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A regulated auxin minimum is required for seed dispersal in *Arabidopsis*

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Local hormone maxima are essential for the development of multicellular structures and organs. For example, steroid hormones accumulate in specific cell types of the animal fetus to induce sexual differentiation¹ and concentration peaks of the plant hormone auxin direct organ initiation and mediate tissue patterning²⁻⁴. Here we provide an example of a regulated local hormone minimum required during organogenesis. Our results demonstrate that formation of a local auxin minimum is necessary for specification of the valve margin separation layer where *Arabidopsis* fruit opening takes place. Consequently, ectopic production of auxin, specifically in valve margin cells, leads to a complete loss of proper cell fate determination. The valve margin identity factor INDEHISCENT (IND) is responsible for forming the auxin minimum by coordinating auxin efflux in separation-layer cells. We propose that the simplicity of formation and maintenance make local hormone minima particularly well suited to specify a small number of cells such as the stripes at the valve margins.

Control of seed dispersal was crucial for crop domestication during the Neolithic revolution⁵ and remains a major problem for young crops of the Brassicaceae family such as oilseed rape⁶. Understanding the mechanism of fruit development in the closely related model species *Arabidopsis thaliana* will probably provide the insight necessary to solve this issue.

The major exterior parts of an *Arabidopsis* fruit include the valves or carpel walls, the replum, a thin structure extending along the length of the fruit, and the valve margins, which form at the valve/replum border (Supplementary Fig. 1a). The valve margins differentiate into narrow stripes of cells consisting of a separation layer and a layer of lignified cells⁷. Immediately before fruit opening, cells in the separation layer secrete cell-wall-degrading enzymes to mediate cell separation in a process known as dehiscence⁸.

Auxin is involved in diverse aspects of plant growth and development, including the patterning of Brassicaceae fruit⁹⁻¹². The *DR5rev::GFP* reporter³ has been used to visualize auxin responses in various *Arabidopsis* tissues. We used this tool to characterize tissue-specific auxin-response levels throughout *Arabidopsis* fruit development (Fig. 1a and Supplementary Fig. 1b). A dynamic tissue-specific green fluorescent protein (GFP) expression pattern was observed (see Supplementary Information and Supplementary Fig. 1b) suggesting that distribution of auxin is under careful regulation, possibly to ensure proper postfertilization growth, and agrees with direct measurements of the naturally occurring auxin, indole-3-acetic acid (IAA; Supplementary Fig. 1c). The separation layer of the valve margins had very low GFP signals especially at later stages (Fig. 1a, developmental stages defined in refs 7 and 13), indicating that these

cells have reduced auxin response and/or levels. In the following text, we will refer to this as an auxin minimum.

Valve margin formation requires the activity of IND, and *ind* mutant fruit fail to open on maturation¹⁴. This defect is known as indehiscence. The *IND* gene encodes a putative basic helix-loop-helix transcription factor and is expressed in stripes where the valve margins will subsequently differentiate (Supplementary Fig. 2). *IND* expression is inhibited in the valves by FRUITFULL (*FUL*)¹⁵ and in the replum by REPLUMLESS (*RPL*)¹⁶; however, little is known about the downstream events that lead to valve margin formation.

In contrast to wild type, no auxin minimum in the separation layer of *ind-2* mutant fruit was observed using the *DR5rev::GFP* reporter (Fig. 1a, b). Conversely, in *rpl* mutants with ectopic *IND* expression in the reduced replum¹⁶, *GFP* signal was greatly decreased, showing that the auxin minimum had expanded to include the replum (Fig. 1c). These results indicate that IND is involved in creating the auxin minimum observed at the separation layer of wild-type fruit (Fig. 1a).

To test whether this spatially restricted auxin minimum is required for valve margin specification, the bacterial auxin biosynthesis gene *iaaM*¹⁷ was used to increase auxin concentration specifically in valve margin cells. For this purpose, we modified the valve-margin-specific *IND::IND:GUS* construct (Supplementary Fig. 2) and created transgenic *IND::IND:iaaM* lines. In these lines, the auxin minimum no longer formed (Fig. 1d). We furthermore found that *IND::IND:iaaM* transgenic fruit (from 22 independent lines out of 23) were indehiscent, but without reduced *IND* expression and with increased auxin levels (Supplementary Fig. 3). Scanning electron microscopy (SEM), lignin staining and marker line analysis revealed that these fruit lacked valve margin differentiation (Fig. 1e-l), similar to what has previously been described for *ind* mutants¹⁴. The results obtained using the *iaaM* gene demonstrate that formation of the auxin minimum is necessary for separation layer development and indicate that the indehiscent phenotype of the *ind* mutant is at least partially due to a failure to direct a local auxin minimum at the valve margins.

To gain further insight into the role of IND in valve margin differentiation, the *IND* gene was expressed under control of the constitutive CaMV35S promoter (*35S::IND*)¹⁴ or ectopically induced with dexamethasone (DEX)¹⁸ using a translational fusion of IND to the glucocorticoid receptor (*35S::IND:GR*). The developmental defects of ectopic IND activity are similar to those obtained when the *IND*-related *HECATE* genes are over expressed¹⁹, and are reminiscent of mutants with defects in polar auxin transport such as *pin-formed* (*pin1*) and *pinoid* (*pid*) (Fig. 2a-d)^{20,21}. The *PIN1* gene encodes a member of the PIN family of auxin efflux carriers²². Polar, subcellular localization of PIN proteins determines the direction

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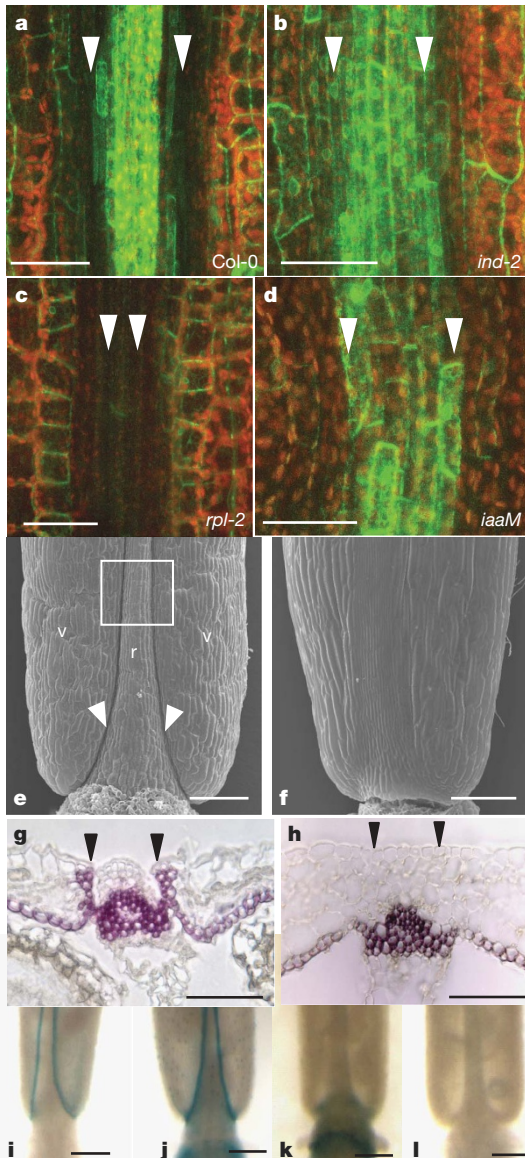


Figure 1 | An auxin minimum forms at the valve margin of *Arabidopsis* fruit. **a–d**, Confocal images of *DR5::GFP* taken at the valve/replum region (white frame in **e**) at stage 17b of Col-0 (**a**), *ind-2* (**b**), *rpl-2* (**c**) and *IND::IND:iaaM* (**d**). GFP signals are in green and plastids are in red. **e, f**, SEM images of the basal part of stage-17b fruit from wild-type (**e**) and *IND::IND:iaaM* (**f**) plants. **g, h**, Phloroglucinol-stained cross sections of stage-17b wild-type (**g**) and *IND::IND:iaaM* (**h**) fruit. Arrowheads indicate valve margin positions. **i–l**, Expression of valve margin markers in stage-15 wild-type (**i, j**) and *IND::IND:iaaM* (**k–l**) fruit. Scale bars: 250 μm (**i–l**), 100 μm (**e, f**), 50 μm (**a–d, g, h**).

of auxin flow²³ and they have been shown to mediate auxin maxima in multiple developmental processes²⁴. The *PID* gene is necessary for proper auxin distribution²⁵ by ensuring polar localization of PIN proteins at the plasma membrane²⁶. The observed phenotypes therefore indicate that IND regulates auxin transport, which is highly consistent with the requirement for IND in generating the auxin minimum at the valve margin (Fig. 1b).

To identify components of the auxin transport machinery that IND interacts with, *PIN* reporter lines were analysed for fruit expression and localization. *PIN3* is apolarly localized in root columella cells and diverts the auxin flux downwards in response to gravity²⁷. Here we find that *PIN3* is also expressed during *Arabidopsis* fruit development. A transcriptional *PIN3::GUS* construct was expressed ubiquitously in mature fruit (Supplementary Fig. 4), whereas a translational *PIN3::PIN3::GFP* fusion only produced signal in the valves and replum

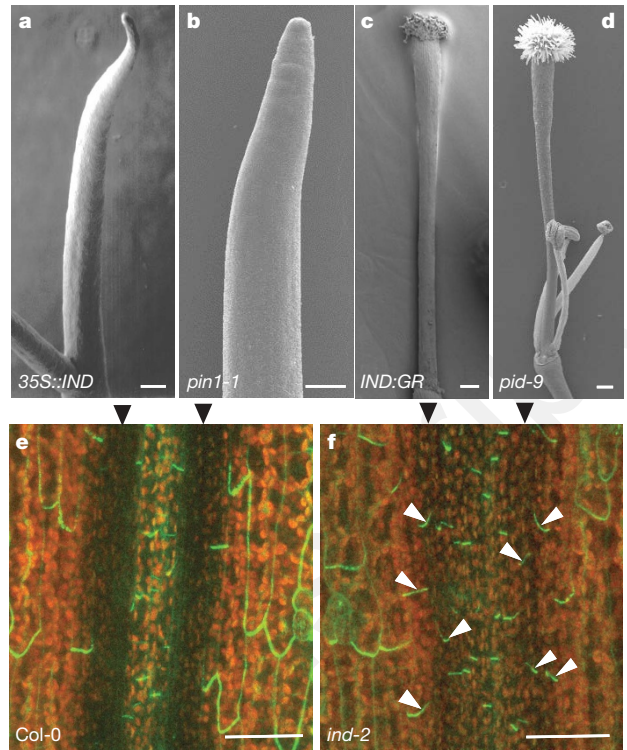


Figure 2 | IND regulates auxin transport. **a–d**, SEM images of *35S::IND* pin-like inflorescence (**a**), a *pin1-1* mutant (**b**), stage-15 fruit from DEX-treated *35S::IND:GR* (**c**) and a *pid-9* fruit at a similar stage (**d**). **e, f**, IND prevents polar *PIN3::PIN3::GFP* localization at the valve margin. *PIN3::PIN3::GFP* in Col-0 (**e**) and *ind-2* (**f**) stage-17 fruit. *PIN3::PIN3::GFP* is basally localized in replum and valve cells, but not observed in the valve margin cells of Col-0 fruit. In *ind-2* fruit, *PIN3::PIN3::GFP* is basally localized in valve margin cells (white arrowheads). For confocal images, $n > 10$. Scale bars: 200 μm (**c, d**), 100 μm (**a, b**), 50 μm (**e, f**).

where it is predominantly basally localized (Fig. 2e). At the separation layer, *PIN3::GFP* is absent in wild-type fruit; however, in *ind-2* mutant fruit, *PIN3::GFP* becomes basally localized in all cell files spanning across from the valves to the replum (Fig. 2f). These data imply that the *PIN3* gene is post-transcriptionally regulated at the separation layer, and that IND is required to prevent polar *PIN3* localization in this region.

The *PIN3::PIN3::GFP* line was crossed to *35S::IND:GR* plants to study the effect of DEX-induced IND activity on *PIN3* localization. In the absence of DEX, we observed a strict polar localization of *PIN3::GFP* in the root endodermis (Fig. 3a, b), whereas 48 h of DEX treatment caused delocalization of *PIN3::GFP* to all sides of the cells (Fig. 3c, d). To check whether the effect of IND on localization is specific to *PIN3*, *35S::IND:GR* was crossed to a *PIN1::PIN1::GFP* line⁴. *PIN1::GFP* is apically localized in valve epidermal cells of stage-10 gynoecia (Fig. 3e); however, 16 h of DEX treatment resulted in a marked *PIN1::GFP* apolarization (Fig. 3f) and occasional anticlinal cell divisions (see Supplementary Information and Supplementary Fig. 5). These results show that IND controls PIN localization and thereby the direction of auxin transport.

After 24 h of DEX treatment, the *PIN1::GFP* signal had almost disappeared, indicating that *PIN1::GFP* proteins in the apolar state are unstable and may be relatively short lived (Fig. 3g). This could explain the absence of *PIN3::GFP* signal at the separation layer of wild-type fruit despite *PIN3* expression (Supplementary Fig. 4).

As a putative basic helix–loop–helix transcription factor, IND is unlikely to directly regulate PIN polarization. Localization of PIN proteins is partially governed by their phosphorylation status, which depends on the antagonistic action of the PP2AA1/2/3 phosphatases²⁸, the *PID* kinase²⁶ and probably the related kinases *WAG1*, *WAG2* and *AGC3–4* (*PID2*) (refs 29 and 30). To test whether IND transcriptionally regulates the expression of any of these factors, we performed quantitative messenger RNA abundance assays (quantitative polymerase

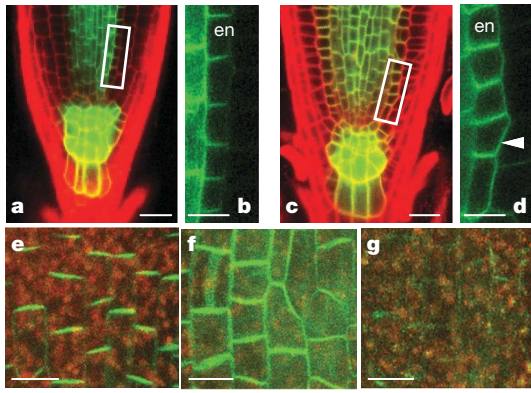


Figure 3 | IND mediates PIN delocalization. Polar PIN3:GFP localization in the root endodermis (en) of two-day-old seedlings (a, b) is shifted to apolar/lateral localization (c, d) in *35S::IND:GR* treated with DEX (indicated with arrowheads). e, Apical PIN1:GFP localization in valve cells of a stage-10 uninduced *35S::IND:GR* gynoecium. f, g, Delocalization of PIN1:GFP in a 16-h DEX-treated *35S::IND:GR* gynoecium (f). The PIN1:GFP signal significantly decreases after 24 h of DEX treatment (g). Control DEX treatments of the *PIN3::PIN3:GFP* and *PIN1::PIN1:GFP* lines did not change PIN polarity (data not shown). For confocal images, $n > 10$. Scale bars: 25 μm (a, c), 10 μm (b, d–g).

chain reaction, qPCR) using tissue from *35S::IND:GR* seedlings treated with DEX (Fig. 4a). Whereas *PP2AA* expression did not significantly change (data not shown), *PID* expression was repressed by DEX treatment and the induction by auxin was blocked in the presence of DEX. Moreover, *WAG2* expression was unaffected by DEX alone, whereas a modest induction by auxin was notably enhanced in the presence of DEX. These data show that IND can inversely regulate the expression of

PID and *WAG2* (Fig. 4a). In contrast, IND did not regulate *PIN1* expression, and *PIN3* was only mildly induced (Fig. 4a). Both *PID* and *WAG2* are key regulators of PIN polar localization (ref. 28 and C.S.G.-A. *et al.*, manuscript in preparation) and thus the IND-dependent regulation of these kinases provides a mechanism for how IND modulates PIN localization during margin formation.

To confirm the relevance of the results obtained in seedlings, endogenous *PID* and *WAG2* expression in stage-15 fruit from wild type (Col-0), *ind-2* and *ful-2* were compared (Fig. 4b). *WAG2* expression was significantly decreased in *ind-2* compared to wild type ($P < 0.05$), whereas no detectable difference in *PID* expression was observed. The lack of change in *PID* expression may be due to minor increases that are masked by using the whole fruit. In contrast, in *ful-2* fruit with an expanded *IND* expression domain¹⁵, *PID* expression is markedly reduced and *WAG2* is induced (Fig. 4b). These data support the suggestion that *IND* is involved in the regulation of PIN-dependent auxin transport by inverse regulation of *PID* and *WAG2* transcription.

To investigate whether IND is a direct regulator of *PID* and *WAG2*, we performed chromatin immunoprecipitation (ChIP) assays using a GR antibody followed by qPCR and found significant enrichments of *PID* and *WAG2* fragments in *35S::IND:GR* plants induced by DEX ($P < 0.05$, Fig. 4c). An induction experiment with DEX and the translational inhibitor cycloheximide showed similar results (Supplementary Fig. 6). We have therefore identified *PID* and *WAG2* as two targets of a potential set of intermediate factors directly regulated by IND to control auxin transport during formation of the separation layer. These results also show that IND interprets cellular auxin levels to modify the direction of auxin flow by acting both as a transcriptional repressor and as an inducer. Additional genetic analysis will be necessary to investigate the specific contributions of *PID*, *WAG2* and potentially other factors to separation layer development.

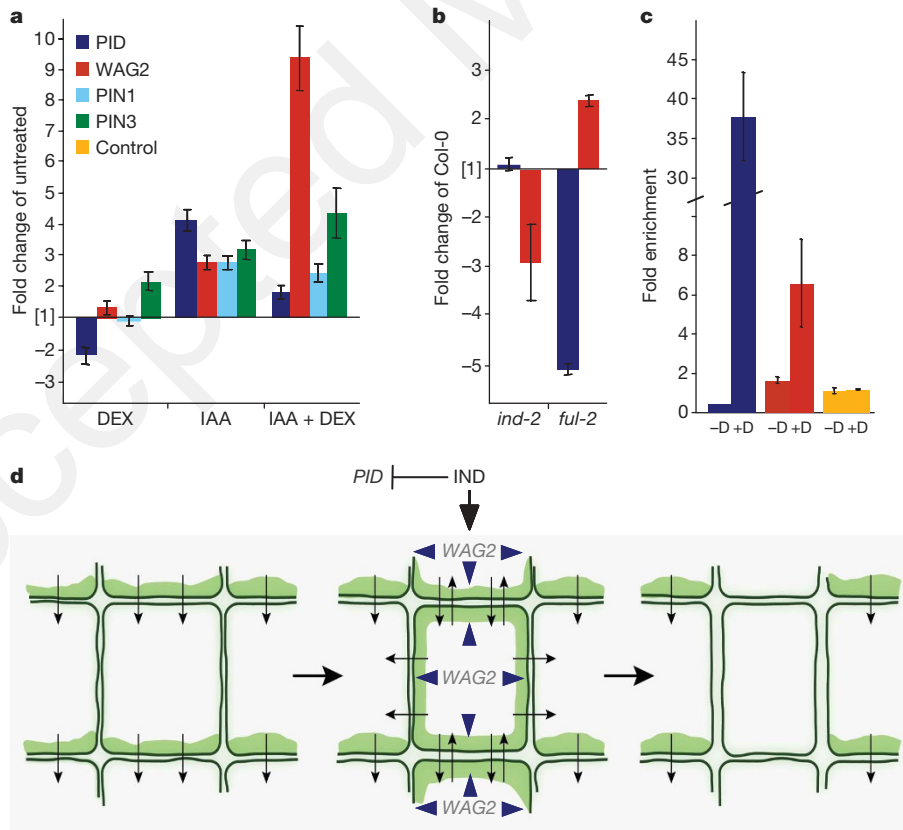


Figure 4 | IND directly regulates *PID* and *WAG2* expression. a, b, Quantitative RT-PCR in 7-day-old *35S::IND:GR* seedlings \pm DEX and \pm IAA ($n = 3$) in wild-type (a) versus *ind-2* and *ful-2* ($n = 4$) (b) stage-15 fruit. c, ChIP experiment against the *PID* and *WAG2* genes. -D and +D indicate absence or presence of DEX, respectively. Standard errors ($n = 3$) of

biological repeats are indicated. The *NRT2.1* gene (At1g08090) serves as a negative control not affected by DEX treatment. Colour coding for genes in a–c is as shown in a. d, Model for IND-mediated auxin minimum formation at the separation layer. PIN localization is shown in green and auxin flow is indicated by arrows from cell to cell.

IND expression is initiated in the presumptive valve margin region of stage-11 flowers just before fertilization (Supplementary Fig. 2). IND is therefore likely to have important functions in setting up fruit patterning from an early stage. Here we have identified a specific role for IND in separation layer formation, which occurs late during fruit development to facilitate seed dispersal. We have summarized our results in a model suggesting that IND is responsible for forming an auxin minimum by regulating polar localization of PIN auxin transporters (Fig. 4d). Our results indicate that IND coordinates auxin transport to specify the separation layer at least partially by repressing *PID* and inducing *WAG2* expression at the valve margins, subsequently leading to PIN relocation from apico-basal to apolar-lateral and resulting in a local auxin minimum.

Great progress has been achieved in recent years on the importance of auxin maxima and gradients in various aspects of plant development²⁻⁴. Our data show that highly regulated local depletion of auxin can have an important role in determining cell fate as well. We propose that the creation of such an auxin minimum is particularly well suited to ensure proper specification of a small number of cells such as the stripes at the valve margin, because coordination of auxin transport to create and maintain an auxin minimum may be a simpler process than for an auxin maximum. To our knowledge, a regulated minimum of a signalling molecule has not previously been reported in plants or animals, and thus provides a new concept in general hormone biology.

METHODS SUMMARY

Confocal microscopy. Confocal microscopy was performed using a Leica SP laser scanning microscope equipped with an Argon krypton laser (Leica Microsystems). The 488-nm excitation line of an argon ion laser was used to excite GFP. GFP emission spectra were collected between 505 and 584 nm and plastid autofluorescence was collected between 601 and 790 nm. Epidermal surface images were captured using laser reflection between 480 and 499 nm. Floral buds were dissected and mounted in water and ~0.05% Tween-20 ($n > 10$). 1–2- μm Z sections were imaged and converted to maximum projections. Images were averaged 2–3 times and were processed using the Leica CONFOCAL software, ImageJ and Adobe Photoshop software (version 7.01; Adobe Systems).

Scanning electron microscopy. Fruit were fixed for approximately 4 h at 25 °C in FAA (50% ethanol, 5% glacial acetic acid and 3.7% formaldehyde). After critical point drying, tissue was coated with gold/palladium and examined in a Quanta 600 microscope using an acceleration voltage of 20 kV.

Quantitative PCR with reverse transcription. Total RNA was extracted using RNeasy Plant mini kit (Qiagen, 74904). Complementary DNA was produced using 2–5 μg total RNA and a polyT(15) primer. Three biological and two technical repeats were performed for the experiments in Fig. 4a and Supplementary Fig. 6a, c. Because the experiments in Fig. 4b, Supplementary Figs 3a and 6b were carried out on cDNA derived from RNA of pooled tissue, three (Fig. 4b and Supplementary Fig. 6b) and two (Supplementary Fig. 3a) technical repeats were performed.

Quantitative PCR was performed with SYBR green jumpstart Taq readymix (Sigma, S4438) on a Chromo4 real-time PCR detector. PCR products were checked by agarose gel electrophoresis. *UBQ10* was used as a normalization control because its expression is not affected by auxin treatment. Student's *t*-test was performed and significant results had *P* values < 0.05.

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Author Contributions This project was conceived by K.S., M.F.Y. and L.Ø. Experiments were designed by K.S. and L.Ø. K.S. performed the confocal microscopy and expression analyses, T.G. carried out the chromatin immunoprecipitations, L.Ø. made the *IND::IND:iaaM* lines and performed plant sections and tissue staining, K.L. performed the IAA measurements, P.R. made the *35S::IND:GR* transgenic line, S.J.L. characterized the *35S::IND* lines and took the *35S::IND* SEM image shown, and C.S.G.-A., J.F. and R.O. analysed the effect of *WAG2* activity on PIN localization. K.S. and L.Ø. analysed the data and wrote the paper.

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METHODS

Constructs and generation of transgenic plants. Generation of transgenic plants harbouring the *35S::IND* construct was previously reported⁵. Out of 101 T1 lines, 8 were observed to have the *pin*-like phenotype.

To construct the *35S::IND:GR* plasmid, we first cloned the *IND* open reading frame into the pCR2.1 vector (Invitrogen) for sequencing and further into the BamHI site of pGREEN0229-*35S:GR*³¹.

Primers INDa and INDb (primer list in Supplementary Information) were used to PCR amplify a 3.2-kb fragment of the *IND* gene containing 2.7 kb of promoter sequence and the 0.5-kb open reading frame. This fragment was cloned into the pCR2.1 vector for sequencing and further into the StuI and AgeI sites of the GFP-containing plant transformation vector pEGAD³², giving rise to *IND::IND:GFP*. A fragment containing the β -glucuronidase (*GUS*) open reading frame was amplified. This fragment was cloned into the pCR2.1 for sequencing and further into the AgeI and EcoRI sites of the *IND::IND:GFP* plasmid exchanging the GFP for *GUS*. Expression of *IND::IND:GUS* at the valve margin was assessed (Supplementary Fig. 2). Out of 100 transgenic lines, 32 were found to cosuppress *IND* expression resulting in indehiscent fruit. This is similar to what was previously reported for an independent *IND::GUS* reporter construct¹⁴.

A fragment containing the entire 2.3-kb open reading frame of the *iaaM* gene was amplified using the vector pMON518 (ref. 33) as a template. This fragment was cloned into the pCR2.1 vector for sequencing and further as an AgeI/EcoRI (blunted) fragment into the AgeI and BamHI (blunted) sites of *IND::IND:GFP*, replacing the GFP open reading frame and giving rise to *IND::IND:iaaM*. Out of 132 T1 lines, 109 showed severe auxin overproduction phenotypes (rosette leaf epinasty, increased apical dominance and senescing shoot apical meristems). These lines never produced fruit. For the remaining 23 lines, 22 were indehiscent. We also constructed a plasmid containing the *IND* promoter directly fused to the *iaaM* gene. Out of 52 T1 lines, all were severely affected by auxin overproduction and only 3 lines produced fruit. These were all indehiscent. On the basis of the higher level of ectopic effects in the *IND::iaaM* plants, we decided to continue our studies using *IND::IND:iaaM* lines with phenotypes restricted to the valve margin.

Generation of the *PIN3::PIN3:GFP* reporter line is described elsewhere (Z. Ding *et al.*, submitted).

Transgenic plants were selected by kanamycin or glufosinate resistance after *Agrobacterium*-mediated transformation into *Arabidopsis* (Col-0 ecotype).

IAA quantification. For each measurement, three independent samples were collected. Frozen samples (containing pooled tissue of 10–20 mg fresh weight) were homogenized in 0.5 ml 50 mM sodium-phosphate buffer, pH 7.0, containing 0.02% diethyldithiocarbamic acid (antioxidant, Sigma) and 2,000 pg [¹³C₆]IAA (Cambridge Isotope Laboratories) internal standard, using the Retsch MM 301 vibration mill (Retsch GmbH & Co. KG) and a 3 mm tungsten carbide bead at a frequency of 30 Hz for 2 min. The samples were then incubated for 15 min at +4°C with continuous shaking. pH was adjusted to 2.7 with 1 M HCl, and the samples were purified by solid phase extraction on a 500 mg Isolute C8 (EC) column (International Sorbent Technology), conditioned with 2 ml methanol and 2 ml 1% acetic acid. After sample application, the column was first washed with 2 ml 10% methanol in 1% acetic acid and then eluted with 2 ml 70% methanol in 1% acetic acid and the sample was evaporated to dryness. The sample was dissolved in 0.2 ml 2-propanol and 1 ml dichloromethane, and 5 μ l 2 M trimethylsilyl-diazomethane in hexane (Aldrich) was added to methylate the sample. After incubation at room temperature (22 °C) for 30 min, 5 μ l 2 M acetic acid in hexane was added to destroy excess diazomethane and the sample was evaporated to dryness. The samples were then silylated by adding 15 μ l acetonitril

and 15 μ l BSTFA plus 1% TMCS (Pierce). After 30 min incubation at 70 °C, the samples were evaporated to dryness, dissolved in heptane and quantified by GC-SRM-MS (gas chromatography-selected reaction monitoring-mass spectrometry) as previously described³⁴.

DEX induction. Flowers from *PIN1::PIN1:GFP 35S::IND:GR* double-transgenic line were sprayed with a solution of 10 μ M DEX and 0.05% Silwet. A DMSO control was included. For expression assays, *35S::IND:GR* seeds (~20) were germinated in 5 ml of 0.5% glucose (w/v) 0.5 \times Mureshige and Skoog medium with constant shaking. After 7 days growth under constant light, seedlings were treated and tissue collected at the time points described before snap freezing in liquid nitrogen. Experiments were performed in seedlings because it was not possible to control the chemical dose in fruit. Dexamethasone (Sigma, D1756) and cycloheximide (Sigma, C4859) were dissolved in DMSO, and indole-3-acetic-acid (Sigma, I2886) was dissolved in 70% ethanol. The no-treatment controls had the equivalent concentrations of solvents.

Primer design. Primers for quantitative RT-PCR assays were designed using Primer3 (ref. 35) or AtRTPrimer³⁶ software. A list of the primers used is provided in Supplementary Information.

ChIP. *35S::IND:GR* seeds were grown for 7 days in 50 ml of 0.5% glucose (w/v) 0.5 \times Mureshige and Skoog medium with constant shaking. Seedlings were then treated with 50 μ M IAA \pm 10 μ M DEX and ChIP was performed as described in ref. 37 with the following modifications. 2 μ g of anti-GR antibody (AB3580, Abcam) was used for approximately 200 mg of tissue. Dynabeads-protein A (100-02D, Invitrogen Ltd) was used. Quantitative PCRs were done using primers Mu-likeF, Mu-likeR, NRT2.1F, NRT2.1R, PID-678F, PID-368R, WAG2F and WAG2R from the list in Supplementary Information. Mu-like retrotransposon was used as an internal control for normalization³⁸.

Lignin staining. Tissue was fixed and sectioned. Sections 8 μ m thick were treated for 2 min with 2% phloroglucinol (in 95% ethanol) and photographed in 50% hydrochloric acid.

Reporter gene activity. Analysis of β -glucuronidase expression in YJ80, YJ161 and *PIN3::GUS* was done as previously described³⁹.

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