Arabidopsis WAT1 is a vacuolar auxin transport facilitator required for auxin homoeostasis

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The plant hormone auxin (indole-3-acetic acid, IAA) has a crucial role in plant development. Its spatiotemporal distribution is controlled by a combination of biosynthetic, metabolic and transport mechanisms. Four families of auxin transporters have been identified that mediate transport across the plasma or endoplasmic reticulum membrane. Here we report the discovery and the functional characterization of the first vacuolar auxin transporter. We demonstrate that WALLS ARE THIN1 (WAT1), a plant-specific protein that dictates secondary cell wall thickness of wood fibres, facilitates auxin export from isolated Arabidopsis vacuoles in yeast and in Xenopus oocytes. We unambiguously identify IAA and related metabolites in isolated Arabidopsis vacuoles, suggesting a key role for the vacuole in intracellular auxin homoeostasis. Moreover, local auxin application onto wat1 mutant stems restores fibre cell wall thickness. Our study provides new insight into the complexity of auxin transport in plants and a means to dissect auxin function during fibre differentiation.

DOI: 10.1038/ncomms3625

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Auxin has a crucial role in multiple aspects of plant growth and development, from controlling plant architecture, directional growth responses and abiotic and biotic stress responses to regulating reproductive and vascular development. Diffusion of auxin across membranes is limited and at least four distinct transporter families have evolved to facilitate intercellular and intracellular transport. The AUX1/LAX influx transporter protein family was originally found through the isolation of the Arabidopsis auxin resistant1 (aux1) mutant. aux1 roots are resistant to the membrane impermeable synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D), and show agravitropic growth as consequences of a defect in cellular auxin uptake. The AUX1 gene codes for a protein homologous to amino-acid permeases that mediates auxin import when heterologously expressed in Xenopus laevis oocytes. The second class of auxin transporters is represented by the ATP-binding cassette subfamily B (ABC)-type transporters of the MULTIDRUG RESISTANCE/phosphoglycoprotein (ABC/MDR/PGP) family of membrane proteins, which mediate ATP-dependent auxin transport through the plasma membrane. ABCB1, ABCB4 and ABCB19 have been identified as proteins with binding affinity to the auxin transport inhibitor 1-naphthylphthalamic acid. The biochemical evidence for these ABCB proteins having a role in auxin transport has been provided by heterologous expression in tobacco cells, HeLa cells and yeast. Finally, PIN-FORMED (PIN) family members have a critical role in determining the directionality of auxin flow in embryonic and post-embryonic development. In contrast to ABC transporters, which occur across all kingdoms, PIN genes are plant specific. The canonical 'long' PIN proteins PIN1–4 and 7 are localized at the plasma membrane and ensure auxin efflux. However, it was shown recently that PIN5 and PIN8, two atypical 'short' PINs with a shorter hydrophilic loop, are localized at the endoplasmic reticulum (ER) and 14–16, highlighting the importance of intracellular mechanisms in controlling auxin homeostasis. Similarly, the PIN-LIKES (PILS) protein family has recently been identified based on in silico structural homology with PIN5. PILS proteins have been suggested to regulate intracellular auxin accumulation at the ER.

A gene expression profiling approach previously identified hundreds of candidate genes for secondary cell wall formation in Zinnia elegans xyleogenic cell cultures. Mutations in an Arabidopsis homologue of one of these genes, WALLS ARE THIN1 (WAT1), caused a drastic reduction in secondary cell wall thickness of stem fibres. The WAT1 protein is similar in sequence to a Medicago truncatula nodulin 21, originally shown to be upregulated in Rhizobium-induced Medicago nodules. The Arabidopsis WAT1 gene family is composed of 46 members. WAT1 has been proposed to be involved in integrating auxin signalling and secondary cell wall formation in Arabidopsis fibres based on transcriptomic, metabolomic and physiological data. The wat1-1 mutation resulted in the massive downregulation of auxin-related gene expression and reduced auxin content in stems, and an increased sensitivity of wat1 seedlings to 5-Methyl-Trp, a toxic analogue of Trp, an auxin precursor.

In this study, we demonstrate that WAT1 is a so far unknown tonoplast-localized auxin transporter, suggesting a critical role for the vacuole in regulating intracellular auxin homeostasis in plants. Further, the fact that the reduction in secondary cell wall thickenings in wat1 mutants can be rescued by exogenous auxin application underpins the role of auxin in secondary growth in plants.

**Results**

**A link between WAT1 and auxin responses.** A microarray-wide search for the most highly co-regulated genes with WAT1 (ref. 21) resulted in the identification of several genes involved in auxin transport and signalling (that is, AUX1, IAA9, IAA13, LAX2, ARF4, ARF11 and PIN1; Fig. 1). Consistent with the cell wall phenotype of wat1 mutants, other WAT1 co-regulated genes are known to be essential for either vascular patterning (ATHB-8 and ATHB-15) or secondary wall deposition (IRX9, MYB43, KNAT7 and FRA1). Strikingly, the expression of several of these genes, including WAT1 itself, is also induced by auxin (Figs 1 and 2).

Phenotypic alterations resulting from the wat1 mutation have been reported at late stages of plant stem development; however, WAT1 is expressed much earlier in plant development (Fig. 2). To elucidate primary effects of the wat1 mutation, we carried out comparative microarray analysis profiling mRNA global expression of 10-day-old in vitro-grown wild-type and wat1-1 mutant seedlings. This revealed 40 genes that exhibited significantly altered expression levels in wat1-1, with 13 genes displaying lower and 27 higher mRNA levels compared with the wild type (Supplementary Fig. S1). The two most downregulated genes, besides WAT1 itself, were IAA19 and an auxin-responsive GH3 family member both known to be associated with auxin responses. Among the 13 downregulated genes, at least 7 are induced by auxin in wild-type plants according to the Genevestigator database. In contrast, among the 27 genes upregulated in wat1, 5 are repressed by auxin in wild-type plants (Supplementary Fig. S1). These results revealed that the wat1-1 mutation modulates the expression levels of several auxin-responsive genes.

Finally, to determine whether there is a causal link between lower auxin content and the fibre phenotype in wat1 stems, we locally applied auxin (2,4-D and NAA) to growing stem segments of wat1. Ten days after auxin application, cell wall thickness of wat1 interfascicular fibres was restored to wild-type levels (Fig. 3). These results confirm the role of auxin in promoting xylem fibre differentiation.

**WAT1 is a tonoplast-localized auxin transporter.** The WAT1 gene encodes a plant-specific protein of 389 amino acids predicted to have 10 transmembrane domains, and it has been classified as a member of the Plant Drug Metabolite Exporter family (Transport Classification Database (http://www.tcdb.org)). Although PINs and AUX1 are members of the auxin efflux carrier and amino acid/auxin permease families, respectively, several of them also possess 10 predicted transmembrane domain. Although WAT1 did not exhibit sequence homology with any of the other auxin transporters, its predicted secondary structure was similar, showing the greatest resemblance with the ER-localized PIN5 and PILS2/5 proteins (Supplementary Fig. S2). Beyond their similarity in size, they have a centrally localized, shorter hydrophilic loop in comparison with the 'long PINs' (PIN1–4, 7).

Although WAT1 has been previously detected in both plasma membrane and tonoplast fractions in systematic proteomic studies, we found very little co-localization of WAT1-GFP (green fluorescent protein) with the plasma membrane marker, yellow fluorescent protein (YFP)-NPSN12 (ref. 29) (Fig. 4a). In contrast, WAT1-GFP co-localized to the greatest extent with the tonoplast marker, YFP-VAMP711 (Fig. 4a), consistent with our previous interpretation that WAT1 may be a tonoplast-localized protein. WAT1-GFP exhibited some overlapping fluorescent signal with the late endosomal marker, YFP-RabG3f, but to a lesser extent than with YFP-VAMP711. As in the case for the tonoplast marker YFP-VAMP711, WAT1-GFP localization was also insensitive to Brefeldin A (BFA) treatment (Supplementary Fig. S3), suggesting that WAT1-GFP was delivered to the tonoplast by a BFA-insensitive, Golgi-independent pathway.
The subcellular localization of WAT1 and its structural resemblance to auxin carriers prompted us to test the capacity of WAT1 to transport indole-3-acetic acid (IAA) across the tonoplast membrane. Therefore, we isolated vacuoles from *wat1* and wild-type mesophyll protoplasts and carried out transport studies using $^{14}$C-IAA. Experiments were conducted at pH 7.6, a condition under which IAA is present mainly in the anionic, unprotonated form, and diffusion is minimal. As a control, $^3$H$_2$O was added to the incubation medium as water readily diffuses through biological membranes. This confirmed the volume, intactness and stability of both wild-type and *wat1* vacuole populations.

Auxin did not accumulate in wild-type vacuoles, whereas in *wat1* vacuoles, the auxin concentration increased in a time-dependent manner (Fig. 4b). If we assume that auxin uptake rates are similar in both vacuole populations, this points to an efficient, WAT1-dependent auxin efflux from wild-type vacuoles, which is obviously absent in *wat1* vacuoles. As exporting anionic auxin from the vacuole is thermodynamically uphill, a likely energy source would be the coupling with downhill transport of protons (for a review, see ref. 31). Therefore, we hypothesize that WAT1 is a tonoplastic-localized transporter that exports auxin from the vacuole to the cytoplasm, perhaps operating as a H$^+:\text{IAA}^-$ symporter.

To test this hypothesis, additional vacuole transport experiments were performed under conditions that ensured a drastic reduction of the driving force for proton-coupled transporters (Fig. 4c). NH$_4^+$, a proton gradient dissipater, was added to the external buffer, whereas Mg-ATP was omitted to avoid compensation of proton gradient dissipation by V-ATPase-mediated proton pumping into the vacuole. In this experiment, NH$_4^+$ should theoretically reduce IAA efflux from wild-type vacuoles, whereas in *wat1* vacuoles, this was hindered and the auxin concentration increased in a time-dependent manner (Fig. 4d).

**Figure 1 | WAT1 coexpression vicinity network.** The most highly co-regulated genes with WAT1 as identified with the Arabidopsis Coexpression Data Mining Tools from the University of Leeds$^{21}$ (http://www.Arabidopsis.leeds.ac.uk/act/coexpanalyser.php). Nodes represent genes; edge width indicates whether two given genes are co-expressed above a certain mutual rank threshold$^{36}$. Nodes are colour coded to reflect the level of induction by auxin of the corresponding genes according to the Genevestigator$^{22}$ (https://www.genevestigator.com) and the Bio-Array Resource$^{27}$ (http://www.bar.utoronto.ca) websites.

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vacuoles, resulting in a net increase in vacuolar IAA concentration over time. This was the case (Fig. 4c), thereby supporting the hypothesis that WAT1 operates as a H\(^+\)-IAA\(^-\) symporter. In the presence of NH\(_4^+\) and absence of Mg-ATP, not only is the pH gradient expected to decrease but also the difference in electrical potential across the tonoplast, which is a driving force for IAA\(^-\) uptake. This explains the decrease in IAA accumulation in \textit{wat1} vacuoles in the presence of NH\(_4^+\) (absence of Mg-ATP; Fig. 4c) versus the absence of NH\(_4^+\) and presence of Mg-ATP (Fig. 4b). It is noteworthy that the reduced difference in membrane potentials should lead to a similar reduction of IAA influx towards the vacuole lumen in wild type. The fact that a net increase of IAA levels in wild-type vacuoles in the presence of NH\(_4^+\) was observed strengthens our conclusion that WAT1 efflux was primarily affected by NH\(_4^+\) application. Finally, to determine whether the radiolabelled \(^{14}\)C detected during vacuolar transport experiments was uniquely associated with IAA or converted to other related metabolites, high performance liquid chromatography (HPLC) analysis was performed on wild-type vacuoles. Only one major peak corresponding to IAA was detected, confirming that the radiolabel was associated uniquely to IAA (Fig. 5). Altogether, these results strongly support the hypothesis that WAT1 mediates IAA export from the vacuole.

These findings raised the question as to whether auxin is actually present in plant vacuoles. Although early physiological studies in the green alga \textit{Hydrodictyon}\(^3\) speculated that the vacuole of higher plants might represent a pool of symplastic auxin\(^3\), experimental evidence for vacuolar auxin in plants is still lacking. To address whether auxin is found in \textit{Arabidopsis} vacuoles, vacuoles were purified from protoplasts of wild-type leaves and IAA and related metabolites were measured (Table 1). Vacuole purity was first confirmed by western blots using antibodies detecting different cellular compartment markers (Supplementary Fig. S4). Strikingly, IAA, several of its precursors, the IAA catabolite oxIAA (oxIAA) and the conjugate IAA-Glc could be detected in purified vacuoles (Table 1), suggesting an important role for the vacuole in auxin storage and regulation of auxin homeostasis in \textit{Arabidopsis}. The apparent discrepancy in our observations that auxin and related metabolites were found in isolated vacuoles while isolated wild-type vacuoles did not take up auxin in transport experiments (Fig. 4b) suggests that WAT1 can act as an efficient auxin exporter but that its activity is likely regulated \textit{in vivo}.

**WAT1 auxin transport activity in yeast and \textit{Xenopus} oocytes.** To confirm WAT1 auxin transport activity, we cloned the complementary DNA of WAT1 into the yeast expression vector pDR196 and introduced it into \textit{Saccharomyces cerevisiae}. As demonstrated by reverse transcriptase–PCR, WAT1 was highly expressed in yeast transformed with pDR196-WAT1 (Fig. 6a). When fused with GFP, WAT1 localized to the plasma membrane of yeast cells (Fig. 6b). WAT1 expression resulted in a significant increase in radiolabelled \(^3\)H-IAA uptake as compared with the empty vector control (Fig. 6c). The specificity of IAA transport was confirmed by performing \(^3\)H-IAA transport in the presence of various non-radioactive potential competitors (Fig. 6d). \(^3\)H-IAA influx in yeast expressing WAT1 was greatly inhibited by unlabelled IAA (69%) and to a lesser degree (25% and 27%) by oxIAA and 1-naphthaleneacetic acid (NAA), respectively. Conjugated forms of IAA (IAA-L-alanine, IAA-L-phenylalanine and IAA-Glc) and other forms of natural or synthetic auxinic

**Figure 2 | WAT1 is induced by auxin.** (a) Reverse transcriptase (RT)-PCR analysis of WAT1 expression in 10-day-old wild-type plantlets transferred for 2 h on MS medium with or without 1\(\mu M\) IAA before RNA extraction. The amount of cDNA template in each RT-PCR reaction was normalized to the signal from the actin-encoding ACT2 gene, and primers were designed to rule out the amplification of genomic DNA. Histograms represent WAT1 expression levels normalized versus ACT2 expression levels. Mean ± s.d., \(n = 3\) biological replicates. (b) GUS activity in the aerial portion (top) and roots (bottom) of 10-day-old \textit{ProWAT1:GUS} plantlets transferred for 2 h on MS medium with or without 1\(\mu M\) IAA before staining.

**Figure 3 | Local auxin application rescues deficiency in secondary cell wall formation in \textit{wat1} mutants.** Phloroglucinol-HCl staining of transverse stem sections, lignified cell walls stained red. Lanolin ± auxin was applied to the second oldest internode of the inflorescence stem. Stems were sectioned 10 days after application. Sections are representative of data from two experimental replicates each with six individuals per genotype and treatment. Scale bars, 50 \(\mu m\).
compounds (indole-3-butyric acid and 2,4-D) did not reduce WAT1-mediated 3H-IAA influx in yeast. Finally, WAT1 did not facilitate 3H-Trp uptake (Fig. 6e). WAT1-mediated IAA influx was further confirmed by testing it in an independent heterologous expression system: Xenopus oocytes (Fig. 6f). As compared with water-injected oocytes, WAT1 complementary ribonucleic acid (cRNA)-injected oocytes incorporated significantly more 3H-IAA. WAT1 did not facilitate Trp import or export in oocytes: influx and efflux of 15N-labelled Trp in WAT1-expressing oocytes were 100.02 ± 0.13 (n = 11) and 101.13 ± 1.09 (n = 8) that of control oocytes, respectively (mean ± s.d.). These data confirm that WAT1 catalyses a net influx of IAA, in agreement with data obtained in yeast. These results demonstrate that WAT1-mediated IAA transport activity does not require any additional, plant-specific components.

It is important to note that transporters maintain their orientation with respect to the cytoplasm so that protein domains facing the cytoplasm in the native context also face the cytoplasm in these heterologous expression systems. Therefore, if the thermodynamic conditions are favourable, they also maintain their direction of transport (with respect to the cytosol) when targeted to a different membrane in heterologous expression systems (in this case, the plasma membrane as opposed to the tonoplast)\textsuperscript{14,34}. The conditions used in our heterologous expression systems are compatible with the thermodynamics between the cytosol and vacuole. Therefore, WAT1-mediated IAA import from the medium towards the cytosol in yeast and oocytes would correspond to vacuolar export towards the cytosol (equivalent to the external solution in isolated vacuoles experiments). Therefore, yeast and oocyte IAA transport data both independently confirm that WAT1 acts as an auxin exporter in vacuoles.

**Discussion**

Herein we demonstrate that *Arabidopsis thaliana* *WAT1* encodes a novel auxin transporter in plants. Although *WAT1* is already
expressed during seed germination and seedling development, the \textit{wat1} phenotype only becomes apparent at later stages of plant development. We show that the most conspicuous phenotype, a decrease in stem fibre cell wall thickness\textsuperscript{19}, could be restored by exogenous auxin application to stems. Mutations in the class III HD ZIP transcription factor REVOLUTA also led to impaired fibre differentiation, and this phenotype was correlated with reduced expression of several \textit{PIN} genes and reduced polar auxin transport\textsuperscript{28}. However, the discovery that \textit{WAT1} possesses auxin transport activity and that the fibre phenotype in \textit{wat1} mutants can be restored by exogenous application of auxin provides the first direct genetic evidence for a role of auxin in fibre differentiation. As vacuole-localized auxin cannot bind to nuclear or ER-localized auxin receptors, active redirection of vacuolar auxin to the cytoplasm might be critical for auxin signalling, especially in strongly vacuolated cells such as interfascicular fibres.

Despite the absence of a seedling phenotype, a mutation in \textit{WAT1} altered the expression of several auxin-regulated genes. As free auxin content in \textit{wat1} seedlings is similar to wild type\textsuperscript{19}, the deregulation of auxin-related genes may be due to \textit{wat1}-mediated changes in the subcellular compartmentation of auxin. The regulation of auxin homoeostasis via intracellular compartmentalization is becoming an increasingly central theme in auxin biology\textsuperscript{35,36}. The role of the ER in auxin metabolism and signalling had already been inferred by the presence of auxin-binding protein 1 (ABP1), an auxin receptor involved in determining cell expansion rate\textsuperscript{37}, gene expression\textsuperscript{38}, \textit{Rho}-GTPase-dependent cytoskeleton organization\textsuperscript{39} and clathrin-dependent endocytosis\textsuperscript{40} and of IAA-amino acid amidohydrolases in the ER lumen\textsuperscript{41}. The recent discovery that PIN5 and PIN8, two atypical members of the PIN family, mediate auxin flow between the cytosol and ER lumen further reinforces the major role of the ER in auxin homoeostasis\textsuperscript{14–16}. It has recently been postulated that PIN5 residence/activity at the ER is regulated by ABP1 in a similar manner that extracellular ABP1 regulates PIN protein activity at the PM\textsuperscript{35,40}. The similarities between \textit{WAT1}, PIN5 and PILS2/5 are striking; the predicted proteins bear structural resemblance to one another and from a functional standpoint, they mediate intracellular auxin homoeostasis. These features, along with the fact that they all have homologues in basal land plants\textsuperscript{42,43} suggest an ancient, conserved, auxin transporter structure and support the theory that intracellular regulation of auxin metabolism is an ancient event in the evolution of land plants\textsuperscript{44}.

To our knowledge, this is the first report demonstrating a role for the vacuole in the regulation of auxin homoeostasis. \textit{WAT1}-mediated auxin transport across the tonoplast membrane uncovers an additional level of intracellular complexity in auxin signalling pathways in plant cells. Paradoxically, the presence of auxin and its metabolites in vacuoles has never been demonstrated, although in earlier physiological studies it has been inferred\textsuperscript{32,33}. \textsuperscript{14}C-IAA transport assays carried out on isolated vacuoles showed that \textit{WAT1} exports IAA from the vacuole to the external medium, the equivalent to the cytoplasm in plant cells. This directionality is strongly supported by the overall decrease in auxin response gene expression in \textit{wat1} seedlings and heterologous expression data in yeast, whereby \textit{WAT1} facilitates auxin uptake from the extracytoplasmic space into the cytosol. Most vacuolar transporters use an electrochemical gradient and act either as vacuolar proton antiporters for import processes or proton symporters to export compounds\textsuperscript{31}. \textit{H}\textsuperscript{+}-\textit{ATPases} and \textit{H}\textsuperscript{+}-\textit{PPases} generate a \textit{trans}-tonoplast \textit{H}\textsuperscript{+} electrochemical gradient, with a positive electrical potential and a lower \textit{pH} in the vacuole with respect to the cytosol or external medium\textsuperscript{31}. In \textit{Arabidopsis}, the vacuolar \textit{pH} is around 6. This means that vacuolar auxin is essentially in the non-permeant, anionic form and its export would be thermodynamically uphill. As \textit{WAT1}-mediated auxin export required energy in isolated vacuole transport experiments, this is also likely to be the case \textit{in vivo}.

Table 1 | IAA and related metabolite content in wild-type vacuoles.

| Concentration (pmol ml\textsuperscript{-1}) |
|-------------------------------------------|
| Anthranilic acid (ANT)                     |
| Tryptophan (TRP)                          |
| Tryptamine (TRA)                          |
| Indole-3-acetamide (IAM)                  |
| Indole-3-acetonitrile (IAN)               |
| Indole-3-acetic acid (IAA)                |
| 2-oxindole-3-acetic acid (oxIAA)          |
| 1-O-indole-3-acetyl-β-D-glucose (IAA-Glc)* |

The mean abundance ± s.d. is given (n = 5).

\textsuperscript{1}IAA-Glc was the only IAA conjugate detected, the concentration of IAA-Ala, IAA-Asp, IAA-Glu and IAA-Leu were all below the detection limit.
Although it is clear that PINs, PILS, ABCBs, AUX-LAXs proteins and now WAT1 are essential factors in auxin transport, there are clearly other potential candidates within plant genomes. Of the 25,498 predicted proteins in the Arabidopsis genome, 5–10% are thought to be transporters45, and among them, only a limited number has been assigned a biological function. Some transporters might even transport several substrates. One example is the previously characterized NRT1.1 nitrate transporter, which also can transport nitrate at low nitrate concentrations46. Although we cannot categorically exclude that WAT1 might also be multifunctional, our results clearly demonstrate its capacity to facilitate auxin transport, and the fact that the wat1 phenotype can be rescued by applying nitrate to other members of the WAT1 family.

**Methods**

**Plant growth conditions and β-glucuronidase assays.** A. thaliana plants (Col-0) were used. The mutant lines wat1-1 and wat1-3 were isolated from a transfer DNA mutagenized population as described45. For in vitro experiments, seeds were surface-sterilized and sown on agar-solidified MS medium including sucrose (10 g l⁻¹) and MES buffer (0.5 g l⁻¹) pH 5.7 in culture rooms with a 8-h photoperiod (120 μmol photons per m s⁻¹) at 22°C. Plants were also routinely grown in Jiffy peat pellets (9 h light, 200 μmol photons per m s⁻¹, 22°C, 65% relative humidity).

**GUS assays on proWAT1::GUS lines.** Tissues were incubated in 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, Euromedex, Souffelweyersheim, France), 0.1% Triton X-100, 50 mM sodium phosphate buffer (pH 7.0) and 1 mM each of potassium ferricyanide and potassium ferrocyanide. After the reaction, the GUS staining was fixed for 1 h in 3% paraformaldehyde, 0.5% glutaraldehyde and 50 mM sodium phosphate buffer (pH 7.0).

**Microarray analysis.** Microarray analysis was performed with a 22-k CATMA Arabidopsis array. Two independent biological comparisons were carried out. RNA integrity check, cDNA synthesis, hybridization and array scanning were done as described46. Statistical analysis and identification of differentially expressed genes were done as described45. Expression data from this article were deposited according to MIAME (Minimum Information About a Microarray Experiment) standards in the GEO (Gene Expression Omnibus) database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE10716 and at CATdb (Complete Arabidopsis Transcriptome database, http://www.urgv.verty.inra.fr/CATdb/) under the project name ‘RS06-04_Noduline-like’. Coexpression analysis was performed using the Arabidopsis Coexpression Data Mining Tools from the University of Leeds45.

**WAT1 cDNA cloning for expression in yeast.** WAT1 cDNA amplified from 10-day-old in vitro Arabidopsis Col-0 plants using N21-PstI/S (5′-AAAACTGCAATGCGGGATAAACCCTACT-3′) and N21-Sall/AS (5′-ACGCTGGTACTCAACATTTGCCTGGTACT-3′) primers was cloned into the pDR196 yeast expression vector. For subcellular localization, the GFP sequence was fused in carboxy terminal to WAT1 before cloning into pDR196 as well.
**Localization of WAT1:GFP and BFA treatments.** For co-localization, F1 seeds obtained from crossovers between p3SS::WAT1:GFP, a line that fully restores the wild-type phenotype\(^29\), and plants expressing either YFP-VAM711, YFP-RabG3f or YFP-NPSN12 (ref. 29) were surface sterilized, stratified for 24 h and grown for 4 days under long-day conditions on half-strength MS plates (5% sucrose, 0.8% agar, pH 5.8). Before observation, seedlings were mounted in liquid half-strength medium (without sugar). Observation was carried out using a Leica DMIRE2 laser scanning confocal microscope. GFP and YFP were, respectively, excited with the 488-nm (GFP) and 514-nm (YFP) line of an Argon laser, and emission was detected between 500 and 530 nm for GFP and between 550 and 600 nm for YFP. For co-localization, the ‘Colocalisation’ plugin for ImageJ was used with default settings.

Four-day-old p3SS::WAT1:GFP seedlings grown on half-strength MS were transferred to liquid medium containing dimethylsulfoxide (DMSO) as control or 50 μM BFA (Sigma-Aldrich). Seedlings were incubated in the dark for 3 h and transferred to fresh liquid medium containing 5 μM of the lipophilic dye FM4-64 (Invitrogen/Molecular Probes) for 5–15 min. Seedlings were rinsed once in MS medium and once in water before mounting them in water on microscope slides. For observation, a Zeiss Observer.Z1 LSM780 confocal microscope was used. GFP and FM4-64 were excited with a 488-nm Argon laser and emission was detected between 490 and 569 nm for GFP and between 668 and 757 nm for FM4-64.

**Local auxin applications.** Anatolian wax (Sigma) was melted at 50°C for 4 h and 50 μM IAA was added to a final concentration of 1 mM from 100 mM stocks in DMSO. For control treatments, lentil was mixed with DMSO (1% final concentration). With the help of sterile wooden toothpicks, rings of wax were then applied onto the second internodes of 5-week-old plants. Plants were grown in long day, 16 h of light and 8 h of dark. Ten days after application internodes were collected, they were hand-sectioned at the site of application and stained in phloroglucinol (in 20% HCl) for 2 min. A Zeiss Axioplan 2 imaging system with a differential interference contrast (DIC) set-up was used for observation and capture of pictures.

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