ER-located PINs generate potent auxin sinks driven by the IAA decarboxylation pathway

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ABSTRACT

Auxin is an essential and well-investigated regulator of plant development. Though, many aspects determining its (sub-)cellular distribution, as well as its metabolic turnover, are obscure. PIN5 is a transporter that resides on the endoplasmic reticulum and is presumed to influence internal auxin homeostasis by direct sequestration and subsequent degradation. Due to its distinct expression pattern and incomplete metabolomics analysis, the exact role of PIN5 protein and the identity of downstream auxin degradation products show significant gaps. To this end, we utilized morphologically homogeneous tobacco BY-2 cell cultures in light of the recently updated knowledge on auxin metabolism. We show that the expression of *Arabidopsis thaliana AtPIN5* in the BY-2 system phenocopies so-called auxin starvation defects. Moreover, we reveal that the activity of *At*PIN5 leads, in extreme cases, to the broad range of processes accompanying programmed cell death. Strikingly, we also show that a significant part of auxin metabolites downstream of the *At*PIN5 activity are part of the re-emerging auxin decarboxylation pathway. Taking together, we report the direct induction of programmed cell death by auxin stimulus and propose the physiological framework of the auxin decarboxylation route.

INTRODUCTION

The phytohormone auxin, or indole-3-acetic acid (IAA), is vital for plant growth and development. The pattern formation at the level of individual tissues and organs is achieved by the formation of local auxin maxima or minima, orchestrated by the cell-to-cell transport of IAA (Weijers *et al.*, 2021). Among the mediators of auxin flow emerge the transporters of the PIN-formed (PIN) family. Showing a remarkable asymmetrical distribution on the plasma

membrane (PM), they coordinate auxin fluxes across the tissues, thereby controlling the downstream developmental programs (Adamowski & Friml, 2015). Recent crystallographic studies of the PIN transporters (Su *et al.*, 2022; Ung *et al.*, 2022; Yang *et al.*, 2022) brought compelling insights into the molecular mechanism of auxin transport over the membrane (Andersen *et al.*, 2023; Ung *et al.*, 2023a,b).

Besides the well-investigated auxin fluxes between cells, mediated by the PM-located PINs, another group of PIN proteins residing at the endoplasmic reticulum (ER) has been described. The so-called ER-PINs regulate internal auxin levels by conveying auxin into the ER lumen for subsequent metabolization (Mravec *et al.*, 2009; Ding *et al.*, 2012). The metabolic products downstream of the ER-located PIN activity have been examined several times and with various degrees of consistency (Mravec *et al.*, 2009; Ding *et al.*, 2012; Abdollahi Sisi & Růžička, 2020). In addition, the pathways accompanying the IAA breakdown have recently undergone revision (Hayashi *et al.*, 2021; Dobrev *et al.*, 2023), highlighting the IAA decarboxylation as another relevant auxin degradation route (Dobrev *et al.*, 2023). Thus, many aspects of the IAA homeostasis controlled by ER-PINs remain unclear (Abdollahi Sisi & Růžička, 2020; Ung *et al.*, 2023a).

The connection between auxin and programmed cell death (PCD) has been repeatedly proposed (Kacprzyk *et al.*, 2022). It was shown that auxin pulses produced from the cells undergoing PCD in the outermost layers of the root cap are required for priming lateral root primordia in the primary root meristem (Xuan *et al.*, 2015, 2016). It was also revealed that auxin can suppress certain instances of PCD (Kerchev *et al.*, 2013). It has even been hypothesized that auxin is a product of tryptophan degradation occurring in defunct cells (Sheldrake, 2021). However, the authentic effect of auxin activity to trigger PCD has never been presented.

Cell-suspension cultures have proved to be an excellent model system, which enables studying the effects of growth substances directly and in a homogeneous manner (Petrášek & Zazímalová, 2006; Petrasek *et al.*, 2006). Here, we explore the impact of conditional expression of the well-characterized ER-located *Arabidopsis thaliana At*PIN5 (Mravec *et al.*, 2009) in the BY-2 tobacco suspension cultures. We comprehensively analyze the immediate IAA metabolites downstream of PIN5 activity and reveal the remarkably elevated presence

of products of the IAA decarboxylation pathway (Dobrev *et al.*, 2023). We also describe the corresponding phenotypic consequences, including the extreme instances of PCD.

RESULTS

Auxin response is required for controlling cell expansion and division rates of the BY-2 tobacco cell cultures

The growth and proliferation of tobacco BY-2 cells depends on the exogenous supply of auxin to the media. Its absence is generally characterized by enhanced cell elongation and lowered cell division, termed collectively as symptoms of auxin starvation (Winicur *et al.*, 1998). Our previous work has revealed that interfering with auxin transport leads to similar morphological defects (Mravec *et al.*, 2008; Müller *et al.*, 2019). Indeed, the induction of the transgene encoding a PM-located *Arabidopsis thaliana At*PIN7 auxin carrier enhances cell elongation (Figure 1a) and division arrest (Figures 1b and S1a), consistent with that occurring on the auxin-free medium (Figure S1b; Mravec *et al.*, 2008; Müller *et al.*, 2019). We observed similar auxin-deficient phenotypes by interfering with auxin signaling by the inducible expression of the stabilized version of the auxin signaling repressor *At*IAA17, *At*axr3-1 (Leyser *et al.*, 1996; Knox *et al.*, 2003; Figures 1c-e) and we were also able to pharmacologically reduce cell proliferation by the dose-dependent application of IAA antagonist PEO-IAA, which competitively binds the TIR1/AFBs auxin receptors (Nishimura *et al.*, 2009; Figure 1f). This indicates that the auxin starvation phenotypes are dependent on the TIR1/AFB signaling, in addition to the availability of auxin in the cells.

Inducible expression of *At*PIN5 leads to defects corresponding to the symptoms of auxin starvation in BY-2 cells

Next, to study the impact of internal auxin fluxes on the cell culture phenotypes, we expressed in the BY-2 cells the characterized *Arabidopsis thaliana* gene *AtPIN5* (Mravec *et al.*, 2009; Abdollahi Sisi & Růžička, 2020), employing two independent transcription inducible systems, XVE (Zuo *et al.*, 2000; Petrasek *et al.*, 2006) and GVG (Aoyama & Chua, 1997; Mravec *et al.*, 2008). Normally, under control conditions, on the third day of the subculture cycle, the cultures turn into the exponential growth phase characterized by intensive cell divisions, reflective of cell files consisting of short cells (Figure 2a, S2a). Upon the induction

of the *At*PIN5 transgene, the cells became remarkably expanded (Figures 2a, b and S2b, c), and their cell division reduced (Figures 2c and S2d), akin to the auxin starvation phenotypes (Figures 1 and S1). We also co-transformed the cells carrying the inducible *AtPIN5-GFP* construct with the transcriptionally-dependent auxin reporter *DR5::RFP* (Barbez *et al.*, 2013). Although the intensity of the induced GFP signal in individual cells varied across the population, the presence of AtPIN5-GFP was generally able to lower the *DR5::RFP* signal (Figures 2d, e). This indicates that the *At*PIN5 activity is capable of changing the internal availability of auxin to control the growth and expansion of the BY-2 cells.

IAA enhances the symptoms of *At*PIN5-induced auxin starvation

The dependence of the BY-2 tissue cultures on the addition of the synthetic auxin (2,4-dichlorophenoxyacetic acid or 2,4-D) to the media is ascribed to the low levels of endogenous IAA in the cells, caused by its native metabolization (Simon *et al.*, 2013; Seifertová *et al.*, 2014; Müller *et al.*, 2021). Accordingly, the cultivation in the presence of IAA also leads to auxin starvation phenotypes (Simon *et al.*, 2013; Figure 3a). To test the role of internal auxin fluxes under conditions where only native auxin is present, we cultivated the cells expressing *AtPIN5* under the supply of IAA, too. The control lines proliferated less intensively and resembled the auxin-starved ones. Surprisingly, the induction of *At*PIN5 led to even stronger auxin starvation phenotypes (Figures 3b, c). Thus, it seems that the PIN5-like transporters can even enhance rapid IAA turnover in BY-2 cells.

To follow the original idea and to compensate for the fast IAA metabolization more efficiently, we were continuously adding IAA to the media every 12 h during the cultivation. We observed a rescue of the cell proliferation in the control cells; they displayed no apparent signs of auxin starvation (Figures 3d, e). Strikingly, though, the *AtPIN5* induction caused strong auxin starvation phenotypes (Figures 3d, e). Under these conditions, PIN5-GFP was present on ER as in cells cultivated on 2,4-D, also reporting the absence of remarkable morphological changes of this compartment (Figure S3). This further underlines that PIN5 is able to generate a potent intracellular IAA sink.

ER-PINs specifically transport both IAA and 2,4-D (Ung *et al.*, 2022). Hence, we measured the cellular accumulation of radioactively labeled [^{3}H]-IAA and [^{3}H]-2,4-D, following the induction of *AtPIN5*. The lines showed a strong cellular uptake of [^{3}H]-IAA (Figure 2f).

Interestingly, we observed no change in the *At*PIN5-mediated [^{3}H]-2,4-D accumulation (Figure 2g). Altogether, our data suggest that *At*PIN5 efficiently channels IAA from the cytosol to be delivered to likely highly active metabolic enzymes inside the ER lumen.

Induction of *AtPIN5* promotes programmed cell death

To validate whether the strong auxin withdrawal from the cytosol by *At*PIN5 corresponds to a native downstream process, we analyzed the viability of the cells examined. As revealed by the trypan blue exclusion test (van Wees, 2008), we observed an increased ratio of cells showing a dye-positive cytoplasm shrinkage as a sign of decreased cell viability as a consequence of the *At*PIN5 induction (Figures 4a, b and S4a, b). Moreover, the internal trypan blue staining was even more apparent in the cells cultured on IAA (Figures 4a, b). This shows that the PIN5-mediated funneling auxin from the cytosol can indeed lead to increased mortality of BY-2 cells.

Further, we analyzed the global transcriptional changes downstream of the *At*PIN5 activity to hint at the possible relevant physiological processes related to auxin starvation and lower cell viability. Indeed, using the PANTHER overrepresentation test (Mi *et al.*, 2019) we found a remarkable overlap with the previously published transcriptional profiles from the BY-2 cells cultured in the auxin-free medium (Müller et al., 2021; Figures 4c and S4c, d). This included pronounced changes in the expression of stress-related genes, particularly peroxidases (Table S1). We further examined the transcriptional response exclusively linked with the *At*PIN5 expression (Table S2), revealing a single enriched gene ontology term (GO:0000041) supported by 6 individual transcripts coding for transporters of transition metals (Peñarrubia *et al.*, 2015). This suggests that the auxin starvation is associated with the enhanced expression of the stress-related peroxidases and that the *AtPIN5* expression leads to the enhanced metal ion transport, presumably activating the response preceding the cell death.

The increased expression of peroxidases following the *AtPIN5* induction suggests elevated activity of produced reactive oxygen species (ROS). The role of auxin has been implicated in the crosstalk with ROS homeostasis (Tognetti *et al.*, 2012; Chen *et al.*, 2014b). Hence, we utilized a sensor carboxy-2',7'-dichloro-dihydro-fluorescein diacetate (H2DCFDA), which indicates the intracellular presence of various ROS, including H_2O_2 (Akter *et al.*, 2021). Indeed, upon induction of *AtPIN5*, the cells displayed significantly increased H2DCFDA

fluorescence, corroborating the enhanced ROS activity (Figures 4d, e, and S4e). PIN5 was previously shown to be a part of ER stress adaptive mechanisms (Chen *et al.*, 2014a). Although we did not notice that the repeated additions of IAA into the media would influence the ER localization of *At*PIN5-GFP or lead to remarkable changes in the overall ER structural pattern (Figures 2d and S3), pathways involving ROS production and ER stress are often preceding PCD (Petrov *et al.*, 2015). Hence, this further highlights that the lower viability of the *At*PIN5-GFP cells accompanied by ROS can be perhaps ascribed to the initiation of processes related to PCD.

Among the main features of PCD is vacuolar leakage and subsequent acidification of cytoplasm (Valandro *et al.*, 2020). To this end, we utilized 2',7'-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM), a fluorescent dye used for analyzing lowered cytosolic pH as a result of disrupted vacuolar integrity (Scheuring *et al.*, 2015). Under control conditions, the cells contained several smaller vacuoles. The induction of *At*PIN5 led to the enlargement of central vacuoles, their occasional collapse and acidification of the whole cytoplasm and nucleus (Figures 4f, g). Finally, we tested the nuclear DNA fragmentation, a hallmark of PCD, using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) (Tripathi *et al.*, 2017). The expression of *AtPIN5* (Figures 4h, i, and S4f) resulted in an increased number of TUNEL-stained nuclei, highlighting that DNA fragmentation takes part in the signaling cascade downstream of the rapid depletion of auxin in the cytoplasm. Taken together, we conclude that the decreased cell viability associated with PCD, different from uncontrolled stress-related necrotic processes (Van Durme & Nowack, 2016), is another hallmark of PIN5-triggered auxin starvation.

The activity of *At*PIN5 stimulates auxin degradation by the IAA decarboxylation pathway

To get insights into how the ER-mediated IAA conversion affects internal auxin homeostasis in light of recent findings (Müller *et al.*, 2021; Hayashi *et al.*, 2021; Dobrev *et al.*, 2023), we have expanded the list of IAA metabolites detected by liquid chromatography-mass spectrometry (LC-MS) following the *AtPIN5* induction. The measurements confirmed a rapid catabolism of applied IAA. 2 hours after adding into standard media, IAA was efficiently converted into several metabolites, mainly to amino-acid conjugates of IAA (Figure 5a; compare Seifertová *et al.*, 2014; Müller *et al.*, 2021). The induction of *At*PIN5 led to a further decrease in IAA levels (Figures 5a and S5a). We also measured the breakdown of IAA in the

cells cultivated in a media supplied with IAA every 12 hours. In the control lines, IAA was converted into its conjugates within 2 hours as well (Figure 5b). Surprisingly, the induction of *AtPIN5* dramatically reduced the internal IAA levels to close to trace amounts. Although we were also able to detect the activity of the common oxidative IAA degradation pathway, we found remarkably increased conversion towards the IAA decarboxylation pathway, documented by elevated levels of ascorbigen (Abg) and dihydro-ascorbigen (DiHAbg) (Dobrev *et al.*, 2023; Figure 5b). It has been proposed earlier that indole-3-carbinol (I3C), an unstable immediate IAA decarboxylation product and precursor of Abg, can stimulate apoptosis antagonistically to auxin (Katz *et al.*, 2015; Katz & Chamovitz, 2017). However, even high doses of I3C did not interfere with the cell growth, regardless of the induction of *AtPIN5* (Figure S5b). Taken together, ER represents a powerful auxin sink for the degradation of endogenous IAA, metabolized by the IAA decarboxylation pathway, and capable of triggering PCD.

DISCUSSION

A possible mechanism underlying PIN5-mediated auxin sinks

The classical concept of polar auxin transport has premised the existence of efflux carriers asymmetrically localized at PM as the central prerequisite for the directional flow of IAA (Rubery & Sheldrake, 1974; Raven, 1975). Later, the PIN auxin transporters were discovered, matching henceforth the proposed attributes of the main IAA carriers (Gälweiler *et al.*, 1998; Petrasek *et al.*, 2006). ER-located PINs were found subsequently, and the relevant works highlighted their capability to influence internal auxin homeostasis (Mravec *et al.*, 2009; Ding *et al.*, 2012; Abdollahi Sisi & Růžička, 2020). Further, the detailed crystallographic studies have evidenced that the transporting mechanism provided by both PM and ER-located PINs is identical (Su *et al.*, 2022; Ung *et al.*, 2022; Yang *et al.*, 2022).

Yet, many aspects of the PIN-mediated transport remain obscure. Compelling arguments supported by the structural models suggest that IAA is transported from the cytosol against concentration gradients at the expense of membrane potential (Su *et al.*, 2022; Ung *et al.*, 2022, 2023a,b; Yang *et al.*, 2022; Andersen *et al.*, 2023). Indeed, in the root apical meristem, PINs concentrate auxin in the uttermost root tip (Adamowski & Friml, 2015). PM-located PINs also establish and maintain auxin maxima, determining the position of flower primordia

in the shoot apical meristem in an equivalent manner (Reinhardt *et al.*, 2003). In contrast, the canalization hypothesis proposes auxin that auxin is funneled away from the site of its external application, i.e., source, towards the sink in the established vascular tissue determining the connective vascular strands (Mitchison, 1980; Sachs, 1991; Ravichandran *et al.*, 2020). Similarly, the root or hypocotyl tropic reactions depend on the transport outwards from the tissue with higher auxin concentration (Friml *et al.*, 2002; Ottenschläger *et al.*, 2003; Rakusová *et al.*, 2011; Band *et al.*, 2012). The expression of PIN5 proteins leads to a remarkable auxin deficiency in BY-2 cells. In the conditions when 2,4-D does not interfere with the internal IAA fluxes (Figure 5b), the excess of IAA leads to even enhanced PIN5-mediated auxin degradation rates. Hence, in this system, the PIN5-like auxin transporters likely operate in the source-sink mode, too, perhaps by the mechanism of facilitated diffusion, as earlier presumed for auxin canalization (Mitchison, 1980; Rolland-Lagan & Prusinkiewicz, 2005).

Auxin deprivation as a trigger for PCD

Along with the auxin-dependent processes, PCD is an inherent part of plant development. The connections between auxin and PCD pathways have been proposed, but rather indirectly (reviewed in Van Durme & Nowack, 2016; Sheldrake, 2021; Kacprzyk *et al.*, 2022). Auxin has been demonstrated as a central regulator of xylem patterning, where PCD gives rise to conductive vessels (Escamez & Tuominen, 2014; Ruzicka *et al.*, 2015); direct transdifferentiation of the tissue cultures into xylem vessels can also be induced by external cues (Fukuda & Komamine, 1980; Kubo *et al.*, 2005; Ruzicka *et al.*, 2015). In addition, various stages of lateral root development are determined by auxin activity in parallel to PCD (Casimiro *et al.*, 2001; Benková *et al.*, 2003; Swarup *et al.*, 2008; Escamez *et al.*, 2020). Auxin serves as a signal produced by the cells undergoing PCD in the root cap to prime the prospective lateral roots in the primary root meristem (Xuan *et al.*, 2015, 2016). Interestingly, Di Mambro et al. (2019) presented the model that the activity of *PIN5* in the lateral root cap affects root meristem size. Though, the direct influence of PIN5 on PCD in the lateral root cap has not been investigated.

Very little is known about the induction of PCD as a result of direct (positive or negative) auxin stimulus. We reveal that auxin deprivation in BY-2 cells can lead, in extreme cases, to cell death. The observed phenotypes are not related to the stress-induced necrosis nor the

direct activity of a relevant auxin metabolite, here I3C (Katz *et al.*, 2015; Katz & Chamovitz, 2017). We also have not noticed any cells resembling those forming xylem vessels following the *AtPIN5* induction. A chemical screen performed by Kerchev *et al.* (2013) revealed that the H_2O_2-dependent cell death caused by defective photorespiration can be reverted by both synthetic auxins and IAA. Accordingly, we found that the expression of *AtPIN5* in BY-2 cells leads to elevated levels of ROS, among other signs of PCD, including (RNA-seq evidenced) imbalance of transition of metal transport, vacuolar acidification, and DNA fragmentation.

IAA decarboxylation pathway downstream of PIN5-mediated auxin sequestration

PIN5 transports auxin into the ER in order to promote auxin degradation (Mravec *et al.*, 2009; Ung *et al.*, 2023a). The levels of IAA-aspartate and IAA-glutamate conjugates are elevated following *At*PIN5 overexpression (Mravec *et al.*, 2009). Paradoxically, the GRETCHEN HAGEN (GH3) enzymes catalyzing the IAA conjugation are generally seen in the cytoplasm (Ludwig-Müller *et al.*, 2009; Ostrowski *et al.*, 2014; Di Mambro *et al.*, 2019; Abdollahi Sisi & Růžička, 2020; Zou *et al.*, 2022). We have recently uncovered a new level of complexity of the IAA metabolome. Among known IAA breakdown routes emerges the auxin decarboxylation pathway, which includes Abg and DiHAbg. Even if occurring across angiosperms, the purpose of auxin decarboxylation is unknown (Dobrev *et al.*, 2023). Strikingly, we found that the relevant metabolic compounds are related to increased AtPIN5 activity. Hence, we conclude that IAA decarboxylation is the main and potent auxin degradation pathway downstream of PIN5.

EXPERIMENTAL PROCEDURES

Plant material and molecular transgenic work

The dexamethasone-inducible gene constructs, *35S>>GVG::AtPIN5* and *35S>>GVG::AtPIN7*, including the corresponding cell cultures, have been described earlier (Petrasek *et al.*, 2006; Mravec *et al.*, 2009). For creating *G10-90>>XVE::AtPIN5*, the corresponding *AtPIN5* open reading frame was cloned with the Gateway system (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) into the pMDC7 vector (Curtis & Grossniklaus, 2003). The respective Gateway entry clones were generated either by the amplification of wild-type DNA template or that originating from the *PIN5-GFP*^{MGS} plants (Verna *et al.*, 2015)

(primer sequences AAAAAGCAGGCTATATGATAAATTGTGGAGAT and AAGAAAGCTGGGTTTCAATGAATAAACTCC). The *G10-90>>XVE::Ataxr3-1* line was made by the recombination of the *Ataxr3-1* Gateway entry clone (Siligato *et al.*, 2016) into the pMDC7 vector.

The tobacco cell lines BY-2 (*Nicotiana tabacum* L., cv. Bright Yellow-2; Nagata *et al.*, 1992) were standardly cultivated in a liquid medium supplemented with 1 µM 2,4-D as described in Petrasek *et al.* (2006) unless stated otherwise. The transformation of cell lines, RT-qPCR validation of the transgene expression, and cell line maintenance were done as described earlier (Petrasek *et al.*, 2006; Müller *et al.*, 2021). The expression of relevant *AtPIN7, AtPIN5* and *Ataxr3-1* genes was induced by 1 µM dexamethasone (GVG lines) or 3 µM β-estradiol (XVE lines) at the beginning of the subculture interval, and the corresponding amount of solvent (DMSO) was added to control cells. The *XVE::AtPIN5-GFP/DR5::RFP* cell line was obtained by transformation of the *XVE::AtPIN5-GFP* line with the *DR5::RFP* construct (Barbez *et al.*, 2013). For all experiments, the cells in the exponential growth stage were used, typically 4-d-old, unless specified otherwise.

Microscopy and image analysis

Nomarski differential interference contrast (DIC) microscopy was performed with the Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with a color digital camera (DVC 1310C, Austin, Texas, USA). Cell length and diameter were measured manually using the Fiji image analysis software (Rueden *et al.*, 2017). 200 cells in each data set were measured. Cell density was determined by counting the cells with the Fuchs-Rosenthal hemocytometer slide, typically in the fourth day of subcultivation or periodically during the entire period; individual values represent the average of at least six aliquots of every sample. The following compounds were used for treatments: 2-(1H-Indol-3-yl)-4-oxo-4-phenyl-butyric acid (PEO-IAA; OlChemIm, Olomouc, Czech Republic) and indole-3-carbinol (I3C; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

For the fluorescence imaging, Zeiss LSM900 laser scanning microscope (Axio Observer 7, inverted) with Airyscan 2 with Multiplex mode (Carl Zeiss, Jena, Germany) with appropriate filter sets to visualize reporter genes GFP (excitation 488 nm and emission 509 nm wavelengths) and/or mRFP (excitation 590 nm and emission 599 nm wavelengths). Detailed images were obtained with the 40x or 63x water/oil immersion objectives.

Assays on cell viability, ROS content and PCD

The trypan blue exclusion test was used to visualize the penetration of the non-viable cells by the dye (van Wees, 2008). Approximately 10 µl of the 0.4 % trypan blue solution (Sigma-Aldrich) was added to the 0.5 ml cell suspension and recorded subsequently. Cell viability was determined as the percentage of dye-negative cells relative to the total number of cells.

For the detection of reactive oxygen species (ROS), the cells were incubated with the 10 µM H2DCFDA (2′,7′-dichlorfluorescein-diacetate; Thermo Fisher Scientific, Waltham, Massachusetts, USA) solution (Akter *et al.*, 2021) for 15 min and washed twice with the BY-2 medium. The relative ROS accumulation is presented as the mean pixel intensity of cells determined by the Zeiss Zen software. In total, about four hundred cells were counted from three independent experiments. The integrity of the vacuoles was determined after 10 min of staining with 10 µM BCECF-AM ((2′,7′-bis-2-carboxyethyl)-5-(and-6) carboxyfluorescein; Sigma-Aldrich; Scheuring *et al.*, 2015). The number of cells with aberrant vacuolar integrity was assessed as a ratio of cells with the cytoplasmic signal to that of the cells showing a normal vacuolar signal. About five hundred cells were processed in three independent experiments. In both cases, the confocal images were acquired with the Plan-Apochromat 10x/0.45 DIC M27 objective.

TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay (Tripathi *et al.*, 2017) was performed in 2-day-old cells according to the manufacturer's protocol (TMR red in situ cell death detection kit, Roche Diagnostics GmbH, Heidelberg, Germany) with modifications as described earlier (Smetana *et al.*, 2012). The labeled cells were examined using a color digital camera (DVC 1310C, Austin, Texas, USA) at the Nikon Eclipse E600 microscope. All cells were analyzed from at least 10 view fields, covering approximately 100 cells each. The quantification is expressed as a percentage of cells carrying the TUNEL signal divided by the total cell number in the visual field.

Auxin uptake assays

Auxin uptake assays were performed in 3-d-old cells as described earlier (Delbarre *et al.*, 1996; Petrásek *et al.*, 2003). ([^{3}H]-2,4-D (10 Ci.mmol^{-1}) or [^{3}H]-IAA (25 Ci.mmol^{-1}) (American Radiolabeled Chemicals, Inc., St Louis, MO, USA) were added to the equilibrated cell

suspension to the final concentration of 2 nM prior to incubation. The presented values are means of each triplicate from a representative experiment out of three biological repetitions.

IAA metabolic profiling

The sampling of the 2-d-old BY-2 cells was done 2 hours after the (last) addition of 1 µM IAA into the media. Auxin metabolites were then extracted and purified according to previously described procedures (Dobrev & Kamínek, 2002; Dobrev *et al.*, 2023), and followed by the LC-MS quantitative analysis further detailed in Dobrev *et al.* (2023). The samples were processed in triplicates. The results are presented as a mean of at least three independent biological repetitions.

Transcriptome analysis

The total RNA was isolated from 50-100 mg of cells using RNeasy Plant Mini kit (Qiagen, Düsseldorf, Germany) and treated with DNA-Free kit (Thermo Fisher Scientific). RNA purity, concentration and integrity were evaluated on 0.8% agarose gels (v/w) and with the Bioanalyzer RNA Nano 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA). RNA sequencing was performed using Illumina NovaSeq 6000 platform. The analysis resulted in at least 15 million 150 bps read pairs. The raw reads were quality-filtered using Rcorrector and Trim Galore scripts (Song & Florea, 2015). Transcript abundances (transcripts per million – TPM) were determined using the Salmon software package (Patro *et al.*, 2017) with parameters --posBias, --seqBias, --gcBias, --numBootstraps 30. The index was built from the *Nicotiana tabacum* v1.0 cDNA dataset (Edwards *et al.*, 2017), by including the *AtPIN5* (*AT5G16530*) gene sequence. Visualization, quality control of data analysis and determination of differentially expressed genes were determined using the sleuth package in R (Love *et al.*, 2014; Pimentel *et al.*, 2017) (version 0.29.0). Transcripts with the q-value <= 0.05 and $\log_2$ fold change >=1 (upregulated) or <= -1 (down-regulated) were considered to be significantly differentially expressed. Other criteria for selection of gene list of interest are specified in corresponding result section.

Gene ontology analysis (statistical overrepresentation test) was done using the Panther Classification System (Mi *et al.*, 2019). Raw sequencing data are available through GEO Series

accession number XXX at the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/).

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AUTHOR CONTRIBUTIONS

JP, KR and MČ designed the research. MČ performed most experiments. KM did RT-qPCR and transcriptome analysis. NK and NAS made DNA constructs. PS provided preliminary results for the initiation of the study. OS performed part of the PCD assays. PID did metabolic profiling. KR, JP and MČ wrote the paper. KR acquired funding. All authors have exercised their right to comment on the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. The hallmarks of auxin starvation in tobacco BY-2 cell cultures. (a, b) The induction of the *XVE::AtPIN7* transgene leads to increased cell elongation (a) and decreased growth, monitored by the cell density in the media (b). (c-e) The induction of *XVE::Ataxr3-1* phenocopies auxin starvation phenotypes, documented by representative image on (c), quantification of reduced proliferation (d) and increased cell elongation ($P < 0.001$ by Student's *t*-test) (e). (f) The cell density of the wild-type BY-2 cells decreases with the growing concentration of PEO-IAA, an inhibitor of auxin-dependent gene expression ($F(5,48) = [11.112]$, $P < 0.001$ by one-way ANOVA). On the dot plots, the open symbols are averages ± S.E. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test. Scale bars: 50 µm.

Figure 2. The conditional expression of *XVE::AtPIN5* in BY-2 cells mimics the auxin starvation phenotypes. (a-c) The induction of *XVE::AtPIN5* in the cell line cultured in a standard medium with 2,4-D leads to increased cell elongation (a), plotted on (b, $P < 0.05$ by Student's *t*-test), and reduced proliferation rate (c). (d, e) The induction of *XVE::AtPIN5-GFP* in the cells co-expressing auxin *DR5::RFP* reporter results in co-localization of PIN5-GFP with the ER-targeted RFP (d) and lowering the RFP expression in a reduction of fluorescent cells in the upper quantiles (e). Scale bars: 50 µm. On (c), the open symbols are averages ± S.E. On (e), the vertical error bars represent median ± 95% confidence intervals. $^{***}P < 0.001$ by Student's *t*-test.

Figure 3. Replacing 2,4-D with the native auxin IAA in media enhances the auxin starvation effects caused by the induction of *XVE::AtPIN5*. (a) The comparison of the cell proportions of the control *XVE::AtPIN5* cells cultivated in the medium supplemented with 2,4-D or IAA reveals the increase of the elongation growth in the presence of IAA ($P < 0.001$ by Student's *t*-test). (b-c) The cell proliferation rate of cells containing the induced *XVE::AtPIN5* transgene is even lower in the medium with IAA added at the beginning of subcultivation, the representative image on (b), the corresponding quantification of cell density on (c). (d, e) The phenotype of the *XVE::AtPIN5* cells cultured in a medium continuously supplemented with IAA every 12 hours during the entire cultivation period leads to the partial reversion of the starvation phenotypes in the control lines, however, the induction of the transgene leads to strong auxin starvation phenotypes, as documented at the representative image on (d)

and by the quantification of the cell growth on (e). (f, g) The induction of *XVE::AtPIN5* results in higher uptake of [^{3}H]-IAA (f), but shows no effect on uptake of [^{3}H]-2,4-D (g). Scale bars: 50 µm. On the bar plots, the open symbols are averages ± S.E. $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

Figure 4. An increased incidence of symptoms of PCD following induction of *XVE::AtPIN5*. (a-b) The replacement of 2,4-D with IAA leads to more frequent cytoplasm shrinkage and hereafter increased accumulation of the trypan blue dye inside cells (arrowheads), where *XVE::AtPIN5* was induced, documented by representative images on (a), the inverse frequency of the trypan-blue intracellular staining on (b). (c) DiVenn diagram shows that a large part of genes differentially expressed genes following the *XVE::AtPIN5* induction is shared with those from the cultures undergoing auxin starvation; red, significantly upregulated genes; blue, downregulated genes; yellow, opposite expression changes. A single dot denotes a group of 10 genes. (d, e) An increased fluorescence intensity on confocal images of the H2DCFDA staining of *XVE::AtPIN5* lines detects the elevated presence of reactive oxygen species (ROS) following the transgene induction (d), as quantified in (e). (f, g) Confocal images of cells stained by BCECF-AM to label acidic areas (f), as quantified in (g), document that the *XVE::AtPIN5* induction leads to decreased acidity in vacuoles but increased acidity in the cytoplasm and nucleus area (arrowheads). (h, i) Confocal images of TUNEL staining, marking the disintegration of nuclear DNA (red channel: TUNEL, blue channel: nuclear DAPI staining) (h), shows an enhanced labeling frequency in the cells where the *XVE::AtPIN5* construct was induced (i). For (d) and (f), the color intensity is indicated by the rainbow color lookup table (inset on (d)). Scale bars: 50 µm. On the dot plots, the open symbols are averages ± S.E. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by Student's *t*-test.

Figure 5. Inducible *XVE::AtPIN5* expression in BY-2 cells promotes IAA deactivation by the decarboxylation pathway. (a) LC-MS quantification of IAA and its metabolites in the cells expressing *XVE::AtPIN5* cultured on the standard 2,4-D-supplemented media shows moderately decreased levels of IAA and its most degradation products. (b) The induction of *XVE::AtPIN5* in the cells cultivated in media supplemented with IAA added every 12 hours leads to a dramatic drop of IAA and its conjugates, while the compounds belonging to the IAA decarboxylation pathway show steadily elevated levels. The cells were harvested 2 hours

after the addition of 1 µM IAA. Open symbols indicate the mean from three independent measurements; the bars are ± S.E. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's t-test, n. s.: not significant. IAA: indole-3-acetic acid, IAA-Asp: IAA-aspartate, IAA-GE: IAA-glucose ester, OxIAA-Asp: Oxo-IAA-aspartate, Abg: ascorbigen, DiHAbg: dihydro-ascorbigen.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. The additional features of auxin starvation of tobacco BY-2 cells. (a) The induction of the *GVG::AtPIN7* transgene leads to decreased cell density, reflecting their low proliferation rate during the whole subcultivation interval. (b) The enhanced cell expansion is typical for cultivation on the auxin-free medium (shown on the *GVG::AtPIN7* line without the chemical inducer). On (a), the open symbols are averages ± S.E. Scale bars: 50 µm.

Figure S2. The conditional expression of *GVG::AtPIN5* in the BY-2 cells mimics the auxin starvation phenotypes. (a-c) The induction of *GVG::AtPIN5* in the cell line cultured in standard medium with 2,4-D leads to increased cell expansion (P < 0.001 by Student's t-test) and reduced proliferation rates (c). On (c), the open symbols are averages ± S.E. Scale bars: 50 µm.

Figure S3. The pattern and signal intensity of the AtPIN5-GFP foci are comparable in both standard 2,4-D-containing medium and that continuously supplemented with IAA every 12 hours. Scale bars: 10 µm.

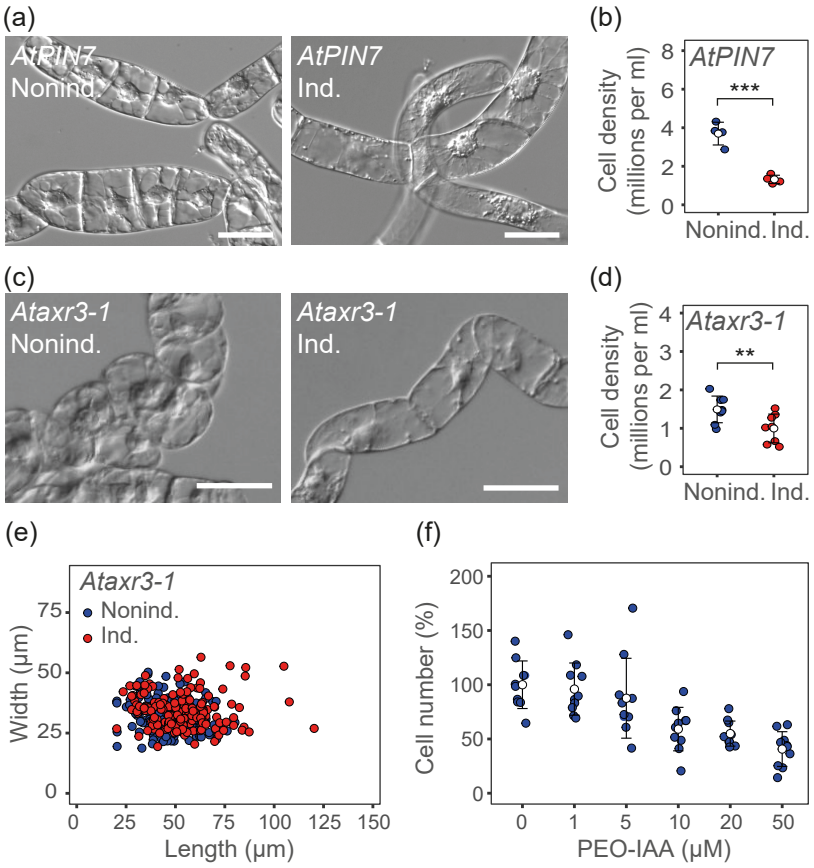
Figure S4. Increased occurrence of symptoms of PCD and its relationship to auxin starvation following induction of *AtPIN5*. (a, b) The representative images of incorporation of the trypan blue inside the cells following induction of *GVG::AtPIN5* (a), quantified on (b). (c, d) Quality control of RNA-Seq data processing, a heat-map representing Jensen-Shannon divergence between individual samples (sorted by internal sample IDs) (c), projections of individual samples onto the principal components (PC) (d). (e) An increased fluorescence on confocal images of the *GVG::AtPIN5* cells stained by 10 µM H2DCFDA to detect reactive oxygen species (ROS), following the transgene induction. (f) TUNEL staining (left: TUNEL, middle: control nuclear DAPI staining, right: merged false-colored images) marks an

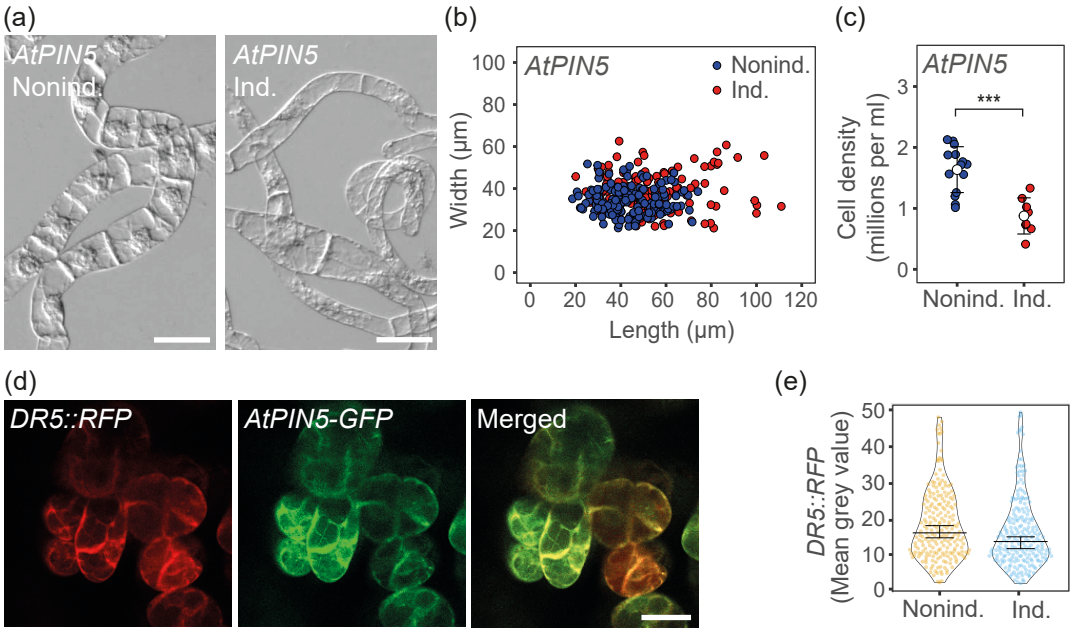
enhanced disintegration of nuclear DNA in the cells where *GVG::AtPIN5* was induced. On (b), the open symbols are averages ± S.E., ***$P < 0.001$ by Student's t-test. Scale bars: 50 µm.

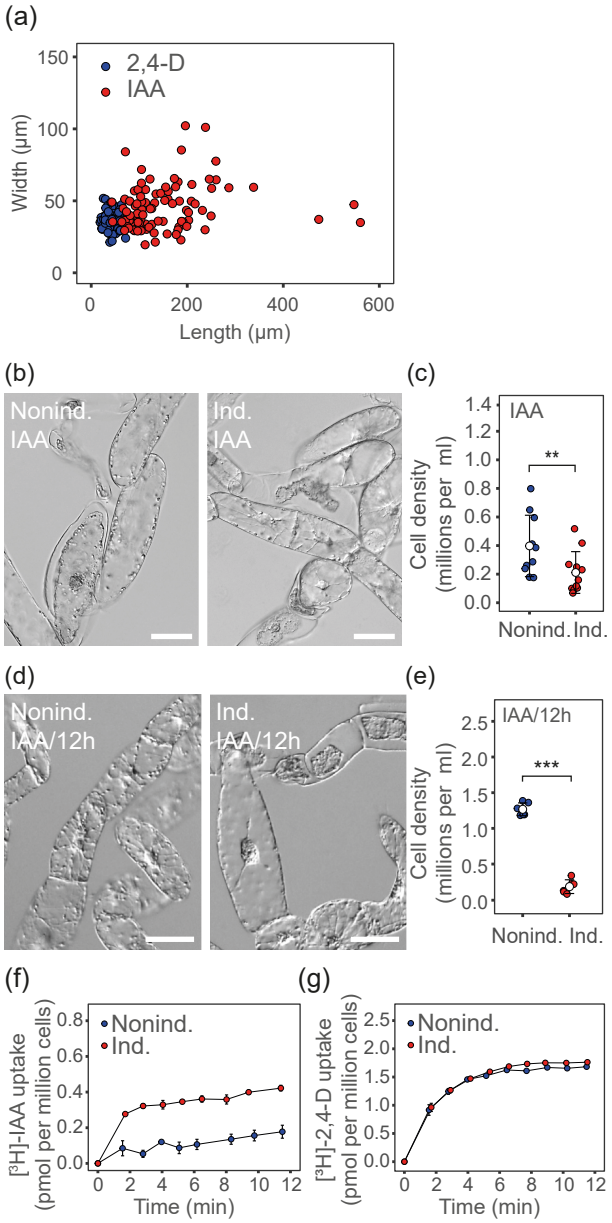
Figure S5. Additional experiments corroborating the role of the IAA decarboxylation pathway following the *AtPIN5* expression. (a) LC-MS analysis of IAA and its metabolites in cells expressing *GVG::AtPIN5* cultured on the standard 2,4-D-supplemented media, 2 hours after adding 1 µM IAA. The induction of *GVG::AtPIN5* leads to decreased levels of IAA and elevation of the IAA-Asp and IAA-Glu conjugate content. (b) In a standard medium supplemented with 2,4-D, I3C does not interfere with the growth rates of the control cells, nor with that following induction of *XVE::AtPIN5*. Open symbols indicate the mean from three independent measurements; the bars are ± S.E. ***$P < 0.001$ by Student's t-test. n. s.: not significant. IAA: indole-3-acetic acid, IAA-Asp: IAA-aspartate, IAA-GE: IAA-glucose ester, IAA-Glu: IAA-glutamate, Abg: ascorbigen, I3C: indole-3-carbinol.

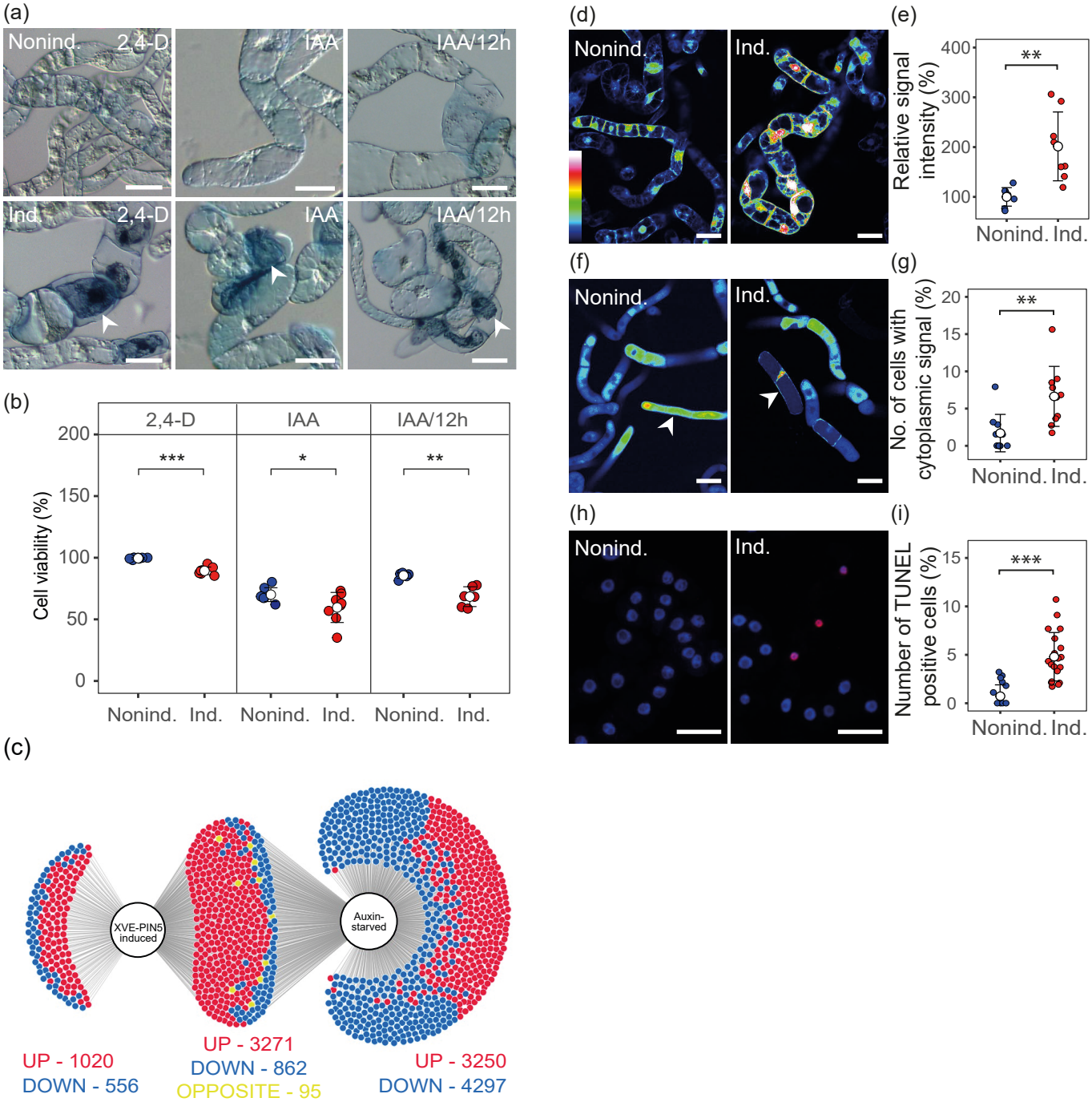
Table S1. Results of PANTHER Overrepresentation test (GO - Molecular function) of transcripts significantly upregulated in both auxin-starved and the *XVE::AtPIN5*-expressing cells.

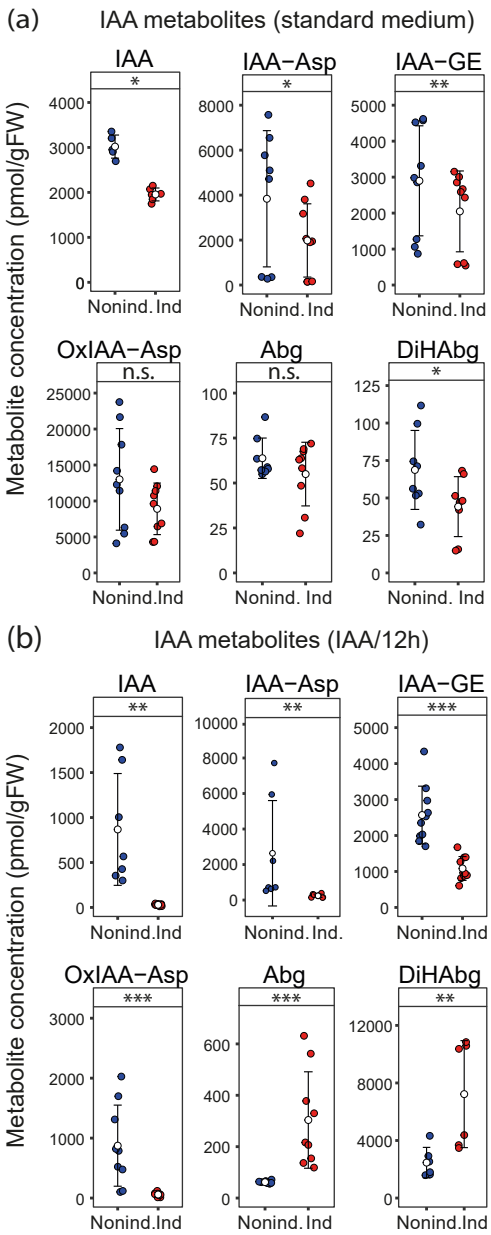
Table S2. Results of PANTHER Overrepresentation test (GO - Molecular function) of transcripts significantly upregulated in BY-2 cells carrying induced *XVE::AtPIN5*, compared to untreated controls and auxin-starved cells.


(a)
AtPIN7
Nonind.
AtPIN7
Ind.
(b)
AtPIN7
Cell density (millions per ml)
***
Nonind.
Ind.
(c)
Ataxr3-1
Nonind.
Ataxr3-1
Ind.
(d)
Ataxr3-1
Cell density (millions per ml)
**
Nonind.
Ind.
(e)
Ataxr3-1
Nonind.
Ind.
Width (µm)
Length (µm)
(f)
Cell number (%)
PEO-IAA (µM)


(a)
AtPIN5
Nonind.
AtPIN5
Ind.
(b)
AtPIN5
Nonind.
Ind.
Width (µm)
Length (µm)
(c)
AtPIN5
***
Cell density
(millions per ml)
Nonind.
Ind.
(d)
DR5::RFP
AtPIN5-GFP
Merged
(e)
DR5::RFP
(Mean grey value)
Nonind.
Ind.


(a)
2,4-D
IAA
Width (µm)
Length (µm)
(b)
Nonind.
IAA
Ind.
IAA
(c)
IAA
Cell density
(millions per ml)
Nonind.
Ind.
(d)
Nonind.
IAA/12h
Ind.
IAA/12h
(e)
IAA/12h
Cell density
(millions per ml)
Nonind.
Ind.
(f)
Nonind.
Ind.
[³H]-IAA uptake
(pmol per million cells)
Time (min)
(g)
Nonind.
Ind.
[³H]-2,4-D uptake
(pmol per million cells)
Time (min)


(a)
Nonind.
2,4-D
IAA
IAA/12h
Ind.
2,4-D
IAA
IAA/12h
(b)
2,4-D
IAA
IAA/12h
Cell viability (%)
200
150
100
50
0
***
*
**
Nonind.
Ind.
Nonind.
Ind.
Nonind.
Ind.
(c)
XVE-PIN5 induced
Auxin-starved
UP - 1020
DOWN - 556
UP - 3271
DOWN - 862
OPPOSITE - 95
UP - 3250
DOWN - 4297
(d)
Nonind.
Ind.
(e)
Relative signal intensity (%)
400
300
200
100
**
Nonind.
Ind.
(f)
Nonind.
Ind.
(g)
No. of cells with cytoplasmic signal (%)
20
15
10
5
0
**
Nonind.
Ind.
(h)
Nonind.
Ind.
(i)
Number of TUNEL positive cells (%)
15
10
5
0
***
Nonind.
Ind.


(a)
IAA metabolites (standard medium)
Metabolite concentration (pmol/gFW)
IAA
IAA−Asp
IAA−GE
OxIAA−Asp
Abg
DiHAbg
*
**
n.s.
Nonind.
Ind
(b)
IAA metabolites (IAA/12h)
***