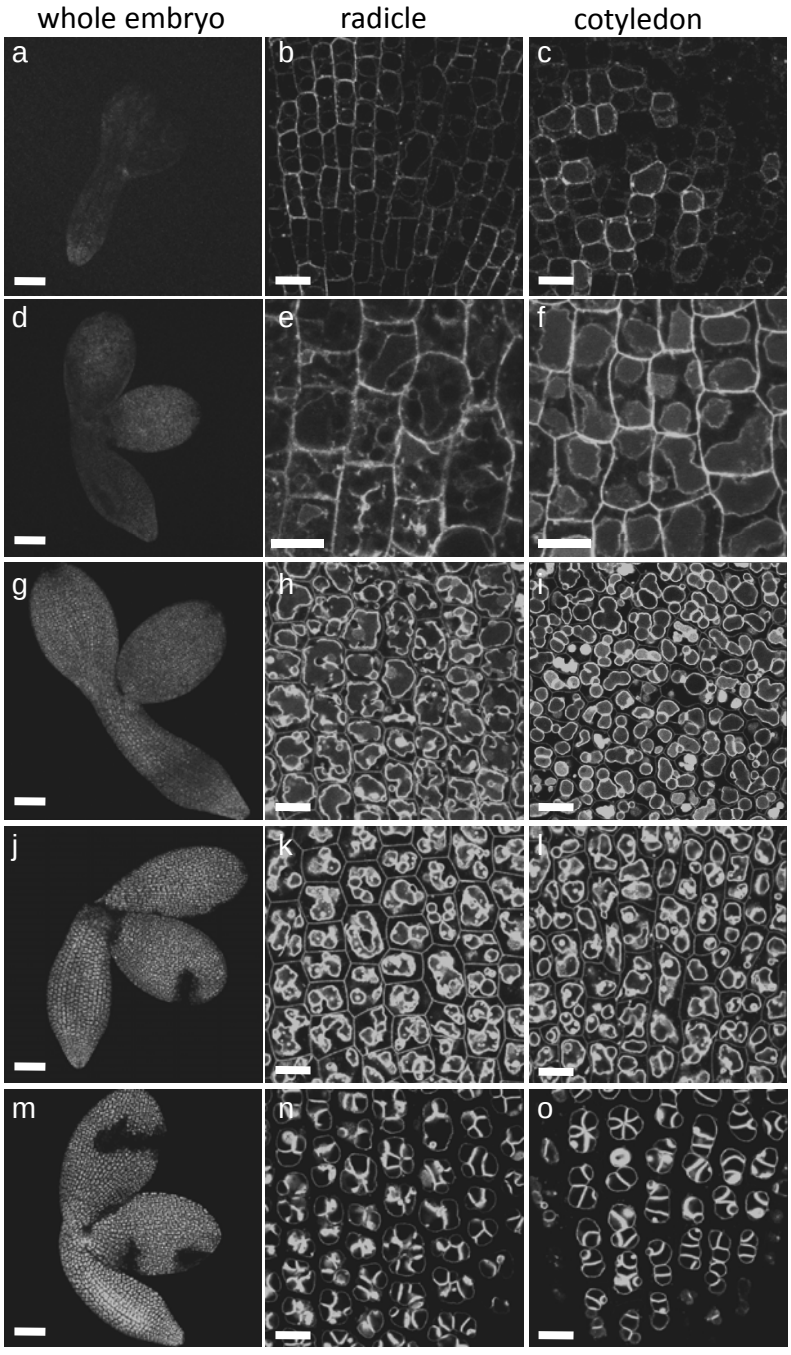

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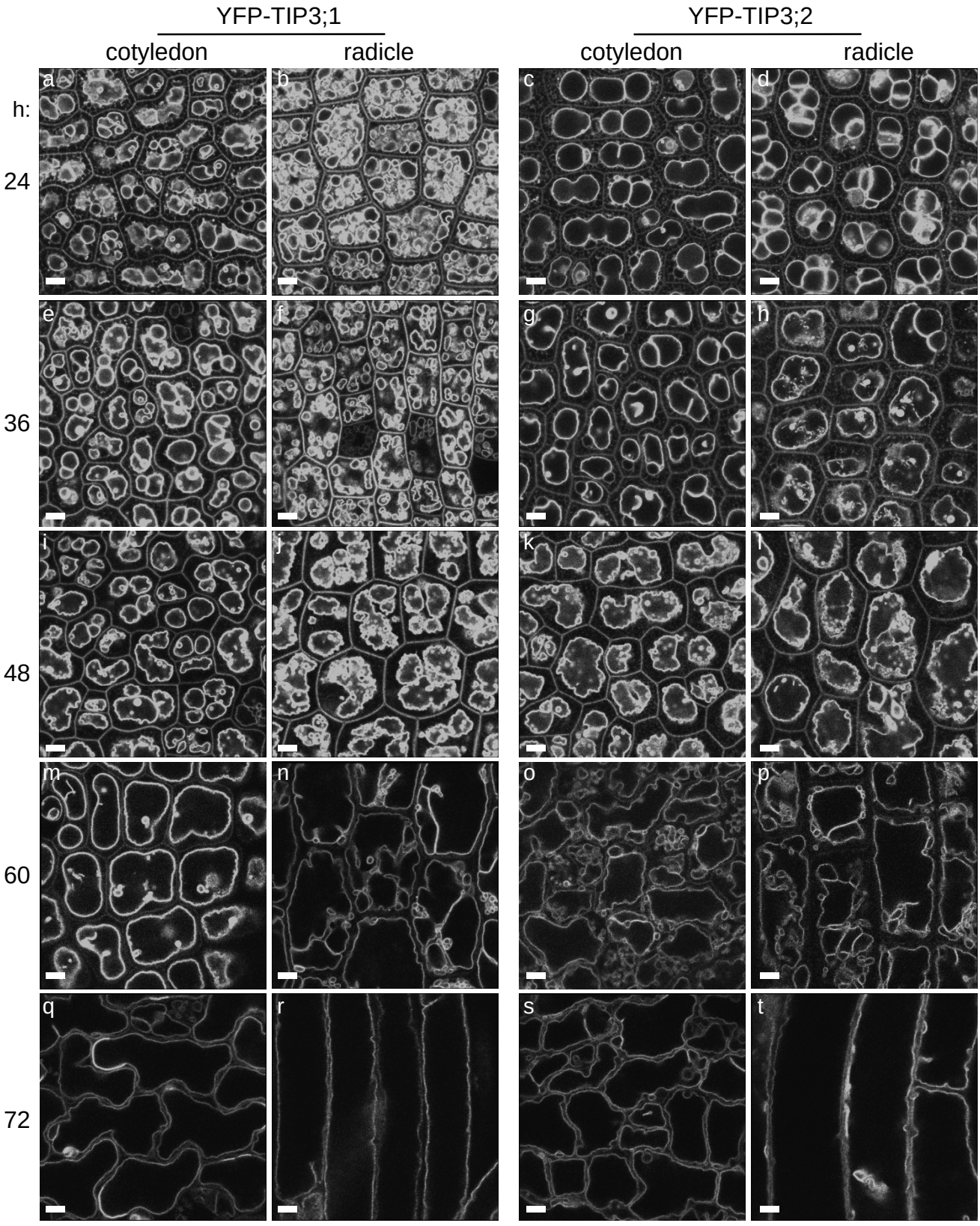
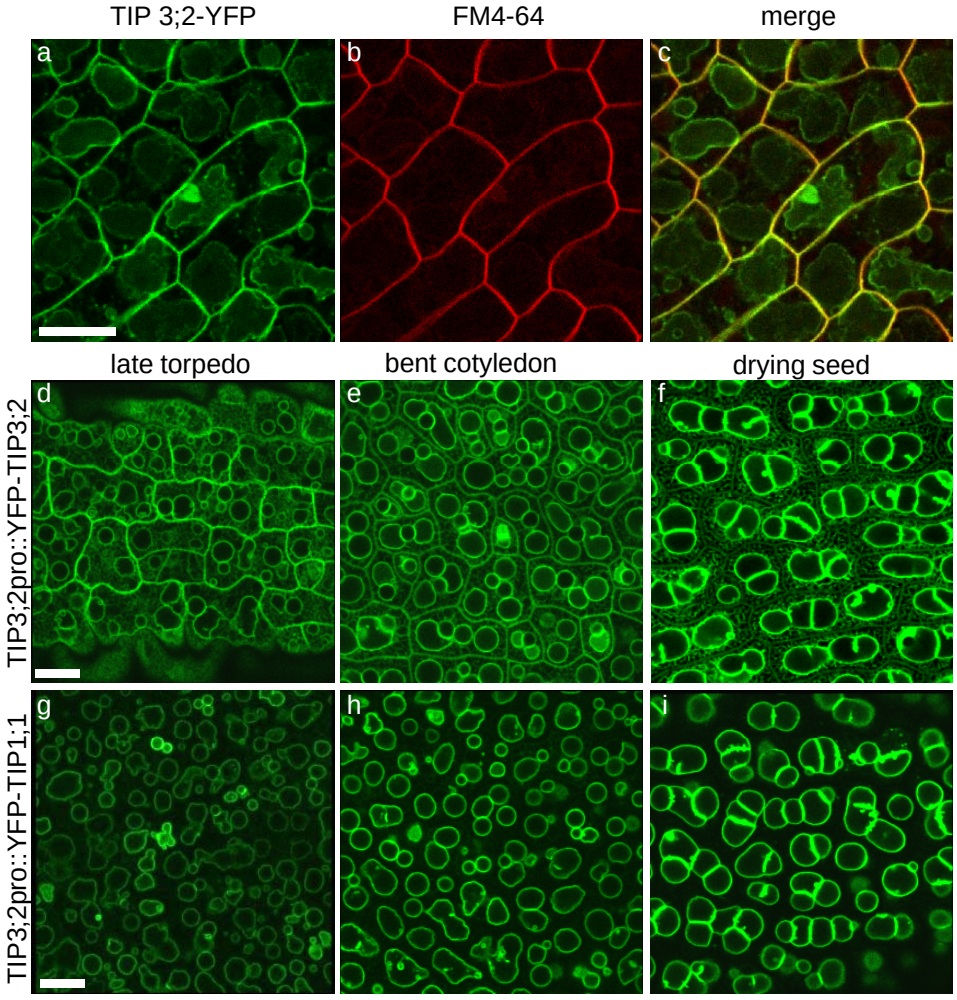
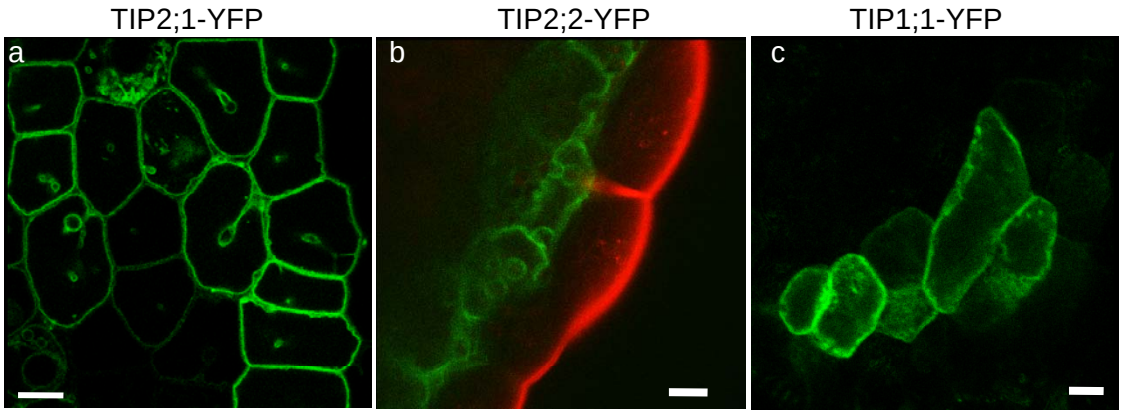
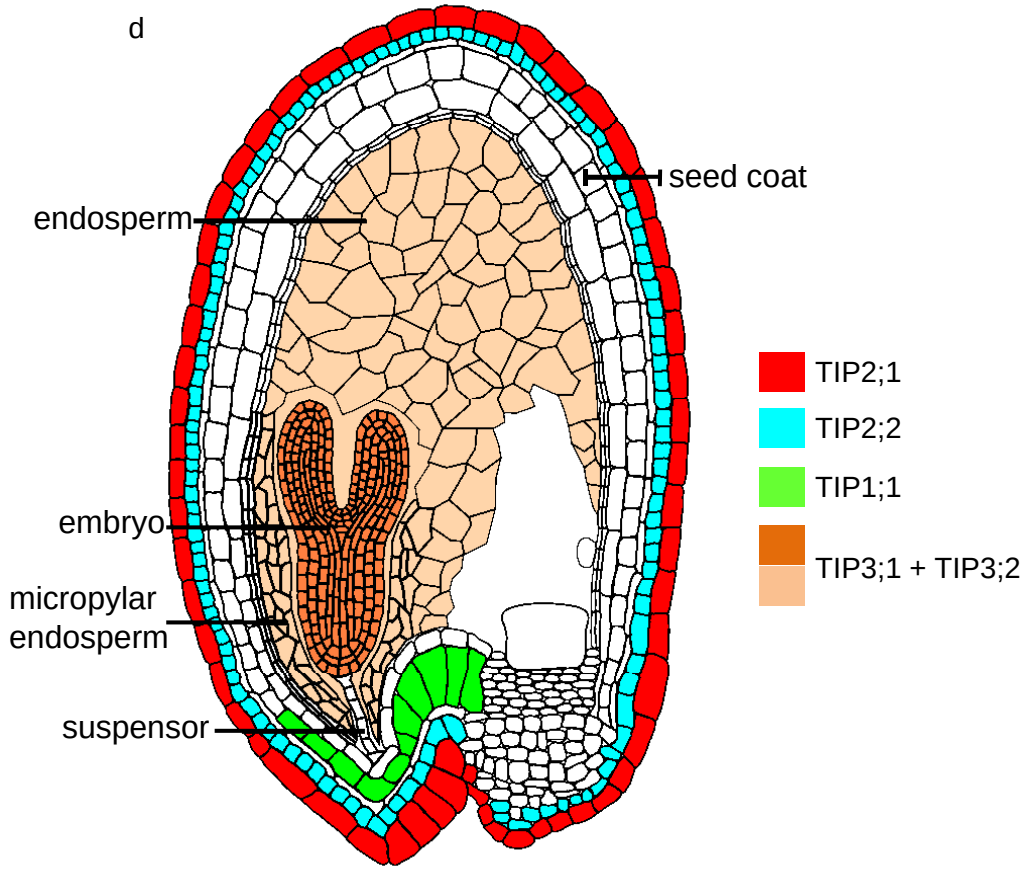

YFP-TIP3;1
YFP-TIP3;2
cotyledon
radicle
cotyledon
radicle
h:
24
36
48
60
72
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t

Figure 7

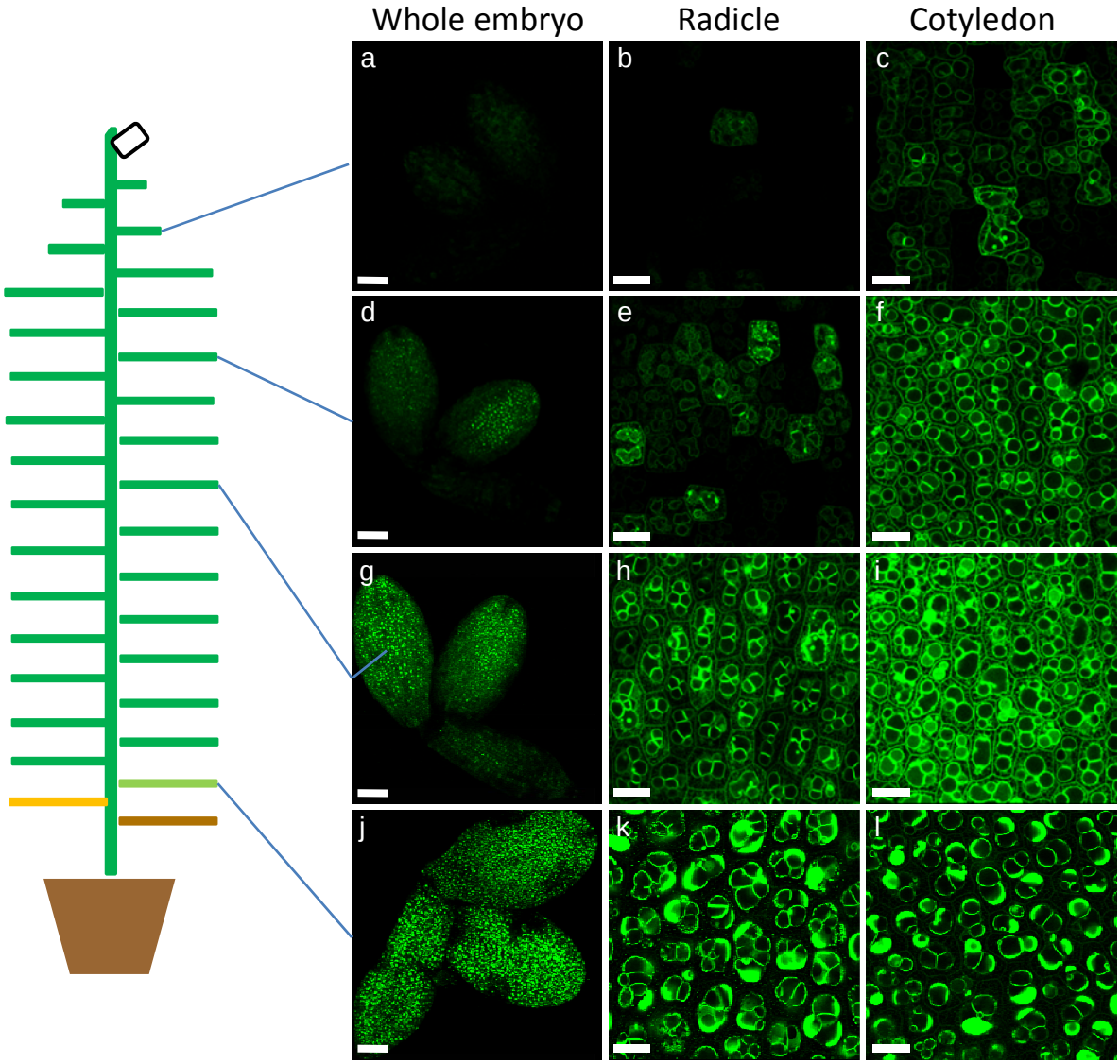

TIP 3;2-YFP
FM4-64
merge
a
b
c
late torpedo
bent cotyledon
drying seed
d
e
f
TIP3;2pro::YFP-TIP3;2
g
h
i
TIP3;2pro::YFP-TIP1;1

Supplementary Fig. S1

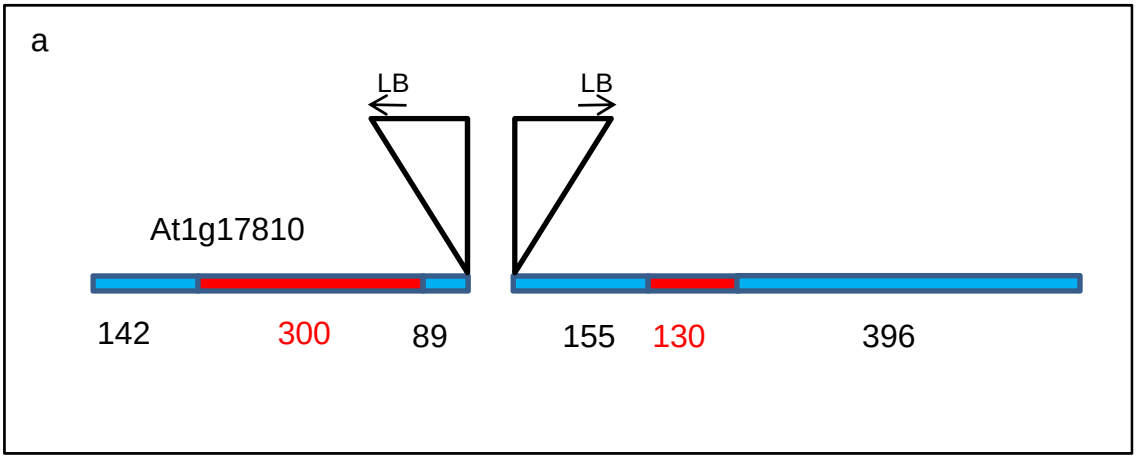




Supplementary Fig. S2



Suppl. Fig. S3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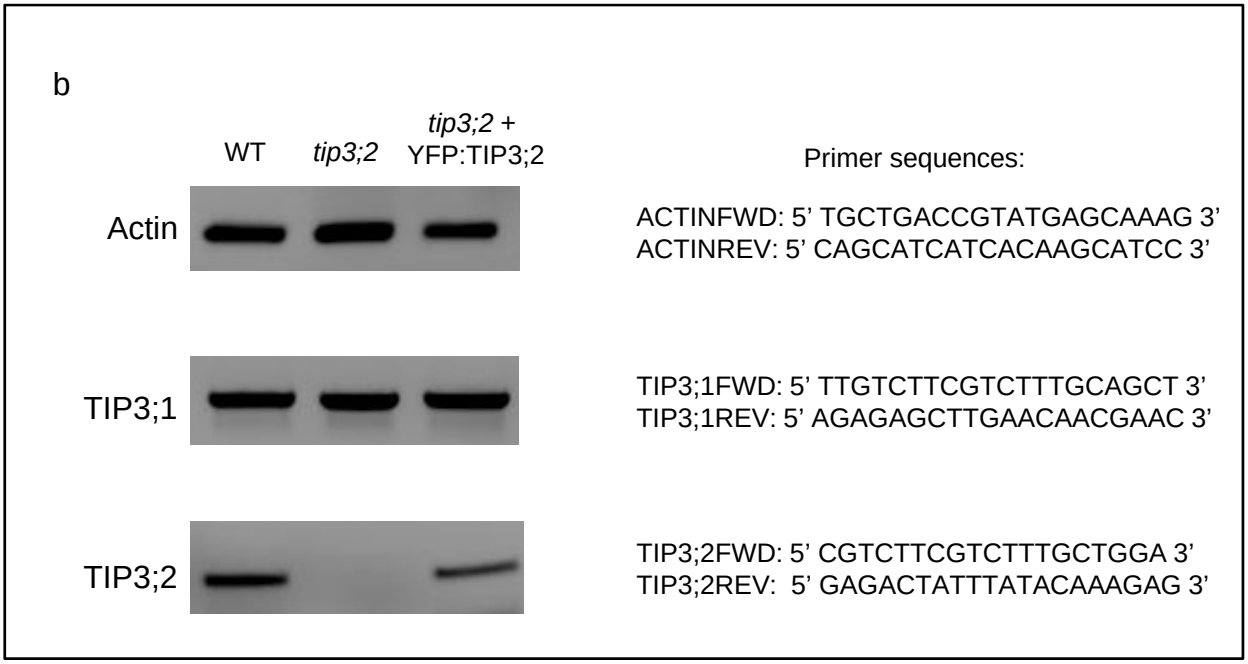
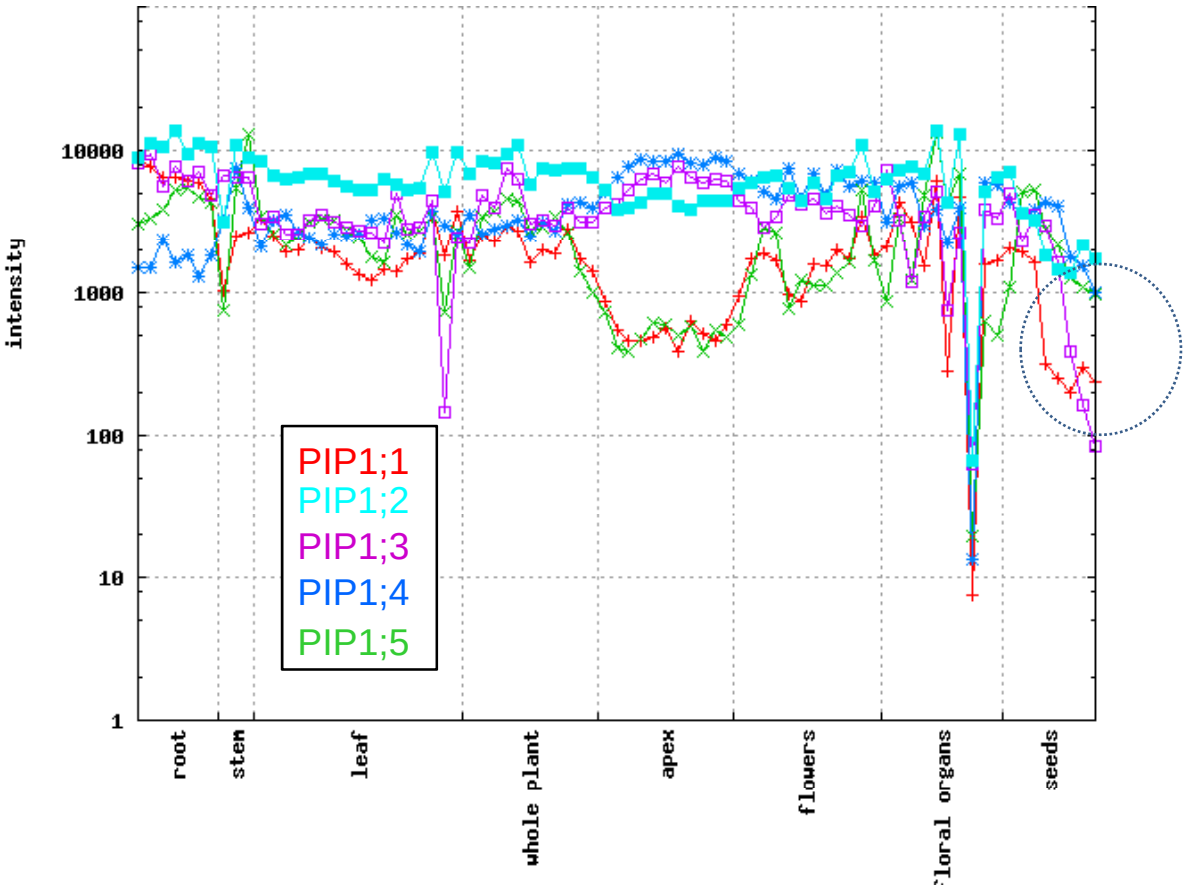
LB
LB
At1g17810
142
300
89
155
130
396


b
WT
tip3;2
tip3;2 + YFP:TIP3;2
Actin
TIP3;1
TIP3;2
Primer sequences:
ACTINFWD: 5' TGCTGACCGTATGAGCAAAG 3'
ACTINREV: 5' CAGCATCATCACAAGCATCC 3'
TIP3;1FWD: 5' TTGTCTTCGTCTTTGCAGCT 3'
TIP3;1REV: 5' AGAGAGCTTGAACAACGAAC 3'
TIP3;2FWD: 5' CGTCTTCGTCTTTGCTGGA 3'
TIP3;2REV: 5' GAGACTATTTATACAAAGAG 3'

Suppl. Fig. S4

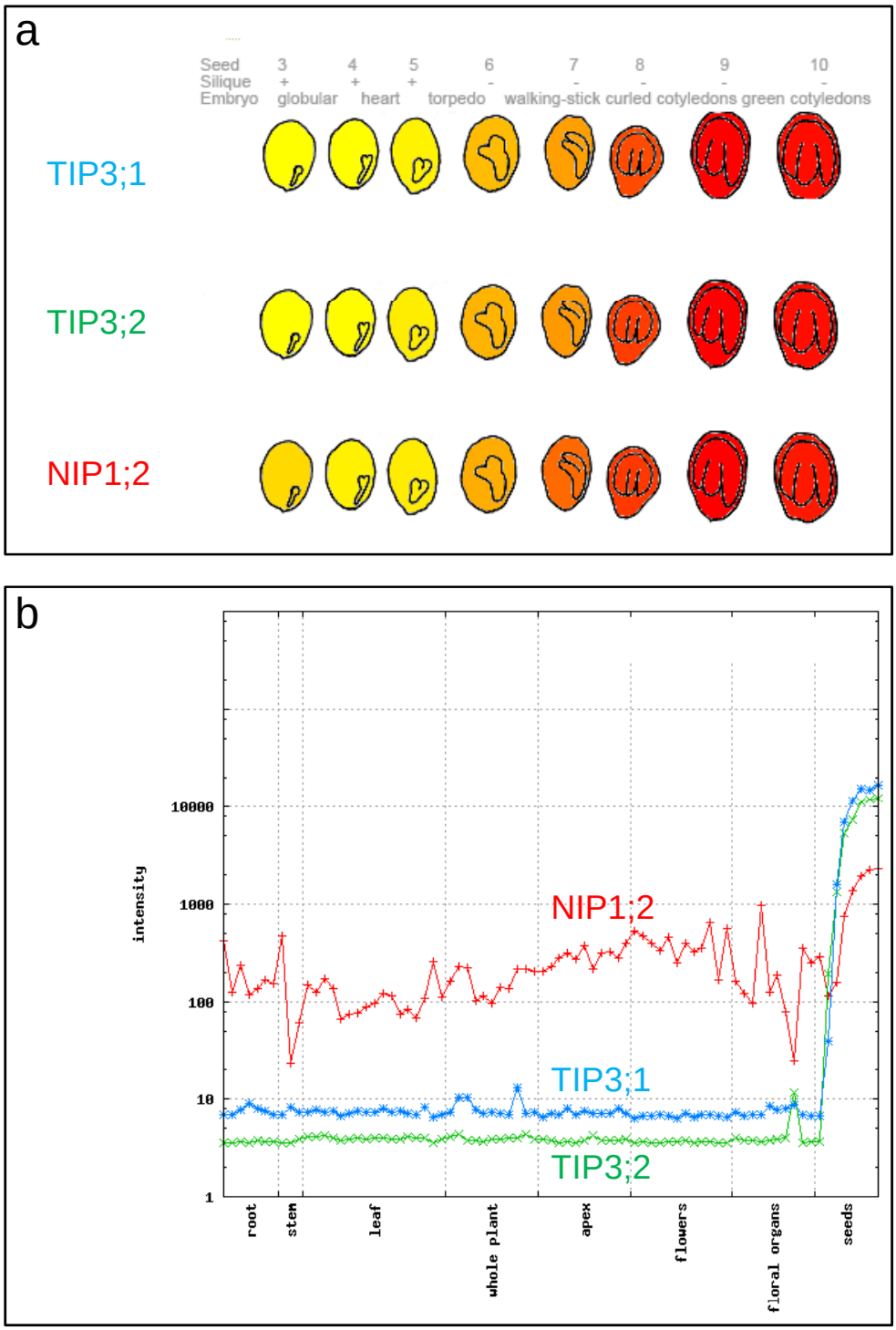

intensity
10000
1000
100
10
1
PIP1;1
PIP1;2
PIP1;3
PIP1;4
PIP1;5
root
stem
leaf
whole plant
apex
flowers
floral organs
seeds


PIP1;2
Seed 3 4 5 6 7 8 9 10
Silique + + + - - - - -
Embryo globular heart torpedo walking-stick curled cotyledons green cotyledons


PIP1;4
Seed 3 4 5 6 7 8 9 10
Silique + + + - - - - -


PIP1;5
Seed 3 4 5 6 7 8 9 10
Silique + + + - - - - -
Embryo globular heart torpedo walking-stick curled cotyledons green cotyledons

Suppl. Fig. S5


a
Seed 3 4 5 6 7 8 9 10
Silique + + + - - - - -
Embryo globular heart torpedo walking-stick curled cotyledons green cotyledons
TIP3;1
TIP3;2
NIP1;2
b
intensity
10000
1000
100
10
1
NIP1;2
TIP3;1
TIP3;2
root
stem
leaf
whole plant
apex
flowers
floral organs
seeds