Local auxin biosynthesis promotes stem cell differentiation and organogenesis in Arabidopsis shoot apex

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Summary

One of the striking aspects of stem cells is their ability to self-renew to maintain the adequate pool over extended periods and differentiate into distinct cell types on demand to sustain the tissue growth and repair by supplying new cells. Arabidopsis plant harbors pluripotent stem cells in the central zone (CZ) of the shoot apex. The daughters of these cells upon cell division move towards the peripheral zone (PZ). At the flanks of the meristem, they form lateral organs. Cells that get displaced underneath CZ become part of the rib-meristem (RM). RM gives rise to stem tissue and vascular cell types in higher plants. Despite the three decades of genetic research work, our understanding of how stem cells differentiate into PZ and RM cell types is inadequate. Here, we show that locally produced auxin in combination with transportis essential for stem cell differentiation and organogenesis in the shoot apex. WUSCHEL, a homeodomain transcription factor, negatively regulates auxin biosynthesis in the stem cell niche to maintain the long-term pluripotency of shoot stem cells. Our findings

reveal the role of local auxin biosynthesis in stem cell differentiation and how WUSCHEL regulates auxin signaling to promote stem cell fate in the shoot apex.

Main text

In shoot apical meristem (SAM), stem cell proliferation and exit are strictly controlled by cell-cell signaling network controlled by receptor like kinase CLAVATA1 (CLV1) and its ligand CLAVATA3 (CLV3)[1,2]. CLV3 binds to CLV1 and initiates the signaling cascade, which restricts the expression of *WUSCHEL* (*WUS*) in the organizing center[3–6].WUS protein moves from its site of synthesis through plasmodesmata and directly activates *CLV3* in CZ and also represses genes involved in differentiation[7–9].Thus, WUS not only specifies stem cells in apical meristem but also serves the central hub around that patterns of gene expression are established to specify CZ, PZ, and RM to form a functional SAM.

Here, we extend our previous observation of live-imaging to show that the culmination of auxin signaling in PZ is contributed by local auxin biosynthesis besides polar transport, and together they control stem cell differentiation in SAM. To maintain the fate of stem cells, WUS negatively regulates auxin responses in the CZ by directly regulating auxin biosynthesis and signaling genes to counter the differentiation pathways. Furthermore, we show that the enlarged CZ in *clv3* mutant plants maintains DII-Venus stably, suggesting that the stem cell fate and auxin signaling are dynamically regulated to maintain the overall SAM organization.

Results

***TAA1* and *TAR2* are expressed in shoot**

A previous live-imaging study has shown that transient downregulation of *WUS* in the stem cell niche results in higher auxin responses in the PZ with ectopic stem cell differentiation,

indicating that the fate of stem cells is directly linked to auxin signaling[10].We analyzed the WUS responsive genes from the Yadav et al., (2013) and identified *ANTHRANILATE SYNTHASE ALPHA SUBUNIT1*(*ASA1*), *TRYPTOPHAN SYNTHASE ALPHA1* (*TSA1*) and *TRYPTOPHAN AMINOTRANSFERASE RELATED2* (*TAR2*). To confirm this finding independently, plants having the *35S::WUS-GR* transgene were treated either with 10 µm dexamethasone (Dex) or ethanol for 4 h as a control. To establish whether the selected genes are direct targets of WUS, in parallel, plants were treated simultaneously with 10 µm Dex plus 10 µm Cycloheximide (Cyc) and Cyc alone for 4 h. Cyc acts as a protein synthesis inhibitor. Therefore, a spurious effect mediated due to gene expression activated of downstream genes can be ruled out. WUS mediated repression of *ASA1*and *TSA1* was found neither in Dex or in Dex+ Cyc treated plants compared with control. Although, we found more than a 2-fold decrease in the *TAR2*transcript levels in treated plants compared to mock (Supplementary Fig. 5a).

In order to understand the role of *TAR2* and its closest paralogues in auxin biosynthesis in the shoot, we analyzed the RNAseq dataset, which was obtained from apetala1/cauliflower double mutant after labeling the *CLV3*, *WUS*, *FILAMENTOUS FLOWER* and *ATHMGB15* expressing cells with fluorescent proteins, respectively (Monika Mahajan and R. K. Yadav unpublished data). Except for *TRYPTOPHAN AMINOTRANSFERASE* (*TAA1*) and *TAR2*, none of the *TARs* were found to be enriched in the SAM. In a previous study, Stepanova et al., (2008) identified *TAA1* as an allele of *weak ethylene insensitive 8*(*wei8*). Based on the RNAseq data, we found that *TAA1* and *TAR2* are expressed in CZ and PZ cell types, whereas *TAR1*does not express in SAM, but *TAR3* and *TAR4* are expressed very weakly. *TARs* catalyze an essential step in the auxin biosynthesis pathway, where they convert Tryptophan into Indole Pyruvic acid (IPyA)[11]. Further, the YUCCA class of enzymes converts IPyA into

Indole 3-Acetic Acid (IAA)[12].*TAA1* and *TAR2* are upstream of YUCCAs in the auxin biosynthesis pathway and not studied in detail.

To understand the role of *TAA1* and *TAR2* in shoot and flower development. First, we examined the expression pattern of *TAA1* and *TAR2* by in situ hybridization in the inflorescence meristem (IM) of Arabidopsis. The transcript of *TAA1*is specifically detected in the epidermal cell layer of CZ cells in 4-week-old WT-L*er* SAM(Fig. 1a; Supplementary Fig. 2a). The expression pattern of *TAA1* was further investigated in IM using a translational fusion construct made by Brumos et al., (2018), where *YPet* sequence was inserted in front of the *TAA1* coding sequence (*pTAA1::YPet-TAA1*) to make an N-terminal fusion. This construct successfully rescued the *taa1 tar2* double mutant phenotype[13]. In comparison to the mRNA expression pattern reported by *in situ* hybridization, we found the expression of translation fusion construct *pTAA1::YPet-TAA1* slightly more broad in the SAM but spatially restricted to the L1 layer (Fig. 1b-c). Similarly, we studied the expression pattern of *TAR2* by in situ hybridization in the 4-week-old shoot apices of WT-L*er*. In situ studies revealed expression of *TAR2* in the PZ zone of SAM, where typically the site of incipient primordia is marked. However, its expression is completely devoid from the CZ and RM cells (Fig. 1d; Supplementary Fig. 2e). To further investigate the expression of *TAR2*, we made a transcriptional fusion construct by amplifying the 3-kb promoter region above the *TAR2* translational start site (TSS). We placed the 3-kb promoter fragment in front of the H2B-YFP translational fusion in a modified pGreen vector. Several independent T1 lines were selected to examine the *TAR2* expression in the shoot (n= 18). The majority of the reporter lines showed the activity of the *TAR2* promoter very similar to native mRNA expression pattern, as reported by in situ studies (Fig. 1e, f). In two lines, we saw the expression of *pTAR2* all over the shoot apex, and these lines were not taken further.

In parallel, we checked the expression of *TAA1* and *TAR2* in embryo and seedling using the *pTAA1::YPet -TAA1*and *pTAR2::H2B-YFP* lines, respectively. Both *pTAA1::Ypet-TAA1* and *pTAR2::H2B*-YFP did not show expression in the globular stage (16-32 cell stage)(Supplementary Fig. 1a, j), however, at 64-cell stage Ypet-TAA1 fluorescence was clearly visible in the epidermal cell layer of apical domain proembryo (Supplementary Fig. 1b; ref. 11). Ypet-TAA1 is retained in the epidermal cell layer of the presumptive shoot apex of the heart stage, and in postembryonic stages, expression was confined to the epidermal cell layer of SAM in 3-DAS seedlings and 4-week-old IM, respectively (Supplementary Fig. 1c-f).In stage-3 and 4 flowers,*TAA1* expression was observed in the center of the meristem (Supplementary Fig. 1g-i). In contrast to *TAA1*, expression of *pTAR2::H2B-YFP* reporter was not detected in the globular stage (Supplementary Fig. 1j-k).*TAR2* promoter activity was noticed first in the heart stage in the presumptive hypocotyl region of proembryo (Supplementary Fig. 1l). In the postembryonic development, *TAR2* expression was seen in the periphery of SAM in 3-DAS seedlings (Supplementary Fig. 1m-n).In flowers and IM, *TAR2* expression is extended beyond the L1 cell layer in the PZ cell types (Supplementary Fig. 1o-r).

Locally produced auxin is essential for lateral organ initiation and patterning

To investigate further the role of *TAA1* and *TAR2* in organogenesis and stem cell differentiation, we analyzed the double mutant plant of *wei8-1 tar2-1* hereafter it will be *taa1 tar2*. It has been reported previously that *taa1 tar2* double mutant plants show a defect in floral organ patterning and flower development[11].Though, it is very well established that mutants having defects in auxin biosynthesis, signaling, and transport effects embryo patterning, root development, and lateral organ formation in SAM[14–16].We dissected the shoot apices of 4-week old WT, *taa1*, and *tar2* single mutant plants along with *taa1 tar2* double mutant. We did confocal imaging to measure the size of the shoot.*taa1* and *tar2*single mutant

SAM look very similar to WT (Fig. 1g-I; Supplementary Fig. 3e-g, m). In contrast, the double mutant plants harboring *taa1 tar2* display spherical SAM (Fig. 1j; Supplementary Fig. 3h, m). The organ boundaries of *taa1 tar2* plants are not patterned. The outgrowth and polarity of floral organ primordia are compromised in double mutant plants in comparison to single mutant and WT, suggesting a critical role of *TAA1* and *TAR2* in lateral organ formation, growth, and patterning (Fig. 1g-j). Since auxin promotes apical dominance, to find out the role of *TAA1* and *TAR2*, we grew the WT Col-0 along with *taa1*, *tar2*single and *taa1 tar2* double mutant, and found that single mutant of both *taa1* and *tar2* are indistinguishable from WT in plant stature and SAM size, while the *taa1 tar2*double mutant plants are dwarf and their SAM size is smaller than the WT and respective mutant (Fig. 3p; Supplementary Fig. 3i-l)

After uncovering that the *taa1 tar2* double mutant has smaller SAM compared to control, we asked whether stem cell self-renewal has got perturbed in these mutants, and that could affect the relative size of CZ and PZ in these plants. To test this, we made crosses between the *taa1-1 -/- tar2-1 -/+* with *pCLV3::mGFP-ER* reporter lines, and isolated *taa1*, *tar2* and *taa1tar2* double mutant, respectively. A closer examination of *CLV3* expression pattern in *taa1 tar2* mutant revealed that the double mutant plants do not show a change in CZ size compared to control and respective single mutants, indicating that in the absence of auxin biosynthesis the relative size of the CZ is not perturbed (Fig. 1j, n). Although in PZ, overall cell number decreased in the auxin biosynthesis mutant, CZ cell number did not decrease significantly. Ratio of cells in CZ to PZ also increased in *taa1 tar2* double mutant plants due to decrease in PZ cell number (Fig. 1s-u). Taken together, the expression analysis of*TAA1* and *TAR2*revealed that both genes are expressed inSAM.*TAR2* expression displays a strong correlation with emerging organ primordia; however, single mutant does not show any defect

in organogenesis and shoot development. Interestingly, *taa1 tar2* double mutant plants show a defect in lateral organ formation, shoot apex patterning, stem growth, and axillary meristem formation, suggesting the role of local auxin biosynthesis in organ formation and shoot development.*TAA1* and *TAR2* can complement each other, and therefore discerning their role in SAM patterning is challenging to entangle. The relative size of PZ is reduced in the plants lacking auxin biosynthesis in SAM.

Locally produced auxin provides robustness to auxin signaling in SAM

To better understand the role of local auxin biosynthesis in auxin responses in SAM, we wanted to know whether the input and output responses are altered in the *taa1*, *tar2* single, and *taa1 tar2* double mutant plants. map auxin responses, we crossed the *pPIN1::PIN1-GFP*; *pDR5rev::3XVENUS-N7* line with *taa1-1* -/- *tar2-1* +/- plants. After making the reporter homozygous in the *taa1*, *tar2*, and *taa1 tar2* double mutant background, respectively, we examined the meristem expressing *pPIN::PIN1-GFP* and *pDR5rev::3XVENUS-N7*. Confocal imaging of the shoot apices of *taa1*, *tar2* single mutant, and *taa1 tar2* double mutant plants revealed polar localization of PIN-GFP. In the *taa1* and *tar2* single mutant, patterns of*DR5* expression and PIN1-GFP polarization were very similar to control plants (Fig. 2 a-c, e-g, i-k, m-o). In *taa1 tar2* double mutant the PIN1-GFP expression was weak, however, it was polarized (fig 2l, p).As reported by *DR5* expression, auxin levels were significantly reduced in *taa1 tar2* double mutant compared to control and single mutant plants (Fig. 2d, h, y).Taken together, we found in *taa1* and *tar2* single mutant expression of *DR5*and PIN1-GFPpolarization comparable to control plant (Fig. 2a-c, e-f, i-j, m-o, y). However, *DR5* expression was significantly down regulated in *taa1tar2* double mutant plants due to lack of auxin signaling output (Fig. 2d, h, y). Despite that PIN1-GFP showed polarization *taa1 tar2* double mutant plants.

Next, we checked the stability of auxin input sensor DII-Venus in the single mutant of *taa1* and *tar2* and compared this with WT control to determine whether the local auxin biosynthesis mediated by *TAA1* and *TAR2* play any role in its stability in SAM. For this, we crossed the line carrying *R2D2*transgene with *taa1-1* -/- *tar2-1* +/-. For both single mutants as well as for double mutant plants were identified after following parents. We found that a single mutant of *taa1* and *tar2* show the DII-Venus expression pattern similar to WT (Fig. 2q-s, u-w, z). In *taa1tar2* double mutant background, the DII-Venus was stable in the CZ cells of both IM and flower meristem, clearly indicating the significance of *TAA1* and *TAR2* mediated auxin biosynthesis in maintaining an optimum threshold of auxin signaling in SAM (Fig. 2t, x, z). Taken together, our findings revealed that auxin biosynthesis mediated by *TAA1* and *TAR2* is essential for maintaining the optimum level of auxin signaling in SAM. If it gets perturbed, the normal growth of the SAM is compromised. This finding is consistent with the observation made with the *DR5* reporter in this genetic background.

Auxin biosynthesis in the incipient organ primordia is critical for lateral organ initiation

In *taa1 tar2* double mutant due to auxin biosynthesis defect differentiation of stem cells got partially affected, but it did not get impeded completely. We asked whether the lack of *DR5*activity or DII stability, if anyhow, is a good indicator of auxin signaling abrogation in SAM. We should see a complete abrogation of lateral organ primordia formation in the *taa1tar2* double mutant. We also reasoned based on the expression pattern that the *TAR2*should be critical in organ initiation than *TAA1*.To explore the role of PIN1 in organogenesis in *taa1 tar2* double mutant and to uncouple the residual auxin signaling maintained by the auxin transport mediated by PIN1 in SAM. We crossed the *taa1-1*-/- *tar2-1*

+/- plants with weak *pin1-5* allele. Since a strong *pin1* mutant does not form lateral organs in the reproductive phase despite having functional CZ, PZ, and RM. *pin1-5* mutant plants form lateral organs with defective flowers and set the seeds (Fig. 3b, f,j).We isolated *pin1 taa1* and *pin1tar2* double mutant plants, respectively, to ascertain their relative contribution in auxin signaling and organogenesis (Fig. 3c, d; Supplementary Fig. 4c, d). In the double mutant plants, the presence of the *pin1-5* allele was confirmed based on the flower organ number and flower morphology, whereas to determine *taa1-1* and *tar2-1* genotype, a T-DNA PCR was setup, respectively. The *pin1-5* single mutant inflorescences make at least 7-8 flowers (n=18), and flower primordia abut in the PZ similar to WT (Fig. 3f, j). Unlike a strong *pin-1* mutant, the *pin1-5* allele also initiates and maintains axillary IM, which further bears the flowers and continues the growth (Fig. 3b, m). In contrast, the main IM of both *pin1taa1* and *pin1tar2* double mutant plants bear a significantly lesser number of flowers before terminating in to pin like SAM (Fig. 3c, d, g, h, k, l, o). Although, both *pin1taa1*and *pin1tar2*double mutant plants bear a fewer number of flowers in comparison to *pin1*, indicating that local auxin biosynthesis is critical for lateral organ formation in the SAM. The emergences of lateral organs further get reduced in *pin1 tar2* double mutant plant in comparison to *pin1 taa1*, suggesting that auxin produced in PZ perhaps has a more direct effect on the organ formation (Fig. 3n-o). *pin1 taa1* mutant plant axillary inflorescences formed flower without producing pin-shaped apices at 35 days, indicating that the lateral organs are still being produced in the *pin1taa1*double mutant while their *pin1tar2* counterparts show exaggeration in pin-shaped apices formation (Fig. 3c-d and m). This genetic evidence makes *TAR2* more critical than *TAA1* in lateral organ formation in the reproductive phase of the plant development, which correlates with its expression in the PZ of SAM. *TAA1*is required in the epidermal cell layer for apical dominance. However, it can rescue the lateral organ defect in the PZ in the absence of *TAR2*(Fig. 3p, Supplementary Fig. 4e).These findings establish the role of local auxin

biosynthesis along with polar transport in SAM patterning and growth, which was not reported earlier.

Auxin signaling control the transition of stem cell progenitors into differentiating cells in PZ

To understand the role of auxin transport and biosynthesis on cell differentiation and phyllotaxy, we identified triple mutant plants for*pin1-5 taa1 tar2.*T-DNA PCR confirmed the *taa1* and *tar2*T-DNA alleles, which was verified by sequencing, while the *pin1-5*EMS mutant was sequenced directly to verify the mutation. The *pin1 taa1 tar2*triple mutant plants did not form rosettes in vegetative development (Supplementary Fig. 4f, g). The majority of the triple mutant plants isolated from these crosses displayed unequal size cotyledons in the beginning and found rootless, but when kept for 2-3 weeks, they formed a mound shape structure in between the two cotyledons (Fig. 4o; Supplementary Fig. 4h, i, k). Interestingly, triple mutant plants fail to produce leaves. We were unable to conclude looking at this phenotype whether the heap of cells is an undifferentiated cell mass or regenerated shoot tissue. To further determine the identity of these cells, we studied the expression of SAM marker genes by in situ hybridization.

CLV3 and *WUS* are usually expressed in CZ and RM cells, respectively. In *pin1 taa1 tar2* triple mutant plants, expression of *CLV3*mRNA was detected in the tip of the mound (Fig. 4q). A closer examination of the in situ images revealed a dramatic increase in *CLV3* transcript levels. However, its expression was expanded significantly in the epidermal and subepidermal cell layers in PZ compared to *taa1 tar2*double mutant plants (Fig. 4c, j). Surprisingly, *WUS* expression level went down dramatically in *pin1 taa1 tar2* triple mutants compared to *taa1 tar2* double mutants, possibly due to overexpression of *CLV3*(Fig. 4i, p).To investigate whether these plants lack functional PZ or they fail to form the PZ cell type in the

absence of auxin signaling. We examined the expression of genes, which are expressed in the PZ and organ boundary regions. The overall expression patterns of *ARF3*, *ARF4*, *ARF5*, and *CUC1* are although maintained similar to WT in *taa1 tar2* double mutant. However, the level of expression has weakened significantly in *taa1 tar2*double mutant SAM (Fig. 4d-g, k-n).This has already resulted in irregular organ boundaries and phyllotactic pattern formations and auxin signaling. In the *pin1 taa1 tar2* triple mutant, both the expression patterns and levels of *ARF3*, *ARF4*, *ARF5* and *CUC1*were either abolished entirely or weakened significantly, suggesting that auxin signaling is not only critical for timely transition of stem cells into differentiating cell types but also regulates the expression of essential CZ and PZ marker genes (Fig. 4r-u).

Auxin levels dynamically regulate stem cell fate in SAM

To understand how local auxin biosynthesis affects the stem cell fate and how WUS mediated down regulation of auxin biosynthesis in niche cells is controlled. First, we systematically analyzed the WUS binding sites having TAAT core in the *TAR2* promoter. We identified two WUS binding sites in the *TAR2* promoter and tested them in electrophoretic mobility shift assay (EMSA) experiments. The oligo containing putative cis-elements that conferred WUS binding in EMSA assay when mutated in the TAAT core (Supplementary Fig. 5d), we observed abrogation of WUS binding as reported in earlier studies (Supplementary Fig. 5c). Second, we deleted the WUS binding cis-element in *TAR2* promoter, and made *pTAR2*Δ(-897, -2094)*::H2B-YFP* construct. It was introduced in WT L*er*, and five independent T1 lines showing H2B-YFP expression driven by *pTAR2*Δwere isolated. Further, plants were made homozygote, and the expression of *pTAR2*Δwas analyzed in T2 (Fig. 5b, d). We found the expression of*pTAR2*Δexpanded towards the CZ in

comparison to control plants in SAM, indicating that in the absence of WUS binding on *pTAR2*Δ, its expression gets aberrated.

Next, we asked, if auxin signaling is so critical for stem cell fate and maintenance in the SAM, then *clv3* mutant SAM should have lower auxin level due to higher dosages of WUS, and a corresponding decrease in auxin signaling and biosynthesis. To test this, we crossed the of *R2D2*line and *pTAR2::H2B-YFP* in the *clv3* mutant. We found the DII-Venus sensor throughout the CZ in the *clv3-2*mutant except in the narrow PZ, where *TAR2* expression is still active (Fig. 5e, h and l). Both the *TAR2* in situ studies and the *pTAR2* reporter expression pattern data revealed that *TAR2* is negatively regulated and maintained in a narrow circumference at the flanks of the meristem in the PZ (Fig. 5e ,h; Supplementary Fig. 5g), where differentiation of stem cells still occur, however, in the CZ auxin signaling threshold has decreased and this has resulted in stable DII-Venus. Thus, WUS counters auxin biosynthesis by regulating *TAR2,*and keep its expression restricted to PZ cell types. In the *clv3-2* mutant overwhelming amount of WUS protein gets accumulated, which further negatively regulates auxin biosynthesis. Taken together, we deciphered the regulation of *TAR2*by WUS in the *clv3-2* mutant, and by deleting WUS binding sites in *pTAR2* in WT.

We found a strong link between *TAR2* mediated auxin biosynthesis on signaling in PZ of SAM and differentiation. If auxin is required for differentiation of stem cell daughters in PZ, then why *TAA1* mediated auxin biosynthesis in the epidermal cell layer unable to promote differentiation of stem cells (Supplementary Fig. 2a, c). To test the influence of auxin on stem cell fate, we drove the expression of *TAR2* under the *35S* promoter. We generated *35S::TAR2* transgenic lines in WT L*er*. We found that the rescued plants did not show termination of the shoot apex in T1 (n=21, Supplementary Fig 6a). However, when the same

lines were put again on the plate in the T2 generation, eleven of them showed termination of shoot apex on average in one quarter segregating plants (Supplementary Fig. 6b, c).Semiquantitative RT-qPCR experiment revealed that plants showing homozygosity for *35S::TAR2*transgene displayed elevated levels of *TAR2* transcript as well as shoot termination phenotype, indicating that beyond a threshold auxin levels in meristem can trigger stem cell differentiation (Supplementary Fig. 6b, c, f).To test this more subtly, we made a *6X-OP::TAR2* operator construct and dipped into *pCLV3::LhG4* and *pWUS::LhG4* driver lines, respectively. Interestingly, T1 plants carrying *pCLV3::LhG4 6X-OP::TAR2* made at least 3-4 true leaves before shoot termination (n=48; Supplementary Fig. 6d). In contrast, T1 plants carrying *pWUS::LhG4 6X-OP::TAR2* made only two true leaves (n=40; Supplementary Fig. 5e), in addition to cotyledons, indicating that early onset of auxin biosynthesis in niche cells causes premature termination of SAM. Taken together, the finding presented here show that elevated level of auxin biosynthesis in SAM is detrimental for stem cell maintenance. Auxin accumulated below a critical threshold in the epidermal cell layer perhaps is not promoting ectopic differentiation of stem cells possibly, due to polar transport of auxin by PIN1 towards the primordia. However, ectopic expression of *TAR2*in the inner cell layers do promote differentiation of SAM stem cells. This finding also supported by a previous study, where DII-Venus was observed in niche cells[17].

To understand the role of auxin biosynthesis and WUS on auxin responsive genes, especially on *ARFs* in SAM. We carried out the qRT-PCR in the *35S::WUS-GR* plant treated with Dex. Among the shoot enriched *ARFs*, *ARF1, 5, 7,*and *11*are upregulated in response to WUS. However, *ARF4* and *ARF18*are downregulated (Supplementary Fig. 7a). To test whether auxin level is critical for *ARF* expression, we also tested their expression in the *taa1 tar2* mutant background. Transcription of *ARF1, 3,5, 6, 8,*and *9*are significantly down regulated,

and hence depended on auxin levels (Supplementary Fig. 7b).Taken together, the differential regulation of auxin signaling gene seen in the SAM not only mediated by *ARFs*, but it is also affected by critical regulators whose expression is not depended upon auxin signaling.

Discussion

In this study, we show that simultaneous disruption of auxin biosynthesis with transport lead to defect in auxin signaling in SAM. As a result of this, stem cells, self-renewal do not get compromised, but they fail to differentiate into PZ and RM cell types. In contrast to strong *pin1*, *taa1tar2pin1-5* triple mutant plants are unable to produce lateral organs even in the vegetative phase[18],demonstratinga previously undiscovered role of local auxin biosynthesis in organogenesis. This data also explains why *DR5::GFP* expression is elevated in the PZ in the *pin1-7* vegetative SAM, as reported by Guenot et al., (2012). This is also significant because when *yuc1yuc4pin1-5* triple mutant plants were analyzed for *CLV3* and *WUS* expression in SAM, their expression patterns did not deviate from the WT[19], suggesting a high redundancy among YUCCAs. This finding also confirms the epistatic nature of *TAA1* and *TAR2* in the IPyA auxin biosynthesis pathway.

Our finding also confirms that in the absence of local auxin biosynthesis, optimum auxin response in the PZ is abrogated. This is also attributed to the SAM patterning and phyllotactic defect observed in these plants. Double mutant analysis of auxin biosynthesis gene with transport revealed that *TAA1* is critically required for stem tissue growth, whereas *TAR2* is required for lateral organ formation and their growth, which correlates well with their spatiotemporal expression patterns (Fig. 1a, d; Supplementary Fig. 1f, o).

Based on the auxin signaling and SAM marker genes analysis in *taa1tar2pin1* triple mutant, we conclude that shoot stem cells do not require auxin signaling for their maintenance and

self-renewal. Still, residual auxin signaling could not be ruled out in the affected plants. However, beyond a threshold, elevated auxin responses in SAM are detrimental for stem cell maintenance. A ring of cells surrounding the CZ was shown to be functional PZ based on the organ initiation in *clv3*mutant[10].Interestingly this small band of cells maintains*TAR2* expression (Fig. 5e).However, DII-Venus is unstable in these cells (Fig. 5l), indicating that auxin signaling still occurs in this narrow region. Thus, WUS can regulate the transition of stem cells into PZ cell types by regulating auxin input in SAM. This notion is also supported by *TIR1*expression[20].This regulation further gets traction by polar transport of auxin by PIN1 towards the PZ cells and further reinforced by auxin signaling[16,21]. Thus, it creates a robust patterning of the shoot into PZ and CZ cell types whose boundaries are not evident but dynamic due to underlying feedback mechanisms[22].

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Author Contributions

R.Y. conceived the project, S.Y. and R.Y. designed experiments. S.Y., H.K., and R.Y. performed the experiments. S.Y., H.K., and R.Y. analysed the data. S.Y. and R.Y. wrote the paper.

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YFP clv3-2 and *pWUS::eGFP-WUS clv3-2* lines. Imaging work was carried out at the confocal microscopy core facility of IISER Mohali. The IISER Mohali core grant funds support confocal imaging facility and robotic facility.

Competing interest statement

The authors declare no competing financial interests.

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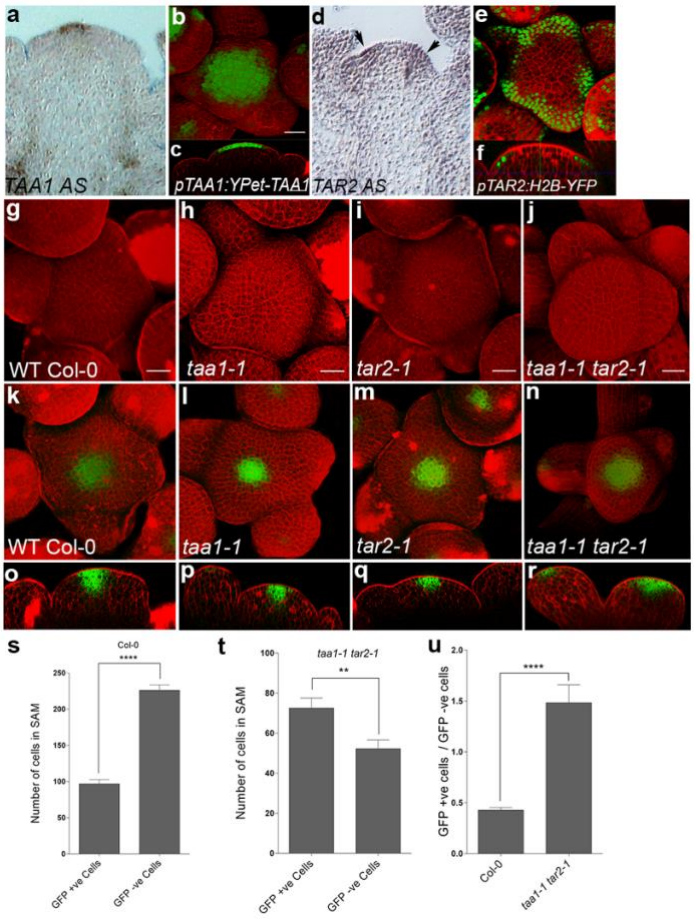

Figure 1. *TAA1* and *TAR2* are expressed in the inflorescence meristem and affect shoot meristem development

TAA1 mRNA expression is detected in the epidermal cell layer of SAM by *in situ* hybridization (a). Three dimensional (3D) reconstrued top view of SAM, showing expression of Ypet-TAA1 driven by *pTAA1* (b). Side view of the 3D image, showing Ypet-TAA1 protein (in green) localized to epidermal cell layer of SAM (c). *TAR2* mRNA is detected in the PZ of SAM (black arrowheads) by *in situ* hybridization, but devoid from CZ cells (d). 3D reconstructed top view of four week old WT-L*er* SAM expressing *pTAR2::H2B-YFP* (in green) (e), and side view (f). Confocal images stacks converted into 3D image to view SAM of WT Col-0, *taa1-1*, *tar2-1* and *taa1-1 tar2-1*, respectively (g-j). Expression pattern of *pCLV3::mGFP-ER* in Col-0, *taa1-1*, *tar2-1* and *taa1-1 tar2-1*, respectively (k-r). 3D top view of SAM (k-n), and side view of the same image (o-r). Propidium iodide (PI, in red) was used to visualize cell outline. Quantification of *pCLV3::mGFP* +ve (CZ cells) and GFP-ve cells (PZ cells) in Col-0 (n=9) and *taa1-1 tar2-1* (n=9) double mutant (s-u), Error bars show SEM, asterisk marks represent statistically significant difference among the tested samples, Statistical test: Student t-Test ($p < 0.05$). Scale bars: 20μM.

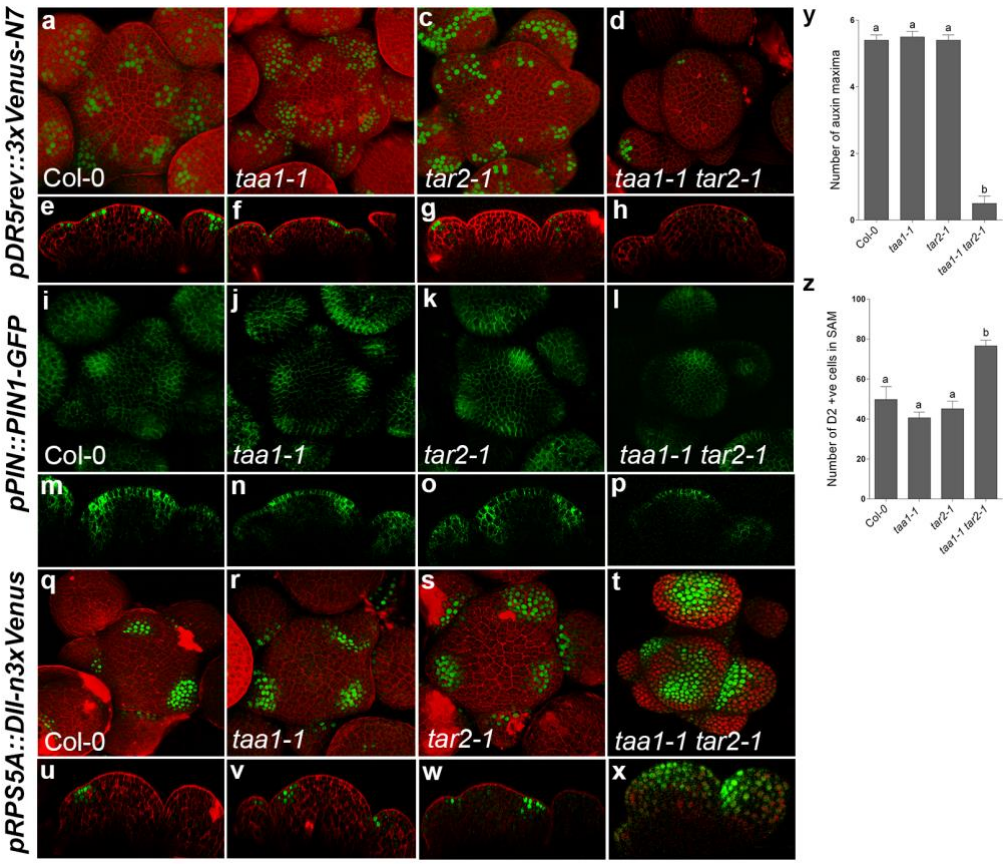

Figure 2. Auxin response in SAM is depended on the local auxin biosynthesis

3D reconstructed top view of Arabidopsis SAMs showing the expression of the auxin response reporter *pDR5rev::3XVENUS-N7* in Col-0, *taa1-1*, *tar2-1* and *taa1-1 tar2-1* double mutant (a-d). Side view of (a-d) in (e-h). *taa1-1 tar2-1* double mutant SAM lacks *DR5* responses. *pPIN::PIN1-GFP* expressing SAM of Col-0, *taa1-1*, *tar2-1*, *taa1-1 tar2-1* rendered into 3D. Top views are shown (i-l), while the side of views of the SAM is displayed in (m-p). Note, despite auxin biosynthesis defect, *taa1-1 tar2-1* double mutant plants show PIN1-GFP polarization and organogenesis. DII-Venus sensor in Col-0, *taa1-1*, *tar2-1*, and *taa1-1 tar2-1* SAM top view (q-t) while the side views are shown (u-x). Note, in *taa1-1 tar2-1* double mutant SAM DII-Venus is stable despite weak auxin signalling. Quantification for auxin maxima in Col-0 (n=9), *taa1-1* (n=9) *tar2-1* (n=9) and *taa1-1 tar2-1* (n=9) (y). Quantification of DII-Venus positive cells in Col-0 (n=8), *taa1-1* (n=8), *tar2-1* (n=8) and *taa1-1 tar2-1* (n=7) (z). Statistical test: one way ANOVA followed by Tukey's multiple comparisons test, different letters represent the statistically significant differences ($p<0.05$).

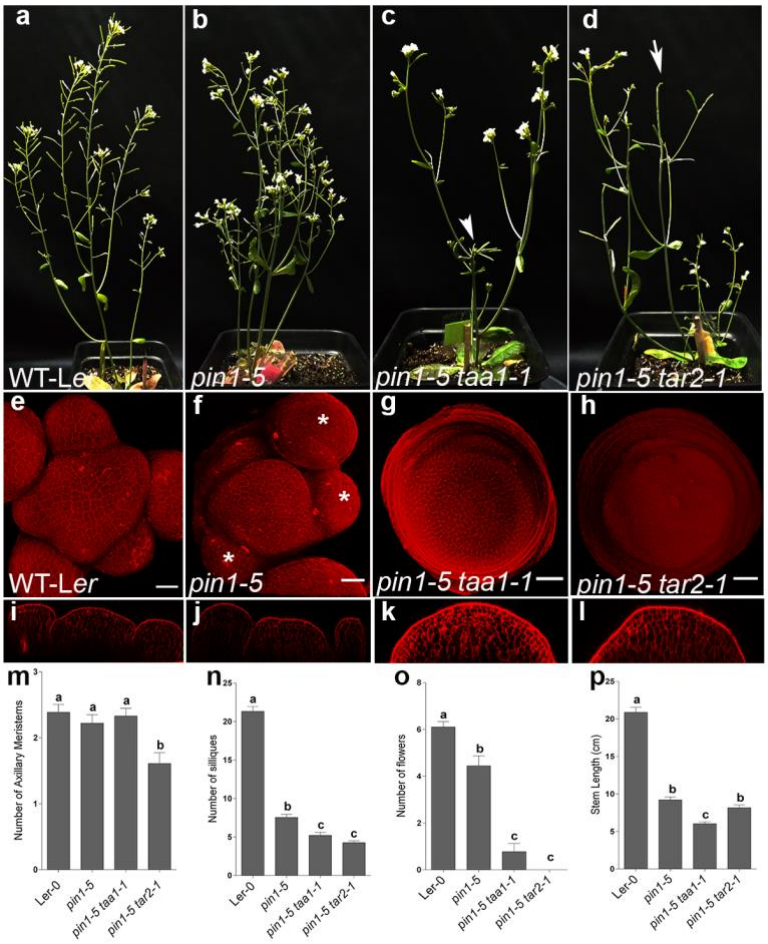

Figure 3. Auxin biosynthesis in emerging organs primordia is critical for organ initiation
From left to right 35 days old WT L*er*, *pin1-5*, *pin1-5 taa1-1*, and *pin1-5 tar2-1* (a-d). 3D top view of WT L*er*, *pin1-5*, *pin1-5 taa1* and *pin1-5 tar2-1* SAM (e-h), and side views (i-l). White arrowhead pointing to pin like inflorescence in *pin1-5 taa1-1*, and arrow in *pin1-5 tar2-1* plants. *pin1-5* makes lateral organs similar to WT (white asterisk). Graphs showing the quantification of axillary meristem, siliques numbers, flower numbers and stem length, respectively, WT L*er* (n=18), *pin1-5* (n=18), *pin1-5 taa1-1* (n=18) and *pin1-5 tar2-1* (n=18) (m-p). Statistical test: one way ANOVA followed by Tukey's multiple comparisons test, different letters represent the statistically significant differences ($p<0.05$). Error bars show SEM. Scale bars: 20µM.

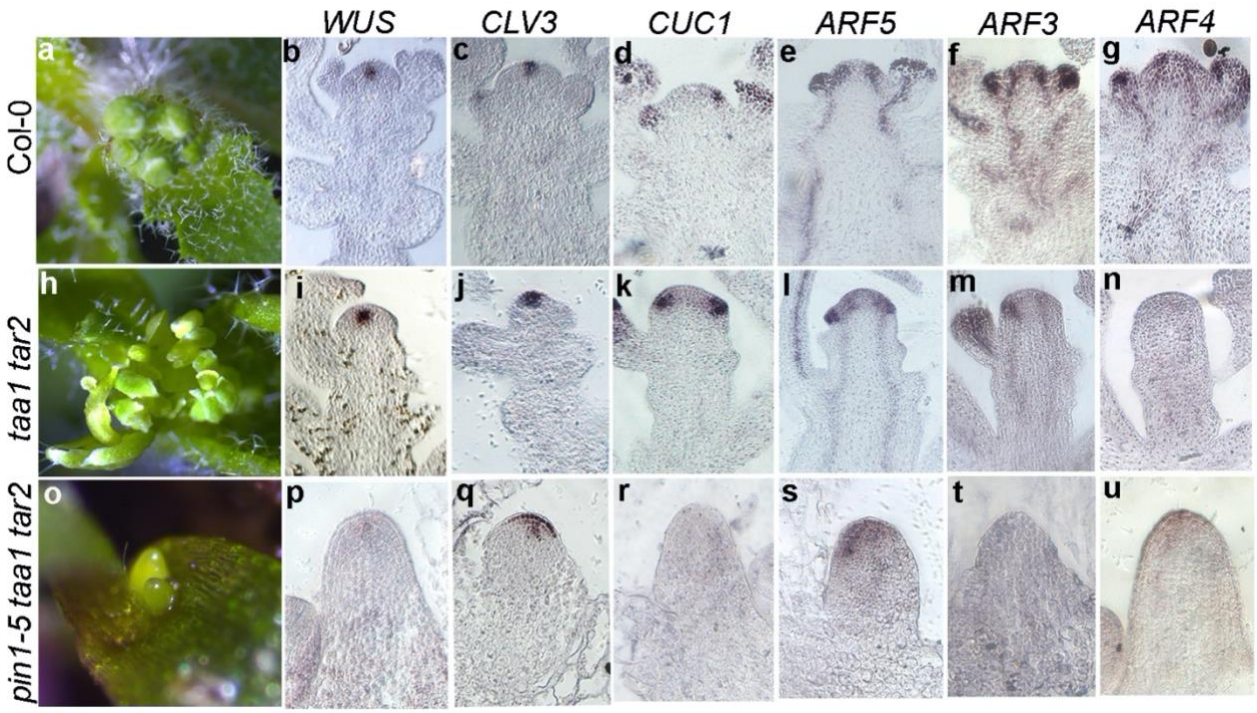

Figure 4. Repeal of auxin transport and biosynthesis inhibits stem cell differentiation

Representative images of 4-week old shoot apex taken under dissecting scope for Col-0 (a), *taa1-1 tar2-1* double mutant (h), and two weeks old for *pin1-5 taa1-1 tar2-1* triple mutant plants (o). SAM marker genes for CZ (*CLV3*), PZ (*ARF3, 4, 5*), RM (*WUS*) and organ boundaries (*CUC1*) were analysed in Col-0 (b-g), *taa1-1 tar2-1* (i-n) and *pin1-5 taa1-1 tar2-1* (p-u) by in situ hybridisation. Marker genes for both PZ and RM were either got miss expressed or reduced in their expression levels, while the CZ marker *CLV3* expanded dramatically in the PZ cells.

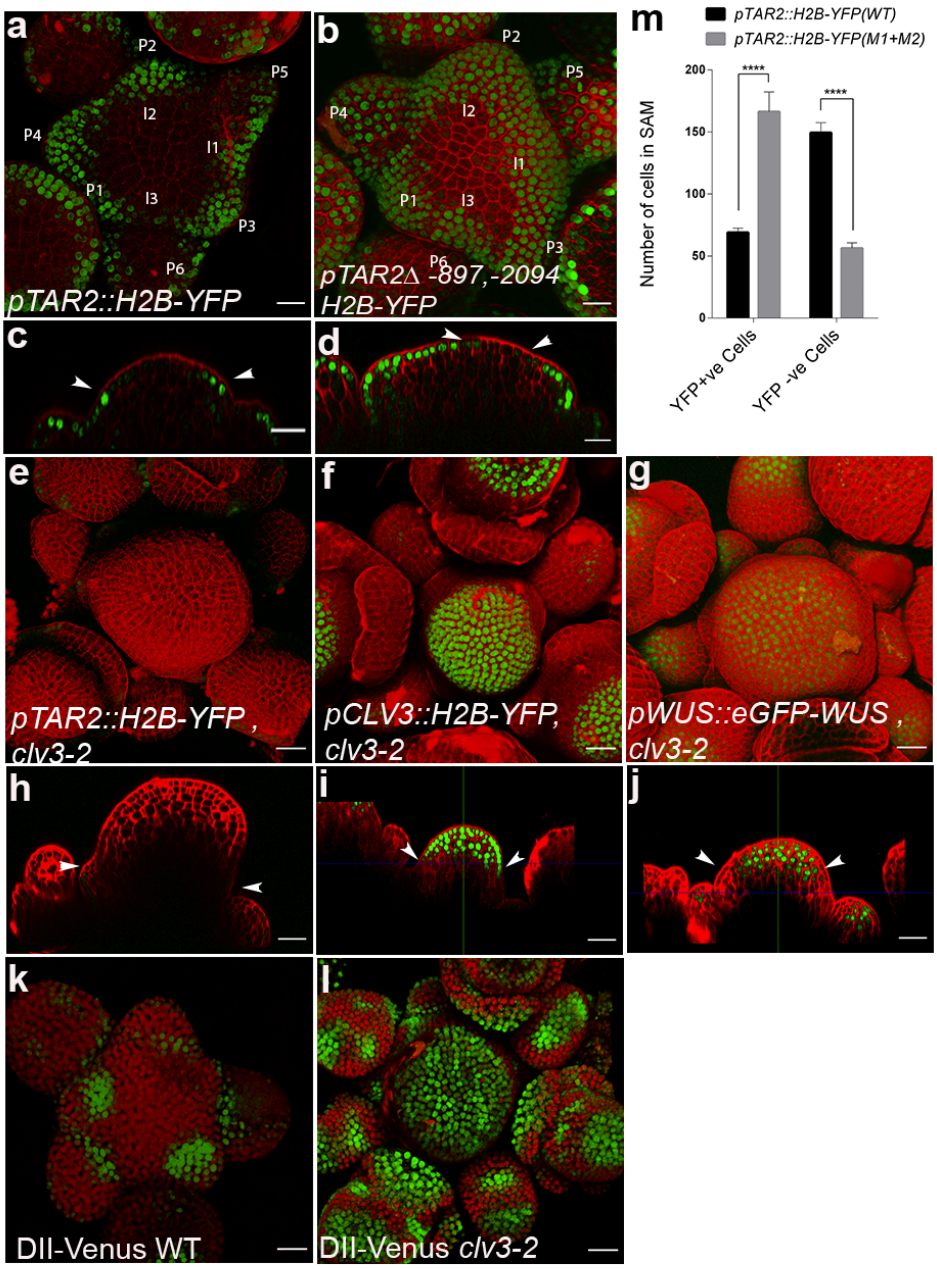

Figure 5: WUS dynamically regulates auxin threshold in SAM

3D reconstructed top view of SAM of four-week old WT-L*er* expressing *pTAR2::H2B-YFP* (green) (a) and *pTAR2Δ(-897,-2094)::H2B-YFP* (green) (b). *pTAR2ΔΔ(-897,-2094)* reporter show broad expression in comparison to control. Side view of (a) and (b) is shown in (c) and (d). Note the expression of *pTAR2Δ(-897,-2094)* expanded dramatically towards CZ in the epidermal cell layer of SAM (indicated by white arrowheads in c, d). The number of YFP -ve cells reduced in WUS binding site deleted construct (m). Expression of *pTAR2::H2B-YFP* in the *clv3-2* auxiliary meristem (AM) (e, h), arrowheads indicate the restriction of *pTAR2* activity in the PZ. *pCLV3::H2B-YFP* (green) in the *clv3-2* (f, i), arrowheads indicate the

expression of *CLV3*. *pWUS::eGFP-WUS* (green) in *clv3-2* (g, j), arrowheads indicate expression and localization of WUS. 3D reconstructed top view of SAM, showing DII-Venus in WT (k) and *clv3-2* (l). Quantification of YFP +ve and YFP -ve cells carried out in *pTAR2::H2B-YFP* (n= 10) and *pTAR2(-897,-2094):H2B-YFP* (n=10). Statistical test: Student t-test. Error bars represent SEM. Asterisk marks represent a statistically significant difference ($p < 0.0001$). Scale bars: 20μM.