Title

Cross-species functional diversity within the PIN auxin efflux protein family

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Abstract

In plants, directional transport of the hormone auxin creates auxin concentration maxima and paths of transport that provide positional, polarity, and growth regulatory cues throughout development. In *Arabidopsis thaliana,* the polar-localized auxin transport protein PIN-FORMED1 (AtPIN1) is required to coordinate development during flowering. However, Arabidopsis has a derived PIN family structure; the majority of flowering plants have retained a clade of PIN proteins phylogenetically sister to PIN1, the Sister-of-PIN1 clade (SoPIN1), which has been lost in the Brassicaceae, including Arabidopsis. Based on PIN localization in the grasses *Brachypodium distachyon* and *Zea mays*, which have both SoPIN1 and PIN1 clades, we previously proposed that the organ initiation and vein patterning roles attributed to AtPIN1 were shared between the SoPIN1 and PIN1 clades in grasses. Here we show that *sopin1* and *pin1b* mutants have distinct phenotypes in Brachypodium and thus the two clades are not functionally equivalent. *sopin1* mutants have severe organ initiation defects similar to Arabidopsis *atpin1* mutants, while *pin1b* mutants initiate organs normally but have increased stem elongation. Heterologous expression of Brachypodium PIN1b and SoPIN1 in Arabidopsis *atpin1* mutants provides further evidence for functional distinction between the two clades. SoPIN1 but not PIN1b can complement null *atpin1* mutants, while both SoPIN1 and PIN1b can complement an *atpin1* missense allele with a single amino acid change. The different localization behaviors of SoPIN1 and PIN1b when heterologously expressed in Arabidopsis provide insight into how PIN accumulation at the plasma membrane, tissue-level protein accumulation, transport activity, and interaction, all contribute to the polarization dynamics that distinguish PIN family members. Combined, these results suggest that the PIN polarization and trafficking behaviors required for organ initiation differ from those required for other PIN functions in the shoot, and that in most flowering plants these functions are split between two PIN clades.

Introduction

The plant hormone auxin is an essential mobile signal controlling growth and patterning throughout plant development (Leyser, 2010). Auxin can passively enter cells, triggering a vast array of downstream signaling events (Wang and Estelle, 2014), but it cannot easily exit the cell without active transport (Raven, 1975; Rubery and Sheldrake, 1974). As a result, directional efflux mediated by the polar-localized PIN-FORMED (PIN) efflux carriers can organize auxin accumulation patterns, creating concentration maxima and paths of transport that regulate growth, position organs, and pattern tissues (Adamowski and Friml, 2015). Because auxin itself feeds back to regulate PIN-mediated transport both transcriptionally and post-transcriptionally (Leyser, 2006), the transport system shows remarkable robustness and plasticity. For example, within the PIN family compensatory changes in PIN abundance can mitigate PIN loss-of-function mutant phenotypes (Blilou et al., 2005; Paponov et al., 2005; Vieten et al., 2005), environmental inputs can trigger tissue-level changes in PIN abundance and polarity leading to altered plant growth (Habets and Offringa, 2014), and auxin transport paths can be reorganized in response to injury (Xu et al., 2006), or spontaneously in tissue culture (Gordon et al., 2007). The self-organizing properties of the auxin transport system thus gives this patterning mechanism extraordinary versatility, and allows it to coordinate local and long range communication in the plant.

The correct initiation and positioning of organs (leaves, flowers, stems) in the growing tip, or shoot apical meristem, of *Arabidopsis thaliana* (Arabidopsis) plants requires the action of the PIN-FORMED1 (AtPIN1) auxin efflux carrier (Okada, 1991). AtPIN1 is targeted to the plasma membrane and polarized in cells (Gälweiler et al., 1998). In the meristem epidermis, polarization of AtPIN1 in neighboring cells converges around the initiation sites of new organs, suggesting that polarized AtPIN1 concentrates auxin into local maxima causing organ initiation (Benková et al., 2003; Heisler et al., 2005; Reinhardt et al., 2003). Accordingly, in *atpin1* loss-of-function mutants, or if auxin transport is pharmacologically inhibited, organ initiation is aborted, but it can be rescued with local auxin application to the meristem flank (Reinhardt et al., 2003; Reinhardt et al., 2000). Organ initiation in *atpin1* mutants can also be rescued with epidermal-specific AtPIN1 expression (Bilsborough et al., 2011) and reducing AtPIN1 function specifically in the epidermis compromises organ positioning and initiation (Kierzkowski et al., 2013), demonstrating the importance of convergent AtPIN1 polarization in the epidermis during organ formation.

The recurrent formation of AtPIN1 convergence points surrounding auxin maxima in the meristem epidermis has been the focus of several computational models that attempt to explain how auxin feeds back on its own transport via AtPIN1 to polarize toward auxin maxima and control organ spacing (Abley et al., 2016; Bayer et al., 2009; Jönsson et al., 2006; Smith, 2006; Stoma et al., 2008). However, AtPIN1 is also expressed during the patterning of the vascular strands formed coincident with organ positioning, and in these sub-epidermal cells AtPIN1 is polarized basally, away from the presumed auxin maxima, suggesting that the control of AtPIN1 polarity with respect to auxin is not consistent across tissues (Bayer et al., 2009).

Indeed, AtPIN1 is also expressed during patterning of leaf veins, and in mature tissues (Gälweiler et al., 1998; Scarpella et al., 2006). During the vegetative phase, AtPIN1 is not required for organ initiation but the organs that do form are misplaced and have severe morphological and vascular defects, suggesting an important role for AtPIN1

in post-initiation morphogenesis and vein patterning in leaves, consistent with the vascular patterning defects observed upon pharmacological inhibition of auxin transport (Guenot et al., 2012; Sawchuk et al., 2013; Verna et al., 2015). Furthermore, in mature tissues, AtPIN1 is expressed in vascular-associated cells and is required for efficient long distance transport of auxin down the shoot in the polar auxin transport stream, and this has been proposed to play an important role in the regulation of shoot branching (Bennett et al., 2016; Bennett et al., 2006; Gälweiler et al., 1998; Shinohara et al., 2013). Mutations in other PIN family members in combination with AtPIN1 mutants suggest further functions in embryo development, root development and during plant growth responses to light and gravity (Leyser, 2005). Unfortunately, the myriad roles for AtPIN1 during plant development are genetically obscured by the severity of *atpin1* mutant organ initiation defects.

We previously showed that all sampled flowering plants outside of the Brassicacea family have a clade of PIN proteins sister to the PIN1 clade (Sister-of-PIN1 or SoPIN1), while Arabidopsis and other Brassicacea species have lost this clade (O'Connor et al., 2014). During organ initiation in the grass *Brachypodium distachyon* (Brachypodium) SoPIN1 is highly expressed in the epidermis, polarizes towards presumed auxin maxima, and forms convergent polarization patterns during the formation of new organs, suggesting a role in creating the auxin maxima required for organ initiation. In contrast, the PIN1 clade members in Brachypodium, PIN1a and PIN1b, are not highly expressed in the epidermis, orient away from presumed auxin maxima, and are primarily expressed during patterning in the sub-epidermal tissues. Thus, the combined expression domains and polarization behaviors of SoPIN1, PIN1a, and PIN1b in Brachypodium largely recapitulate those observed for AtPIN1 in Arabidopsis.

The localization and polarization of the Brachypodium SoPIN1 and PIN1 clades can be modeled with two different polarization modes with respect to auxin; SoPIN1 polarizes “up-the-gradient”, towards the neighboring cell with the highest auxin concentration, while PIN1a and PIN1b polarize “with-the-flux”, accumulating in the membrane with the highest auxin flux (O'Connor et al., 2014). Both polarization modes were previously applied to AtPIN1 in order to capture the switch in polarity observed during organ initiation and vein patterning, first orienting toward auxin maxima during convergence point formation, then orienting away from maxima during vein patterning below the epidermis (Bayer et al., 2009). These localization and modeling results suggest that in most angiosperm species the organ placement and vascular patterning functions attributed to AtPIN1 in *Arabidopsis* are split between the PIN1 and SoPIN1 clades, and that these two clades have different polarization properties with respect to auxin.

Here we present the functional analysis of both SoPIN1 and PIN1 protein clade members in Brachypodium, a species with the canonical two-clade family structure. We show that SoPIN1 and PIN1b have different functions during Brachypodium development, with SoPIN1 being required for organ initiation during the flowering phase, and PIN1b regulating stem elongation. Using heterologous expression in Arabidopsis, we show that the two proteins have different accumulation, polarization and transport behaviors that result in different functional properties independent of transcriptional context. These results suggest that the Arabidopsis AtPIN1 protein represents an example of an evolutionary phenomenon the opposite of subfunctionalisation in which protein functions are amalgamated into a single protein rather than diversified amongst paralogs. AtPIN1 has a repertoire of roles, and

associated polarization behaviors that are distributed among several clades of PIN proteins in most flowering plants.

Results

SoPIN1 and PIN1b have different functions in Brachypodium

During organ formation in the Brachypodium shoot, both SoPIN1 and PIN1b expression precede PIN1a, which only accumulates significantly at the site of midvein formation after the organ begins to grow. In the earliest stages of initiation, prior to the periclinal cell divisions that are the hallmark of morphogenesis, SoPIN1 forms convergent polarization patterns around the presumed auxin maxima in the meristem epidermis, while PIN1b is expressed internally and orients away from the maxima (O'Connor et al., 2014). Because of their early expression and opposing polarization patterns, we focused on characterizing SoPIN1 and PIN1b as representatives of the SoPIN1 and PIN1 clades.

As a first step towards understanding the functional differences between the SoPIN1 and PIN1 clades, we targeted Brachypodium SoPIN1 and PIN1b with CRISPR and recovered loss-of-function mutants in both genes (see methods). Both *sopin1-1* and *pin1b-1* mutants have single base-pair lesions that result in frame-shifts and premature stop codons (Figure 1A). *sopin1-1* mutants show severe organ initiation defects in the inflorescence remarkably similar to loss-of-function *atpin1* mutants in *Arabidopsis* (Figure 1B-C, Figure 1 – supplement 1)(Okada, 1991). The indeterminate flowering shoots of the Brachypodium inflorescence, called spikelets (Figure 1B, inset), often fail to initiate in *sopin1-1* despite sometimes clear definition of node vs internode tissue (n, and i, in Figure 1C). When spikelets do form, the spikelet meristems are often devoid of new organs (Arrows in Figure 1C inset).

In wildtype spikelet meristems, SoPIN1 convergence point formation is coincident with a decrease in the nuclear auxin response reporter protein DII-Venus (Brunoud et al., 2012) (DII) (Figure 1E), which functions in Brachypodium and is degraded in the presence of auxin in spikelets (Figure 1 - supplement 2). In *sopin1-1* meristems DII accumulation is uniformly high for long stretches of the epidermis, and the patterned reduction of DII both in the meristem epidermis and internally fails to occur, suggesting a failure to organize auxin maxima (Figure 1F arrow).

In contrast to the severe defects of *sopin1-1*, organ initiation in *pin1b-1* mutants is unaffected (Figure 1D). However, *pin1b-1* mutants show minor shoot twisting and increased internode (stem tissue) length, especially in the basal few internodes (Figure 1G, 1H). The longer internodes of *pin1b-1* lead to an overall increase in plant height (Figure 1H). The internode defects of *pin1-b* are consistent with the abundant PIN1b expression observed in wildtype stem tissue (Figure 1I). The difference in phenotypes between *sopin1-1* and *pin1b-1* Brachypodium mutants suggests a functional distinction between the SoPIN1 and PIN1 clades, and reveals that while PIN1b is expendable for organ initiation, it is involved in the regulation of internode growth.

SoPIN1 and PIN1b accumulate differently in Arabidopsis

The difference in the *sopin1-1* and *pin1b-1* phenotypes in Brachypodium may be due to their different expression patterns and not necessarily to differences in polarization behavior as previously hypothesized (O'Connor et al., 2014). In order to explore what functional differences exist between the proteins themselves, we expressed both Brachypodium proteins in wildtype (Columbia, Col-0) Arabidopsis under the control of a 3.5kb Arabidopsis *PIN1* promoter fragment known to drive PIN1 expression sufficient to complement *pin1* mutants (*proAtPIN1*) (Heisler et al., 2005). Remarkably, despite the loss of SoPIN1 from Arabidopsis, Brachypodium SoPIN1 created clear convergent polarization patterns around the sites of organ initiation in inflorescence meristems (Figure 2I asterisk, 25 of 27 meristems from 4 independent transgenic events). SoPIN1 protein abundance was highest in the meristem epidermis and SoPIN1 convergence points were most clearly observed surrounding I2 and I1 primordia (Figure 2A). Immediately below the apex, SoPIN1 accumulated in an ill-defined ring shape within which the vascular bundles will form (Figure 2J, 15 of 23 meristems from 4 independent events).

In contrast, significant PIN1b accumulation was absent from the meristem epidermis in 19 of 29 meristems from 7 independent transgenic events. In the few meristems where PIN1b was significantly expressed in the epidermis, it did not show clear convergent polarization patterns, and its polarity was often unclear (Figure 2B). Within initiating organs PIN1b often localized to punctate vesicular bodies inside cells not the cell membrane (Figure 2B arrow). The PIN1b expression level remained low just below the meristem apex, but in contrast to SoPIN1, PIN1b formed defined domains around the presumptive developing vascular bundles (Figure 2L). The lack of PIN1b protein in the meristem epidermis was not due to silencing of the transgene in these lines because we observed abundant PIN1b protein in the developing vasculature below the apex, even in plants where the meristem had no epidermal expression (Figure 2D) (8 samples from 4 events). In the same tissues SoPIN1 accumulated in both the vasculature and the epidermis (Figure 2C) (5 samples from 2 events).

In order to determine whether there were also tissue-level differences in protein accumulation in mature tissues, we imaged SoPIN1 and PIN1b in the basal internode. Here, AtPIN1 normally accumulates in a highly polar manner in the root-ward plasma membranes of cambium (c) and xylem parenchyma (xp) tissues of the vasculature (Bennett et al., 2016; Gälweiler et al., 1998). PIN1b accumulated in a similar pattern to AtPIN1 (Figure 2F, 2H. 10 samples from 5 independent transgenic events). In contrast, in addition to accumulating in the cambium and xylem parenchyma, SoPIN1 accumulated in the central pith tissue (p) (Figure 2E, 2G. 15 samples from 4 independent transgenic events). AtPIN1 is not normally observed in the pith (Bennett et al., 2016; Gälweiler et al., 1998). In the basal internode both proteins showed the characteristic AtPIN1 root-ward polarization pattern regardless of tissue-level abundance (Figure 2K, 2M).

Taken together, these results show that even under the same transcriptional control SoPIN1 and PIN1b show distinct tissue-level accumulation patterns in Arabidopsis. While the overall behavior of the two Brachypodium proteins is similar to AtPIN1 in many tissues, there are behaviors unique to each Brachypodium PIN even under the AtPIN1 promoter. PIN1b fails to accumulate in the epidermal tissues where AtPIN1 and SoPIN1 remain high, whereas SoPIN1 accumulates in the pith tissue where

AtPIN1 and PIN1b do not. The convergent polarization patterns of SoPIN1 and the vascular accumulation of PIN1b in Arabidopsis are remarkably similar to their native behaviors in Brachypodium (O'Connor et al., 2014).

SoPIN1 but not PIN1b can restore organ initiation and bulk auxin transport in AtPIN1 null mutants

To determine whether the observed differences in SoPIN1 and PIN1b polarization and accumulation have functional consequences in Arabidopsis, we used the *proAtPIN1* driven SoPIN1 and PIN1b constructs to complement the Arabidopsis *pin1-613* mutant (also known as *pin1-7)*. The *pin1-613* allele is a putative null T-DNA insertion mutant with severe organ initiation defects in the inflorescence (Bennett et al., 2006; Smith, 2006; Zourelidou et al., 2014). Given that epidermal PIN1 function is important for organ initiation (Bilsborough et al., 2011; Kierzkowski et al., 2013), as expected only SoPIN1 was able to complement the *pin1-613* mutation and mediate organ initiation (Figure 3A) (3 out of 6 independent transgenic events showed complementation). However, phenotypic complementation of *pin1-613* by SoPIN1 was incomplete, and mature plants showed a variety of phenotypic defects (Figure 3A, Figure 3 - supplement 1). Most notably, each flower produced more sepals and petals than wild-type, but almost no stamens (Figure 3B, 3C, Figure 3 - supplement 2). SoPIN1 complemented *pin1-613* plants were thus sterile. We wondered if these phenotypes could be explained if SoPIN1 had poor auxin transport function in Arabidopsis. However, SoPIN1 restored wild-type levels of bulk auxin transport to *pin1-613* basal internodes (Figure 3D). Thus SoPIN1 is at least in part functionally capable of initiating organs and mediating root-ward auxin transport in the stem, but it is not functionally identical to AtPIN1 under the same promoter.

In SoPIN1 complemented *pin1-613* mutants, SoPIN1 accumulation increased in the meristem epidermis relative to wild-type or heterozygous plants, but the pronounced convergent polarization patterns observed in the WT background were less clear (Figure 4A, Figure 4 - supplement 1) (16 of 16 meristems). SoPIN1 complemented meristems showed a variety of phyllotactic defects and had highly variable morphologies (Figure 4 – supplement 1) (16 of 16 meristems). Similar to the pattern observed in the wild-type background, sub-epidermal SoPIN1 in *pin1-613* mutants accumulated in a loosely defined ring within which individual vein traces were difficult to discern (Figure 4I) (13 of 16 meristems). In the mature tissues, SoPIN1 accumulated in the epidermis, vasculature, and mature pith tissues similar to the wild-type background (Figure 4C, 4E, 4G).

In contrast to SoPIN1, PIN1b-expressing *pin1-613* plants had pin-formed inflorescences that were indistinguishable from *pin1-613* alone (Figure 3A) (all 7 events failed to complement). The lack of complementation mediated by PIN1b was not caused by silencing or low expression level because abundant PIN1b signal was observed in *pin1-613* meristems (23 of 26 *pin1-613* meristems from 7 independent events). In most PIN1b expressing *pin1-613* samples, expression increased in the epidermis relative to wildtype, forming a ring-shaped domain around the meristem apex (Figure 4B, 4D arrow, Figure 4 - supplement 2) (14 of 19 meristem apices from 6 independent events). Unlike in the wildtype background, PIN1b in the epidermis of *pin1-613* meristems was more consistently targeted to the membrane and polar

(Figure 4K). However, even with this elevated polar expression in the meristem epidermis, PIN1b was unable to mediate organ initiation. Below the apex, PIN1b was polarized root-ward in *pin1-613* meristems (Figure 4J), forming defined traces associated with the vasculature (Figure 4F, 4L). In the basal stem of *pin1-613* mutants PIN1b accumulated in a pattern similar to wild-type, although the arrangement of vascular bundles was irregular (Figure 4H). Remarkably, despite clear polar PIN1b expression in *pin1-613* mutant stems (Figure 4M), PIN1b was unable to rescue bulk auxin transport in this tissue (Figure 3D).

Although PIN1b was incapable of supporting organ formation or mediating bulk transport in *pin1-613,* when an auxin maximum was created artificially by addition of lanolin paste infused with IAA, PIN1b epidermal accumulation increased during the initiation of the resultant primordia (Figure 4 – supplement 3) (4 of 6 samples from 2 independent transgenic events). Thus, in the absence of AtPIN1, PIN1b accumulation in the epidermis is still auxin responsive and capable polar localization in *pin1-613* meristem epidermal tissue, but it is not able to mediate organ initiation itself. These results demonstrate that when expressed in *Arabidopsis,* there is a clear functional separation between SoPIN1 and PIN1b independent of transcriptional control.

SoPIN1 and PIN1b show different behaviors when expressed in the meristem epidermis

Epidermal-specific AtPIN1 expression is sufficient to rescue organ initiation in *atpin1* mutants (Bilsborough et al., 2011). In order to drive increased PIN1b expression in the epidermis, and to help reduce transgene position-effect variation of expression level, we utilized a two-component expression system in the Landsberg *erecta* (L*er*) background to drive SoPIN1 and PIN1b under the control of the epidermis-enriched Arabidopsis ML1 promoter (Hereafter designated *proAtML1>>*) (Lenhard, 2003; Sessions et al., 2002). Under the control of *proAtML1* we achieved consistently high epidermal expression of both SoPIN1 and PIN1b, but similar to the *proAtPIN1* driven localization described above, only SoPIN1 showed clear convergent polarization patterns around the sites of organ initiation (Figure 5A-5D, Figure 5 supplement 1 and 2) (9 of 9 meristems). Despite consistently high epidermal expression with this system, PIN1b polarity remained difficult to determine, and in many cells the abundance of protein on the membrane remained low (Figure 5D) (10 of 10 meristems). Instead, PIN1b accumulated in intracellular bodies, especially in the cells of the apical dome and the central domain of initiating organs (Figure 5B, 5D arrow). PIN1b abundance and polarity was highest at the boundaries of lateral organs (Figure 5 - supplement 2). Thus SoPIN1 and PIN1b show consistent behaviors in the meristem epidermis when expressed under either *proAtPIN1* or *proAtML1.* Despite increased PIN1b expression under *proAtML1,* and a resulting increase in protein accumulation in the apex, PIN1b was still unable to form convergent polarization patterns in wildtype plants.

Both SoPIN1 and PIN1b can rescue the Arabidopsis *pin1-4* mutation when expressed in the meristem epidermis

In order to determine whether the increased PIN1b abundance in the meristem epidermis achieved by the *proAtML1* two-component system had functional consequences, we crossed these transgenes into *pin1-4*. The *pin1-4* allele is in the Landsberg *erecta* background and has a single P579 to L amino acid change in the second-to-last transmembrane domain of AtPIN1 (Bennett et al., 1995), but the phenotype is similarly severe to *pin1-613* (Figure 6A). Remarkably, both SoPIN1 and PIN1b driven by *proAtML1* were able to rescue the organ formation defects of *pin1-4* (Figure 6A). In contrast to the SoPIN1-mediated complementation of *pin1-613* described above, both SoPIN1 and PIN1b-complemented *pin1-4* plants made WT flowers that produced seed (Figure 6 – supplement 1). In addition, both *proAtML1* SoPIN1 and PIN1b expressing *pin1-4* lines were able to rescue bulk auxin transport in the basal internode, although PIN1b was less effective than SoPIN1 (Figure 6B). Compared to wildtype and SoPIN1-complemented plants, PIN1b-complemented *pin1-4* plants showed a significant increase in stem diameter (Figure 6C). Thus while both proteins can complement the *pin1-4* organ initiation phenotype, the SoPIN1 and PIN1b complemented lines have differing auxin transport properties and different plant morphologies, suggesting once again that SoPIN1 and PIN1b are not functionally identical.

SoPIN1-complemented *pin1-4* meristems were slightly smaller than wildtype, and in rare cases showed defects in phyllotaxy (Figure 5 – supplement 1), but the protein localization was similar to the pattern observed in the WT background, with clear convergent polarization around initiating organs (Figure 5E, 5G). In contrast, compared to the WT background, PIN1b localization in *pin1-4* was dramatically altered (Compare Figure 5B with Figure 5F). Most obvious was an increase in membrane targeted PIN1b and a corresponding reduction in intracellular PIN1b (Figure 5H). PIN1b polarity in the *pin1-4* background was more apparent than in wildtype, and convergent polarization patterns clearly marked incipient organs (Figure 5H). PIN1b-complemented meristems accumulated less PIN protein in the apical dome compared to SoPIN1-complemented meristems, and the meristems were larger (Figure 5 – supplement 2).

In the basal internode, both PINs had similar accumulation patterns in the outer few cell layers (Figure 5I-J arrows), and both showed basal polarization in the epidermis (Figure 5K-L arrows). Despite this expression domain being drastically different than the wildtype vascular-associated pattern of AtPIN1 (Bennett et al., 2006; Gälweiler et al., 1998), expression in these few cortex layers and epidermis was apparently sufficient to drive wildtype levels of rootward bulk auxin transport in *pin1-4* (Figure 6B).

Discussion

SoPIN1 and PIN1b have different functions in Brachypodium

During spikelet development in Brachypodium SoPIN1 forms convergent polarization patterns surrounding the sites of organ initiation and strong expression of the auxin response reporter DR5 (O'Connor et al., 2014). We provide additional evidence here that SoPIN1 polarizes towards sites of high auxin concentration by showing that a DII-Venus minimum occurs at SoPIN1 convergence points. In *sopin1* mutants the reduction of DII does not occur, suggesting that SoPIN1 functions to concentrate auxin at epidermal maxima, and similar to *Arabidopsis,* this is required for organ initiation in the inflorescence. The specificity of SoPIN1 for the outer tissues in Brachypodium provides further support for the idea that maxima formation is necessary for organ initiation, and that this is primarily mediated by convergent PIN in the meristem epidermis (Jönsson et al., 2006; Kierzkowski et al., 2013; Smith, 2006).

SoPIN1 clade mutants have been reported in the legume *Medicago truncatula* and in tomato (*Solanum lycopersicum*), and these mutants show pleiotropic phenotypes involving phyllotaxy, organ initiation, inflorescence branching, leaf serrations, and leaf compounding, but they do not form barren pin meristems (Martinez et al., 2016; Zhou et al., 2011). These wider morphogenetic events also involve epidermal PIN convergence points and associated auxin maxima, suggesting a general role for SoPIN1 clade members in generating such maxima (Barkoulas et al., 2008; Bilsborough et al., 2011). But the lack of barren pin-formed meristems in these mutants may suggest that different species are variably dependent on SoPIN1-generated maxima for organ initiation.

In contrast to *sopin1*, loss of PIN1b function has no clear organ initiation defects, despite being expressed in developing organs (O'Connor et al., 2014). Given that auxin drainage is thought necessary for proper organ size and placement (Deb et al., 2015), the lack of an organ initiation phenotype in *pin1b* is surprising. However, it is possible that PIN1a performs some of this function, and double mutants are needed to address the redundancy of PIN1a and PIN1b during organ initiation.

The increased internode elongation in *pin1b* mutants provides new genetic tractability to address how PINs regulate tissue growth in the shoot independent of organ initiation. Grasses contain intercalary meristems, bands of indeterminate tissue separated from the apical meristem that are responsible for internode growth after organ initiation. PIN1b expression around this meristematic tissue (Figure 1I) suggests that PIN1b may regulate growth by influencing auxin distribution in this meristem. This is consistent with evidence that loss of the ABCB1 auxin exporter in maize results in dwarfism associated with reduced activity of intercalary meristems (Knöller et al., 2010). How PIN1b alters auxin dynamics to control internode growth will be an important direction for future research.

The properties that define PIN behavior and function

Membrane accumulation. Because of their differing phenotypes in Brachypodium, we used heterologous expression of SoPIN1 and PIN1b in Arabidopsis to explore the ways in which different PIN family members may have different properties post-transcription (Summarized in Figure 7). When expressed in the meristem epidermis in

wild-type Arabidopsis, SoPIN1 is localized to the membrane in most cells while PIN1b often accumulates internally (Compare Figure 5C and D). Thus, with the same transcriptional control different PINs can vary in the degree to which, after protein production, they accumulate at the plasma membrane. The differential membrane targeting of PIN1b and SoPIN1 is a tissue-specific phenomenon however, because unlike in the epidermis, in the basal internode both PINs accumulate at the plasma membrane (Figure 2K, 2M). The regulation of PIN plasma membrane polar targeting and endocytic recycling has been an important avenue for understanding PIN function and general membrane protein biology (Luschnig and Vert, 2014). Our results provide further evidence that at least some of the signals governing membrane accumulation are inherent in, and vary between different PIN family members.

Tissue accumulation. Under the same transcriptional control SoPIN1 and PIN1b show different tissue-level accumulation patterns in Arabidopsis. In wildtype plants *proAtPIN1*-expressed PIN1b shows less overall accumulation in the epidermis compared to SoPIN1 (Compare Figure 2C and 2D). The punctate PIN1b signal in the meristem epidermis may be PIN1b protein being actively targeted for degradation as has been shown for PIN2 in the root (Abas et al., 2006). In contrast, SoPIN1 is abundant in the epidermis and accumulates in an expanded expression domain in the sub-epidermal meristem tissues (Compare Figure 2J and 2L). Also, in the basal internode SoPIN1 accumulates in the pith tissue where AtPIN1 and PIN1b do not (Figure 2E, 2G). The presence of SoPIN1 in the pith is not easy to explain because AtPIN1 shows no protein accumulation in this tissue. It is possible however that the sub-epidermal SoPIN1 protein accumulation in the meristem persists into the mature internode.

In Arabidopsis, endogenous PIN family members show a degree of cross-regulation where loss-of-function mutations in one PIN family member result in ectopic accumulation of a different PIN in a compensatory pattern (Blilou et al., 2005; Paponov et al., 2005; Vieten et al., 2005). We observed similar behavior in the *pin1-613* null background where SoPIN1 and PIN1b accumulation in the meristem epidermis was increased in the absence of AtPIN1 (Figure 4 supplements 1 and 2). However, we did not observe the same cross-regulation in the *pin1-4* background where SoPIN1 and PIN1b tissue-level accumulation seemed similar between *pin1-4* mutant and wild-type meristems (Figure 5 - supplements 1 and 2). These variable tissue-level abundances, and PIN cross-regulation behaviors highlight the overall redundancy of some PIN behaviors, and further demonstrate the importance of PIN post-transcriptional regulation for controlling PIN abundance.

Transport activity. In Arabidopsis, phosphorylation of PINs by several different families of protein kinases is necessary for efficient auxin transport (Barbosa et al., 2014; Jia et al., 2016; Willige et al., 2013; Zourelidou et al., 2014). The necessity for PIN activation by phosphorylation may explain the inability of PIN1b to mediate bulk auxin transport in the basal stem of *pin1-613* plants despite being expressed, accumulating at the membrane, and being polarized rootward in this tissue (Figure 4M). It is possible that in the *proAtPIN1* domain PIN1b does not interact with the appropriate activating kinase, and it is thus inactive. Indeed, a partially un-phosphorylatable form of AtPIN1 fails to complement fully the bulk auxin transport defect of *pin1-613* mutants in the basal internode (Zourelidou et al., 2014). However, when expressed using *proAtML1,* PIN1b expression in the outer tissue layers of the basal internode appears sufficient to mediate bulk auxin transport in *pin1-4* (Figure

6B), suggesting that PIN1b activity may be tissue dependent, perhaps because of the differing expression domains of activating kinases (Zourelidou et al., 2014). Indeed, Arabidopsis PIN4 and PIN7 are present in the *proAtML1* domain (Bennett et al., 2016), making it conceivable that these PINs are the normal targets of activating kinases in this tissue. Regardless, the behavior of PIN1b in *pin1-613* Arabidopsis provides a clear indication that even once a PIN has accumulated at the cell membrane in a tissue it may not be active.

Interaction. A particularly striking result is the ability of PIN1b to form convergent polarization patterns and mediate organ initiation in the *pin1-4* missense mutant background when it is unable to do so in the null *pin1-613* background. The strong influence of *pin1-4* on PIN1b membrane targeting and polarity in the meristem epidermis (Compare Figures 6D and 6H) suggests that PIN1b may be cooperating with a partially functional *pin1-4* protein and together they recapitulate the functions of wildtype AtPIN1. The presence of some *pin1-4* function is supported by the result that SoPIN1 complementation of the null *pin1-613* allele is partial and because of flower defects the plants are sterile (Figure 3B, 3C), while complementation of *pin1-4* is complete and flowers are phenotypically normal and set seed (Figure 6 - supplement 1). Consistent with these different complementation phenotypes, SoPIN1 convergent patterns are more evident in the presence of *pin1-4* then they are in the null *pin1-613* background (Compare 4A and 5E). If PIN1b is indeed inactive in null *pin1-613* mutants as we hypothesized above, then it is possible *pin1-4* facilitates the interaction of PIN1b with the appropriate activating kinase, and this allows PIN1b to perform organ initiation. Alternatively, *pin1-4* may provide polarity information that PIN1b lacks, and even though *pin1-4* is non-functional, it is able to target or stabilize PIN1b on the appropriate membrane to mediate convergent polarization patterns and organ initiation. However, *pin1-4* interaction with PIN1b cannot explain the ability of PIN1b to rescue bulk transport in the basal internodes of *pin1-4* mutants, because the two proteins presumably do not overlap in this tissue. In this case the necessary interaction between PIN1b and *pin1-4* may be set up early during organ initiation, and protein modifications propagated to the basal internode. Direct PIN interaction has so far never been shown, but if one PIN type can convey polarity or activity information to another through direct interaction this may be important for understanding auxin transport in tissues where multiple PINs overlap, such as in the root meristem (Blilou et al., 2005), or in Brachypodium where multiple PINs are present during organ initiation in the shoot (O'Connor et al., 2014).

Polarity. We previously showed that the polarization dynamics of SoPIN1, PIN1a, and PIN1b in *Brachypodium* could be modeled by assigning two different polarization modes to the SoPIN1 and PIN1 clades (O'Connor et al., 2014). In the model, SoPIN1 orients toward the adjacent cell with the highest auxin concentration, thus transporting auxin up the concentration gradient and providing a positive feedback to concentrate auxin into local maxima. In contrast, in the model PIN1a and PIN1b proteins are allocated in proportion to auxin flux, thus providing a positive feedback where flux through the tissue is amplified by the allocation of PIN1a/b in the direction of that flux. The assignment of two different polarization modes was previously used to describe the behavior of AtPIN1 during organ placement and vein patterning utilizing an auxin-concentration based switching mechanism between the up-the-gradient (UTG) and with-the-flux (WTF) polarization modes (Bayer et al., 2009). However, it has also been suggested that a flux-based mechanism alone can account for both convergence points and vein patterning (Abley et al., 2016; Stoma et al., 2008).

There are still no known mechanisms for direct sensing of intercellular auxin gradients or flux across membranes, but the *sopin1* and *pin1b* phenotypes in Brachypodium are consistent with the different polarization modes. SoPIN1 is required for organ initiation and the formation of auxin maxima in Brachypodium, which is primarily modeled using UTG polarization (Bayer et al., 2009; Jönsson et al., 2006; Smith, 2006). On the other hand, *pin1b* mutant plants do not show organ initiation defects, but rather only have internode elongation defects, a tissue where WTF models have been used to explain PIN dynamics and measured auxin transport kinetics during vein patterning and the regulation of branch outgrowth (Bayer et al., 2009; Bennett et al., 2016; Mitchison, 1980; Mitchison et al., 1981; Prusinkiewicz et al., 2009).

In wild-type Brachypodium the SoPIN1 and PIN1b expression domains are almost entirely mutually exclusive (O'Connor et al., 2014), making it possible that the observed polarization differences between the two clades are due to expression context and not functional differences between the proteins themselves. More specifically, perhaps an UTG mechanism dominates the epidermis while a WTF mechanism is utilized in the internal tissues, and different PINs interact equally with these context-dependent mechanisms. Our heterologous expression studies do not exclusively support context-dependent or protein-dependent mechanisms for SoPIN1 and PIN1b polarity. It is clear that alone, only SoPIN1 and AtPIN1 show the convergent polarization patterns associated with UTG polarization, and alone only SoPIN1 and AtPIN1 are thus able to mediate organ initiation, while PIN1b cannot. On the other hand, all three PINs are capable of root-ward polarization in the basal internode tissue. The results presented here do not demonstrate whether within a single cell PIN1b and SoPIN1 would orient differently with respect to auxin as might be expected for the dual polarization model (O'Connor et al., 2014). However such context-independent polarization behavior was previously observed for PIN1 and PIN2 in the root where both PINs can polarize in opposing directions within a single cell type when expressed in the PIN2 domain (Kleine-Vehn et al., 2008; Wisniewska et al., 2006).

Outlook

In total, our Brachypodium mutant phenotypes and heterologous expression results point to multiple levels at which PIN family members can be functionally distinct. Differential membrane targeting, tissue level accumulation, transport activity, indirect or direct interaction, and the resultant polarity may all contribute to the dynamics of PIN action during plant development. In most flowering plants two PIN clades, SoPIN1 and PIN1, with differing properties post-transcription mediate auxin transport in the shoot, but these properties are seemingly combined into AtPIN1 in Arabidopsis and other Brassicaceae species. Because PIN1b is unable to mediate organ initiation while AtPIN1 can, and these two PINs are both members of the same clade, AtPIN1 may have gained the ability to form convergent polarization patterns and mediate organ initiation after, or coincident with, the loss of the SoPIN1 clade. Indeed, when comparing Brassicaceae PIN1 proteins against a broad sampling of other angiosperm PIN1 proteins, the Brassicaceae PIN1 proteins have several divergent protein domains (Figure 7 - supplement 1), suggesting possible neofunctionalization within the Brassicacea family. Alternatively, an expansion of the PIN3,4,7 clade is also characteristic of Brassicacea species (O'Connor et al., 2014), making it possible duplicated members of this clade buffered the loss of SoPIN1. However, there is no indication that PIN3,4,7 have a role in organ initiation in the inflorescence (Guenot et al., 2012). Regardless, we believe the combination of SoPIN1 and PIN1

characteristics into AtPIN1 coincident with the loss of the SoPIN1 clade represents a form of reverse-subfunctionalization, the combination of functions originally split between homologs into a single protein after gene loss. It is not surprising that PINs may be particularly amenable to this kind of functional evolution because, as described above, there are several post-transcriptional regulatory steps that ultimately combine to control PIN function in plants. The output of auxin transport is the sum of a vast network of post-transcriptional interactions that all act to regulate auxin transport itself, and this gives the system plasticity during development, and perhaps also over evolutionary time.

Materials and Methods

sopin1-1 and *pin1b-1* creation with CRISPR

SoPIN1 (Bradi4g26300) and PIN1b (Bradi3g59520) were targeted with CRISPR using vectors developed for rice (Miao et al., 2013). CRISPR constructs were transformed into Brachypodium inbred line Bd21-3 using previously published methods (Bragg et al., 2015).

sopin1-1 CRISPR

The SoPIN1 guide was AGGCTGTCGTACGAGGAGT. This guide was shorter than the typical 20bp in an effort to provide greater target specificity for SoPIN1 (Fu et al., 2014). In the T0 regenerated plants, 5 out of 9 independent transgenic events showed severe organ initiation defects, and all 5 contained lesions in the SoPIN1 CRIPSR target site. Unfortunately, only one of the events with a T0 phenotype set seed. In the T1 progeny of this event only those individuals that contained the CRISPR transgene showed lesions in the SoPIN1 CRISPR target site, and these plants showed the *sopin1* phenotype and thus failed to set seed, suggesting active editing by the SoPIN1 CRISPR transgene in this event.

Not all events showed such efficient editing however, and we identified an independent T1 family where a C insertion in the SoPIN1 CRISPR target site co-segregated with the barren inflorescence phenotype. We designated this allele, which causes a premature stop codon before the end of the third exon codon 739 base pairs downstream from the target site, *sopin1-1*. We backcrossed a heterozygous *sopin1-1* plant to the Bd21-3 parental line and all F1 progeny (N=4) were wild type. In the F2 generation, the *sopin1-1* lesion co-segregated with the barren inflorescence phenotype (N=60: 32 het, 18 homo,10 wt). Amongst these plants, 16 did not have the Cas9 transgene, and the barren inflorescence phenotype still co-segregated with the *sopin1-1* lesion (N=16: 8 het, 3 homo, 5 wt). We crossed the T1 *sopin1-1* heterozygous plant with a line homozygous for the SoPIN1-Citrine genomic reporter line (O'Connor et al., 2014). In the F2 we identified families homozygous for *sopin1-1* but segregating for the SoPIN1-Citrine transgene. Only individuals that lacked the SoPIN1-Citrine transgene showed a *sopin1-1* phenotype, while those that contained the SoPIN1-Citrine transgene made spikelets and set seed. This complementation was independent of the presence of Cas9.

pin1b-1 CRISPR

The PIN1b guide was AGGGCAAGTACCAGATCC. We identified a single plant from the regenerating T0 PIN1b CRISPR population that had longer basal internodes and twisted leaves. This plant was homozygous for an A deletion in the PIN1b CRISPR target site causing a premature stop in the second exon 502 base pairs downstream, here designated *pin1b-1*. All T1 progeny showed the *pin1b* phenotype and were homozygous for the *pin1b-1* lesion. We backcrossed these T1 plants to Bd21-3 and all F1 progeny had a wild-type phenotype (N=11). In the F2, the *pin1b* phenotype co-segregated with the *pin1b-1* lesion (N= 215, 91 het, 39 homo, 26 wt). Amongst these plants, 24 did not have the Cas9 transgene, and the *pin1b* phenotype co-segregated perfectly with the *pin1b-1* lesion (N=24: 10 het, 6 homo, 8 wt).

Reporter Constructs

All constructs were cloned using Multi-site Gateway (Invitrogen). For pZmUbi::DII-Venus, we first cloned the maize ubiquitin promoter into pDONR P4-P1R (Primer IDs 1-2 Table 1) and this was subsequently recombined with pDONR 221 containing Arabidopsis DII and pDONR P2R-P3 containing VENUS-N7 (Brunoud et al., 2012) into the Multi-site Gateway binary vector pH7m34GW (http://gateway.psb.ugent.be/). This construct was then transformed into Brachypodium Bd21-3 using previously published methods (Bragg et al., 2015). In the T3 generation, degradation of DII-Venus in the presence of auxin was validated by treating excised Brachypodium spikelet meristems with 1 µM 1-naphthaleneacetic acid (NAA) or mock treatment in 70% ethanol, and imaging every 30 min (Figure 1 – figure supplement 2).

For *proAtPIN1* complementation, a 3.5kb Arabidopsis PIN1 promoter region was amplified from a genomic clone previously reported to complement the *pin1* (Heisler et al., 2005) and cloned into Gateway vector pDONR P4-P1R (Primer IDs 3-4 Table 1). For each Brachypodium PIN-Citrine fusion construct the entire coding region, including the Citrine insertion, was amplified from the previously published reporter constructs (O'Connor et al., 2014) and cloned into pENTR /D-TOPO (Primer IDs 5-8 Table 1). The *proAtPIN1* pDONR P4-P1R and PIN coding region vectors were then recombined into Gateway binary vector pH7m24GW (http://gateway.psb.ugent.be/) and transformed by floral dip into both Col-0 and plants heterozygous for *pin1-613* (also known as *pin1-7,* SALK_047613) (Bennett et al., 2006; Smith, 2006). Complementation was assessed in the T3 generation, and all plants were genotyped for both the *pin1-613* mutation (Primer IDs 9-11 Table 1) and for presence of the PIN transgene (Primer IDs 12-14 Table 1).

For the *proAtML1* lines the PIN coding regions with Citrine insertion pENTR /D-TOPO Gateway vectors were recombined downstream of the two-component OP promoter in vector pMoA34-OP (Moore et al., 1998) and then transformed into the *proAtML1* driver line in the Landsberg *erecta* background (Lenhard, 2003). Lines homozygous for both the *proAtML1* driver and OP::PIN were crossed to het *pin1-4* and complementation was assessed in the F2 and F3 generations. All complemented plants were genotyped for *pin1-4* (Primer IDs 15-16 Table 1 with AciI digestion), the Brachypodium PINs (Primer IDs 12-14 Table 1), and the presence of the ML1 driver transgene (Primer IDs 17-18 Table 1).

Confocal Imaging

All confocal images were captured on a Zeiss 780 laser scanning confocal using 514nm excitation. Detection wavelengths: 517-570nm for Citrine tagged PINs, 631-717nm for Propidium Iodide, and 646-726 for chlorophyll A auto-fluorescence. The pinhole was set to 1 airy unit for all meristem stacks and details of sub-epidermal polarization, but was open to the maximum setting for tiled longitudinal and cross sections of the basal internode (Figures 2C-H, 4C-H and 5I-J). Detection gain and laser power were varied according to signal strength unless direct comparisons between genotypes were made as indicated in figure legends.

Auxin Transport Assays

Auxin transport assays were carried out as described in (Crawford et al., 2010). Briefly, 17 mm long basal internodes were excised and the apical end submerged in 30 µl Arabidopsis salts (ATS) without sucrose (pH = 5.6) containing 1 µM ^{14}C-IAA (American Radiolabeled Chemicals). After 6 hours incubation, the basal 5 mm segment was excised, cut in half, and shaken overnight at 400 RPM in 200 µl scintillation liquid prior to scintillation counting. 10µM N-1-Naphthylphthalamic Acid (NPA), an auxin transport inhibitor, was added prior to incubation for negative controls.

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838 **de, S., et al.** (2014). Auxin efflux by PIN-FORMED proteins is activated by
839 two different protein kinases, D6 PROTEIN KINASE and PINOID. *eLife* **3**.

840

841

Figures and Legends

Figure 1. SoPIN1 and PIN1b have different functions in Brachypodium. (A) SoPIN1 (Bradi4g26300) and PIN1b (Bradi3g59520) CRISPR-derived *sopin1-1* and *pin1b-1* mutant alleles. Exons are indicated by grey boxes. Arrowheads indicate CRISPR target sites and are labeled with the type of DNA lesion (C insertion or A deletion). Both alleles have premature stop codons at the positions indicated by lines. **(B-D)** Inflorescence phenotypes of CRISPR-derived *sopin1-1* and *pin1b-1* mutants. **(B)** Wildtype (WT) (inbred line Bd21-3) Brachypodium inflorescences usually have several spikelets (s) on each inflorescence. Inset shows spikelet meristem (arrow), with several floral meristems being initiated. **(C)** *sopin1-1* plants have severe organ initiation defects in the inflorescence, and spikelets often abort (asterisk), or fail to initiate despite a clear distinction between the white node (n) and green internode (i) tissue. Inset shows two barren spikelet meristems (arrows) with organ initiation defects. **(D)** *pin1b-1* plants have normal organ initiation, but internode tissue often bends. A phenotypically normal spikelet (s) is indicated. **(E)** Medial confocal Z-section of pZmUbi::DII-Venus (DII) expression in a WT spikelet co-expressing SoPIN1 tagged with Cerulean (a CFP variant) under the native SoPIN1 promoter. Organ primordia are numbered I3, I2, I1 from youngest to oldest prior to morphogenesis, and P1, P2, P3, etc. youngest to oldest after morphogenesis. DII is normally degraded at SoPIN1 convergence points in I2 and I1 primordia (asterisks). Inset shows color look-up-table for all PIN images. **(F)** Medial confocal Z-section of pZmUbi::DII-Venus expression in a *sopin1-1* spikelet meristem. DII degradation does not occur in *sopin1-1* meristems, and organs fail to initiate (arrow). Inset shows color look-up-table for DII. **(G-H)** Basal internodes are elongated in *pin1b-1* mutants compared to WT. Lines in **(G)** connect equivalent nodes in WT (left) and *pin1b-1* (right) primary stems. Axillary shoots and leaves have been removed from the main axis. **(H)** Mean internode lengths along WT (left) and *pin1b-1* (right) primary stems. Nodes are numbered from 1 starting from below the inflorescence. (n=11-12 each internode, error bars +/- standard error of the mean). **(I)** Maximum projection of PIN1b tagged with Citrine under its native promoter in a longitudinal hand-section through several immature vegetative nodes**.** Arrows indicate the shoot apical meristem (SAM), node (n), and internode (i) tissues. Scale bars: 1cm in **(B-D)** and **(G)**. 25µm in **(E)**, **(F)** and **(I)**.

Figure 1 – supplement 1. Mature whole-plant phenotypes of Brachypodium CRISPR-derived *sopin1-1* and *pin1b-1* mutants. Left WT (Inbred Bd21-3), middle *sopin1-1,* and right *pin1b-1.* Scale bar: 1cm.

Figure 1 – supplement 2. DII-Venus is degraded in the presence of auxin in Brachypodium spikelet meristems. (A) 1 µM NAA-treated, and **(B)** mock-treated spikelet meristems expressing pZmUbi::DII-Venus imaged every 30 min after treatment. Images from left to right, pre-treatment expression, 30 min, 60 min and 90 min time-points. Scale bars: 25µm.

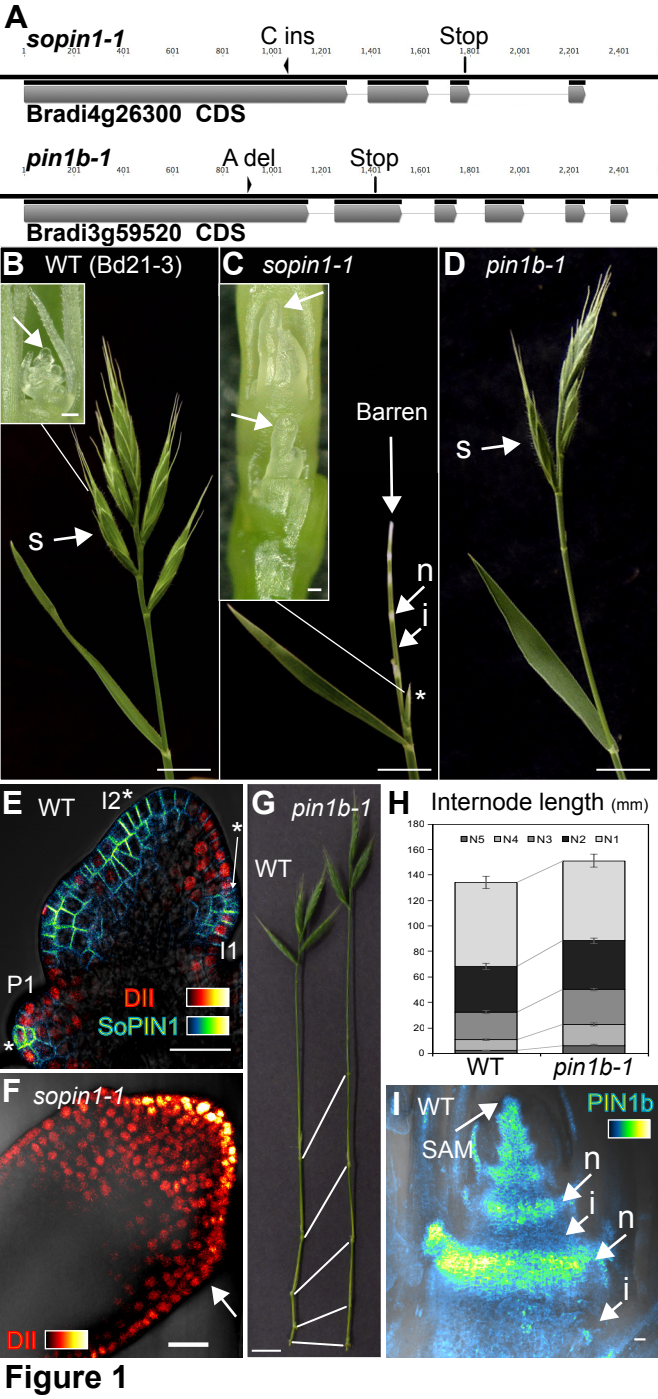

A
sopin1-1
C ins
Stop
Bradi4g26300 CDS
pin1b-1
A del
Stop
Bradi3g59520 CDS
B WT (Bd21-3)
s
C sopin1-1
Barren
n
i
*
D pin1b-1
s
E WT
I2*
I1
P1
DII
SoPIN1
F sopin1-1
DII
G pin1b-1
WT
H Internode length (mm)
N5 N4 N3 N2 N1
WT
pin1b-1
I WT
SAM
PIN1b
n
i
n
i

Figure 1

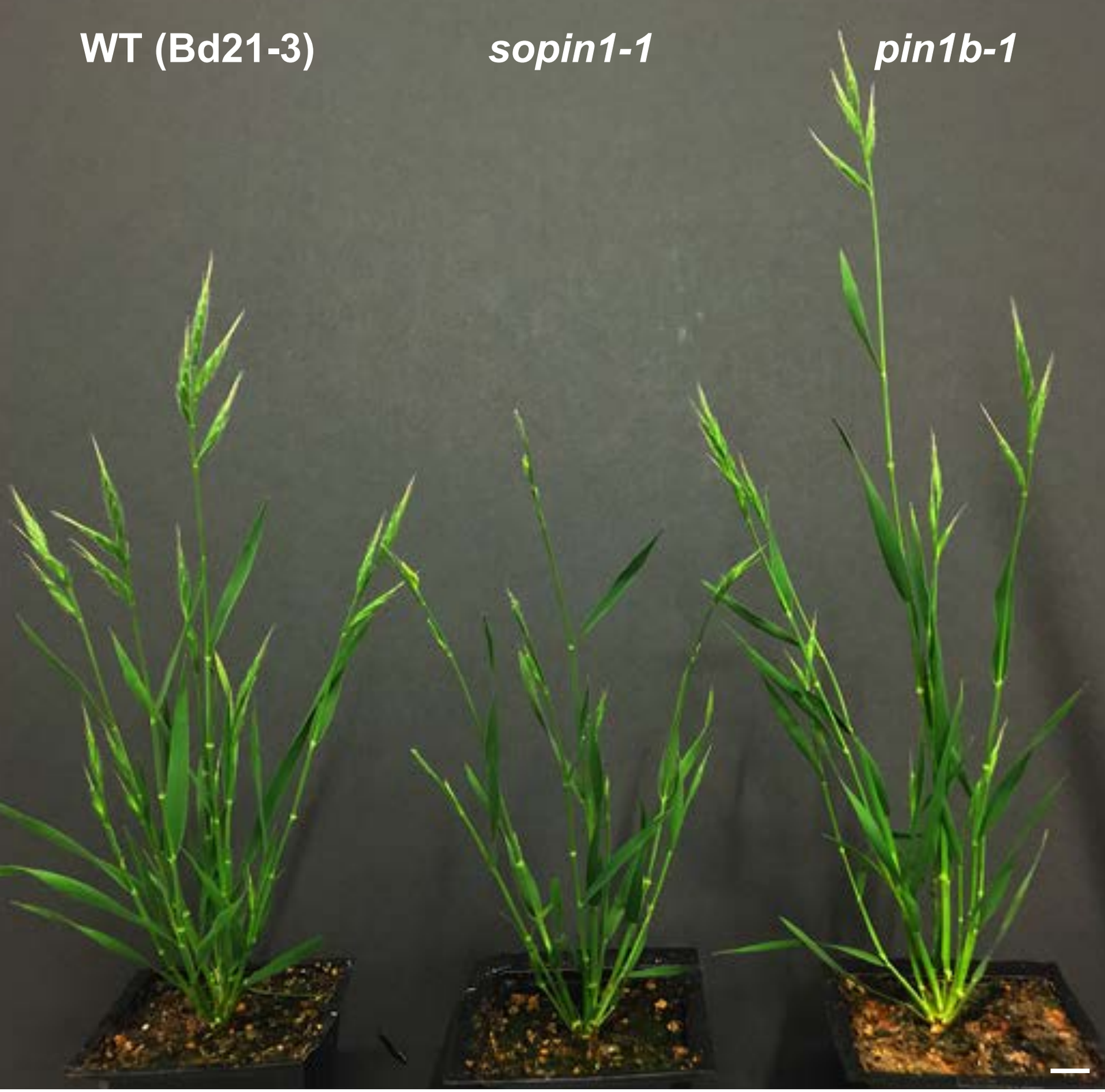


Figure 1 - supplement 1

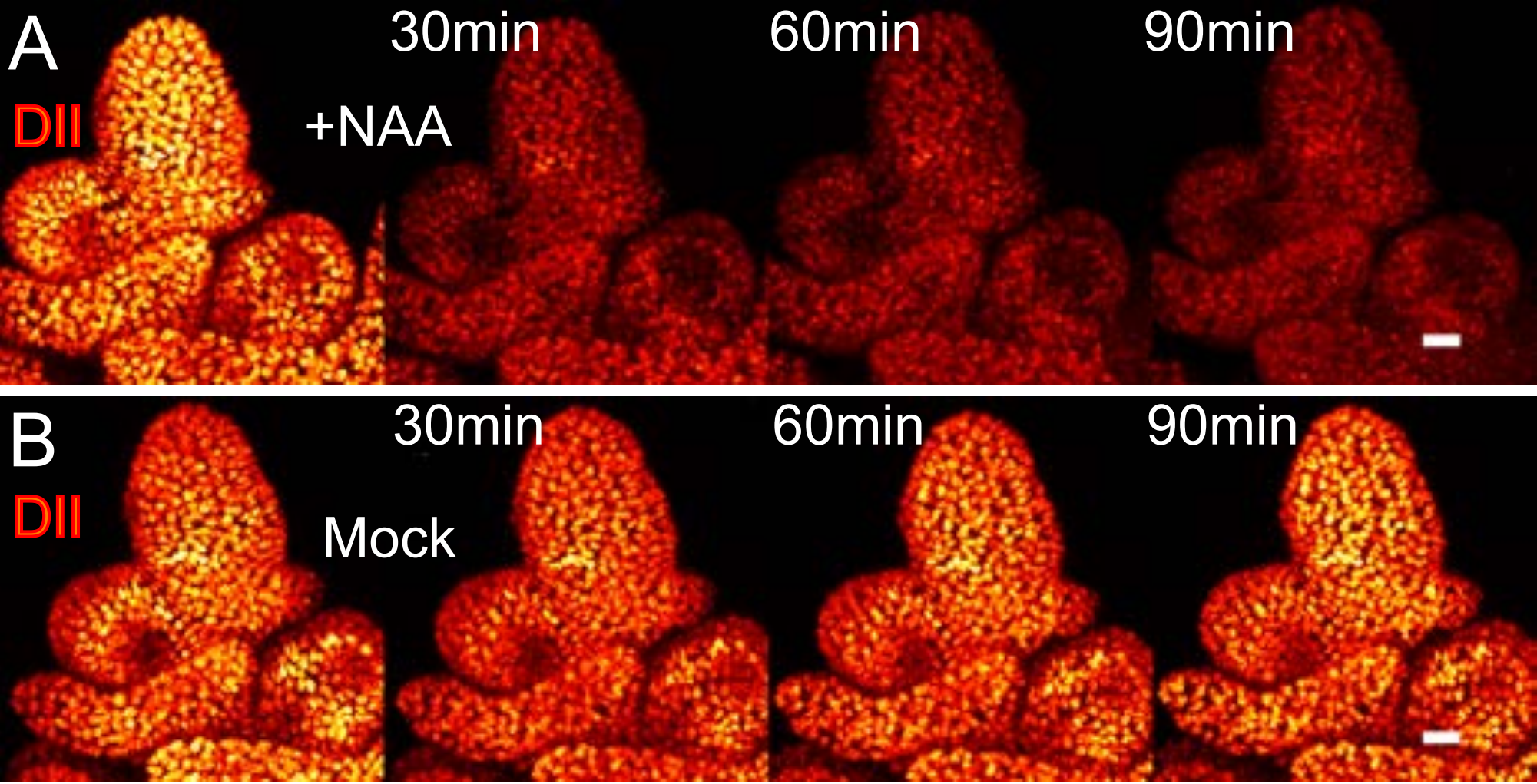


Figure 1 - supplement 2

Figure 2. SoPIN1 and PIN1b show different behaviors when expressed in wildtype Arabidopsis. Arabidopsis *AtPIN1* promoter (*proAtPIN1*) -driven expression of Citrine-tagged (a YFP derivative) SoPIN1 and PIN1b in wildtype Columbia (Col-0) Arabidopsis. **(A,C,E,G,I,J,K)** SoPIN1, **(B,D,F,H,L,M)** PIN1b. **(A-B)** Maximum projections of meristem apexes. Arrow in **(A)** indicates SoPIN1 convergence point (cp) in an I2 primordium. Arrow in **(B)** indicates internalized PIN1b in punctate membrane bodies. **(C-D)** Tiled confocal maximum projections of longitudinal hand-sections through apexes. Arrows indicate SoPIN1 epidermal expression in sepal primordia and flower pedicels in **(C)** and the lack of PIN1b epidermal expression in the same tissues in **(D)**. **(E-F)** Tiled confocal maximum projections of longitudinal sections through basal inflorescence stem internodes 1cm above the rosette. **(G-H)** Tiled confocal maximum projections of cross-sections through basal internodes 1cm above the rosette. Signal at the edge of each section (arrows) is cuticle auto-florescence. The cambium (c), xylem parenchyma (xp), and pith (p) tissues are indicated in **(E-H). (I)** Maximum projection detail of SoPIN1 convergence point (Asterisk) in I2 primordia in meristem shown in **(A)**. **(J)** Confocal z-section of SoPIN1 accumulation in a ring-shaped domain just below the apex of the meristem shown in **(A)**. **(K)** Maximum projection detail of root-ward polarized SoPIN1 (arrows) in a longitudinal section of the basal internode pith tissue. **(L)** Confocal z-section of PIN1b accumulation in distinct vascular-associated domains just below the apex of the meristem shown in **(B)**. **(M)** Maximum projection detail of root-ward polarized PIN1b (arrows) in a longitudinal section of the basal internode xylem parenchyma tissue. Red signal in all panels is chlorophyll auto-florescence. Scale bars: 25μm in **(A-B),** 100μm in **(C-H),** and 25μm in **(I-M)**.

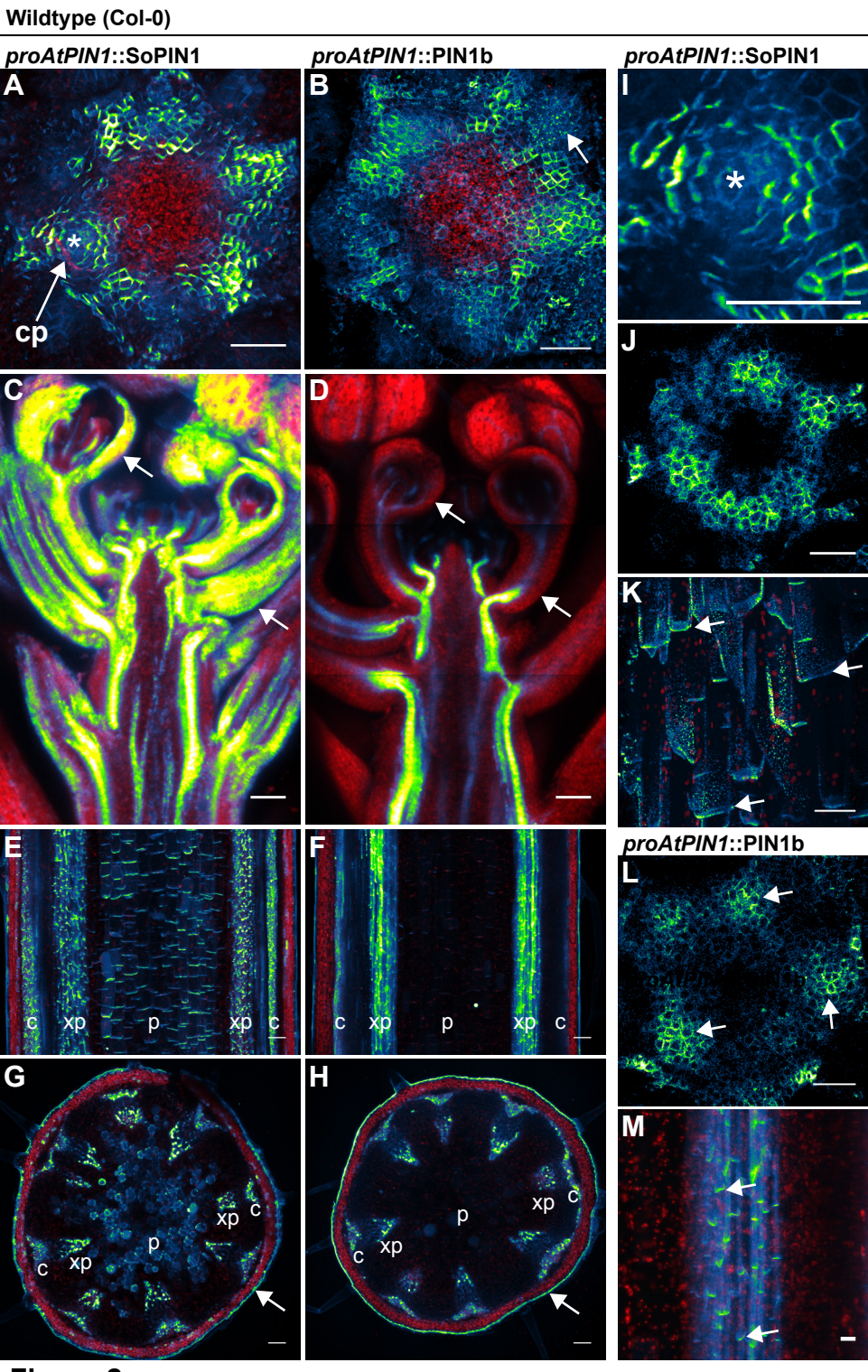
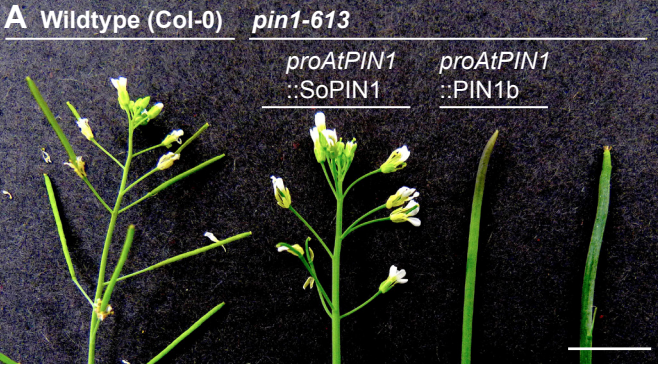

Wildtype (Col-0)
proAtPIN1::SoPIN1
proAtPIN1::PIN1b
proAtPIN1::SoPIN1
A
B
I
*
cp
C
D
J
K
E
F
proAtPIN1::PIN1b
L
c
xp
p
xp
c
G
H
M

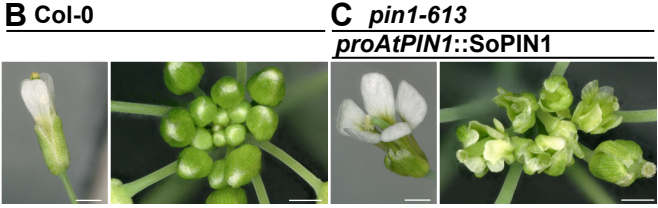
Figure 2

Figure 3. SoPIN1 but not PIN1b can partially complement the Arabidopsis *pin1-613* mutant organ initiation and bulk transport defects. (A) From left to right, inflorescence phenotypes of WT (Col-0), *proAtPIN1::*SoPIN1 in *pin1-613, proAtPIN1*::PIN1b in *pin1-613,* and *pin1-613* alone. Note that PIN1b-expressing *pin1-613* plants are indistinguishable from *pin1-613* alone. **(B)** Flower (left), and inflorescence apex (right) of WT (Col-0). **(C)** Flower (left), and inflorescence apex (right) of *proAtPIN1::*SoPIN1 complemented *pin1-613* mutants. Note the increase in petal number in the flower, see Figure 3 - supplement 2 for organ counts. **(D)** Box-plot of bulk auxin transport (counts per minute, CPM) through basal internodes 1cm above the rosette of 40-day-old Arabidopsis inflorescence stems. (n=16 each genotype). Samples with different letters are significantly different from each other (ANOVA, Tukey HSD, $p < 0.05$). Scale bars: 1cm in **(A)**, 1mm in **(B-C)**.

Figure 3 - supplement 1. Whole-plant phenotypes of *proAtPIN1*-driven complementation of *pin1-613.* From left to right, Col-0, *proAtPIN1::*SoPIN1 complemented *pin1-613*, *proAtPIN1::*PIN1b expressing *pin1-613, pin1-613.*

Figure 3 - supplement 2. Floral organ number in *proAtPIN1::*SoPIN1 complemented flowers. Mean and standard-error of sepal, petal, stamen and carpel organ numbers in heterozygous *pin1-613* or wildtype (white bars), and *proAtPIN1::*SoPIN1-complemented *pin1-613* flowers (grey bars) (n=30).


A Wildtype (Col-0)
pin1-613
proAtPIN1 ::SoPIN1
proAtPIN1 ::PIN1b


B Col-0
C pin1-613
proAtPIN1::SoPIN1

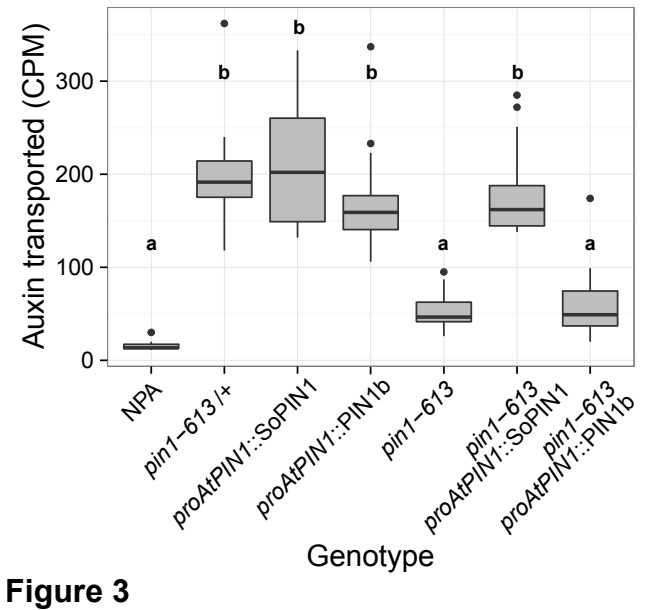

D Stem auxin transport
Auxin transported (CPM)
0
100
200
300
a
b
b
b
a
b
a
NPA
pin1−613/+
proAtPIN1::SoPIN1
proAtPIN1::PIN1b
pin1−613
pin1−613 proAtPIN1::SoPIN1
pin1−613 proAtPIN1::PIN1b
Genotype

Figure 3

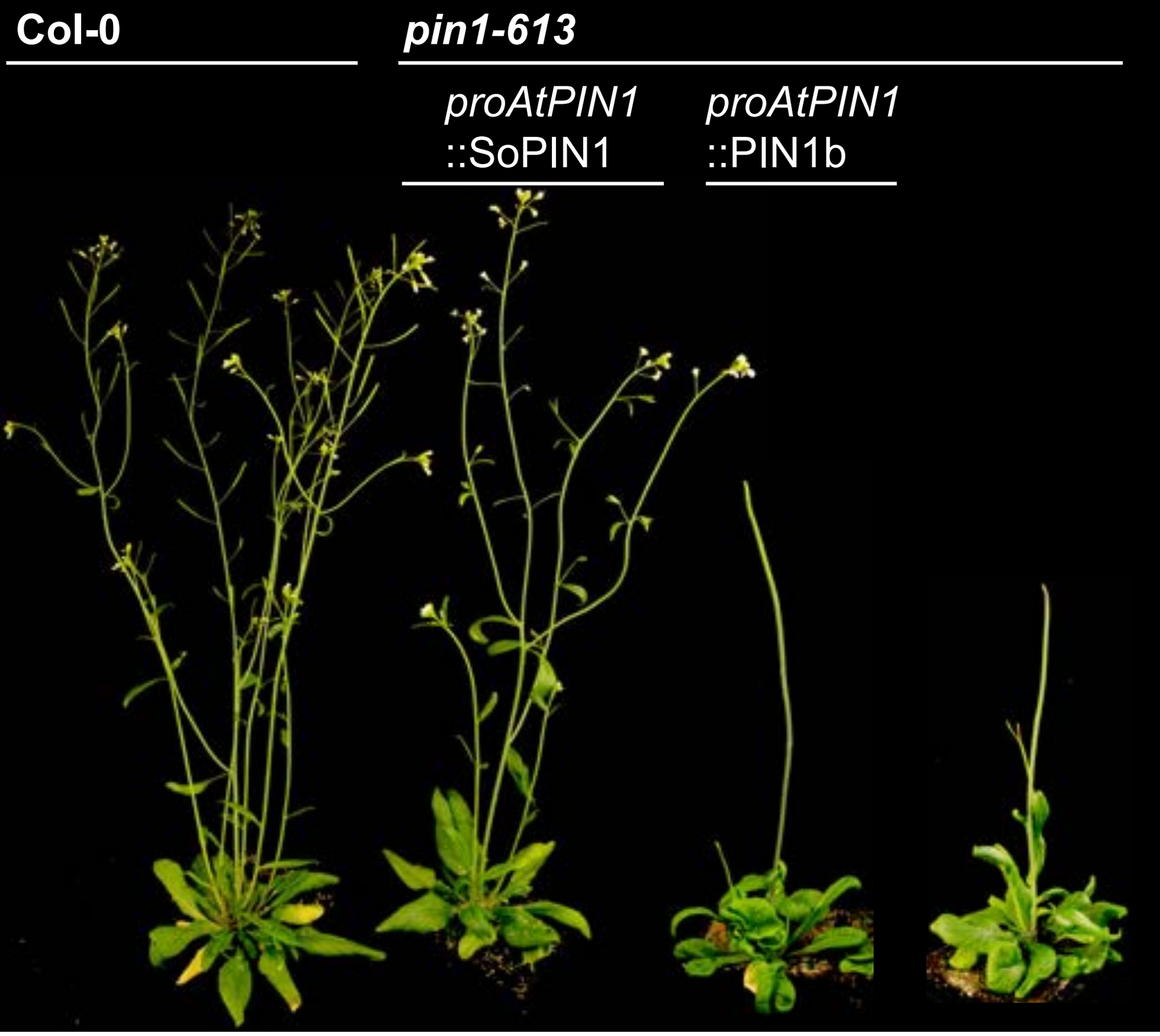


Figure 3 - supplement 1

Floral organ number - *proAtPIN1::*SoPIN1

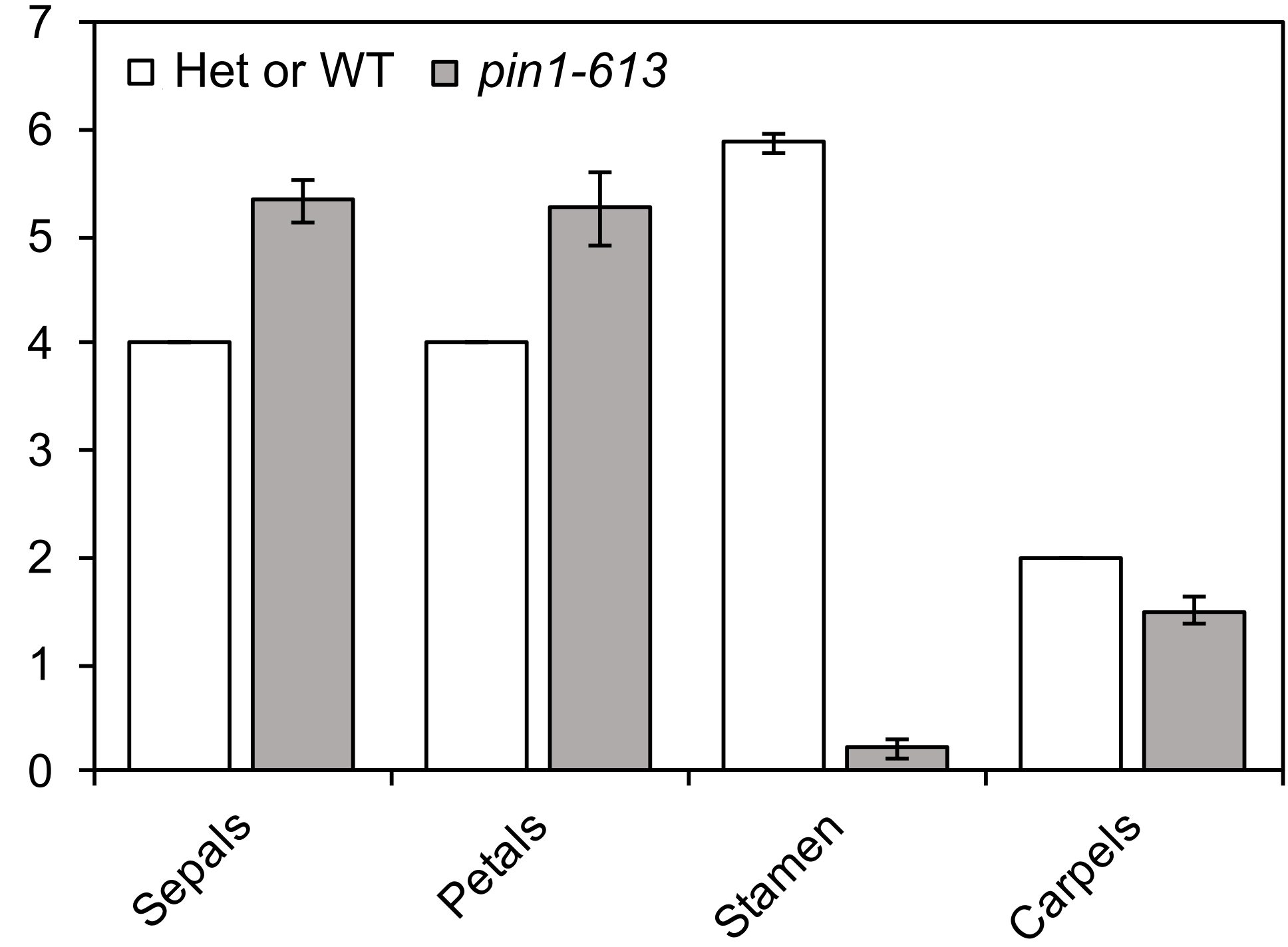


Figure 3 - supplement 2

Figure 4. SoPIN1 but not PIN1b can mediate organ initiation and bulk transport in null *pin1-613 mutants*. Arabidopsis *PIN1* promoter (*proAtPIN1*) -driven expression of Citrine-tagged (YFP derivative) SoPIN1 and PIN1b in null *pin1-613* mutant tissue. **(A,C,E,G,I)** SoPIN1, **(B,D,F,H,J,K,L,M)** PIN1b. **(A-B)** Maximum projections of meristem apexes. Arrow in **(B)** indicates PIN1b ring shaped epidermal domain. **(C-D)** Tiled confocal maximum projections of longitudinal hand-sections through apexes. Arrow in **(D)** indicates increased PIN1b in the epidermis in the *pin1-613* background. **(E-F)** Tiled maximum projections of longitudinal sections through basal inflorescence stem internodes 1cm above the rosette. **(G-H)** Tiled maximum projections of cross-sections through basal internodes 1cm above the rosette. Signal at the edge of each section (arrows) is cuticle auto-florescence. The cambium (c), xylem parenchyma (xp), and pith (p) tissues are indicated in **(E-H). (I)** Confocal z-section of SoPIN1 accumulation in a ring-shaped domain just below the apex of a complemented *pin1-613* meristem. **(J)** Longitudinal hand-section of PIN1b just below a *pin1-613* meristem apex. Arrow shows root-ward polarized PIN1b. **(K)** Detail of polarized PIN1b in the meristem epidermis of a *pin1-613* meristem apex. **(L)** Cross-section of PIN1b in distinct bundles 2mm below a *pin1-613* meristem apex. **(M)** Root-ward polarization of PIN1b (arrow) 3-4 mm below the apex of a *pin1-613* meristem. Red signal in all panels is chlorophyll auto-florescence. Scale bars: 25μm in **(A-B),** 100μm in **(C-H),** and 25μm in **(I-M)**.

Figure 4 – supplement 1. *proAtPIN1*-driven SoPIN1 expression in *pin1-613* segregating family. (A) *proAtPIN1::*SoPIN1 expression in 6 different wt or het *pin1-613* meristem samples. **(B)** *proAtPIN1::*SoPIN1 expression in 6 different complemented *pin1-613* meristems. All samples were imaged with identical settings to show the increase in SoPIN1 in the *pin1-613* mutant background. Red signal is chlorophyll auto-florescence. Scale bars: 25μm.

Figure 4 – supplement 2. *proAtPIN1*-driven PIN1b expression in *pin1-613* apexes. Two representative meristems each from four different transgenic events. All samples were imaged with identical settings. Scale bars: 25μm.

Figure 4 – supplement 3. *proAtPIN1*-driven PIN1b dynamics during organ formation induced by addition of lanolin containing 1mM IAA on *pin1-613* apexes. From left to right, pre-treatment, 24, 48, 72, and 96 hours after treatment. Four representative samples are shown top to bottom. Arrows indicate the lanolin paste at the 24-hour time-point. All samples were imaged with identical settings. Scale bars 25μm.

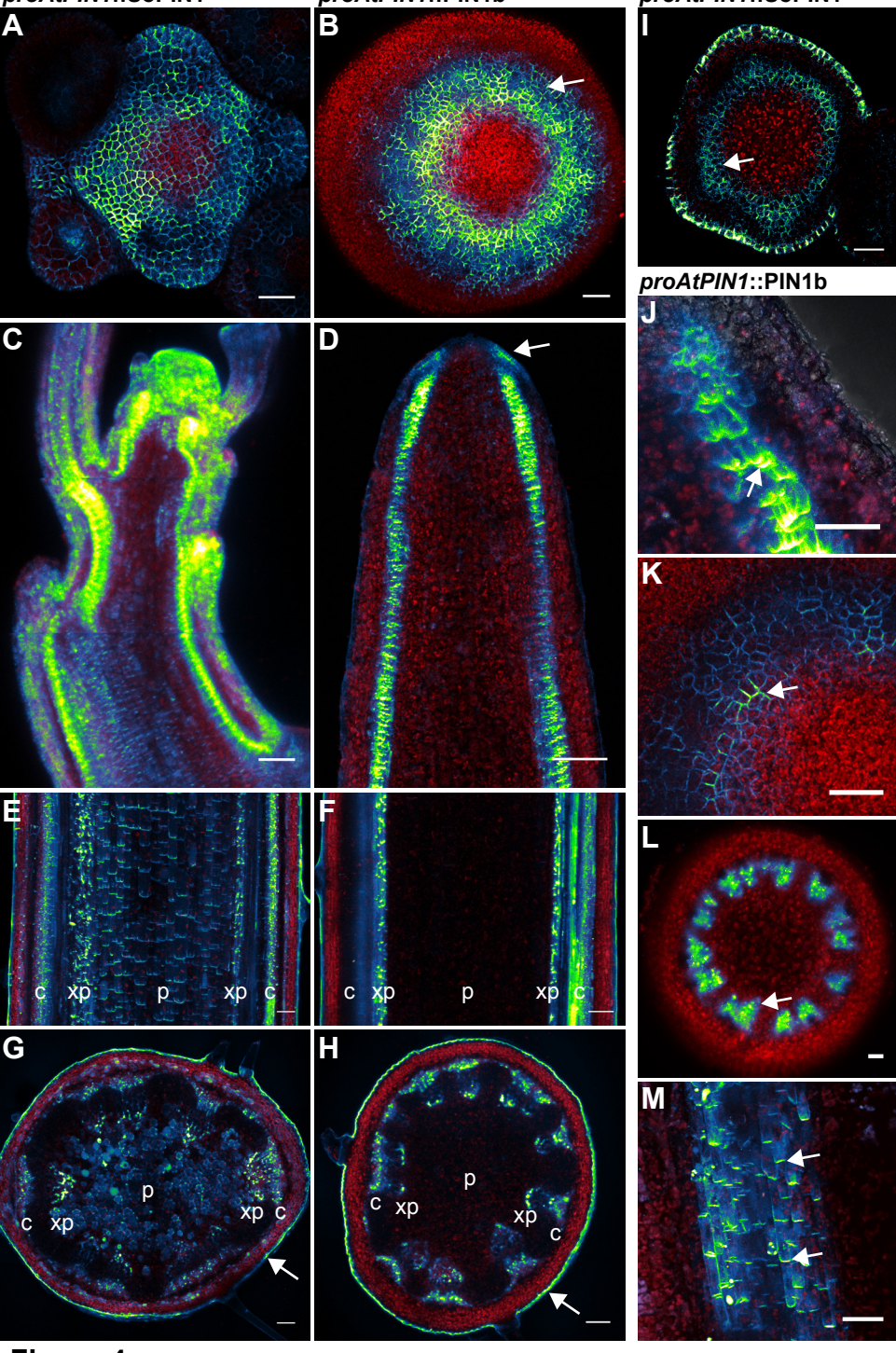

proAtPIN1::SoPIN1
proAtPIN1::PIN1b
proAtPIN1::SoPIN1
proAtPIN1::PIN1b
A
B
I
C
D
J
K
E
F
L
G
H
M
c
xp
p
xp
c

Figure 4

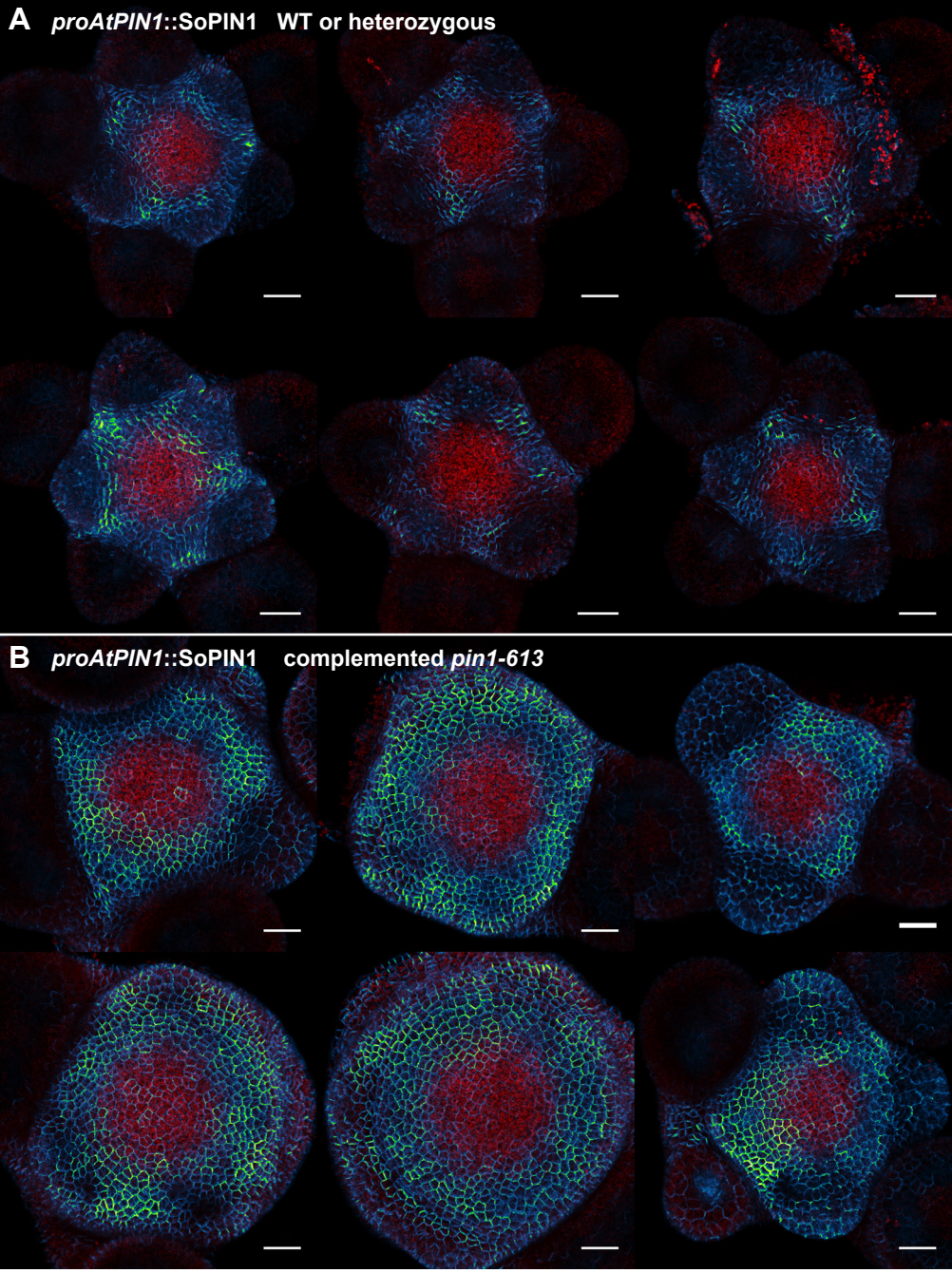


Figure 4 - supplement 1

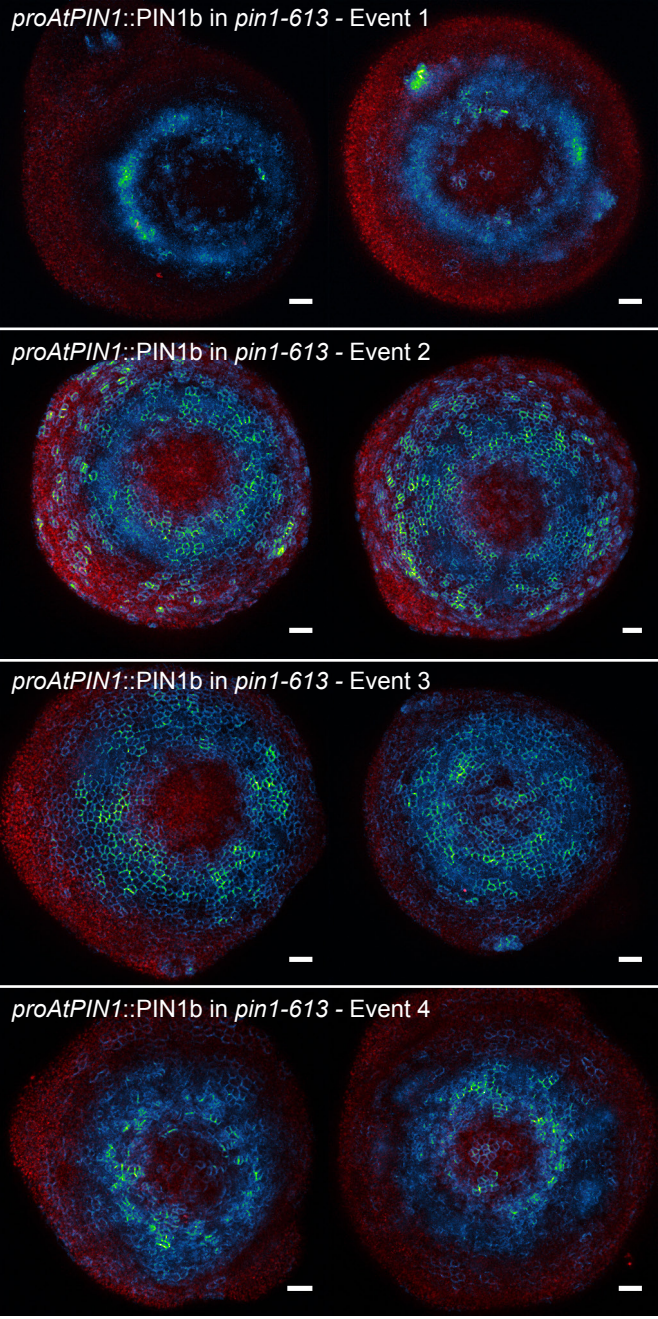


Figure 4 - supplement 2

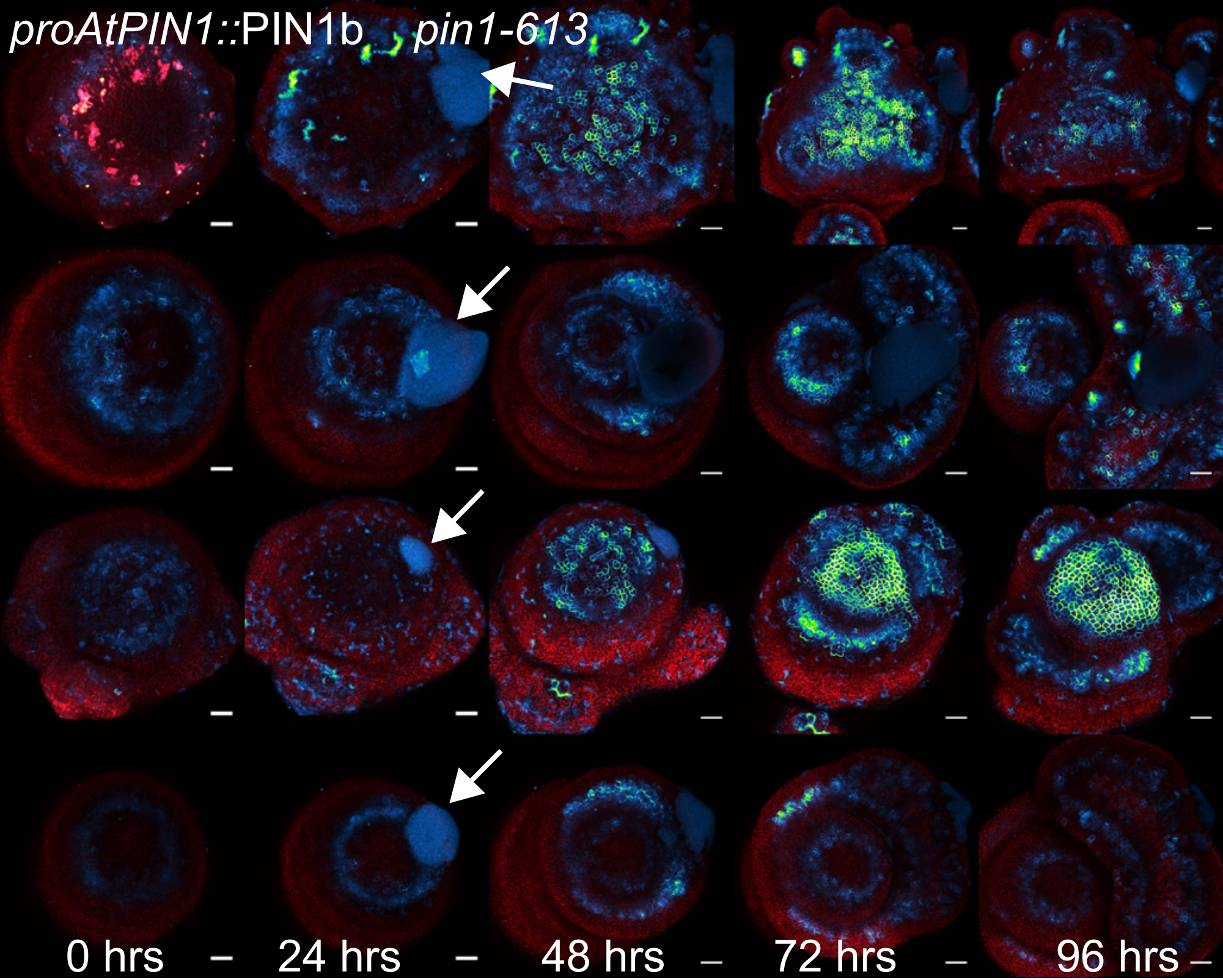


Figure 4 - supplement 3

Figure 5. SoPIN1 and PIN1b show different behaviors under *proAtML1*-driven expression. Maximum projections of *proAtML1::*LhG4 driving pOP::SoPIN1 or pOP::PIN1b (*proAtML1*>>SoPIN1 and *proAtML1*>>PIN1b) in wildtype Landsberg *erecta* (L*er)* **(A-D),** and *pin1-4* **(E-L)** inflorescence meristems and basal internodes. **(A)** SoPIN1 and **(B)** PIN1b maximum projections of wildtype L*er* inflorescence meristems. I3, I2, I1, and P1 primordia are indicated. White boxes around each I2 primordium indicate the regions detailed in **(C-D).** Asterisk in **(C)** indicates convergence point. Arrow in **(D)** indicates punctate PIN1b. **(E)** SoPIN1 and **(F)** PIN1b maximum projections of complemented *pin1-4* meristems. I3, I2, I1, and P1 primordia are indicated. White boxes around each I2 primordia indicate the regions detailed in **(G-H)**. Asterisks mark convergence points in **(G)** and **(H)**. Red signal in **(C,D,G,H)** is cell wall propidium iodide staining. **(I-J)** Tiled maximum projections of cross hand-sections of the basal internode of SoPIN1 **(I)** and PIN1b **(J)** -complemented *pin1-4* plants showing PIN signal in the outer cortex layers (arrows). Red signal in **(I-J)** is chlorophyll auto-florescence. **(K-L)** Epidermal maximum projections showing root-ward polarized PIN localization (arrows) in the basal internode of SoPIN1 **(K),** and PIN1b **(L) -**complemented *pin1-4* plants. Scale bars: 25µm in **(A-H)**. 100µm in **(I-L)**.

Figure 5 – supplement 1. *proAtML1*>>SoPIN1 representative meristem maximum projections. (A) *proAtML1*>>SoPIN1 expression in three different wildtype L*er* meristems. **(B)** *proAtML1*>>SoPIN1 expression in three different complemented *pin1-4* meristems. Capture settings are identical in all samples. Scale bars: 25µm.

Figure 5 – supplement 2. *proAtML1*>>PIN1b representative meristem maximum projections. (A) *proAtML1*>>PIN1b expression in three different wildtype L*er* meristems. **(B)** *proAtML1*>>PIN1b expression in three different complemented *pin1-4* meristems. Capture settings are identical in all samples. Scale bars: 25µm.

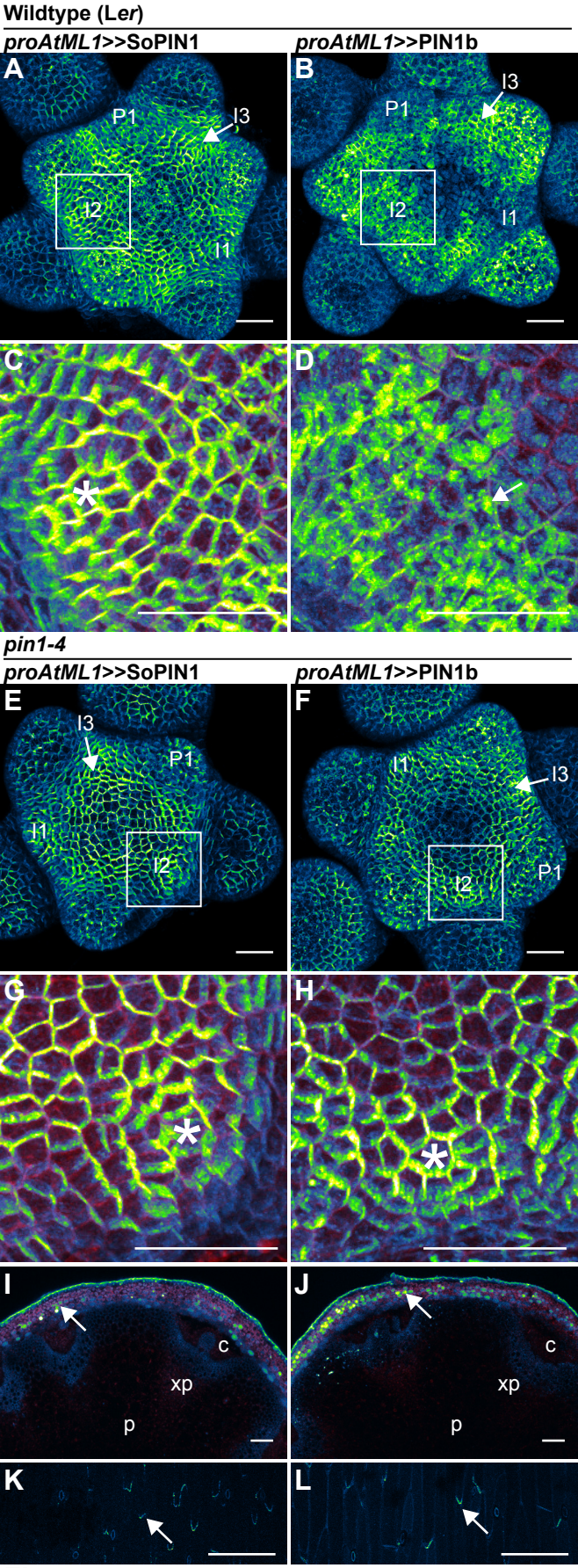

Wildtype (Ler)
proAtML1>>SoPIN1
proAtML1>>PIN1b
A
P1
I3
I2
I1
B
P1
I3
I2
I1
C
D
pin1-4
proAtML1>>SoPIN1
proAtML1>>PIN1b
E
I3
P1
I1
I2
F
I1
I3
I2
P1
G
H
I
c
xp
p
J
c
xp
p
K
L

Figure 5

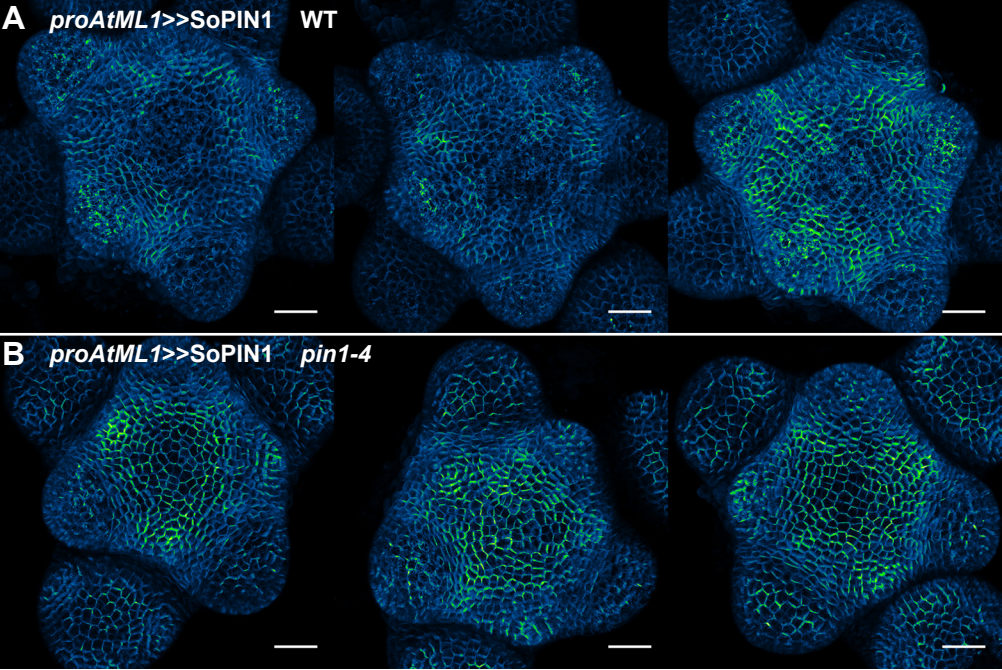


Figure 5 - supplement 1 - *proAtML1*>>SoPIN1

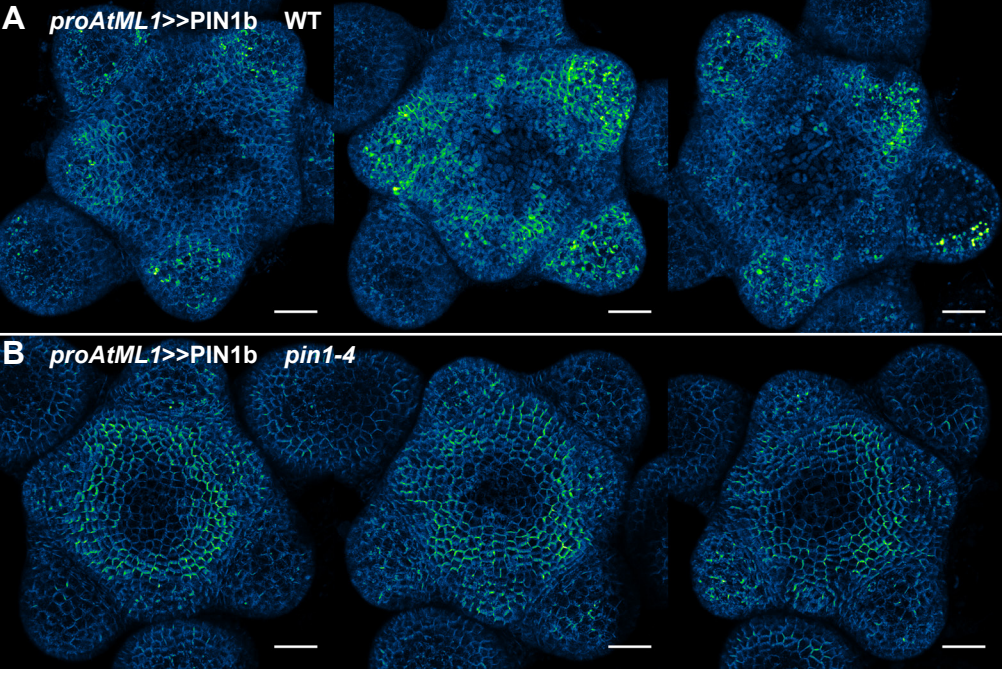
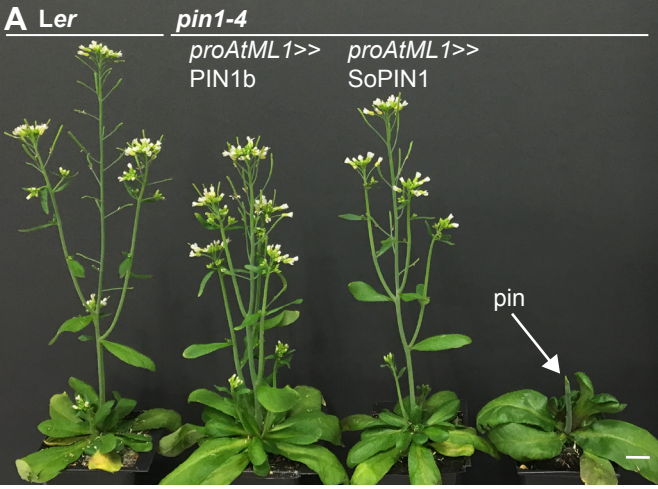
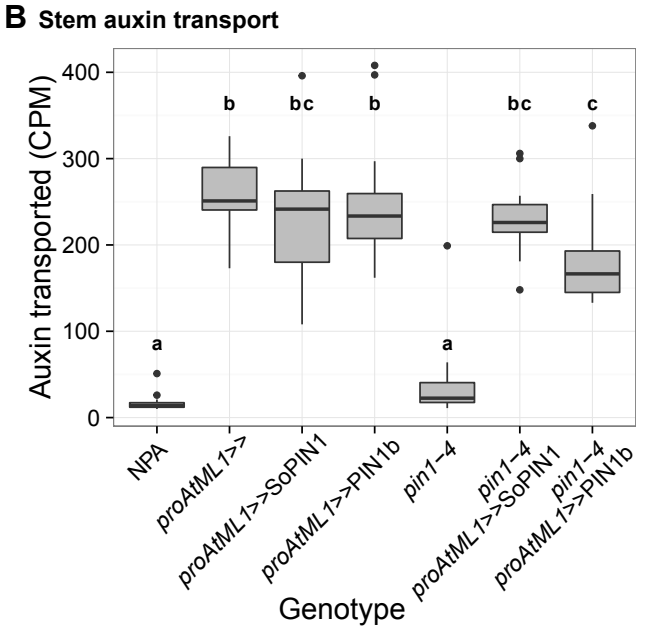


Figure 5 - supplement 2 - *proAtML1*>>PIN1b

Figure 6. Both SoPIN1 and PIN1b can complement *Arabidopsis pin1-4* under *proAtML1*-driven expression. (A) From left to right, wildtype *Ler, proAtML1*>>PIN1b complemented *pin1-4, proAtML1*>>SoPIN1 complemented *pin1-4, and pin1-4 alone.* Arrow indicates barren pin inflorescence in *pin1-4*. **(B)** Box-plot of bulk auxin transport (counts per minute, CPM) through basal internodes 1cm above the rosette of 40-day-old Arabidopsis inflorescence stems (n=16 each genotype). Samples with different letters are significantly different from each other (ANOVA, Tukey HSD, $p < 0.05$). **(C)** Box-plot of stem cross-sectional area (square mm) of the basal internode 1cm above the rosette (n=12 each genotype). Representative Toluidine Blue O stained hand cross-sections are shown above each box. Samples with different letters are significantly different from each other. (ANOVA, Tukey HSD, $p < 0.05$). *Scale* bars: 1cm in **(A)**. 500µm in **(C).**

Figure 6 – supplement 1. *proAtML1*>> complemented *pin1-4* inflorescence phenotypes. (A) Wildtype Ler, **(B)** *proAtML1*>>SoPIN1 complemented *pin1-4,* and **(C)** *proAtML1*>>PIN1b complemented *pin1-4* inflorescence apexes. Scale bars: 1mm.


A Ler
pin1-4
proAtML1>>
PIN1b
proAtML1>>
SoPIN1
pin


B Stem auxin transport
Auxin transported (CPM)
400
300
200
100
0
a
b
bc
b
a
bc
c
NPA
proAtML1>>
proAtML1>>SoPIN1
proAtML1>>PIN1b
pin1−4
pin1−4 proAtML1>>SoPIN1
pin1−4 proAtML1>>PIN1b
Genotype

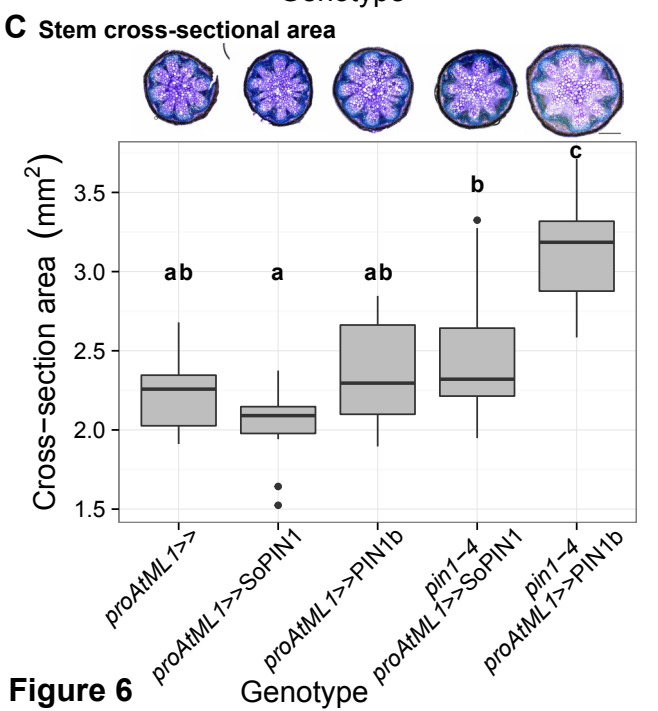

C Stem cross-sectional area
Cross−section area (mm2)
3.5
3.0
2.5
2.0
1.5
ab
a
ab
b
c
proAtML1>>
proAtML1>>SoPIN1
proAtML1>>PIN1b
pin1−4 proAtML1>>SoPIN1
pin1−4 proAtML1>>PIN1b
Genotype

Figure 6

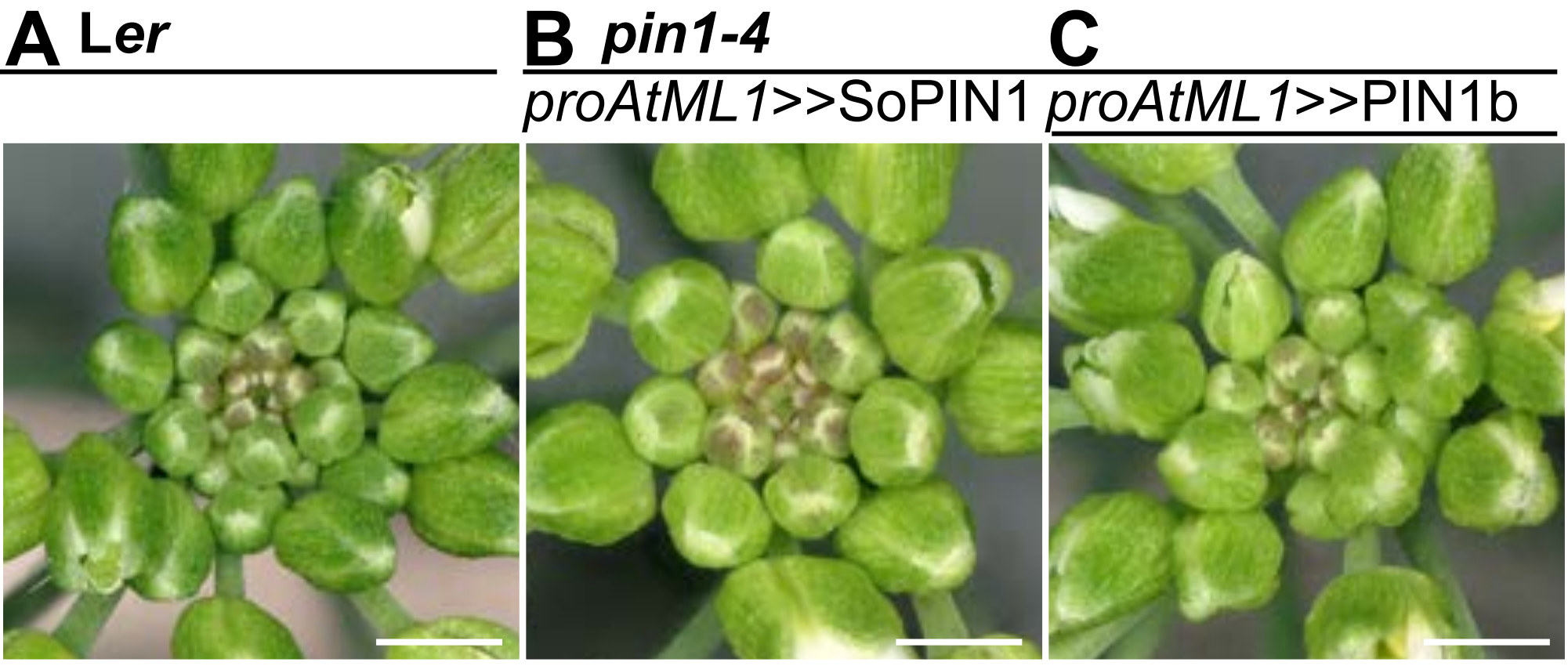


Figure 6 - supplement 1

Figure 7. Heterologous expression summary: Functional distinction between PIN auxin efflux proteins during development. SoPIN1 is in green, PIN1b in blue, and the putative partially functional *pin1-4* protein is in grey. Red arrows indicate measured auxin transport in the basal internode, while red bar-headed lines indicated reduced transport. Black lines represent polarized PIN patterns. Convergence points are marked with asterisks. **(A)** When expressed in both the epidermis and internal tissues with *proAtPIN1* in wildtype Col-0*,* SoPIN1 forms convergent polarization patterns in the epidermis and is partially able to rescue the organ initiation phenotypes and bulk transport in null *pin1-613* mutants. **(B)** When SoPIN1 is expressed only in the epidermis from the *proAtML1* promoter, it forms convergence points in the wild type background and is able to rescue more fully the organ initiation phenotypes of the *pin1-4* single amino acid change mutation in L*er.* **(C)** In contrast, when PIN1b is expressed in both the epidermis and internal tissues from the *proAtPIN1* promoter in wildtype Col-0, it accumulates mostly in the internal tissues, and is unable to complement the *pin1-613* organ initiation phenotype. It is also unable to transport auxin through stem segments, despite apparently AtPIN1-like accumulation and polarization in the stem. **(D)** When PIN1b is expressed in the epidermis from *proAtML1* promoter, it does not form convergent polarization patterns in the wildtype *Ler* background (blue ovals), but it does in the *pin1-4* background, where it is able to rescue the defective organ initiation phenotype and mediate bulk transport.

Figure 7 - supplement 1. Brassicaceae-specific PIN1 domains. (A) A wrapped protein alignment showing PIN1 clade members from across the angiosperms. Grass PIN1a proteins are indicated in grey, grass PIN1b proteins are indicated in black and Brassicaceae PIN1 proteins are indicated in red. Domains that are unique to the Brassicaceae family are indicated by transparent red boxes over the alignment. **(B)** Sequenced angiosperm species and version numbers, from https://phytozome.jgi.doe.gov. Species used in the alignment in **(A)** are indicated with green circles.

Table 1. Oligos. See methods for usage.

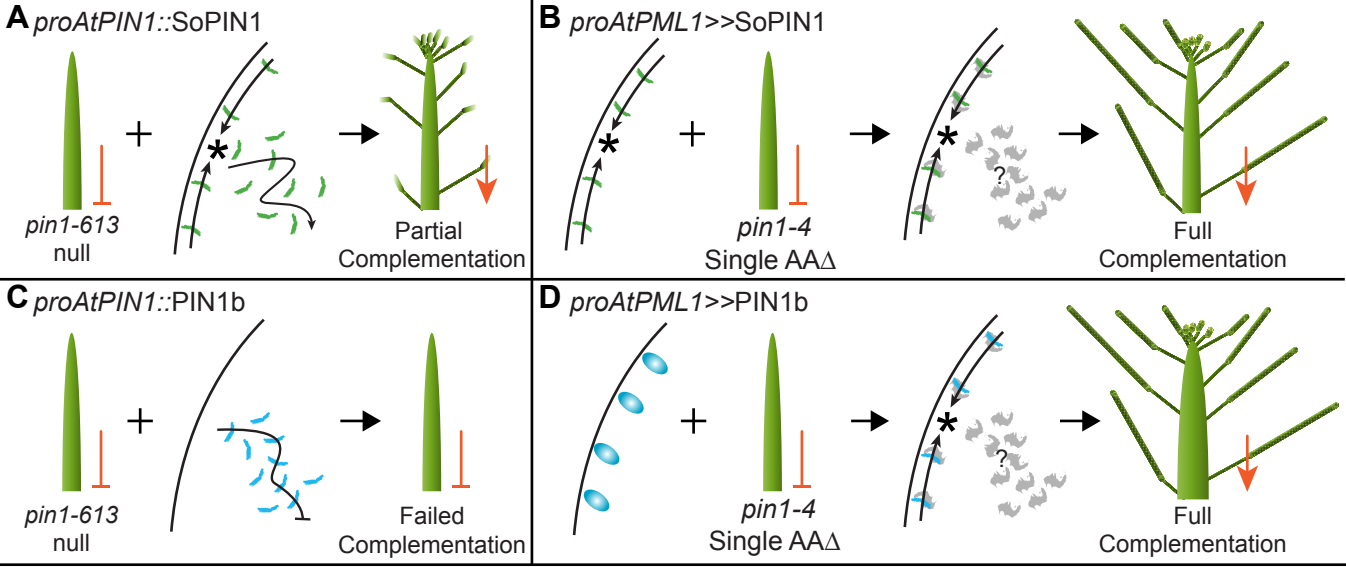

A proAtPIN1::SoPIN1
pin1-613
null
Partial
Complementation
B proAtPML1>>SoPIN1
pin1-4
Single AAΔ
Full
Complementation
C proAtPIN1::PIN1b
pin1-613
null
Failed
Complementation
D proAtPML1>>PIN1b
pin1-4
Single AAΔ
Full
Complementation
SoPIN1
PIN1b
pin1-4
Polarity
Convergence Point
Transport

Figure 7

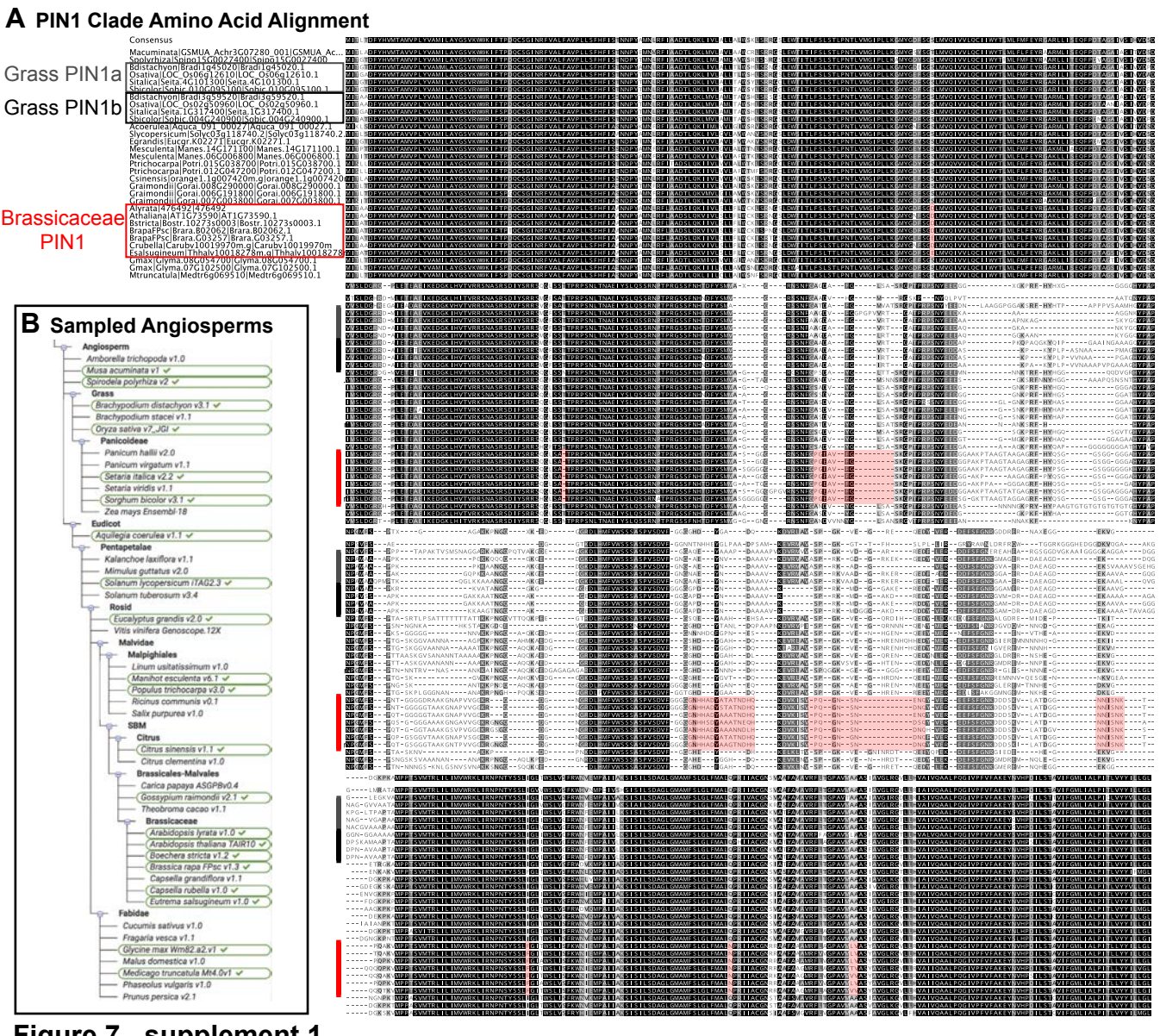


Figure 7 - supplement 1

Table 1: Primers

ID#	Name	Sequence	Purpose
1	019 - Ubi-1 Prom attB4 F	GGGGACAACTTTGTATAGAAAAGTTGCTGCAGTGCAGCGTGACCCGG	pZmUbi amplification for cloning
2	020 - Ubi-1 Prom attB1 R	GGGGACTGCTTTTTTGTACAAACTTGCTGCAGAAGTAACACCAAACA	pZmUbi amplification for cloning
3	PIN1pro-GW-F	GGGGACAACTTTGTATAGAAAAGTTGTTACCCTCATCCATCATTAACTT	*proAtPIN1* amplification
4	PIN1pro-GW-R	GGGGACTGCTTTTTTGTACAAACTTGTCTTTTGTTCGCCGGAGAAGAGA	*proAtPIN1* amplification
5	455 BdSoPIN1 cacc mRNA	TCACATCTGCTGCCGCTGCC	SoPIN1-Citrine coding region amplification
6	302 - PIN_7 qPCR UTR R2	AATCCCAAAAGCCGACATTG	SoPIN1-Citrine coding region amplification
7	466 BdPIN1b cacc mRNA-2	CACCTGTACACACTGCGGCGCT	PIN1b-Citrine coding region amplification
8	308 - PIN_5 qPCR UTR R1	ACTCGCTAACCAACCCCTTAATT	PIN1b-Citrine coding region amplification
9	MVR087 - pin1-613 RP (SALK_047613)	AATCATCACAGCCACTGATCC	*pin1-613* genotyping
10	MVR086 - pin1-613 LP (SALK_047613)	CAAAAACACCCCCAAAATTTC	*pin1-613* genotyping
11	MVR036 - LBb1.3	ATTTTGCCGATTTCGGAAC	*pin1-613* genotyping
12	344 - Citrine Seq R	GAAGCACATCAGGCCGTAG	PIN1b-Citrine and SoPIN1-Citrine genotyping
13	524_Bradi4g26300_4230_F	CGTTCCGTGTTGATTCCGATG	SoPIN1-Citrine genotyping
14	541_Bradi3g59520_PIN1b_5084_F	TGATGCTCTTCATGTTCGAGTACC	PIN1b-Citrine genotyping
15	543_pin1-4_Aci_F	GCTTTTGCGGCGGCTATGAGATTTGT	*pin1-4* genotyping
16	544_pin1-4_Aci_R	GCTTCTGATTTAATTTGTGGGTTTTCA	*pin1-4* genotyping
17	076 - BASTA_F2	CTTCAGCAGGTGGGTGTAGAG	ML1::LhG4 genotyping
18	077 - BASTA_R2	GAGACAAGCACGGTCAACTTC	ML1::LhG4 genotyping