

# The endoplasmic reticulum localized PIN8 is a pollen-specific auxin carrier involved in intracellular auxin homeostasis

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## SUMMARY

The plant hormone auxin is a mobile signal which affects nuclear transcription by regulating the stability of auxin/indole-3-acetic acid (IAA) repressor proteins. Auxin is transported polarly from cell to cell by auxin efflux proteins of the PIN family, but it is not as yet clear how auxin levels are regulated within cells and how access of auxin to the nucleus may be controlled. The Arabidopsis genome contains eight PINs, encoding proteins with a similar membrane topology. While five of the PINs are typically targeted polarly to the plasma membranes, the smallest members of the family, PIN5 and PIN8, seem to be located not at the plasma membrane but in endomembranes. Here we demonstrate by electron microscopy analysis that PIN8, which is specifically expressed in pollen, resides in the endoplasmic reticulum and that it remains internally localized during pollen tube growth. Transgenic Arabidopsis and tobacco plants were generated overexpressing or ectopically expressing functional PIN8, and its role in control of auxin homeostasis was studied. PIN8 ectopic expression resulted in strong auxin-related phenotypes. The severity of phenotypes depended on PIN8 protein levels, suggesting a rate-limiting activity for PIN8. The observed phenotypes correlated with elevated levels of free IAA and ester-conjugated IAA. Activation of the auxin-regulated synthetic DR5 promoter and of auxin response genes was strongly repressed in seedlings overexpressing PIN8 when exposed to 1-naphthalene acetic acid. Thus, our data show a functional role for endoplasmic reticulum-localized PIN8 and suggest a mechanism whereby PIN8 controls auxin thresholds and access of auxin to the nucleus, thereby regulating auxin-dependent transcriptional activity.

**Keywords:** PIN8, endoplasmic reticulum, pollen, auxin, *Arabidopsis thaliana*, *Nicotiana tabacum*.

## INTRODUCTION

Plant growth and development are both driven by instructive auxin gradients, whose establishment once

auxin is synthesized can be explained solely by the sub-cellular and tissue localization of auxin transporters

(Grieneisen *et al.*, 2007; Benjamins and Scheres, 2008). The subcellular localization of members of the PIN family of auxin efflux carriers is particularly dynamic, localizing to specific membrane domains through polar recycling (Geldner *et al.*, 2001; Dhonukshe *et al.*, 2007; Wabnik *et al.*, 2011). The genome of *Arabidopsis thaliana* contains eight PINs; most of the PIN proteins which have been characterized to date (PIN1–4 and PIN7) are targeted to the apical, basal or lateral plasma membranes (PM) depending on the protein, the cell type, the developmental context and environmental input as reviewed in Grunewald and Friml (2010). PIN5 is an atypical member of the PIN family as it is localized not to the PM but to the endoplasmic reticulum (ER) (Mravec *et al.*, 2009). This localization of PIN5, as well as its effect on indole-3-acetic acid (IAA) metabolism, suggested the existence of PIN-dependent auxin transport at the ER and the possibility that the ER acts as a recycling station for IAA (Friml and Jones, 2010).

The PIN proteins are predicted to contain between eight and ten trans-membrane helices, distributed between two hydrophobic domains located at the proteins' N- and C-termini and separated by a central hydrophilic region. This central region may either be long or short. PIN5 has a short central loop; a potentially important factor in its subcellular localization (Mravec *et al.*, 2009). According to phylogenetic analysis (Paponov *et al.*, 2005) PIN8 has a short hydrophilic middle region and, like PIN5, is more distantly related to the PM-localized PIN proteins, which all contain a long central hydrophilic loop (Mravec *et al.*, 2009). Recently, evidence for the internal localization of PIN8, when specifically over-expressed in root hair cells, has been reported (Ganguly *et al.*, 2010). In this case, PIN5 and PIN8, were shown to be localized internally, although partial co-localization of PIN8 with the PM staining dye FM4-64 indicated a broader distribution of PIN8 in tobacco BY-2 and *Arabidopsis* root hair cells. Different auxin transport activities of PIN8 and PIN5 have been shown by a [<sup>3</sup>H]-naphthalene-1-acetic acid (NAA) retention experiment in BY-2 tobacco cells. The induction of PIN5 expression resulted in insignificant changes in auxin transport rates, whereas PIN8 expression significantly decreased NAA retention (Ganguly *et al.*, 2010).

In this report, we investigate the expression pattern of PIN8 and show that PIN8 is the only member of the PIN family specifically accumulated in pollen. In its expression domain PIN8 resides in the ER and it also remains internally localized upon pollen tube germination, thus supporting the hypothesis that PIN8 is involved in intracellular auxin homeostasis in the male gametophyte. Further, we characterized PIN8 overexpressing mutants and show that PIN8 can alter the auxin pool and affect the auxin-dependent activation of nuclear transcription.

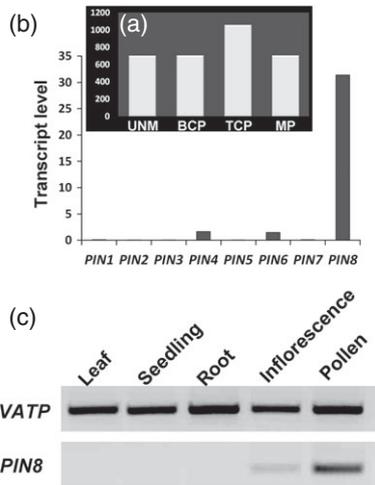
## RESULTS

### The PIN family in *Arabidopsis* pollen: PIN8 is specifically up-regulated in the male gametophyte

Analysis of publicly available microarray data for mature pollen (Pina *et al.*, 2005) revealed that several auxin-related genes are involved in gametophyte development (Table S1 in Supporting Information). Among the auxin biosynthesis genes, two YUCCA genes are expressed in pollen while no gene transcripts from the TAA1 family are present in pollen. GH3-5, a member of the GH3 family which participates in auxin conjugation and the IAA amino acid conjugate hydrolase ILL3 are expressed in pollen. This expression pattern demonstrates that the machinery for conjugation and de-conjugation of auxin is expressed in these cells. All of the well-known components of the auxin signaling network, e.g. members of the AFB, TPL, ARF and Aux/IAA families, are also expressed in pollen. Interestingly, only two members of the ARF and Aux/IAA families, out of 20 and 28 members represented on the *Arabidopsis* gene chip, respectively, are expressed in pollen. With regard to the mechanism for auxin transport, three PINs, namely PIN3, PIN4 and PIN8, are expressed in the male gametophyte but no AUX1/LAX genes are expressed at significant levels.

A further comparison of transcriptomic data (Honys and Twell, 2004; Pina *et al.*, 2005; Wang *et al.*, 2008) showed that of the PINs, PIN8 is widely and highly expressed during pollen development, from microspore to mature pollen (Figure 1a) (Honys and Twell, 2004; Pina *et al.*, 2005) as well as during pollen germination (Wang *et al.*, 2008). Since the relative expression and the identity of the expressed PINs varied among the different transcriptomic studies, we first quantified transcript levels of all PINs in *A. thaliana* pollen (Figure 1b). PIN4 and PIN6 were confirmed to be expressed in pollen, although at very low levels, and PIN8 proved to be the most strongly expressed PIN found in pollen.

We further characterized the PIN8 expression pattern by RT-PCR in different tissues. We performed RT-PCR on RNA isolated from seedlings, roots, leaves, inflorescences and pollen, revealing that PIN8 is specifically expressed in pollen (Figure 1c) as predicted by the microarray data. To analyze PIN8 expression *in planta*, the green fluorescent reporter gene (GFP) was inserted in the genomic sequence of PIN8 flanked with its native promoter and untranslated regions (UTRs) (PIN8::PIN8GFP). The fluorescent PIN8 fusion protein, with GFP inserted in the small central loop of PIN8, was monitored during flower development from stages 9 to 13 according to the method of Cecchetti *et al.* (2008) (Figure 2). Microscopic analysis revealed that PIN8 accumulated significantly starting from stage 11 until pollen dehiscence. This implies a role for PIN8 at later stages of microgametogenesis, after the pollen has reached the tricellular stage.



**Figure 1.** *PIN8* expression analysis.

(a) Details of *PIN8* expression during pollen development retrieved from microarray data (Hony and Twell, 2004). UNM, uninucleate microspores; BCP, bicellular pollen; TCP, tricellular pollen; MP, mature pollen.

(b) Real-time PCR analysis of *AtPINs* from cDNA of *Arabidopsis thaliana* pollen. Transcript level is expressed as relative quantification where *VATP* was used as the reference gene.

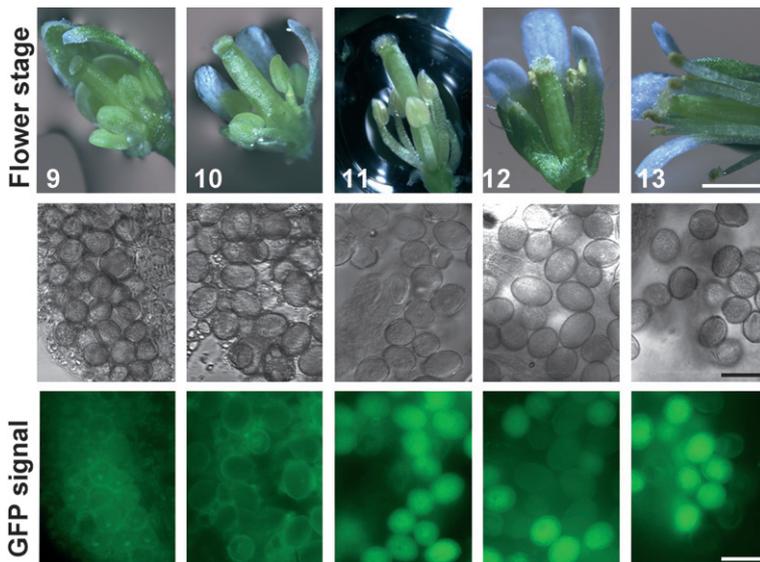
(c) Real-time PCR analysis of *PIN8* from cDNA from different tissues of *A. thaliana* indicates that *PIN8* is specifically expressed in the male gametophyte. The *VATP* gene was used as a control.

### ***PIN8* is localized in the endoplasmic reticulum in the pollen grain and growing pollen tube**

We next investigated the subcellular localization of *PIN8* in the male gametophyte. We transiently co-transformed tobacco pollen with the *PIN8::PIN8GFP* construct described above along with different subcellular markers (Figure 3). *PIN8*-GFP co-localized with the ER marker (Figure 3g). The

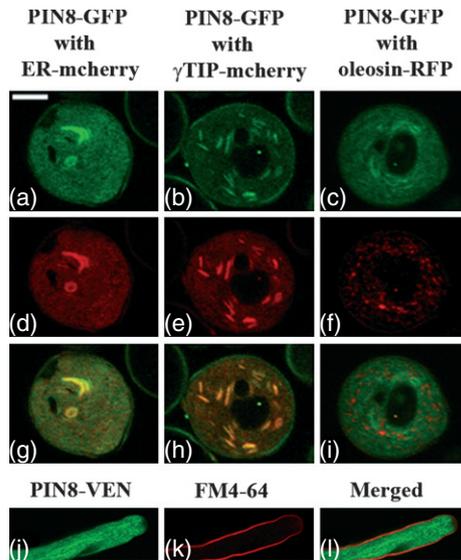
co-localization was particularly conspicuous in ellipsoidal shaped structures within the pollen grains. Similar structures have previously been reported as ER membranes surrounding lipid bodies and enlarged tubular ER around the vacuoles (Yamamoto *et al.*, 2003). We examined the nature of these structures using the tonoplast marker  $\gamma$ -tonoplast intrinsic protein ( $\gamma$ TIP) (Figure 3b) and red fluorescent protein (RFP)-tagged oleosin as the marker for lipid bodies (Figure 3c). Spherical structures containing *PIN8* co-localized with the tonoplast marker (Figure 3h) but not with the lipid body marker (Figure 3i).

The peak of *PIN8* accumulation during the later stages of pollen development (Figure 2) and the presence of *PIN8* transcripts in the pollen tube microarray data (Wang *et al.*, 2008) suggested a function for *PIN8* during pollen germination and growth. Therefore, we studied the localization of *PIN8* in pollen grains after induction of germination *in vitro* (Figure 3j–l). Since a possible localization of *PIN8* at the PM has been reported (Ganguly *et al.*, 2010), we investigated whether such a localization might be germination-dependent. Using the PM marker FM4-64 in germinating pollen from tobacco lines overexpressing a fluorescent fusion of *PIN8* in pollen (Lat52::*PIN8VEN*) we were able to exclude this possibility, and confirmed that *PIN8* is internally localized not only in pollen grains but also in growing pollen tubes (Figure 3j). Consistent co-localization of *PIN8* with ER structures surrounding electron-transparent vesicles was confirmed on thin plastic sections after high-pressure freeze cryofixation and freeze substitution followed by anti-GFP immunogold labeling (Figure 4). Pollen grains from Lat52::*PIN8VEN* tobacco lines were used, and the analysis of several sections showed that the immunogold particles were restricted to the ER membranes (Figure 4d,h). Most noticeably, ER structures



**Figure 2.** Analysis of accumulation of *PIN8* during male gametophyte development.

Transgenic *Arabidopsis* plants carrying a genomic sequence of *PIN8* under its native regulatory elements and a translation fusion with GFP inserted in the central hydrophilic loop were analyzed. Flowers at different developmental stages (scale bar = 1 mm) and the corresponding pollen in bright field and the GFP fluorescence channel (scale bar = 20  $\mu$ m), are shown. *PIN8* accumulates in pollen starting from flower stage 11; flower stages are noted as previously described (Cecchetti *et al.*, 2008).



**Figure 3.** Localization of PIN8 fluorescent fusion proteins in tobacco pollen grains and pollen tubes.

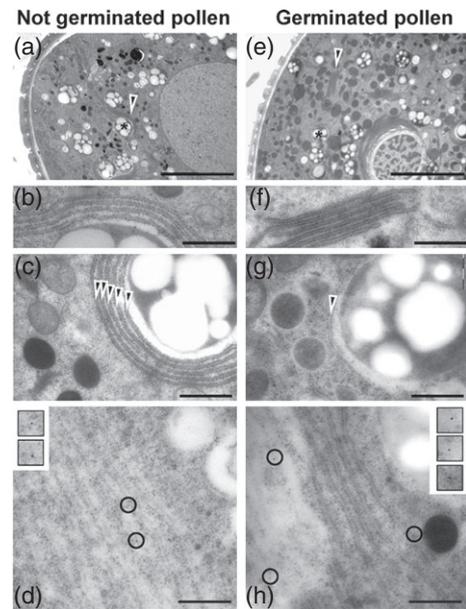
Fluorescence confocal micrographs of tobacco pollen grains transiently expressing PIN8-GFP, endoplasmic reticulum (ER)-mCherry marker,  $\gamma$ TIP-mCherry tonoplast marker and oleosin-red fluorescent protein (RFP) lipid marker (a–i). For each co-localization experiment the PIN8 fluorescence images (a–c), the cellular marker images (d–f) and the merged images (g–i) are shown. Images were taken 48 h after transformation. Confocal microscopy images of plasma membrane marker FM4-64 staining were obtained with germinated pollen expressing PIN8VEN (j–l). The red signal for FM4-64 (j), the green one for PIN8VEN (k) and the merged fluorescence signals (l) are shown. Scale bar = 10  $\mu$ m.

surrounded electro-transparent vesicles, possibly vacuoles (Figure 4c).

We observed that the morphology of the ER during pollen tube growth (Figure 4e–h) and noted that the ER mostly wrapped around storage granules before germination (Figure 4c) and changed upon germination to form free-floating stacks and dispersed tubular ER within the pollen grain (Figure 4e,f). Despite the changes in ER morphology during germination, PIN8 was still localized to the ER. No labeling could be detected when the same immunolocalization method was applied to wild-type (WT) pollen grains. Thus we conclude that PIN8 resides in the ER of pollen.

#### PIN8 overexpression causes prominent auxin-related phenotypes by altering auxin homeostasis

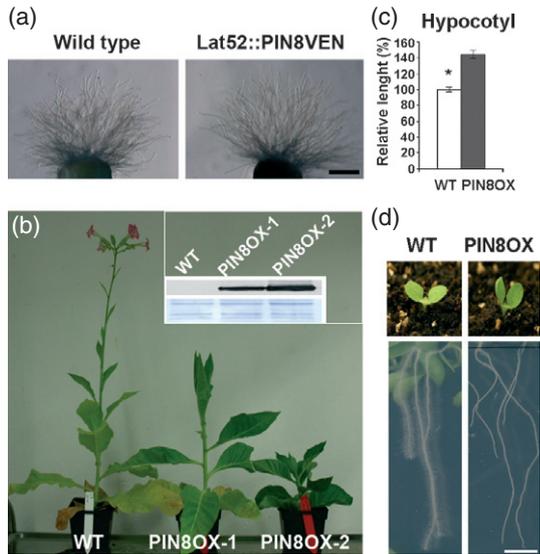
In order to elucidate the function of PIN8 we first analyzed two T-DNA insertion lines of the *AtPIN8* locus (Figure S1a). Both SALK lines homozygous for the insertion and with affected *PIN8* expression (Figure S1b) showed, under standard greenhouse conditions, normal sporophyte and pollen development. The meiosis and mitotic divisions, as assayed by 4',6-diamidino-2-phenylindole (DAPI) staining, occurred at comparable flower stages to WT flowers and the competence of *pin8* pollen for germination and fertilization was confirmed by the observed normal seed set (Figure S1d).



**Figure 4.** Endoplasmic reticulum (ER) morphology and PIN8 localization in ungerminated and germinated tobacco pollen. Overview of the pollen grain (a, e). Scale bar = 5  $\mu$ m. Note the typical appearance of ER (arrowheads), being mostly wrapped around storage granules (asterisks) before germination (a–c) and forming free-floating stacks after germination (e–g). Immunostaining against GFP shows the localization of PIN8VEN at the ER both in germinated and ungerminated pollen (d, h). Gold particles are highlighted by circles and are shown in higher magnification in the insets. Scale bar in (b)–(d) and (f)–(h) = 0.5  $\mu$ m.

The absence of a phenotype for *pin8* knock-out plants might be explained by a fine-tuning function for PIN8 such that it affects the overall pollen fitness only modestly. PIN5, the other ER-localized PIN, also resulted in only minor plant defects in the T-DNA insertion lines (Mravec *et al.*, 2009). Based on these results, we decided to also follow an overexpression strategy to characterize PIN8. Translational fusions of PIN8 with VENUS fluorescent protein under the control of a strong pollen-specific promoter (*Lat52*) were used to generate stable transgenic tobacco plants overexpressing PIN8 in the gametophyte (*Lat52::PIN8VEN*). Additionally, since little is known about auxin transport in pollen and other PINs have been studied in the sporophyte, we also generated transgenic tobacco plants ectopically expressing PIN8 under a constitutively active promoter (*35S::PIN8VEN*).

The sporophyte of the *Lat52::PIN8VEN* plants, in which fluorescently tagged PIN8 protein was expressed specifically at late stages of pollen development, looked morphologically indistinguishable from WT. Semi-*in vivo* pollen germination (Shivanna *et al.*, 1991) did not result in us seeing a substantial difference between the two genotypes (Figure 5a). In contrast, *35S::PIN8VEN* (*PIN8OX*) plants exhibited strong auxin-related phenotypes. Plants were smaller in comparison to WT (Figure 5c), had cup-shaped



**Figure 5.** Phenotypic analysis of the gametophyte and sporophyte of PIN8 overexpression lines.

(a) Semi-*in vivo* pollen germination performed with Lat52::PIN8VEN pollen versus wild type pollen. After pollination the pistil was excised 1 cm from the stigma and the images of the emerging tubes were taken 22 h later. Scale bar = 0.5 mm.

(b) Developmental defects induced by PIN8 overexpression in the sporophyte (PIN8OX) include smaller and greener plants with epinastic leaves where the strength of the phenotype correlates with the accumulation of PIN8 protein (western blot, in inset).

(c), (d) Two-week-old seedlings grown on agar plates show longer hypocotyls (c) and longer primary root than the wild type (WT) (d). Scale bar = 5 mm. Cotyledons are cup shaped (d). Error bars represent SEM,  $n = 20$ ,  $*P < 0.05$ .

cotyledons (Figure 5d), hyponastic leaves which failed to expand completely and remained juvenile for a long period (Figure 5b). One-week-old seedlings grown on agar plates showed longer hypocotyls (Figure 5c), shorter root hairs and a longer primary root (Figure 5d). Interestingly, a stronger phenotype was also associated with an accumulation of higher amounts of PIN8 protein (Figure 5b inset), suggesting a rate-limiting activity of PIN8 is associated with the WT phenotype.

To gain insight into how the observed phenotypes correlated with auxin content, and thus with PIN8 activity, we quantified the levels of IAA in pollen of Lat52::PIN8VEN plants and in leaves, cotyledons and roots of PIN8OX tobacco plants and also analyzed the effect of PIN8 overexpression on auxin levels (Figure 6). Comparison of auxin content in the different tissues of tobacco (Table S2) revealed that mature pollen grains contained relatively high amounts of auxin – more than 10 times the content found in young leaves. Additionally, the free IAA content, which typically represents 1–2% of total IAA pool in other tissues, was increased up to 8–10% in mature pollen. Despite the apparent absence of a morphological phenotype due to PIN8 overexpression in pollen, analysis of the auxin content

demonstrated a statistically significant decrease (1.6-fold) of free IAA in Lat52::PIN8VEN pollen, suggesting that PIN8 in pollen, where it is localized to the ER, is involved in the regulation of auxin homeostasis.

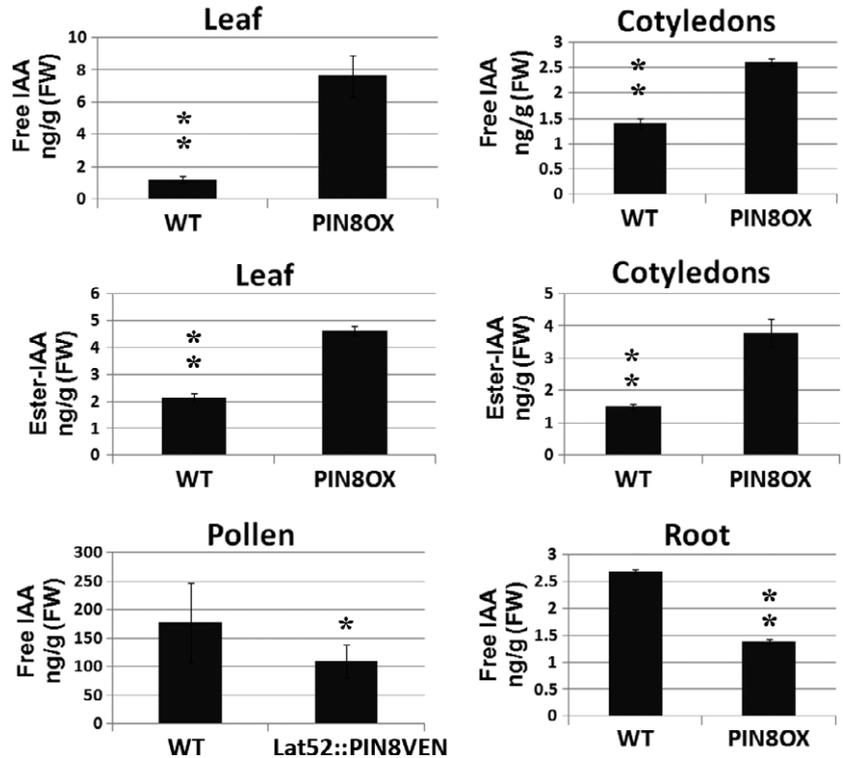
Analysis of the IAA content in cotyledons of 2-week-old seedlings and of the first expanded leaves from 5-week-old plants showed higher levels of free IAA and ester-conjugated IAA in PIN8OX plants. PIN8OX leaves, in which the overall content of auxin was higher, accumulated six times more free IAA and twice as much ester-conjugated IAA as WT leaves. In roots, PIN8 overexpression resulted in lower levels of free IAA.

#### Induction of the natural auxin response genes and the *DR5::GUS* synthetic reporter are constitutively repressed in seedlings overexpressing *PIN8*

The previously reported endomembrane localization of PIN8 (Mravec *et al.*, 2009; Ganguly *et al.*, 2010) and the phylogenetic relationship with PIN5 (Mravec *et al.*, 2009) suggested that the alteration of auxin pools observed in PIN8OX tissues could be the result of transport activity at the ER. In particular, the capacity of PIN8OX leaves to accumulate elevated amounts of free and conjugated IAA reinforces the hypothesis of the ER as a recycling station for auxin (Friml and Jones, 2010). To clarify whether the IAA content reflected an increased storage capacity or induced stronger auxin signaling, we employed the *DR5::GUS* auxin-responsive reporter. First we generated transgenic Arabidopsis plants constitutively overexpressing PIN8 with (35S::PIN8VEN) or without (35S::PIN8) a fluorescent tag. Both overexpression lines (AtPIN8OX) displayed similar characteristics to those reported for tobacco PIN8OX plants, i.e. smaller plants, curly leaves, longer hypocotyls, shorter root hairs and longer juvenile plants (Figure S2). This result confirmed that the fluorescent tag did not interfere with the protein function. Next, we analyzed the expression of the synthetic auxin reporter *DR5::GUS* in AtPIN8OX plants (Figure 7). Compared to *DR5::GUS* control seedlings (Figure 7e–h), the tissue expression pattern in leaf margins, cotyledons, primary root and hypocotyls, as revealed by GUS staining, appeared not to be affected (Figure 7a–d). This result excludes a role for PIN8 in intercellular auxin transport and the consequent generation of auxin gradients; instead it suggests defects in auxin signaling. Further, the intensity of GUS staining appeared weaker in AtPIN8OX plants, indicating that the higher free IAA levels in PIN8OX leaves do not correspond to a stronger auxin-mediated gene expression. The effect of PIN8 on auxin signaling was particularly evident when the exogenous auxin NAA was applied to the seedlings (Figure 7i–p) and resulted in strong suppression of GUS gene (*uidA*) expression in AtPIN8OX (Figure 7i–l). In order to further illustrate that the inhibition of auxin response was due to the activity of PIN8, a set of auxin response genes (Li *et al.*, 2009) was selected and

**Figure 6.** Quantification of indole-3-acetic acid (IAA) in PIN8OX leaves, cotyledons and roots and in Lat52::PIN8VEN pollen.

Free IAA and ester-IAA quantification in different tissues of wild type (WT), PIN8OX and Lat52::PIN8VEN plants. Cotyledons of 2-week-old seedlings ( $n = 5$ ), first expanded leaves of 5-week-old plants ( $n = 4$ ), roots of 2-week-old seedlings ( $n = 4$ ) and mature pollen from freshly dehiscent anthers ( $n = 9$ ) were analyzed. FW, fresh weight. \*\* $P < 0.0001$ ; \* $P < 0.05$ .

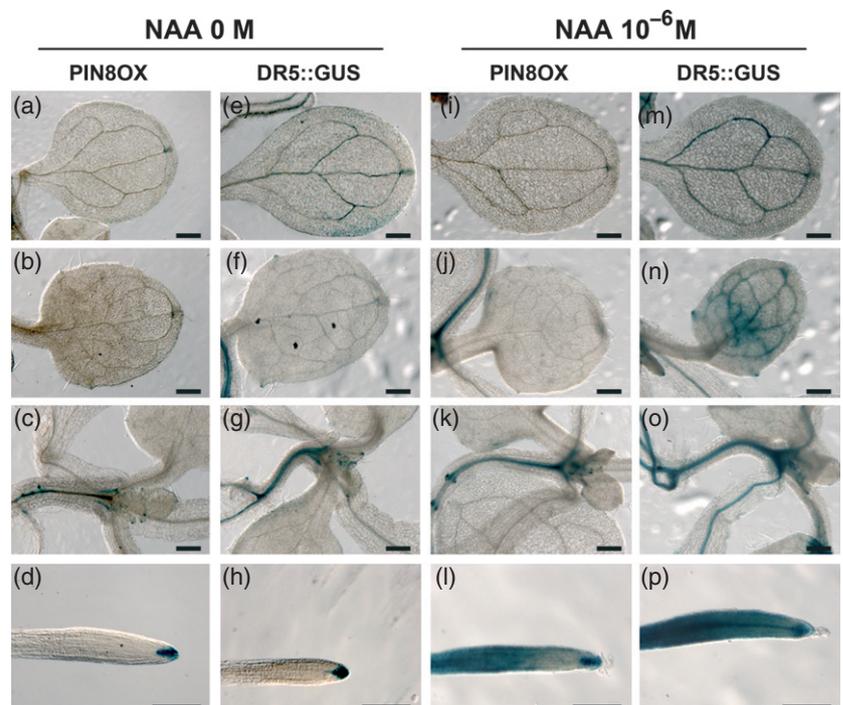


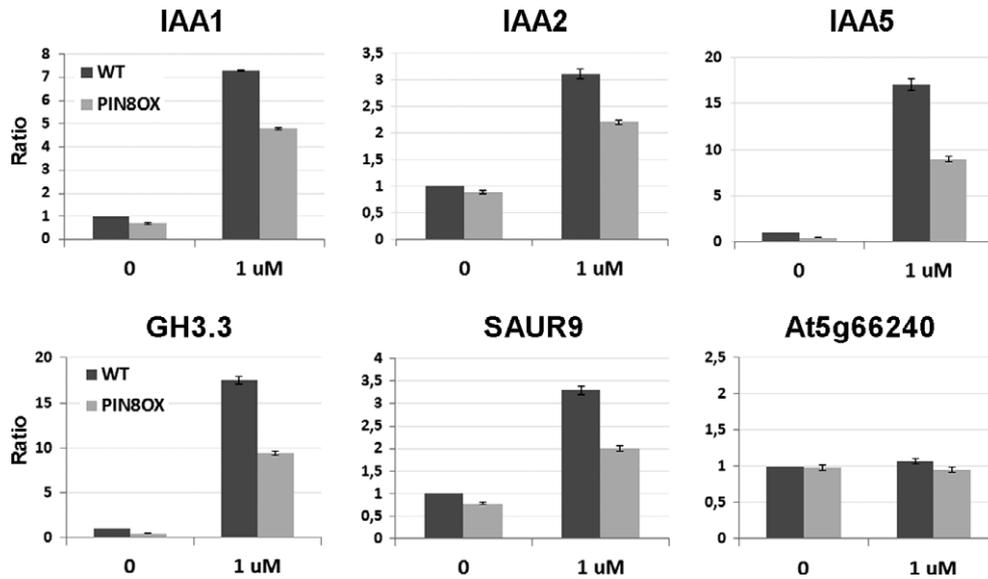
quantitative gene expression analysis was performed (Figure 8). The data showed a down-regulation of auxin response genes in AtPIN8OX (47–89% of the corresponding expression level in WT) and a weaker activation of auxin

responsive gene expression upon application of NAA. Localization analysis of PIN8 in AtPIN8OX (Figure S2) confirmed that PIN8 resides in endomembranes with a distinct perinuclear ER pattern. This suggested that PIN8 affects

**Figure 7.** Auxin-responsive GUS staining in control (DR5::GUS) and PIN8OX seedlings.

Ten-day-old seedlings were removed from agar plates and incubated in water (a–h) or in a solution containing  $10^{-6}$  M naphthalene-1-acetic acid (NAA) (i–p) for 6 h and then histochemically stained for GUS. Representative pictures of cotyledons, leaves, hypocotyls and primary roots are shown. Scale bar = 200  $\mu$ m.





**Figure 8.** Auxin-induced expression of selected auxin response genes in wild type (WT) and PIN8OX seedlings.

Twelve-day-old seedlings were removed from agar plates and incubated for 1 h in water (0) or in a solution containing  $10^{-6}$  M naphthalene-1-acetic acid (NAA) (1  $\mu$ M). For each gene, the expression levels in WT incubated in water were set at 1.0 and the gene expression levels for PIN8OX and treatments are presented as ratio to these values. Expression levels of the non-auxin response gene *At5g66240* are also shown. Error bars represent SEM,  $n = 2$ . The auxin-responsive genes are statistically significantly down-regulated in PIN8OX seedlings ( $P < 0.05$ ).

auxin-regulated gene transcription by either preventing access of auxin to the nucleus or altering the biologically active auxin pool within the nucleus.

## DISCUSSION

Strong evidence has accumulated that show an essential role for PINs in various physiological and developmental processes in plants (Müller *et al.*, 1998; Friml *et al.*, 2002a,b; Billou *et al.*, 2005; Mravec *et al.*, 2009) through the direct regulation of polar auxin transport (Petrášek *et al.*, 2006; Wisniewska *et al.*, 2006). It is thought that through distinct auxin-mediated transcriptional and post-translational regulation, PINs enable the plant to adjust growth and development in response to internal and environmental cues (Abas *et al.*, 2006; Sauer *et al.*, 2006; Kleine-Vehn and Friml, 2008). Thus specific PINs display changes in transcript level under specific conditions or in specific organs or cell types (Paponov *et al.*, 2008).

Thus far the role of auxin in microgametogenesis remains relatively poorly understood. Here we demonstrate specific up-regulation of *PIN8* mRNA in the male gametophyte. As *PIN8* expression levels were at their highest during microgametogenesis and in comparison with other PINs, our data suggest a specific role for PIN8 in regulating auxin transport in pollen. Analysis of publicly available microarray data (Pina *et al.*, 2005) showed that all canonical components of auxin signaling, such as members of *AFB*, *TPL*, *ARF* and *Aux/IAA* families, were represented in the pollen. Increased capacity for auxin accumulation in pollen grains was shown by expression analysis of auxin biosynthesis *YUCCA* genes

and a response as expected for higher levels of auxin was shown using the synthetic auxin reporter *DR5::GUS* in anthers and pollen (Aloni *et al.*, 2006; Cheng *et al.*, 2006; Cecchetti *et al.*, 2008). In particular, *DR5::GUS* expression in tapetum cells could be detected prior to the reporter activity shown in the developing pollen grains (Aloni *et al.*, 2006). The *TAA1* family of IAA biosynthesis genes, which has recently been shown to encode proteins that function upstream of the *YUCCA* genes, in the same biosynthetic pathway (Mashiguchi *et al.*, 2011; Phillips *et al.*, 2011; Stepanova *et al.*, 2011; Won *et al.*, 2011) are not expressed in pollen. These data collectively suggest that the tapetum layer supplies the developing pollen grains with either IAA or its precursors, such as indole-3-pyruvate, which could be converted to IAA by the *YUCCA* flavin monooxygenases. Our direct quantification of IAA levels revealed that mature pollen grains not only accumulated free IAA, but did so at concentrations significantly higher than in leaves, where active auxin biosynthesis occurs. Yet, PIN8 overexpression in pollen resulted in only moderately altered auxin levels with no phenotypic changes. The *Lat52* promoter is active in starting gene expression from the late, bicellular stage of pollen development. Presumably, at this stage the auxin pools provided by the tapetum were already formed, therefore elevated levels of PIN8 could not result in severe effects.

The capacity of PIN8 to alter the composition of the auxin pool and to limit auxin availability for transcriptional gene regulation was demonstrated in the reference plants tobacco and *Arabidopsis* using dominant interference by

overexpression. Auxin quantification in tobacco leaves, roots and cotyledons proved that pleiotropic phenotypes caused by PIN8 ectopic expression were correlated with a significant alteration of free and ester-conjugated IAA in a tissue- and organ-specific manner. Interestingly, in both reference species the strength of the phenotypes reflected the amount of PIN8 accumulation, i.e. a stronger phenotype was associated with higher PIN8 accumulation supporting rate-limited transport activity performed by PIN8.

The decreased response of the auxin reporter *DR5::GUS* and of the expression of the auxin-induced genes in the AtPIN8OX genotype suggest that PIN8 prevents auxin signaling for transcriptional regulation of gene expression. Similarly, the inhibitory effect of PIN8 overexpression on root hair elongation was attributed to a PIN8-mediated reduced availability of auxin for the transcription of root hair morphogenetic genes (Ganguly *et al.*, 2010). The dual localization reported in root hairs, to both ER and to the PM, did not allow discrimination between the contribution of PIN8 to auxin sequestration into intracellular compartments and to auxin export from the cytosol (Ganguly *et al.*, 2010). Here, the predominant endomembrane localization of PIN8 with a distinct perinuclear ER signature and the *DR5::GUS* expression pattern in AtPIN8OX suggest that the PIN8-mediated auxin transport at the ER is responsible for the observed phenotypes. The auxin quantification data showed that, in addition to alteration of auxin homeostasis, PIN8 overexpression results in a differential accumulation of auxin along the plant. This results in elevated levels of free IAA in auxin source tissues, such as leaves, and decreased levels of free IAA in auxin sinks, such as roots. This finding further suggests that lowered auxin signaling caused by PIN8 function is associated with the accumulation of IAA in storage compartments, possibly the ER itself. The development of methods for auxin quantification within subcellular compartments will be essential to clarify and quantify the effect of PIN8 on intracellular auxin pools.

Typically, PINs are localized polarly within their expression domain, indicating the direction of auxin efflux (Wisniewska *et al.*, 2006). A different role was assigned to ER-localized PIN5, suggesting, though indirectly, its contribution to the regulation of intracellular auxin homeostasis by transporting auxin to the ER lumen. Localization at the ER was proposed to be a common feature for short-loop PINs including PIN8 (Mravec *et al.*, 2009). Preliminary data showed an endomembrane localization of PIN8; while several lines of evidence also supported an additional plasma membrane localization in Arabidopsis root hair cells and tobacco BY-2 cells (Ganguly *et al.*, 2010). In order to clarify the localization of PIN8 in those cells where it is naturally expressed, namely mature pollen and growing pollen tubes, co-localization with different subcellular markers was performed and exclusive ER-targeting was resolved by electron microscopy. These results suggest that PIN8 is involved in regulation of intracellular

auxin homeostasis rather than in efflux of auxin out of pollen. Moreover, the finding that PIN8, the only gametophyte-specific member of the PIN protein family, is not localized at the poles suggests that polar auxin transport in the sporophyte and gametophyte (if indeed present) is mediated by different mechanisms.

So far it is not clear why the loss of *PIN8* function does not cause an obvious morphological phenotype. Although *PIN8* is specifically up-regulated in pollen, the *PIN8* expression level is relatively low when compared to other pollen-expressed genes (Honys and Twell, 2004; Pina *et al.*, 2005). Similarly, *PIN5* has a very low expression level in its expression domain, and relatively minor defects were observed in the knock-out plants (Mravec *et al.*, 2009), while the overexpression resulted in significant phenotypes. This might indicate a fine-tuning function performed by the ER-localized PINs at endogenous low protein levels. However, it is possible that changes in auxin homeostasis could be compensated by other regulatory mechanisms such as biosynthesis, degradation, transport and conjugate formation (Tromas and Perrot-Rechenmann, 2010) which ensure that free IAA and concentrations of metabolites remain at the optimum levels. Among these mechanisms the activity of the auxin conjugate hydrolases might be particularly significant; most of these have an ER retention sequence (Ludwig-Müller, 2011) and thus localize as PIN8 to the ER.

## EXPERIMENTAL PROCEDURES

### Plant material and growth conditions

*Arabidopsis thaliana* (accession Colombia) wild-type and mutants and tobacco plants (cultivar Petit Havana) were grown on soil under long-day conditions (16 h light/8 h dark) at 21°C. *Pin8* Salk lines (SALK 107965 and SALK 044651) were obtained from the Nottingham Arabidopsis Stock Centre. For measurements of root growth and hypocotyl length, tobacco plants were grown on SCN medium as previously described (Dovzhenko *et al.*, 1998). For experiments involving GUS staining and auxin-induced gene expression analysis Arabidopsis plants were grown on SCA medium (Dovzhenko *et al.*, 2003).

### DNA construct

For the *PIN8::PIN8GFP* construct the full-length coding sequence of *AtPIN8* including the promoter and 3' UTR was amplified by PCR from Arabidopsis accession Columbia genomic DNA with the primer pairs 5'-CAAAGTTTCTGTTCACCT-3' and 5'-AGGGGAAGAAGAAAGGGTAAC-3'. The *GFP* coding sequence (CDS) was inserted in-frame into the *PIN8* CDS at position 688 from the start codon (ATG). The construct was then introduced in the binary vector pGJ-Bar. For the *35S::PIN8VEN* construct the CDS of the yellow fluorescence protein (VENUS) was inserted in-frame into the intronless *PIN8* cDNA at position 553 from the start codon by overlap extension PCR. The corresponding PCR product was first cloned in the pDONOR207 vector (Invitrogen,) and then transferred into the binary vector pB2GW7,0 under control of the cauliflower mosaic virus 35S promoter generating the pB2GW7,0-PIN8VENUS vector. For *Lat52::PIN8VENUS*, the 35S promoter was excised from pB2GW7,0-PIN8VENUS with the restriction enzymes *SacI* and *SpeI*

and substituted with the sequence of the Lat52 promoter (Twell *et al.*, 1991).

*Arabidopsis* and tobacco transgenic lines were obtained via *Agrobacterium tumefaciens*-mediated transformation according to previously reported methods (Clough and Bent (1998) and Clemente (2006) respectively). *35S::ER-mcherry* and *35S::tonoplast-mcherry* were as previously reported (Nelson *et al.*, 2007); lipid body marking was according to De Domenico *et al.* (2007).

### GUS staining

The GUS staining was carried out as previously described (Ditengou *et al.*, 2008). Seedlings were incubated in staining solution overnight at 37°C. For visualization the chlorophyll was removed by incubation in 70% ethanol.

### Arabidopsis RNA isolation and RT-PCR analysis

Total RNA from 1-week-old seedlings, roots of 2-week-old plantlets, rosette leaves of 4-week-old plants, and inflorescence of freshly blooming plants was isolated using the plant RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA from *Arabidopsis* pollen was performed using the RNeasy Micro Kit (Qiagen). First-strand cDNA was synthesized from 1 µg RNA using RevertAid M-MuLV reverse transcriptase (Fermentas, St Leon-Rot, Germany). The resulting cDNA was subjected to RT-PCR or quantitative (q)RT-PCR. For RT-PCR the primer pairs used were 5'-TTAGGTCTGTTTCATGGCATCAC-3' and 5'-TATGCCAAAGTTGTTGGTAAGG-3' for *PIN8* and 5'-GGATTCGCCAACCTTGTATG-3' and 5'-AGAAATGTGGACCTCGGTG-3' for *VATP*. For qRT-PCR a Light cycler480 (Roche, Mannheim, Germany) Real-Time system and Maxima SYBR Green (Fermentas) were used. The following primer pairs were used: for *PIN1* 5'-GGTGGTGGTCGGAAGCTCTAA-3' and 5'-TAGCAGGACCACCGTCTCT-3', for *PIN2* 5'-CTACGCAATGTTTAAACGCAAGC-3' and 5'-TTCTGCCTCTCTCTGGT-3', for *PIN3* 5'-GCACCTGACAACGATCAAGG-3' and 5'-AGACCATTCTCGGCGTCTTT-3', for *PIN4* 5'-AACCAACGAACTGCAAGAG-3' and 5'-AAGACCGCCGATATCATCAC-3', for *PIN5* 5'-CGTCTCGTTTGTATTTCACC-3' and 5'-ACCAACGACAAGAGAGTTAGTAAGAG-3', for *PIN6* 5'-TAAGTCTCCA CCCATTCGCGGAT-3' and 5'-TCTTTGGTGCATGTCTTTG-3', for *PIN7* 5'-GCTTCTCGCAAGATTCCG-3' and 5'-TGTTTCATTGGCTCATTATCAAC-3', for *PIN8* 5'-CTGGCATCCAACAGGAGGAAGAGG-3' and 5'-GCCCTTGCGGCGTTAAGCTC-3'.

The efficiency of each primer pair was determined by examination of a standard curve using serial dilutions of genomic DNA. The PCR was performed using a three-step protocol including melting curve analysis. For analysis of auxin response genes primer sequences were as described (Li *et al.*, 2009).

### Pollen isolation, transformation, germination and staining

Flower buds from freshly blooming flowers were collected for *Arabidopsis* pollen isolation and gently squashed using a plastic piston in isolation medium (1.49 g L<sup>-1</sup> KCl, 0.25 g L<sup>-1</sup> MgSO<sub>4</sub> × 7H<sub>2</sub>O, 0.11 g L<sup>-1</sup> CaCl<sub>2</sub>, 127.5 g L<sup>-1</sup> mannitol, 1 ml L<sup>-1</sup> 1 M phosphate buffer pH 7.0 with 0.1 M KOH).

The pollen-containing suspension was filtered through 32 µm mesh and centrifuged at 50 g for 5 min. The pellet was washed twice and then used for total RNA isolation.

*Nicotiana tabacum* pollen isolation was performed as described (Touraev and Heberle-Bors, 1999). For pollen transient transformation 60 µl of a sterile stock solution of microparticles (50% glycerol, 30 mg ml<sup>-1</sup>, gold particles of diameter 0.3–3 µm) were thoroughly vortexed and mixed with 3 µl DNA in H<sub>2</sub>O (1 µg µl<sup>-1</sup> concentration). While thoroughly mixing, 60 µl of 2.5 M CaCl<sub>2</sub> was added and afterwards 20 µl of 1 M spermidine. The suspension was incubated

on ice for 30 min and then centrifuged at 17 000 g. The pellet was washed twice with ethanol and then suspended in 22 µl ethanol. Eleven microliters was applied to the macrocarriers per shot.

Sixty microliters of the pollen suspension was dropped in the middle of a wet 3-cm Petri dish and the biolistic transformation was performed using a helium-driven PDS-1000/He particle delivery system (Bio-Rad Laboratories GmbH, Vienna, Austria), according to the following parameters: target distance 9 cm, 1000 p.s.i. helium rupture pressures and 27 in Hg vacuum pressure. After bombardment, pollen grains were cultured in maturation medium (400 g L<sup>-1</sup> sucrose, 11.5 mg L<sup>-1</sup> L-proline, pH 7) at 25°C. To induce germination of the bombarded pollen, the pollen suspension was centrifuged for 3 min at 200 g and the pellet suspended in germination medium [100 mg H<sub>3</sub>BO<sub>3</sub>, 100 mg KNO<sub>3</sub>, 708 mg Ca(NO<sub>3</sub>)<sub>2</sub> × 4H<sub>2</sub>O, 200 mg MgSO<sub>4</sub> × 7H<sub>2</sub>O, 100 g L<sup>-1</sup> sucrose, 400 mg L<sup>-1</sup> 2-(*N*-morpholine)-ethanesulfonic acid (MES), 1 g L<sup>-1</sup> casein hydrolysate, pH 5.9] and incubated at 25°C.

Semi-*in vivo* pollen germination was performed as-described (Shivanna *et al.*, 1991). After a pollination time of 3 h, 10 mm of the style was cut and implanted in agar medium and the emerging pollen tubes were recorded 24 h later. Ten styles for each genotype were analyzed and the experiment was repeated twice. Growing tobacco pollen tubes for FM4-64 staining were incubated in germination medium supplied with 10 µM FM4-64 for 15 min. and immediately imaged.

### Quantification of free and conjugated indole-3-acetic acid

Tobacco leaf tissue, cotyledons and pollen grains from plants grown on soil and roots from seedlings grown on plates were collected, weighed and immediately frozen in liquid nitrogen. Each type of frozen tissue was homogenized using a Mixer Mill (MM 300; Qiagen), with a 3-mm tungsten carbide bead in 300 µl homogenization buffer (35% of 0.2 M imidazole, 65% isopropanol, pH 7) containing a known amount of [<sup>13</sup>C<sub>6</sub>]-IAA as an internal standard. After 1 h on ice, 150 µl of the homogenate was purified through two sequential solid phase extraction (SPE) columns, anion exchange and plastic affinity, using a Gilson SPE 215 system (Gilson, Inc., Middleton, WI, USA), methylated, dried and dissolved in ethyl acetate exactly as previously described (Barkawi *et al.*, 2010). The samples were then analyzed using gas chromatography-selected ion monitoring-mass spectrometry (GC-SIM-MS) on an Agilent 6890/5973 system (Agilent Technologies, Inc., Santa Clara, CA, USA). The level of free IAA was quantified by isotope dilution analysis based on the [<sup>13</sup>C<sub>6</sub>]-IAA internal standard.

For the rest of the homogenate, 100 µl was hydrolyzed in 1 N NaOH (1 h at 22°C) for measurement of free plus ester-linked IAA and 50 µl was hydrolyzed in 7 N NaOH (3 h, 100°C under nitrogen gas) for measurement of total IAA (Bialek and Cohen, 1992). After hydrolysis, the pH of the homogenate was adjusted to 2.7 and it was desalted by passing through a C18 SPE column (100 mg; Varian, Harbor City, CA, USA), washed with 3 × 0.6 ml water, eluted with 3 × 0.3 ml methanol, evaporated to dryness and dissolved in 150 µl of homogenization buffer. The purification of the IAA released from the conjugates was subsequently the same as the method used for the purification of free IAA. The samples were analyzed using GC-SIM-MS, and the levels of free plus ester-linked IAA and total IAA were determined by isotope dilution analysis based on the [<sup>13</sup>C<sub>6</sub>]-IAA internal standard.

### Microscopy

For electron microscopy, isolated tobacco pollen, germinated and not germinated, was soaked into nitrocellulose tubes and high-pressure-frozen in a Bal-Tec HPM 010 (ABRA-Fluid AG, Widnau,

Switzerland), using hexadecene as filler. Freeze substitution was carried out in a Leica AFS2 at 90°C in acetone containing 1.5% uranyl acetate for 3 days, followed by stepwise infiltration with a Lowicryl HM20 at 40°C for 2 days (Polysciences Europe GmbH, Eppelheim, Germany). Polymerization was 2 days at 40°C and 2 days at 22°C, by UV light. Sections of 90 nm were cut on a Leica UC6 ultramicrotome. Incubation steps for immunostaining were 10 min blocking, 1 h primary and 45 min secondary antibody. Antibodies were diluted 1:50 in 5% skim milk in PBST (50 mM PBS containing 0.05% Tween-20). Washing was done in between and afterwards twice on PBST. Micrographs were taken with a 1024 × 1024 CCD detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems, Scheuring, Germany) in a Zeiss EM 902A operated in the bright field mode (Carl Zeiss AG, Oberkochen, Germany).

Whole plant images were taken using Nikon D60 digital camera (Nikon, Tokyo, Japan) and detailed plant images were acquired using a stereomicroscope Stemi SV11 Apo (Carl Zeiss AG). For fluorescence microscopy a Axiovert 200M inverted microscope (Carl Zeiss AG) and appropriate emission filters (DIC, DAPI, GFP) were used. For confocal laser scanning microscopy pollen images were acquired using a LSM5Live microscope (argon 488 nm laser for the GFP channel and a 561-nm diode laser for the mCherry channel) and root images were acquired using a iMORE microscope (TILL Photonics GmbH, Gräfelfing, Germany) coupled with a 470-nm laser (Picoquant GmbH, Berlin, Germany).

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** PIN8 T-DNA insertional mutants (*pin8*).

**Figure S2.** Phenotypic analysis induced by PIN8 overexpression and PIN8 localization in *Arabidopsis thaliana*.

**Table S1.** Auxin-related genes expressed in pollen (Pina *et al.*, 2005).

**Table S2.** Free and total quantification of indole-3-acetic acid in different tissues of *Nicotiana tabacum*.

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