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Identification of a Novel Putative Auxin Carrier Family and its role in Cellular Auxin Homeostasis

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Contents

Proloog

Charles Darwin bestudeerde op het einde van de 19e eeuw, samen met zijn zoon Francis, hoe coleoptielen (embryonale bladeren) van rietgras naar het licht toe groeien. Wanneer het bovenste topje van het blad werd afgeknipt of afgedekt, groeide het blad niet langer naar het licht toe. Een historisch experiment, geniaal in z'n eenvoud, die nog steeds dagelijks ontelbare keren bevestigd wordt want bijvoorbeeld ook steeltjes van afgeknipte basilicumblaadjes groeien niet langer naar het venster toe. Charles Darwin postuleerde in zijn boek "The Power of Movement in Plants" het bestaan van een stof die aangemaakt wordt in de tip van het blad en die, afhankelijk van het licht, naar beneden wordt getransporteerd. Die stof zou dan de groei van het blad naar het licht reguleren (Darwin, 1880). In 1926 kon de Nederlandse wetenschapper Fritz Went die stof isoleren en noemde deze auxine, naar het griekse "*auxein*" wat "groeien" betekent (Went, 1926).

Nederlandse samenvatting

In de natuur is het plantenhormoon auxine een essentiële regulator voor de groei en ontwikkeling van planten. Tientallen jaren intensief wetenschappelijk onderzoek bracht het belang van auxine voor vele ontwikkelingsprocessen in planten aan het licht. Enkele voorbeelden zijn embryovorming, ontwikkeling van nieuwe organen zoals zijwortels en zijtakken, fruitvorming en tropismen. Die laatsen zorgen ervoor dat wortels naar beneden groeien en scheuten naar het licht (beschreven in Vanneste and Friml, 2009). De betrokkenheid van deze eenvoudige molecule in zo veel verschillende processen vereist een strikte regulatie in iedere cel. Om de auxine-niveaus in elke cel op het juiste peil te houden hebben planten een aantal slimme strategieën ontwikkeld waarvan een overzicht wordt gegeven in hoofdstuk 1 (Beschreven in Barbez and Kleine-Vehn, 2013).

Dit eerste hoofdstuk beschrijft onder meer het belang van de lokale auxine-productie en -afbraak alsook het auxinetransport doorheen de plant. Dit laatste proces wordt gestuurd door auxinetransporteiwitten in het plasmamembraan rond de cel waardoor auxine van cel naar cel getransporteerd kan worden. De nadruk wordt gelegd op de recente ontdekking van vermoedelijke auxinetransporteiwitten op het membraan van het endoplasmatisch reticulum, een apart compartiment binnenin de cel. Deze ontdekking suggereert dat auxine niet enkel van cel naar cel wordt getransporteerd, maar ook tussen de verschillende compartimenten binnenin een cel.

Eén familie van vermoedelijke auxine-transporteiwitten, op de rand van dit apart compartiment in de cel, zijn de PIN-likes of PILS eiwitten die beschreven worden in hoofdstuk 2 (Barbez et al., 2012). Dit hoofdstuk beschrijft de ontdekking en de karakterisering van deze nieuwe eiwitfamilie in de veelgebruikte modelplant, de zandraket (*Arabidopsis*

thaliana). Computeranalyses van diverse andere plantgenomen tonen aan dat PILS-eiwitten voorkomen in zowat alle landplanten en dus ook in planten die belangrijk zijn voor de landbouw zoals graangewassen, groenten en fruit. De PILS-familie bestaat uit 7 eiwitten die elk een specifieke activiteit vertonen tijdens de verschillende groeistadia van de plant. Ons onderzoek toont aan dat PILS-eiwitten belangrijk zijn voor de groei en de ontwikkeling van planten gezien zaailingen waarin een aantal PILS eiwitten ontbreken, defecten vertonen in onder meer wortelgroei en zijwortelvorming. Bovendien zorgt PILS-activiteit voor een verminderde auxine signalisatie en het vasthouden van auxine in de cel. Deze resultaten doen vermoeden dat PILS-eiwitten het auxinetransport stimuleren van het cytoplasma (het hoofcompartiment van de cel) naar het endoplasmatisch reticulum, een apart compartiment in de cel. Dit mechanisme zou de plant in staat stellen om overmatige auxine-niveaus onschadelijk te maken door ze af te zonderen.

Tijdens het onderzoek naar de PILS-eiwitten werden we geconfronteerd met een aantal vaak voorkomende technische beperkingen die eigen zijn aan genetisch onderzoek. Om de locatie van een eiwit in de cel te bepalen kan men het gen (dit is het stukje DNA dat de code draagt voor het eiwit), isoleren en verlengen met een stukje DNA dat de code draagt voor een klein fluorescerend eiwit. Door een techniek die stabiele transformatie heet en destijds ontwikkeld werd door de bekende Belgische moleculaire biologen Jef Schell Marc Van Montagu, kan men het gemodificeerde stuk DNA terug in de plant brengen. De locatie van het eiwit met het fluorescerend verlengstukje in de cel, kan men nu bekijken onder een microscoop met blauw licht. Deze elegante methode heeft als nadeel dat ze veel tijd in beslag neemt. Het modificeren van een gen gaat relatief snel, maar een getransformeerde plant moet 2 tot 3 generaties (3 tot 6 maanden) lang groeien vooraleer die geanalyseerd kan worden. Een

stabiele transformatie van planten is de belangrijkste manier om de locatie en de functie van een eiwit te achterhalen. Toch werden er in het verleden reeds methodes beschreven voor het transiënt (of kortstondig) transformeren van planten. Deze transformatie, die enkel in een paar cellen van de plant plaatsvindt en voorbijgaand van aard is, gebeurt echter vrij snel zodat men de plant reeds na één of meerdere dagen kan analyseren. Een van deze technieken heet "particle bombardment" waarbij het gemodificeerde DNA op kleine goudpartikels wordt gekleefd, en dan onder grote druk (bombardement) in het plantweefsel wordt gebracht (Sanford et al., 1987; Klein et al, 1988). In hoofdstuk 3 beschrijf ik een toepassing van deze techniek waarbij we in tabak-celculturen, twee verschillende soorten DNA binnen brengen d.m.v. particle bombardment. Deze techniek maakt het mogelijk om snel na te gaan of een gen correct gemodificeerd werd, en ook waar het eiwit zich in de cel bevindt (aangezien er nu een fluorescerend eiwitje aan verbonden is). De techniek die we beschrijven geeft bovendien een initieel idee van het effect dat het bestudeerde eiwit heeft op de auxinehuishouding in de cel.

Naast de productie en de afbraak van het hormoon auxine, kunnen planten ook auxine verbinden met andere moleculen zoals suikers, eiwitten of aminozuren. Aminozuren zijn de bouwstenen waaruit eiwitten worden gevormd. Planten kunnen deze verbindingen, ook wel auxine-conjugaten genoemd, vormen om verschillende redenen (beschreven in Lüdwig-Müller et al., 2011). In zaden van de grove den *(Pinus sylvestris)* bevinden zich auxine-glucose conjugaten waarvan auxine wordt vrijgesteld tijdens het kiemen van de zaden (Ljung et al., 2001). Dit is een slimme strategie om snel het auxineniveau in bepaalde cellen aan te passen aan de noden van de plant. Toch is de reden waarom planten zoveel verschillende soorten conjugaten vormen tot op de dag van vandaag, nog grotendeels onbekend. De binding van auxine aan het

aminozuur aspartaat is onomkeerbaar, er kan dus geen auxine vrijgesteld worden uit dit conjugaat. Men vermoedt dat dit een strategie is van planten om een overmaat aan auxine onschadelijk te maken vooraleer het wordt afgebroken. Maar men weet niet of auxine-aspartaat nog andere functies vervult tijdens de groei en de ontwikkeling van planten. Hoofdstuk 4 gaat dieper in op het conjugaat, auxine-aspartaat of meer specifiek: indol-3-acetyl-aspartaat (IA-Asp). Hoewel voorgaand onderzoek suggereert dat IA-Asp een inactieve verbinding is (Bartel and Fink, 1995), tonen wij aan dat IA-Asp wel degelijk een effect heeft op de groei en de ontwikkeling van de modelplant *Arabidopsis thaliana*. IA-Asp zal net zoals vrij auxine wortelgroei remmen en het vormen van zijwortels stimuleren maar kan in tegenstelling tot vrij auxine, niet doorheen de plant getransporteerd worden. Ons onderzoek suggureert dat IA-Asp meer is dan een ‘vuilnisbak’-molecule en wel degelijk van belang is voor de groei en de ontwikkeling van planten. De zoektocht naar de exacte functie van IA-Asp gaat momenteel nog steeds verder maar kan bijdragen tot de kennis over hoe een eenvoudige molecule als auxine de groei en de ontwikkeling kan sturen van complexe levensvormen zoals planten. Planten die onmisbaar zijn voor de landbouw, de geneeskunde, de productie van groene energie maar ook als bouwstof voor meubels, huizen, kledij...

Ik hoop dat ik met dit werk een steentje kan bijdragen aan de kennis over auxine en zijn rol in de groei en de ontwikkeling van planten.

Scope

Scope

Since more than a century, the plant hormone auxin is known to be indispensable for plant growth and development. Many years of research unveiled that the correct transport/distribution of auxin through the plant, is crucial for its function as a regulator of many physiological processes (reviewed in Vanneste and Friml, 2009; reviewed in Sauer et al., 2013). Auxin transport through plant tissues is mediated by a multitude of auxin transport carriers at the plasma membrane (reviewed in Zazimalova et al., 2010). However, also on subcellular level auxinic compounds have been suggested to require transport across organelle membranes (e.g. peroxisomes and chloroplasts), but the underlying carriers are elusive (Sauer et al., 2013). Moreover, recent data revealed the presence of auxin transport carriers at the endoplasmic reticulum (Mravec et al., 2009; Bosco et al., 2012; Ding et al., 2012). Considering such a high subcellular complexity of auxin biology, it is to be expected that yet unknown, putative auxin transport carriers contribute to the establishment and maintenance of cellular auxin homeostasis.

We intended to screen for proteins that resemble the predicted protein topology of the prominent PIN auxin carriers. The online SMART tool of EMBL enables to predict the topology of a protein of interest as well as to screen for proteins with a similar predicted protein topology. Our *in silico* screen has led to the identification of an uncharacterized protein family, which we later called the PIN-likes (PILS) proteins. Although PIN and PILS proteins do not share high levels of amino acid sequence similarities, both families show very similar predicted protein domain structures. Moreover, both families carry a computationally defined auxin transport InterPro domain pinpointing a potential auxin transport function. Based on these findings, we hypothesized that PILS proteins are putative auxin carriers.

The aim of my Master- and subsequent PhD project was to characterize this new protein family and to unravel its role in plant growth and development.

Brief Summary

The phytohormone auxin is an essential regulatory substance for adaptive plant growth and development. Decades of intensive research revealed the importance of auxin in many plant developmental processes such as embryonic apical-basal axis formation, vascular development, postembryonic organogenesis, tropistic growth, fruit development, stress response, senescence, and apical dominance (reviewed in Vanneste and Friml, 2009). These multitude of responses require defined regulation of cellular auxin levels. To meet these requirements, plants developed a number of strategies from which an overview is given in **chapter 1** (Barbez and Kleine-Vehn, 2013). Chapter 1 reviews the importance of polar auxin distribution through the plant as well as its local metabolism. We highlight the recent identification of putative auxin transport carriers at the endoplasmic reticulum (ER), pinpointing the importance of auxin transport between the different compartments within the cell.

We identified and characterized the PIN-LIKES (PILS) putative auxin carriers in *Arabidopsis thaliana* (Barbez et al., 2012), which are addressed in **chapter 2**. We show that PILS proteins localize at the ER, display specific expression patterns in different growth stages and are involved in several plant developmental processes, including main root growth and lateral root development. Our data suggests that PILS proteins regulate cellular responsiveness to auxin and influence intracellular auxin uptake and metabolism. This indicates an additional complexity in the regulation of cellular auxin homeostasis.

Chapter 2 shows that the activity of PILS2 and PILS5 decreases the levels of nuclear auxin signaling. However, the PILS protein family contains seven members, which might not all have the same working mechanism. In order to efficiently address the activity of the different PILS proteins as well as to confirm the functionality of fusion proteins, we

developed a method, which is described in **chapter 3** (Barbez et al., 2013). The presented method assumes that auxin carrier activity affects cellular auxin homeostasis and, hence, also auxin signaling. The construct of interest gets transiently co-transformed in tobacco Bright Yellow-2 (BY-2) cells, with a construct containing the synthetic highly auxin responsive promoter DR5rev fused with RED FLUORESCENT PROTEIN (RFP) (Marin et al., 2010). The difference in *DR5rev:RFP* signal intensity between those BY-2 cells and BY-2 cells co-transformed with *DR5rev::RFP* and the inert ER-marker *HDEL-GFP*, gives an idea how putative carrier activity affects cellular auxin response.

PILS activity has an impact on the levels of cellular free indole-3-acetic acid (IAA), the most abundant auxin in *A. thaliana*, via its conjugation to for instance amino acids (chapter 2). It has been previously described that the majority of cellular auxin appears in a non-free form, mainly bound to sugars, peptides, proteins or, single amino acids (reviewed in Lüdwig-Müller, 2011). The biological relevance of this high number of different auxin-appearances is until today only poorly understood. Most of the identified auxin conjugates are assumed to be IAA-storage molecules or intermediates of auxin degradation and, therefore, biologically inactive (reviewed in Lüdwig-Müller, 2011). However, in **Chapter 4**, we show preliminary unpublished data suggesting that the non-hydrolysable auxin amide conjugate indole-3-aspartic-acid (IA-Asp) displays biological activity in the model plant *Arabidopsis thaliana*. Exogenous application of IA-Asp affects seedling growth, which is partially due to its effect on nuclear auxin signaling mediated by the well described auxin receptor TRANSPORT INHIBITOR RESPONSE 1 (TIR1).

The 4 upcoming chapters give insight into the recently uncovered PILS-protein mediated intracellular auxin transport and its effect on cellular auxin homeostasis (Chapter 2). This novel mechanism pinpoints towards the existence of compartmentalized auxin metabolism (Chapter 1). Moreover, the observation that not only free IAA but also IAA metabolites might show biological activity (Chapter 4) could shed new light on the complex nature of auxin biology.

Chapter 1

General introduction

Modified from

Divide Et Impera

Cellular Auxin Compartmentalization

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Current Opinion in Plant Biology, 2013

&

Cellular auxin homeostasis:

Gatekeeping is housekeeping

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Current opinion in Plant Biology: EB wrote and JKV edited the manuscript.

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Abstract

The phytohormone auxin is an essential regulator for plant growth and development. Decades of intensive research revealed the mutual importance of auxin metabolism and intercellular cell-to-cell transport for the regulation of spatiotemporal auxin distribution. Just recently, intracellular putative auxin carriers, such as the PIN-FORMED (PIN)5/PIN8 and the PIN-LIKES (PILS)2/PILS5 were discovered at the endoplasmic reticulum and seem to limit nuclear auxin signalling via an auxin sequestration mechanism. Moreover, these auxin carriers at the endoplasmic reticulum (ER) might provide a link between auxin compartmentalization and auxin conjugation-based metabolism.

Here we review the recent findings on auxin compartmentalization at the ER and discuss its potential contribution to cellular auxin homeostasis and its importance for plant development.

Auxin and its perception

At the end of the 19th century, the German botanist Julius von Sachs postulated in his *'Theorie der organbildende Stoffe'* the existence of moving factors enabling cell-to-cell communication in plants (von Sachs, 1880). By studying tropisms in Canary grass, Charles Darwin and his son Francis anticipated in the same period a growth regulatory compound, which is transported through the plant (Darwin and Darwin, 1881). This growth substance was later on identified as the first plant hormone called auxin (Went, 1926). More than 100 years of research revealed the importance of auxin in a multitude of plant developmental processes, such as embryogenesis, vascular development, post embryonic organogenesis such as lateral root formation, tropisms, apical dominance, stress response and senescence (Sundberg and Ostergaard, 2009; Grunewald and Friml, 2010; Peris et al., 2010; Scarpella et al., 2010; Zazimalova et al., 2010).

Indole-3-acetic acid (IAA) is the most abundant natural auxin, but also others, such as indole-3-butyric acid (IBA), 4-chloro-indole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA), are naturally occurring in plants (Woodward and Bartel, 2005; Simon and Petrasek., 2010; Strader and Bartel, 2011). Auxin seems to exert most of its action by binding to the nuclear receptors TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) proteins (for detailed reviews, see Leyser, 2006; Chapman and Estelle, 2009). Auxin stabilizes the co-receptor complex of TIR1/AFB and AUXIN RESISTANT/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) proteins, which triggers the proteasome-dependent degradation of the Aux/IAA transcriptional regulators. The Aux/IAAs regulate auxin-dependent gene transcription by forming dimers with the AUXIN RESPONSE FACTOR (ARF) proteins. The auxin-dependent release of the ARF transcription factors leads to the

onset of auxin-mediated transcriptional reprogramming (for a review, see Chapman and Estelle, 2009).

Given the relatively simple nature of this signaling cascade, the power of auxin to guide such a variety of developmental aspects is still puzzling. The existence of multiple Aux/IAA-ARF combinations may provide a platform for specific responses (De Smet et al., 2010). Additionally, the auxin-signaling cascade could be specified by differences in the binding affinity of particular TIR1/AFB-Aux/IAA co-receptor combinations, thus possibly resulting in auxin concentration-dependent transcriptional outputs. In fact, auxin has been suggested to act as a threshold-specific trigger of plant development (Cheng et al., 2006; Lau et al., 2011). Further complexity might result from the binding of auxin to a particular AFB subpopulation, which possibly represses auxin signaling (Greenham et al., 2011). Other auxin receptors, such as the AUXIN BINDING PROTEIN 1 (ABP1) (Jones and Venis, 1989) and the S-PHASE KINASE-ASSOCIATED PROTEIN 2A (SKP2A) (Jurado et al., 2010), additionally contribute to rapid non-genomic reactions and to the regulation of the cell cycle, respectively. However, the underlying auxin receptor crosstalk and its interweaved contribution to plant development is largely unknown (reviewed in Sauer and Kleine-Vehn, 2011). Finally, the tissue-specific distribution of all these auxin-signaling components may largely determine the cellular sensitivity to the hormone. Such a complex signaling scenario could lead to concentration and cell-type-dependent cellular responses.

These concentration-dependent perception mechanisms would require tightly controlled cellular auxin levels. Auxin maxima and even graded auxin concentration have been repeatedly suggested in various plant tissues (Kuhlemeier, 2007; Benjamins and Scheres, 2008; Grunewald and Friml, 2010). However, most findings are based on the

synthetic auxin responsive promoter element DR5 or the novel auxin-signaling sensor DII (Vernoux et al., 2011) and direct experimental proof for auxin gradients and its morphogen-like action is still missing. Nevertheless, defined, local auxin signaling maxima and minima control various developmental processes, including embryonic axis establishment, tropic growth, lateral organ initiation, fruit formation, root meristem patterning, tissue regeneration and vascular tissue differentiation (Sundberg and Østergaard, 2009; Grunewald and Friml, 2010; Llavata Peris et al., 2010; Scarpella et al., 2010; Zažímalová et al., 2010; Zhao, 2010).

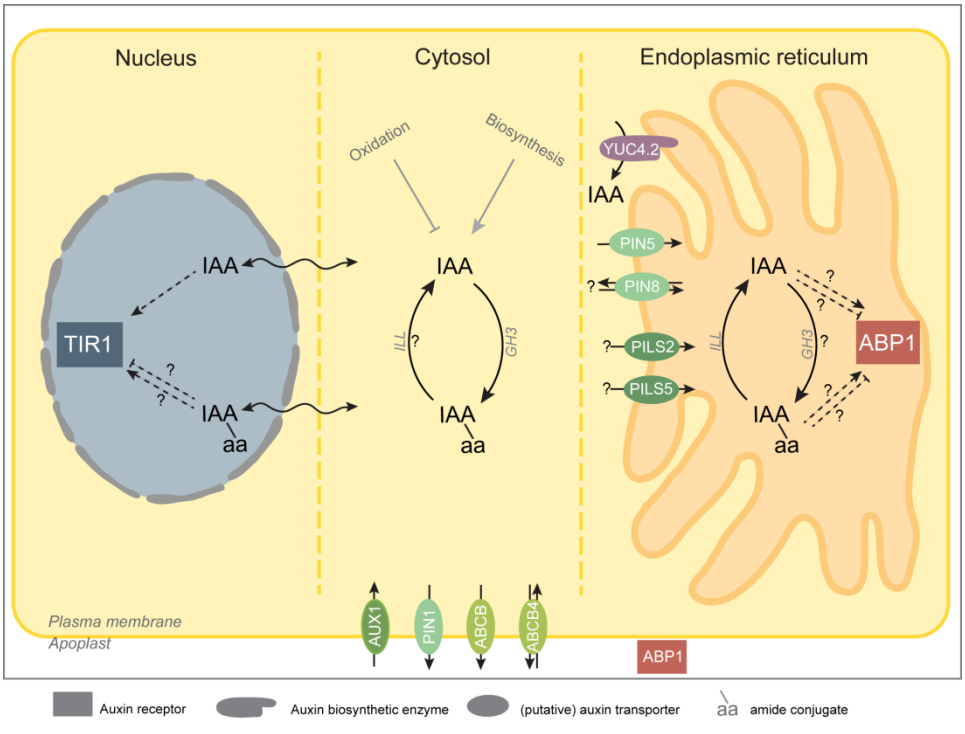

Figure 1. Hypothetical model on the carrier dependent assignment of distinct auxin pools in the nucleus, cytosol and endoplasmic reticulum.

Straight arrows depict carrier mediated auxin transport while wavy lines represent the passive diffusion of auxin into the nucleus. Enzymatic activity is given by bended arrows and dashed lines depict the possible binding of auxin (conjugates) on the auxin receptors TIR1 or ABP1.

Abbreviations: TIR1: TRANSPORT INHIBITOR RESPONSE1; IAA: Indole-3-acetic acid; GH3: GRETCHEN HAGEN 3; ILL: IAA-LEUCINE RESISTANT1-like; AUX1: AUXIN RESISTANT1; YUC4.2: YUCCA4.2; PIN: PIN-FORMED; ABCB: ATP BINDING CASSETTE B; PILS: PIN-LIKES; ABP1: AUXIN BINDING PROTEIN1.

Auxin distribution

Taken into account that auxin is involved in almost all plant developmental processes, not only auxin signaling, but also the levels of cellular auxin need to be strictly controlled. During the entire life time of a plant, auxin will form local auxin maxima and minima, which trigger the onset of developmental and physiological processes, such as new organ formation (Benkova et al., 2003; Sorefan et al., 2009). Therefore, plant cells developed a multitude of strategies to establish defined auxin levels in particular cell types. Research efforts in the past decades mainly focused on cellular auxin metabolism and intercellular (cell-to-cell) auxin transport as means to fine tune cellular auxin levels. However, the recent identification of two distinct protein families of putative auxin transport carriers within the cell pinpoints the importance of intracellular auxin compartmentalization for cellular auxin homeostasis (Mravec et al., 2009; Barbez et al., 2012). The carrier-dependent sequestration of auxin seems to enable compartmentalized auxin metabolism and limits availability of auxin for nuclear signaling. Here we shortly review the general aspects of auxin metabolism and intercellular auxin transport. Afterwards we focus on the recently discovered cellular auxin compartmentalization in the ER (Figure1), which is of unexpected importance for plant growth and development.

Auxin metabolism

IAA is structurally related to tryptophane (Trp) and its biosynthesis occurs either via several Trp-dependent pathways or via a Trp-independent pathway (Normanly, 2010; Zhao, 2010). The distinct Trp-dependent auxin biosynthesis pathways are distinguished based on their major intermediates (Zhao, 2010; Zhao, 2012). Intrinsic and extrinsic factors will define the activity of the respective biosynthetic pathways and overall rate of auxin production, ultimately contributing to developmental and environmental auxin responses (Ruiz Rosquete et al., 2011). Auxin biosynthesis mainly takes place in aerial tissues, such as apical meristems and young leaves (Ljung et al., 2001), however, more recent studies revealed the developmental importance of local auxin biosynthesis in various tissues (Ljung et al., 2005: Cheng et al., 2006; Cheng et al., 2007; Pagnussat et al., 2009; Chandler et al., 2009).

Cellular IAA is mainly present as amide derivatives and to lower extent as ester-linked conjugates in *Arabidopsis thaliana* (Tam et al., 2000). Most common auxin conjugates in *Arabidopsis* are IA-Ala, IA-Leu,, IA-Asp, IA-Glu, IA-glucose, and protein/peptide conjugates (Sztein et al, 1995; Tam et al., 2000; Kowalczyk and Sandberg, 2001; Ljung et al., 2002; Seidel et al., 2006).

Auxin conjugates are generally considered as temporary reservoirs of inactive IAA, releasing the free active hormone upon hydrolysis (Figure 2) (Fluck et al., 2000; Jakubowska and Kowalczyk, 2005). However, conjugation to particular moieties, such as Asp and Glu, seems to be irreversible (Östin et al, 1998; Ljung et al., 2001b; Rampey et al., 2004), eventually implying alternative functions for these auxin conjugates.

In *Arabidopsis thaliana*, IAA conjugation with glucose requires the UDP-glucose transferase UGT84B1 (Jackson et al., 2001). IBA-glucose is formed via another hydrogen peroxide-inducible UGT74E2 (Tognetti et al., 2010). However, not much is known about the enzymes involved in auxin conjugation with sugar molecules. ATP-dependent synthesis of auxin amide conjugates is catalyzed by members of the Gretchen Hagen 3 (GH3) family of synthetases (Staswick et al. 2005). The *GH3* genes are induced by auxin or auxinic herbicides (Hagen and Guilfoyle, 1985; Kelley et al., 2004) and after infections with plant pathogens that increase cellular auxin levels (Deeken et al., 2006; Siemens et al., 2006), hinting at a regulatory loop to balance elevated free IAA levels. The importance of GH3 proteins during plant development, as well as during stress and defense responses, has been documented (for a review, see Ludwig-Müller, 2011). The *GH3/WES1* overexpressors display developmental defects, such as reduced hypocotyl and root growth. *WES1* expression is under the control of the stress hormones salicylic acid and abscisic acid and is induced by stress conditions, such as drought, high salinity, or cold (Park et al., 2007). A *WES1* gain-of-function mutant is resistant to both biotic and abiotic stresses, whereas loss-of-function mutants showed a reduced stress resistance (Park et al., 2007), suggesting that auxin conjugation is involved in protective mechanism against stressful conditions.

Most auxin conjugations are reversible via the action of specific hydrolases with the subsequent release of free IAA. Whereas little is known about the hydrolysis of auxin sugar conjugates, the process is better understood for amino-acidic conjugates. Members of the IAA-LEUCINE RESISTANT 1 (ILR1)-like family of IAA amidohydrolases regulate free cellular auxin levels by deconjugating auxin amides (Figure

2) (Bartel and Fink, 1995; Davies et al., 1999; LeClere et al., 2002). These enzymes play an important role during plant development, as suggested by their dynamic and differential expression patterns and the phenotypes of multiple mutants in *Arabidopsis* (Rampey et al., 2004). Additional evidence for their developmental impact comes from the rapid hydrolysis of auxin amide conjugates in seeds in response to imbibition (Bialek and Cohen, 1992). The regulation of conjugate hydrolases has received special interest in particular contexts, such as during auxin-mediated symbiosis and nodule organogenesis (Campanella et al., 2008). Jasmonic acid activates auxin conjugate hydrolases, thereby increasing the release of free IAA (Davies et al. 1999) and it is tempting to speculate that also other phytohormones could regulate free IAA levels via these pathways.

The regulation of auxin conjugation and subsequent hydrolysis is still molecularly ill defined, but seems to have fundamental importance to fine-tune auxin responses (Figure 2).

Notably, conjugates have been also described for the endogenous auxin IBA (Epstein and Lüdwig-Müller, 1993). In contrast to IAA, ester-linked IBA-conjugates prevail over amide derivatives in *Arabidopsis* (Ludwig-Müller et al., 1993). The physiological importance of IBA remains a matter of controversy that also applies for its corresponding conjugates. IBA might either act/signal independently or might mainly function as IAA storage/precursor form (Ludwig-Müller, 2000; Woodward and Bartel, 2005).

Different species and even plant organs have distinct profiles of IAA conjugates (Barratt et al., 1999; Walz et al., 2008; Bajguz and Piotrowska, 2009). In maize kernels, ester-conjugates are most abundant including IA-glucose and IA-myo-inositol (Bandurski et al., 1995). Ljung et al also described in 2001 the presence of ester conjugates in the seeds of

Scot Pine. These conjugates get hydrolyzed during germination which contributes to the increased free IAA levels (Ljung et al., 2001). It is difficult to envision that such a wide spectrum of derivatives act mainly as storage molecules or catabolic precursors. In non-vascular plants, such as mosses, auxin conjugation seems to be slower, less abundant and the pathways display lower substrate specificity compared to those in vascular plants (Sztein et al., 1995, 1999; Rensing et al., 2008). *In silico* studies suggest even the absence of *GH3* orthologues in unicellular algae (*Chlorophyta* lineage), despite the presence of orthologues of the IAA amidohydrolases (De Smet et al., 2011). The elaborate auxin conjugation machinery in higher plants compared to lower plants may suggest the evolution of (an) additional function(s). One potential role for IAA conjugates could be to antagonize auxin effects. IA-Ala treatments inhibit IAA-induced shoot growth and root initiation in tomato (*Solanum lycopersicum*) cell cultures (Magnus et al., 1992). Furthermore, IA-Trp has been shown to confer resistance to IAA, IBA and the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) (Staswick, 2009). The functional importance of non-hydrolysable conjugates, such as IA-Asp and IA-Glu, (Kowalczyck and Sandberg, 2001; Ljung et al., 2002) remains to be addressed. Besides the potential effects on IAA steady states, it is tempting to speculate that some IAA conjugates may compete with the free hormone for common binding site(s) in transporters and/or receptors, or might even directly regulate rate-limiting steps in auxin metabolism.

IAA catabolism has been proposed to occur either by an oxidative decarboxylation pathway, leading to modifications of both the side chain and the indole ring (Barceló et al., 1990; Östin et al., 1995; Reinecke and Bandurski, 1987), or through non-decarboxylative oxidation of the indole nucleus (Östin et al., 1998). The non-decarboylative pathway

reduces free IAA, but also initiates the degradation of IA-Asp conjugates (Tuominen et al., 1994; Riov and Bangerth, 1992) (Figure 2). Hence, auxin derivatization to IA-Asp may represent another metabolic strategy to "en route" excessive auxin into catabolic pathways. However, it remains to be seen whether the oxidation of IA-Asp has distinct regulatory functions compared to the direct oxidation of free-IAA. Also the oxidation of other conjugates, such as oxIAA-hexose, might be linked to auxin catabolism (Östin et al., 1998).

Oxidative degradation of auxin appears to be developmentally important, such as during fruit ripening (Frenkel et al., 1975; Purgatto et al., 2002) and during plant responses to oxidative stress (Jansen et al., 2001; Chaoui and El Ferjani, 2005; Tognetti et al., 2011). However, the cellular mechanisms that control the oxidative degradation of auxin remain to date poorly understood.

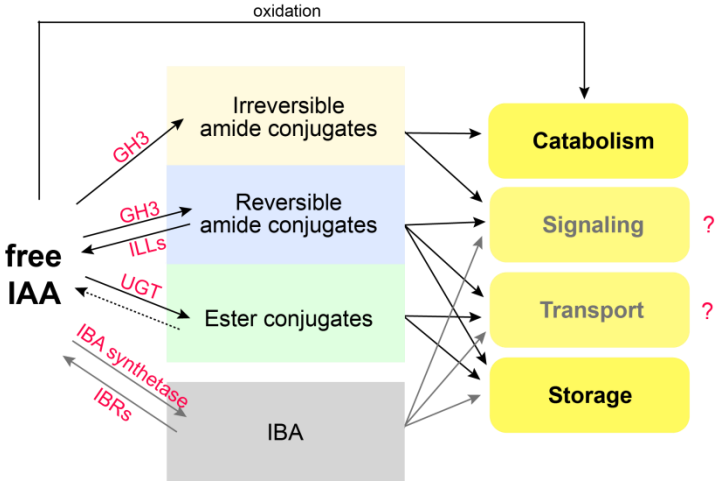

Figure 2. Conversion and inactivation of auxin

Active free IAA levels can be modulated via conversion into IBA or IAA conjugation to amides or esters. Both conjugation and conversion to IBA might provide a mechanism for the temporary storage of reversibly inactivated auxins. Free IAA and amide conjugates (irreversible), such as IA-Asp and IA-Glu, eventually undergo oxidation-

based degradation. Besides catabolism and storage of auxin, conversion into IBA and IAA conjugation could also be implicated and important for auxin signaling and transport.

Asp: Aspartic acid; Glu: Glutamic acid; GH3: GRETCHEN HAGEN 3; IAA: Indole-3-acetic acid; ILL: IAA-LEUCINE RESISTANT (ILR)-LIKE; IBA: Indole-3-butyric acid; IBR: INDOLE-3-BUTYRIC ACID RESPONSE; UGT: UDP-GLUCOSYL TRANSFERASE.

Intercellular auxin transport

Defined carrier-dependent spatiotemporal distribution of auxin in various plant tissues is essential for plant development as suggested by the multiple developmental defects in auxin transport mutants and upon pharmacological interference with the auxin transport machinery (Bennet et al., 1996; Gälweiler et al., 1998; Luschnig et al., 1998; Friml et al., 2002, Friml et al., 2002b; Murphy et al., 2002). Auxin transport through the plant occurs either via a long-distance transport through the phloem (Friml and Palme, 2002) or via a strictly regulated directional (polar) cell-to-cell transport mediated by a multitude of auxin transport carriers at the plasma membrane, such as the AUXIN RESISTANT1/LIKE AUX1 (AUX1/LAX) influx carriers, the ATP BINDING CASSETTE (ABC) transporters of the MULTIDRUG RESISTANCE (MDR) subfamily and the PIN-FORMED (PIN) auxin efflux carriers (Bennet et al., 1996; Luschnig et al., 1998; Geisler et al., 2005) (Figure 1). The last decades thoroughly boosted our knowledge and understanding of polar auxin transport during plant development. For further depiction of polar auxin transport, we refer to several excellent reviews on this topic (Zazimalova et al., 2010; Peer et al., 2011; Kramer and Bennet, 2006).

Intracellular auxin transport

ER localized PIN-proteins

PIN1-type (PIN1-PIN4 and PIN7) auxin efflux carriers regulate intercellular auxin transport at the plasma membrane, while PIN5 and PIN8 function at the ER (Mravec et al., 2009; Bosco et al., 2012; Ding et al., 2012) (Figure 1). Notably, the role of PIN6 remains to be elucidated. PIN5 activity increases cellular auxin retention in *A. thaliana* protoplasts presumably by catalyzing auxin transport from the cytosol into the ER lumen (Mravec et al., 2009). *PIN5* is abundantly expressed *in planta* and loss- and gain-of-function mutants display defects in auxin-dependent processes including root- and hypocotyl growth and lateral root development (Mravec et al., 2009). Moreover, PIN5 activity decreases free IAA and increases the cellular levels of auxin amino conjugates, such as IA-Asp and IA-Glu and the ester conjugate IA-Glc, suggesting a role for PIN5 in possibly compartmentalized auxin metabolism (Figure 1).

The pollen specific PIN8 also localizes to the ER and got recently characterized by two independent research groups (Bosco et al., 2012; Ding et al., 2012). Ectopic expression of PIN8 (under the strong viral constitutive 35S promoter) causes auxin related phenotypes in *N. tabacum* and *A. thaliana*, such as deviating root, root hair and hypocotyl length, as well as altered flowering time (Bosco et al., 2012; Ding et al., 2012). Ding and colleagues show that the *pin5* mutation largely rescues the *A. thaliana pin8* pollen defects and also *PIN5* overexpression equalizes the *PIN8* overexpression phenotypes, indicating antagonistic roles for PIN5 and PIN8 (Ding et al., 2012). In alignment with this genetic interaction data, ectopic PIN8 activity decreases cellular auxin accumulation in *A. thaliana* protoplasts and reduces the cellular levels of IA-Asp and IA-Glu, which is opposite to the effect of PIN5 (Ding et al., 2012).

However, Ding et al. and Bosco et al. describe that PIN8 overexpression either enhances (Ding et al., 2012) or reduces (Bosco et al., 2012) nuclear auxin signaling in *Arabidopsis* root tips, respectively. One could speculate that the different PIN8 overexpression levels in the respective lines may have an impact on the PIN8 transport direction/activity. Dynamics in auxin transport directionality have been previously described for the auxin transport carrier ABCB4 that alters its transport direction according to the cellular auxin levels (Yang et al., 2009) (Figure 1). Ding et al. observed higher free IAA levels in leaves, hypocotyls and roots of *35S:PIN8-GFP* expressing *A. thaliana* plants. Bosco et al. also revealed higher free IAA levels in leaves but lower free IAA levels in roots of *35S:PIN8-GFP* expressing *N. tabacum* plants. These contrasting results suggest a complex function of PIN8 in plant development and the underlying mechanism still needs to be elucidated.

PILS proteins

An *in silico* screen for proteins with a predicted protein topology similar to PIN proteins enabled the recent identification and initial characterization of the PILS protein family of putative auxin carriers at the ER (Barbez et al., 2012) (Figure 1). Despite the similarities in predicted protein topology, PIN and PILS proteins display only limited sequence similarities and bioinformatics analysis revealed the evolution of PILS proteins in plants to be distinct from PIN proteins (Figure 3) (Feraru et al., 2012; Viaene et al., 2012). Most *PILS* genes are abundantly expressed in roots and/or shoot tissues and *pils2/pils5* loss- and gain-of-function mutants display auxin related phenotypes, including defects in hypocotyl/root growth and postembryonic organogenesis (Barbez et al., 2012). Similar to PIN5, PILS2 and PILS5 activity at the ER increases

cellular auxin accumulation, but reduces nuclear auxin signaling. Hence, PILS2 and PILS5 might facilitate the sequestration of cytosolic auxin into the ER where it is unavailable for nuclear auxin signaling (Barbez et al., 2012) (Figure 1). Moreover, PILS2 and PILS5 proteins affect free IAA to auxin conjugate (IA-Asp and IA-Glu) ratios, indicating a regulatory role for PILS2 and PILS5 in cellular auxin metabolism (Barbez et al., 2012) (Figure 1). The in depth characterization of the individual PILS1-PILS7 proteins will reveal their specific roles in cellular auxin homeostasis and their importance for plant development.

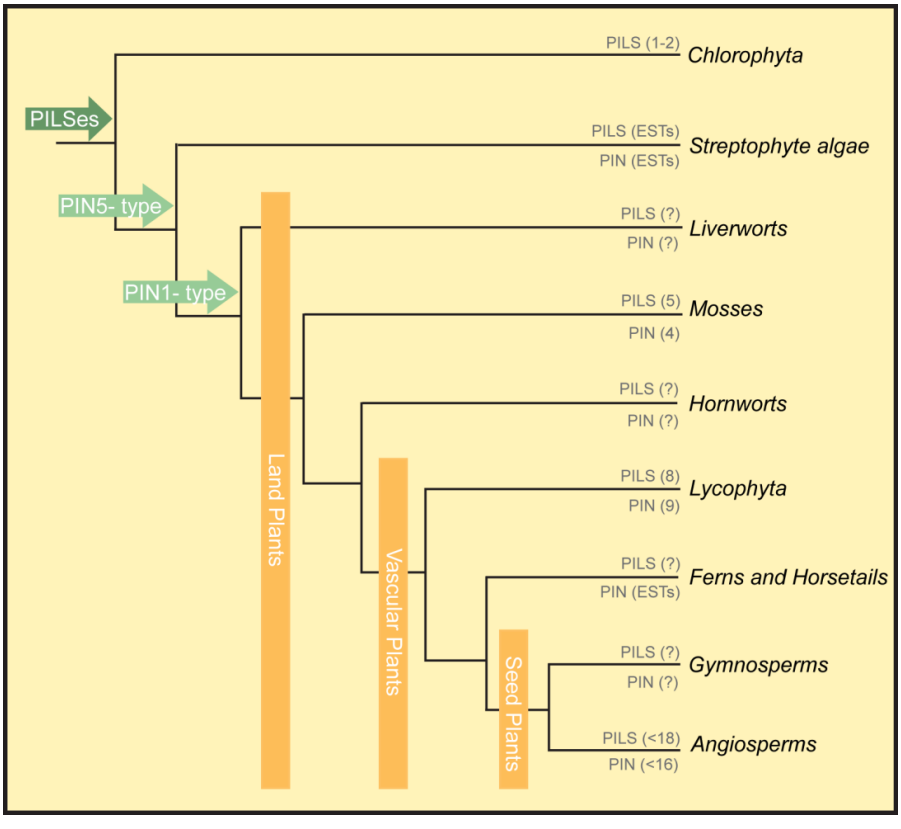

Figure 3. Depiction of the evolutionary origin of the PILS- and the two classes of PIN-genes (arrows) according to Feraru et al., 2012 and Viaene et al., 2012. The current known number of PIN and PILS genes (or the existence of ESTs) for each lineage are given in brackets. A question mark indicates the lack of sequence data for this particular lineage.

Compartmentalized auxin metabolism

PIN5, PILS2 and PILS5 activity at the ER increases cellular auxin accumulation and seem to increase the rate of auxin conjugation presumably by auxin transport from the cytosol into the ER (Mravec et al., 2009; Barbez et al., 2012) (Figure 1). PIN8 counteracts PIN5 activity possibly by decreasing cellular auxin compartmentalization and auxin conjugation rates (Ding et al., 2012). Considering this current model on intracellular auxin transport and its link to auxin metabolism, we may speculate that auxin conjugation takes place in the ER. Until now, the localization of the auxin conjugases from the GH3 family has not been experimentally addressed in *A. thaliana*, however, the *Physcomitrella patens* PpGH3.1-GFP protein fusion displays a cytosolic localization (Ludwig-Müller et al., 2009, Ludwig-Müller et al., 2009b). However, GH3 functionality in the cytosol remains to be demonstrated and it still needs to be seen whether GH3 proteins (or so far unknown auxin conjugating enzymes) reside in the ER of higher plants. On the other hand, *in silico* analysis of the ILR1-like amidohydrolases revealed that some family members carry an ER-retention motif (Campanella et al., 2003; Bitto et al., 2009). Experimental elucidation of the subcellular localization of auxin conjugating and hydrolyzing enzymes will contribute to our mechanistic understanding of how auxin metabolism is compartmentalized within the cell (Figure 1).

Nevertheless, there are additional evidences that the ER could have particular importance for cellular auxin homeostasis/metabolism. YUCCA4 is involved in tryptophan-dependent auxin biosynthesis and a particular splice variant localizes to the outer surface of the ER (Kriechbaumer et al., 2012) (Figure 1). The functional importance remains so far unclear, but YUCCA4 activity at the ER surface might supply auxin

for transportation into the ER. Moreover, the auxin receptor ABP1 resides to a large extent in the ER (Jones et al., 1993; Henderson et al., 1997). Although ABP1 has poor *in vitro* auxin binding capacity in the presumed pH range of the ER (Tian et al., 1995), it has to be investigated whether ABP1 has a function in the ER with a potential link to compartmentalized auxin metabolism. Further work on auxin biosynthesis, transport, metabolism and perception in and around the ER is needed to depict the importance of this organelle for cellular auxin homeostasis and signaling.

The endoplasmic reticulum (ER) is an indispensable organelle in eukaryotic organisms and is responsible for posttranslational modifications of proteins as well as their correct entrance into the secretory pathway which will target them to their final destinations (Bassham et al., 2008). Besides the recently uncovered importance of this compartment for auxin biology, many more ER-membrane resident proteins have been repeatedly described to be involved in a wide spectrum of plant developmental processes. The receptors of the phytohormone cytokinin are described to localize mainly at the ER in *Arabidopsis thaliana* (Wulfetange et al., 2011). Also the perception and signal transduction of the plant hormone ethylene partially takes place at the membrane of the ER (Qiao et al., 2012; reviewed in Ji et al., 2013). Besides, the ER hosts several members of the aquaporin family, which is a large protein family of integral membrane proteins mainly facilitating the passage of water molecules over membranes (reviewed in Maeshima and Ishikawa., 2008). The PLASMAMEMBRANE INTRINSIC PROTEIN 1;1 (RhPIP1;1), from the aquaporin family, resides in the ER membrane and is important for ethylene regulated petal expansion in rose, even though RhPIP1;1 does not seem to mediate water transport (Chen et al., 2013). However, members of the other ER localized small and basic intrinsic proteins (SIP)-subclass of the aquaporin family are shown to

possess water channel activity when heterologously expressed in yeast (Ishikawa et al., 2005). Other described transport proteins at the ER are: MALE GAMETOGENESIS IMPAIRED ANTHERS (MIA), a P-type ATPase important for pollen development (Jakobsen et al., 2005), ER-TYPE CA2+-ATPASE 1 (ECA1) which is required to confer tolerance to exogenous Mn(2+) (Wu et al. 2002), CALCIUM ATPASE 2 (ACA2) (Hong et al., 1999) as well as the fatty acid transporter AtABCA9 supplying fatty acids for lipid synthesis in the ER (Kim et al., 2013).

The presence of a high number of important proteins at the ER, emphasizes the central role of this organelle for plant growth and development.

Conclusion

The postulation of Julius von Sachs together with Darwin's pioneering experiments, more than 100 years ago, anticipated the presence of moving compounds through the plant required for its correct growth and development. On the contrary, the complex nature of intracellular auxin compartmentalization remained all those years entirely unforeseen and got just recently uncovered. The gross of auxin molecules seem to be assigned into i.) nuclear, ii.) cytosolic, and iii.) ER auxin pools and might enable defined signaling events (Figure1). Such a subdivision is reminiscent to the old maxim *divide et impera* (divide and rule), used by many ancient emperors including Julius Caesar and Napoleon. *Divide et impera* depicts a political and military strategy by which a bigger population gets divided into smaller subgroups in order to structure and/or maintain the regency. Such a comparison is particularly intriguing, because of the existence of nuclear (TIR1) and mainly ER (ABP1) localized auxin receptors (Figure1). These receptors could read out the distinct auxin pools in the nucleus and the ER. The cytosol connects both pools and in this regard

also the auxin carrier activity at the plasma membrane influences both auxin signaling and homeostasis.

PIN5, PILS2 and PILS5 activity down regulates nuclear auxin signaling. However, it still needs to be seen whether this effect on auxin signaling is solely due to the decreased levels of cytosolic auxin (available for diffusion in the nucleus) or whether certain signaling events in the ER might affect nuclear auxin signaling. Another open question concerns the direct or indirect impact of carrier-dependent changes in the auxin metabolite profile and its contribution to cellular auxin responses. It needs to be seen whether only the carrier induced reduction in free auxin levels or whether also the increased auxin conjugate levels contribute to the auxin signaling output (Figure1).

Besides, it is interesting to question whether polar and/or intracellular auxin transport are conserved during evolution or whether they are rather recent acquisitions. PIN orthologues are found in streptophyte algae and land plants (Figure 2) (Mravec et al., 2009; Viaene et al., 2012). Moss PpPINs exclusively localize to the ER when expressed in tobacco BY-2 cells, indicating PIN mediated intracellular auxin transport to be evolutionary older than PIN mediated cell-to-cell auxin transport (Mravec et al., 2009). Compared to PINs, the PILS protein family is also conserved in evolutionary older species, including the unicellular algae *Ostreococcis tauri* and *Chlamydomonas reinhardtii* (Barbez et al., 2012) (Figure 2). In accordance, phylogenetic analyses suggest that the PILS proteins originated earlier than the PINs (Barbez et al., 2012; Feraru et al., 2012; Viaene et al., 2012). Hence, PILS-dependent intracellular auxin compartmentalization appears to be evolutionary older than PIN-dependent intra- and intercellular auxin transport (Barbez et al., 2009; Mravec et al., 2009) (Figure 2). Moreover, computer simulations

revealed the remarkable possibility that auxin sequestration in the ER might have contributed to the evolution of directional, spatiotemporal auxin distribution through plant tissues and, hence, might have evolutionary importance for vascular development (Wabnik et al., 2011). Further investigation of the auxin transport and metabolism machinery in evolutionary older species, such as mosses and algae, will unveil the evolutionary conserved importance of intracellular auxin transport for auxin homeostasis.

Here we reviewed the current knowledge on carrier-mediated auxin compartmentalization at the ER. Although the exact mechanism of how intracellular auxin transporters regulate cellular auxin homeostasis still needs to be unraveled, recent findings implicate an unexpected molecular complexity and importance for plant growth and development.

Acknowledgements

We apologize to our colleagues whose work have been inadvertently omitted or could not be reviewed in depth. We are grateful to the Wiener-, Wissenschafts-, Forschungs- und Technologiefonds (WWTF), Agentschap voor Innovatie door Wetenschap en Technologie (IWT), and Fonds Wetenschappelijk Onderzoek (FWO) for funding our scientific quests for knowledge.

Chapter 2

PILS Putative Auxin Carrier Family

Modified from:

A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants

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Author contributions: E.B. and J.K.V. conceived the project. E.B. carried out most of the experiments. M.K., E.Z. and J.P. performed auxin metabolite profile and auxin accumulation in BY-2. C.B. analyzed auxin-dependent PILS expression and contributed to phenotype analysis. M.R.R. contributed to PILS cloning. J.R. and A.P. measured auxin content in Arabidopsis. B.W., J.Z. and M.G. performed auxin accumulation in yeast and protoplasts. Y.L. modified the estradiol inducible vector. All authors analyzed and discussed the data; E.B. and J.K.V. wrote the paper and all authors saw and commented on the manuscript.

Abstract

The phytohormone auxin acts as a prominent signal, providing, by its local accumulation or depletion in selected cells, a spatial and temporal reference for changes in the developmental program (Benkova et al., 2003, Friml et al., 2003; Reinhardt et al., 2003; Leyser et al., 2006; Dubrovski et al., 2008; Sorefan et al., 2009; Prasad et al., 2001). The distribution of auxin depends on both auxin metabolism (biosynthesis, conjugation and degradation) (Woodward and Bartel, 2005; Ikeda et al., 2009; Zhao et al., 2010) and cellular auxin transport (Bennet et al., 1996; Luschnig et al., 1998; Geisler et al., 2005; Petrasek et al., 2006; Zazimalova et al., 2010). We identified *in silico* the novel putative auxin transport facilitator family, called PIN-LIKES (PILS). Here, we illustrate that PILS proteins are required for auxin-dependent regulation of plant growth by determining the cellular sensitivity to auxin. PILS proteins regulate intracellular auxin accumulation at the endoplasmic reticulum (ER) and thus auxin availability for nuclear auxin signalling. PILS activity affects the level of endogenous auxin indole-3-acetic acid (IAA) presumably via intracellular accumulation and metabolism. Our findings reveal that the transport machinery to compartmentalize auxin within the cell is of an unexpected molecular complexity and demonstrate this compartmentalization to be functionally important for a number of developmental processes.

Results and discussion

Novel PILS protein family

Prominent auxin carriers with fundamental importance during plant development are PIN-FORMED (PIN) proteins(Benkova et al., 2003, Friml et al., 2003; Reinhardt et al., 2003; Sorefan et al., 2009; Ikeda et al., 2009; Zazimalova et al., 2010). PIN1-type auxin carriers regulate the directional intercellular auxin transport at the plasma membrane. In contrast, atypical family member PIN5 regulates intracellular auxin compartmentalization into the lumen of the ER and its role in auxin homeostasis just surfaced (Mravec et al., 2009; Zazimalova et al., 2010). PIN proteins display a predicted central hydrophilic loop, flanked at each side by 5 transmembrane domains. We screened *in silico* for novel PIN-like putative carrier proteins with a predicted topology similar to PIN proteins (Fig. 1a; Supplementary Fig. 2) and identified a protein family of 7 members (Fig. 1b) in *Arabidopsis thaliana*, which we designated as the PILS proteins. In contrast to the similarities in the predicted protein topology, PIN and PILS proteins do not show pronounced protein sequence identity (10%-18%), which limits the identification of PILS proteins by conventional, reciprocal Basic Local Alignment Search Tool (BLAST) approaches. However, the distinct PIN and PILS protein families contain both the InterPro auxin carrier domain which is an *in silico* defined domain, aiming to predict auxin transport function (*www.ebi.ac.uk/panda/InterPro.html*). The PILS putative carrier family is conserved throughout the whole plant lineage, including unicellular algae (like *Ostreococcus tauri* and *Chlamydomonas reinhardtii*) (Supplementary Fig. 3) where PIN proteins are absent (Mravec et al., 2009), suggesting that PILS proteins are evolutionarily older.

PILS genes are broadly expressed in various tissues (Fig. 1c) and *PILS2-7* were transcriptionally up-regulated by auxin application in wild type (WT) seedlings (Fig. 1d-f; Supplementary Fig. 4), suggesting an involvement in auxin-dependent processes.

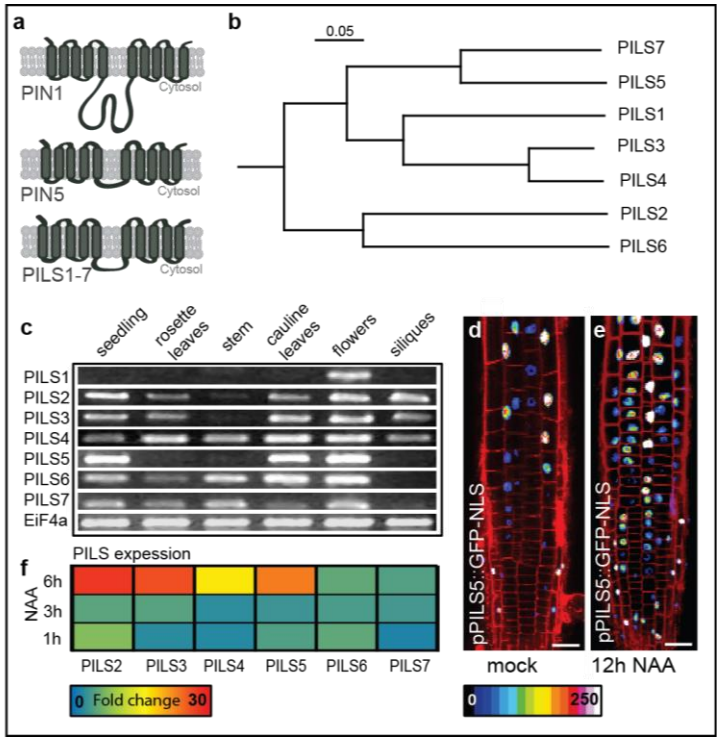


Figure 1: Novel PILS protein family

a, Predicted topology of both PIN1-/PIN5-type PIN subfamilies and PILS proteins. **b,** Phylogenetic tree of the *A. thaliana* PILS proteins. Scale depicts 0.05 substitutions per position. **c.** RT-PCR of the seven PILS genes and EIF4a (control) in several plant tissues. **d-e,** NAA (10µM; 12h) induced pPILS5::GFP-NLS (GFP fused to a nuclear localization signal) expression in the root transition zone. Color-code (black to white) depicts (low to high) GFP signal intensity. Propidium iodide stained cell walls in red. Scale bar: 25µm. **f,** qRT-PCR of PILS2-7 after 10µM NAA treatment for 1, 3 and 6 h. Color code from blue (low) to red (high) depicts fold changes (0-30) (see also Supplemental Fig. S4).

PILS loss- and gain-of function mutants

To investigate the potential function of the putative PILS auxin flux facilitators in plant development, we overexpressed PILS proteins using the constitutive, viral *35S* promoter. Ectopic expression of *PILS* genes, such as *PILS1* or *PILS3*, resulted in dwarfed and/or bushy plants displaying severe defects in flower development, leading to sterility in the T1 generation (Fig. 2a-d). Flowers of these *PILS* overexpressing plants showed severe patterning defects, such as homeotic transformation of flower organs into new flower buds, triplication of the gynoecium or non-fused carpels (Fig. 2b-d). To circumvent sterility, we screened for weaker *p35S::PILS* lines and isolated moderately *PILS5* overexpressing lines showing fertile flower development.

To further assess the developmental importance and potential redundancy of PILS proteins in auxin regulated processes, we focused on *PILS2* and *PILS5*, since they are the most abundantly expressed *PILS* genes in seedlings (Fig. 1c) and display partially overlapping expression domains (Fig. 2g). Initially, we investigated PILS function in auxin-dependent hypocotyl and root growth. Dark grown *p35S::PILS5::GFP* expressing and *pils2 pils5* double mutant seedlings showed reduced and enhanced hypocotyl growth, respectively (Fig. 2e; Supplementary Fig. 5). *PILS5* gain-of-function also resulted in agravitropic hypocotyl growth (Fig. 2f). *PILS2* and *PILS5* showed a particular overlapping expression in the root transition zone (Fig. 2g), suggesting a redundant role in regulating root growth. Indeed, *pils2* single and more pronounced *pils2 pils5* double mutant seedlings showed significantly longer roots compared to WT seedlings, while seedlings overexpressing *PILS5* had shorter roots (Fig. 2h). Collectively, our data indicate the requirement of defined PILS protein activity for auxin-dependent growth regulation.

Beside the root and shoot organ growth, auxin tightly controls *de novo* organ formation such as lateral root organogenesis (Benkova et al., 2003). Intriguingly, *pils2* and *pils5* single and more pronounced *pils2 pils5* double mutants showed higher lateral root density (Fig. 2i). On the contrary, *PILS5* gain-of-function reduced lateral rooting (Fig. 2i).

These findings indicate developmental importance of PILS proteins in auxin-regulated processes, such as *de novo* organ formation and growth regulation.

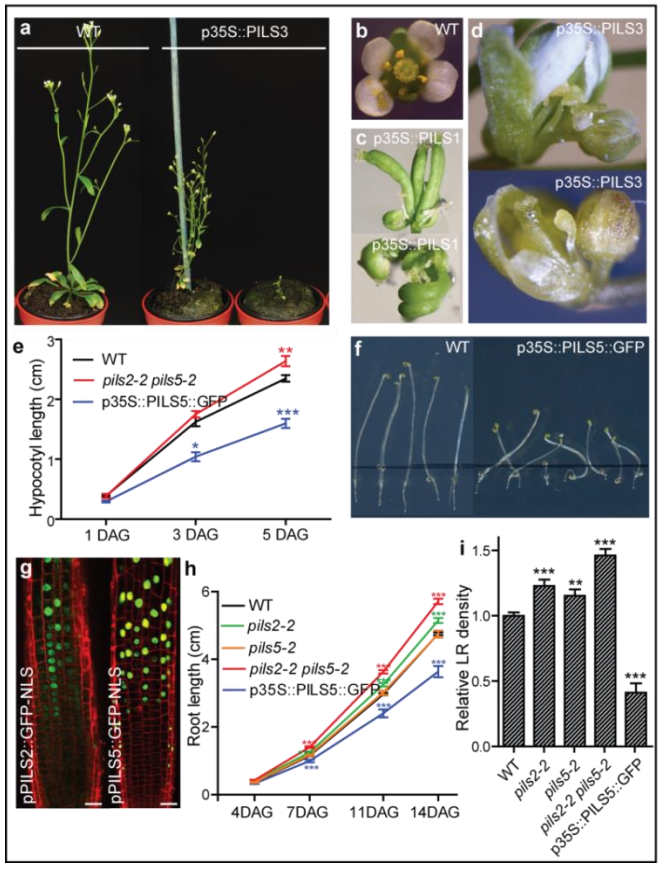


Figure 2: Phenotypes of PILS loss- and gain-of function mutants

a-d, Wild type (WT) and T1 transgenics strongly expressing p35S::PILS, such as p35S::PILS3 (a), are sterile and show dwarfy growth. Flowers of WT (b) p35S::PILS1 (c) and p35S::PILS3 (d). **e,** Hypocotyl length of dark grown *pils2-2 pils5-2*,

p35S::PILS5::GFP and WT seedlings (n>15). **f,** Dark grown WT and p35S::PILS5 seedlings (5 DAG). **g,** pPILS2::GFP-NLS (left) and pPILS5::GFP-NLS (right) expression in the root transition zone (green). Propidium iodide stained cell walls in red. Scale bar: 25µm. **h-i,** Root length (h) and lateral root density (14 DAG) (i) of *pils2-2, pils5-2, pils2-2 pils5-2,* p35S::PILS5::GFP and WT seedlings (n>40). DAG: days after germination, LR: lateral root. Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001

PILS proteins affect auxin dependent cellular growth

Next we investigated whether auxin responses are affected in *pils* mutants using the auxin response reporter DR5 (Ulmasov et al., 1997). *pils2-2* knock down and *pils5-2* knock out mutants did not show altered DR5 activity in the main root tips (Supplementary Fig. 6a), but showed higher pDR5rev::GFP signal intensity in lateral roots (Fig. 3a). Moderately *p35S::PILS5::GFP*-expressing seedlings displayed a visibly reduced auxin response maximum in the very root tip of main (Fig. 3b) and lateral roots (Supplementary Fig. 6b). To distinguish between direct and indirect effects, we investigated the effect of PILS proteins on nuclear auxin signalling at the cellular level. Therefore, we transiently co-expressed *PILS2* or *PILS5* together with the auxin response reporter *pDR5rev::mRFPer* (Expressing the red fluorescent protein in response to auxin signaling) in tobacco Bright Yellow 2 (BY-2) cells. *PILS2* or *PILS5* expression in BY-2 cells reduced auxin signalling as visualized by *pDR5rev::mRFPer* activity (Fig. 3c,d). These findings indicate that PILS proteins negatively affect nuclear auxin signalling presumably by affecting cellular auxin homeostasis. To address whether cellular PILS action on auxin signalling affects cellular growth, we expressed *PILS1, PILS3* and *PILS5* under a root hair specific promoter. Deviations in free (active) IAA levels or in auxin signalling induce (high levels) or repress (low levels) root hair growth (Lee et al., 2006). As expected, the root hair specific expression of *PILS1, PILS3* and *PILS5* significantly reduced root hair

length (Fig. 3e, f; Supplementary Fig. 7a-c), possibly due to PILS-dependent regulation of auxin homeostasis and signalling.

Next we tested whether PILS proteins affect auxin-dependent cellular growth responses. We treated PILS gain- and loss-of-function mutants with auxin that inhibits primary root growth. *pils2 pils5* loss- and *PILS5* gain-of-function mutants showed hyper- and hyposensitive root growth, respectively (Fig. 3g). In agreement with these observations, also the auxin promoted root hair growth was enhanced in *pils2 pils5* double mutants and reduced in *PILS5* overexpressors (Fig. 3h). This set of data indicates that PILS putative auxin facilitators modulate auxin-dependent growth responses during plant development.

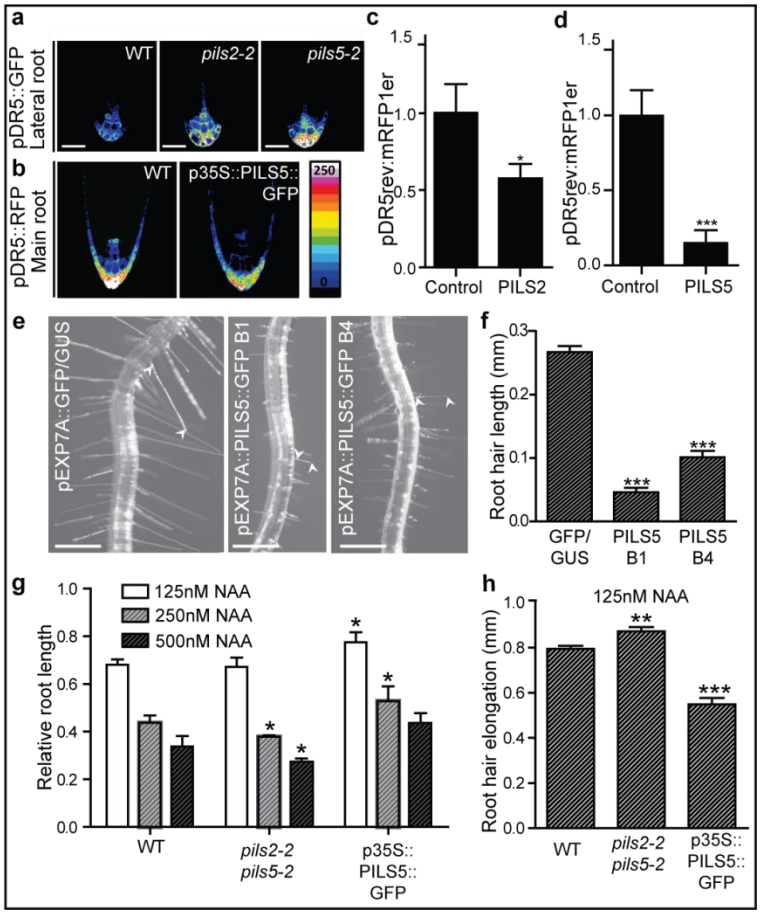


Figure 3: PILS proteins affect auxin dependent cellular growth

a-b, DR5 promoter activity in *pils2-2* and *pils5-2* (lateral roots) (a) and p35S::PILS5::GFP (main root) (b) compared to wild type (WT) (see also Supplementary Fig. S6). Color-code (black to white) depicts (low to high) pDR5rev::GFP and *pDR5rev::mRFP1er* signal intensity. Scale bars, 25µm. **c-d,** Mean grey value of *pDR5rev::mRFP1er* signal intensity in BY-2 cells expressing *p35S::PILS2* (c) or *p35S::PILS5* (d) compared to control cells (*p35S::GFP::HDEL)* (n>60 cells). **e-f,** Root hair length (e, arrowheads) and quantification (f) of transgenic lines expressing *pEXP7A::GFP::GUS* (control) and *pEXP7A::PILS5:GFP* (Independent lines B1 and B4) (n=20 seedlings with 400 counted root hairs in total). Scale bars, 25µm. **g,** Relative root length of NAA treated *pils2-2 pils5-2* and p35S::PILS5::GFP seedlings compared to WT (n>20). **h,** Auxin-induced root hair elongation (n=20 seedlings with 400 counted root hairs in total). Mean untreated root hair length was subtracted from the treated values. Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001.

PILS involvement in cellular auxin homeostasis

To unravel the mechanism by which PILS proteins regulate auxin-dependent plant development, we investigated the subcellular localization of PILS proteins. We introduced N- or C-terminal GREEN/RED FLUORESCENT PROTEIN (GFP/RFP) fusions with PILS proteins and transiently or stably expressed these fusion proteins in tobacco BY-2 cell culture, *Arabidopsis* seedlings and heterologously in yeast (Fig. 4a-c; Supplementary Fig. 8). PILS1-3 and PILS5-7::G/RFP fusion proteins localized to the ER in all analyzed systems (Fig. 4a-c; Supplementary Fig. 8 and Fig. 10c). In contrast, N and C terminal PILS4 fusions did not show detectable fluorescence. The transgenic *pPILS5::PILS5::GFP* also showed ER localization (Fig. 4a) and complemented the *pils2 pils5* double mutant to the *pils2* single mutant level (Supplementary Fig. 9), indicating PILS function at the ER.

Next, we addressed whether putative PILS auxin carriers affect cellular auxin accumulation. We generated *PILS2* estradiol-inducible tobacco BY-2 cell cultures and performed ^{3}H-IAA accumulation assays. *PILS2* induction increased the accumulation of radioactivity in BY-2 cells (Fig. 4d). In accordance with the auxin accumulation assays in BY-2 cells, *pils2 pils5* mutant protoplasts showed significantly higher auxin export (Fig. 4e; Supplementary Fig. 10a), indicating reduced auxin retention capacity in *pils2 pils5* loss-of-function mutants. These gain and loss-of-function studies consistently illustrate that PILS2 and PILS5 function at the ER controls cellular accumulation of auxin.

To additionally address PILS protein function in a non-plant system, we expressed *PILS3, PILS5* and *PILS7* (tagged to GFP or HA) in *Saccharomyces cerevisiae* yeast cells. In accordance with the effect of PILS2 and PILS5 in plant cells, also the *AtPILS3, AtPILS5* and *AtPILS7* expression in yeast increased retention of exogenously applied auxin (Fig. 4f; Supplementary Fig. 10b). To assess the specificity to auxin we used the common organic control benzoic acid (BA). Notably, PILS3, PILS5 and PILS7 did not affect the cellular accumulation of BA (Fig. 4f; Supplementary Fig. 10b). These findings indicate that PILS proteins specifically regulate cellular auxin accumulation.

PILS proteins increase cellular auxin accumulation, but decrease auxin signalling, which is reminiscent to the regulation of auxin metabolism shown for the PIN5 auxin transporter (Mravec et al., 2009). To assess the potential action of PILS proteins on auxin metabolism, we analyzed the effect of PILS proteins on the auxin metabolism using the inducible *PILS2* BY-2 cell line induced for 48h. The high performance liquid chromatography (HPLC) chromatogram of [^{3}H]IAA after the exogenous addition of [^{3}H]IAA showed a PILS2-dependent reduction of

free [^{3}H]IAA within 20 minutes (Figure 4g, h), indicating that PILS2 activity affects auxin metabolism.

In accordance to the auxin metabolite profiling in BY-2 cell cultures, *pils2*, *pils5* and *pils2 pils5* mutant *Arabidopsis* seedlings showed significantly higher free IAA levels compared to WT seedlings (Fig. 4i). Intriguingly, also the ratio of amid auxin conjugates IAA-Glutamate (Glu) and IAA-Aspartate (Asp) to free IAA was significantly shifted towards free IAA in *pils2* and *pils5* loss-of-function mutants (Fig. 4j, k). Moderately *p35S::PILS5::GFP* expressing seedlings displayed a visibly reduced auxin response maximum in the very root tip (Fig. 3b), but only a mild and statistically non-significant reduction in free IAA levels at the whole seedling level (Fig. 4i). However, the ratio of IAA-Glu and IAA-Asp to free IAA was significantly shifted towards the conjugates in *p35S::PILS5::GFP* seedlings (Fig. 4j, k), indicating a higher rate of auxin conjugation.

These findings are indicative for a PILS function in cellular auxin homeostasis by regulating auxin metabolism. Hence, we propose a model in which PILS proteins at the ER membrane facilitate intracellular auxin accumulation, which appears to contribute to the possibly compartmentalized regulation of auxin metabolism (Supplemental Fig. 1). It is tempting to speculate that also auxin conjugation could take place in the ER, although the molecular components remain to be identified.

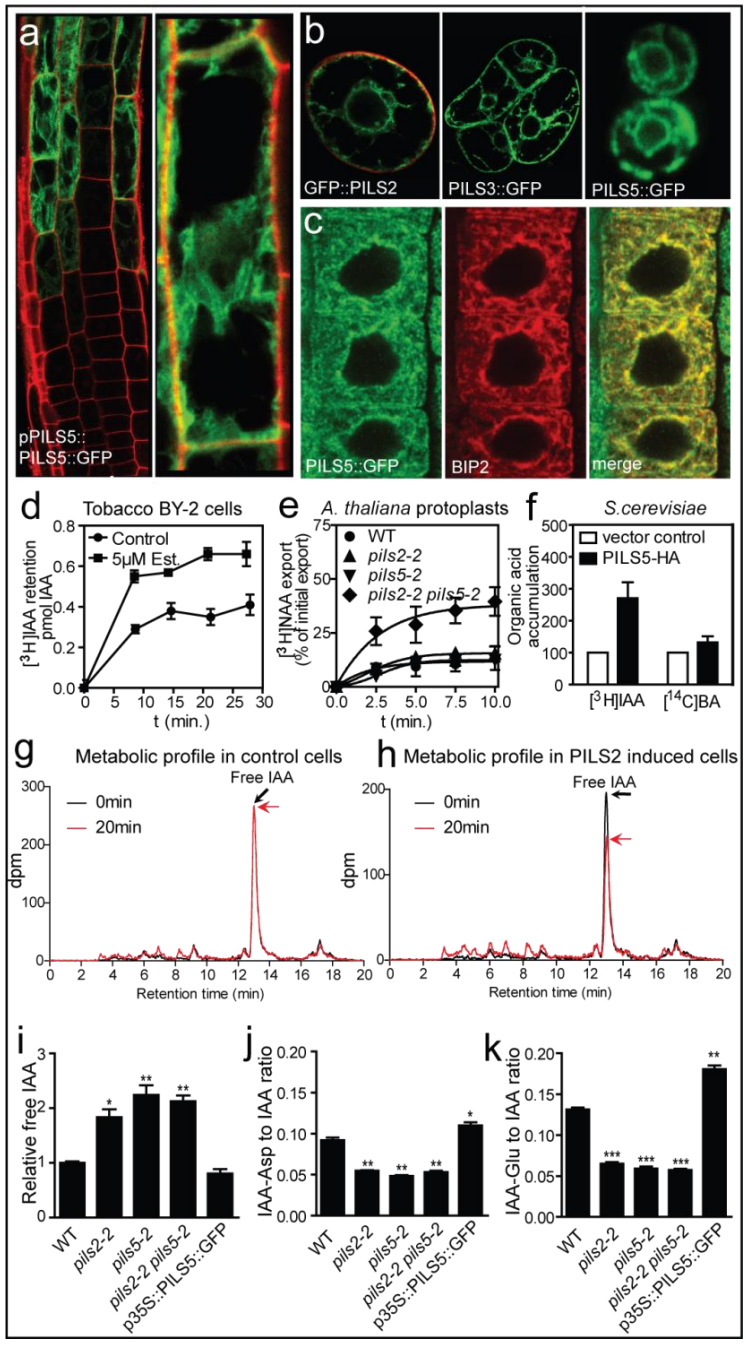


Figure 4: PILS involvement in cellular auxin homeostasis

a-c, GFP::PILS2 (b), PILS3::GFP (b), PILS5::GFP (a, b, c) under endogenous (a) or constitutive promoter (b and c) in *A. thaliana* root (a, c), tobacco BY-2 cells (b, left panels) and *Saccharomyces cerevisiae* yeast cells (b, right panel). Immunocytochemistry of PILS5::GFP and the ER marker BIP2 (c). **d,** ^{3}H-IAA retention in tobacco BY-2 cells

upon estradiol induced *PILS2* expression (n=3 repetitions). **e,** ^{3}H-NAA export assay in *A. thaliana* protoplasts of wild type (WT), *pils2-2*, *pils5-2* and *pils2 pils5* mutants (n=3 repetitions; see also Supplementary Fig. S10a). **f,** Accumulation of auxin and benzoic acid in *Saccharomyces cerevisiae* yeast cells transformed with pGPD::PILS5::HA or an empty vector (n=3 repetitions; see also Supplementary Fig. S10b). **g-h,** ^{3}H-IAA metabolic profile (HPLC chromatogram) in PILS2 non- (g) and estradiol-induced (h) tobacco BY-2 cells after 0 (black line) and 20 min (red line). **i-k** Liquid chromatography-mass spectrometry (LC-MS) derived free IAA levels (i) and ratios of IAA-Asp (j) and IAA-Glu (k) to free IAA in WT, *pils2-2*, *pils5-2*, *pils2-2 pils5-2* and p35S::PILS5::GFP (n=3 repetitions). Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001. Scale bar, 10µm.

Conclusion

Our *in silico* and reverse genetics approaches led to the identification of a novel family of putative auxin transport facilitators. All our genetic, pharmacological, cell biological, physiological and biochemical approaches consistently suggest that PILS function at the ER membrane, regulate intracellular auxin accumulation and affects free IAA levels presumably via conjugation-based auxin metabolism. The PILS action on cellular auxin homeostasis is reminiscent to the function of the atypical PIN family member PIN5 (Mravec et al., 2009). We uncovered that an additional, distinct protein family regulates intracellular auxin homeostasis. Our analyses of the PILS proteins suggest that intracellular auxin transport and, hence, auxin compartmentalization might be evolutionarily older than directional, cell-to-cell PIN-dependent auxin transport mechanisms. The identification of a novel protein family for the regulation of intracellular auxin homeostasis highlights the evolutionary and developmental importance of intracellular auxin transport. Further studies will address the potential interplay or possible diversified function of ER localized PIN5 and PILS1-7 proteins.

Methods

PILS gene accession codes

Sequence data from this article can be found in The Arabidopsis Information Resource (TAIR) *(http://www.arabidopsis.org/)* or GenBank/EMBL databases under the following accession numbers: PILS1 (At1g20925), PILS2 (At1g71090), PILS3 (At1g76520), PILS4 (At1g76530), PILS5 (At2g17500), PILS6 (At5g01990), PILS7 (At5g65980).

Plant material, growth conditions and DNA constructs

We used *Arabidopsis thaliana* of ecotype Columbia 0 (Col-0). The *Nicotiana tabacum* L. cv. Bright Yellow-2 cell line (Nagata et al., 1992) was used as suspension cultured cells. *pils2-1 (SALK_024808), pils2-2 (SALK_125391), pils5-1(SALK_070653) and pils5-2 (SALK_072996)* were obtained from the Nottingham *Arabidopsis* Stock Center *(NASC)*. Insertion sites were verified, homozygous lines selected and the decrease or absence of the *respective PILS* transcript was shown by RT–PCR. The *pils2-2* and the *pils5-2* mutants were crossed into *DR5rev::GFP*[2]. Gateway cloning was used to construct *pPILS2::GFP::GUS, pPILS5::GFP::GUS, p35S::PILS1-7, p35S::GFP::PILS1-7, p35S::PILS1-7::GFP, pPILS5::PILS5::GFP* and *pMDC7_B(pUBQ)::PILS2*. The PILS full length genomic fragments, cDNA and promoter regions were amplified by PCR from genomic DNA and cDNA, respectively. The PCR was performed using the high fidelity DNA polymerase "I proof" (Biorad). The used primers are given below. The full genomic and cDNA fragments were cloned into the pDONR221 (Invitrogen) vector and the promoter regions into the pDONR-P4P1 using Invitrogen BP-clonase according to manufacturer's instructions. Coding sequences were transferred from the entry clones to gateway compatible destination vectors (given below)

using the Invitrogen LR clonase(+) according to manufacturer's instructions. The resulting constructs were transformed into Col-0 plants by floral dipping in *Agrobacterium tumefaciens* liquid cultures. Yeast vectors were transformed into budding yeast *(Sacharomyces cerevisiae)* via electroporation. The *p35S::PILS5::GFP* line was crossed into *pDR5rev::mRFP1er* (Marin et al., 2010). The following lines and constructs have been previously described: *pDR5rev::mRFP1er* (Marin et al., 2010), *pDR5rev::GFP*^{2}*; p35S::GFP::HDEL* (Langhans et al., 2008). Seeds were stratified at 4 °C for 2 days in the dark. Seedlings were grown vertically on half Murashige and Skoog medium. Plants were grown under long-day (16 h light/8 h dark) conditions at 20–22 °C

Chemicals

1-Naphtaleneacetic acid (NAA) was supplied by Duchefa, 2,4-dichlorophenoxy acetic acid (2,4-D), Estradiol and propidium iodide (PI) by Sigma-Aldrich and ^{3}H-indole-3-acetic acid (^{3}H-IAA), ^{3}H-naphtalene-1-acetic acid (^{3}H-NAA) and ^{14}C-benzoic acid (^{14}C-BA) (specific radioactivity 20 Ci mmol^{-1}) by American Radiolabeled Chemicals, Inc. (St. Louis, USA).

RNA extraction and quantitative real time PCR (qPCR)

Whole RNA of seedlings was extracted using the Rneasy Mini Kit (Qiagen) in technical triplicates, the extracted RNA samples were treated with DNAse (Ambion). Q-PCR analysis was performed using ICycler (Bio-Rad) with the Platinum SYBR Green qPCR Super-UDG kit (Invitrogen) following recommendations of the manufacturer. Q-PCR was carried out in 96-well optical reaction plates heated for 10 minutes to 95°C to activate

hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 seconds at 95°C and annealing-extension for 60 seconds at 58°C. Target quantifications were performed with specific primer pairs (given below) designed using Beacon Designer 4.0 (Premier Biosoft International, Palo Alto, CA). Expression levels were normalized to the expression levels of translation initiation factor EiF4a. The used primers are given below.

Phenotype analysis

For analysis of the root length and lateral root density, plates were scanned on a flat-bed scanner. Root hairs were imaged at a binocular (Leica). For hypocotyls analysis, seeds on plates were exposed to light for 3 hours at 18°C, and cultivated in the dark at 20°C. Seedlings were imaged in real time with an infra-red camera (Canon, Tokio) to define the exact moment of germination and analysed 1, 3 and 5 days after germination. Hypocotyls, root and root hair lengths were measured with the ImageJ (*http://rsb.info.nih.gov/ij/*) software. Lateral root density for each seedling was obtained by calculating the number of lateral roots per root length unit 14 days after germination. For analysis of hypocotyls length, a minimum of 15 hypocotyls per condition or mutant line were analysed in each experiment. For analysis of root length and lateral root density, a minimum of 40 plants per condition or mutant line were analysed in each experiment. Means and standard errors were calculated and the statistical significance was evaluated by the student t-test using the GraphPad Prism5 (*www.graphpad.com*) software. For the analysis of root hair growth, 20 seedlings per transgenic line were imaged by binocular (Leica) and 20 root hairs (randomly chosen in the root hair zone) per seedling were measured with the ImageJ *(http://rsb.info.nih.gov/ij/)* software. The

mean and standard error of the mean per transgenic line were calculated and the statistical significance was evaluated by the student t-test. To obtain the auxin dependent root hair elongation, the same number of root hairs per seedling, seedlings per mutant line and condition were analysed as described above. The untreated mean average root hair length of the respective genotype was subtracted from the individual auxin treated root hair length to obtain auxin induced growth in millimeter. The mean and standard error of the mean of the respective genotype were calculated and the statistical significance was evaluated by the student t-test using the GraphPad Prism5 (*www.graphpad.com*) software. All experiments were performed at least in three independent biological repetitions.

BY-2 Plant material

Cells of tobacco line BY-2 (*Nicotiana tabacum* L., cv. Bright Yellow-2) (Nagata et al., 1992) transformed with *pMDC7_B(pUBQ)::PILS2* were cultured in liquid cultivation medium (3% (w/v) sucrose, 4.3 g l^{-1} Murashige and Skoog salts, 100 mg l^{-1} inositol, 1 mg l^{-1} thiamin, 0.2 mg l^{-1} 2,4-D, and 200 mg l^{-1} KH_2PO_4 (pH 5.8)). BY-2 cell lines were cultivated in darkness at 26°C on an orbital incubator (Sanyo Gallenkamp, Schöeller Instruments Inc., Prague, Czech Republic; 150 rpm, 32 mm orbit) and subcultured weekly. Stock BY-2 calli were maintained on media solidified with 0.6% (w/v) agar and subcultured monthly.

Transient transformation of BY-2 cells and monitoring of cellular auxin signalling in BY2

Ten ml of three-day-old cells were harvested on filter paper by vacuum filtration and kept on plates with solid BY-2 medium. The cells were

transformed via particle bombardment with a PDS 1000/He biolistic system (Bio-Rad) according to the manufacturer's instructions *(http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9075.pdf)*. 2 µl of plasmid DNA (0.05 µg/µl of the *pDR5rev::mRFP1er* contruct and 0.1µg/µl of *p35S::PILS2* and *p35S::PILS5*) was added to 6.25 µl of 1.6-µm diameter gold particles (dissolved in 50% glycerol). The suspension was supplemented with 2.5 µl spermidine (0.1 M stock solution) and 6.25 µl $CaCl_2$ (2.5 M stock solution). The particles were pelleted by centrifugation, washed twice with 70% and 100% ethanol and, subsequently, resuspended in 10 µl of 100% ethanol. Cells were bombarded under a pressure of 1100 punds per square inch (psi). The plates were sealed with parafilm and kept in the dark for 18 h at 25°C. For microscopic analysis, cells were gently transferred from the filter to a microscopy slide (in water) and subsequently covered with a cover slip. Samples were analyzed via confocal microscopy. The *pDR5rev::mRFP1er* expression was evaluated by defining the mean gray value (MGV) of each imaged cell (middle sections). For each experiment, confocal settings were defined based on the *p*DR5rev::mRFP1er signal of the control cells and remained unchanged during the respective experiments. Transformants were identified based on the fluorescence of both proteins and imaged with a 40x objective. Every experiment/transformation was done in triplicate and for each condition a total number of at least 60 transformed cells were imaged. For each experiment, the means and standard errors were calculated and the statistical significance (independence between the two populations) was obtained by the student t-test using the GraphPad Prism5 (*www.graphpad.com*) software.

Immunocytochemistry

Whole-mount immunological staining on 5-day-old seedlings was done in an Intavis robot according to the described protocol (Sauer et al., 2006). The antibodies used at the final dilutions were monoclonal mouse anti-BIP2 (Hsc70) at 1:200 (Stressgen Bioreagents), monoclonal rabbit anti-GFP at 1:600 (Invitrogen). The secondary anti-mouse (Invitrogen) and anti-rabbit (Sigma-Aldrich) antibodies conjugated with Cy3 and Alexa488 respectively were used at 1:600 dilution.

Microscopy

Confocal microscopy was done with a Zeiss 710 microscope (Zeiss) or Leica SP5 (Leica). Fluorescence signals for GFP (excitation 488 nm, emission peak 509 nm), mRFP1 (excitation 561 nm, emission peak 607 nm) and propidium iodide (PI) staining (excitation 536nm, emission peak 617nm) were detected with a 20x, 40x (water immersion) or 63x (water immersion) objective. Sequential scanning was used for double labeling to avoid crosstalk between channels. Fluorescence signal intensity was analyzed with Image J *(http://rsb.info.nih.gov/ij/)* software and data were statistically evaluated with Microsoft Excel 2007.

Auxin transport assays in tobacco BY-2 cells, baker's yeast and Arabidopsis thaliana protoplasts

Auxin accumulation with two-day-old BY-2 cells was measured as previously described (Delbarre et al., 1996; Petrasek et al., 2006). The ^{3}H-IAA was added to give a final concentration of 2 nM. Accumulation results were expressed as pmols of particular auxin accumulated per 10^{6}

cells. The 0.5-ml aliquots of cell suspension were collected continuously and accumulation of label was terminated by rapid filtration under reduced pressure on 22-mm-diameter cellulose filters. The cell cakes and filters were transferred to scintillation vials, extracted in 0.5 ml of 96% ethanol for 30 min, and afterwards 4 ml of scintillation solution (EcoLite Liquid Scintilation Fluid, MP Biomedicals, Solon, USA) was added. Radioactivity was determined by liquid scintillation counter Packard Tri-Carb 2900TR (Packard-Canberra, Meridian, CT, USA). Yeast ^{3}H-IAA loading was quantified with the unspecific ^{14}C-benzoic acid (BA) as control assayed in parallel and performed as previously described (Bailly et al., 2008). Relative export is calculated from yeast retained radioactivity as follows: (radioactivity in the yeast at time t=10 min.) - (radioactivity in the yeast at time t=0)) * (100%)/ (radioactivity in the yeast at t=0 min.). Unspecific loading due to diffusion was eliminated by vector control subtraction. IAA export from *Arabidopsis thaliana* mesophyll protoplasts was analyzed as described[16].

HPLC metabolic profiling in tobacco BY-2 cells

Two days old BY-2 cells were prepared for the experiment by equilibration in uptake buffer as already described for accumulation assays (Petrasek et al., 2006). Experiments were done in uptake buffer and under standard cultivation conditions. Cells were incubated with 20 nM ^{3}H-IAA for a period of 0 and 20 min. Cells and media (uptake buffer) were collected and frozen in liquid nitrogen (100 mg of fresh weight and 5 ml per sample). Extraction and purification of auxin metabolites in cells and media were performed as described (Dobrev et al., 2005; Dobrev et al., 2002). The metabolites were separated on HPLC consisting of autosampler and 235C diode array detector (Perkin Elmer, Shelton, USA),

column Luna C18 (2), 150 × 4.6 mm, 3 µm (Phenomenex, Torrance, USA), mobile phase A: 40 mM CH_3COONH_4, (pH 4.0) and mobile phase B: CH_3CN/CH_3OH, 1/1, (v/v). The flow rate was 0.6 ml min^{-1} with linear gradient 30–50% B for 10 min, 50–100% B for 1 min, 100% B for 2 min, 10–30% B for 1 min. The column eluate was monitored on 235C DAD followed by Ramona 2000 flow-through radioactivity detector (Raytest GmbH, Straubenhardt, Germany) after online mixing with three volumes (1.8 ml min^{-1}) of liquid scintillation cocktail (Flo-Scint III, Perkin Elmer). The radioactive metabolites were identified on the basis of comparison of their retention times with authentic standards. For the results presentation the total integrated area of chromatogram plots has been normalized based on the equalization of total accumulated radiolabel.

In silico and phylogenetic analysis.

PILS genes were identified via defining the predicted protein domain organization (topology) of PIN2 and subsequent screening for proteins with a similar predicted protein domain organization. Therefore, we used the SMART-protein tool from EMBL *(http://smart.embl-heidelberg.de/)* (Schultz et al., 1998; Letunic et al., 2009). The phylogenetic tree of AtPILS was constructed with the DNA-man software version 4.0. PILS topologies were defined by the online HMM-top tool *(http://www.enzim.hu/hmmtop/)* (Tusnady et al., 2001) and visualized by the TMRPres2D software *(http://biophysics.biol.uoa.gr/TMRPres2D/download.jsp)* (Spyrolpoulos et al., 2004). PILS orthologues were identified with the online tool Plaza *(http://bioinformatics.psb.ugent.be/plaza/)* (Proost et al., 2009).

Free IAA and conjugate measurements in Arabidopsis thaliana

For the quantification of free IAA and its amino acid conjugates, approximately 10 mg of plant material was taken into analysis. The samples were processed as previously described (Pencik et al., 2009) and quantified by UHPLC-MS/MS

Used primers and vectors

Genotyping primers

pils2-1 RP: CTGGAGAAACCTGACATCTCG
pils2-1 LP: GATTGAAGCCGGCTTAAATTC
pils2-2 RP: CTGGAGAAACCTGACATCTCG
pils2-2 LP: TACCATTGATCTGTCTTCGGG
pils5-1 RP: TTGAGACCCGTATCATTGGAG
pils5-1 LP: TGTCCTGATAAAACCTTTTCAGG
pils5-2 RP: TACTGCACCGAAAATGAAACC
pils5-2 LP: TTGTACTATTTGCACCGGCTC
Insert primer (LBb) (combine with RP): GCGTGGACCGCTTGCTGCAACT

RT-PCR primers used for insertion lines

PILS2 Fw: GCGATCATTATCGGATCAGT
PILS2 Rev:TTGCATACCTTGGACAGTAGTC
PILS5 Fw: TGTTGAAGCCCGTAATTCCATGAAC
PILS5 Rev: TTCATTGCGGACCCTTTAATCAGC

qPCR primers

PILS1 Fw: CGGTAACACAGCTCCACTTC
PILS1 Rev: GCAACAAGTAACGCACAACC

PILS2 Fw: GTGATGCTTGTACTTGGTGGTATG
PILS2 Rev: AACTTGAACATTGGATCTGCTGAG
PILS3 Fw: AGGCGACCATGCAAGTGTTG
PILS3 Rev: GTGGTACAGCTAGATGACAGTGAG
PILS4 Fw: TGTCATAACTAAGCCTCCTTCAC
PILS4 Rev: CTCGCAACTCTCAGAATCTCC
PILS5 Fw: CTTGGAATAGTCTGTGTTCGGTAC
PILS5 Rev: GCACTGAGCATTCGTCTTGAG
PILS6 Fw: GCCTACATCAGTGCTCTCAG
PILS6 Rev: GCACTGAGCATTCGTCTTGAG
PILS7 Fw: TCCTCCAGACCCTCTCTTTCG
PILS7 Rev: ACAAGAAGATGACCGAGCACTC
EiF4a Fw: CTGGAGGTTTTGAGGCTGGTAT
EiF4a Rev: CCAAGGGTGAAAGCAAGAAGA

Cloning primers

gDNA/cDNA:

PILS1_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGAGGATGAGGC
TTTTGGATC
PILS1_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)GGCTACGAGC
CACATGAAGAATG
PILS2_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGTCAGGTTTCTC
CAGTGGAA
PILS2_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)TTGCATACCTT
GGACAGTAGTCTC
PILS3_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGTGAAGCTTTT
GGAGCTGTTC
PILS3_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)AGCTACAAGC
CACATGAAGAATG
PILS4_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGAAGCTTTTGGA
GTTGTTCA
PILS4_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)TGTCACAAGCC
ACATGAAGAATG

PILS5_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGGATTCTGGTC GTTGTTGGA
PILS5_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)GACTAACAAG TGAAGGAAGATGG
PILS6_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGATTGCTCGGAT CCTTGCCG
PILS6_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)GAAGAGTATG TTAATGTAGAGTAC
PILS7_Fw_GGGGACAAGTTTGTACAAAAAAGCAGGCTCGATGGGTTTCTTAGA GTTGTTGGA
PILS7_Rev_GGGGACCACTTTGTACAAGAAAGCTGGGTC(TCA)GGAGAGGATG GAGAGGAAGATGG

Promoter:

PILS2_Fw_GGGGACAACTTTGTATAGAAAAGTTGCGAACTCCATTGTTAACAG TAATAGC
PILS2_Rev_GGGGACTGCTTTTTTGTACAAACTTGCCTCGATCTCACTATGTAA AGCTCG
PILS5_Fw_GGGGACAACTTTGTATAGAAAAGTTGCGCAATATACGTGACGTGG TCCACT
PILS5_Rev_GGGGACTGCTTTTTTGTACAAACTTGCCTTTTTATGTGGTTCTTTA GAC

Destination vectors:

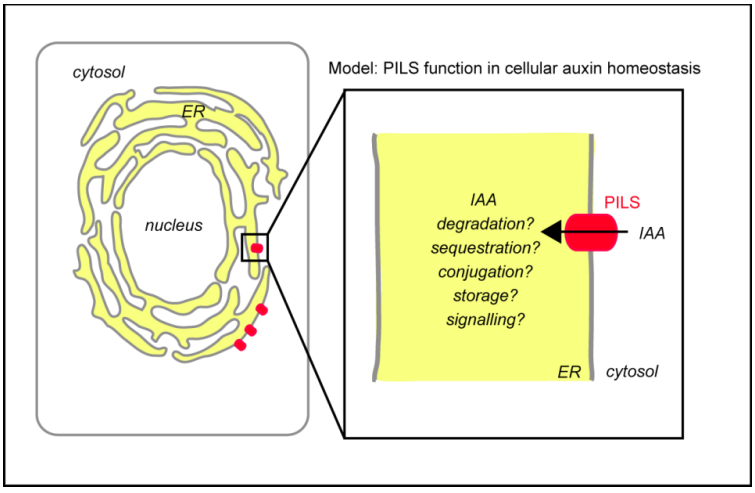
pPILS::GFP/GUS: pKGWFS7 (Karimi et al., 2005)
p35S::PILS::GFP: pH7FWG2,0 (Karimi et al., 2005)
p35S::GFP::PILS: pH7WGF2,0 (Karimi et al., 2005)
p35S::PILS::RFP: pK7RWG2,0 (Karimi et al., 2005)
p35S::RFP::PILS: pK7WGR2,0 (Karimi et al., 2005)
p35S::PILS_D: pH7WG2D,1 (Karimi et al., 2005)
pPILS::PILS::GFP: pK7m34GW,0 (Karimi et al., 2002)

Estradiol inducible PILS: *pMDC7_B(pUBQ)* (Curtis and Grossniklaus, 2003) (p35S promoter was exchanged by the pUBQ10 promoter. Construct will be in detail described in (Lee and Geldner., unpublished)

Acknowledgements

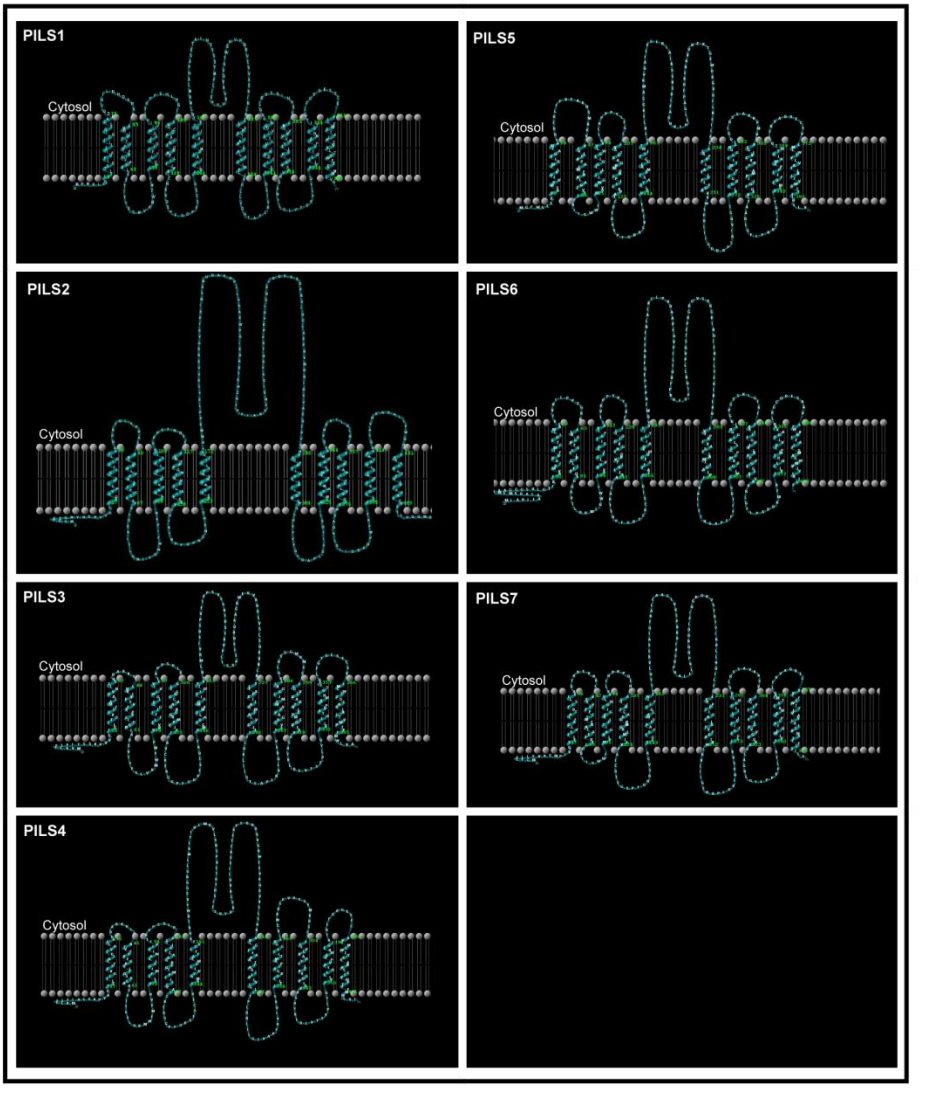
We are grateful to Carina Braeckman for plant transformation; Wilson Ardiles for sequencing support; Laurence Charrier for excellent technical assistance; Alexis Maizel, Niko Geldner and Peter Pimpl for providing material; KV group members for critical reading of the manuscript and the BOKU-VIBT Imaging Center for access and expertise. This work was supported by the Vienna Science and Technology Fund (WWTF) (to J.K.-V.), the Agency for Innovation by Science and Technology (IWT) (predoctoral fellowship to E.B.), the Odysseus program of the Research Foundation-Flanders (to J.F.), the Swiss National Funds (to M.G), the Ministry of Education, Youth and Sports of the Czech Republic (LC06034) (to E.Z.), Grant Agency of the Czech Republic project P305/11/2476 (to J.P.) and P305/11/0797 (to E.Z).

Supplementary information



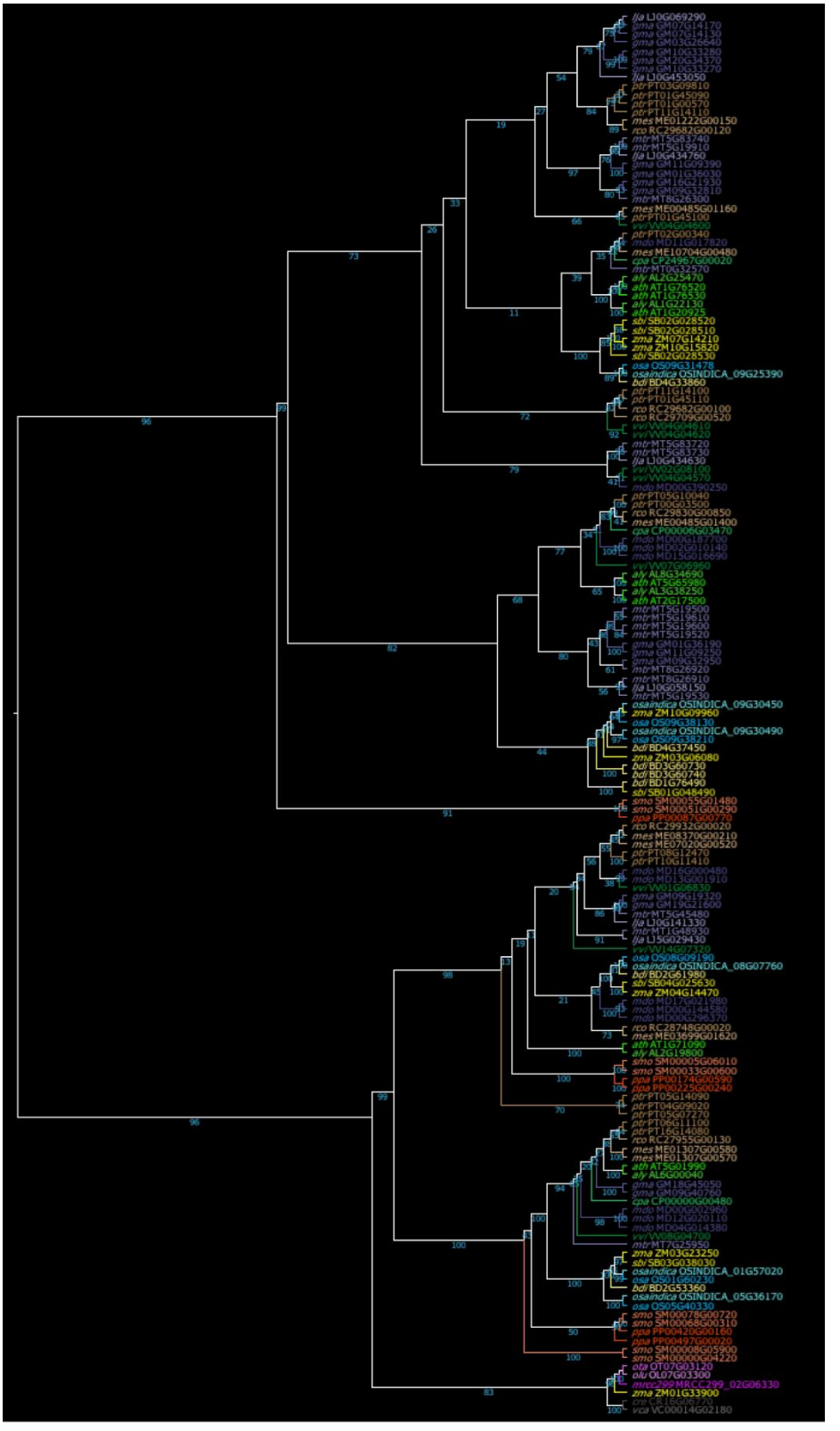
Supplemental Figure S1 Model on cellular PILS function.

PILS proteins localize to the endoplasmic reticulum (ER) and regulate cellular auxin accumulation, presumably by regulating auxin transport from the cytosol into the ER lumen. PILS function increases auxin conjugation-based inactivation of auxin and negatively regulates nuclear auxin signaling. We assume that both auxin sequestration into the ER and auxin conjugation might affect the availability of auxin for nuclear signalling. While our data may support a potential auxin conjugation in the ER, it remains to be seen whether known or yet to be discovered auxin amide conjugate synthetases function in the ER. To our knowledge, potential candidates of the GRETCHEN HAGEN (GH3) IAA amido synthetase family have not been localized in *Arabidopsis*, but in silico analysis of GH3 sequences do not suggest their residence in the ER (Ludwig-Müller et al., 2011). In *Physcomitrella*, cytoplasmic localization was suggested for GH3-1 tagged with GFP (Ludwig-Müller et al., 2009). Nevertheless, auxin metabolism is likely to be compartmentalized in the ER. Most known auxin conjugate hydrolases, such as IAA-LEUCINE RESISTANT1 LIKE (ILL) proteins, contain ER retention motifs (Leclere et al., 2002; Campanella et al., 2003), but their localization remains to be experimentally proven. Moreover, isoforms of YUCCA4 catalyzes auxin biosynthesis at the outer surface of the ER (Kriechbaumer et al, 2012), possibly providing substrate for the auxin transport into the ER. Notably, also the auxin receptor AUXIN BINDING PROTEIN1 (ABP1) mainly resides in the lumen of the ER (Jones et al., 1997; Henderson et al., 1997). These results strongly suggest that auxin compartmentalization is important for plant development, but the unifying mechanism remains to be discovered.



Supplemental Figure S2 PILS protein topologies.

In silico predicted transmembrane domain organization of PILS1-7 proteins.

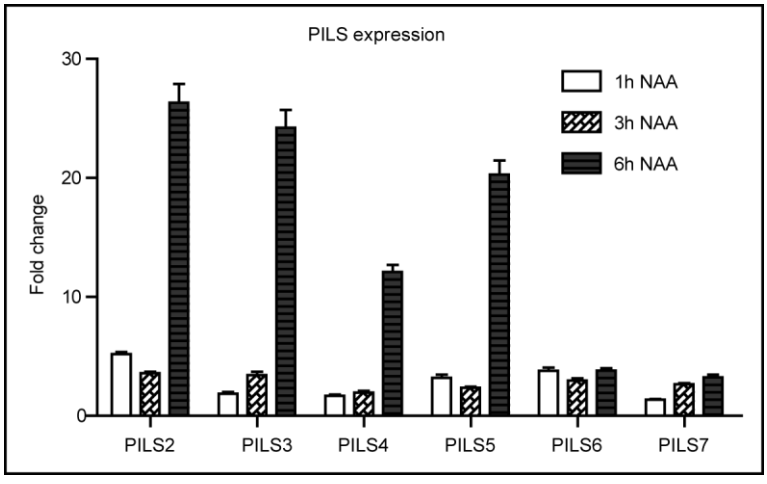


http://bioinformatics.psb.ugent.be/plaza/gene_families/explore_trees/HOM000497

Supplemental Figure S3 Phylogenetic tree of the PILS orthologues.

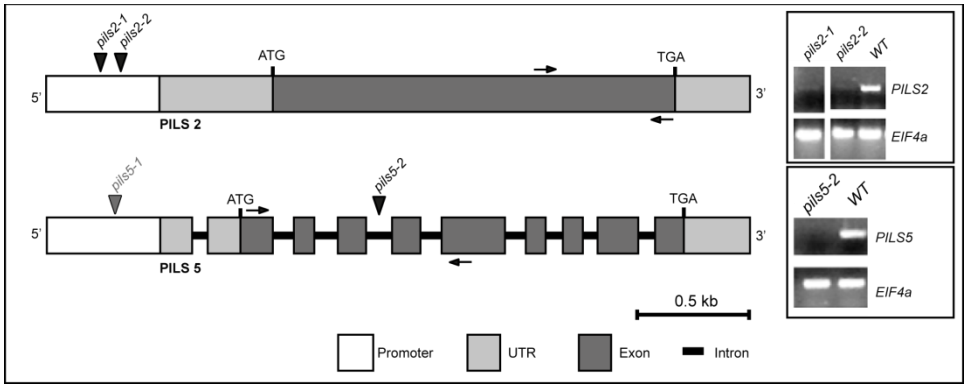
PILS orthologues in several plant species are identified and represented in a phylogenetic tree using the Plaza2.0 online platform (*http://bioinformatics.psb.ugent.be/plaza/)* (Proost et al., 2009; Zmasek et l., 2001). Bootstrap values are given as percentages on the nodes.

Taxonomy abbreviations: *aly: Arabidopsis lyrata (light green) ; ath: Arabidopsis thaliana (light green); cpa: Carica papaya (green); gma: Glycine max (dark purple); lja: Lotus japonica (light purple); mes: Manihot esculenta (light brown); mtr: Medicago truncatula (purple); osa: Oryza sativa (blue) spp. Japonica; osaindica: Oryza sativa spp. Indica (dark blue); ppa: Physcomitrella patens (red); ptr: Populus trichocarpa (dark brown); rco: Ricinus communis; sbi: Sorghum bicolor (yellow); vvi: Vitis vinifera (dark green); zma: Zea mays (yellow); mdo: Malus domestica; cre: Chlamydomonas reinhardtii (dark gray); mrcc299: Micromonas sp. RCC299 (dark bordeaux); ota: Ostreococcus tauri (bordeaux); smo: Selaginella moellendorfii (orange); vca: Volvox carteri (light gray); olu: Ostreococcus lucimarinus (light bordeaux); bdi: Brachypodium distachyon (dark yellow)*



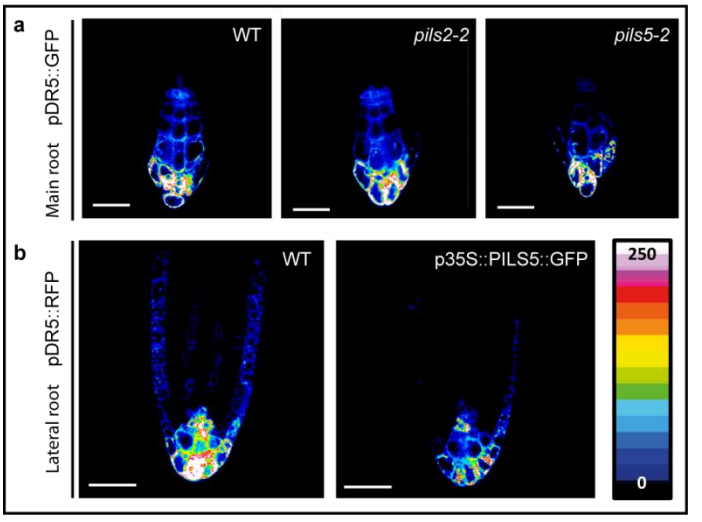
Supplemental Figure S4 qPCR analysis of auxin induced PILS expression.

The effect of 10µM NAA treatment for 1, 3 and 6 h on PILS expression levels was analyzed via quantitative RT-PCR. Graph depicts fold change in PILS expression upon auxin treatment compared to PILS expression levels in untreated seedlings. (n=3 biological repetitions). The PILS expression values were normalized towards the expression values of the housekeeping gene EIF4a. Error bars depict standard deviation of 3 technical replications.



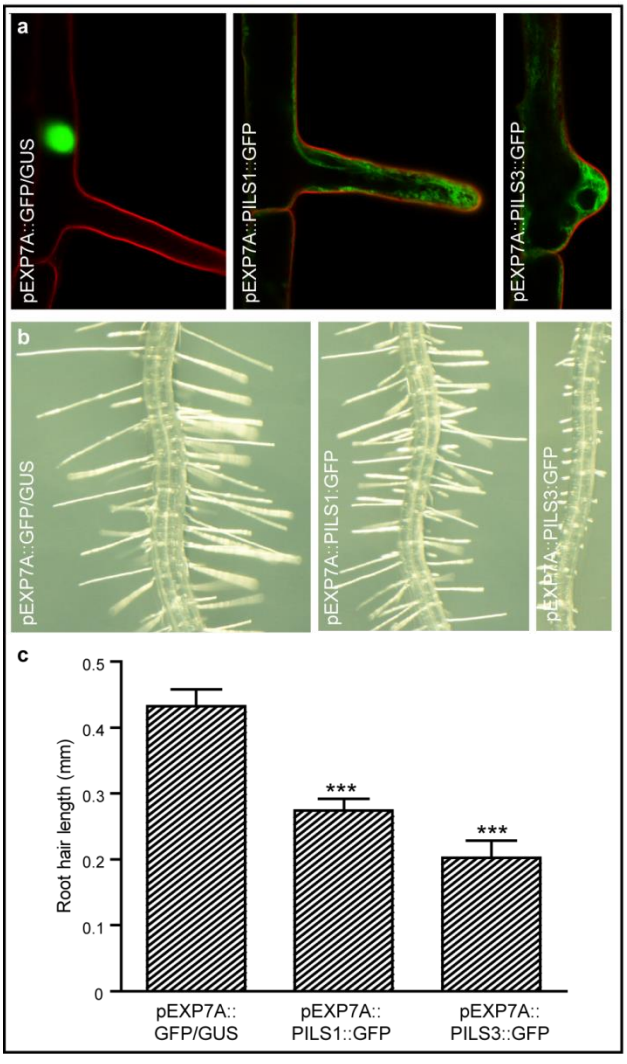
Supplemental Figure S5 PILS2 and PILS5 gene structure.

The PILS2 and PILS5 genes contains 1 and 9 exons, respectively. The analyzed T-DNA insertions are depicted in the figure (arrow heads). The PILS2 and PILS5 expression levels in the respective insertion lines were analysed by RT-PCR (given in the right panels). Arrows depict the annealing position of the used RT-PCR primers. *pils2-1* and *pils2-2* showed a strong reduction in transcription level. We did not detect PILS5 mRNA in the *pils5-2*, suggesting a full knock-out mutant. In contrast, PILS5 mRNA levels in the *pils5-1* mutant where similar to wild type (not shown). Scale bar, 500bp.



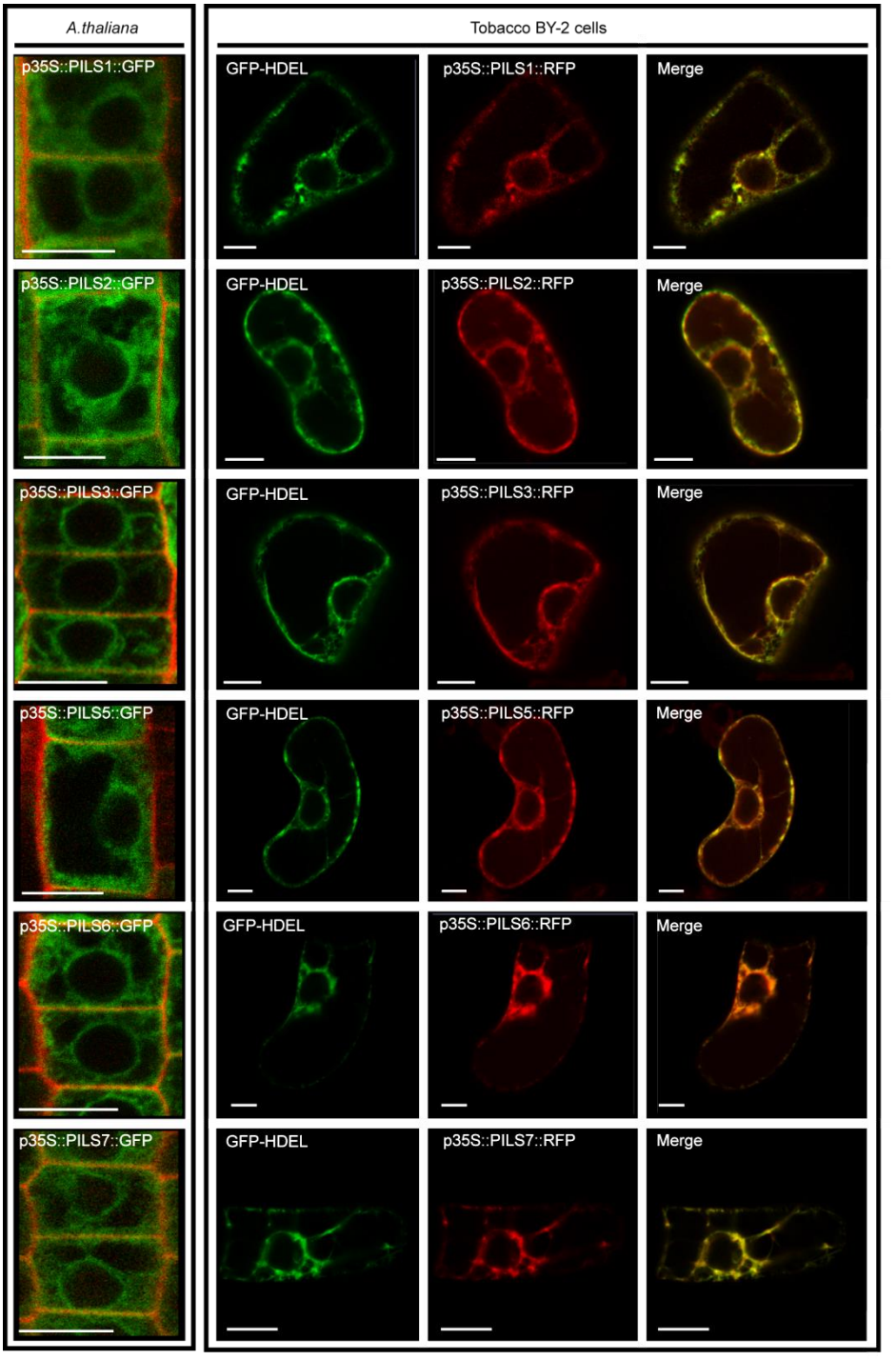
S**upplemental Figure S6** Nuclear auxin signaling in pils loss- and gain-of-function mutants.

a,b Analysis of the auxin reporter *DR5rev:GFP* (a) or *DR5rev:mRFP1er* (b) expression reveals no change in auxin signaling in the main root of *pils2-2* and *pils5-2* single mutants compared to wild type (*Col-0*) (a). Lateral roots of seedlings overexpressing PILS5 display decreased auxin signaling compared to wild type (*Col-0*). Representative pictures are shown and a color-coded heat map (black to white) was used to visualize (low to high) DR5rev:mRFP1er and DR5*rev*:GFP signal intensity**.**



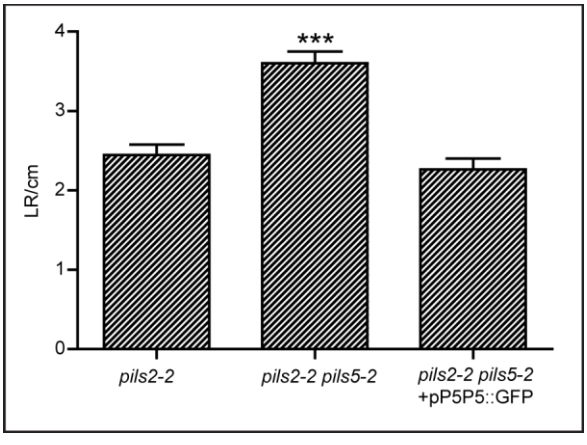
Supplemental Figure S7: Root hair cell-specific PILS activity represses root hair growth.

a, Root hair specific expression of NLS-GFP/GUS (left), PILS1:GFP (middle) and PILS3:GFP (right) **b-c,** Root hair specific PILS1 and PILS3 (pEXP7A:PILS1:GFP ad pEXP7A:PILS3:GFP) expression represses root hair growth. **c,** Graph depicts the root hair length of seedlings expressing pEXP7A:GFP/GUS or pEXP7A:PILS1/3:GFP in the root hair cells (n=20 seedlings with 400 counted root hairs in total). Error bars represent s.e.m.


A.thaliana
Tobacco BY-2 cells
p35S::PILS1::GFP
GFP-HDEL
p35S::PILS1::RFP
Merge
p35S::PILS2::GFP
GFP-HDEL
p35S::PILS2::RFP
Merge
p35S::PILS3::GFP
GFP-HDEL
p35S::PILS3::RFP
Merge
p35S::PILS5::GFP
GFP-HDEL
p35S::PILS5::RFP
Merge
p35S::PILS6::GFP
GFP-HDEL
p35S::PILS6::RFP
Merge
p35S::PILS7::GFP
GFP-HDEL
p35S::PILS7::RFP
Merge

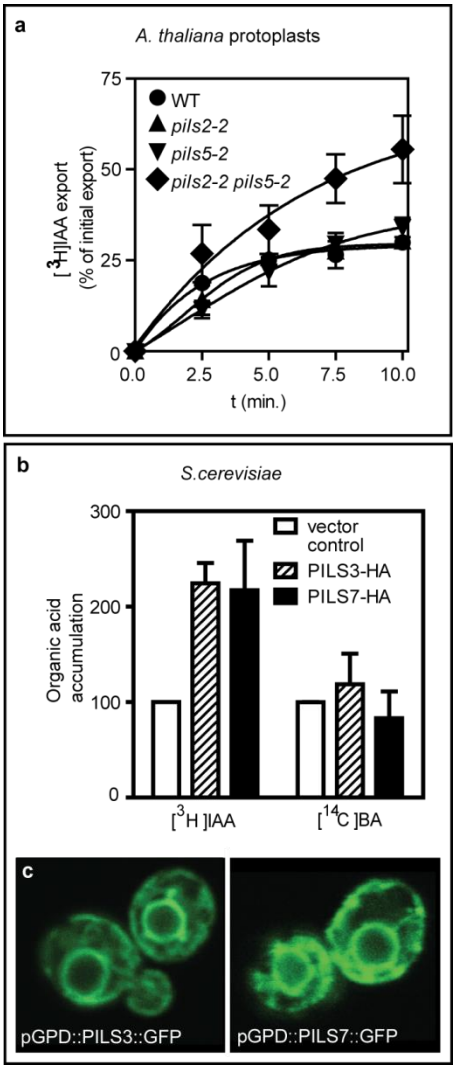
Supplemental Figure S8 PILS intracellular localization.

PILS(1-3)-GFP and PILS(5-7)-GFP fusion proteins reveal intracellular PILS localization to the endoplasmic reticulum (ER) as shown by live cell imaging of 35S:PILS:GFP in *A.thaliana* root cells (left panel). Propidium iodide stained cell walls in red. Transient co-expression of PILS(1-3)-RFP and PILS(5-7)-RFP protein fusions and the ER-marker GFP-HDEL in tobacco BY-2 cells shows complete colocalization (right panel). PILS4-GFP/RFP did not show detectable fluorescence (not shown). Scale bar, 10µm.



Supplemental Figure S9 *pils2-2/pils5-2* mutant complementation.

PILS5 expression under its endogenous promoter complements the enhanced lateral root density phenotype of the pils2-2/pils5-2 loss-of-function mutant (n=40 seedlings). Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001.



Supplemental Figure S10 PILS-dependent auxin accumulation in plant and non-plant cells

a, Arabidopsis protoplasts of *pils2-2/pils5-2* mutant leaves show higher ^{3}H-IAA export compared to protoplasts from wild type (Col-0) leaves (n=3 repetitions). Error bars represent s.e.m. **b,** Auxin accumulation assay in the *Saccharomyces cerevisiae* yeast cells. Cells transformed with GPD:PILS3:HA or GPD:PILS7:HA display higher auxin but not benzoic acid accumulation compared to cells transformed with an empty vector (n=3 repetitions). **c,** PILS3:GFP and PILS7:GFP localization in *Saccharomyces cerevisiae*. Error bars represent s.e.m.

Chapter 3

Carrier-Driven Cellular Auxin Homeostasis

Modified from:

Single-Cell-Based System to Monitor Carrier Driven Cellular Auxin Homeostasis

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Author contributions: EB and JKV conceived the project, EB carried out most of the experiments, ML and MP performed the auxin accumulation assays and imaged the stably transformed BY-2 cell lines. AM supplied the DR5rev:mRFP construct. EB, JKV, ML, MP, EZ, JP, AM and JF discussed the results, EB and JKV wrote the manuscript. All authors read and approved the final manuscript.

Abstract

Background: Abundance and distribution of the plant hormone auxin play important roles in plant development. Besides other metabolic processes, various auxin carriers control the cellular level of active auxin and, hence, are major regulators of cellular auxin homeostasis. Despite the developmental importance of auxin transporters, a simple medium-to-high throughput approach to assess carrier activities is still missing. Here we show that carrier driven depletion of cellular auxin correlates with reduced nuclear auxin signaling in tobacco Bright Yellow-2 (BY-2) cell cultures.

Results: We developed an easy to use transient single-cell-based system to detect carrier activity. We use the relative changes in the signaling output of the auxin responsive promoter element DR5 to indirectly visualize auxin carrier activity. The feasibility of the transient approach was demonstrated by pharmacological and genetic interference with auxin signaling and transport. As a proof of concept, we provide visual evidence that the prominent auxin transport proteins PIN-FORMED (PIN)2 and PIN5 regulate cellular auxin homeostasis at the plasma membrane and endoplasmic reticulum (ER), respectively. Our data suggest that PIN2 and PIN5 have different sensitivities to the auxin transport inhibitor 1-naphthylphthalamic acid (NPA). Also the putative PIN-LIKES (PILS) auxin carrier activity at the ER is insensitive to NPA in our system, indicating that NPA blocks intercellular, but not intracellular auxin transport.

Conclusions: This single-cell-based system is a useful tool by which the activity of putative auxin carriers, such as PINs, PILS and WALLS ARE THIN1 (WAT1), can be indirectly visualized in a medium-to-high throughput manner. Moreover, our single cell system might be useful to investigate also other hormonal signaling pathways, such as cytokinin.

Background

The phytohormone auxin is crucial to control plant growth and development. At the cellular level, auxin regulates cell division, cell expansion, and cellular differentiation (Perrot-Rechenmann, 2010). Auxin largely exerts its action through a multistep signaling pathway: Aux/IAA proteins are repressors of the AUXIN RESPONSE FACTOR (ARF) transcription factors. Auxin directly binds to the nuclear co-receptors TRANSPORT INHITOR RESPONSE/AUXIN F-BOX PROTEIN (TIR/AFB) and the Aux/IAA. Auxin binding causes the subsequent degradation of Aux/IAA transcriptional repressors (Gray et al., 2001; Dharmasiri et al., 2005; Kepinski and Leyser, 2005). Subsequently, auxin perception leads to the de-repression of the ARF transcription factors, initiating transcriptional reprogramming.

The spatial and temporal distribution of auxins depends on auxin metabolism (biosynthesis, conjugation, and degradation) and the activity of cellular auxin transporters (Ruiz Rosquete et al., 2011). To date, various auxin carriers have been identified (Zazimalova te al., 2010), among which the most prominent are auxin influx carriers of the AUXIN RESISTANT1/LIKE AUX1 (AUX/LAX) class, ABC transporters of the MULTIDRUG RESISTANCE (B-type) subfamily, and PIN-FORMED (PIN) auxin carriers (Bennet et al., 1997; Luschnig et al., 1998, Gesiler et al., 2005). Pharmacological and genetic interference with auxin carriers have illustrated the importance of auxin transport mechanisms for various aspects of plant development (Tanaka et al., 2006). In particular, classical auxin transport inhibitors, such as 1-naphthylphthalamic acid (NPA) (Katekar and Geissler, 1980; Rubery, 1974; Fujita et al., 2008), are valuable tools to assess various auxin carrier-mediated developmental processes. Typically, auxin carriers mediate the cellular auxin import or export at the plasma membrane and, thus, regulate the auxin availability

for nuclear auxin signaling (carrier-driven cellular auxin homeostasis). However, recently, a subclass of PIN proteins, such as PIN5 and PIN8, has been shown to reside at the endoplasmic reticulum (ER) and to control cellular auxin homeostasis presumably via the regulation of intracellular auxin compartmentalization into the ER lumen (Mravec et al., 2009; Bosco et al., 2012; Ding et al., 2012). Yet another evolutionary distinct PIN-LIKES (PILS) putative auxin carrier family functions at the ER, indicating broad developmental and evolutionary importance of intracellular auxin transport (Barbez et al., 2012; Feraru et al., 2012; Barbez and Kleine-Vehn, 2012).

The transport capacity of a multitude of auxin carriers and their sensitivity to auxin transport inhibitors has been analyzed in plant cell systems, such as *Arabidopsis* protoplasts or Bright Yellow-2 (BY-2) cell cultures of tobacco (*Nicotiana tabacum*), and in heterologous cell systems, such as yeast and mammalian cells (Imhoff et al., 2000; Geisler et al., 2005; Petrasek et al., 2006; Yang et al., 2006; Mravec et al., 2009; Yang et al., 2009). These elaborate auxin transport assays are important tools to study transport activities and mechanisms. However, it would be desirable to develop easier methods to asses auxin carrier activity. An alternative approach has been proposed for the indirect visualization of the carrier-driven auxin homeostasis (Lee et al., 2006; Ganguly et al., 2010). This bioassay utilizes the stimulating effect of free auxin levels on root hair elongation. The root hair-specific expression of an auxin carrier and its action on the root hair length is used to indirectly visualize carrier driven auxin homeostasis. However, auxin fluxes in neighboring tissues also contribute to the regulation of root hair growth (Jones et al., 2009), preventing the combined use of ectopic carrier expression and its sensitivity to auxin transport inhibitors. Moreover, the time-consuming

generation of stable transgenic lines might limit the use of this bioassay for high-throughput applications.

Another frequently used tool to monitor auxin signaling is the synthetic, highly auxin responsive promoter DR5, created by tandem repeats of the auxin responsive element (AuxRE) from the soybean GH3 promoter (Ulmasov et al., 1997). Previously, the DR5 promoter activity has been suggested to indicate the relative rate of nuclear auxin signaling in various tissues (Casimiro et al., 2001; Benkova et al., 2003; Friml et al., 2003; Dubrovski et al., 2008; Petersson et al., 2009; Sorefan et al., 2009). DR5 has been used to visualize auxin signaling maxima and minima which, however, do not correlate in all cells with the actual auxin levels possibly due to cell type-dependent cues (Nakamura et al., 2003; Nemhauser et al., 2000; petersson et al., 2009).

Here, we present a novel single cell based system, using the DR5 promoter, to address cellular mechanisms that affect cellular auxin homeostasis and ultimately auxin signaling. We show the correlation between DR5 promoter activity and fluctuations in cellular auxin levels in tobacco BY-2 cells. Our data suggest that the transient expression of *DR5rev:monomeric RED FLUORESCENT PROTEIN (mRFP)* reporter for nuclear auxin signaling can be used to illustrate the relative state of cellular auxin signaling in tobacco BY-2 cells. By means of this single-cell-based system, the relative auxin carrier induced changes in cellular auxin signaling were monitored, indirectly indicating auxin carrier activity. As a proof of concept, we assessed the prominent PIN auxin carrier activity and confirmed their effects on cellular auxin homeostasis/signaling (Petrasek et al., 2006, Mravec et al., 2009). Moreover, a pharmacological approach revealed that the activity regulation of PIN2 at the plasma membrane and PIN5 at the ER are distinct. Furthermore, we show that this single-cell-based system could be

analogously used to investigate other putative carriers, such as PILS and WAT1, or potentially even other hormonal pathways, such as cytokinin.

Results

Indirect visualization of auxin carrier activity in tobacco BY-2 cells.

In previous studies, the synthetic auxin-responsive promoter element DR5 fused to the monomeric *RFP* or *GFP* reporter gene (*DR5rev:mRFP/GFP*) (Galavotti et al., 2010; Marin et al., 2010; Friml et al., 2003) was used to visualize the auxin response maxima within tissues and it was proposed to indirectly estimate auxin distribution (Benkova et al., 2003; Friml et al., 2003; Sorefan et al., 2009). However, auxin distribution and DR5-based auxin signaling do not always correlate in plant tissues presumably due to cell type-specific sensitivities to auxin (Petersson et al., 2009).

To reduce cell type dependent effects, we tested whether the DR5 promoter could be used in tobacco BY-2 cell cultures to indirectly estimate auxin carrier activity. In order to address the correlation between auxin carrier activity and DR5 promoter activity in tobacco BY-2 cells, we stably transformed the *DR5rev:mRFP* construct into transgenic BY-2 lines carrying the construct for inducible *PIN1-GFP* (Lankova et al., 2010) or *PIN7* (Petrasek et al., 2006) expression. PIN1 and PIN7 are plasma membrane localized auxin efflux carriers important for plant growth and development (Petrasek et al., 2006; Zazimalova et al., 2010). Induction of *PIN1-GFP* and *PIN7,* which causes cellular auxin depletion (Petrasek et al., 2006), decreased DR5rev:mRFP signal intensity compared to non-induced cells, reflecting lower nuclear auxin signaling (Figure1A-1F, Supplemental Figure S1A). The DR5rev:RFP signal intensity is

represented by the average mean gray value (MGV) of the induced cell population (n>150 single cells) relative to the average MGV of the uninduced control population (n>150).

Within the estradiol induced *PIN1-GFP* expressing BY-2 cell population, we observed a negative correlation between DR5rev:mRFP and PIN1-GFP signal intensity (Supplemental Figure S1B and S1C), suggesting lower levels of nuclear auxin signaling in case of high PIN1-GFP activity.

To unambiguously depict auxin carrier activity of PIN1-GFP and PIN7, we performed auxin accumulation assays on the same cell lines and observed lower accumulation of the radiolabelled synthetic auxin 1-naphtylacetic acid ([^{3}H]NAA) in estradiol induced *PIN1-GFP* expressing cells compared to non-induced cells (Figure 1G). Our findings indicate that DR5rev:mRFP signal intensity (Fig1A-F) correlates with cellular auxin accumulation (Figure 1G), presumably due to carrier induced changes in cellular auxin content and subsequent alterations in auxin signaling.

We conclude that under our experimental conditions the DR5 promoter activity can be used in BY-2 cells to indirectly visualize auxin carrier-dependent regulation of cellular auxin homeostasis.

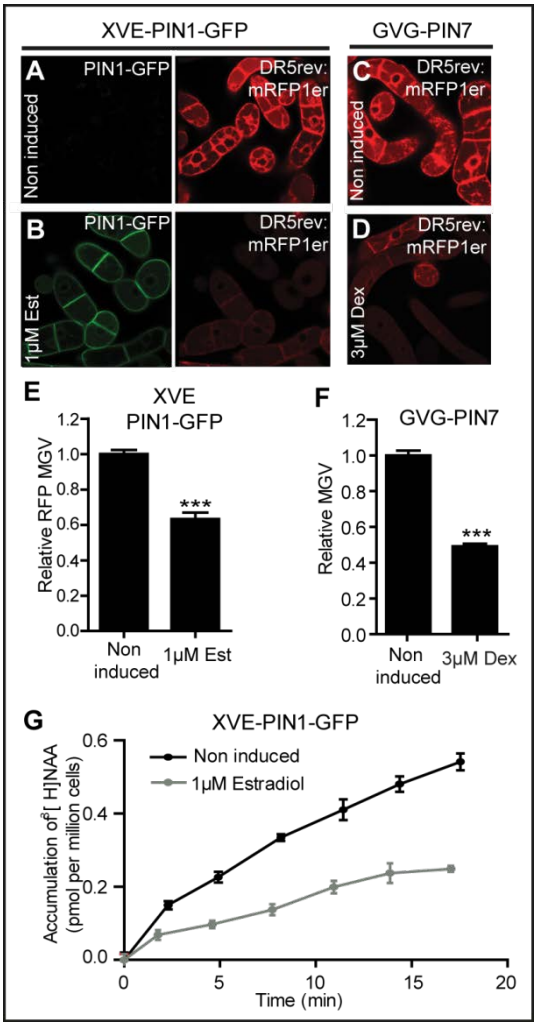

Figure 1. Correlation between altered DR5 promoter activity and cellular auxin accumulation in stably transformed tobacco BY-2 cells.

Tobacco BY-2 cells stably transformed with the auxin responsive *DR5rev:mRFP* and the estradiol (Est) inducible *XVE-PIN1-GFP* or the dexamethasone (Dex) inducible *GVG-PIN7* construct.

(A,B) Induction of *PIN1-GFP* (in green) with estradiol visibly decreases DR5rev:mRFP (in red) signal intensity compared to non-induced cells as visualized by confocal imaging. **(C,D)** Dexamethasone-dependent induction of *PIN7* visibly decreases DR5rev:mRFP signal intensity compared to non-induced cells. **(E)** Graph depicts mean gray values (MGV) of the DR5rev:mRFP signal intensity in *PIN1* induced and non-induced cells (n = 150). **(F)** MGV of the DR5rev:mRFP signal intensity in *PIN7* induced and non-induced

cells (n = 150). **(G)** [^{3}H]NAA accumulation assays in non-induced and estradiol-induced (*XVE-PIN1-GFP)* cells. Error bars represent standard error. Statistical significance was evaluated with the unpaired student T-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$).

Procedure for transient auxin carrier expression in a single-cell-based system

In BY-2 cells, the visualization of auxin signaling could be used to indirectly monitor carrier driven cellular auxin homeostasis. To establish a medium-to-high throughput assay, we elaborated on procedures to transiently express auxin carriers. Particle bombardment is an easy to use procedure that enables high transformation efficiencies at low plasmid concentrations and has, in case of partial automatization, the potential for high-throughput use (Sanford et al., 1987; Klein et al., 1988). We adjusted the previously described particle bombardment procedure (Sanford et al., 1987; Klein et al., 1988) for efficient, transient tobacco BY-2 transformation (see Materials and Methods). To obtain high protein expression levels, BY-2 cell cultures in the exponential growth phase were used. DNA concentrations ranging from 0.05 µg/µl to 1 µg/µl were sufficient for transient expression and resulted in a correlation between DNA concentration and expression levels, allowing fine-tuning of the gene expression (data not shown). The co-transformation efficiency was tested by transformation of two plasmids at different concentration ratios and the co-transformation levels were calculated (Supplemental Table S1). Although dependent on concentration, in general the co-transformation efficiency was very high (approximately 90% at 0.05 µg/µl for both plasmids), enabling the two plasmids to be efficiently co-transformed (Supplemental Table S1). Thus, BY-2 particle bombardment can be used as a suitable method to efficiently co-express genes of interest.

Transient single-cell-based-system to monitor auxin signaling

To initially test whether the transient DR5 expression in BY-2 cells could be used to visualize qualitative differences in levels of auxin signaling between two samples of interest, we transiently co-transformed tobacco BY-2 cells with the auxin responsive *DR5rev:mRFP* construct and the stabilized auxin signaling repressor *IAA17mImII* fused to the activator domain of the herpes simplex virus VP16. This construct leads to constitutive auxin signaling in plant cells (Tiwari et al., 2003; Li et al., 1991). As expected, the mean gray value (MGV) of DR5rev:mRFP (reflecting auxin signaling) was higher in cells co-transformed with *DR5rev:mRFP* and *35S:VP16-IAA17mImII* than in control cells expressing *DR5rev:mRFP* and the inert endoplasmic reticulum (ER) marker *35S:HDEL-GFP* (Figure 2A-2C). To further elaborate on the relative changes in DR5/auxin signaling, we subdivided the transformed cell population in 4 classes according to the relative MGV (the MGV relative to the MGV of the control population). Individual cells were scored as low (-) with a relative MGV below 0.5 (2^{-1}), medium (+) with a relative MGV above 0.5 (2^{-1}) and below 1 (2^{0}), high (++) with a relative MGV between 1 (2^{0}) and 2 (2^{1}) and very high (+++) with a relative MGV value higher than 2 (2^{1}). (Figure 2E and Supplemental Figure S2). This alternative visualization allowed us to trace the shifts in relative cell numbers with low, medium, high and very high RFP signal intensity between two samples and to compare even more subtle differences in DR5rev:mRFP signal intensities. 35S:VP16-IAA17mImII enhanced auxin signaling in our system and accordingly reduced the relative cell numbers with low/medium and increased the cell numbers with strong, or very strong RFP signal intensity (Figure 2E).

These findings indicate that the transient *DR5rev:mRFP* expression in BY-2 cells can be used to monitor the qualitative differences in nuclear auxin signaling.

Next, we examined whether our single-cell-based system can be used to address mechanisms of auxin transport. Therefore we treated the *DR5rev:mRFP*-transformed cell population with the auxin transport inhibitor NPA that reduces cellular auxin efflux and, hence, increases cellular auxin levels (Murphy et al., 2002). As expected, NPA treatment significantly increased the relative rate of DR5 signaling in our transient assay (Figure 2B, 2D and 2F), revealing that NPA action on cellular auxin efflux and cellular auxin homeostasis can be monitored in our single-cell-based system.

Our findings suggest that the DR5- and single-cell-based system can be used to qualitatively monitor changes in auxin signaling. However, in order to use this system in a meaningful way, the experimental design needs to be carefully chosen, because DR5 activity has been suggested not to solely reflect auxin signaling. The phytohormone brassinolide affects the expression of several auxin responsive genes as well as the activity of the DR5 promoter in *Arabidopsis thaliana* (Nakamura et al., 2003; Nemhauser et al., 2000) (Supplemental Figure S3A and S3B). Nevertheless, in our experimental conditions, brassinolide treatment did not increase the average MGV of *DR5rev:mRFP* transformed tobacco BY-2 cells (Supplemental Figure S3C-S3E). This finding indicates that either brassinosteroids do not increase auxin signaling in BY-2 cells or that our approach is not sensitive enough to trace subtle differences, such as brassinosteroid-induced auxin signaling.

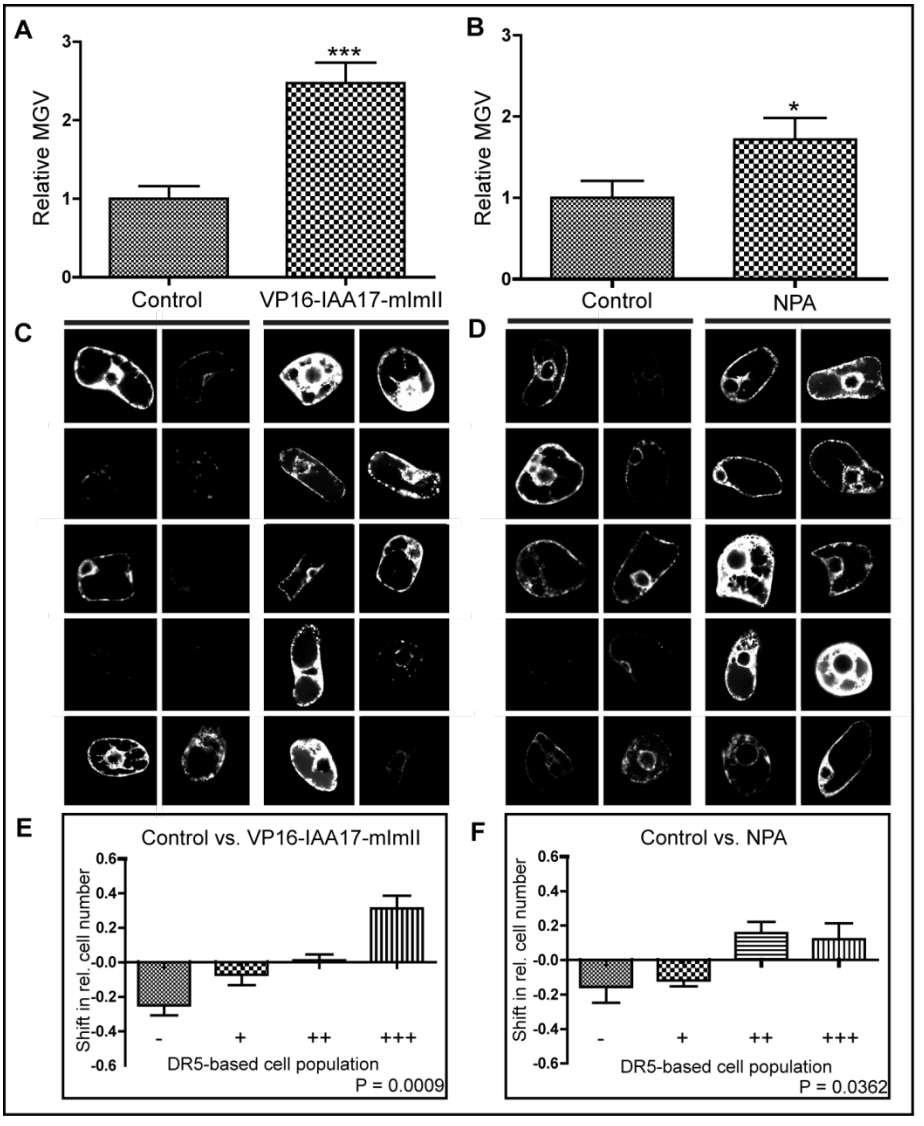

Figure 2. Effect of altered auxin signaling and transport capacity on cellular auxin homeostasis.

(A,B) Graphs represent the relative average mean gray values (MGV) of the DR5rev:mRFP signal intensity. Error bars represent standard error (n = 60). Statistical significance was evaluated with the unpaired student T-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). **(A)** Coexpression of *DR5rev:mRFP* and the stabilized version of *IAA17* fused to a VP16 activator domain (*35S:VP16-IAA17mImII*), causing constitutive auxin signaling, significantly increased the relative average MGV compared to the *35S:HDEL-GFP-*

expressing control cells. **(B)** NPA treatment leads to an increased MGV/DR5 signaling compared to transformants maintained in standard cultivation medium. **(C)** 10 representative pictures of DR5rev:mRFP are shown for the control cells (left panel) and the cells overexpressing *VP16-IAA17-mImII* (right panel). **(D)** 10 representative pictures are shown for the untreated control cells (left panel) and the cells treated with NPA (right panel). **(E,F)** Graphs depict the changes in relative number of transformed cells displaying a low (-), medium (+), high (++), and very high (+++) DR5rev:mRFP signal between the two samples addressing *VP16-IAA17-mImII* expression or NPA treatment (for detailed description of the quantification, see Supplemental Figure S2). Error bars represent standard error (n=3 repetitions with at least 50 counted cells). Statistical significance was evaluated with the ANOVA test; the P- value is indicated.

Auxin carrier trafficking and localization in the single-cell-based system

To further assess the usability of the method, we studied the effect of the PIN auxin carrier activity on the cellular auxin signaling. Initially, a time-frame of presumably high PIN protein activity was defined by investigating the PIN trafficking/localization after transient BY-2 transformation. Transmembrane proteins, such as PIN proteins, are co-translationally inserted into the ER membrane. Plasma membrane-localized PIN proteins, such as PIN1, are exported subsequently from the ER and sorted to the plasma membrane (Boutte et al., 2006). In contrast, PIN5 proteins remain at the ER membrane, where they function as regulators of (intra)cellular auxin homeostasis (Mravec et al., 2009).

Ten hours after transformation, we observed colocalization of PIN1-RFP with the inert ER marker HDEL:GFP in most of the transformed cells (87%) (Figure 3A and 3D), implying high levels of newly synthesized PIN proteins, whereas 16 h after transformation, in most analyzed cells, PIN1-RFP was absent from the ER and solely visible

(at the given confocal setting) at the plasma membrane (Figure 3B and 3D) where it is active (Petrasek et al., 2006). Afterwards, the percentage of cells with a strong PIN1-RFP signal at the plasma membrane diminished over time (Figure 3D). This decrease in PIN1 localization at the plasma membrane correlated with an increase of the PIN1-RFP occurrence in the vacuole (Figure 3C and 3D), hinting at a PIN1 turnover by lytic degradation (Kleine-Vehn et al., 2008).

Altogether, PIN1 proteins displayed a pronounced localization and, presumably, high activity at the plasma membrane between 16h-18h after transformation. At that time point (17 h after transformation), also other PIN proteins, such as PIN2-GFP and PIN5-GFP, strongly localized at the plasma membrane and ER (Figure 4A and 4B), respectively. We conclude 16 h to 18 h as a suitable time frame for the analysis of PIN auxin carrier activity.

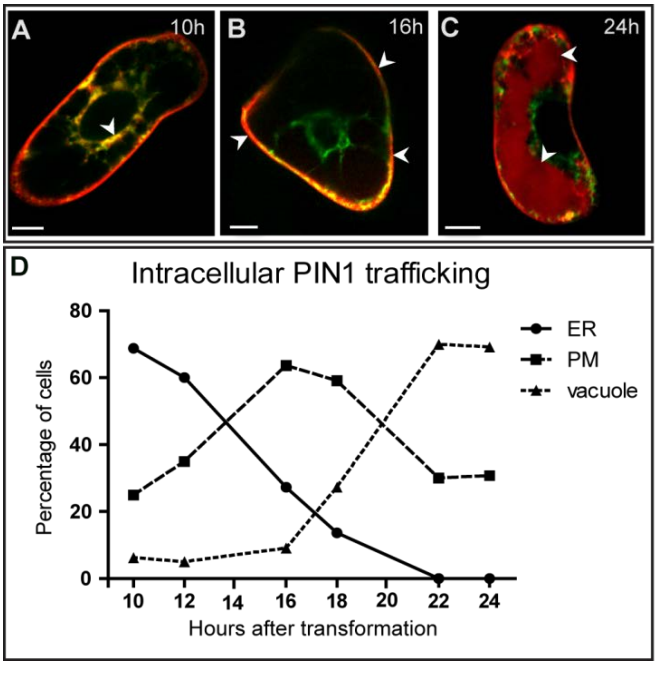

Figure 3. Cellular localization and trafficking of PIN1-RFP.

(A) Colocalization of PIN1 proteins (in red) with the ER marker HDEL-GFP (in green) 10 h after transformation. **(B)** PIN1-RFP preferentially localizes to the plasma membrane (PM) after 16 h. **(C)** 24 h after transformation, PIN1-RFP shows vacuolar localization indicating lytic turnover. **(D)** Relative number of cells showing pronounced PIN1-RFP signal in the ER, PM, and in the vacuole over a time course of 24 h after transformation. Cells showing PIN1-RFP both at PM and either at the ER or vacuole were ascertained as ER or vacuole positive, respectively. Bars = 10 μm.

Activity of the PIN auxin transport proteins affects auxin signaling

Next, we investigated the effect of the PIN auxin carrier activity on the cellular auxin signaling using the DR5- and single-cell-based system. Cells cotransformed with *DR5rev:mRFP* and the plasma membrane localized auxin efflux carrier *35S:PIN2-GFP* had a lower DR5rev:mRFP signal intensity than the control cells expressing *DR5rev:mRFP* and the inert ER marker *35S:HDEL-GFP* (Figure 4A, 4E and 4I). These observations are in agreement with the PIN induced decrease in cellular auxin accumulation (Figure 1A-I) and indicate a decreased auxin signaling due to the enhanced PIN2-auxin efflux carrier activity at the plasma membrane.

PIN5 is an ER localized auxin carrier described to facilitate auxin transport from the cytosol into the ER. This auxin sequestration into the ER presumably reduces the availability of auxin for nuclear auxin signaling (Mravec et al., 2009). In agreement with these assumptions, the *PIN5-GFP* expression caused a significant decrease in the DR5rev:mRFP signal intensity (Figure 4B, 4F and 4J)

We conclude that the DR5- and single-cell-based system can be used to indirectly monitor the activity of plasma membrane and ER localized PIN auxin transporters.

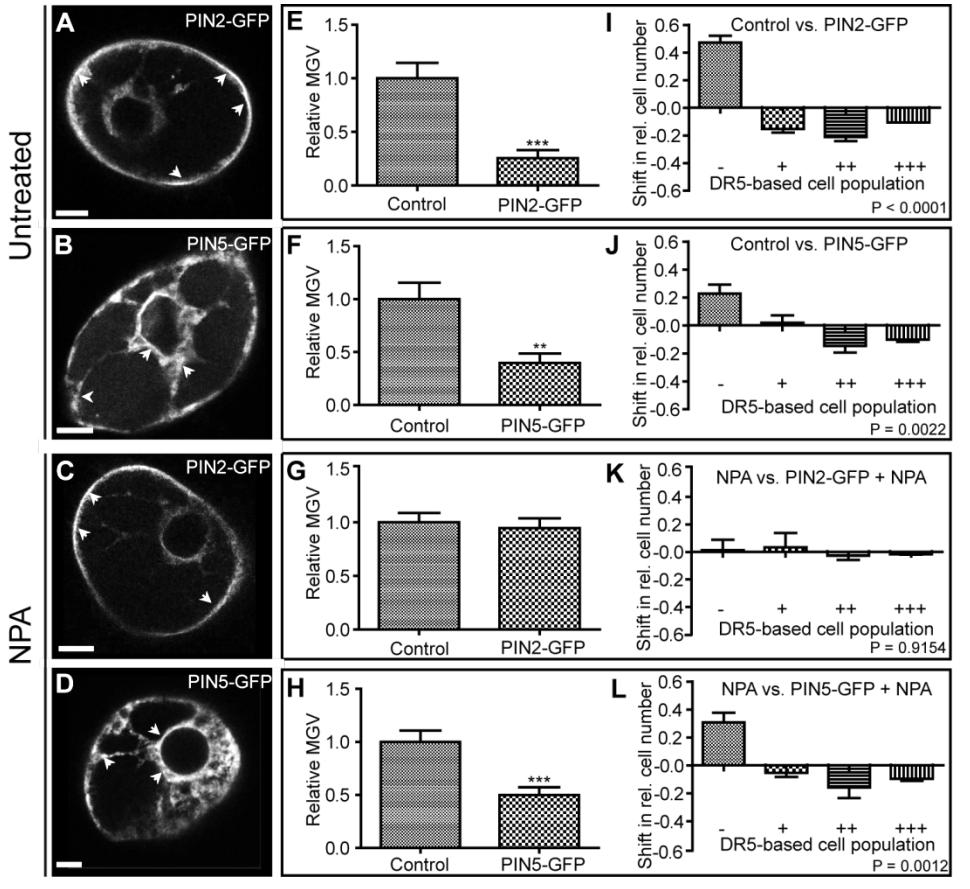

Figure 4. Effect of PIN protein activity on cellular auxin homeostasis.

(A) Preferential PIN2-GFP localization at the plasma membrane. **(B)** Typical perinuclear ER localization of PIN5-GFP. **(C,D)** PIN2-GFP and PIN5-GFP localization is not affected in the presence of the auxin transport inhibitor NPA (application with 10 µM NPA-enriched medium). Arrowheads depict preferential PIN2 and PIN5 localizations at the PM and ER, respectively. Bars = 10 µm. **(E,I,F,J)** Cells co-transformed with *DR5rev:mRFP* and either *35S:PIN2-GFP* or *35S:PIN5-GFP* had a lower DR5rev:mRFP signal intensity than the control cells expressing *DR5rev:mRFP* and the inert ER marker

35S:HDEL-GFP. **(G,H,K,L)** In the presence of NPA, PIN5-GFP but not PIN2-GFP expression decreases DR5rev:mRFP signal intensity. **(E-H)** Graphs represent the relative average mean gray values (MGV) of the DR5rev:mRFP signal intensity. Error bars represent standard error (n = 60). Statistical significance was evaluated with the unpaired student T-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). **(I-L)** Graphs depict the change in relative number of transformed cells displaying a low (-), medium (+), high (++), and very high (+++) DR5rev:mRFP signal intensity between the two samples (for detailed description of the quantification, see Supplemental Fig S2). Error bars represent standard error (n=3 repetitions with at least 50 counted cells). Statistical significance was evaluated with the ANOVA test; the P- value is indicated.

Auxin transport inhibitor NPA inhibits PIN2, but not PIN5 action in the single cell system

As described above, the expression of *PIN2-GFP* facilitates the auxin efflux from cells and, hence, lowers the levels of intracellular auxin signaling (Figure 4E and 4I). After treatment with the auxin transport inhibitor NPA, *PIN2* expression did not decrease auxin signaling compared to NPA treated *HDEL-GFP* expressing control cells (Figure 4G and 4K). Importantly, NPA did not visibly affect the transient PIN2 localization at the plasma membrane (Figure 4C), indicating that our single-cell-based system monitors the inhibitory effect of NPA on the auxin transport activity of PIN2 (Petrasek et al., 2006; Yang et al., 2009).

In contrast to PIN2, the *PIN5-GFP* expression (Figure 4D) reduced the auxin signaling even in the presence of NPA (Figure 4H and 4L). This difference in NPA sensitivity indicates that the mechanisms of NPA action and/or auxin transport mechanisms of PIN2 and PIN5 are distinct. This finding is in agreement with a NPA binding activity at the plasma membrane (Cox et al., 1996). To further assess the NPA insensitivity of putative auxin carriers at the ER, we analyzed PILS putative auxin carrier

activity in our system. PILS5 localizes to the ER and was recently described to decrease nuclear auxin signaling presumably due to auxin sequestration into the ER (Barbez et al., 2009). Similar to PIN5, we observed a PILS5 dependent decrease in DR5 signaling in the presence of NPA (Supplemental Figure S4). Hence, we assume that NPA inhibits intercellular, but not intracellular auxin transport at the ER in BY-2 cell cultures.

We conclude that the single-cell-based system is a sensitive approach not only to monitor the carrier-driven auxin homeostasis, but also to assess the auxin carrier sensitivity to auxin transport inhibitors. These data demonstrate that our transient approach can be used to investigate the genetic or pharmacological interferences with auxin carrier function.

WAT1 protein activity affects cellular auxin homeostasis

To assess whether the approach is suitable to monitor also the activity of other putative transporters, we co-expressed *DR5rev:mRFP* with *WALLS ARE THIN1* (*WAT1*) (Figure 5A). WAT1 is a tonoplast-localized transmembrane protein that belongs to the drug/metabolite transporter superfamily. WAT1 activity has an impact on auxin homeostasis by affecting tryptophan and/or auxin metabolism via an unknown mechanism (Ranocha et al., 2010). We used our single-cell-based system to investigate whether WAT1 affects the cellular auxin signaling in BY-2 cells. Cells co-transformed with *DR5rev:mRFP* and *35S:WAT1:GFP* had a lower DR5rev:mRFP signal intensity than control cells expressing *DR5rev:mRFP* and *35S:GFP:GFP* (Figure 5B and 5C), implying a negative effect of WAT1 protein activity on nuclear auxin signaling. These data show that our single-cell-based system visualizes the effect of

WAT1 on cellular auxin homeostasis and that it could be in principle used to indirectly asses the activity of a wide range of carrier proteins.

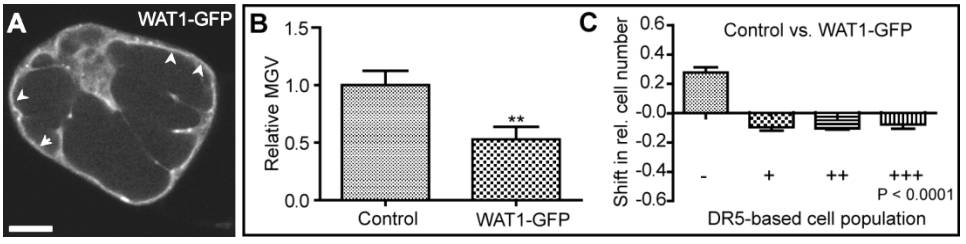

Figure 5. Effect of WAT1:GFP protein activity on cellular auxin homeostasis.

(A) Preferential WAT1:GFP localization at the tonoplast (arrowheads). Bar = 10 µm. (b,c) Lower DR5rev:mRFP intensity in cells expressing *35S:WAT1:GFP* than control cells co-expressing *DR5rev:mRFP* and *35S:GFP:GFP*. **(B)** Graph represents the relative average mean gray values (MGV) of the DR5rev:mRFP signal intensity. Error bars represent standard error (n = 60). Statistical significance was evaluated with the unpaired student T-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). **(C)** Graph depicts the change in relative number of transformed cells displaying a low (-), medium (+), high (++), and very high (+++) DR5rev:mRFP signal intensity between the two samples (for detailed description of the quantification, see Supplemental Figure S2). Error bars represent standard error (n=3 repetitions with at least 50 counted cells). Statistical significance was evaluated with the ANOVA test; the P-value is indicated.

Indirect visualization of the cellular cytokinin homeostasis

As the single-cell-based system enables the indirect monitoring of carrier driven cellular auxin homeostasis, we tested whether the method could be used analogously for other hormonal pathways. The synthetic cytokinin-responsive promoter TWO-COMPONENT-OUTPUT-SENSOR (TCS):GFP was expressed transiently in tobacco BY-2 cells for indirect

visualization of the cellular cytokinin signaling (Muller et al., 2008). The distribution characteristics of the cells transiently transformed with TCS:GFP were similar to those previously observed for DR5rev:mRFP. Analogously, we measured the average MGV of the transformed cell population and furthermore categorized the cells in subpopulations with very strong, strong, medium, and low TCS:GFP signal intensity (Figure 6 A-C). The TCS:GFP activity was higher in transformants treated with 6-benzylaminopurine (BAP), a native aromatic cytokinin (Perrot-Rechenmann, 2010), than in those grown in standard cultivation medium (Figure 6A and 6B), suggesting an enhanced cytokinin signaling in BY-2 cells upon cytokinin application.

These results suggest that our single-cell-based system could eventually be extended to other applications, such as the indirect visualization of cellular cytokinin signaling.

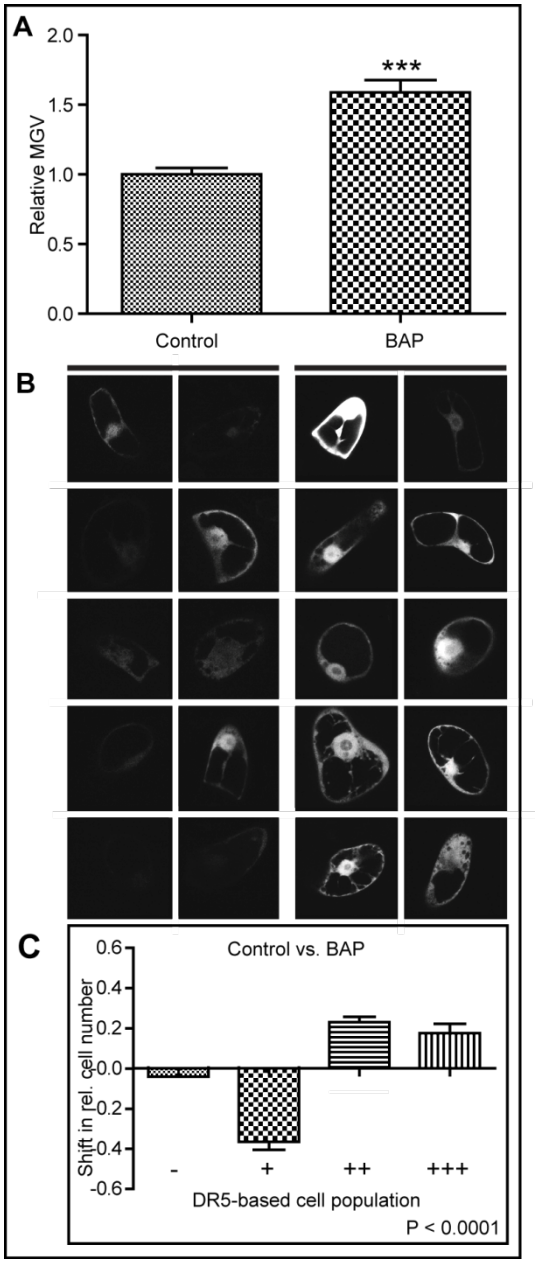

Figure 6. Effect of exogenous 6-benzylaminopurine (BAP) on cellular cytokinin signaling.

BAP treatment (application with 10 µM BAP-enriched medium) led to an increased TCS:GFP signaling compared to transformants maintained in the standard cultivation medium (control). **(A)** Graph represents the relative average mean gray values (MGV) of

the TCS:GFP transformed BY-cells. Error bars represent standard error (n = 60). Statistical significance was evaluated with the unpaired student T-test (* P < 0.05, ** P< 0.01, *** P < 0.0001). **(B)** Representative pictures show the TCS:GFP signal intensities of 10 untreated (left) and BAP treated (right) transformed cells. **(C)** Graph depicts the change in relative number of transformed cells displaying a low (-), medium (+), high (++), and very high (+++) TCS:GFP signal intensity between the two samples (for detailed description of the quantification, see Supplemental Figure S2). Error bars represent standard error (n=3 repetitions with at least 50 counted cells). Statistical significance was evaluated with the ANOVA test; the *P*-value is indicated.

Discussion and conclusion

The phytohormone auxin plays a key role in many aspects of plant growth and development. The cellular auxin content is tightly controlled by local auxin metabolism (biosynthesis, conjugation/deconjugation, or oxidation) and auxin transport facilitators (Casimiro et al., 2001; Blakeslee et al., 2005; Ljung et al., 2005; Woodward and Bartel, 2005; Grunewald and Friml, 2010, Ruiz Rosquete et al., 2011). Whereas the complex interplay of these factors still needs to be unraveled, it is clear that various transporters have pronounced effects on the cellular auxin homeostasis (Bennett et al., 1996; Geisler et al., 2005; Petrasek et al., 2006; Krouk et al., 2010; Ranocha et al., 2010; Barbez et al., 2012). Furthermore, the steady release of new annotated genomes increases the number of putative auxin carriers and enables the study of their molecular evolution. The scientific progress in auxin carrier identification emphasizes the growing demand for suitable approaches to assess carrier-driven cellular auxin homeostasis.

Here, we present a single-cell-based system that allows us to monitor qualitative differences in nuclear auxin signaling between two samples of interest. Thanks to this easy approach, carrier-driven auxin

homeostasis and its sensitivity to auxin transport inhibitors can be visualized. The transient approach enables (possibly in combination with automated imaging systems) medium-to-high throughput work flows that can be used for chemical genomic or gain- and loss-of-function screens. The DR5rev:mRFP signal intensity can be easily estimated by measuring the mean grey values. Ratiometric imaging of DR5 signaling and a constitutive (auxin independent) marker could furthermore increase the sensitivity of the approach. Also the usage of the so-called novel auxin signaling sensor (Aux/IAA-based) termed DII-VENUS (Brunoud et al., 2012) could be useful to improve the temporal resolution of the system, because DII is not based on gene regulation, but on auxin-dependent protein degradation. For high-throughput work flows, automation, such as qRT-PCR or luciferase-based detection, might be most beneficial.

Various transient expression approaches, such as gold particle bombardment, micro-injection, polyethylene glycol (PEG)-mediated DNA uptake, and electroporation of protoplasts (Schnorf et al., 1991; Takeuchi et al., 1992; Datta et al., 1998; Niemes et al., 2010) have been successfully used to transiently transform plant cells. Whereas transient transformation of protoplasts has been proven to be highly efficient in high-throughput work flows, particle bombardment of plant cells might be preferable for investigating auxin carrier activity, because it does not affect the cell wall integrity that might be required for auxin carrier trafficking and function (Robert et al., 2010., Wabnik et al., 2010; Feraru et al., 2011). Accordingly, here, we utilized particle bombardment of BY-2 cells as a transient transformation system to establish a single-cell-based system to monitor cellular auxin homeostasis.

As a proof of concept, we investigated prominent PIN auxin efflux carriers and visualized their action on the cellular auxin signaling. In stably transformed BY-2 cell lines, we illustrate that PIN-dependent

reduction in cellular accumulation of exogenous auxin correlates with decreased (DR5-based) nuclear auxin signaling. Using the single-cell-based system, we reveal the differential sensitivities of PIN2, PIN5 and PILS5 to the auxin transport inhibitor NPA. Under our experimental condition, NPA blocks PIN2 action at the plasma membrane, but does not diminish PIN5 and PILS5 function at the ER, indicating that auxin transport mechanisms at the plasma membrane and at the ER could be partially distinct. These findings are in agreement with the assumption that NPA action on auxin carrier activity might be restricted to the plasma membrane (Cox et al., 1996). As such, NPA could be applied to distinguish between intercellular and intracellular auxin transport. We assume that the differential sensitivity of PIN2 and PIN5 to NPA indicate the suitability of the system for chemical genetic approaches.

Besides the analysis on PIN2, PIN5 and PILS5, we also confirmed that WAT1 negatively affects cellular auxin signaling. WAT1 localizes to the tonoplast and has been suggested to regulate cellular auxin homeostasis (Ranocha et al., 2010), possibly by sequestering a yet to be identified auxinic compound into the vacuole. How WAT1 affects auxin homeostasis is still unclear and the mechanism awaits in-depth characterization. Nevertheless, WAT1 activity could be visualized indirectly with our system that might be helpful to further characterize its functionality.

In summary, we established an easy and useful tool to visualize carrier activities that affect cellular auxin signaling. This complementary method bridges the gap between highly elaborated direct auxin transport assays and indirect approaches such as root hair-based visualization of carrier-driven cellular auxin homeostasis (Imhoff et al., 2000; Geisler et al., 2005; Lee et al., 2006; Petrasek et al., 2006; Yang et al., 2006; Mravec et al., 2009; Yang et al., 2009; Ganguly., 2010). Given the transient nature

of our approach, it allows, for instance, the rapid and systematic pre-screening of several mutant versions of an auxin carrier of interest. Subsequently, interesting candidates could be analyzed in depth in other, more elaborated systems.

This single-cell-based system could be also used to analyze other molecular components involved in auxin homeostasis, such as regulators of the auxin signaling or metabolism. Moreover, it could be eventually extended to investigate other hormonal pathways by means of different reporter constructs, such as the cytokinin-responsive element TCS:GFP (Müller et al., 2008). However, compared to the DR5 auxin reporter, further in depth characterization of TCS:GFP activity in BY-2 cells is needed to use the system analogously.

Methods

Plant material and growth conditions

Nicotiana tabacum L. cv. Bright Yellow-2 cell line (Nagata et al., 1992) was cultivated at 25°C in darkness on an orbital incubator at 150 rpm in liquid medium (3% sucrose, 4.3 g L^{-1} Murashige and Skoog salts, 100 mg L^{-1} inositol, 1 mg L^{-1} thiamin, 0.2 mg L^{-1} 2,4-dichlorophenoxacetic acid (2,4-D) and 200 mg L^{-1} KH_2PO_4, pH 5.8) and subcultured weekly (50x dilution). The used constructs for transient BY-2 cell transformation have been described previously: *DR5rev:mRFP* (Marin et al., 2010), *XVE::PIN1:GFP* (Lankova et al., 2010), *GVG-PIN7* (Petrasek et al., 2006), *35S:PIN2-GFP* (Abas et al., 2006), *35S:PIN5-GFP* (Mravec et al., 2009), *35S:PILS5_D* (Barbez et al., 2012), *35S:HDEL-GFP* (Langhans et al., 2008), *35S:PIN1-RFP* (Robert et al., 2019), *35S:VP16-IAA17mImII* (Tiwari et al., 2003), *35S:WAT1:GFP* (Ranocha et al., 2010), *35S:GFP:GFP* (Ranocha et al., 2010), and

TCS:GFP (Muller et al., 2008). Expression of *PIN1-GFP* in *XVE:PIN1-GFP/DR5:mRFP1* genes was induced by the addition of β-estradiol (1 μM, 48 h) and *PIN7* in *GVG:PIN7/DR5:mRFP* by the addition of dexamethasone (3 μM, 48 h) at the beginning of the subculture interval. The solvent DMSO (estradiol) or H_2O (dexamethasone) were also added to control samples. We used *Arabidopsis thaliana* of ecotype Columbia 0 (Col-0). Seedlings were grown vertically on half Murashige and Skoog medium. Plants were grown under long-day (16 h light/8 h dark) conditions at 20–22 °C. The *Arabidopsis thaliana* DR5rev:GFP line was described previously (Friml et al., 2003). Treatment with 1μM brassinolide for 18h was performed on 7 day old seedlings in liquid growth medium.

Stable Transformation of BY-2 cells

The basic transformation protocol of An (An et al., 1985) was used. For the transformation, BY-2 lines carrying *PIN1:GFP* gene, under the estradiol-inducible transactivator XVE, (XVE-PIN1:GFP) (Lankova et al., 2010) or *PIN7* gene under dexamethasone-inducible promoter (line GVG-PIN7) (Petrasek et al., 2006) were used. Three-day-old BY-2 cells were co-incubated with *Agrobacterium tumefaciens* strain GV2260 carrying *DRrev5:mRFP* construct. Resulting double transformed lines were maintained in culture media containing 100 μg mL^{-1} kanamycin, 100 μg mL^{-1} hygromycin and 100 μg mL^{-1} cefotaxim.

Verification of transgene expression using Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Tobacco total RNA was extracted from stably transformed BY-2 cells (induction of expression GVG-PIN7 by the dexamethasone (3 µM, 24 h)) using SpectrumTM Plant Total RNA Kit (Sigma - Aldrich) and treated with DNAse from DNA-freeTM Kit (Ambion). M-MLV Reverse Transcriptase (H-) (Promega) was used to generate cDNA, according to the manufacturer's instructions. qPCR was performed using DyNAmoTM Flash SYBR® Green qPCR Kit (Finnzymes). Specific primers: AtPIN7 forward 5‘-GGGAAGAAGAGTCGGAGAG-3‘, reverse 5‘-AAGAGCCCAAATGAGACCAA-3‘; Ta = 56ºC. Resulting values are expressed as a ratio of relative expression of particular gene in induced cells against relative expression of this gene in non-induced cells. Actin was used as reference gene

Auxin accumulation measurements

Auxin accumulation in 2-day-old cells was measured using radioactively labelled auxins according to (Delbarre et al., 1996), as modified by (Petrasek et al., 2006). Treatments were replicated at least three times and the average values (± standard errors) were expressed as pmols of the particular auxin accumulated per million cells. At the beginning of the accumulation assay [^{3}H]NAA (20 Ci mmol^{-1}; American Radiolabeled Chemicals, Inc., St Louis, MO, USA) (as a good substrate of auxin efflux carrier) was added to the PIN1-GFP induced BY-2 cell line XVE-PIN1:GFP/DR5rev:mRFP (non-induced line was used as a control) to give a final concentration 2nM of [^{3}H]NAA.

Transient transformation of BY-2 cells

Adjusted from previously described procedures (Sanford et al., 1987; Klein et al., 1988) 10 ml of three-day-old cells were harvested on filter paper by vacuum filtration and kept on plates with solid BY-2 medium. The cells were transformed via particle bombardment with a PDS 1000/He biolistic system (Bio-Rad) according to the manufacturer's instructions *(http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_9075.pdf)*. To coat the gold particles with DNA, 2 µl of plasmid DNA (if not indicated differently, 0.05 µg/µl of each construct to transform) was added to 6.25 µl of 1.6-µm diameter gold particles (dissolved in 50% glycerol). The suspension was supplemented with 2.5 µl spermidine (0.1 M stock solution) and 6.25 µl $CaCl_2$ (2.5 M stock solution). For *35S:PIN2-GFP*, *35S:PIN5-GFP*, *35S:HDEL:GFP*, and *35S:VP16-IAA17mImII*, 0.1 µg/µl was used for the transformation and 0.05 µg/µl for *3S5:WAT1:GFP* and *35S:GFP:GFP*. The particles were pelleted by centrifugation, washed twice with 70% and 100% ethanol and, subsequently, resuspended in 10 µl of 100% ethanol. Cells were bombarded under a pressure of 1100 psi. Pharmacological treatments were done by applying 0.5 ml of BY-2 growth medium, enriched with 10 µM NPA, 10 µM 6-benzylaminopurine (BAP) (Duchefa) or 1 µM brassinolide (BR) (Fuji Chemical Industries) directly after transformation. The plates were sealed with parafilm and kept in the dark for 18 h at 25°C. For microscopic analysis, cells were gently transferred (with a spatula) from the filter to a microscopy slide (in water) and subsequently covered with a cover slip. Samples were analyzed via confocal microscopy.

Microscopy

Live-cell confocal microscopy was done with a Zeiss 710 microscope (Zeiss). Fluorescence signals for GFP (excitation 488 nm, emission peak 509 nm) and mRFP1 (excitation 561 nm, emission peak 607 nm) were detected. Sequential scanning was used for double labeling to avoid crosstalk between fluorescence channels. The *DR5rev:mRFP* expression was evaluated by defining the mean gray value (MGV) of each imaged cell (middle sections). For each experiment, confocal settings were defined based on the DR5rev:mRFP signal of the control cells and remained unchanged during the respective experiment. Transformants were identified based on the fluorescence of both proteins, imaged with a 40x objective, and subdivided into four clusters (very low, low, medium, and high) according to the relative MGV (See also Supplemental Figure 2). Every experiment was done in triplicate (independent transformations) and for each condition, a total number of at least 60 transformed cells were imaged. The means and standard errors were calculated and the statistical significance (independence between the two populations) was obtained by student t-test (for the analysis of the MGV) and ANOVA analysis (for the subdivision into clusters).

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We thank T.J. Guilfoyle, C. Luschnig, D. Goffner, J. Sheen and P. Pimpl for sharing published materials and M. De Cock for help in preparing the manuscript. This work was supported by the Vienna Science and Technology Fund (WWTF) (to J.K.-V.), the Agency for Innovation by Science and Technology (IWT) and Fonds voor Wetenschappelijk Onderzoek (FWO) (predoctoral fellowship and travel grant to E.B.), the Odysseus program of the Research Foundation-Flanders (to J.F) Grant Agency of the Czech Republic, projects CZ.1.07/2.3.00/20.0043, CZ.1.05/1.1.00/02.0068 (to J.F.), P305/11/2476 (to J.P.) and P305/11/0797 (to E.Z.) and the Land Baden-Württemberg, the Chica und Heinz Schaller Stiftung and the CellNetworks cluster of excellence of the Heidelberg university (to A.M.)

Supplementary information

concentration plasmid 1 (µg/µl)	concentration plasmid 2 (µg/µl)	cotransformation efficiency
0.05	0.01	32/40 (80%)
0.05	0.05	39/43 (91%)
0.05	0.1	40/40 (100%)

Table S1. Cotransformation efficiencies.

The cotransformation efficiency was measured for two constructs transformed at several concentration ratios. Transformants were identified based on the presence of plasmid 1 and the percentage of cells carrying both plasmids was calculated.

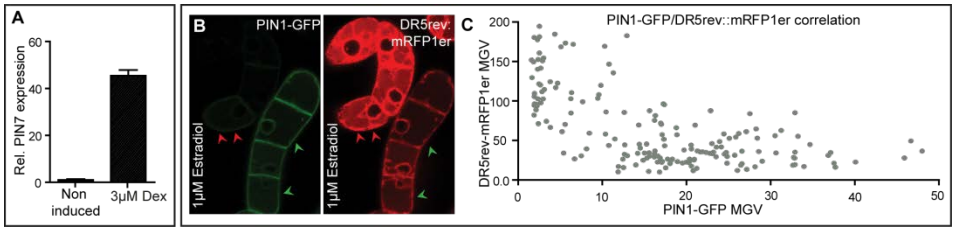

Figure S1: Correlation between PIN1-GFP and DR5rev:mRFP signal intensity.

(A) Graph depicts relative *PIN7* expression levels of dexamethasone induced *GVG-PIN7* and non-induced cells analysed by quantitative-RT-PCR (n=3). **(B)** Estradiol induced BY-2 cells shows individual variability of *PIN1-GFP* expression. Cellular intensity of PIN1-GFP reveals a negative correlation between PIN1-GFP and DR5rev:mRFP signal intensity. Strongly *PIN1-GFP* expressing cells show a strong decrease *in* DR5rev:mRFP signal intensity (green arrow heads) compared to cells with weaker *PIN1-GFP* expression (red arrow heads). **(C)** Scatterplot depicts single cell mean gray value (MGV) of the PIN1-GFP and the corresponding DR5rev:mRFP fluorescent intensity (n=178).

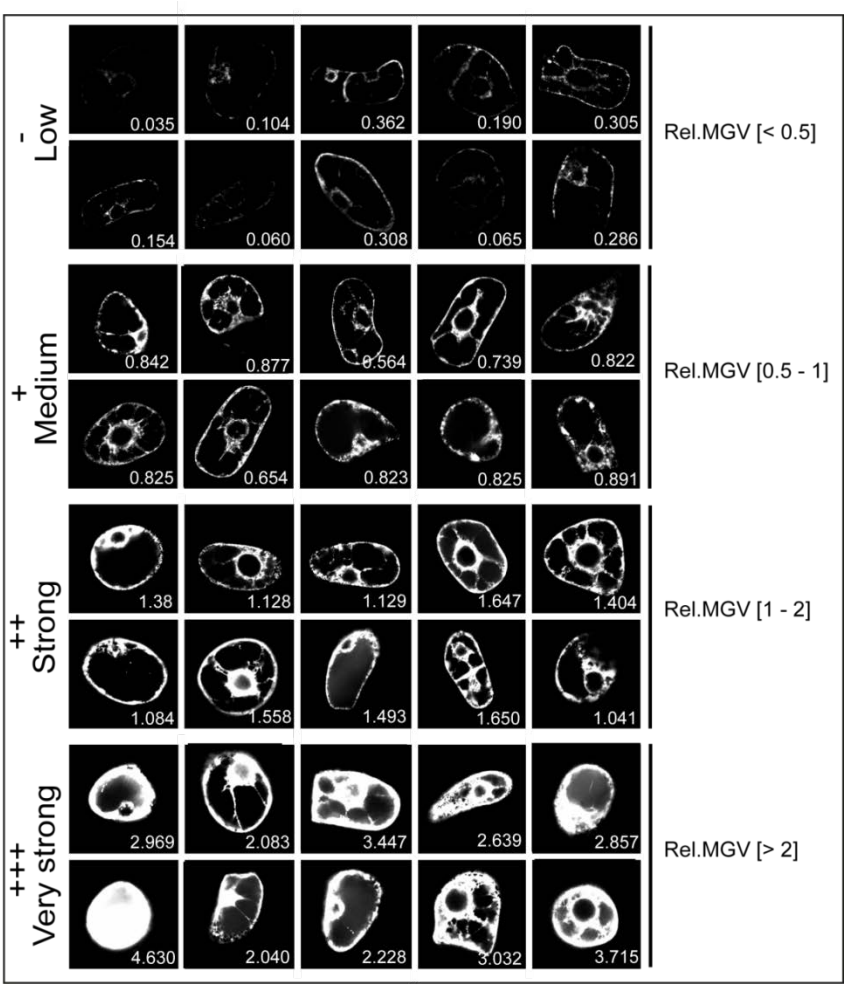

Figure S2. DR5rev:mRFP signal intensity quantification.

DR5rev:mRFP signal intensity is visualized by gray scale representation and the mean gray value (MGV) of each transformed cell is measured using Image J. The relative MGV of each cell is calculated according to the average MGV of the control sample. Individual relative MGV are depicted in the pictures.

The transformed cell population of each sample is subdivided in 4 classes according to the relative MGV. Cells were scored as low (-) with a relative MGV below 0.5 (= 2^{-1}), medium (+) with a relative MGV between 0.5 (= 2^{-1}) and 1 (= 2^{0}), high (++) with a relative MGV between 1 (= 2^{0}) and 2 (= 2^{1}) and very high (+++) with a mean grey value higher then 2 (= 2^{1}). This evaluation visualizes the variability of DR5rev:RFP1er signal intensity within the transformed cell population. In the used confocal settings, most of the visualized cells clustered in the categories medium and strong.

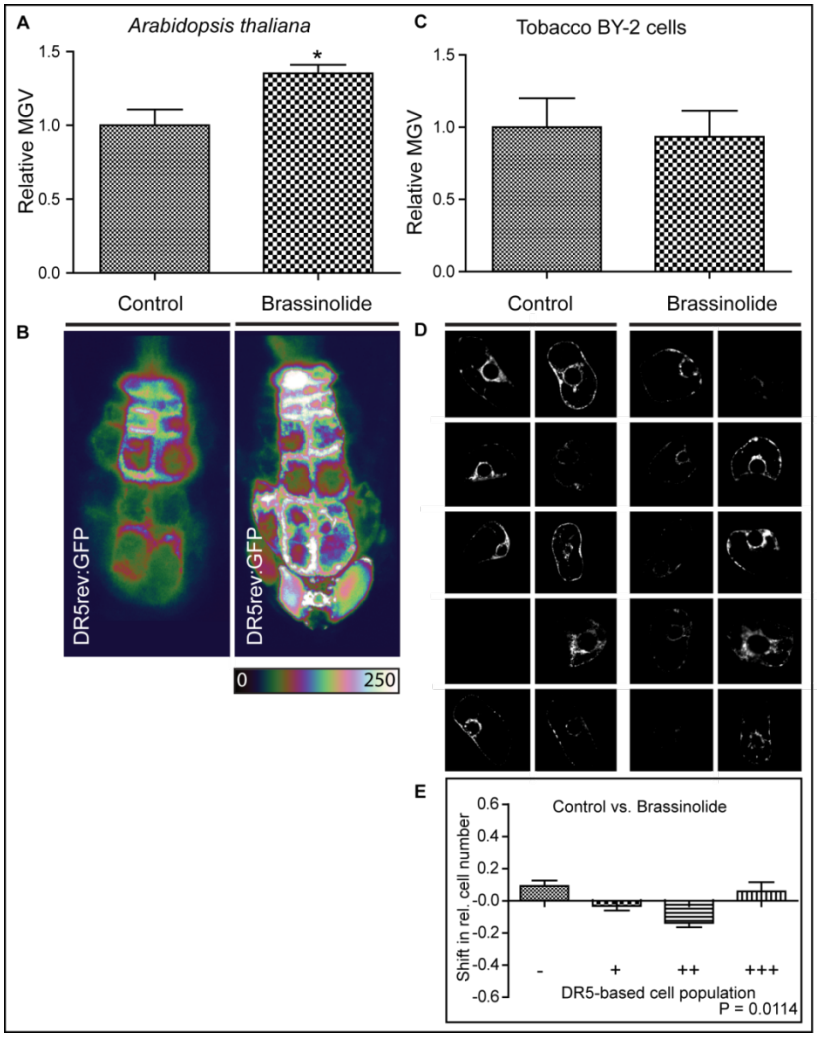

Figure S3. Effect of Brassinolide on cellular auxin homeostasis.

(A) *DR5rev:GFP* expression in the root tip of brassinolide (1µM; 18 hours) treated and untreated *Arabidopsis thaliana* seedlings. Graph represents the relative average mean gray values (MGV) of DR5rev:GFP intensity. Error bars represent standard error (n > 20). **(B)** Representative pictures display DR5rev:GFP signal intensity of untreated (left) and brassinolide treated (right) seedlings. Color-code (black to white) depicts (low to high) DR5*rev*:GFP signal intensity. **(C)** Graph represents the relative average MGV of the *DR5rev:mRFP* transformed BY-cells. Error bars represent standard error (n > 50). Application with 1µM brassinolide-enriched medium did not lead to a significant change in the average relative MGV of DR5rev:mRFP. Statistical significance was evaluated with the unpaired student T-test (* P < 0.05, ** P< 0.01, *** P < 0.0001). **(D)**

Representative pictures show the DR5rev:mRFP signal intensities of 10 transformed control (left) and brassinolide treated (right) cells. **(E)** Graph depicts the change in relative number of transformed cells displaying a low (-), medium (+), high (++), and very high (+++) DR5rev:mRFP signal intensity between the two samples. For detailed description of the quantification, see Supplemental Figure S2. Brassinolide treatment (application with 1µM brassinolide-enriched medium) leads to a significant change in relative number of cells displaying a low, medium, high, and very high DR5rev:mRFP signal intensity indicating that brassinolide affects the variability of relative MGV within the transformed cell population. Error bars represent standard error (n=3 repetitions with at least 50 counted cells). Statistical significance was evaluated with the ANOVA test; The P value is indicated.

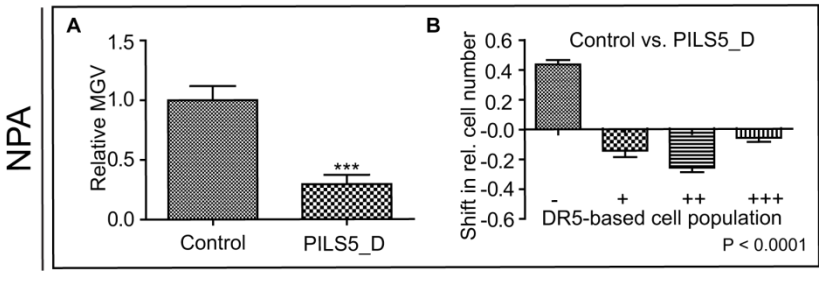

Figure S4. PILS5 sensitivity to NPA.

In the presence of NPA, PILS5_D expression decreases DR5rev:mRFP signal intensity. **(A)** Graphs represent the relative average mean gray values (MGV) of the DR5rev:mRFP signal intensity. Error bars represent standard error (n = 60). Statistical significance was evaluated with the unpaired student T-test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). **(B)** Graphs depict the change in relative number of transformed cells displaying a low (-), medium (+), high (++), and very high (+++) DR5rev:mRFP signal intensity between the two samples (for detailed description of the quantification, see Supplemental Figure S2). Error bars represent standard error (n=3 repetitions with at least 60 counted cells). Statistical significance was evaluated with the ANOVA test; the P- value is indicated.

Chapter 4

IA-asp Involvement in Plant Development

Non-hydrolysable Auxin Conjugates affect Auxin Signaling and Plant Development

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Unpublished

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Author contributions: EB and JKV conceived the project. EB carried out most of the experiments. AH performed the *in vitro* IA-asp binding assay. J.R. measured the auxin and conjugate content in *Arabidopsis thaliana*. MG performed the computational TIR1 modeling. EB, JKV, AH, LCV, MG and CO discussed the results, EB wrote the manuscript.

Abstract

The phytohormone auxin is generally known to play a crucial role in plant growth and development. Decades of research intensively focused on the most abundant auxin molecule, indole-3-acetic acid (IAA), which has shed an enormous light on the mechanisms of its perception, signaling, biosynthesis and transport through the plant. However, free IAA gets largely further metabolized via its conjugation to other molecules, such as sugars, amino acids, peptides and even proteins. Recent publications report the importance of auxin conjugate enzymes for plant development. However, the specific biological roles for the wide spectrum of auxin conjugates is currently unknown. Here, we show preliminary data suggesting that the auxin amide conjugate indole-3-acetyl-aspartic acid (IA-asp) possess biological activity similar to free IAA. Exogenous application of IA-asp inhibits main root growth and stimulates nuclear auxin signaling in *Arabidopsis thaliana* possibly via a direct impact on the TIR1-dependent auxin signaling pathway.

Introduction

Auxin is one of the most studied phytohormones due to its unambiguous importance for plant growth and development (reviewed in Sauer et al., 2013). Many decades of research unraveled a high number of plant developmental processes in which auxin is involved, such as embryogenesis, post embryogenic organogenesis, tropisms and biotic- as well as abiotic stress responses (reviewed in Vanneste and Friml, 2009). Considering its versatile role in plant development, auxin needs to be strictly controlled on all levels including, its biosynthesis, metabolism, transport and intracellular sequestration (Barbez and Kleine-Vehn, 2013). These processes establish and maintain the correct levels of *active auxin* in a particular organ, tissue or cell. The most abundant endogenous auxin, indole-3-acetic-acid (IAA), is currently described to be only active in its free form which, however, covers only a small portion of the total auxin amount in the cell (Östin et al., 1998; Kowalczyk and Sandberg, 2001). The majority of auxin is covalently bound/conjugated to different molecules such as sugars, amino acids, peptides and/or proteins (Cohen and Bandurski, 1982). Most of the cellular IAA in *Arabidopsis thaliana* is present as amid-linked conjugates of which the most abundant are IA-alanine (IA-ala), IA-leucine (IA-leu), IA-aspartate (IA-asp) and IA-glutamate (IA-glu) (Tam et al., 2000), however, also others have been detected (Kowalczyk and Sandberg, 2001; Kai et al., 2007). IA-ala and IA-leu are described as IAA storage molecules which can be hydrolyzed when free IAA is needed (Hangarter and Good, 1981; Bartel and Fink, 1995; Davies et al., 1999; Rampey et al., 2004). However, the conjugation of IAA to aspartate or glutamate is considered to be irreversible as an intermediate step in the degradation pathway (Tuominen et al., 1994; Östin et al., 1998). Except from IAA-tryptophane (IA-trp), which displays anti-auxinic characteristics (Staswick et al., 2005), IAA amide conjugates

are assumed to be biologically inactive (Leclere et al., 2002). However, the high number of distinct auxin conjugates makes it difficult to envision that none of them exerts a relevant biological function in plants (Rosquete et al., 2012).

Results

Effect of exogenous IA-asp on seedling development

Similar to free IAA, IA-ala and IA-leu inhibit main root growth in *Arabidopsis thaliana* seedlings presumably by the hydrolysis of these conjugates causing the subsequent release of free IAA (Bartel and Fink, 1995; Fig. 1a and b). Previous work described that 40µM IA-asp does not affect main root growth in *A. thaliana* seedlings (Leclere et al., 2002). Under our growth conditions, also seedlings grown on the non-hydrolysable conjugates IA-asp and IA-glu display shorter roots (Fig. 1a and c). This difference in observations could be due to the IA-asp compound used for the analysis. In Leclere et al., 2002, the IA-asp was used in a L/R isomeric mix obtained from Sigma Aldrich. For our analysis, we use the (only naturally occuring) L-isomer of the IA-conjugates of interest purchased from Olchemim (Czech Republic). We could confirm that the IA-L/R-asp chimer is biologically inactive at a concentration of 40µM (data not shown). All below described experiments were performed with IA-L-conjugate isomers.

We tested whether IA-asp affects also other auxin regulated processes in Arabidopsis seedlings. Indeed, IA-asp also increases the lateral root density and stimulates root hair elongation suggesting biological activity for IA-asp similar to free IAA (Fig. 1d and e). However, the concentration of exogenous IA-conjugates needed to alter seedling growth is much higher than in the case of free IAA, which might

indicate low cellular uptake rates (Bartel and Fink, 1995; Rampey et al., 2004). By performing endogenous IAA and IA-asp measurements after IAA and IA-asp treatment of *A thaliana* seedlings, we observed that, in contrast to free IAA, the cellular IA-asp accumulation after exogenous IA-asp application is rather poor (Fig 2). This explains the need of high exogenous IA-asp concentrations to trigger effects on plant development. However, seedlings germinated on normal growth medium and transferred to IA-asp supplemented medium 4 days after germination display root growth inhibition upon 2.5µM IA-asp (Fig 1d). Taken together, our findings indicate that the IAA amino acid conjugate IA-asp displays biological activity.

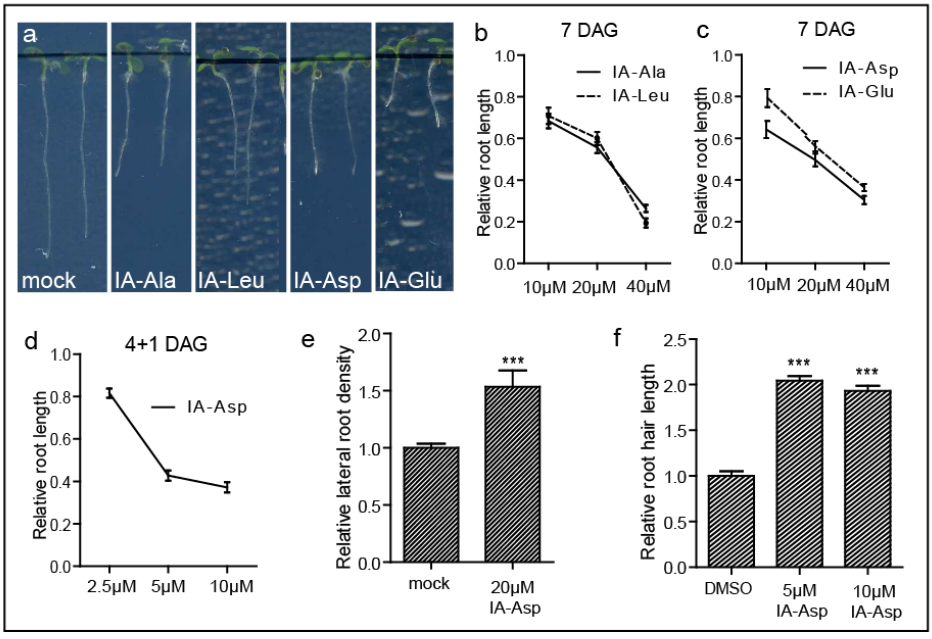

Figure 1. Effect of exogenous IA-Asp on seedling development

a, 7 day old wild type (WT) seedlings grown on unsupplemented or 20µM IA-conjugate supplemented growth medium.

b, c, Relative root length of WT seedlings grown on IA-ala (b), IA-leu (b), IA-asp (c) or IA-Glu (d) supplemented growth medium compared to WT seedlings grown on

unsupplemented growth medium (7 days after germination) Student t-test P-value < 0.001. (n>30)

d, Relative seedling growth of WT seedlings germinated on unsupplemented growth medium and transferred to IA-asp supplemented medium 4 days after germination compared to seedlings transferred to unsupplemented growth medium. The root growth was measured, 1 day after seedling transfer. (n>30).

e, Lateral root density of seedlings grown on IA-asp supplemented medium compared to seedlings grown on unsupplemented medium. (n> 15)

f, Relative root hair length of seedlings grown on IA-asp supplemented medium compared to seedlings grown on un-supplemented growth medium. (n> 200)

Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001

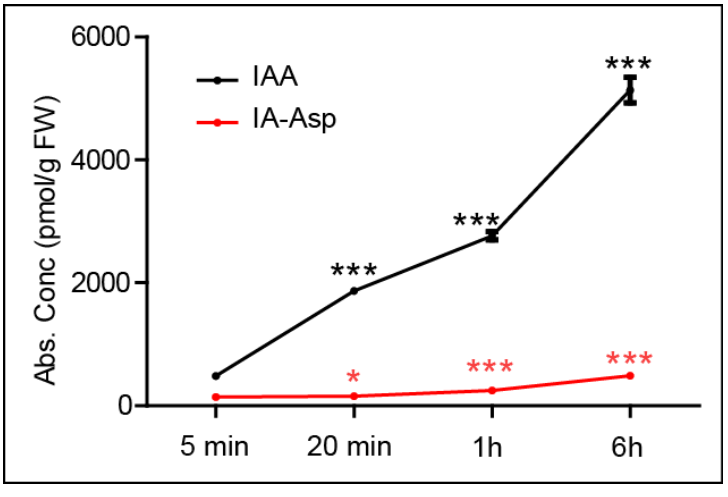

Figure 2. Effect of IAA and IA-Asp op the endogenous levels of IAA and IA-Asp respectively.

Absolute increase of endogenous IAA and IA-Asp levels upon exogenous application of 1µM IAA and 10µ IA-Asp respectively. Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001

IA-asp effect on nuclear auxin signaling

Next, we investigated whether IA-asp can affect nuclear auxin response using the auxin responsive promoter-reporter line DR5rev::GFP (Ulmasov et al., 1997). IA-asp treatment increased the *DR5rev::GFP* signal intensity as well as the expression of several auxin responsive genes as shown by qRT-PCR (Fig. 3a and b). We also analyzed the effect of IA-asp on the recently described, rapidly reacting auxin response reporter DII::Venus showing auxin dependent degradation (Brunoud et al., 2012). After 10 min of IA-asp treatment, we observed a significantly decreased *DII-Venus* signal intensity indicating increased auxin signaling (Fig. 3c and d). Treatment with IA-leu also resulted in a partial *DII-Venus* degradation (Fig. 3c and d). Since IA-asp is not-hydrolyzable, this data indicates that, unlike IA-Leu that requires hydrolysis for the release of free IAA, IA-asp itself might be able to rapidly induce auxin signaling leading to the alteration of auxin dependent gene expression.

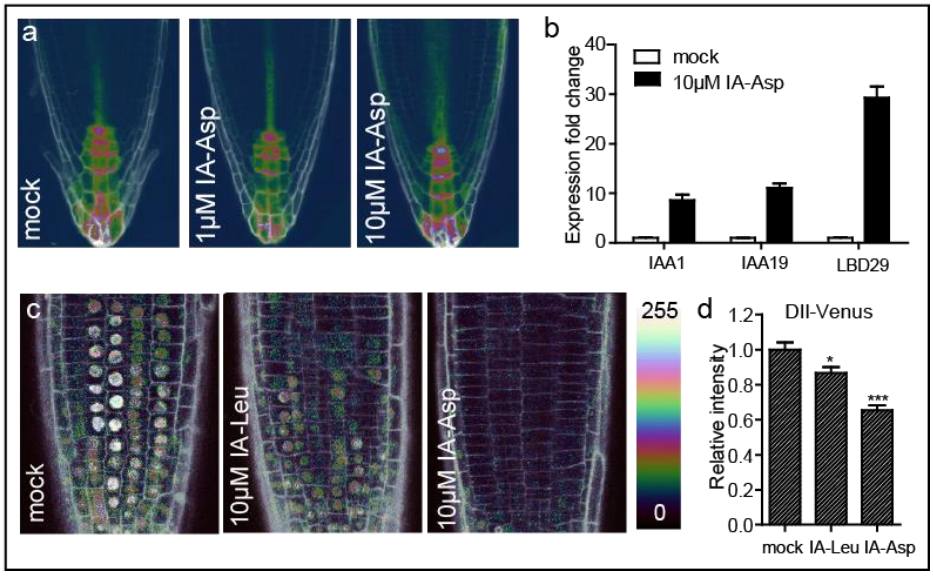

Figure 3. IA-asp effect on nuclear auxin signaling.

a, Auxin responsive DR5 promoter activity in WT *pDR5::GFP* seedlings treated with IA-Asp for 3h. Colour-code (black to white) depicts (low to high) *pDR5rev::GFP* signal intensity.
b, qRT-PCR reveals the expression of auxin responsive genes IAA1 and IAA19 and LBD29 in WT seedlings treated with 10μM IA-asp for 30min. Graph depicts the relative expression of the auxin responsive genes compared to their basal expression in the untreated seedlings. The auxin responsive gene expression values were normalized towards the housekeeping gene EIF4a using the Livak method.
c,d, *DII::Venus* signal intensity in WT *DII-Venus* seedlings treated with IAA, IA-leu or IA-asp for 10 min. Colour-code (black to white) depicts (low to high) *DII-Venus* signal intensity (c). Graph depicts the relative average of *DII-Venus* signal intensity (d). Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001.

The involvement of auxin receptor TIR1 in IA-asp signalling

Most nuclear auxin is perceived by auxin receptors such as TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) that, upon auxin binding, triggers the ubiquitination and subsequent degradation of the AUX/IAA auxin signaling repressors affecting the expression of specific target genes (Gray et al., 1999). To investigate whether IA-Asp acts via the TIR1/AFB pathway, we grew wild type and *tir1afb2afb3* triple mutant seedlings on IA-asp supplemented medium. The mutant seedlings displayed a partial resistance towards the root growth inhibiting effect of IA-asp compared to wild type seedlings, suggesting that TIR1/AFB proteins are involved in mediating the effect of IA-asp on seedling main root growth (Fig. 4a and b). Next, we used a synthetic yeast system expressing a TIR1/AFB protein as well as a YFP-tagged AUX/IAA protein from *A. thaliana* enabling to monitor their auxin dependent degradation (Havens et al., 2012). We analyzed

yeast cells containing AtTIR1 or AtAFB2 both co-expressed with IAA1-YFP and observed that, besides free IAA, also IA-asp is able to trigger a partial degradation of IAA1-YFP, which was most prominent in the AtAFB2 expressing yeast cells. In contrast, a mutation in TIR1, aborting its co-receptor function, fully inhibited the IA-asp effect on IAA1-YFP degradation. These data suggest that, in the presence of AtTIR1/AFB2, IA-asp is able to trigger AtAux/IAA degradation in a heterologous system. Yeast cells lack any further auxin machinery, which pinpoints a direct interaction between TIR1/AFB proteins and IA-asp (Fig 4c and d). Moreover, computational molecular modeling predicts 2 possible orientations for IA-asp to fit into the TIR1 binding pocket (Fig. 4e). To test whether IA-asp can bind to an auxin co-receptor complex, we performed in vitro competition binding assays. TIR1-ASK1 and GST-IAA14 were recombinantly expressed and IA-asp was tested for its ability to compete with radiolabeled IAA to bind the receptor complex. We observed that IA-Asp can indeed outcompete the radiolabelled IAA from the TIR1-IAA14 co-receptor complex (Fig. 4f). To assess the specificity of IA-asp for the receptor complex, we included the organic control benzoic acid, which was not able to compete with IAA binding on the complex. These data indicate that IA-asp displays a specific binding capacity to the TIR1-IAA14 receptor complex *in vitro.*

Our data indicate that auxin receptor TIR1 is required for IA-asp effect on plant development presumably via a direct interaction of IA-asp with the TIR-AUX/IAA receptor complex.

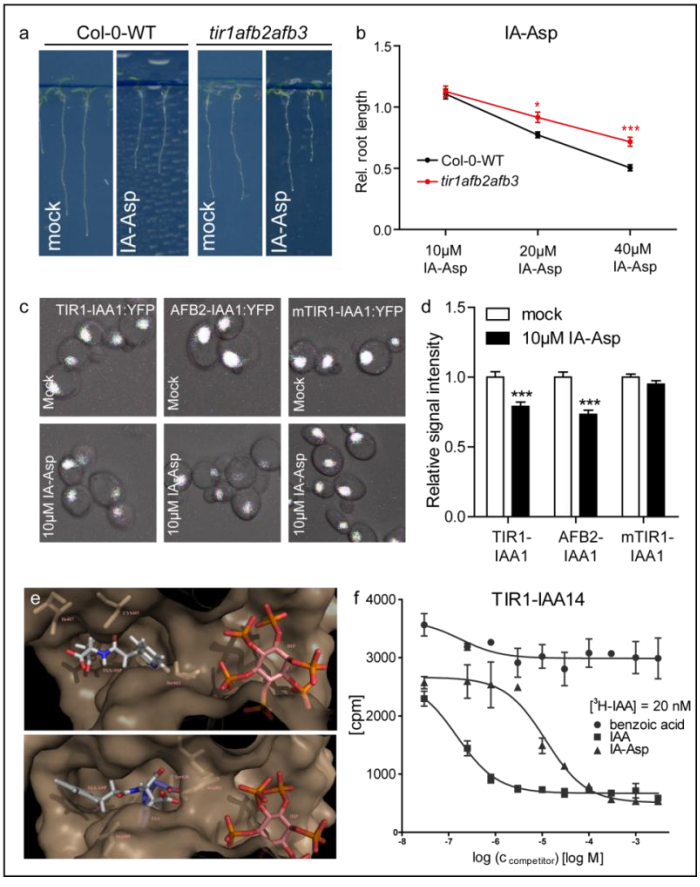

Figure 4. The involvement of auxin receptor TIR1 in IA-asp signalling

a,b, 7 day old WT and *tir1afb2afb3* triple mutant seedlings grown on un-supplemented or IA-asp supplemented growth medium. Graph depicts the root length of seedlings grown on IA-asp supplemented medium relative to seedling grown on un-supplemented medium (b). Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001.

c,d, IAA1-YFP signal intensity in *Saccharomyces cerevisiae* yeast cells expressing the *A.thaliana* auxin receptor AtTIR1, AtAFB2 or mTIR1 after a 3 hours IA-asp treatment. Graph shows the relative average IAA-YFP signal intensity (n>50). Error bars represent s.e.m. Student t-test P-values: *P< 0.05, **P<0.001 ***P<0.0001. This experiment was performed twice.

e, Computational representation of two binding possibilities for IA-asp in the TIR1 ligand binding pocket.

f, Levels of [^{3}H]IAA binding to TIR1-IAA14 co-receptor complex upon competition with benzoic acid, IAA and IA-asp. This experiment was performed twice.

Discussion

The indispensability of auxin for plant growth and development requires a strict regulation on the level of signaling, biosynthesis, metabolism and transport through the plant. Many decades of intensive research have provide a better understanding of these processes and have shed more light on the complex nature of auxin biology (reviewed in Vanneste and Friml, 2009). The recent elucidation of auxin transport between different compartments within the cell (Mravec et al., 2009; Bosco et al., 2012; Ding et al., 2012; Barbez et al., 2012) and its impact on cellular auxin metabolism opened the discussion on the biological relevance of the high number of auxin metabolites (Rosquete et al., 2012). The IAA amide conjugate indole-3-acetyl-aspartic-acid (IA-asp) has been previously described to have no effect on *A. thaliana* seedling main root growth at a concentration of 40µM (Leclere et al., 2002). However, for these analyses, the L/R isomeric mix of IA-asp has been used which was also biologically inactive in our hands (data not shown). In this manuscript, we show preliminary data, suggesting that IA-L-asp affects plant development in a similar manner as free IAA. Under our growth conditions, IA-L-asp inhibits main root growth, increases the lateral root density and stimulates root hair elongation in *Arabidopsis thaliana* seedlings via a positive effect on nuclear auxin signaling. It could be speculated that the IA-R-asp isomer is biologically inactive and therefore higher concentrations of the IA-L/R-asp are needed to observe the phenotypes we obtain with the IA-L-asp isomer.

We could show that IA-asp action requires the auxin receptor TIR1 (and/or one of its AFB homologues) since the *tir1afb2afb3* triple mutant displays partial resistance to the root growth inhibiting effect of IA-asp. IA-asp also induces degradation of the auxin co-receptor IAA1-YFP

heterologously in *Sacharomyces cerevisiae* yeast cells indicating IA-asp binding on the TIR1/AFB-IAA1 receptor complex. Moreover, in vitro competition assays on recombinant TIR1-IAA14 proteins indicates the ability of IA-asp to bind this receptor complex. The concentration of IA-asp, needed to outcompete radiolabelled IAA bound at the complex is much higher compared to non-labeled free IAA in our in vitro assay, which pinpoints at low *in vitro* affinity of IA-asp for this particular receptor complex. However, the presence of 4 different TIR1/AFB proteins in *Arabidopsis thaliana*, and 33 AUX/IAA proteins allow the formation of 132 TIR1/AFB-AUX/IAA combinations which could have different binding affinities for IA-asp. Tissue specific expression of certain TIR1/AFB-AUX/IAA combinations as well as the different distributions of IAA and IA-asp through the plant could provide an additional level in the regulation of cellular auxin homeostasis. However, more competition assays with different TIR1/AFB-AUX/IAA combinations are needed to strengthen this hypothesis.

Another, and actually historically earlier, described auxin receptor is AUXIN BINDING PROTEIN 1 (ABP1) which localizes at the plasma membrane as well as in the endoplasmic reticulum. ABP1 is expressed from a single copy gene and plants lacking ABP1 are embryo lethal (Chen et al., 2001; reviewed in Sauer and Kleine-Vehn, 2011). Although the molecular function of ABP1 has been until today only partially elucidated, it is described to mainly mediate fast and non-genomic responses. Recent articles describe the stimulating effect of non-auxin-bound-ABP1 on clathrin dependent endocytosis at the plasma membrane (Robert and Kleine-Vehn et al., 2010). Auxin inhibits endocytosis (Paciorek et al., 2005) via its binding to ABP1 (Robert and Kleine-Vehn, 2010). It needs to be seen whether IA-asp inhibits the stimulating effect of ABP1 on endocytosis. Preliminary *in silico* molecular simulations suggests the

ability of IA-asp to bind ABP1 and to form even more hydrogen bridges than free IAA, suggesting a more favorable binding (data not shown).

The poor uptake of exogenous IA-asp as well as preliminary results on IA-asp polar transport assays (data not shown) suggest the absence of cell-to-cell IA-asp transport, which is in big contrast to free IAA. These data indicate a cell autonomous signaling function for IA-asp allowing a very accurate regulation of developmental processes on a cellular level.

The question remains what could be the functional importance of IA-asp in plant growth and development since most of the performed experiments in this work are based on exogenous treatments with high IA-asp concentrations. It has been previously described that the IA-asp levels in different parts of *A. thaliana* plants are around a 5 fold lower than the levels of free IAA (Kowalczyk and Sandberg, 2001). However, IA-asp formation could be upregulated upon certain biotic or abiotic stimuli, during a specific developmental process or in a limited number of cells. Böttcher et al described in 2010 that the levels of IA-asp exceed the levels of free IAA in 4-fold during grape berry fruit ripening (Böttcher et al., 2010). Besides, the IAA and jasmonate (JA) conjugating enzymes, GH3.5 and GH3.6 seem to play a role in adventitious root formation (Gutierrez et al., 2012). It is currently under investigation whether IA-asp might play a more specific role in plant development than being just a sink for excessive IAA. The data obtained from the performed micro-array will give insight in which plant developmental processes IA-asp might play a role. The array data needs to be validated by testing the expression of the candidate genes in mutants containing higher or lower levels of IA-asp (Park et al., 2007, Staswick et al., 2005, Gutiérrez et al., 2012).

Even though IA-asp is generally assumed to be an intermediate product of IAA degradation, our preliminary data show that this *'degradation intermediate'* might display biological activity. Further research will give insight into the functional importance of IA-asp for plant growth and development.

Methods

Plant material, growth conditions and DNA constructs

We used *Arabidopsis thaliana* of ecotype Columbia 0 (Col-0) and WS-WT. The *wes1_D* mutant was crossed into *pDR5rev::GFP.* Seeds were stratified at 4 °C for 2 days in the dark. Seedlings were grown vertically on half Murashige and Skoog medium. Plants were grown under long-day (16 h light/8 h dark) conditions at 20–22 °C. Previously described lines: *tir1afb2afb3* triple mutant (Dharmasiri et al., 2005).

Yeast strains

pG5G-TIR1, pG5G-AFB2, pG5G-mTIR in W814-29B and pG4GY-IAA1, pG4GY-IAA6, pG4GY-IAA18 in W303-1A were described previously (Havens et al., 2012).

Chemicals

1-Naphtaleneacetic acid (NAA) was supplied by Duchefa, Indole-3-acetic acid (IAA), 2,4-dichlorophenoxy acetic acid (2,4-D), Estradiol, Brefeldin A and propidium iodide (PI) by Sigma-Aldrich. indole-3-acetyl-L-aspartic acid (>98% pure), our MS analysis (detection limit of 0,1 %) did not detect IAA contamination, indole-3-acetyl-L-alanine (>98% pure), indole-

3-acetyl-leucine (>98% pure) and indole-3-acetyl-L-glutamic acid (>98% pure) by Olchemim (Czech Republic).

RNA extraction and quantitative real time PCR (qPCR)

Whole RNA of seedlings, seedling roots or shoots was extracted using the innuprep Plant RNA kit (Analytik Jena) from which cDNA was synthesised using the iScript cDNA synthesis kit (Biorad). Q-PCR analysis was performed using an Biorad CFX96 Real time system with the IQ SYBRgreen super mix (Biorad) following manufacturers recommendations. Q-PCR was carried out in 96-well optical reaction plates heated for 3 minutes to 95°C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 10 seconds at 95°C, annealing for 30 seconds at 55°C and extension for 30 seconds at 72°C. Target quantifications were performed with specific primer pairs (given below) designed using Quantprime (http://quantprime.mpimp-golm.mpg.de/main.php?page=home). Expression levels were normalized to the expression levels of translation initiation factor EiF4a using the Livak method. If not mentioned different, experiments were performed at least 3 times.

Used qPCR primers:

EIF4a_FW: CTGGAGGTTTTGAGGCTGGTAT

EIF4a_Rev: CCAAGGGTGAAAGCAAGAAGA

IAA1_FW: GTCAAAAACTCAGAATCATGAAAGGA

IAA1_Rev: TGCCTCGACCAAAAGGTGTT

IAA19_FW: GTGGTGACGCTGAGAAGGTT

IAA19_Rev: CGTGGTCGAAGCTTCCTTAC

LBD29_FW: ACTGGAAGTTCTGGGACGGTTC

LBD29_Rev: TGCCTGAGGAGGTTTCGTTGTG

Phenotype analysis

If not mentioned differently, seedlings were germinated and grown on sugar free growth medium possibly supplemented with a compound in the given concentration. To avoid possible photodegradation of the used auxinic compounds, plates were grown under a yellow filter. For the analysis of the lateral root density and if mentioned elsewhere, seedlings were germinated on a nylon mesh (SilkAndProgress, www.silkandprogress.cz) in contact with the growth medium. The mesh with the seedlings was transferred to a mock or compound supplemented growth plate 4DAG. For analysis of the hypocotyl length, root length and lateral root density, plates were scanned on a flat-bed scanner. Lateral root density for each seedling was obtained by calculating the number of lateral roots per root length unit 10 (4+6) days after germination. . For analysis of hypocotyl length, root length and lateral root density, a minimum of 30 plants per condition or mutant line were analysed in each experiment. Means and standard errors were calculated and the statistical significance was evaluated by the student t-test using the GraphPad Prism5 (*www.graphpad.com*) software. For the analysis of root hair elongation, 20 seedlings per condition were imaged by binocular (Leica) and 20 root hairs (randomly chosen in the root hair zone) per seedling were measured with the ImageJ *(http://rsb.info.nih.gov/ij/)* software. The untreated mean average root hair length of the respective genotype was subtracted from the individual auxin treated root hair length to obtain auxin induced growth in millimeter. The mean and standard error of the mean of the respective genotype were calculated and the statistical significance was evaluated by the student t-test using the GraphPad Prism5 (*www.graphpad.com*) software. If not mentioned different, experiments were performed at least 3 times.

Compound treatments

5 day old *pDR5rev::GFP and DII-Venus* seedlings were transferred to mock (DMSO), IAA or IAA-conjugate (both dissolved in DMSO) supplemented liquid or solid growth media respectively for the given time and concentration. Brefeldin A treatments were performed as previously described (Paciorek et al., 2005).

Microscopy

Confocal microscopy was performed with a Leica SP2 or Leica SP5 microscope. Fluorescence signals for GFP (excitation 488 nm, emission peak 509 nm), mRFP1 (excitation 561 nm, emission peak 607 nm) and propidium iodide (PI) staining (excitation 536nm, emission peak 617nm) were detected with a 63x (water immersion) objective. Fluorescence signal intensity was analyzed with Image J *(http://rsb.info.nih.gov/ij/)* software and data were statistically evaluated with Microsoft Excel 2010.

Heterologous Competitive Auxin Binding Assays

Radioligand binding assays were performed as previously described in Calderón Villalobos et al. (2012). In brief, binding assays were carried out using highly pure recombinant TIR1-ASK1 protein complex and N-terminal GST-tagged IAA14 protein. Three independent experiments with two replicates each were performed using 20 nM or 50 nM [^{3}H]IAA and increasing concentrations of cold competitors IA-asp, IAA or benzoic acid. Samples containing proteins, radiolabeled IAA and cold competitor were incubated for 1 hour on ice, subsequently filter-immobilized and washed with binding buffer. Filters were incubated overnight in scintillation buffer and retained radiolabeled auxin was measured via scintillation counting. Data analysis was performed using GraphPad Prism 5 software. IC50 values were obtained applying One site - Fit logIC50

model. K_i was calculated via Cheng-Prusoff equation based on a K_d of TIR1-IAA14 for IAA of 10.1 nM (Calderón Villalobos et al. 2012).

Free IAA and conjugate measurements in Arabidopsis thaliana

For the quantification of free IAA and its amino acid conjugates, approximately 60 mg of plant material was taken into analysis. IAA and IAA derivatives were isolated and enriched by immune-affinity extraction and subsequently quantified by a LC-MS/MS analysis. The use of internal standards for the compounds of interest makes the method very sensitive and selective. The used protocol has been previously described in detail (Pencik et al., 2009).

Yeast assays

The yeast assay to address potential IA-asp and TIR1/AFB auxin receptor induced AUX/IAA-YFP degradation was performed as described previously (Nemhauser et al., 2012).

Molecular modeling

For the docking of IA-asp in the TIR1 receptor: the crystal structure of the TIR1 (PDB: 2P1Q) was used. TIR1 was simulated with the co-receptor IAA7 and the co-factor Inositol Hexakisphosphate (IHP), with free IAA and IA-asp respectively. Free IAA has been crystallized with TIR1. IA-asp was docked to TIR1 without giving a specific site, using the molecular operating environment (MOE 2011.10) program. During the docking the protein was kept rigid, while the ligand was flexible.

Conclusion & Perspectives

International research efforts unveiled the unambiguous importance of the plant hormone auxin and its polar distribution for plant growth and development (Vanneste and Friml., 2009; Sauer et al., 2013). At the start of this project, in 2008, only plasma membrane (PM) localized auxin transport carriers were described, including the auxin export carriers from the PIN protein family (Zazimalova et al, 2010). As PILS proteins share a similar predicted protein topology with the PINs, we assumed that PILS proteins would represent an additional family of auxin transport carriers at an unknown membrane. Only in 2009, it got published that a subgroup of the PINs, including PIN5 and PIN8, localizes intra-cellularly to the endoplasmic reticulum (ER). This was a novel finding which unveiled the presence of auxin transport within the cell for the very first time (Mravec et al., 2009). In the same period, we found that the 7 members of the PILS family also reside in the ER, which emphasized the significant importance of intracellular auxin transport for plant growth and development. The observation that ER localized (putative) auxin transport carriers affect cellular auxin metabolism pinpoints towards a mechanism of compartmentalized auxin metabolism in the cell (Barbez and Kleine-Vehn, 2013). Compartmentalized auxin metabolism, such as conjugation and conjugate hydrolysis, could enable a more accurate regulation of auxin homeostasis in particular tissues and cell types. However, it still needs to be seen whether certain auxin metabolic enzymes, including auxin conjugating enzymes from the GH3 family and hydrolases from the ILR family, indeed reside in intracellular compartments, such as the ER (Reviewed in Barbez and Kleine-Vehn, 2013). We are currently cloning the GH3.5 and GH3.6 enzymes, which conjugate IAA to aspartate and glutamate, fused with a N- or C-terminal GFP tag, in order to unveil the intracellular compartment where the conjugation process takes place. We are furthermore generating an estradiol inducible PILS5 line in the

gh3.1,2,3,5,6 multiple mutant background in which no IA-asp can be formed. This line will enable us to test, via qPCR, whether PILS5 activity decreases nuclear auxin signaling in the absence of GH3 dependent auxin conjugation. Measurements of endogenous IAA and IA-conjugate levels in this line will moreover reveal whether PILS5 induction/activity stimulates GH3 independent IA-asp and IA-glu formation or not. We are also generating estradiol inducible GH3.5, GH3.6 and MtIAR33(IA-asp hydrolyzing enzyme from *Medicago truncatula*) lines in the PILS loss- and gain-of-function mutant backgrounds. We will test IAA conjugation to aspartate and glutamate in PILS loss-of-function mutants and whether IA-asp hydrolysis can reverse the PILS5 overexpression phenotypes. In order to further unveil the specific impact of PILS activity on plant growth and development, we performed a micro-array on PILS5 induced *A. thaliana* seedling roots which is currently under computational analysis.

Our in depth analysis of the role of PILS proteins in plant growth and developmental as well as the dissection of their molecular mechanism might be of significant importance for industrial applications. In silico analysis reveals the presence of PILS orthologues in land plants as well as evolutionary ancient species such as mosses and algae (Barbez et al.,2012; Feraru et al., 2012), indicating a conserved role/function for PILS proteins in plant development. The involvement of auxin in such a broad spectrum of plant developmental processes makes it difficult to modulate a process of interest without affecting others. Many post-embryonic plant developmental processes such as lateral organ formation require the local accumulation of auxin (reviewed in Vanneste and Friml, 2009) while the onset of others, such as fruit opening, require an auxin minimum (Sorefan et al., 2009). The negative impact of PILS activity on nuclear auxin signaling by the intracellular sequestration and conjugation of auxin could

be used as a tool to specifically modulate auxin signaling in a tissue and cell type of choice with minimal or no impact on neighboring cell files.

PILS protein activity affects cellular auxin metabolism by increasing the levels of certain auxin conjugates, such as IA-asp and IA-Glu. This data triggered our curiosity about the nature of auxin conjugates and their function in plant development, which is until now only poorly understood. It is generally assumed that IA-asp and IA-glu, in contrast to for instance IA-ala and IA-leu, are not hydrolyzed back to free IAA and are inactive intermediates of auxin degradation (reviewed in Ludwig-Müller, 2011). Except from IA-trp, which has been suggested to display anti-auxinic effects (Staswick et al., 2009), no other auxin conjugates have been described to alter auxin signaling. Therefore, our recent preliminary observation that IA-asp might possess biological activity could shed new light on the complex nature of cellular auxin homeostasis. We show that IA-asp displays auxin like effects on seedling growth and nuclear auxin signaling, which is at least partially mediated by the auxin receptor TIR1. A micro array on IA-asp treated seedlings is currently under computational analysis and will give a better insight in which plant developmental processes IA-asp could be involved.

Auxin has been decribed to inhibit endocytosis (Paciorek et al., 2005) via its binding on the auxin receptor ABP1 (Robert and Kleine-Vehn, 2010). However, this effect was only observed with the more stable synthetic auxin analogues naphtyl-actic-acid (NAA) and 2,4D and not with exogenous application of the naturally occurring free IAA. Paciorek et al. described the decreased endocytosis in the auxin overproducing mutant YUCCA1, however, this mutant contains, besides higher levels of free IAA, also substantially higher levels of IA-asp (Zhao et al., 2002). We are currently testing whether IA-asp and not free IAA inhibits the

stimulating effect of ABP1 in endocytosis. Preliminary *in silico* molecular simulations suggests the ability of IA-asp to bind ABP1 and to form even more hydrogen bridges than free IAA, possibly suggesting a more favorable binding (data not shown). We will test whether IA-asp inhibits ABP1 dependent PIN1-YFP endocytosis, which can be visualized by the use of chemical Brefeldin A (BFA). BFA inhibits exocytosis and thus causes the intracellular accumulation of PIN1-YFP. Besides, we will compare the rates of endocytosis, visualized by the uptake of the dye FM4-64, in various mutants containing more or less IA-asp. These experiments will provide insight into the impact of cellular auxin metabolism/conjugation on ABP1 regulated processes.

The poor uptake of exogenous IA-asp as well as preliminary results on IA-Asp polar transport assays (data not shown) suggest the absence of cell-to-cell IA-asp transport, which is in big contrast to free IAA that requires cell-to-cell transport for its function . This data indicates a cell autonomous signaling function for IA-asp allowing a very accurate regulation of developmental processes on a cellular level. We are currently generating transgenic *Arabidopsis thaliana* lines expressing the auxin conjugating enzymes GH3.5 and GH3.6 fused to a GFP tag in order to unveil the intracellular localization of IA-asp formation. It further needs to be seen whether IA-asp can be transported over the different compartments/organelles within the cell which might be required to reach its receptors. To address this question, the substrate specificity of the currently known intracellular auxin carriers needs to be tested and further research is required to identify potential auxin- or auxin metabolite-transport carriers on the membrane of other organelles in the cell.

In 1955, Andreae and Good described the conversion of exogenously applied IAA into IA-asp and IA-glu (Andreae and Good,

1955). Since plants cannot flee from unfavorable conditions, their growth and development is enormously flexible and aimed to deal with the constantly changing environment. Certain pathogens including *Agrobacterium tumefaciens* and plant parasitic nematodes have strategies to elevate the levels of endogenous auxin of the host, resulting in tissue growth, which is favorable for the pathogen (Bevan et al., 1982; Grunewald et al., 2009). Conjugating the over amount of free IAA to Aspartate could be a way to deal with pathogen invasion. However, specific roles for IA-asp in plant development and stress response have not been described so far. Nevertheless, the roles of the conjugating enzymes from the GH3 family in biotic and abiotic stress responses have been described multiple times (reviewed in Ludwig-Müller, 2011). It is a matter of discussion whether GH3 protein activity only aims to lower down the levels of free IAA. More specific roles for GH3 proteins and their reaction products (conjugates) in these stress responses cannot be ruled out. The formed IA-asp could activate genes involved in multiple stress responses. To identify IA-asp regulated genes, we performed a micro-array on IA-asp treated *A. thaliana* seedlings, which is currently under computational analysis. Due to the enormous conversion of exogenously applied IAA into IA-asp, it is to be expected that part of the previously identified late IAA responsive genes (Paponov et al., 2008) could actually respond to IA-asp.

It has been previously described that the conjugation of auxin to aspartate and glutamate is irreversible in *Arabidopsis thaliana* since no radiolabeled IAA has been detected after the exogenous application of radiolabeled IA-asp (Tuominen et al., 1994; Östin et al, 1998). However, Leclere et al described in 2002 that the IA-amide conjugate hydrolases ILR1 and ILL2 (specific for mainly IA-ala, IA-phe and IA-leu) display a certain IA-asp and IA-glu hydrolysis activity *in vitro* (Leclere et al.,

2002). Others have shown a symbiont induced upregulation of an IA-amide hydrolase in *Medicago truncatula*, with a high *in vitro* IA-asp specificity (Campanella et al. 2008). This suggests possible IA-asp hydrolysis in higher plants, which might be conditional and species specific. It could be that small levels of IA-asp hydrolysis in *A. thaliana* were not detectable during former studies and this should be re-evaluated since the development of more sensitive methodology (Pencik et al., 2009). Alternatively, IA-Asp could elevate the endogenous IAA level via an additional mechanism. To address the effect of IA-asp on cellular auxin homeostasis in *A. thaliana*, the levels of endogenous free IAA after IA-Asp treatment should be measured. The synthetic auxin analogue conjugate 2,4D-Asp could be added as a control to distinguish between potential IA-asp/2,4D-asp hydrolysis and the increase of endogenous IAA formation.

The ability of plants to produce such a wide spectrum of auxins and auxin conjugates makes it difficult to envision that solely free auxin exerts a functional importance in plant development (Rosquete et al, 2012). Plants contain several different auxin analogues and conjugates and moreover display a high versatility in cell specific auxin perception and signaling. These aspects create an infinite number of developmental output possibilities for only 1 simple molecule like auxin. These numerous mechanisms to regulate auxin homeostasis are possibly central to the complexity of higher plants.

In this thesis, I described the identification and characterization of a novel putative auxin transport carrier family and the impact of intracellular auxin transport on auxin metabolism. I also developed a method to qualitatively address the impact of effector proteins on nuclear auxin signaling in tobacco BY-2 cells. Besides, I have proposed a potential involvement of non-hydrolysable auxin amide conjugates in plant growth and development. With this PhD work, I hope to contribute a little piece to the puzzle of auxin biology.

General Remarks

Abbreviations

2,4-D	2,4-dichlorophenoxy acetic acid
4-Cl-IAA	4-chloroindole-3-acetic acid
ABCB	ATP BINDING CASSETTE B
ABP1	AUXIN BINDING PROTEIN 1
ACA2	CALCIUM ATPASE 2
AFB	AUXIN SIGNALLING F-BOX
ANOVA	analysis of variance
ARF	AUXIN REPONSE FACTOR
AUX	AUXIN RESISTANT
AUX1	AUXIN RESISTANT1
AuxRE	auxin responsive element
BA	benzoic acid
BAP	6-benzylaminopurine
BIP	BINDING IMMUNOGLOBULIN PROTEIN
BLAST	Basic Local Alignment Search Tool
BR	brassinolide
BY-2	Bright Yellow 2
cDNA	complementary DNA
DAG	days after germination
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECA1	ER-TYPE CA2+-ATPASE 1
ER	endoplasmic reticulum
FWO	Fonds Wetenschappelijk Onderzoek
GFP	GREEN FLUORESCENT PROTEIN
GH3	GRETCHEN HAGEN 3
GPD	The constitutive yeast Glyceraldehyde-3-phosphate-dehydrogenase promoter.
GUS	ß-Glucuronidase
GVG	A chimeric transcription factor consisting of the DNA-binding domain of the yeast transcription factor GAL4, the transactivating domain of the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor (GR) (Aoyama et al., 1997).
HPLC	High pressure liquid chromatography
IAA	indole-3-acetic acid
IAA	INDOLE-3ACETIC ACID INDUCIBLE
IAA-Glc	IAA-glucose

IA-Ala	IAA-alanine
IA-Asp	IA-aspartate
IA-Glu	IA-glutamate
IA-Leu	IAA-leucine
IA-Phe	IA-phenylalanine
IA-Trp	IA-tryptophan
IBA	indole-3-butyric acid
IBR	INDOLE-3-BUTYRIC ACID RESPONSE
IC50	the half maximum inhibitory concentration
ILL	IAA-LEUCINE RESISTANT1-like
ILR	IAA-LEUCINE RESISTANT1
IWT	Agentschap voor Innovatie door Wetenschap en Technologie
LAX	LIKE AUX1
LC-MS	liquid chromatography-mass spectrometry
MDR	MULTIDRUG RESISTANCE
MGV	mean gray value
MIA	MALE GAMETOGENESIS IMPAIRED ANTHERS
mRFP	monomeric RFP
NAA	1-naphtaleneacetic acid
NPA	1-naphthylphthalamic acid
PAA	phenylacetic acid
PCR	polymerase chain reaction
PEG	polyethylene glycol
pEXP7A	promoter EXPANSIN 7A
PI	propidium iodide
PILS	PIN-likes
PIN	PIN-FORMED
PIN	PIN-FORMED
PM	plasma membrane
qRT-PCR	quantitative real time PCR
RFP	RED FLUORESCENT PROTEIN
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
s.e.m.	standard error of the mean
SKP2A	S-PHASE KINASE-ASSOCIATED PROTEIN 2A
T1,T2	generation after Transformation
TAIR	The Arabidopsis Information Resource
TCS	TWO-COMPONENT-OUTPUT-SENSOR

TIR1	TRANSPORT INHIBITOR RESPONSE1
Trp	tryptophane
UBQ	ubiquitin
UGT	UDP GLUCOSYL TRANSFERASE
WAT1	WALLS ARE THIN1
WT	wild type
WWTF	Wiener-, Wissenschafts-, Forschungs- und Technologiefonds
XVE	A chimeric transcription activator, consisting of the DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor (E; ER) (Zuo et al., 2000).
YFP	YELLOW FLUORESCENT PROTEIN
YUC	YUCCA

Author contributions

Here I address my contribution to the projects, described in chapters 1-4 as well as my contribution to other publications which are not described in this thesis.

ABP1 Mediates Auxin Inhibition of Clathrin-Dependent Endocytosis in *Arabidopsis*

Stéphanie Robert,[1,2,11] Jürgen Kleine-Vehn,[1,2,11] Elke Barbez,[1,2] Michael Sauer,[1,2,12] Tomasz Paciorek,[1,2] Pawel Baster,[1,2] Steffen Vanneste,[1,2] Jing Zhang,[1,2] Sibu Simon,[3] Milada Čovanová,[3] Kenichiro Hayashi,[4] Pankaj Dhonukshe,[5] Zhenbiao Yang,[6] Sebastian Y. Bednarek,[7] Alan M. Jones,[8] Christian Luschnig,[9] Fernando Aniento,[10] Eva Zažímalová,[3] and Jiří Friml[1,2,*]

(Cell, 2010)

This research article describes the involvement of the auxin receptor AUXIN BINDING PROTEIN 1 (ABP1) in the clathrin mediated internalization of PIN auxin efflux carriers from the plasma membrane. This mechanism regulates the levels of PIN proteins at the plasma membrane and thus the levels of cell-to-cell auxin transport.

At this time, I was optimizing the method of transient transformation of tobacco BY-2 cells via particle bombardment in the lab of Prof. Dr. Jiri Friml. The ease and the speed of this method allowed me to quickly test the effect of transgenes on the localization and stability of molecular markers. I was very happy and proud when I got the chance to contribute to this interesting story. For the Robert and Kleine-Vehn et al., 2010 paper, I visualized the effect of ABP1-GFP and the truncated ABP1ΔKDEL-GFP overexpression on the plasma membrane localization of PIN1-RFP via the transient transformation of tobacco BY-2 cells (Fig. 4e-h in Robert and Kleine-Vehn et al., 2010). Truncated ABP1ΔKDEL-GFP but not ABP1-GFP full length stimulates the internalization of PIN1-RFP, an effect which could be inhibited by exogenous auxin application (Fig. 5a-g in Robert and Kleine-Vehn et al., 2010). When the auxin

binding pocket of ABP1ΔKDEL-GFP was mutated, exogenous auxin could no longer inhibit the ABP1 stimulating effect on PIN1-RFP internalization (Fig. 5 m-o in Robert and Kleine-Vehn et al., 2010). I also show that the ABP1ΔKDEL-GFP effect on PIN1-RFP internalization could be blocked by inhibiting clathrin mediated endocytosis, in a genetic as well as pharmacological way, indicating that the ABP1 regulated PIN internalization is clathrin dependent (Fig7 a-d in Robert and Kleine-Vehn et al., 2010).

Besides, I also contributed to this story by cloning the mutated 35S:ABP1-5ΔKDEL-GFP construct used in figure 5 (Robert and Kleine-Vehn et al., 2010) and by the plant transformation and selection of the previously cloned 35S: ABP1ΔKDEL-GFP.

Cellular Auxin Homeostasis: Gatekeeping Is Housekeeping

Michel Ruiz Rosquete[a,b], Elke Barbez[a,b] and Jürgen Kleine-Vehn[a,b,1]

(Molecular Plant, 2011)

This review provides a superficial overview of the diverse topics of plant biology including auxin biosynthesis, conjugation, degradation and transport and how these processes are regulated in response to environmental inputs. Moreover, we highlight the recent findings on how intracellular auxin transport and metabolism jointly regulate cellular auxin homeostasis. I mainly contributed to this review by writing about auxin conjugation, conjugate hydrolysis and local auxin biosynthesis.

A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants

Elke Barbez[1,2], Martin Kubeš[3], Jakub Rolčík[4], Chloé Béziat[1,2], Aleš Pěnčík[5], Bangjun Wang[6], Michel Ruiz Rosquete[1,2], Jinsheng Zhu[6], Petre I. Dobrev[3], Yuree Lee[7], Eva Zažímalová[3], Jan Petrášek[3], Markus Geisler[6], Jiří Friml[1] & Jürgen Kleine-Vehn[1,2]

(Nature, 2012)

This article (Chapter 2) is very close to my heart since it is the result of intensive work which started in September 2008 at the beginning of my master thesis. This research project was the very first research project I would ever work on. By knowing where this project starts but not knowing where it would end, I started the characterization of the PILS protein family via a reversed genetic approach. After some *in silico* work (defining the predicted protein topology of the PILS proteins and the identification of PILS homologues genes in other plant species) I analyzed the expression levels of the PILS genes in different *Arabidopsis thaliana* plant organs. I genotyped insertion lines and confirmed the loss of PILS expression via RT-PCR. Besides, I cloned the 7 *A.thaliana* PILS genes in different expression vectors in order to generate gain-off-function transgenic 35S:PILS lines and to unveil the subcellular localization of the PILS proteins in *A. thaliana*, tobacco BY-2 cells and yeast, via confocal microscopy. I also generated transgenic lines expressing a GUS and GFP construct under the regulation of the PILS promoters in order to visualize the PILS expression patterns. I performed all phenotyping experiments described in the paper, analyzed the effect of PILS expression on nuclear auxin signaling in *A. thaliana* and tobacco BY-2 cells and contributed to the writing of the manuscript.

Besides, I'm very grateful to all our collaborators for their fantastic contributions to this project, which made it possible to publish this work in *Nature*.

***Divide Et Impera*—cellular auxin compartmentalization**

Elke Barbez[1,2] and Jürgen Kleine-Vehn[1]

(Current Opinion in Plant Biology, 2013)

In the summer of 2012, I got the chance to write a review on intracellular auxin transport (Chapter 1). The opportunity came on an interesting moment considering the recent publications describing auxin carrier activity at the endoplasmic reticulum (Bosco et al., 2012; Ding et al., 2012; Barbez et al., 2012). We review the recent findings on auxin compartmentalization and its impact on cellular auxin metabolism. This review opportunity gave me the chance to discuss and speculate on the potential presence of compartmentalized auxin metabolism as an additional level in the regulation of auxin homeostasis. This, until today, unexplored hypothesis triggered my curiosity which formed the base for the project, described in Chapter 4.

METHODOLOGY ARTICLE Open Access

Single-cell-based system to monitor carrier driven cellular auxin homeostasis

Elke Barbez[1,2], Martina Laňková[3], Markéta Pařezová[3], Alexis Maizel[4], Eva Zažímalová[3], Jan Petrášek[3], Jiří Friml[1,5] and Jürgen Kleine-Vehn[1,2*]

(BMC Plant Biology, 2013)

This article (Chapter 3) originated from the simple idea to quickly visualize the overexpression effect of a auxin carrier of interest on nuclear auxin signaling by the transient transformation of tobacco BY-2 cells. This method enabled me to obtain an initial idea about the effect of PILS activity on nuclear auxin signaling in a cellular context (Barbez et al., 2012). It gave moreover an idea on the localization and functionality of the GFP tagged PILS proteins. Although I mainly intended to use this

method for my own purposes, my supervisor Dr. Jürgen Kleine-Vehn supported me to validate this method and to share it with the scientific community. It was for me an exciting plan to publish my initially own idea, which gave me right away the opportunity to write my very first paper. The overall process of publishing this article, which started in the beginning of 2011, took longer than expected, but eventually led to its publication in BMC plant biology this year. This experience made me very happy.

Initially I started with the optimization of the transient transformation of tobacco BY-2 cells and defined a time frame in which the protein under study displays the right subcellular localization and presumed impact on the *DR5::RFP* signal intensity. I validated the method mainly based on the effect of previously described auxin carriers and pharmacological compounds on nuclear auxin signaling. I moreover worked out a suitable way for the quantification and visualization of the obtained data and wrote the manuscript.

I am very grateful for the help we obtained from our collaborators in Prague who performed the stable transformation of tobacco BY-2 cells, the visualization of these stable transformants and the auxin accumulation experiments given in figure 1.

An Auxin Transport Mechanism Restricts Positive Orthogravitropism in Lateral Roots

Michel Ruiz Rosquete,[1,2] Daniel von Wangenheim,[3,4]
Peter Marhavý,[2,5] Elke Barbez,[1,2] Ernst H.K. Stelzer,[3]
Eva Benková,[2,5] Alexis Maizel,[4] and Jürgen Kleine-Vehn[1,2,*]

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(Current Biology, 2013)

Plants grow in response to gravity resulting in upwards growing shoots and roots that penetrate the soil. However the response of lateral roots to gravity is incomplete, enabling radial expansion of the root system. This paper describes an auxin mediated mechanism that regulates the angular growth of lateral roots, which is called the gravitropic setpoint angle (GSA). I was involved in this project at the very start in the beginning of 2011 when I performed lugol stainings on freshly emerging lateral roots. Lugol is a starch staining dye that is commonly used to visualize columella cells in the root tips since these cells contain starch granules. We observed that the establishment of the columella goes hand in hand with the initial elongation of the young lateral root. This nice project, ran by my colleague Dr. Michel Ruiz-Rosquete, became a very interesting story that unveils a molecular mechanism in which a temporal limitation of auxin transport in the tip of lateral roots defines the GSA.

The biological relevance of auxin conjugates: IA-aspartate

Elke Barbez, Jakub Rolcik, Antje Helmut, Melanie Grandits, Chris Oostenbrink, Luz Irina Calderon-Villalobos, Jürgen Kleine-Vehn

(Unpublished)

The activity of PILS intracellular putative auxin transport carriers affects the levels of free and conjugated IAA (Barbez et al., 2012). This observation triggered our interest to find out whether the high number of different auxin conjugates plays a specific role during plant growth and development. Even though the IAA amide conjugates are described to be biologically inactive, we observed that the non-hydrolysable indole-3-acetyl-aspartic-acid (IA-asp) affects *A. thaliana* seedling growth. In this project, we address the potential physiological importance of IAA amide conjugates.

Initially, I tested the effect of exogenous IA-asp on *A. thaliana* seedling growth by analyzing its effect on main root growth, lateral root density and root hair elongation. Besides, I visualized the effect of IA-asp on nuclear auxin signaling by the use of the auxin response reporter lines *DR5rev:GFP* and *DII-Venus* and via q-PCR. I tested the sensitivity of the auxin receptor *tir1/afb quadruple* mutant towards IA-asp and performed the yeast assays shown in Fig. 3. Moreover, I prepared the RNA samples for the micro-array and went to Olomouc (Czech Republic) to prepare the samples for the endogenous IAA and IA-conjugate measurements. I have cloned the GH3.6 gene fused to a GFP tag and I am waiting for the first transformants. I am currently analyzing the effect of IA-asp on ABP1 mediated processes.

Besides, I am very grateful to the collaborators who contributed to this work by performing in vitro binding assays, computational IA-asp binding simulations, HPLC based endogenous auxin measurements and the performance and the computational analysis of the micro array.

CURRICULUM VITAE

PERSONAL INFORMATION

Name:	Elke Barbez
Date of birth:	24.06.1986
Place of birth	Brugge
Nationality:	Belgian

EDUCATION

1992-1998:	Elementary school Sint-Jozef , Blankenberge (Belgium)
1998-2004:	High school Sint-Pieterscollege, Blankenberge (Belgium)
2004-2009:	Master in Biochemistry and Biotechnology at the Ghent University, Gent (Belgium)
2008-2009	Master thesis in the lab of Prof. Dr. Jiri Friml at the VIB, department of Plant Systems Biology, Ghent university, Gent (Belgium)
2009-2013:	PhD student at the VIB, department of Plant Systems Biology, Ghent university, Gent (Belgium) Promoters: Prof. Dr. Jiri Friml (2009-2011) and Prof. Dr. Dirk Inzé (2011-2013), Co-promoter: Dr. Jürgen Kleine-Vehn (2009-2013)
Since August 2011:	Visiting student in the lab of Dr. Jürgen Kleine-Vehn at the BOKU University in Vienna (Austria)

ABROAD EXPERIENCES

August 2008:	5 weeks of internship in the lab of Prof. Dr. Eva Zažímalová at the Institute of Experimental Botany, Prague (Czech Republic)
September 2009:	4 weeks of collaborative research in the lab of Prof. Dr. Eva Zažímalová at the Institute of Experimental Botany, Prague (Czech Republic)
Since August 2011:	Visiting student in the lab of Dr. Jürgen Kleine-Vehn at the BOKU University in Vienna (Austria)

AWARDS

July 2009:	DevGen Master thesis award

FUNDING

2009-2013:	PhD fellowship from the 'Agentschap voor Innovatie door Wetenschap en Techniek' (IWT)
2012-2013:	'Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO)'- mobility grant for a long stay abroad.

TALKS

10/2010:	XVII Congress of the Federation of European Societies of Plant Biology (FESPB) in Valencia, Spain. Title: A novel clathrin-dependent trafficking mechanism mediates tyrosin motif-dependent PIN export from the endoplasmatic reticulum.
09/2011:	European Network for Plant Endomembrane Research (ENPER) meeting in Assisi (Italy). Title: PILS proteins, novel putative auxin transport facilitators at the endoplasmic reticulum.
07/2012:	23th International Conference of Arabidopsis Research. (ICAR) in Vienna (Austria). Title: PILS proteins, novel putative auxin transport facilitators at the endoplasmic reticulum.
04/2013:	Seminar lecture at the Palacky University in Olomouc (Czech Republic). Title: The impact of intracellular auxin transport and metabolism on cellular auxin homeostasis and plant development.
06/2013:	2nd International Meeting of Early Auxin Research, Leiden (The Netherlands). Title: Auxin metabolism, a new angle to an old story.

CONFERENCE POSTERS

09/2012:	European Network for Plant Endomembrane Research (ENPER) meeting in Madrid (Spain). PILS putative auxin carrier family plays a regulatory role in cellular auxin metabolism.
12/2013:	Auxin meeting 2012, Waikoloa (Hawai'i). Title: The PILS novel putative auxin carrier family regulates intracellular auxin homeostasis in plants

PUBLICATIONS

Rosquete MR, von Wangenheim D, Marhavy P, **Barbez E**, Stelzer EH, Benkova E, Maizel A, Kleine-Vehn J (2013) An auxin transport mechanism restricts positive orthogravitropism in lateral roots. *Current biology : CB* **23:** 817-822

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Robert S, Kleine-Vehn J, **Barbez E**, Sauer M, Paciorek T, Baster P, Vanneste S, Zhang J, Simon S, Covanova M, Hayashi K, Dhonukshe P, Yang Z, Bednarek SY, Jones AM, Luschnig C, Aniento F, Zazimalova E, Friml J (2010) ABP1 mediates auxin inhibition of clathrin-dependent endocytosis in Arabidopsis. *Cell* **143:** 111-121

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