



Clathrin-mediated trafficking and PIN trafficking are required for auxin canalization and vascular tissue formation in Arabidopsis

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ABSTRACT

The flexible development of plants is characterized by a high capacity for post-embryonic organ formation and tissue regeneration, processes, which require tightly regulated intercellular communication and coordinated tissue (re-)polarization. The phytohormone auxin, the main driver for these processes, is able to establish polarized auxin transport channels, which are characterized by the expression and polar, subcellular localization of the PIN1 auxin transport proteins. These channels are demarcating the position of future vascular strands necessary for organ formation and tissue regeneration. Major progress has been made in the last years to understand how PINs can change their polarity in different contexts and thus guide auxin flow through the plant. However, it still remains elusive how auxin mediates the establishment of auxin conducting channels and the formation of vascular tissue and which cellular processes are involved. By the means of sophisticated regeneration experiments combined with local auxin applications in *Arabidopsis thaliana* inflorescence stems we show that (i) PIN subcellular dynamics, (ii) PIN internalization by clathrin-mediated trafficking and (iii) an intact actin cytoskeleton required for post-endocytic trafficking are indispensable for auxin channel formation, *de novo* vascular formation and vascular regeneration after wounding. These observations provide novel insights into cellular mechanism of coordinated tissue polarization during auxin canalization.

1. Introduction

The development of plants is a very flexible and dynamic process, which is characterized, among others, by post embryonic organ formation of new leaves, flowers and roots and a high capability of regeneration after wounding [1]. *De novo* tissue patterning and re-patterning typically depend on the coordinated polarization of individual cells leading to polarization of the whole tissues. The important driver for polarity and patterning in plant development is the intercellular morphogen-like plant signaling molecule auxin, which, in some developmental contexts, is able on its own to establish polarized auxin transporting channels [2]. The canalization hypothesis describes the unique property of auxin being transported actively from cell to cell in a directional manner by regulating the polarity of its own flow [3–5]. This feed-back regulation has been proposed to be a key prerequisite for a spontaneous formation of these auxin transport channels [6]. The direction and throughput of the auxin flow depend on the polar localization of the auxin transport proteins PIN (PIN-FORMED) at the plasma membrane (PM) [7,8]. During vascular formation in leaves [9],

shoot apical meristem organogenesis [10,11], shoot branching [12] or regeneration of wounded vasculature [13], it has been shown that localized and polarized PIN1 expression and thus directional auxin transport routes demarcate the position of future vascular strands. To ensure correct tissue patterning and vascular development, PIN subcellular localization must be tightly regulated. PIN proteins have the ability to change their subcellular localization dynamically thus allowing for a flexible rise of new, polarized routes for auxin. Especially when the polar auxin flow is disrupted by wounding they can adapt dynamically and re-establish auxin flow by the formation of new channels and ultimately leading to the formation of new vasculature strands [5,12].

The dynamic changes in the subcellular PIN localization are presumably related to the constitutive cycling of PINs from and to the PM [14], involving clathrin-mediated endocytosis (CME) [15]. Additionally, auxin itself can increase its own efflux by stabilizing PINs at the PM by interfering with their internalization [16,17]. Mutations within the clathrin heavy chain (CHC), an essential protein for CME and some intracellular trafficking pathways [18], interfere with polar PIN

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distribution, auxin distribution patterns and lead to auxin transport-related phenotypes linking PIN polar localization with endocytosis [19]. Further it was shown that many post-endocytic processes and trafficking of cargoes, amongst others PINs, strongly depend on an intact actin cytoskeleton [20] despite the CME can operate [21] and PIN polarity itself can be generated at least within polarized tissues also without intact actin cytoskeleton [22].

Even though major progress was made in the last years to understand how PINs can change their polarity and thus guide auxin flow through the plant it still remains elusive, which cellular processes related to PIN subcellular dynamics are involved in the establishment of auxin conducting channels and the formation of vascular tissue.

An elegant way to study canalization is to observe vasculature regeneration after wounding or vasculature formation from a place of local auxin application. This allows to follow *de novo* auxin channel and vasculature formation, which are either formed around the wound or between an external auxin source and pre-existing vasculature. Performing this in the model *Arabidopsis thaliana*, allows to exploit the large genetic and pharmacological toolbox [13,23].

By the use of genetic and pharmacological methods applied to the model of vascular tissue regeneration after wounding or *de novo* vasculature formation from an artificial auxin source, this work demonstrates that clathrin-mediated and actin-dependent trafficking of PIN proteins are crucial for auxin canalization and vascular strand formation.

2. Material and methods

2.1. Plant material and plant growth condition

Wild-type Col-0 (NASC, The Nottingham Arabidopsis Stock Centre; <http://www.arabidopsis.info>, N1092) and reporter lines: *DR5rev::GFP* [24], *pPIN1::PIN1-GFP* [10] were used as controls. All mutants and transgenic lines used in this study are in the *Arabidopsis thaliana* ecotype Columbia (Col-0) background. Endocytosis-defective mutants: *chc2-1* (SALK_028826) and *chc2-2* (SALK_042321) [19], *XVE > > AUXILIN-LIKE2 x DR5rev::GFP*, *XVE > > AUXILIN-LIKE2* (At4g12770) [25], *tir3-101* (At3g02260), [26], *doc1-1* (At3g02260)*xpPIN1::PIN1-GFP* and actin filament mutants: *act2* (SALK_048987), *act7* (SALK_131610), *act8* (GABI_480B07) [27] were used for the experiments. All seeds were sterilized with 70% aqueous ethanol solution (2 min) and in 10 % sodium hypochlorite solution (15 min), washed 3 times in water (2 min for each change) and next refrigerated at 4 °C for stratification for 48 h. Seeds were germinated in pots with soil and seedlings with second pair of leaves were individually planted and grown in pots with soaked peaty rings in a growth chamber with 16 h light/8 h dark cycle at 20 °C. For the experiments, plants with 10 cm tall inflorescence stem were chosen.

2.2. Experimental design

Inflorescence stems (10 cm tall) having primary tissue architecture were used for experiments with vasculature regeneration after wounding and with local application of compounds. The flowering parts of the stems were dissected (stems were 7 cm tall after decapitation) and an external weight (a leaden ball, 2.5 g) was applied to them for subsequent 6 days, to obtain secondary growth on the stem circumference. Finally, the mechanically stimulated stems were incised transversally in the basal parts (above the leaf rosette) as described previously [13]. An incision was made with a razor blade in the transversal plane to disturb basipetal transport of endogenous auxin. Six days after compound application auxin canalization and vasculature formation from the local sites of application were analyzed. Experiments were conducted two times for each line, with at least 15 plants analyzed.

2.3. Treatment conditions

All the treatments were carried out with mechanically stimulated inflorescence stems, which remained under the external weight during the experiments.

Dependent on the experiment, material was analyzed either 6 days after wounding (tests for vasculature regeneration around wounds) or a droplet of lanolin paste with natural auxin (IAA, Indole-3-Acetic Acid, Sigma-Aldrich, cat. no 15148-2 G), synthetic auxin (PISA, Pinstatic Acid, Alfa Aesar, Santa Cruz Biotechnology and Cross Organics, CAS Registry Number: 4919-33-9), or actin cytoskeleton inhibitor Latrunculin B (LatB, Sigma-Aldrich, cat. no L5288) was applied locally together with auxin below the cut (tests for auxin channels and vascular strand formation from the local sites of application). The applied compounds were replaced during the experiments every 2 days in a fresh droplet of lanolin paste. For the local application, 10 µM water solutions of all compounds mixed with a droplet of lanolin paste were used. Stock solutions of auxin and compounds (LatB, PISA) were dissolved in DMSO (Sigma, cat. no. D5879-500ML).

To induce the overexpression of AUXILIN-LIKE2, inflorescence stems of *XVE > > AUXILIN-LIKE2* mutants were sprayed with 2 µM β-estradiol (Sigma-Aldrich, cat. no E8875) aqueous solution. The stems were under chemical induction 3 days before the experiment and during the whole duration of the experiments. Plants were sprayed once per day. Non-induced *XVE > > AUXILIN-LIKE2* mutants and Col-0 sprayed with β-estradiol were used as controls.

2.4. Imaging and image analysis

The samples of wounded inflorescence stems after treatments were collected under a stereomicroscope (NIKON MSZ1500), manually sectioned with a razor blade and mounted in a 50 % glycerol aqueous solution onto microscopy slides. Visualization of the auxin response reporter *DR5rev::GFP* and PIN1 protein *pPIN1::PIN1-GFP* was performed using a Zeiss Observer.Z1 or an Olympus FLUOVIEW FV1000 confocal laser-scanning microscope. GFP fluorescence was excited by an argon-ion laser light of 488 nm and detected at 510 nm. Acquired images were processed with ZEN 2012 Light Edition and FLUOVIEW software. Figures were created by CorelDraw X6.

2.5. Quantification and statistical analysis

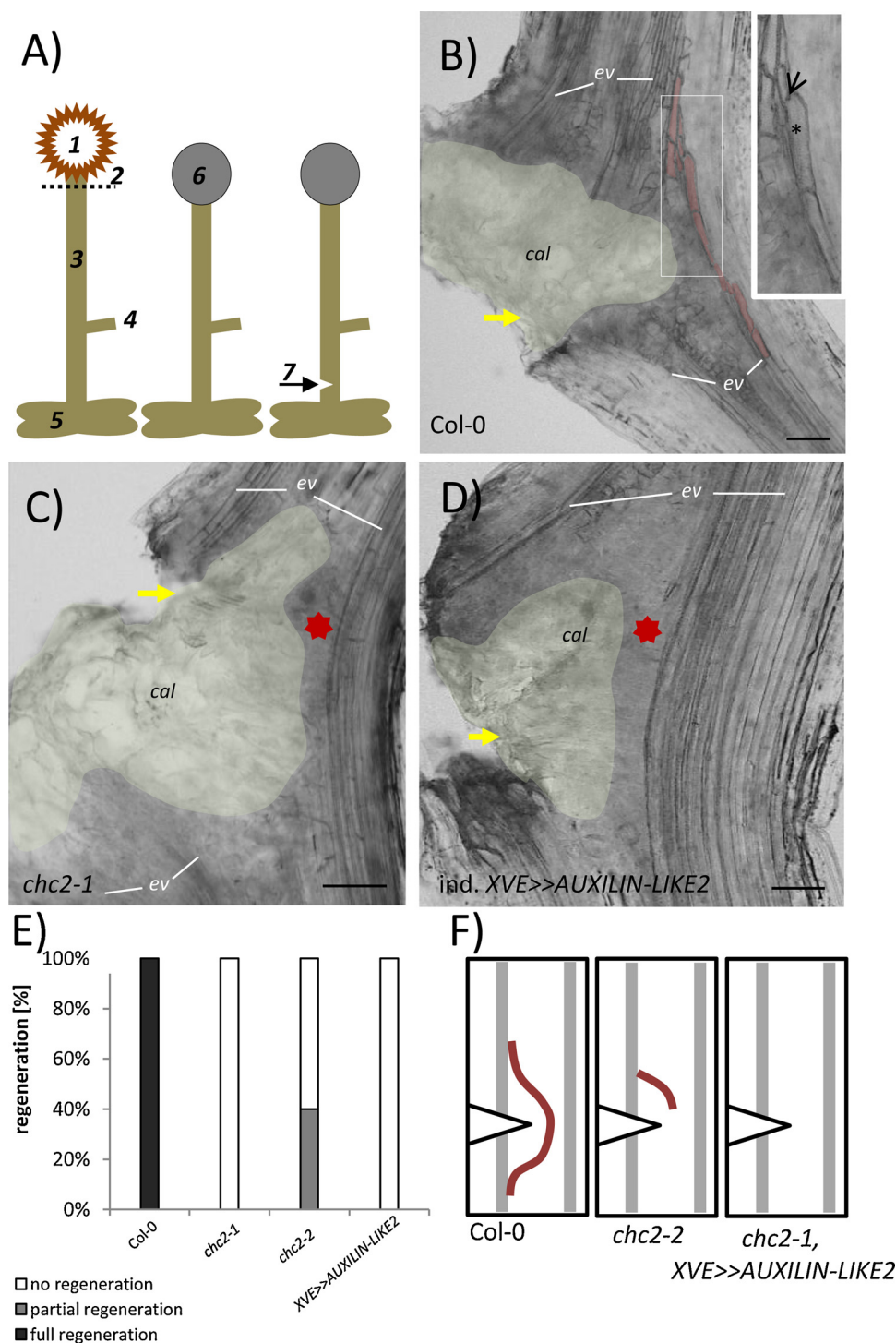
All calculations and graphs were made with Microsoft Office Excel software (Microsoft 2010).

3. Results

3.1. Vasculature regeneration after wounding in mutants defective in clathrin-mediated trafficking

Stem vasculature regeneration after wounding is associated with the induction of polarized PIN1-expressing auxin transport channels, however, it is not clear yet, which cellular processes are involved in the establishment of these channels.

Given that PIN proteins undergo clathrin-mediated constitutive endocytic recycling, and this was proposed to be important for various PIN relocations [15], we first tested the involvement of CME in vasculature regeneration. To this end, we wounded inflorescence stems of Col-0, *chc2-1* and *chc2-2* mutants defective in the clathrin heavy chain, a critical component for CME and some intracellular trafficking pathways [18,19,28]. Wounding was performed in the basal part of the stems, which were stimulated mechanically to obtain secondary growth [13] (Fig. 1A). In analyzed control stems regenerated vasculature developed 6 days after wounding around the inner side of the wound connecting the incised pre-existing vasculature (Fig. 1B). Regenerated vasculature exhibited all features recognized in mature vessels; they



were arranged in continued strands and connected together by perforation plates (Fig. 1B, inset). Secondary cell wall patterning was recognizable (Fig. 1B, inset). In contrast, both of the analyzed clathrin heavy chain-defective mutants *chc2-1* and *chc2-2* either did not show any signs of vasculature regeneration near the wound (Fig. 1C) or the regenerated vasculature was highly defective as seen in case of *chc2-2* (Supplementary Fig. S1A).

Next, we tested the line *XVE >> AUXILIN-LIKE2*, where clathrin-mediated endocytosis is conditionally inhibited by the overexpression of *AUXILIN-LIKE2*, a putative uncoating factor in clathrin-mediated processes [25]. Also in this case, development of vasculature was completely stopped following *AUXILIN-LIKE2* induction (Fig. 1D). In

the non-induced *XVE >> AUXILIN-LIKE2* stems vasculature developed in the neighborhood of the wound (Supplementary Fig. S1B). To evaluate the effect of β -estradiol alone on vasculature regeneration, Col-0 wild type plants were treated and the regeneration of vasculature after wounding was observed and quantified (Supplementary Fig. S1C, D).

Quantification of the regeneration capacity revealed no vasculature formation around a wound in *chc2-1* (0 % regeneration, N = 15) and β -estradiol induced *XVE >> AUXILIN-LIKE2* stems (0 %, N = 15) and only limited regeneration in incised *chc2-2* stems (40 %, N = 15) compared to the Col-0 control (100 %, N = 15) (Fig. 1E-F).

These results show that mutants interfering with clathrin and

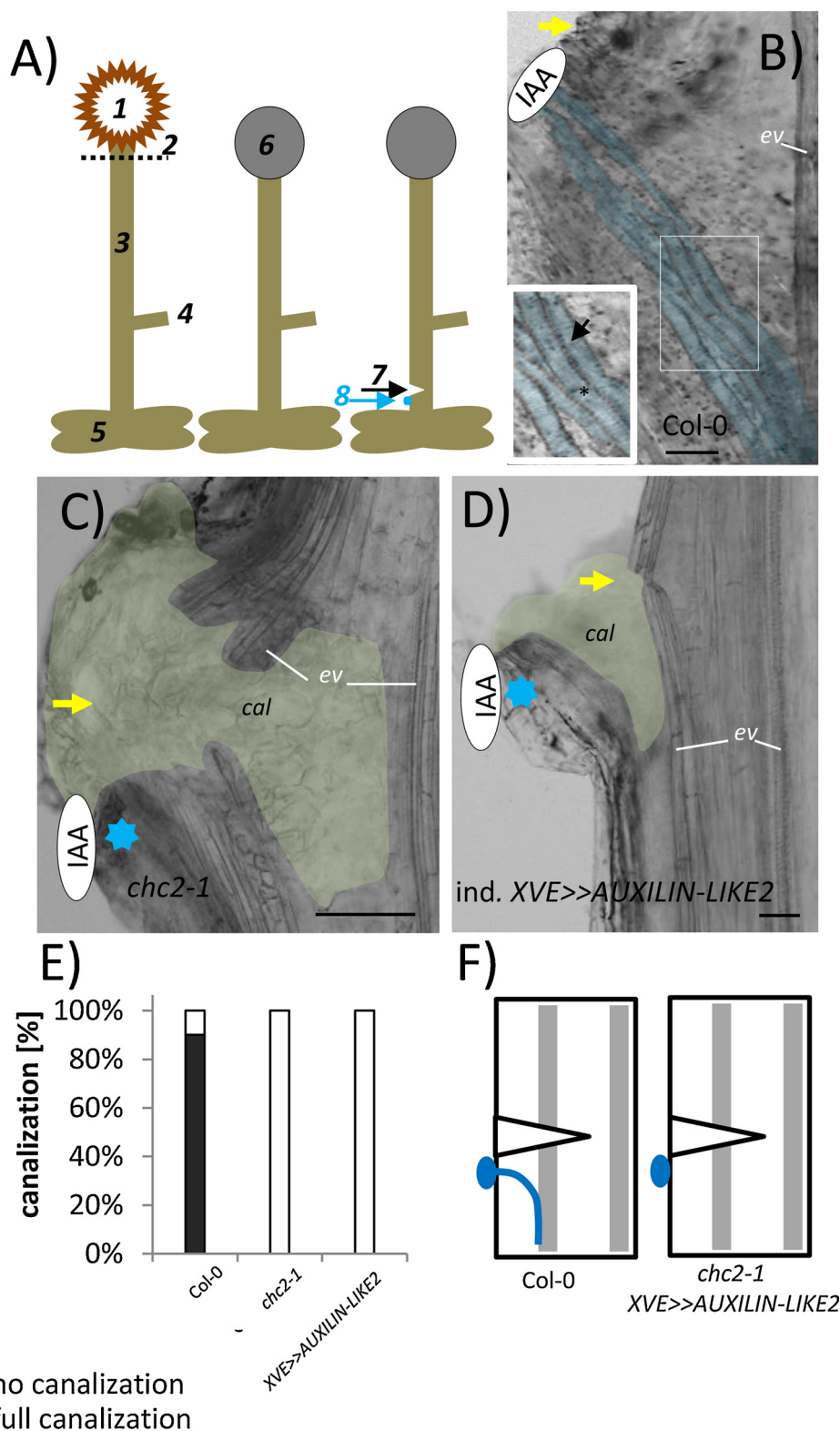


Fig. 2. Vasculature formation from local, external sources of auxin in mutants defective in clathrin-mediated trafficking.

A) Schematic view of the local auxin application experiments: 1 – inflorescence, 2 – place of inflorescence decapitation, 3 – stem, 4 – axillary bud, 5 – leaf rosette, 6 – external weight (2.5 g), 7 – wounding, 8 – local application of a droplet of lanolin paste together with auxin.

B) Vasculature formation from auxin application site (blue outlines). Vessels with typical vascular features, such as secondary cell walls and perforations connecting vessel elements (inset, arrowhead and asterisk, respectively), extending from the external auxin source to pre-existing stem vasculature.

C-D) No vascular strand formation from the local source of auxin in analyzed *chc2-1* and *XVE >> AUXILIN-LIKE2* mutants (blue asterisks), 6 days after application [DAA]. Abundant callus is observed in both of the mutants.

E) Quantification of the results shows complete stop of vasculature formation from external auxin sources in each of the analyzed mutants compared to the control (0 % developed vasculature in mutants and 90 % developed vasculature in control).

F) Schematic visualization of vasculature formation from local, external auxin sources.

Abbreviations: *cal* – callus, *ev* – existing vasculature. Yellow arrows indicate wounds. Blue colors indicate developed vasculature from the local auxin application sites.

therefore with CME are defective in stem vasculature regeneration after wounding.

3.2. Vasculature formation from external auxin sources in mutants defective in clathrin-mediated trafficking

Next we tested requirement of clathrin-mediated trafficking in canalization processes more directly by looking at the vasculature

formation from the place of local auxin application. In these experiments we applied auxin and inhibitors in a droplet of lanolin paste at the side of the stem below the wound as established previously [29] (Fig. 2A).

Natural auxin (IAA, indole-3-acetic acid, 10 μM water solution) externally applied onto the Col-0 inflorescence stems led to the formation of vascular strands, extending from the site of auxin application to the pre-existing vasculature (Fig. 2B). Mature vessels, recognized by

the features typical for the tracheary elements, such as secondary cell wall patterning and open perforation plates, developed 6 days after application (DAA) (Fig. 2B, inset). In turn, in all of the analyzed mutants defective in clathrin-mediated transport (*chc2-1*, *chc2-2*, induced *XVE >> AUXILIN-LIKE2*), no vasculature developed in response to the locally applied auxin (0 %, N = 15 for each of analyzed samples; Fig. 2C-D, Supplementary Fig. S2A). In contrast, in almost all analyzed Col-0 (90 %, N = 15) and in non-induced *XVE >> AUXILIN-LIKE2* stems (70 %, N = 10) vascular strands developed from the sites of auxin application (Fig. 2E-F, Supplementary Fig. S2B). To evaluate the effect of β -estradiol alone on vasculature formation from an external source, Col-0 wild type plants were treated with β -estradiol in a drop of lanolin paste and the vasculature formation was observed and quantified (Supplementary Fig. S2C, D).

These results show that formation of the vascular strands from the place of the local auxin application, similar to vasculature regeneration, requires functioning clathrin-mediated trafficking.

3.3. Auxin channel formation from external auxin sources in mutants defective in clathrin-mediated processes

Next we tested whether the defects in vasculature formation in mutants defective in clathrin-mediated trafficking are due to defects in the establishment of auxin-conducting channels which can be visualized by the auxin response and auxin transport reporters *DR5rev::GFP* and *pPIN1::PIN1-GFP*, respectively.

Local application of natural auxin onto wounded inflorescence stems of *DR5rev::GFP* and *pPIN1::PIN1-GFP* transgenic plants is accompanied by the formation of PIN1-mediated, DR5-positive channels from the exogenous source, 4 DAA (Fig. 3A-B). Moreover, application of natural auxin onto the surface of these stems led to the development of vascular strands extending from the sites of auxin application to the pre-existing vasculature (Supplementary Fig. S3A). In addition to these, in the non-induced *XVE >> AUXILIN-LIKE2 x DR5rev::GFP* transgenic lines, we also observed establishment of DR5-positive channels 4 DAA (Supplementary Fig. S3B) resulting in vascular strand formation in the following 2 days, like it was shown in Supplementary Fig. S2B. In contrast, in the β -estradiol induced *XVE >> AUXILIN-LIKE2 x DR5rev::GFP* stems, neither DR5-positive auxin channels nor vasculature development was observed (Fig. 3C, F).

Next, we tested the effect of PISA, a synthetic auxin analog, which stabilizes PINs at the PM by inhibiting their internalization [30]. In these experiments we locally applied PISA together with auxin or without auxin onto stems of *DR5rev::GFP* and *pPIN1::PIN1-GFP* plants and analyzed them 4 DAA. No formation of auxin-channels from the site of locally applied PISA was observed (Fig. 3D-E and Supplementary Fig. S3C-D). Elevated auxin response in the outer tissues, neighboring the application sites, but no auxin channel formation was visible when PISA + IAA were applied (Fig. 3D). A similar situation was observed in the *pPIN1::PIN1-GFP* stems. In this case, application of PISA together with IAA shows no PIN1-positive channel formation from the sources of local applications (Fig. 3E). Non-polar PIN1 expression was found only in the cells of the outer tissue neighboring the sites of the compound application (Fig. 3E, inset). Definitely, in both of the experiments performed with PISA + IAA application, neither PIN1-mediated auxin channels nor vascular strand development was observed (0 % in each of tested samples) (Fig. 3G).

These results reveal that already PIN1-expressing, DR5-positive auxin channel formation from the local auxin source is defective in mutants or following treatments interfering with clathrin-mediated trafficking in general or PIN1 internalization more specifically. Overall, this suggests that it is specifically the functional PIN internalization, which is required for formation of auxin channels leading to vasculature formation.

3.4. Vasculature regeneration and auxin canalization in actin-defective mutants

Next we tested whether also subcellular trafficking is required for vascular tissue regeneration and canalization. Given that not endocytosis itself but post-endocytic processes and trafficking of PIN proteins and other cargoes in plants are strongly dependent on the actin cytoskeleton [20,21], we tested *actin2*, *actin7*, *actin8* (*act2*, *act7*, *act8*) mutants for their capacity to regenerate vasculature and to form auxin channels from an external auxin source.

We observed that *act7* and *act8* mutants exhibited a complete block of vasculature regeneration (0 % of analyzed *act7* and *act8* stems; N = 10), whereas *act2* showed some regeneration, by forming defective vascular strands around the wound (50 %, N = 10) comparing to Col-0 controls (90 %, N = 10) (Fig. 4A-C, J and Supplementary Fig. S4A).

Next, we locally applied auxin onto the stems of actin-defective mutants and Col-0 controls and analyzed vasculature formation 6 DAA. The vasculature formation from the sites of auxin application was highly defective in *act2*, *act7* and *act8* mutants (Fig. 4D-F), characterized by unshaped vessels arranged in disorganized and typically non-continued vascular-like strands (Fig. 4D, inset) whereas in analyzed Col-0 controls, continuous vascular strands developed from the sites of local auxin application (100 %; N = 10) (Supplementary Fig. S4B). In this experimental set-up, vasculature formation was not completely stopped, but strongly defective development was observed in the tested mutants (80 % in *act2*; 30 % in *act8* and 20 % in *act7* samples; N = 10 for each of the actin-defective mutants) (Fig. 4K).

To complement these observations, we also interfered with actin cytoskeleton pharmacologically using Latrunculin B (LatB), a well-established drug depolymerizing actin cytoskeleton [22,31]. When LatB together with IAA were applied at the stem side of either actin mutants or Col-0, *DR5rev::GFP* or *pPIN1::PIN1-GFP*, vasculature formation and formation of DR5-positive and PIN1-GFP-positive auxin channels was also completely inhibited by the co-treatment (0 % in both actin mutants and control samples, N = 10 for each of the lines) (Fig. 4G-I, L). In addition, we tested Col-0, *DR5rev::GFP* and *pPIN1::PIN1-GFP* stems treated with LatB without auxin and analyzed 6 DAA. Also here, neither PIN1-mediated, DR5-positive auxin channels nor vascular strand development was observed (Supplementary Fig. S4C-E).

Altogether, these observations reveal that an intact actin cytoskeleton is required for establishment of PIN1-expressing, DR5-positive auxin channels and for vascular tissue formation from an external auxin source as well as for vasculature regeneration after wounding.

3.5. Vasculature regeneration and auxin canalization in big mutants

To test the requirement of PIN trafficking more specifically and by an independent method, we tested mutants in the Callosin-like protein, BIG, that have been shown to have defects in polar auxin transport regulation and PIN trafficking [16,26].

Two independent alleles of *big*, *doc1* and *tir3*, were tested for their capability of vasculature regeneration and auxin channel formation. The *tir3* mutant failed to regenerate vasculature after wounding and to form vasculature from an external source of auxin (0 % for both experiments, N = 15) (Fig. 5A, E-G). Also *doc1 x pPIN1::PIN1-GFP* failed to regenerate vasculature or to form vasculature from an external source of auxin. Additionally no PIN1-mediated auxin channels could be observed (0 % for both experiments, N = 15) (Fig. 5B, C, E-G). Only in the control line *pPIN1::PIN1-GFP* PIN1-positive channels and new vasculature strands developed from the external source of auxin (Fig. 5D, E-G).

The analysis of these mutants confirmed that polar auxin transport and PIN trafficking is important for auxin channel establishment and vasculature formation as well as regeneration after wounding.

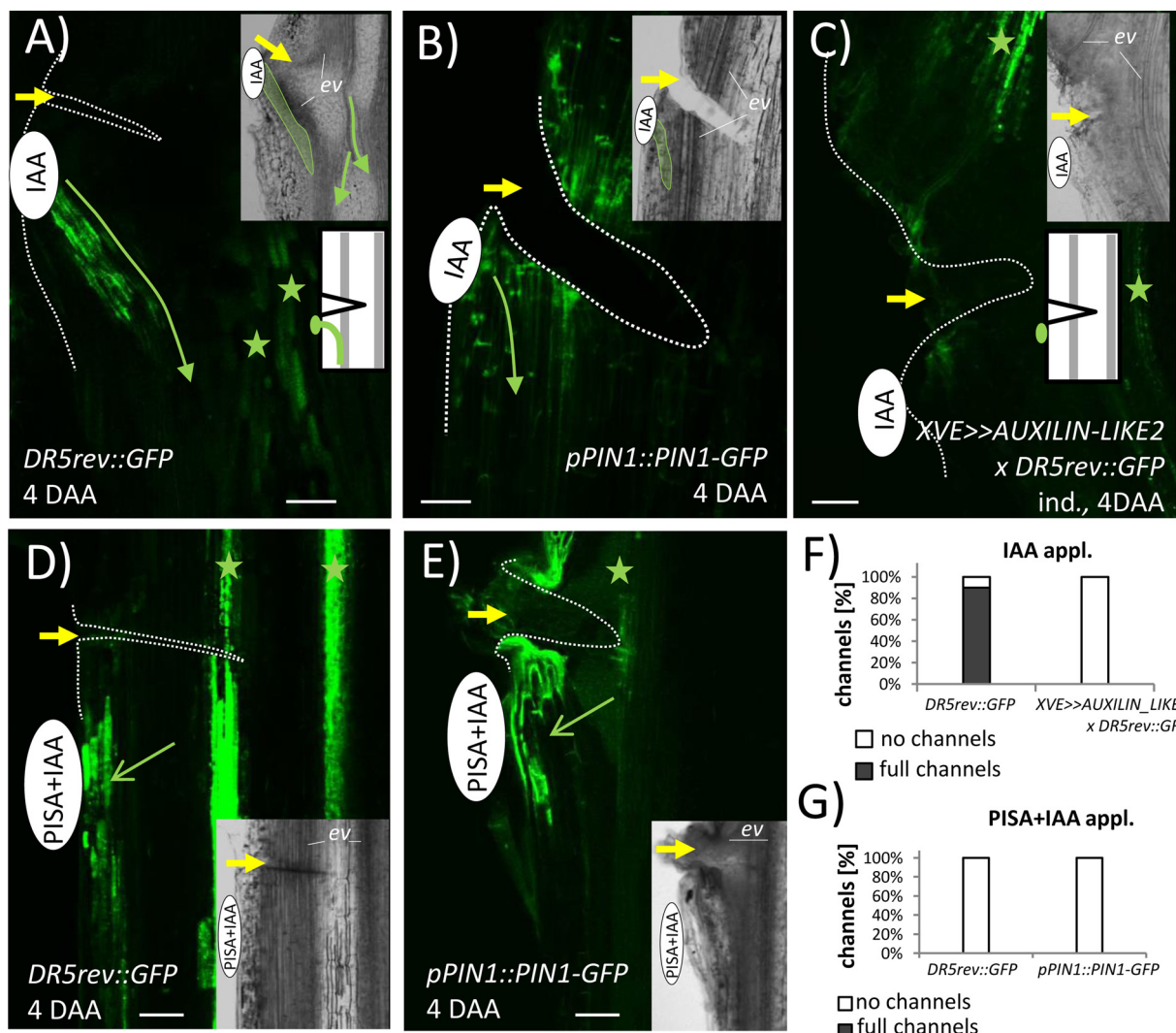
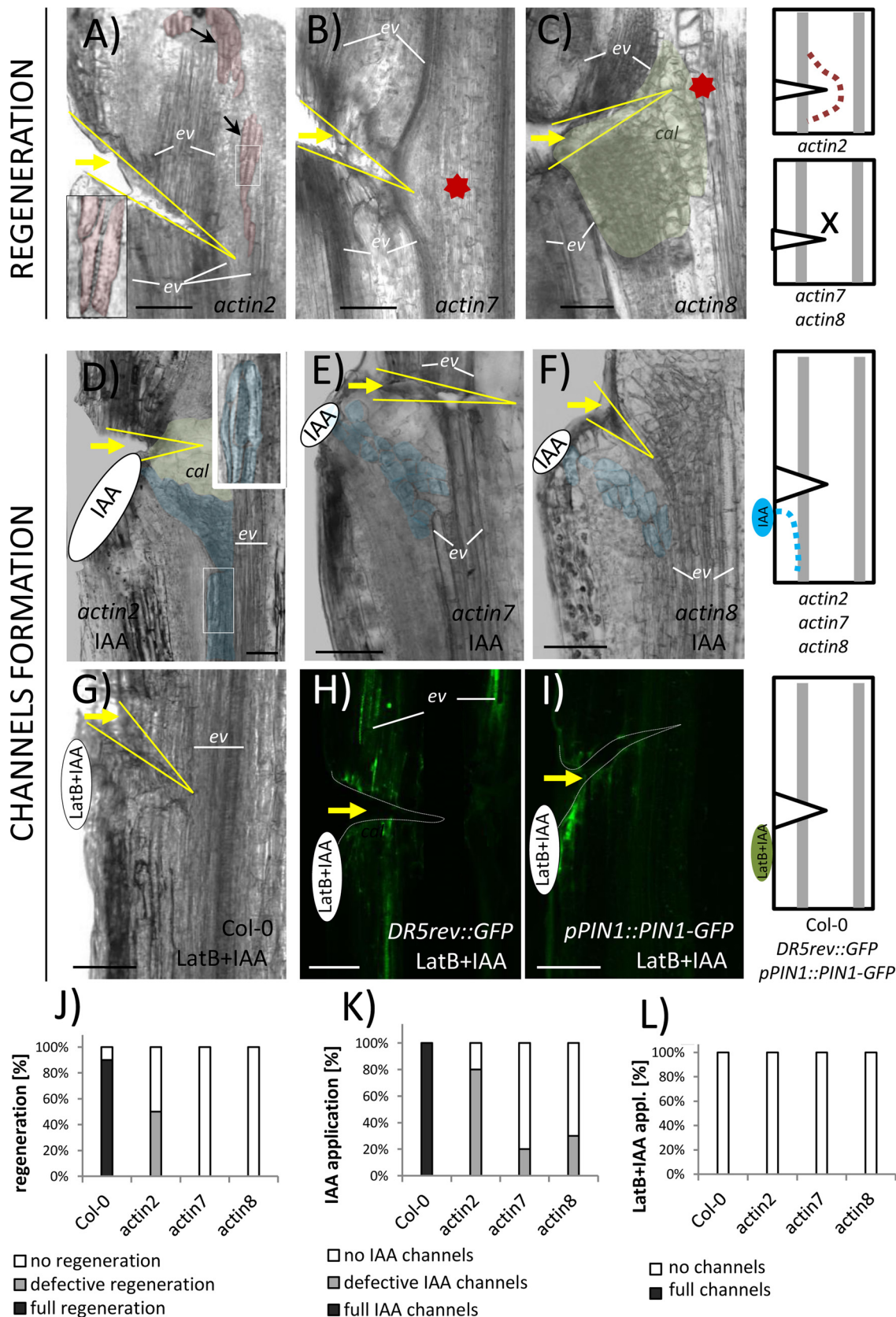


Fig. 3. Auxin channel formation from a local, external auxin source in mutants defective in clathrin-mediated trafficking and after PISA treatment. A) Formation of an auxin channel in *DR5rev::GFP* stems (green arrow), 4 DAA. Channels developed from the external sources of auxin and connected with pre-existing vasculature (inset, green outline and scheme). Elevated auxin response is also observed in regenerating vasculature around a wound (green asterisks and green arrows in inset). Development of the auxin channel is schematically visualized. B) Development of PIN1-positive channel from source of auxin application towards the pre-existing vasculature (green arrow and green outline in inset). C) No auxin-channel formation from the source of locally applied auxin in induced *XVE >> AUXILIN-LIKE2 x DR5rev::GFP* mutant (inset and scheme). Elevated auxin response is visible in pre-existing vasculature (green asterisks). D) Application of PISA together with auxin, 4 DAA. No auxin-channel formation from the source of locally applied compounds was observed in *DR5rev::GFP* stems. Elevated auxin response extending in the outer tissues (green arrow and inset), but no auxin canalization was visible at the site of PISA + IAA application. High auxin response was also observed in pre-existing vasculature (green asterisks). E) Application of PISA together with auxin, 4 DAA. No PIN1-positive channel formation from the source of locally applied compounds was observed in *pPIN1::PIN1-GFP* stems (green arrow). Non-polar PIN1 localization was found in the outer tissues neighboring the sites of the compound application (inset). Elevated auxin response was observed in pre-existing vasculature (green asterisk). F) Quantification of the results shows complete stop of auxin channel formation from external auxin sources in induced *XVE >> AUXILIN-LIKE2 x DR5rev::GFP* line compared to the control (0 % channel formation and 90 % in control). G) Co-treatment with IAA and PISA resulted in complete block of auxin channel formation in *DR5rev::GFP* and *pPIN1::PIN1-GFP* lines. Abbreviations: *ev* – pre-existing vasculature. Yellow arrows indicate wounds. Contours of wounded stems are indicate with dotted lines. Green colors indicate formation of PIN1-positive auxin channels. Scale bars: 50 μ m.

4. Discussion

Formation and regeneration of auxin-conducting channels demarcating vascular strands in plants is a spectacular example of the plant's flexible and self-organizing development. These processes involve tightly regulated intercellular communication, hormonal signaling and coordinated tissue repolarization. Experimental data have proven a key role of auxin in these processes [32]. Nonetheless, the

molecular and cellular mechanisms underlying auxin channel formation and vasculature patterning remain mostly unrevealed. Our study is part of the decades long efforts aimed to elucidate the so-called canalization hypothesis [3]. The observations show the crucial importance of subcellular, actin-mediated PIN mobility and PIN internalization by clathrin-mediated trafficking mechanisms in processes linked to canalization including auxin channel formation, *de novo* vascular formation and vascular regeneration after wounding.



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Fig. 4. Vasculature regeneration and auxin channel formation in actin-defective mutants.

A) Defective vasculature regeneration in *actin2* mutants. Development of discontinuous vessel strands in the neighborhood of the wound was observed (black arrows and red outlines). Vasculature was recognized with secondary cell wall patterning (inset). Defective vasculature regeneration is visualized on the scheme.

B–C) No vascular strand formation around the wound in analyzed *actin7* and *actin8* mutants (red asterisks). No vasculature regeneration is visualized on the scheme.

D–F) Defective vasculature formation from local source of external auxin in all analyzed actin defective mutants (blue outlines and scheme). Vessels developed in the mutants are strongly defective visible by the shapes and connections. However, the secondary cell wall patterning is recognizable in the *act2* mutant (D, inset). Defective vasculature formation is visualized on the scheme.

G–I) No vasculature development in Col-0 stems (G) and no positive auxin channel formation in *DR5rev::GFP* and *pPIN1::PIN1-GFP* stems (H, I) after local application of Latrunculin B together with auxin. Experimental results are visualized on scheme.

J–L) Quantification of the results shows defective regeneration of vasculature around the wound in *actin2* (in 50 % of analyzed plants comparing to control), and absent regeneration in *actin7* and *actin8* mutants (0 %, N = 10) (J), defective vasculature formation after local IAA application (80 % in *act2*; 30 % in *act8* and 20 % in *act7* samples; N = 10) or no vasculature formation after local IAA application (20 % in *act2*; 70 % in *act8* and 80 % in *act7* samples; N = 10) (K) and no vasculature and channel formation after local LatB + IAA application (L) in all analyzed mutants.

Abbreviations: *cal* – callus, *ev* –existing vasculature. Yellow lines and arrows indicate wounds. Red colors indicate regenerated vasculature around a wound. Blue colors indicate developed vasculature from the local auxin application sites. Contours of wounded stems are indicate with dotted lines (H, I).

Scale bars: 100 μ m.

4.1. Subcellular PIN dynamics are important for auxin canalization and vasculature formation

PIN proteins undergo constitutive subcellular dynamics of repeated steps of clathrin-mediated endocytosis and recycling back to the PM [15,19,20]. The physiological role of this dynamics remains unclear but it can be important for both, maintaining PIN polar distribution at the PM [22,33] or dynamically changing PIN polarity, for example in response to environmental signals such as gravity or endogenous signals, presumably including auxin [14,27,34]. Here we tested whether this PIN dynamics is also required in auxin canalization-related contexts such as vasculature formation from an auxin source or vasculature regeneration since these processes require coordinated PIN polarization in individual cells [5,13,35].

Indeed, if correct trafficking of PINs is impaired either genetically, for example by a mutation in the gene coding for the PIN trafficking and polar auxin transport regulator BIG or pharmacologically, by PISA, which stabilizes PINs at the PM, plants display phenotypes associated with a decrease in polar auxin transport [26,30]. This suggests that PINs need to be in a state where they are, on the one hand, mobile or capable of a re-localization and, on the other hand, this mobility must be coordinated in a way that the proteins end up in the right location. By the stabilization of PINs at the PM by the use of PISA, we could show that no auxin channel formation from an external source is initiated, indicating that PINs at the PM are not sufficient and need to undergo subcellular dynamics to allow for this process. Further we show that in *big*, a mutant with a defect in polar auxin transport which is affecting the localization of PIN1, neither channel formation nor vascular regeneration is occurring, further suggesting that not only subcellular dynamics itself but coordinated trafficking is necessary for auxin canalization and vasculature formation.

4.2. Endocytosis in auxin canalization and vasculature formation

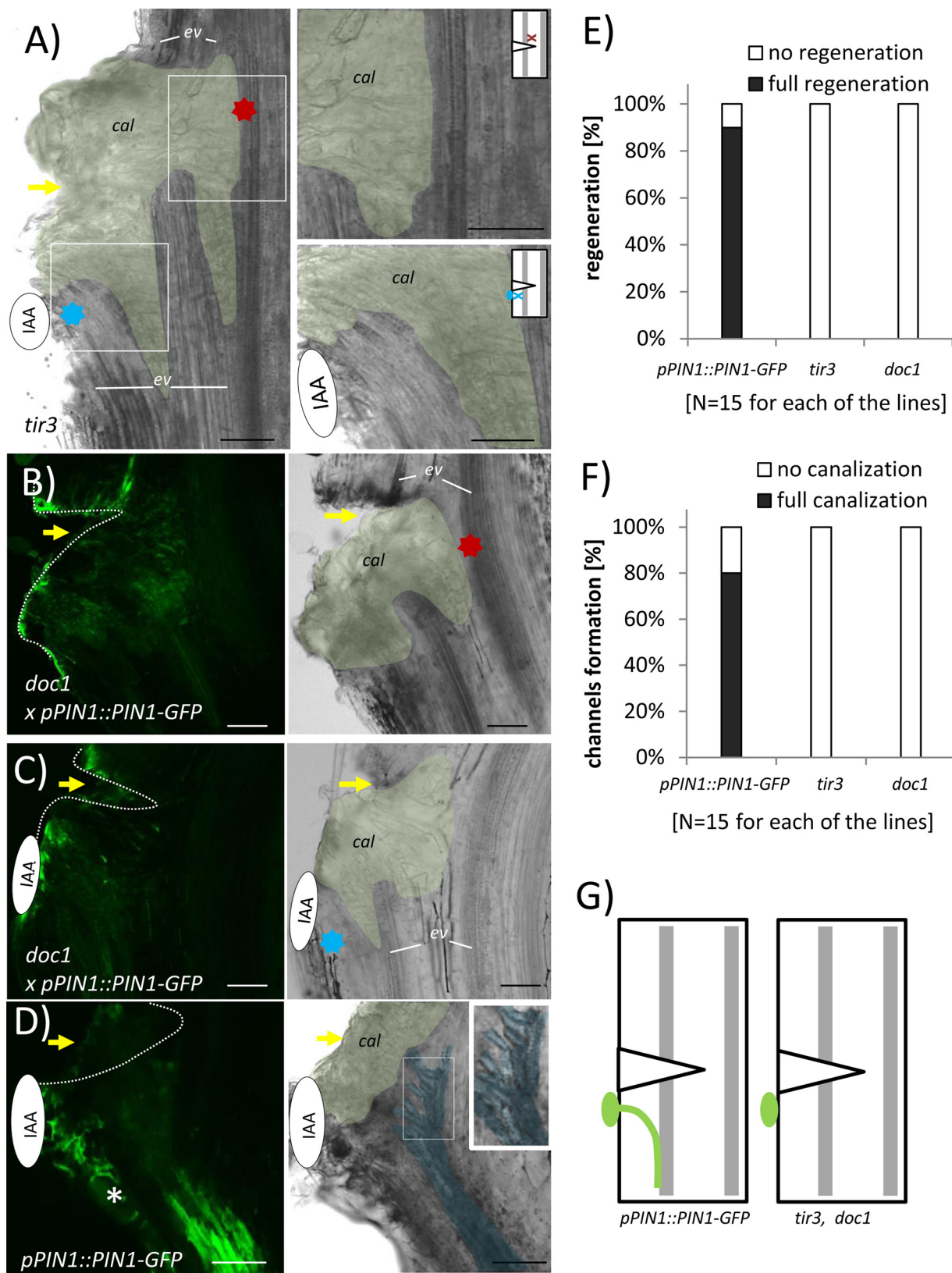
The key process in auxin canalization – formation of polarized auxin channels away from the localized auxin source is conceptually unclear. How is the auxin signal propagated across the tissue and how does it allow for coordinated PIN polarization in individual cells? Studies in the shoot apical meristem proposed mechanical transmission of the signal [36] or other modelling efforts proposed that auxin-mediated PIN polarization can be theoretically realized by cooperation of intracellular and extracellular auxin perception with the latter inhibiting PIN internalization leading to selective retention of PINs on the cell side away from the auxin source [37]. A possible experimental support for this model provided observations that auxin interferes with endocytosis and thus also internalization of PINs from the cell surface [15–17,38]. The PIN internalization is mediated by a clathrin-mediated mechanism and it has been experimentally shown that CME plays a key role in the regulation of PIN polarity and its interference significantly disturbs

plant development [14,25].

Therefore, we tested whether functional CME is required for auxin canalization. We show that interference with endocytosis, shown by the use of mutants with defects in the coat protein clathrin and over-expression of the uncoating factor AUXILIN-LIKE2, significantly blocks auxin canalization, *de novo* vascular formation and regeneration. Due to their cell toxicity in long term experiments, the use of specific CME inhibitors, like IKA and Dyngo [39], was not possible for this study. So mutants interfering with clathrin itself or processes associated with clathrin-mediated endocytosis were used. Clathrin is, however, not only involved in processes at the PM but also in intracellular trafficking from the TGN [18], which makes the mutants not necessarily specific to CME. However, the interference with clathrin is blocking the major route for cargo internalization from the PM in plants [15], therefore the used mutants highlight the importance of clathrin-mediated trafficking and most likely also endocytosis for the processes analyzed in this study. As shown, the process of regeneration is blocked in one of the very first steps, the formation of PIN1-expressing auxin channels, presumably due to failure of PIN polarity establishment but this would require detailed observations of PIN polarities, which is in the stem system not easily possible. Nonetheless, the observations that clathrin-mediated trafficking is required during formation of auxin channels and ultimately for vascular strand formation support the model that auxin feed-back on PIN endocytosis is a part of the canalization mechanism.

4.3. Actin-mediated trafficking in auxin canalization and vasculature formation

After providing evidence that endocytosis, the first step of PIN internalization and thus polarity establishment, is crucial for the formation of PIN1-expressing auxin channels and vasculature regeneration we aimed to test whether also post-endocytic trafficking is required. To this end, we decided to genetically and pharmacologically interfere with the actin cytoskeleton since the CME does not require intact actin whereas post-endocytic processes and trafficking of PIN proteins do [20,21]. We used several actin defective mutants and the actin depolymerizing drug Latrunculin B and these manipulations consistently show that not only endocytosis but also an intact actin cytoskeleton are a prerequisite for both, the formation of auxin-conducting channels and the regeneration of vasculature. Similar to the mutants defective in clathrin-mediated trafficking, regeneration is also blocked already in the step of auxin-conducting channel formation which could be arguably due to a failure in the establishment of PIN polar localization. Thus, whereas in stably polarized tissues such as root tip, the cytoskeleton is not needed for PIN polarity establishment, for example after cell division [20], the dynamic establishment of PIN-conducting channels for vasculature formation and regeneration strictly depends on the intact actin cytoskeleton.



(caption on next page)

5. Conclusions

In conclusion, our observations provide novel insights regarding the cellular mechanisms underlying auxin canalization-mediated vascular

tissue formation and regeneration. With the present work, we show that the formation of PIN1-expressing auxin channels either in context of vascular tissue regeneration or *de novo* vascular strand formation from the place of local auxin application requires intact subcellular dynamics

Fig. 5. Vasculature regeneration and auxin channel formation in *big* PIN trafficking-defective mutants.

A) No regeneration around a wound and no vasculature formation from a local auxin source in *tir3* (red and blue asterisk, respectively). Frames are shown with magnification.

B–C) No regeneration around a wound (B, red asterisk), no vasculature formation and no PIN1-positive channel formation from local auxin source (C, blue asterisk) in the *doc1* mutant.

D) PIN1-positive channels (asterisk) and new vessel strands developed from the external source of auxin in stems of *pPIN1::PIN1-GFP*. New vessels developed extending from the site of auxin application to the pre-existing vasculature (blue outlines and inset).

E–F) Quantification shows no vasculature formation and no auxin channel formation from the sources of local auxin application in both of the tested alleles of *big: doc1* and *tir3* (0 % in *tir3* and *doc1*).

G) Schematic visualization of PIN1 channel formation in analyzed *pPIN1::PIN1-GFP* stems and PIN trafficking-defective mutants.

Abbreviations: *cal* – callus, *ev* – existing vasculature. Yellow arrows indicate wounds. Contours of wounded stems are indicated with dotted lines.

Scale bars: 100 μ m.

of PIN auxin transporters; functional clathrin-mediated trafficking and an intact actin cytoskeleton. If one of these steps is impeded, canalization and vascularization are blocked at an early stage of auxin channel formation highlighting the importance of a properly functioning trafficking machinery in these processes. These observations support a model in which auxin feed-back on endocytosis and trafficking of PIN auxin transporters is a crucial part of the mechanism of coordinated PIN polarization underlying auxin channel formation.

Author contribution

E.M., M.G., H.H. and J.F. designed and conducted experiments and analyzed data. M.A. and H.S.R. contributed with the generation of the genetic material used in the study. E.M., M.G. and J.F. wrote the manuscript, with the assistance of H.H.

Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.plantsci.2020.110414>.

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