A tightly regulated auxin signaling landscape is required for spatial accommodation of lateral roots in Arabidopsis

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ABSTRACT

In *Arabidopsis thaliana*, lateral root (LR) development requires spatial accommodation responses in overlying endodermal cells. This includes loss of cell volume whilst maintaining membrane integrity to allow the expansion of the underlying LR primordia (LRPs). These accommodation responses are regulated by auxin-mediated signaling, specifically through Aux/IAA proteins, involving *IAA3/SHY2*. Plants that express a stabilized version of *SHY2* (*shy2-2*) in differentiated endodermal cells, *CASP1*$_{pro}$*::shy2-2* plants, fail to make LRs. Exogenous treatment with 1-naphthaleneacetic acid (NAA) was reported to partially restore LR formation in this spatial accommodation mutant. Using treatments with auxins having different transport properties, such as NAA, indole-3-acetic acid (IAA), and 2,4-dichlorophenoxyacetic acid (2,4-D), we assessed the ability of each auxin to rescue LR formation in *CASP1*$_{pro}$*::shy2-2* roots. This revealed that IAA is the most effective in partially restoring LR development, NAA is effective in inducing LRPs but cannot maintain their canonical phenotype, whereas 2,4-D induces non-controlled cell divisions. In addition, we show that in *CASP1*$_{pro}$*::shy2*-2 roots, *AUX1* appears to be repressed in the zone where oscillation of the auxin response has been described. Our study advances the understanding of auxin-regulated spatial accommodation mechanisms during LRP formation and highlights the complex interplay of auxin transport and signaling in bypassing the endodermal constraints.

1-INTRODUCTION

The root system plays an important role in plant growth and development. The regulated formation of LRs gives rise to a belowground network, which is a major determinant for nutrition uptake, anchorage, storage and rhizosphere interactions (Péret *et al.*, 2009; Schäfer *et al.*, 2022; Gifford *et al.*, 2024). In addition, the LR development pathway is able to perceive and react to environmental cues to contribute to plant adaptation (Perotti *et al.*, 2020; Schäfer *et al.*, 2022). In Arabidopsis, LRs initiate from a set of the xylem pole pericycle (XPP) cells called LR founder cells (Casimiro *et al.*, 2001). LR founder cell division forms a new organ called LR primordium (LRP), which then undergoes eight developmental stages and traverses three overlying cell layers: endodermis, cortex and epidermis, to become a fully emerged LR (stage VIII of LR development) (Malamy & Benfey, 1997). Multiple aspects of LR development, from positioning to initiation, outgrowth and emergence, have been known to be regulated by the phytohormone auxin and its signaling pathways (Lavenus *et al.*, 2013; Du & Scheres, 2018). One branch of auxin signaling is marked with the binding of IAA, the plant endogenous auxin, to the co-receptor complex consisting of the F-box TRANSPORT INHIBITOR RESPONSE1 (TIR1) protein and Aux/IAA proteins, leading to the ubiquitination and degradation of Aux/IAAs by the 26S proteasome (Zenser *et al.*, 2001; Gray *et al.*, 2003; Dharmasiri *et al.*, 2005). Since Aux/IAAs are repressors of AUXIN RESPONSE FACTORS (ARFs), transcription factors that regulate auxin-dependent gene expression, their degradation releases the inhibition of ARFs, triggering auxin responses (Guilfoyle & Hagen, 2007; Goh *et al.*, 2012; Cancé *et al.*, 2022).

In Arabidopsis, LR formation requires Aux/IAA-mediated signaling in the XPP as well as in the neighboring endodermis (Vanneste *et al.*, 2005; De Smet *et al.*, 2007, 2010; De Rybel *et al.*, 2010; Vermeer *et al.*, 2014; Santos Teixeira & ten Tusscher, 2019). In the LR founder cells, Aux/IAA-mediated signaling regulates the cytoskeleton's remodeling to promote asymmetric cell expansion and nuclear migration to execute the formative divisions resulting in a stage I LRP (Vilches Barro *et al.*, 2019). Concomitantly, Aux/IAA-mediated responses in the endodermis include remodeling of the microtubule organization as well as a regulated loss of cell volume (Stoeckle *et al.*, 2022). These responses in the endodermis were coined spatial accommodation responses, as these cellular adjustments accommodate the expansion growth of the new LRP (Vermeer *et al.*, 2014; Stoeckle *et al.*, 2018, 2022). The importance of Aux/IAA-mediated signaling in the endodermis was revealed via the expression of a stabilized allele of *SHORT HYPOCOTYL2* (*SHY2*)/*IAA3*, a dominant repressor of auxin-mediated gene expression, in differentiated endodermal cells (Vermeer *et al.*, 2014). In this mutant, *CASP1*$_{pro}$*::shy2-2*, LR formation was completely abolished due to the expression of *shy2-2* in the differentiated endodermis and not to the movement of shy2-2 into the pericycle. The ectopic expression of a callose synthase specifically in differentiated endodermal cells to block plasmodesmata-mediated symplastic movement did not alter the phenotype (Vermeer *et al.*,

2014). Additionally, using different promoters to drive *shy2-2* expression in the XPP, instead of the originally used *CASP1* promoter, resulted in a weaker phenotype (Vermeer *et al.*, 2014). It was proposed that these auxin-mediated responses are required to trigger mechanical feedback from the endodermis to sustain the LRP expansion growth (Vermeer *et al.*, 2014; Vermeer & Geldner, 2015). Interestingly, the application of NAA was shown to be able to partially restore LR formation in *CASP1$_{pro}$::shy2-2* (Vermeer *et al.*, 2014). However, most of the resulting LRs were often malformed and their emergence was strongly impaired, but the reason why NAA only partially rescued the *CASP1$_{pro}$::shy2-2* LR phenotype remains unknown (Vermeer *et al.*, 2014).

Thus, we treated the *CASP1$_{pro}$::shy2-2* mutant with different auxin analogues (IAA, NAA and 2,4-D) to explore their potential in rescuing its LRP formation. Their unique stability levels and transport requirements (Delbarre *et al.*, 1996; Ma *et al.*, 2018; Fig. S1) allowed us to characterize auxin responses in *CASP1$_{pro}$::shy2-2*. Our results revealed that IAA is the most potent of the tested auxins to restore LRP formation, whereas NAA and 2,4-D were less capable. In addition, the exogenous auxin treatments could readily overcome the block of cell divisions in the XPP but appeared to be less efficient in restoring the Aux/IAA-mediated responses in the endodermis. Our results suggest that the block in auxin-mediated responses in the endodermis of *CASP1$_{pro}$::shy2-2* is so strong that, even with exogenous auxin treatments, the recovery of auxin signaling is limited. Furthermore, we also investigated whether auxin signaling, cytokinin signaling and the expression of some of the major auxin transporters known to be involved in LR formation, like PIN-FORMED1 (PIN1) and AUXIN RESISTANT 1 (AUX1) (Bennett *et al.*, 1996; Benková *et al.*, 2003), were modified in *CASP1$_{pro}$::shy2-2*. The observed delayed expression of AUX1 in the XPP of *CASP1$_{pro}$::shy2-2* roots suggested that auxin-mediated communication between the XPP and endodermis could be required for AUX1 accumulation in the XPP to potentiate LR formation.

2-MATERIALS AND METHODS

2.1-Plant materials

We used *Arabidopsis thaliana* Columbia-0 (Col-0), *CASP1$_{pro}$::shy2-2* (Vermeer *et al.*, 2014) and several reporter lines, including *UBQ10$_{pro}$::EYFP:NPSN12* (Wave131Y; Geldner *et al.*, 2009), *DR5::NLS-3xVENUS* (Heisler *et al.*, 2005), *TCSn::GFP* (Zürcher *et al.*, 2013), *AUX1::VENUS* (Swarup *et al.*, 2004) and *PIN1::GFP* (Benková *et al.*, 2003). All transgenic lines were in Col-0 background and introgressed into *CASP1$_{pro}$::shy2-2*.

2.2-Culture conditions

Seeds were sterilized in chlorine gas and sown on half-strength Murashige and Skoog (MS) medium with 2.4 g L^{-1} MS basal salt (Duchefa Biochemie) and 0.8% (m/v) plant agar (Duchefa Biochemie). After 48 hours of stratification in the dark at 8°C, they were transferred into a growth chamber (Aralab, 22°C, continuous white lighting at 120 µmol m^{-2} s^{-1}).

2.3-Auxin treatment assay

IAA, NAA or 2,4-D were dissolved in dimethyl sulfoxide (DMSO) at 10 mM and were separately added into the half-strength MS medium to the concentration of 0.1, 1 or 10 µM. DMSO was accordingly factored into the medium for the control condition. 5-day-old Arabidopsis seedlings were transferred into auxin treatment plates and kept in the growth chamber (Aralab, 22°C, continuous white lighting at 120 µmol m^{-2} s^{-1}) for 24 or 48 hours prior to harvest. Since plants were incubated for 24 or 48 hours with auxin, much less than the lighting duration shown by Yamakawa *et al.* (1979), we did not account for the degradation of IAA in our experiment design.

2.4-Root clearing and phenotyping

Harvested seedlings of Col-0 and *CASP1$_{pro}$::shy2-2* were cleared by immersion in 20% (v/v) methanol/ 4% (v/v) HCl solution at 57°C for 20 minutes, then in 7% (w/v) NaOH/ 60% (v/v) ethanol solution at room temperature with gentle agitation for 15 minutes, followed by a three-step rehydration using 40%, 20% and 10% (v/v) ethanol solutions. Cleared samples were stored in 50% (v/v) glycerol at 4°C and mounted onto glass slides for phenotyping. LRP number and developmental stages of Col-0 and *CASP1$_{pro}$::shy2-2* plants were determined on cleared samples ($n \geq 18$ seedlings per condition, in three replicates) and imaged using a Leica DM4 B microscope with a K5 sCMOS camera, operating in differential interference contrast (DIC) mode. LRP width and height were measured using LAS X software [n = 20 LRPs per stage (stage I – IV, prior to LRP crossing the endodermis), per condition, in three replicates]. Statistical analysis was done using RStudio Desktop software (open-source edition, Posit PBC).

2.5-Multiphoton microscopy and image analysis

LRP morphology, auxin and cytokinin signaling, and auxin transport were investigated by using a Leica SP8 multiphoton confocal microscope with Hybrid detectors (4Tune) operating in photon counting mode with 960 nm excitation wavelength and 510 – 600 nm detection window for green/yellow fluorescent proteins, running Leica LAS X software (Stoeckle *et al.*, 2022; de Jesus Vieira Teixeira *et al.*, 2024). Seedlings of the plasma membrane reporter lines (*UBQ10$_{pro}$::EYFP:NPSN12*) were imaged live. Seedlings of the other reporters (*DR5::NLS-3xVENUS*, *TCSn::GFP*, *AUX1::VENUS* and *PIN1::GFP*) were fixed and cleared following the ClearSee method (Kurihara *et al.*, 2015) prior to being imaged. All obtained images were processed and analyzed using Fiji software (Schindelin *et al.*, 2012). DR5::NLS-3xVENUS and TCSn::GFP fluorescence intensity were quantified as mean grey values of the image pixels.

3-RESULTS

3.1-Exogeneous IAA, NAA and 2,4-D addition can only partially restore LR formation in *CASP1$_{pro}$::shy2-2* roots

The inability of *CASP1$_{pro}$::shy2-2* plants to form LRs is due to a dominant repression of Aux/IAA-mediated auxin signaling in differentiated endodermal cells (Vermeer *et al.*, 2014). Here, we applied

treatment with different auxins to investigate their capacity to rescue the LR phenotype in *CASP1$_{pro}$::shy2-2* roots. Specifically, 5-day-old Col-0 (wild type) and *CASP1$_{pro}$::shy2-2* seedlings were treated with IAA, NAA and 2,4-D at different concentrations (Fig. 1). We observed that untreated wild type seedlings exhibited emerged LRs with wide spacing and asymmetrical distribution patterns, as described by others, along the primary root. However, none of the treatments with the tested auxins could evoke a similar LR phenotype in *CASP1$_{pro}$::shy2-2* (Fig. 1).

Although 2,4-D massively induced cell divisions in the XPP in both backgrounds, the treatment did not result in the formation of LRs with the canonical dome-shaped morphology (Fig. 1A,D). Even in wild type, 10 μM 2,4-D triggered an upsurge of cell proliferation in the pericycle without maintaining proper spacing between successive LRPs/LRs to a point where individual LRPs could no longer be distinguished (Fig. 1A,D). Occasionally, we observed LRPs traversing the endodermis or emerging from the cell proliferation zone along the primary root (Figs 1A, D, G, J and S2C). In *CASP1$_{pro}$::shy2-2*, 10 μM 2,4-D application also resulted in a cell proliferation zone spanning along the primary root without separate LRPs, but this zone appeared flattened compared to wild type (Fig. 1A,D). Prolonged treatment could trigger more cell divisions, but *CASP1$_{pro}$::shy2-2* plants rarely formed distinct LRPs and the dividing cells struggled to traverse the endodermis compared to wild type (Figs 1G, J and S2C).

NAA treatment also resulted in a strong induction of cell divisions in the XPP, but unlike 2,4-D, it did not completely disrupt the organization of LRPs in *CASP1$_{pro}$::shy2-2*. NAA treatments significantly increased the number of LRPs in both backgrounds (Fig. 1I). Indeed, LRPs are distinguishable, though very densely distributed on a cell proliferation zone along the primary root, with reduced spacing distance between each two successive LRPs (Fig. 1A,C). Interestingly, in *CASP1$_{pro}$::shy2-2*, none of the formed LRPs managed to traverse the endodermis after 48 hours of 0.1 μM NAA treatment. However, raising the NAA concentration led to a higher percentage of LRPs successfully traversing the endodermis and emerging (Figs 1F and S2B). Nonetheless, independent of the used concentration of NAA, we observed inconsistent LRP morphology and a strong delay in LR emergence, most likely due to difficulties to traverse the endodermis (Fig 1A, C).

IAA treatment was the most potent in reverting the LR phenotype of *CASP1$_{pro}$::shy2-2* plants. Treatment with 10 μM IAA did not trigger extensive cell divisions in the XPP, nor did it produce a cell proliferation zone along the primary root comparable to that observed in roots treated with NAA or 2,4-D. In fact, LRP shape, spacing distance and distribution in *CASP1$_{pro}$::shy2-2* treated with 10 μM IAA were even more resemblant to wild type (Fig. 1A, B). The LRP count of IAA- and NAA-treated *CASP1$_{pro}$::shy2-2* plants increased significantly, albeit the IAA-treated displayed a much smaller margin of increase (Fig. 1H, I). In *CASP1$_{pro}$::shy2-2*, 0.1 μM IAA treatment induced very few LRPs, all of which appeared to be arrested in their early stages of development, whereas treatments with 1 μM and 10 μM IAA resulted in a higher percentage of LRPs traversing the

endodermis and emerging (Figs 1E and S2A). Additionally, LR emergence in *CASP1$_{pro}$::shy2-2* recovered by IAA treatment was also delayed compared to the wild type (Figs 1A, B and S2A).

Overall, by assessing the responsiveness of *CASP1$_{pro}$::shy2-2* to different auxins, we have shown that the LR phenotype in *CASP1$_{pro}$::shy2-2* could only be partially recovered by exogenous auxin treatments, with IAA being the most efficient and 2,4-D the least. Furthermore, the observed delay in LR emergence in *CASP1$_{pro}$::shy2-2*, despite treatment with moderate to high concentrations of auxins (Figs 1A-D and S2), suggests that the spatial accommodation responses in the endodermis are not easily restored by treating them with exogenous auxins.

3.2-Auxin treatment fails to restore stereotypical LR morphology in *CASP1$_{pro}$::shy2-2* roots

To analyze more directly the effect of a resistant endodermis on restricting LR formation in *CASP1$_{pro}$::shy2-2*, we crossed the *UBQ10$_{pro}$::EYFP:NPSN12* plasma membrane reporter (Geldner *et al.*, 2009) into the *CASP1$_{pro}$::shy2-2* background (Fig. 2A). Treating seedlings with the different auxins all resulted in a loss of the canonical dome shape of LRPs until stage IV. In *CASP1$_{pro}$::shy2-2*, the LRPs mostly remained flattened against the endodermis compared to wild type (Fig. 2A). The only exception was seen in IAA-treated *CASP1$_{pro}$::shy2-2* plants, in which we observed dome-shaped LRPs, though slightly flatter compared to wild type (Fig. 2A). In addition, we noticed that stage II and IV primordia in *CASP1$_{pro}$::shy2-2* were wider than in Col-0 when treated with IAA or NAA (Fig. 2A). Regarding 2,4-D treatment, the cell proliferation zone in *CASP1$_{pro}$::shy2-2* appeared completely flattened beneath the endodermis, in which we could not distinguish individual LRs (Fig. 2A), consistent with our observation (Fig. 1A, D).

We analyzed the auxin-induced LRP morphology by quantifying the variation in the morphology of stage I to IV LRPs in both backgrounds after auxin treatment (*CASP1$_{pro}$::shy2-2* plants do not make any LRs without addition of auxin). Particularly, we measured the dimensions of the auxin-induced LRPs prior to stage V, before they have traversed the endodermis. The quantification of LRP dimensions of up-to-stage-IV demonstrates a significant increase in LRP width in *CASP1$_{pro}$::shy2-2* compared to Col-0 whether they are treated with 1 µM IAA or NAA (Fig. 2B, C). Moreover, the ratio between LRP width and height is significantly higher in *CASP1$_{pro}$::shy2-2* compared to wild type with 1 µM IAA and NAA treatments (Fig. 2F, G), confirming that auxin treatments result in wider and flattened LRPs in *CASP1$_{pro}$::shy2-2*. On the other hand, LRP height in *CASP1$_{pro}$::shy2-2* roots after IAA and NAA treatments either was not significantly affected or was lower (stage-IV; IAA-induced LRPs) compared to wild type (Fig. 2D, E). This suggests that, although exogenous auxin treatments can induce cell division and presumably LRP initiation from the XPP, the lack of spatial accommodation responses of the overlying endodermis in *CASP1$_{pro}$::shy2-2* is not easily overcome by auxin treatment and appears to restrain LRP outgrowth, resulting in wider and flatter LRP with a delayed emergence.

3.3-Transport properties of auxins affect induction of auxin-mediated transcriptional responses in *CASP1$_{pro}$::shy2-2*

Aux/IAA-mediated signaling plays a key role throughout LR development. Due to the strong impairment in endodermal Aux/IAA-mediated signaling, *CASP1$_{pro}$::shy2-2* endodermal cells are not responsive to the initiation of expansion growth in the XPP and this results in a complete absence of LRs (Vermeer *et al.*, 2014). Since we showed that treatment with auxins with different transport properties leads to various levels of reversion of the LR phenotype in *CASP1$_{pro}$::shy2-2*, we tested if there was a correlation between the level of rescue with the expression of the auxin response marker *DR5::NLS-3xVENUS* (Heisler *et al.*, 2005; De Smet *et al.*, 2007; Moreno-Risueno *et al.*, 2010) (Fig. 3A). Overall, auxin treatments induced *DR5::NLS-3xVENUS* in *CASP1$_{pro}$::shy2-2* roots, but rather weak compared to wild type roots (Figs 3A and S3). IAA and NAA treatments resulted in an increased *DR5::NLS-3xVENUS* fluorescence in the *CASP1$_{pro}$::shy2-2* root apical meristem, but significantly lower than Col-0, whereas the effect of 2,4-D on *DR5::NLS-3xVENUS* fluorescence was slightly lower (Figs 3A and S3D). Aside from that, the induction of *DR5::NLS-3xVENUS* in *CASP1$_{pro}$::shy2-2* roots was more pronounced in the LR formation zone compared to the root branching zone, independent of the type of auxin used, albeit the effect of 2,4-D was slightly weaker than that of IAA and NAA (Figs 3A and S3A-C). Notably, we observed that NAA treatment affected a notably larger area in which *DR5::NLS-3xVENUS* fluorescence accumulated compared to IAA and 2,4-D (Fig. 3A). Nevertheless, the auxin-induced increase in *DR5::NLS-3xVENUS* is mostly restricted within the vasculature of *CASP1$_{pro}$::shy2-2* (Fig. 3A), suggesting that auxin treatments can modify auxin signaling in *CASP1$_{pro}$::shy2-2* but cannot efficiently bypass the repression of Aux/IAA-mediated signaling in the endodermis. In addition, we also observed that the induction of *DR5::NLS-3xVENUS* fluorescence in the cortex and epidermis of *CASP1$_{pro}$::shy2-2* roots was weaker compared to wild type (Fig. 3A). This suggests that the block in Aux/IAA-mediated signaling in the endodermis also has a non-cell autonomous effect on the outer cell layers in addition to the effect on the XPP.

3.4-*CASP1$_{pro}$::shy2-2* roots display altered levels of the cytokinin response marker TCSn::GFP

The phytohormone cytokinin acts antagonistically in concert with auxins to co-regulate multiple plant biological processes, including LR development (Laplaze *et al.*, 2007; Bielach *et al.*, 2012; Marhavý *et al.*, 2014; Kieber & Schaller, 2018; Nenadić & Vermeer, 2021). Since SHY2 has been linked to the regulation of cytokinin responses (Dello Ioio *et al.*, 2008), we mapped cytokinin signaling outputs in *CASP1$_{pro}$::shy2-2* using the *TCSn::GFP* reporter (Zürcher *et al.*, 2013). Under control conditions, we observed a slight increase in TCSn::GFP signal in the root tip and a significant increase in the pericycle of the differentiated part of roots of *CASP1$_{pro}$::shy2-2* plants compared to wild type roots (Figs 3B and S4A, D). Treatment with the different auxins repressed cytokinin signaling both in Col-0 and *CASP1$_{pro}$::shy2-2* compared to control conditions (Figs 3B and S4). Stronger suppression of TCSn::GFP was observed towards the LR formation zone (Figs 3B and S4B). In particular, NAA-treated *CASP1$_{pro}$::shy2-2* displayed a larger zone where TCSn::GFP signal was subdued compared to the wild type (Fig. 3B). In the root branching zone, we observed a stronger

TCSn::GFP signal in 2,4-D-treated *CASP1$_{pro}$::shy2-2* compared to IAA- and NAA-treated plants (Figs 3B and S4A).

3.5-Endodermal Aux/IAA-mediated signaling regulates the onset of AUX1::VENUS in the XPP

IAA, NAA and 2,4-D have different transport characteristics in plants (Fig. S1). We hypothesized that their different efficiency in rescuing the *CASP1$_{pro}$::shy2-2* LR phenotype could be due to mis-regulation of auxin transporters. To test this, we characterized the expression and localization of the well-characterized auxin efflux carrier PIN-FORMED 1 (PIN1) and importer AUXIN RESISTANT 1 (AUX1) (Bennett *et al.*, 1996; Gälweiler *et al.*, 1998; Benková *et al.*, 2003). Using the *AUX1::VENUS* reporter line (Swarup *et al.*, 2004) crossed into *CASP1$_{pro}$::shy2-2*, we observed that the expression of this major auxin influx carrier was almost absent in the LR formation zone and lower compared to the wild type, whereas we observed no differences in *AUX1::VENUS* expression towards the LR branching zone between the two genotypes (Fig. 4A). This correlates with the described expression pattern of the CASP1 promoter that regulates the expression of *shy2-2* to inhibit Aux/IAA-mediated signaling in differentiated endodermal cells (Vermeer *et al.*, 2014). Interestingly, treatment with any of the tested auxins was able to recover the *AUX1::VENUS* expression level in *CASP1$_{pro}$::shy2-2* to match the AUX1::VENUS pattern in wild type roots (Fig. 4A), suggesting a possible correlation between the absence of LR phenotype and AUX1 levels in the LR formation zone in *CASP1$_{pro}$::shy2-2*. In contrast, the expression of PIN1, appeared similar to the wild type and did not seem to be influenced by any of the auxin treatments in *CASP1$_{pro}$::shy2-2* (Fig. 4B).

4-DISCUSSION

Several studies have used synthetic, and often more stable, auxins such as NAA and 2,4-D to mimic the effects of IAA due to their comparable affinity in TIR1 binding (Kepinski & Leyser, 2004; Dharmasiri *et al.*, 2005; Calderón Villalobos *et al.*, 2012; Karami *et al.*, 2023). Nevertheless, their effects are not strictly the same because IAA, NAA and 2,4-D have dissimilar intercellular transport mechanisms (Delbarre *et al.*, 1996; Fig. S1). IAA can enter the cell partially by diffusion or by active transport via AUXIN RESISTANT (AUX1)/ LIKE-AUX1 (AUX/LAX) influx carriers (Bennett *et al.*, 1996; Delbarre *et al.*, 1996). NAA can freely diffuse through the plasma membrane into the cells due to its high lipophilicity (Delbarre *et al.*, 1996). The accumulation level of IAA or NAA in the cell is controlled by the auxin efflux transporters like the PIN-FORMED (PINs) family or the ATP-BINDING CASSETTE sub-family B (ABCBs), whereas 2,4-D can be imported via the influx carriers, but cannot be exported via the efflux carriers as efficiently (Delbarre *et al.*, 1996; Cho & Cho, 2013).

4.1-Different efficacy in LR induction versus emergence by auxin treatment in *CASP1$_{pro}$::shy2-2*

IAA was the most effective in rescuing the LR phenotype in *CASP1$_{pro}$::shy2-2*. IAA induced the formation of LRPs with a morphology that resembled the wild type, albeit LRP patterning and cell

division were not fully preserved (Figs 1A-B, 2A and 5A). However, it is likely that this could be a result of a potential disturbance of the endogenous auxin gradient that is required for proper LR morphogenesis. NAA, despite sharply increasing LR initiation, did not maintain either the standard distributing pattern or the morphological properties of LRPs in *CASP1$_{pro}$::shy2-2* compared to Col-0 (Figs 1A, C, F, I, 2 and S2). 2,4-D, by contrast, severely disturbed LRP morphology as well as LR emergence in *CASP1$_{pro}$::shy2-2* (Figs 1 and 2A). Thus, it appears that the differences in the auxin signaling (intensity, duration and feedback) and intercellular transport pathways of IAA, NAA and 2,4-D are relevant to the responses in *CASP1$_{pro}$::shy2-2* roots observed after their treatments. Indeed, NAA and 2,4-D, as non-native auxins to plants, could not rescue the LR phenotype in *CASP1$_{pro}$::shy2-2* (Fig. 1). NAA, with its high cell-to-cell mobility, was able to trigger XPP cell divisions along the primary root and give rise to some dome-shaped LRPs in *CASP1$_{pro}$::shy2-2* roots. In contrast, 2,4-D induced massive cell proliferations in the XPP of *CASP1$_{pro}$::shy2-2* roots compared to wild type and was incapable of inducing dome-shaped LRP. This is most likely due to an even more inefficient export of 2,4-D from XPP cells in *CASP1$_{pro}$::shy2-2* roots (Fig. S1). As a result, 2,4-D remains "trapped" within XPP cells, causing repeated divisions. Moreover, the strong endodermal mechanical resistance in *CASP1$_{pro}$::shy2-2* roots might have favored anticlinal division rather than periclinal, resulting in larger auxin-induced cell proliferation zone and wider LR-like structures (Fig 2A-C). In addition, the observed *CASP1$_{pro}$::shy2-2* phenotypes after NAA and 2,4-D application resemble the phenotype of the *gnom^{R5}* mutant treated with the same auxins (Geldner *et al.*, 2004). This could imply that the recycling of the PINs or other membrane proteins could be affected in *CASP1$_{pro}$::shy2-2*. Interestingly, a weak allele of *GNOM* has been described in the regulation of the LR founder cell specification in Arabidopsis (Wachsman *et al.*, 2020). This strong effect could be attributed to the fact that, when the 5-day-old seedlings were transferred, the *CASP1$_{pro}$::shy2-2* plants already exhibited a non-responsive endodermis. The interrupted endodermis–pericycle communication would likely continue to generate increased endodermal resistance towards the growing LRPs, resulting in the observed problems with LRP morphology in auxin-treated *CASP1$_{pro}$::shy2-2* roots.

4.2-Auxin and cytokinin signaling in *CASP1$_{pro}$::shy2-2*

Being a determinant of cell division, elongation, and differentiation, auxin has been widely considered a key signaling molecule involved in the regulation of LR organogenesis. In Arabidopsis, overexpression of a stabilized version of *SHY2*, *shy2-2*, in differentiated endodermal cells disrupts local auxin signaling required to regulate LR formation (Vermeer *et al.*, 2014). However, SHY2 has already been linked to cytokinin signaling in the root apical meristem (Dello Ioio *et al.*, 2008). Because the transduction of auxin and cytokinin signals have counteracting yet complementary effects on LR development (Laplaze *et al.*, 2007; Bielach *et al.*, 2012; Kieber & Schaller, 2018), the deviating *CASP1$_{pro}$::shy2-2* responses could be partly due to different behaviors in auxin and cytokinin signaling upon each auxin treatment condition. Moreover, the auxin-induced flattened and

widened LRPs prior to traversing the endodermis in *CASP1$_{pro}$::shy2-2* roots (Fig. 2) clearly demonstrates that auxin treatments could partially override the impaired endodermal auxin signaling in *CASP1$_{pro}$::shy2-2*. Although we did not observe major differences in the expression pattern of the cytokinin signaling response marker TCSn::GFP (Fig. 3B), it is plausible that cytokinin signaling contributes to spatial accommodation, as TCSn::GFP was shown to be induced in the overlying endodermis during the later stages of LR development (Zürcher *et al.*, 2013). However, in general, auxin treatments resulted in an increase in DR5::NLS-3xVENUS signal and a decrease in the TCSn::GFP signal, similarly in both genotypes. Based on our results, we propose that a tightly controlled, auxin transport module acting in the XPP and overlying cell layers is required to accommodate LR development. Treatment with auxins with altered transport properties and stability will disrupt this auxin signaling landscape resulting in altered LR morphogenesis.

4.3-Aux/IAA-mediated signaling in the endodermis is required for timing AUX1 expression in the XPP

Directional intercellular transport is required to deliver auxin from young aerial tissues, where it is biosynthesized to distant targets within a tissue or across the plants (Petrášek & Friml, 2009). This transport is co-handled by a number of integral plasma membrane proteins, most prominently including AUX/LAX influx carriers, PIN efflux carriers and ABCB transporters (Bennett *et al.*, 1996; Casimiro *et al.*, 2001; Benková *et al.*, 2003; Santelia *et al.*, 2005; Swarup *et al.*, 2008; Péret *et al.*, 2009; Petrášek & Friml, 2009; Cho & Cho, 2013). Here, we provide evidence that the altered auxin responsiveness of *CASP1$_{pro}$::shy2-2* (Figs 1 and 2) could be linked to a mis-regulation of auxin transporters in this mutant. We did not observe significant changes in *PIN1* expression in the LR formation zone between Col-0 and *CASP1$_{pro}$::shy2-2* (Fig. 4B). However, the fact that 2,4-D cannot efficiently be transported via auxin efflux carriers suggests that the strong cell proliferation in *CASP1$_{pro}$::shy2-2* after 2,4-D treatment is unlikely caused by altered expression or activity of the PIN family members (Figs 1A and 2A). Unlike 2,4-D, both IAA and NAA are normally exported from the cell via PINs; thus the PIN1-mediated polar transport of these auxins might have remained unchanged in treated *CASP1$_{pro}$::shy2-2.* This could direct cell division toward the endodermis, so LRPs and LRs could be formed consequently (Figs 1A, 2A & 5A). Interestingly, we showed that *AUX1::VENUS* was repressed in the LR formation zone of *CASP1$_{pro}$::shy2-2* roots and that auxin treatment could restore this expression (Figs 4A and 5B). This suggests that an early signal dependent on Aux/IAA-mediated signaling in the endodermis is required to potentiate the XPP, leading to AUX1 accumulation, hence initiating the LR developmental program. It would be tempting to speculate that this could be a mechanical signal as it was recently shown that re-organization of the cortical microtubule cytoskeleton in the overlying endodermis is required to accommodate LR initiation (Stoeckle *et al.*, 2022).

AUTHOR CONTRIBUTIONS

TXB and VS performed auxin treatment assays. TXB performed analysis of LR morphogenesis, responses marker dynamics and localization of auxin transporters. SMM provided new reagents. KB and JEMV supervised the project. TXB, KB, and JEMV wrote the manuscript.

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DATA AVAILABILITY STATEMENTS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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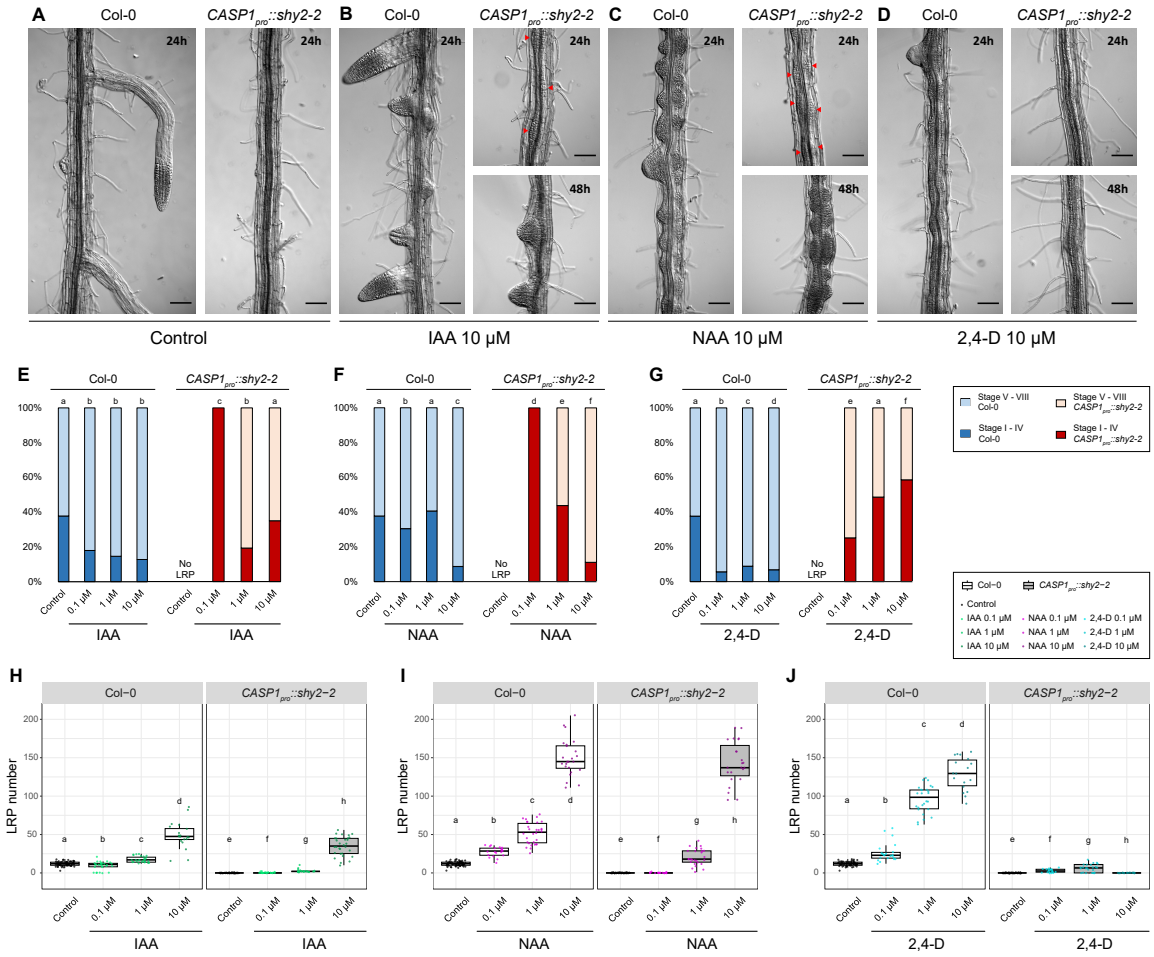

Figure 1. IAA and NAA induce LR formation in *CASP1*$_{pro}$*::shy2-2* with a delay, whereas 2,4-D is less efficient. **A-D**. LRP induction after 24-hour and 48-hour of 10 µM auxin treatments in Col-0 and *CASP1*$_{pro}$*::shy2-2*. Red arrowheads indicate LRPs induced by IAA and NAA after 24-hour treatment. Scale bar = 100 µm. **E-G**. Proportion of LRP stages before traversing the endodermis (stage I - IV) in Col-0 (dark blue) and *CASP1*$_{pro}$*::shy2-2* (dark red) and after (stage V - VIII) in Col-0 (light blue) and *CASP1*$_{pro}$*::shy2-2* (light red), induced by IAA (**E**), NAA (**F**) and 2,4-D (**G**). Different letters indicate Pearson's χ^2 test significant difference (p-value < 0.05). **H-J**. LRP number of Col-0 and *CASP1*$_{pro}$*::shy2-2* after 48 hours of IAA (**H**, green), NAA (**I**, magenta) and 2,4-D (**J**, cyan) treatments. Different letters indicate Wilcoxon rank-sum test significant difference (p-value < 0.05). 5-day old seedlings of Col-0 and *CASP1*$_{pro}$*::shy2-2* were treated with IAA, NAA and 2,4-D at 0.1, 1 or 10 µM for up to 48 hours. Root phenotyping was performed on no less than 18 plants per condition in 3 independent experiments.

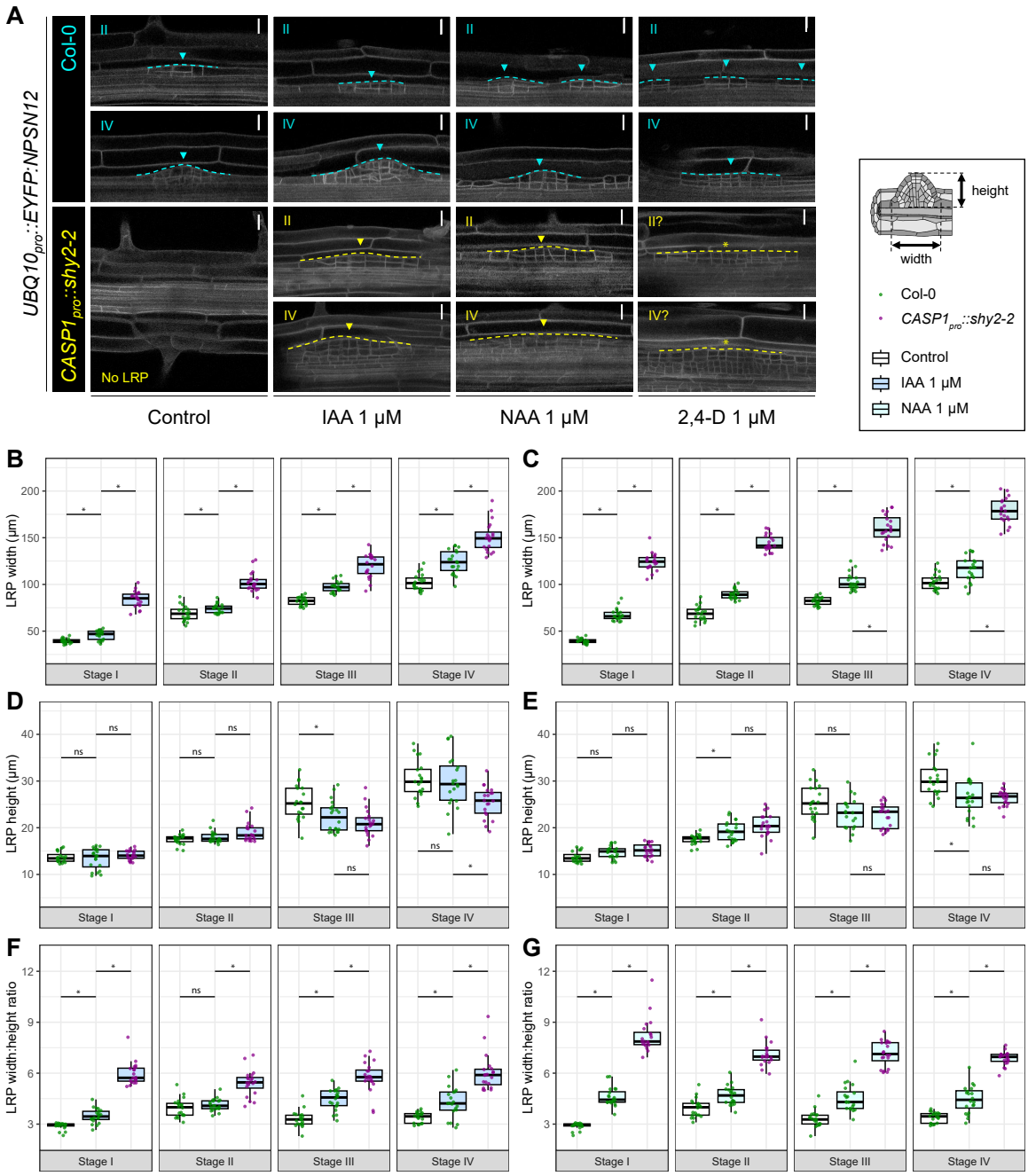

Figure 2. Auxin treatments affect LRP morphology in *CASP1$_{pro}$::shy2-2*. **A**. 2,4-D induces uncontrolled cell divisions without maintaining proper LRP morphology, whereas IAA- and NAA-mediated LRPs in *CASP1$_{pro}$::shy2-2* (yellow) are deformed compared to wild type (cyan). LRP morphology is visualized using the plasma membrane marker *UBQ10$_{pro}$::EYFP:NPSN12* (grey) in Col-0 and *CASP1$_{pro}$::shy2-2* backgrounds. 5-day-old seedlings were treated with IAA, NAA and 2,4-D at 1 µM for 48 hours. Roman numerals, dashed lines and arrowheads of the same colors indicate LRP stages and positions in Col-0 (cyan) and *CASP1$_{pro}$::shy2-2* (yellow). Asterisks indicate uncontrolled cell division. Scale bar = 20 µm; min. 5 images per observation, 3 independent experiments. **B-G**. IAA and NAA treatments result in widened and flattened LRPs in *CASP1$_{pro}$::shy2-2*. 5-day old seedlings of Col-0 and *CASP1$_{pro}$::shy2-2* were treated with IAA (**B**, **D**,

F) and NAA (**C**, **E**, **G**) at 1 µM for 48 hours. LRP dimensions [width (**B**, **C**), height (**D**, **E**) and ratio between LRP width and height (**F**, **G**)] through every stage before they cross the endodermis (stage I - IV) in Col-0 (green) and *CASP1*$_{pro}$*::shy2-2* (magenta) were quantified on 20 LRPs per stage, per condition, in 3 independent experiments. Untreated *CASP1*$_{pro}$*::shy2-2* is omitted due to absence of LRPs. ANOVA post hoc Student's *t*-test significant difference is represented by asterisks (p-value ≤ 0.05) or "ns" (non-significant difference, p-value > 0.05).

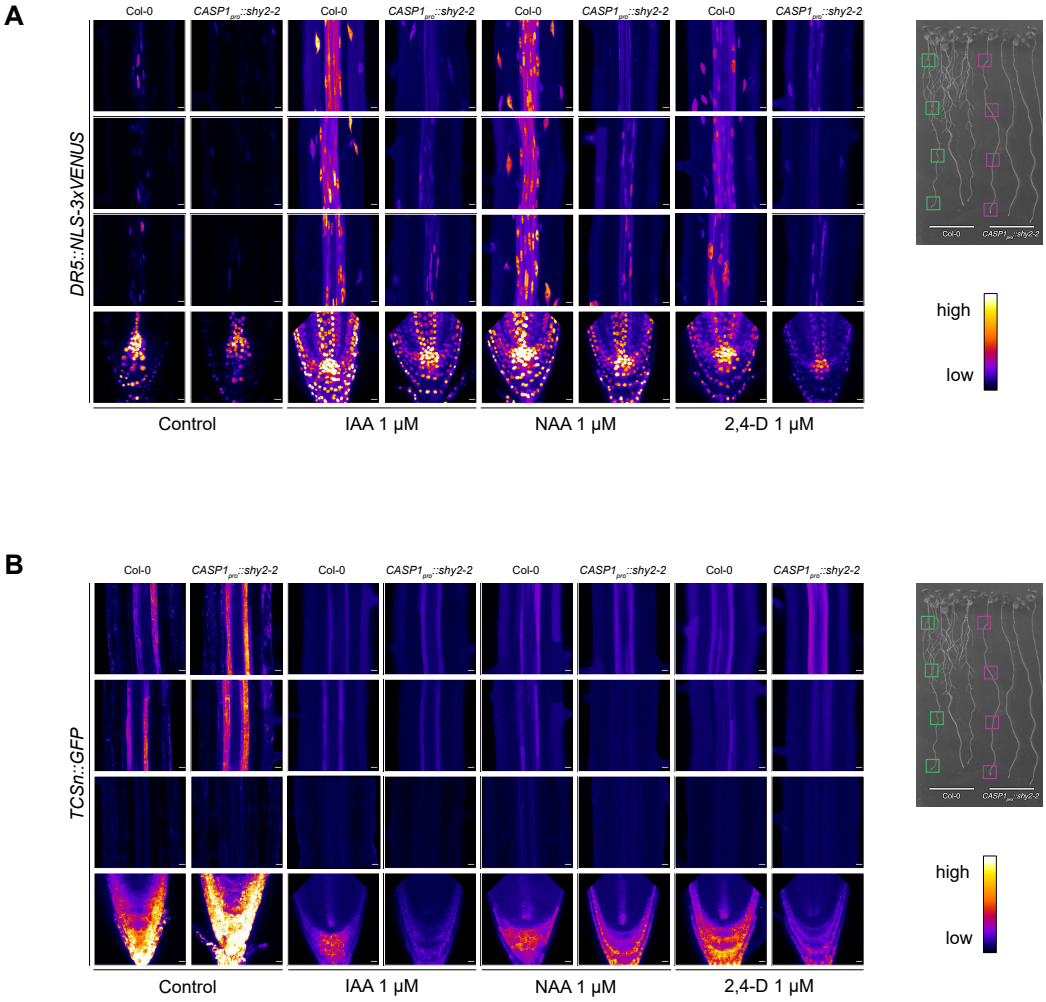

Figure 3. Mapping auxin and cytokinin responses after IAA, NAA or 2,4-D treatment in *CASP1$_{pro}$::shy2-2*. **A**. Auxin signaling outputs are restricted in the vasculature and down-regulated in *CASP1$_{pro}$::shy2-2*. **B**. Cytokinin signaling outputs seem unaffected in *CASP1$_{pro}$::shy2-2*. 5-day-old seedlings of reporter lines for auxin signaling, *DR5::NLS-3xVENUS* (**A**), and of cytokinin signaling, *TCSn::GFP* (**B**), with Col-0 and *CASP1$_{pro}$::shy2-2* backgrounds, treated with IAA, NAA and 2,4-D at 1 µM for 4 hours, were fixed and cleared with ClearSee, and imaged using multiphoton microscopy (min. 5 plants per condition, 3 independent experiments). Relative positions of observation along the primary root are marked with green (Col-0) and magenta (*CASP1$_{pro}$::shy2-2*) boxes. Scale bar = 10 µm. See Figures S3 and S4 for the quantification of DR5::NLS-3xVENUS and TCSn::GFP fluorescence intensity.

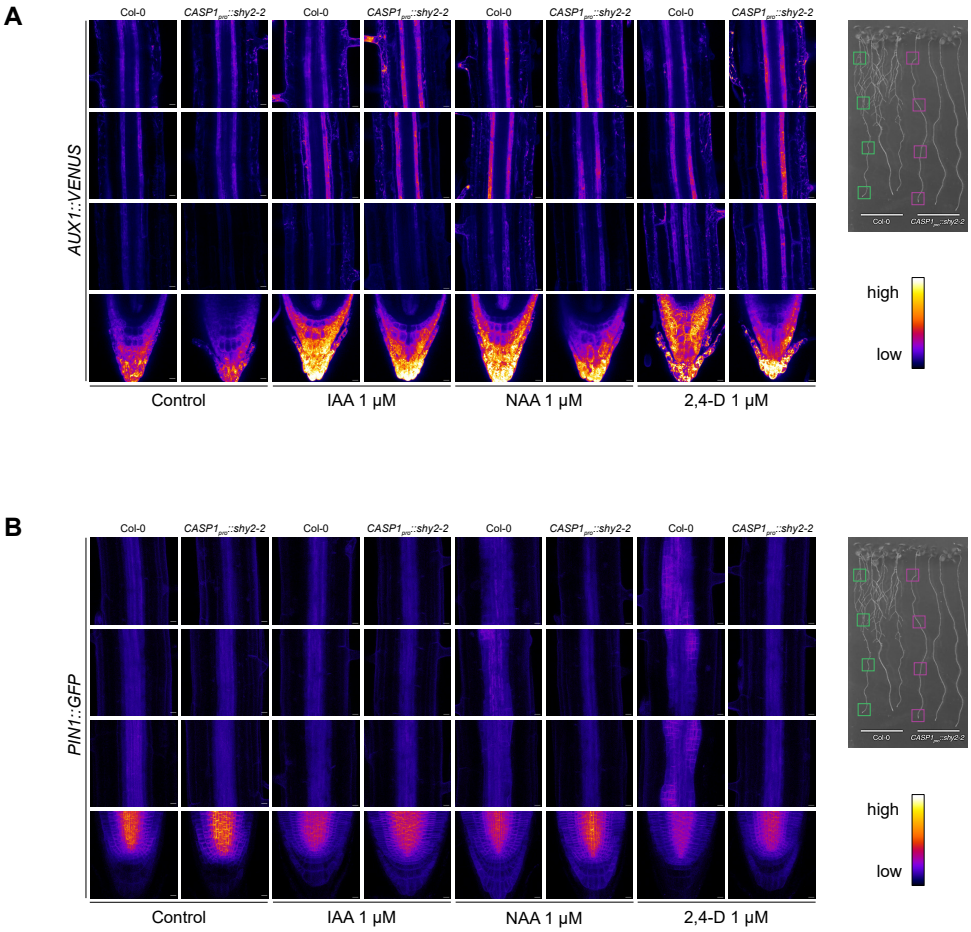

Figure 4. Mis-regulation of auxin transporters in *CASP1$_{pro}$::shy2-2*. 5-day-old seedlings of auxin transporter reporter lines, *AUX1::VENUS* (**A**) and *PIN1::GFP* (**B**), with Col-0 and *CASP1$_{pro}$::shy2-2* backgrounds, treated with IAA, NAA and 2,4-D at 1 µM for 24 hours, were fixed and cleared with ClearSee, and imaged using multiphoton microscope. Relative positions of observation along the primary root are marked with green (Col-0) and magenta (*CASP1$_{pro}$::shy2-2*) boxes. Scale bar = 10 µm; min. 5 images per condition, 3 independent experiments.

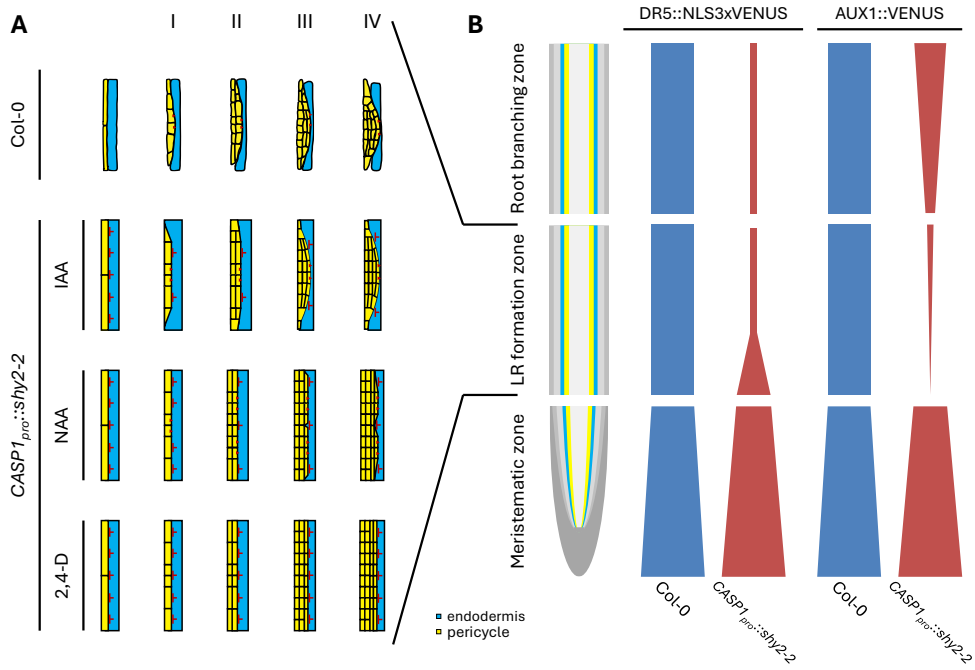

Figure 5. A tightly regulated auxin signaling landscape is required for spatial accommodation of lateral roots in Arabidopsis.

A. Schematic representation of LRP developmental stages prior to traversing the endodermis (stage I - IV) in Col-0 (adapted from Stoeckle *et al.*, 2018) and auxin-treated *CASP1$_{pro}$::shy2-2*. Red perpendicular lines indicate endodermal mechanical block against LRP outgrowth. Red arrowheads indicate PIN1-mediated auxin flow. Untreated *CASP1$_{pro}$::shy2-2* is omitted due to absence of LRPs. **B.** Levels of auxin-induced auxin signaling and AUX1 expression across the root branching zone, the LR formation zone and the meristematic zone in *CASP1$_{pro}$::shy2-2* (red) compared to Col-0 (blue). In auxin-treated *CASP1$_{pro}$::shy2-2*, stronger DR5::NLS3xVENUS signal and weaker AUX1::VENUS signal correlate at the younger end of the LR formation zone. Root branching zone: from shoot base to the most rootward emerged LR; LR formation zone: from below the most rootward emerged LR to the youngest and most rootward LRP; meristematic zone: from below the most rootward LRP to the root cap (Dubrovsky & Forde, 2012).

A tightly regulated auxin signaling landscape is required for spatial accommodation of lateral roots in Arabidopsis

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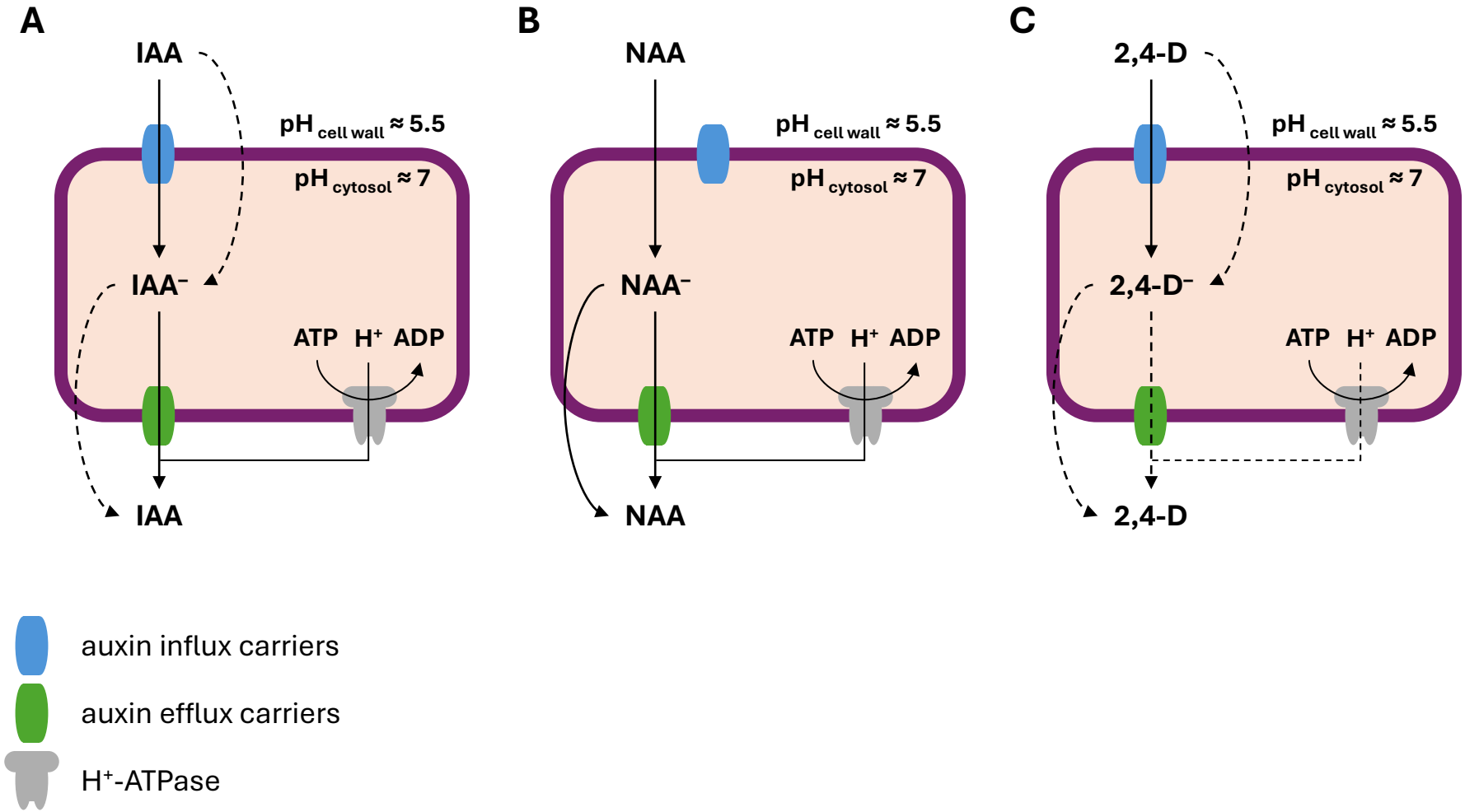
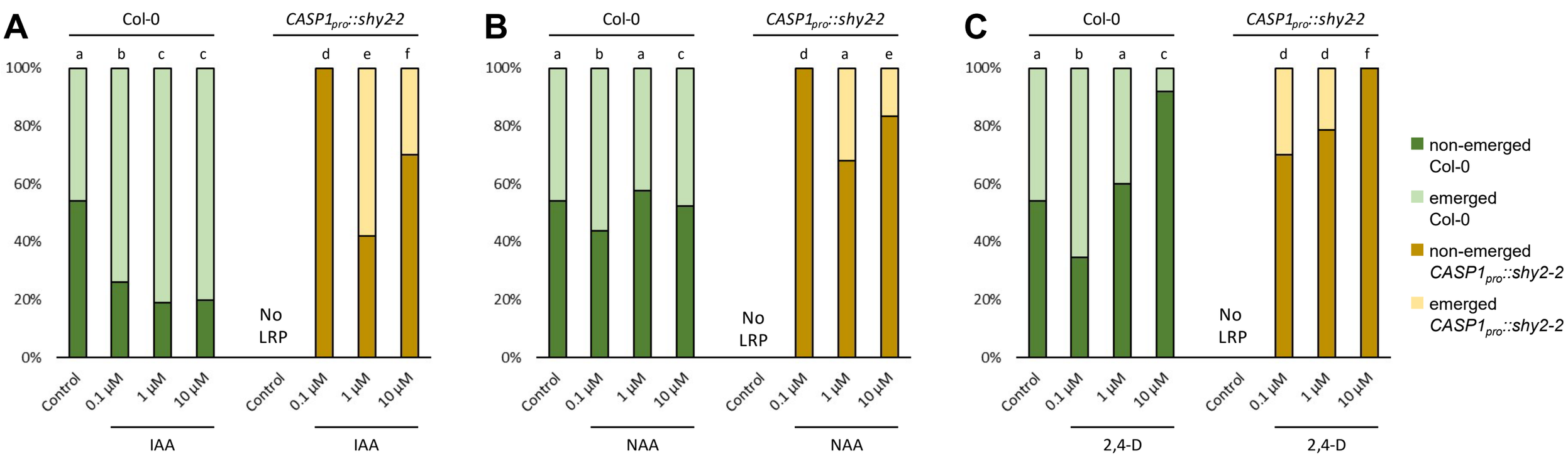


Figure S1. Different paths of cellular transport of IAA, NAA and 2,4-D. A. IAA can be transported into and out of the cells via auxin carriers, or via less efficient passive diffusion. **B.** NAA can be transported via passive diffusion and auxin efflux carriers. **C.** 2,4-D can enter the cells via auxin influx carriers but cannot be efficiently transported via passive diffusion and auxin efflux carriers. Solid arrows: main paths of auxin transport; dashed arrows: less efficient paths of auxin transport. (Schematic representation was inspired by Delbarre *et al.*, 1996)



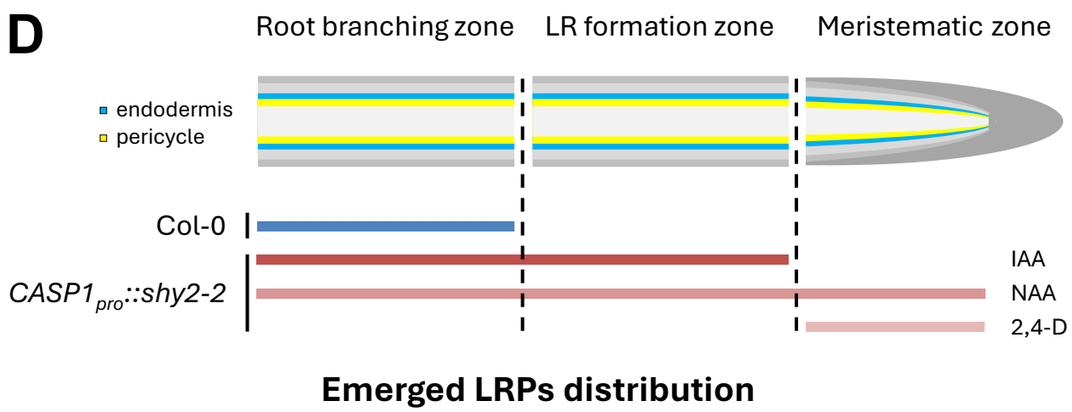


Figure S2. LR emergence is not fully rescued in auxin-treated *CASP1$_{pro}$::shy2-2*. A-C. Proportion of non-emerged LRs (stage I - VII) in Col-0 (dark green) and *CASP1$_{pro}$::shy2-2* (dark yellow) and emerged LRs (stage VIII) in Col-0 (light green) and *CASP1$_{pro}$::shy2-2* (light yellow), induced by IAA (**A**), NAA (**B**) and 2,4-D (**C**). **D.** Observed distribution of emerged LRs in correspondence with the root branching zone, the LR formation zone and the meristematic zone in *CASP1$_{pro}$::shy2-2* after extended auxin treatments (red) compared to untreated Col-0 (blue). 5-day old seedlings of Col-0 and *CASP1$_{pro}$::shy2-2* were treated with IAA, NAA and 2,4-D at 0.1, 1 or 10 µM for 72 hours. Root phenotyping was performed on no less than 18 plants per condition in 3 independent experiments. Different letters indicate Pearson's χ^2 test significant difference (p-value < 0.05). Root branching zone: from shoot base to the most rootward emerged LR; LR formation zone: from below the most rootward emerged LR to the youngest and most rootward LRP; meristematic zone: from below the most rootward LRP to the root cap (Dubrovsky & Forde, 2012).

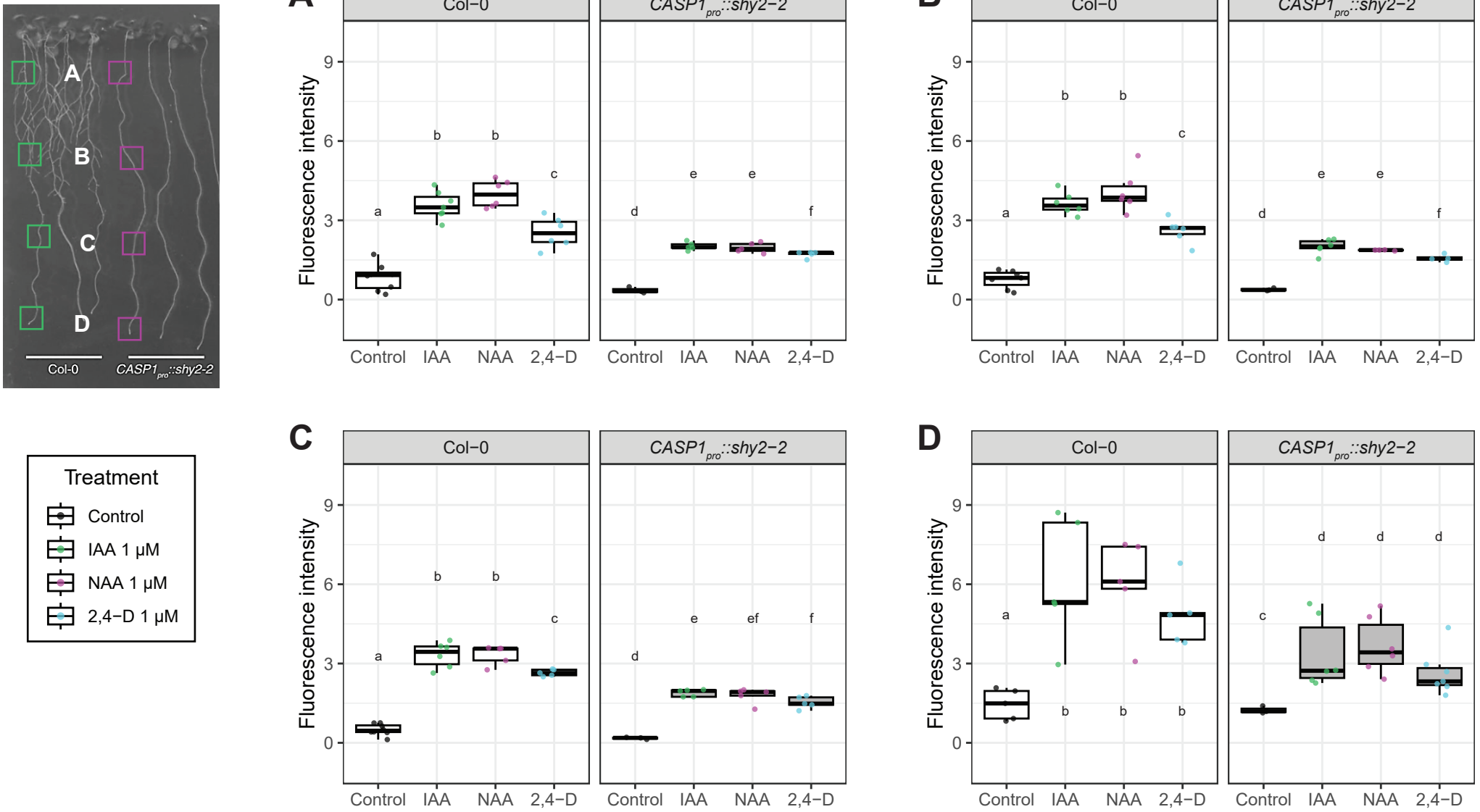


Figure S3. Auxin signaling output was repressed in treated *CASP1$_{pro}$::shy2-2* compared to Col-0. 5-day old seedlings of auxin signaling reporter line, *DR5::NLS-3xVENUS*, with Col-0 and *CASP1$_{pro}$::shy2-2* backgrounds, treated with IAA (green), NAA (magenta) and 2,4-D (cyan) at 1 µM for 4 hours, were fixed and cleared with ClearSee, and imaged using multiphoton microscopy using Hybrid detectors operating in photon counting mode (min. 5 plants per condition, 3 independent experiments). The mean grey values of the image pixels were quantified via Fiji software as DR5::NLS-3xVENUS fluorescence intensity ($n \geq 3$ images per position of observation, per condition and per genotype). Relative positions of observation and quantification are marked along the primary root (**A-D**). ANOVA post hoc Student's *t*-test significant difference is represented by different letters (p-value ≤ 0.05).

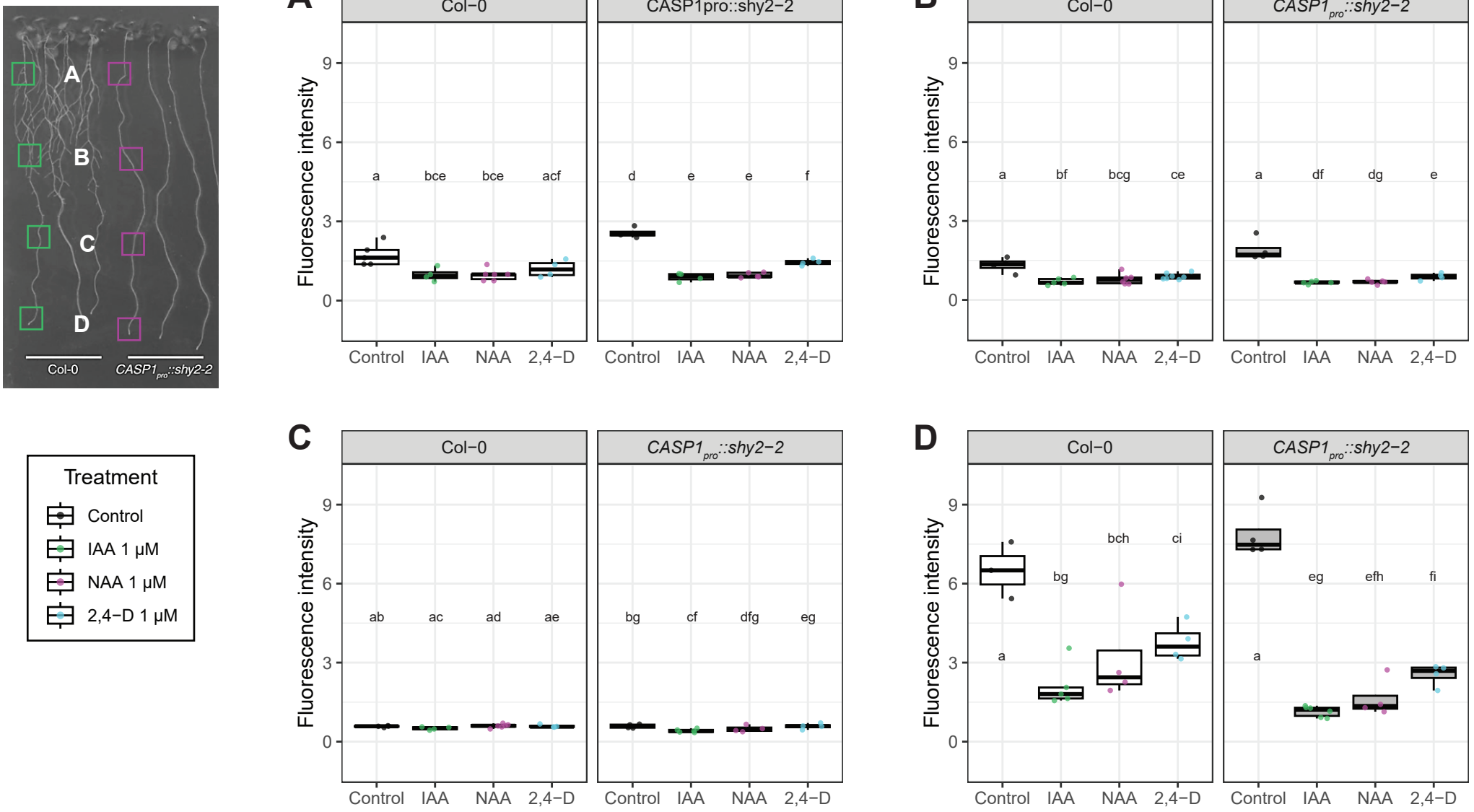


Figure S4. Cytokinin signaling output was unaffected in treated *CASP1$_{pro}$::shy2-2* compared to Col-0. 5-day old seedlings of cytokinin signaling reporter line, *TCSn::GFP*, with Col-0 and *CASP1$_{pro}$::shy2-2* backgrounds, treated with IAA (green), NAA (magenta) and 2,4-D (cyan) at 1 µM for 4 hours, were fixed and cleared with ClearSee, and imaged using multiphoton microscopy using Hybrid detectors operating in photon counting mode (min. 5 plants per condition, 3 independent experiments). The mean grey values of the image pixels were quantified via Fiji software as TCSn::GFP fluorescence intensity ($n \geq 3$ images per position of observation, per condition and per genotype). Relative positions of observation and quantification are marked along the primary root (**A-D**). ANOVA post hoc Student's *t*-test significant difference is represented by different letters (p-value ≤ 0.05).