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ADP1 affects abundance and endocytosis of PIN-FORMED proteins in *Arabidopsis*

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Auxin, as a vital plant hormone, regulates almost every aspect of plant growth and development. We previously identified a dominant mutant, *adp1*-D, displaying loss of apical dominance. We also demonstrated that down-regulation of local auxin biosynthesis in *adp1*-D was responsible for the bushy phenotype of this mutant. Consistent with the reduction of local auxin biosynthesis, we recently discovered that protein abundance of PIN1, PIN3, and PIN7 was reduced in *adp1*-D without accompanying transcription level changes. Additionally, subcellular analysis revealed that over-expression of ADP1 inhibited endocytosis of PIN proteins. Taken together, we conclude that ADP1 regulates plant architecture through the fine-tuning of local auxin biosynthesis and through post-transcriptional regulation of auxin transporters.

Flowering plants display a variety of distinguished architectures, which, to a large extent, are determined by their branching patterns. The formation of branches generally takes place in 2 steps: 1) initiation of a new shoot axillary meristem (AM); and 2) subsequent outgrowth and further development of the newly formed AM.

Auxin,[1,2] cytokinins (CKs)[1,2] and strigolactones (SLs),[3,4,5] have been implicated to date in the regulation of the 2 steps of branch development. Auxin is the most extensively studied of these 3 hormones.[6] Although its role remains elusive, auxin, especially polar auxin transport, is suggested to be indispensable to AM formation.[7] According to recent reports, vegetative AM formation in *Arabidopsis* requires a minimum auxin presence in the leaf axils.[8,9]

The role of auxin as an inhibitor of bud outgrowth has been well established for over 80 y Rather than acting as a direct inhibitory signal, auxin is believed to exert its power by regulating CKs and SLs, the other 2 hormones. On the one hand, auxin prevents bud outgrowing by transcriptionally down-regulating CK synthesis.[10] On the other hand, the presence of auxin can also relieve the repression of SL biosyntheticgenes.[11,12,13] Furthermore, analysis of auxin biosynthetic,[14,15] signaling[16] and polar transport[10] mutants clearly reveals a tight correlation between auxin and branching pattern, since most of those mutants display abnormal branching patterns. Recently, it has been reported that sugar demand of the shoot tip is responsible for apical dominance in pea (*Pisum sativum*), thereby unraveling perhaps an another type of regulator in terms of bud release.[17]

To discover new genetic players underlying the branching pattern mechanism, we identified a dominant mutant with an increased number of lateral organs from an activation tagging mutant collection.[18] The mutant was later designated as *adp1*-D (*altered development program1*- Dominant). Further analysis revealed that not only was the DR5 signal decreased in this mutant, but also that the expression levels of all 11 YUCCA members were significantly down-regulated in its axillary buds.[19] Consistent with this observation, the bushy phenotype of *adp1*-D could be partially rescued by increasing local auxin biosynthesis. These results clearly demonstrate that ADP1 plays an important role in auxin biosynthesis,[19] probably through the YUC/TAA pathway.[20,21] Because we identified a new determinant of local auxin biosynthesis, this finding is novel. Furthermore, the discovery of low auxin levels in *adp1*-D axillary buds is consistent with the revelation that auxin depletion is prerequisite for AM formation.[8,9]

Because the *adp1*-D branching phenotype was only partially rescued by an increase in local auxin biosynthesis, we hypothesized that other factors may also be responsible for this severe phenotype. The fact that the *pin1* mutant is devoid of lateral organs[7] promoted us to investigate whether auxin efflux transporters are affected in *adp1*-D mutants.

We first analyzed expression levels of several PINs, namely, PIN1, PIN2, PIN3, and PIN7, in young seedlings of *adp1*-D. No significant difference was found between *adp1*-D and the wild type (**Fig. 1A**). Additionally, pro*PIN1*:GUS was crossed with *adp1*-D, with GUS activity then checked in the

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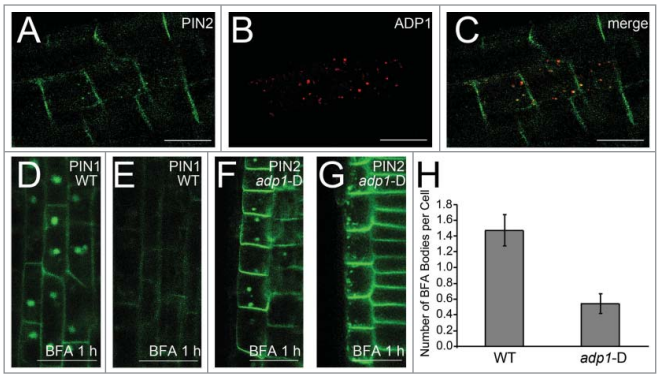

Figure 1. PIN proteins were not transcriptionally regulated in *adp1*-D. (**A**) RT-PCR results of *PIN1*, *PIN2*, *PIN3*, *PIN7*from 7-day-old light grown seedlings of wild type and *adp1*-D. (**B**, **D and F**) GUS staining results of pro*PIN1*:GUSin wild type. DAG: day after germination. Bars = 2 mm. (**C**, **E and G**) GUS staining results of pro*PIN1*:GUSin*adp1*-D. DAG: day after germination. Bars = 2 mm.

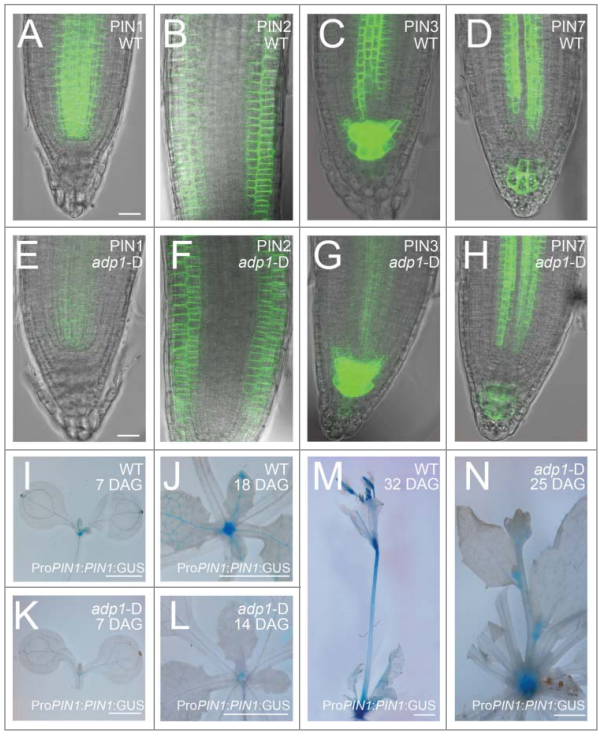

Figure 2. Protein abundances of PIN1, PIN3, PIN7 were down-regulated in *adp1*-D. Pro*PIN1*:*PIN1*:*GFP* signal from 7-day-old seedlings of wild type (**A**) and *adp1*-D (**E**). Pro*PIN2*:*PIN2*:*GFP* signal from 7-day-old seedlings of wild type (**B**) and *adp1*-D (**F**). Pro*PIN3*:*PIN3*:*GFP* signal from 7-day-old seedlings of wild type (**C**) and *adp1*-D (**G**). Pro*PIN7*:*PIN7*:*GFP* signal from 7-day-old seedlings of wild type (**D**) and *adp1*-D (H). From (**A**) to (**H**), bars = 20 μm. (**I**, **J and M**) GUS staining results of Pro*PIN1*:*PIN1*:GUS in wild type.DAG: day after germination. Bars =2mm. (**K**, **L and N**) GUS staining results of Pro*PIN1*:*PIN1*:GUS in *adp1*-D.DAG: day after germination. Bars = 2 mm.

homozygous F_3 generation. Neither the GUS distribution pattern nor signal intensity differed in *adp1*-D seedlings or adults, compared with the wild type (**Fig. 1B–1G**), suggesting that the PIN proteins were not transcriptionally regulated in *adp1*-D.

Next, we detected PIN protein levels in *adp1*-D by crossing *adp1*-D with GFP-tagged PIN protein fusion lines. Intriguingly, fluorescent intensities of several PIN proteins were significantly reduced in *adp1*-D root tips, whereas the polar distribution of PINs was unchanged (**Fig. 2A–2H**). In *adp1*-D root steles, the protein levels of PIN1, PIN3, and PIN7 were decreased to various extents, with PIN1 showing the most dramatic decrease, while the PIN2 signal remained the same. To validate the above observation, we introduced Pro*PIN1:PIN1*:GUS into *adp1*-D and conducted GUS staining in the homozygous F_3 generation. In the wild type, GUS signals were detected in various tissues, such as shoot apical meristem (SAM), leaf tips, emerging axillary buds, and flower primordia; in *adp1*-D, in contrast, only weak signals were observed, especially in SAM regions and axillary buds, where ADP1 is highly expressed. Taken together, these findings strongly indicate that post-transcriptional PIN protein levels are downregulated in *adp1*-D, which may be a cause of the bushy phenotype of the mutant.

Because PIN proteins undergo constitutive endocytosis and recycling to maintain a polar distribution pattern in the plasma membrane, we also examined whether ADP1 could affect endomembrane trafficking of PINs. An inducible RFP-tagged ADP1 overexpression line was crossed with a PIN2-GFP reporter line, and a homozygous F_3 generation was obtained for further subcellular observation. Notably, ADP1 protein localized in endosomes after 30 μM estradiol induction overnight, a result observed previously (**Fig. 3B**).[19] Interestingly, ADP1 over-expression also

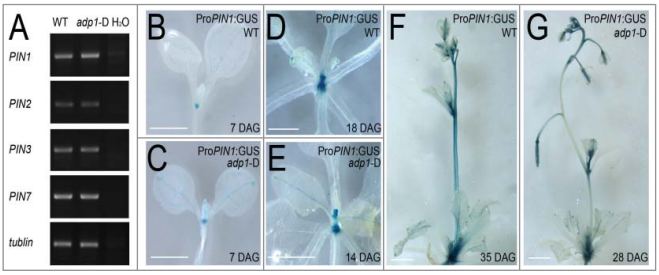

Figure 3. Subcellular trafficking of PIN1 and PIN2 proteins were affected in *adp1*-D. (**A–C**) Overexpression of *ADP1* induces internalization of PIN2-GFP. Note that almost all the vesicles of ADP1 (red) could colocalize with internalized PIN2 signals (green). Arrowheads indicate colocalized signals. (**D**) Pro*PIN1*:*PIN1*:*GFP* signal in the root of wild type, after treatment of 50 μM BFA for 1 h. Arrowheads indicate BFA compartments. (**E**) Pro*PIN1*:*PIN1*:*GFP* signal in the root of *adp1*-D, after treatment of 50 μM BFA for 1 h. The BFA compartments could be hardly detected in *adp1*-D, as also in (**H**). (**F**) Pro*PIN2*:*PIN2*:*GFP* signal in the root of wild type after treatment of 50 μM BFA for 1 h. Arrowheads indicate BFA compartments. (**G**) Pro*PIN2*:*PIN2*:*GFP* signal in the root of *adp1*-D after treatment of 50 μM BFA for 1 h. Arrowheads indicate many smaller particles, instead of large BFA compartments. From (**A**) to (**G**), bars = 20 μm. (**H**) Statistic analysis of numbers of BFA compartments in wild type and *adp1*-D, after treatment with BFA for 1 h. 500 root cells of each genotype were used for analysis, bars represent the SD.

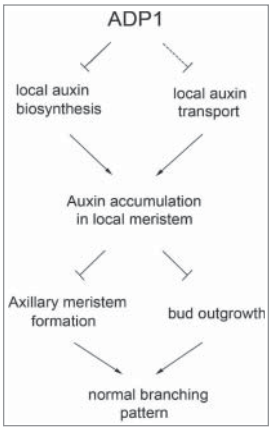

Figure 4. Working model of ADP1. ADP1, which is mainly expressed in the local meristem regions, fine-tunes auxin output by down-regulation of local auxin biosynthesis, probably through local auxin transport.

induced aggregation of PIN2 within cells (**Fig. 3A**). Simultaneous imaging revealed that almost all of the subcellular vesicles of ADP1 co-localized with the PIN2 signal in the root epidermis (**Fig. 3A–3C**), indicating that ADP1 affects endomembrane trafficking of PIN2. Regulation of subcellular trafficking of PIN proteins has been considered to be an important aspect of the auxin efflux through which PIN abundance and activity at the cell surface are modulated.[22,23,24] Consequently, we used the fugal toxic brefeldin A (BFA) to investigate whether ADP1 regulates PIN endocytosis. BFA treatment blocks recycling by inhibiting vesicle formation at the Golgi apparatus and induces the formation of an ER-Golgi hybrid compartment with stacked domains (BFA body).[25] After treatment with BFA for 1 h, PIN1-GFP was aggregated into the large BFA bodies, as shown in **Figure 3D**. However, the number of BFA bodies was greatly reduced in *adp1*-D compared with the wild type, implying that ADP1 affects the endocytosis of PIN1 proteins (**Fig 3D, 3E, 3H**). In regard to PIN2-GFP, however, we found many small particles scattered throughout the epidermis of *adp1*-D, in contrast to the large BFA bodies formed in the wild type. Prolonged treatment with BFA (up to 3 h) did not cause the small particles to aggregate into large BFA bodies (data not shown), further implying that endocytosis of PIN proteins is impaired in *adp1*-D. Taken together, these results imply that ADP1 regulates endocytosis of PIN proteins.

Given the above evidence, we propose a working model (**Fig.** 4), in which ADP1 fine-tunes final auxin output by the regulation of the TAA/YUC pathway and by auxin transport via post-transcriptional regulation of PIN proteins.

As PIN proteins can be sorted into the lytic vacuolar compartment after internalization,[26,27,28] one plausible explanation for reduced PIN abundance is that the abnormal endomembrane trafficking of PIN1 in *adp1*-D results in more PIN1 protein targeted for lytic degradation. Alternatively, because ADP1 is a putative transporter, the transport of specific substrates into endomembrane compartments might change the local cellular environment (*e.g.*, the pH level), thus affecting PIN1 stability. Furthermore, the observations that auxin up-regulates PIN transcription[29] and inhibits internalization of PIN proteins from the plasma membrane[22,30] suggest the positive feedback between auxin and PIN proteins. Therefore, the reduced abundance of PIN proteins in *adp1*-D might be caused by downregulation of local auxin biosynthesis in this mutant, since auxin itself seems to be one of the most prominent regulators of its transport. It would be interesting to explore in future studies whether decreased levels of PIN proteins in *adp1*-D can alter local auxin transport, especially in the axillary buds where ADP1 is highly expressed. Such investigations would represent a new area of research focusing on auxin-regulated developmental patterning via fine-tuning of local auxin biosynthesis and transport.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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