A role for the auxin precursor anthranilic acid in root gravitropism via regulation of PIN-FORMED protein polarity and relocalisation in Arabidopsis

Siamsa M. Doyle1*, Adeline Rigal1*, Peter Grones1†, Michal Karady1,2†, Deepak K. Barange1,3†, Mateusz Majda1, Barbora Parízková2,4, Michael Karampelas5,6, Marta Zwiewka7, Aleš Pencík1,4, Fredrik Almqvist2, Karin Ljung1†, Ondřej Novák1,4† and Stéphanie Robert1†

1Umeå Plant Science Centre (UPSC), Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences (SLU), 90183 Umeå, Sweden; 2Department of Chemical Biology and Genetics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, 783 71 Olomouc, Czech Republic; 3Department of Chemistry, Umeå University, 907 36 Umeå, Sweden; 4Laboratory of Growth Regulators, Institute of Experimental Botany at The Czech Academy of Sciences and Faculty of Science at Palacký University, 78371 Olomouc, Czech Republic; 5Department of Plant Systems Biology, Vlaams Instituut voor Biotechnologie (VIB), 9052 Ghent, Belgium; 6Department of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium; 7Central European Institute of Technology (CEITEC), Masaryk University, 62500 Brno, Czech Republic

Author for correspondence:
Stéphanie Robert
Tel: +46 90 786 8609
Email: stephanie.robert@slu.se

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Summary

• distribution of auxin within plant tissues is of great importance for developmental plasticity, including root gravitropic growth. Auxin flow is directed by the subcellular polar distribution and dynamic relocalisation of auxin transporters such as the PIN-FORMED (PIN) efflux carriers, which can be influenced by the main natural plant auxin indole-3-acetic acid (IAA). Anthranilic acid (AA) is an important early precursor of IAA and previously published studies with AA analogues have suggested that AA may also regulate PIN localisation.

• Using Arabidopsis thaliana as a model species, we studied an AA-deficient mutant displaying agravitropic root growth, treated seedlings with AA and AA analogues and transformed lines to over-produce AA while inhibiting its conversion to downstream IAA precursors.

• We showed that AA rescues root gravitropic growth in the AA-deficient mutant at concentrations that do not rescue IAA levels. Overproduction of AA affects root gravitropism without affecting IAA levels. Treatments with, or deficiency in, AA result in defects in PIN polarity and gravistimulus-induced PIN relocalisation in root cells.

• Our results revealed a previously unknown role for AA in the regulation of PIN subcellular localisation and dynamics involved in root gravitropism, which is independent of its better known role in IAA biosynthesis.

Introduction

Auxin distribution in controlled concentration gradients within certain tissues plays an important role in regulating the dynamically plastic growth and development of plants (Vanneste & Friml, 2009). An intense research effort has revealed many of the complex mechanisms by which plasma membrane-localised auxin carrier proteins are polarly distributed to direct the flow of auxin in plant tissues and maintain these gradients (reviewed by Luschnig & Vett, 2014; and Naramoto, 2017). These proteins, including the well studied PIN-FORMED (PIN) auxin efflux carriers, are remarkably dynamic in that they rapidly relocalise within the cell in response to signals, resulting in changes in their polarity. This dynamic responsiveness, which is facilitated using vesicular cycling and complex endomembrane trafficking pathways, is essential for altering the direction and strength of cell-to-cell auxin flow and redistributing auxin in response to external cues, therefore regulating cell and tissue growth and plasticity.

Root development in Arabidopsis thaliana has received particular attention as a model system, demonstrating the importance of auxin gradients for plant development (Clark et al., 2014). Mutations affecting auxin transporters often disturb root gravitropism and specific PIN proteins within the root tip have been shown to relocalise in response to changes in the gravity vector, leading to altered auxin flow and consequently, organ growth adjustment (reviewed by Geisler et al., 2014). In the root columella, the cellular relocalisation of PIN3 and PIN7 plays an important role in root gravitropic growth responses. While these proteins are generally apolar in columella cells, they redistribute toward the downward-facing plasma membranes upon horizontal reorientation of the root (Friml et al., 2002b; Kleine-Vehn et al., 2010), which is presumed to redirect the flow of auxin within the columella, therefore contributing to auxin accumulation at the lower root side. PIN2 is also involved in the root gravitropic response.
Displaying shootward (apical) polarity within root epidermal cells (Müller et al., 1998), PIN2 transports auxin upward, balancing the correct auxin maximum required in the root apical meristem for root development (Adamowski & Friml, 2015). However, in the case of a horizontal reorientation of the root, PIN2 is rapidly redistributed from the plasma membranes to the vacuoles within epidermal cells at the upper organ side (Abas et al., 2006; Kleine-Vehn et al., 2008). This results in the accumulation of auxin and consequent inhibition of cell elongation at the lower root side, contributing toward the root tip bending downward.

In our previous work, we employed a chemical biology approach in which we isolated and characterised small synthetic molecules selectively altering the polarity of specific PIN proteins, to dissect the trafficking pathways involved in regulating their localisation (Doyle et al., 2015a). This approach led us to identify a potential role for the endogenous compound anthranilic acid (AA) in PIN polarity regulation, which we investigated in the current study. AA is an important early precursor of the main natural plant auxin indole-3-acetic acid (IAA) (Maeda & Dudareva, 2012) and as auxin itself has been shown to regulate PIN polarity in a feedback mechanism to control its own flow (Paciorek et al., 2005), we hypothesised that AA may play a similar regulatory role. Here, using Arabidopsis root gravitropism as a model system for auxin-regulated plastic growth, we provide strong evidence in favour of this hypothesis. Ultimately, we revealed a previously unknown role for AA in the regulation of PIN polarity and relocalisation required for root gravitropic responses and furthermore, we show that this role of AA is distinct from its well known role in IAA biosynthesis.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana was grown vertically on half-strength Murashige and Skoog (½MS) medium at pH 5.6 with 1% sucrose, 0.05% 2-(N-morpholino)ethanesulfonic acid (MES) and 0.7% plant agar for 5 or 9 d at 22°C, on a 16 h : 8 h, light : dark photoperiod. The Columbus-0 (Col-0) accession was used as wild-type (WT). See Supporting Information Table S1 for the previously published Arabidopsis lines used and Table S2 for the genotyping primers used. All mutants/marker lines on the wei2wei7 background were generated in this study by crossing (Methods S1). For generation of 35S::ASA1 (35S::WEI2) and XVE::amiRNA-PAT1 lines and root growth measurements, see Methods S1.

Chemical treatments and IAA metabolite analysis

Stock solutions of Endosidin 8 (ES8) (ID 6444878; ChemBridge, San Diego, CA, USA), AA (Sigma-Aldrich, St. Louis, MO, USA), ES8.7 (ID 6437223; ChemBridge) and ES8.7-Trp (Methods S2) were made in dimethyl sulfoxide (DMSO) and diluted in liquid medium for short-term (2 h) treatments or growth medium for long-term (5 or 9 d) treatments, in which case seeds were directly sown on chemical-supplemented medium. Equal volumes of solvent were used as mock treatments for controls. For quantification of endogenous IAA and its metabolites, 20–30 whole seedlings per sample were flash frozen in liquid nitrogen and c. 20 mg of ground tissue was collected per sample. Extraction and analysis were performed according to Novák et al. (2012) (Methods S2). See Methods S3 for compound degradation analysis.

qPCR, immunolocalisation and confocal microscopy

Quantitative real-time PCR (qPCR) was performed as described previously (Doyle et al., 2015a) (Methods S4). See Table S2 for the qPCR primers used. For β-glucuronidase (GUS) staining, see Methods S4. Immunolocalisation was performed as described previously, using an InsituPro Vsi (Intavis Bioanalytical Instruments AG, Köln, Germany) (Doyle et al., 2015a). Primary antibodies used were anti-PIN1 at 1 : 500 (Nottingham Arabidopsis Stock Centre; NASC), anti-PIN3 at 1 : 150 (NASC), anti-PIN4 at 1 : 400 (NASC) and anti-PIN7 at 1 : 600 (Methods S4). Secondary antibodies used were Cy3-conjugated anti-rabbit and anti-sheep at 1 : 400 and 1 : 250, respectively (Jackson Immunoresearch, Cambridgeshire, UK). Confocal laser scanning microscopy was performed using a Zeiss (Oberkochen, Germany) LSM 780 confocal microscope (see Methods S5 for microscopy image quantifications).

Statistical analyses

For all experiments, at least three biological replicates were performed and always on different days. Occasionally, extra biological replicates were performed, due to poor growth of wei2wei7 that sometimes resulted in a low number of seedlings or quantifiable roots in certain replicates. Unless indicated otherwise, Wilcoxon rank sum test (Mann–Whitney U-test) or Student’s t-test were performed on full, raw datasets of nonparametric or parametric data, respectively, to determine statistically significant differences and the means of the biological replicates are displayed on charts.

Results

AA rescues root gravitropic growth and length differently in an AA-deficient mutant

Using a chemical biology approach, we previously isolated the small synthetic molecule Endosidin 8 (ES8), which disturbs the polarity of selective PIN proteins in Arabidopsis roots, leading to altered auxin direction patterns and defective root growth (Doyle et al., 2015a). Intriguingly, the chemical structure of ES8 revealed that this molecule is an analogue of the endogenous plant compound AA (Fig. 1a), a precursor of tryptophan (Trp), the main precursor of the predominant plant auxin IAA (Ljung, 2013; Zhao, 2014). This prompted us to question whether endogenous AA might play a role in growth and development of the root. We therefore investigated a loss-of-function Arabidopsis mutant in both ANTHRANILATE SYNTHASE SUBUNIT ALPHA1 (ASA1, also known as WEAK ETHYLENE INSENSITIVE2, WEI2) and ANTHRANILATE SYNTHASE

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SUBUNIT BETA1 (ASB1, also known as WEI7). In the double mutant, we2wei7 (Stepanova et al., 2005; Ikeda et al., 2009), the AA level is presumed to be reduced. To confirm this finding, we analysed the levels of several IAA precursors/catabolites, revealing that AA content was indeed significantly reduced in we2wei7 compared with the WT Columbia-0 (Col-0), as were the levels of the IAA precursors Trp, indole-3-acetonitrile (IAN) and indole-3-acetimide (IAM) and IAA itself (Fig. S1), most likely to be due to the decreased AA content. However, neither the IAA precursor tryptamine (Tra) nor catabolite 2-oxoindole-3-acetic acid (oxIAA) showed altered content in the mutant compared with the WT (Fig. S1).

We were interested in the strong agravitropic and short phenotypes of we2wei7 roots compared with Col-0 seedlings (Fig. 1b, c), considering that ES8 treatment reduced both gravitropic root growth and root length in Col-0 (Doyle et al., 2015a). To investigate AA-mediated rescue of these root phenotypes in we2wei7, we performed long-term AA treatments by growing WT and mutant seedlings on medium supplemented with a range of AA concentrations. In Col-0, none of the tested concentrations affected root gravitropic growth, while concentrations of 10 μM or more decreased root length in a dose-dependent manner (Fig. S2a), possibly due to increased IAA biosynthesis. As expected, in we2wei7 both root gravitropic growth and length...
were rescued by AA (Fig. 1c,d), however we observed a striking difference between the AA rescue patterns of these two root phenotypes. While root gravitropic growth in the mutant was almost fully rescued to the WT level at all AA concentrations applied, root length rescue was only partial and was concentration-dependent, with maximal rescue at 5 μM (Fig. 1d). We hypothesised that these different rescue patterns in wei2wei7 might reflect two different roles of AA, one known role in auxin biosynthesis and a distinct, as yet unknown, role in regulating auxin distribution, considering that ES8 disturbs auxin distribution patterns in the root (Doyle et al., 2015a).

We next investigated whether ES8, as an analogue of AA, could rescue either root gravitropic growth or length in wei2wei7. While long-term treatments with ES8 decreased both root gravitropic growth and length in a dose-dependent manner in Col-0 (Fig. S2b), only the highest ES8 concentrations (15 and 20 μM) decreased root gravitropic and length in wei2wei7 (Fig. 1e). Moreover, while root length was not rescued in the mutant at any ES8 concentration, 5 μM ES8 partially rescued the root gravitropic phenotype of the mutant (Fig. 1c,e). The partial root gravitropic rescue of wei2wei7 by ES8 without any effect on root length suggested, considering that ES8 is known to affect auxin distribution in the root (Doyle et al., 2015a), that the root gravitropic rescue of wei2wei7 by AA may occur via a previously unknown role of AA in auxin distribution.

To further test our hypothesis, we used another analogue of both ES8 and AA – ES8 analogue no. 7 (ES8.7; Fig. S3a) and its analogue ES8.7-Trp – in which AA was exchanged for a Trp (Fig. S3b). In Col-0, long-term ES8.7 treatment revealed a similar but weaker effect than ES8 on dose-dependent reduction of root gravitropic growth and length (Fig. S3c). ES8.7 rescued root gravitropic growth in wei2wei7 at a range of concentrations from 1 to 15 μM, with almost no effects on root length (Fig. S3a,d). Moreover, ES8.7-Trp did not rescue root gravitropic growth or length at any concentration in either Col-0 (Fig. S3e) or wei2wei7 (Fig. S3b,f), strongly suggesting that it is the AA part of ES8 and ES8.7 that rescues gravitropic growth of wei2wei7 roots. Together, these results suggested that a potential role for AA in auxin distribution may regulate root gravitropic growth, while the well known role of AA in auxin biosynthesis may be more important for root length regulation.

To investigate any possible degradation or metabolism of the ES8 compounds to release AA or Trp, we performed both short-term and long-term treatments of Col-0 and wei2wei7 seedlings with the ES8 compounds, followed by compound analysis (Fig. S4). We measured the concentrations of the relevant ES8 compound, AA or Trp and the non-AA or non-Trp part of the ES8 compound in planta as well as in ES8 compound-supplemented treatment medium to which no seedlings were added. After short-term treatment (5 h incubation in liquid treatment medium) with 5 μM ES8, high levels of ES8 were detectable in the seedlings and the seedling-free treatment medium remained at c. 5 μM ES8 (Fig. S4a). Importantly, these findings confirmed that ES8 readily enters plant tissues during short-term treatment. After long-term treatment (9 d growth on solid treatment medium), the concentrations of ES8 in the seedlings and the seedling-free treatment medium had lowered considerably, suggesting degradation of ES8 over time, and/or slower uptake from solid than liquid treatment medium. Compared with ES8, much lower levels of ES8.7 and ES8.7-Trp were present in the seedlings after short-term treatment (Fig. S4b,c), suggesting that ES8 may be more efficiently taken up into seedling tissues or ES8.7 and ES8.7-Trp may be degraded or metabolised during the short-term treatment. Degradation of ES8.7-Trp was supported by our measurements of its concentration in the seedling-free treatment medium, which had already lowered to 3.3 μM after short-term incubation and to 0.5 μM after long-term incubation (Fig. S4c).

Moreover, the levels of ES8.7-Trp were considerably lower in the seedlings after long-term compared with short-term treatment. While these results suggested that ES8 and ES8.7-Trp are likely to be degraded over time, the levels of AA and Trp in the seedlings after ES8 compound treatment were not different to the levels after mock treatment and neither AA nor Trp were detected in the treatment medium samples (Fig. S4d,e). Furthermore, we did not detect non-AA or non-Trp parts of the ES8 compounds at any time point, neither in the seedlings nor in the seedling-free treatment medium (Fig. S4f). Therefore, the observed activities of the ES8 compounds were not due to the release of AA or Trp leading to increased IAA biosynthesis.

AA and ES8 can rescue root gravitropic growth in wei2wei7 without rescuing IAA level

As AA is a precursor of IAA, we investigated the possibility that the rescue of root gravitropic growth by ES8 and AA might indirectly result from increased IAA biosynthesis. First, we measured IAA concentrations after long-term treatments with AA. In Col-0, only 10 μM AA significantly increased the IAA level (Fig. 2a), which is likely to have explained why treatments of Col-0 with 10 μM and higher AA resulted in significantly shorter roots (Fig. S2a). While treatment of wei2wei7 with 1 or 10 μM AA rescued the IAA level to that of mock-treated Col-0, treatment with 0.5 μM AA had no effect on IAA content (Fig. 2a), despite this concentration having almost fully rescued root gravitropic growth and partially rescued root length in wei2wei7 seedlings (Fig. 1d). Next, we measured IAA content in seedlings treated long-term with 5 μM ES8, ES8.7 or ES8.7-Trp. While the IAA level was slightly but significantly reduced in mock-treated wei2wei7 compared with Col-0, none of the ES8 compounds significantly affected IAA content compared with mock treatment in either genotype (Fig. 2b). As our IAA analysis was performed on whole seedlings, we cannot rule out small, local changes in IAA levels in specific regions of the root. However, taken together, our results suggested that ES8 and ES8.7 rescue wei2wei7 root gravitropic growth without affecting general IAA content, therefore supporting the hypothesis that AA plays a role in the regulation of root gravitropic growth independently from its function in IAA biosynthesis and potentially via a previously unknown role in auxin distribution.
transformations, which we named AxP expression in the progeny that were homozygous for both PAT1 (3B6 × 2D4 line no. 4) in which ASA1 was five-fold overexpressed compared with the nontreated WT without affecting noninduced PAT1 expression and AxP2 (3B7 × 2D4 line no. 21), in which ASA1 was 10-fold overexpressed, resulting in three-fold overexpression of PAT1 in noninduced conditions (Fig. S5c, d). Additionally, an estradiol-inducible five- and three-fold reduction in PAT1 expression compared with nontreated Col-0 was shown for AxP1 and AxP2, respectively (Fig. S5d).

We analysed the levels of IAA and several IAA precursors/conjugates/catabolites in WT and AxP lines that had been treated in the long-term with oestradiol (grown on supplemented medium) (Fig. S6a). Importantly, AA levels were significantly higher in both AxP lines compared with the WT, while the IAA content was not affected. The levels of Trp, IAN, IAM and oxIAA were also not significantly affected in the lines, while the levels of the IAA conjugates IAA-aspartate (IAAsp) and IAA-glutathione (IAGluz) showed rather variable results (Fig. S6a). These analyses suggested that simultaneous overexpression of ASA1 and silencing of PAT1 resulted in significantly increased AA levels, but did not alter IAA levels.

We next investigated the root phenotypes of the AxP lines. Under control conditions, both lines displayed similar root gravitropic growth to, but slightly shorter roots than, the WT (Fig. S6b,c). After long-term oestradiol treatment, the gravitropic growth of WT and AxP roots was slightly reduced, to a similar extent (Fig. S6b), while the root length of all genotypes was reduced, but more severely in AxP lines than the WT (Fig. S6c). To analyse root gravitropic responses in the AxP lines, we turned the seedlings 90° and subsequently measured the gravistimulated root bending angles (Fig. S6d). We divided the total number of roots, by percentage, into several categories of bending angles (Fig. 3). Under control conditions, Col-0 and both AxP lines responded to the gravistimulus with a very similar range of root bending angles, with most roots bending 75–105° (Fig. 3). Estradiol treatment inhibited the gravitropic response of Col-0 roots, reducing their bending angles, resulting in a significant reduction in the proportion of total roots bending 75–105° and a significant increase in the proportion bending <75° (Fig. 3a,b). The AxP lines, however, responded differently to oestradiol than the WT. As for the WT, oestradiol treatment resulted in both a significant reduction in the proportion of AxP roots bending 75–105° and, in the case of AxP1, a significant increase in the proportion bending <75°, but additionally resulted in a significant increase in the proportion bending >105° in both AxP lines (Fig. 3c–f). Therefore, while estradiol treatment specifically reduces root bending in the WT, this treatment resulted in both under- and over-bending in AxP1 roots and over-bending in AxP2 roots, in response to a gravistimulus. This suggests that increased AA levels in these lines interferes with root gravitropic responses, although we cannot rule out potential effects of changes in other IAA metabolite levels in root gravitropic responses.
PIN polarity in the stele is altered in wei2wei7 and partially rescued by ES8

ES8 has been shown to disturb auxin distribution patterns in the root by altering PIN polarity (Doyle et al., 2015a). Considering that IAA itself can influence its own transport by regulating PIN abundance at the plasma membrane (Paciorek et al., 2005; Robert et al., 2010), we reasoned that AA, as a precursor of IAA, might also play such a role. To investigate this possibility, we first studied the effects of long-term ES8 and AA treatments on the expression pattern of the auxin-responsive promoter DR5 in the root. To observe the effects of ES8 more easily, we used treatment at a high concentration of 15 μM, which led to a strong decrease in green fluorescent protein (GFP) signal in the stele of DR5::GFP WT roots (Fig. S7a), in agreement with previously published work (Doyle et al., 2015a). Furthermore, DR5::GFP crossed into the wei2wei7 background showed a similarly low GFP signal in the stele in control conditions, which was reduced...
even further using 15 μM ES8 treatment (Fig. S7a). While 10 μM AA treatment did not noticeably affect the GFP signal in the stele of the WT, the signal in the wei2wei7 stelle was rescued using this treatment (Fig. S7a). These results suggested that AA may play a role in auxin distribution in the stele.

Next, we focused on the GFP signal in the root tip, particularly around the quiescent centre (QC) and in the columella (Fig. S7b). We used both 5 and 15 μM ES8 treatment concentrations, which, in agreement with previously published work (Doyle et al., 2015a), led to an accumulation of GFP signal in cell file initials surrounding the QC in DR5::GFP WT, which were not labelled under control conditions (Fig. S7b). This accumulation of signal was rather striking, extending into lateral columella and root cap cells, at the higher ES8 treatment concentration of 15 μM. As found for the stelle, DR5::GFP crossed into the wei2wei7 background showed a similar GFP signal pattern in the root tip in control conditions as that induced by ES8 in the WT, with an accumulation of signal in the file initials surrounding the QC (Fig. S7b). This signal pattern was also apparent in the wei2wei7 background after 5 μM ES8 treatment and was enhanced after 15 μM ES8 treatment. While 0.5 μM and 10 μM AA treatment did not noticeably affect the GFP signal in the root tip of Col-0, the signal in the wei2wei7 root tip was slightly increased using 0.5 μM AA treatment and rescued to that of the control WT using 10 μM AA treatment (Fig. S7b). Therefore, we observed a negative correlation between the DR5::GFP signal strength in the root stele and tip. We suggested that a balanced AA level is important for proper auxin distribution in both the root stele and tip, as both addition of exogenous AA/ES8 and deficiency in endogenous AA levels disturbed DR5::GFP signal patterns. Together, these results indicated that AA may indeed play a role in auxin distribution in the root stele and tip, which is likely to affect gravitropic growth. However, it is important to note that we cannot rule out the possible effects of AA/ES8 on local auxin biosynthesis within specific groups of cells.

Our observations of the DR5::GFP signal in the stele prompted us to investigate the rootward-to-lateral plasma membrane fluorescence ratio (hereafter referred to as rootward polarity) of PIN1, PIN3 and PIN7 in the provascular cells of Col-0 and wei2wei7 root tips. We treated seedlings short term (2 h) with 15 μM ES8 or 10 μM AA, performed immunolabelling to observe endogenous PIN1 and PIN7 and used the PIN3::PIN3–GFP line crossed into the wei2wei7 background due to poor labelling of antibodies against PIN3. The fluorescence signals for these PIN proteins were consistently weaker in the mutant than in the WT (Fig. 4a–c), suggesting decreased abundance at the plasma membranes. As previously reported by Doyle et al. (2015a), short-term ES8 treatment significantly, albeit slightly, reduced immunolocalised PIN1 rootward polarity in Col-0 and importantly, AA treatment produced a similar result (Fig. 4d). By contrast, PIN1 rootward polarity was significantly increased by c. 20% in untreated wei2wei7 compared with Col-0, while ES8 treatment appeared to rescue this hyperpolarity of PIN1 in the mutant back to almost that of the WT (Fig. 4d). Although PIN3–GFP rootward polarity was not affected by ES8 or AA treatments in either the Col-0 or wei2wei7 backgrounds, it was increased by over 20% in the mutant compared with the WT (Fig. 4e). Finally, although PIN7 rootward polarity was not affected by ES8 or AA treatment in Col-0, it was strongly increased in the mutant compared with the WT and, like PIN1, was rescued in the mutant back to the level of the WT by ES8 treatment (Fig. 4f). These results suggested that AA may play a role in maintenance of PIN polarity in root provascular cells. One possible speculation on why treatment with AA, by contrast with ES8, did not rescue PIN1 or PIN7 polarity in the mutant may be a rapid conversion of AA to downstream IAA precursors within the seedlings.

As AA is a precursor of auxin, which is known to affect transcription of PIN genes (Vieten et al., 2005; Paponov et al., 2008), we investigated gene expression levels for all the plasma membrane-localised PIN proteins (PIN1, PIN2, PIN3, PIN4 and PIN7) in WT and mutant seedlings at 9 d old, the age at which we performed our root gravitropic growth and length studies. The expression levels of PIN1, PIN2 and PIN4 were strongly decreased in wei2wei7 compared with Col-0, while PIN3 and PIN7 expression levels were somewhat decreased, but not significantly (Fig. S8a). We next investigated the expression levels of PIN1, PIN3 and PIN7 under the same conditions used for our PIN polarity studies in root provascular cells (5-d-old seedlings treated with ES8 and AA for 2 h). At this stage, expression levels of PIN1, PIN3 and PIN7 were somewhat decreased in the mutant compared with the WT, but not significantly (Fig. S8b). Furthermore, treatment with ES8 and AA did not significantly affect the expression of these genes (Fig. S8b). These results implied that while transcription of PIN genes is decreased in wei2wei7, the effects of ES8 and AA on PIN polarity are not due to PIN gene transcriptional changes. Overall, our data suggest that endogenous AA may play a role in regulating the polarity of PIN1, PIN3 and PIN7 in root provascular cells through a mechanism unrelated to PIN gene expression levels. We previously determined that ES8 targets a secretory pathway delivering newly produced PIN1 toward the rootward plasma membranes of root provascular cells (Doyle et al., 2015a) and it is tempting to speculate that AA might play regulatory roles in similar PIN trafficking routes to guide auxin distribution in the root.

AA regulates root gravitropism via repolarisation of PIN3 and PIN7 in the columella

Our observations of the DR5::GFP signal in the columella (Fig. S7b) indicated that AA may also play a role in auxin distribution specifically in this particular root tissue. Additionally, previous studies of the expression patterns of AS1 (WEI2) and ASB1 (WEI7) promoter—GUS fusions in dark-grown Arabidopsis roots revealed strong expression in the root meristem and columella (Stepanova et al., 2005). Plasma membrane-localised PIN3 and PIN7 in the columella are thought to act in the redistribution of auxin in response to gravistimulus (Friml et al., 2002b; Kleine-Vehn et al., 2010), potentially redundantly with PIN4, which was also localised in columella cells (Friml et al., 2002a; Vieten et al., 2005). We therefore reasoned that high
PIN4 and PIN7 in the columella of Col-0 and cells (Fig. S9c).

ASB1::GUS expression was limited to the innermost columella tip (Fig. S9a,b). We observed strong inhibition was detected in the lower part of the root excluding the root wei2wei7 mutant. The antibodies against these PIN proteins did not label the outermost columella cells, in agreement with previous studies using PIN3 and PIN4 antibodies (Friml et al., 2002a,b). We therefore continued our studies using PIN3::PIN3–GFP and PIN7::PIN7–GFP lines crossed into the wei2wei7 background (Fig. 5a,b). We performed long-term treatments of these lines with ES8 and AA and investigated the shootward-plus-rootward to lateral-plus-lateral fluorescence ratio (hereafter referred to as shootward-rootward polarity) of the GFP-labelled PIN proteins. While the shootward-rootward polarity of PIN3–GFP was similar in wei2wei7 and Col-0 backgrounds regardless of compound treatment (Fig. 5c), PIN7–GFP was over 20% more polarly shootward-rootward localised in the mutant than in the WT (Fig. 5d). Moreover, 10 µM AA treatment partially rescued PIN7–GFP polarity in the mutant toward the WT level (Fig. 5d).

Next, we investigated the localisation of endogenous PIN3, PIN4 and PIN7 in the columella of Col-0 and wei2wei7. Interestingly, the fluorescence intensity of these proteins was consistently increased in the innermost cells of the columella in wei2wei7 compared with Col-0 (Fig. S9d–f), suggesting that the abundance and/or localisation of these proteins are altered in the mutant columella. The antibodies against these PIN proteins did not label the outermost columella cells, in agreement with expression of anthranilate synthase genes in the columella may reflect a role of AA in regulating gravity-responsive polarity of these PIN proteins. First, to investigate the expression patterns of the ASA1 and ASB1 promoters in light-grown roots, we performed GUS staining of ASA1::GUS (WEI2::GUS) and ASB1::GUS (WEI7::GUS) seedlings. We observed strong expression of the ASB1 promoter, but not the ASA1 promoter, in the stele of the upper root, while neither ABA1 nor ASB1 promoter expression was detected in the lower part of the root excluding the root tip (Fig. S9a,b). We observed strong ASA1 and ASB1 promoter expression in the tip of the root meristem and in the columella, with ASA1::GUS expressed throughout the columella, while ASB1::GUS expression was limited to the innermost columella cells (Fig. S9c).

Next, we investigated gravity-induced relocalisation of PIN3–GFP and PIN7–GFP in the columella. After a 90° gravistimulus for 30 min, c. 15% more PIN3–GFP and PIN7–GFP were present on the now downward-facing (formerly lateral-facing) plasma membranes of the columella cells in WT seedlings (Figs 5e,f, S10a,c). Long-term treatment of the WT with 5 µM ES8 or 10 µM AA strongly reduced PIN3–GFP relocalisation to only c. 5–10% (Figs 5e, S10a). Strikingly, gravistimulus-induced...
The almost total absence of gravistimulus-induced PIN3- and PIN7 relocalisation in the WT and 5 μM AA (Figs 5e, S10b) appears to correlate with the partial rescue of root gravitropic growth by the same treatment (Fig. 1e). These results implied that endogenous AA may play a role in regulating relocalisation of PIN3 and PIN7 proteins in the columella in response to gravity.

To further investigate a potential role for PIN proteins in AA-regulated root gravitropism, we analysed root gravitropic growth in a range of pin mutants and their crosses with wei2wei7. Interestingly, while the ethylene insensitive root1-4 (ein1-4, a pin2 allele) mutant showed intermediate root gravitropic growth...
between wei2wei7 and Col-0, crossing these mutants caused an additive effect, with wei2wei7/eir1-4 being more severely agravitropic than wei2wei7 (Fig. S11a). Of the tested pin3, pin4 and pin7 alleles, none of the single mutants was affected in root gravitropic growth compared with the WT and introduction of the pin3-4 or pin7-2 mutations into the wei2wei7 background did not affect the gravitropic growth. Interestingly, by contrast with the eir1-4 mutation, introduction of the pin3-5 or pin4-3 mutations into wei2wei7 partially rescued the root gravitropic growth compared with wei2wei7 (Fig. S11a). While pin1-501 showed increased root gravitropic growth compared with the WT, this mutation also partially rescued the root gravitropic growth of wei2wei7 (Fig. S11a).

We next tested the effects of long-term treatments with high concentrations of AA on root gravitropic growth in the mutants. Similarly to the WT, none of the pin mutants tested showed any sensitivity to AA in terms of changes in root gravitropic growth (Fig. S11b). While the introduction of pin3-4 or pin7-2 to wei2wei7 did not alter its sensitivity to AA in terms of increase in gravitropic index, crossing eir1-4 into wei2wei7 significantly increased its sensitivity to AA (Fig. S11b). By contrast, the introduction of pin3-5, pin4-3 or pin1-501 to wei2wei7 reduced its sensitivity to AA, resulting in decreased rescue of root gravitropic growth (Fig. S11b).

The recovery of, as well as the reduction in AA-induced rescue of, wei2wei7 root gravitropic growth by introducing pin1, pin3 or pin4 mutations, provided further evidence for the involvement of PIN1 and PIN3, as well as suggesting the involvement of PIN4, in AA-regulated root gravitropism. It is unclear why pin3-5, but not pin3-4, partially rescues wei2wei7 gravitropism and suppresses the rescue of wei2wei7 gravitropism by AA, but these different effects may be due to potential secondary mutations in one or both mutants, and/or the different positions of the T-DNA insertions. These insertions occurred in an exon in pin3-4 and in the untranslated region (UTR) preceding the start codon in pin3-5. The well known important role of PIN2 in root gravitropism (Abas et al., 2006; Kleine-Vehn et al., 2008), however, is most likely to be not related to AA-regulated root gravitropism, considering the strong additive effect of eir1-4 and wei2wei7 mutations in reducing root gravitropic growth and increasing sensitivity to AA.

Taken together, our results strongly supported a new role for endogenous AA in root gravitropism via regulation of selective PIN protein polarity and dynamics and therefore auxin distribution in both the stele and columella and that this role of AA is independent of its well known function in IAA biosynthesis.

**Discussion**

We provided evidence in favour of a role for AA in root gravitropic growth through regulation of the subcellular localisation of auxin transporter proteins, which is likely to have influenced the flow of auxin within the organ. Following their synthesis, most plasma membrane-targeted proteins are sorted and packaged into selective secretory trafficking routes (Gendre et al., 2014). It has been shown, for instance, that the auxin importer AUXIN-RESISTANT1 (AUX1) and exporter PIN1, when targeted to shootward or rootward plasma membranes of root tip cells, respectively, are transported in distinct endosomes, subject to the control by different regulatory proteins (Kleine-Vehn et al., 2006). The trafficking routes of such proteins may be distinct even if targeted to the same plasma membrane, as is the case, for example, for AUX1 and PIN3 in epidermal hypocotyl cells of the apical hook (Boutté et al., 2013). Such a remarkably complex system of endomembrane trafficking pathways is thought to allow for a high level of control, suggesting the likely existence of an array of selective endogenous compounds and/or signals regulating these trafficking routes.

Once polar plasma membrane-targeted auxin carriers have reached their destination, they remain remarkably dynamic, being subject to constant vesicular cycling (Geldner et al., 2001) to enable rapid retargeting in response to external stimuli (reviewed by Luschnig & Vert, 2014; and Naramoto, 2017). Auxin itself promotes its own flow by inhibiting clathrin-mediated endocytosis of PIN transporters, therefore enhancing their presence at the plasma membrane (Paciorek et al., 2005). Our results suggested that AA, an important early precursor in IAA biosynthesis, may also act on PIN plasma membrane localisation to regulate the flow of auxin, through currently unknown mechanisms.

The use of pharmacological inhibitors, identified through chemical biology approaches, has proven to be a powerful strategy that has greatly assisted in unravelling the details of auxin transporter trafficking mechanisms (reviewed by Hayashi & Overvoorde, 2013; and Doyle et al., 2015b). We previously employed such a strategy, revealing that the AA analogue ES8 selectively inhibits an early endoplasmic reticulum (ER)-to-Golgi secretory pathway, regulated by the adenosine diphosphate (ADP) ribosylation factor guanine nucleotide exchange factors (ARF-GEFs) Gnom and Gnom-Like 1 (GNL1), involved in rootward targeting of PIN1 without affecting the polarity of shootward plasma membrane proteins (Doyle et al., 2015a). We suggest that AA itself is likely to act endogenously on PIN trafficking regulation in a similar way to ES8, but detailed studies on AA mechanisms may prove difficult due to the potential conversion of AA to other IAA precursors in plant tissues. As ES8 appears to mimic the effects of AA on PIN localisation and root gravitropic growth without releasing AA through degradation and without affecting IAA levels, this synthetic compound provided great potential for understanding the mechanisms of AA on PIN localisation in more detail, having already been extremely useful for distinguishing this newly discovered role of AA from its better known role in auxin biosynthesis.

Any pharmacological treatments of biological tissues raise the question of compound uptake efficiency. Based on our analysis of the ES8 compounds inside plant tissues, we can conclude that uptake, either passive or active, of all these compounds occurs, with ES8 being taken up c. 10 times faster than ES8.7 or ES8.7-Trp during short-term treatments. Although we currently do not know how these compounds enter plant tissues, one may speculate that AA and Trp transporters are likely to exist in planta, which might also transport ES8/ES8.7 and ES8.7-Trp, respectively. It will be of great interest in future studies to investigate
the distribution and dynamics of compound uptake, which could potentially be observed by their labelling, fluorescently or otherwise.

Interestingly, the amino acid para-aminobenzoic acid, which has a similar structure to AA and is produced from the same precursor, has also recently been shown to play a role in root gravitropism, distinct from its better known role in folate biosynthesis (Nziengui et al., 2018). However, unlike AA, para-aminobenzoic acid promotes gravitropic root growth in WT plants as well as promoting gravistimulated root bending by enhancing the asymmetric auxin response between the two root sides (Nziengui et al., 2018). Another study linking AA, or more specifically Trp, with root bending, revealed that a mutation in the ASA1 gene conferred a more compressed wavy root phenotype than the WT when seedlings are grown on agar surfaces tilted 30° from the vertical (Rutherford et al., 1998). As the mutant roots respond normally to gravistimuli when grown in nonwaving conditions (within agar), it could be concluded that the root waving phenotype is not caused by agravitropism. Furthermore, the phenotype is rescued in the mutant by supplementing with AA or Trp but not IAA. Mutations in the TRYPTOPHAN SYNTHASE α and β1 genes conferred similar phenotypes, suggesting that a Trp deficiency is responsible. While these results suggested that Trp may be involved in regulating nongravitropic root waving, potentially related to thigmotropism, independently of IAA, our study did not use tilted plates and, moreover, under our conditions (vertical plates), single mutants in ASA1 and ASB1, namely wei2-2 and wei7-1, did not show differences in root growth compared with the WT (Fig. S12). We would therefore argue that the reduced gravitropic index of wei2wei7 roots is indeed due to a gravitropic defect, caused by AA deficiency. It is however very interesting that Trp, another IAA precursor, appeared to regulate root directional growth in response to another stimulus. Indeed, the existence of several complex root growth regulatory mechanisms is hardly surprising, considering the remarkable plasticity of this organ, the growth of which must respond to a wide array of internal signals and external stimuli, including gravity, touch, light, temperature, humidity and various chemical substances.

The agravitropic growth of wei2wei7 roots may be due to a combination of decreased auxin content caused by reduced AA levels and the AA deficiency itself, as both auxin and AA affected the localisation of PIN proteins. As was shown previously for ES8 (Doyle et al., 2015a), AA appears to act selectively depending on the PIN protein and the root tissue. PIN1, PIN3 and PIN7 all displayed increased rootward polarity in provascular cells of wei2wei7 compared with Col-0, suggesting increased flow of auxin toward the root tip in the mutant. Correspondingly, we found decreased expression of the auxin-responsive promoter DR5 in the root stele and increased expression around the root tip QC in the mutant, a pattern that was also observed in WT roots upon ES8 treatment. PIN7, but not PIN3, is also abnormally polarised in columella cells of wei2wei7, while both these proteins appeared to be completely unresponsive to gravstimulus in the mutant columella. Furthermore, the high expression of ASA1 (WEI2) and ASB1 (WEI7) in the root columella of the WT suggested the importance of AA in this tissue in particular, which our results suggested was due to a role for this compound in gravity-regulated PIN distribution amongst the plasma membranes. The particular importance of PIN1 and PIN3 in AA-regulated root gravitropism was further supported by the rescue, as well as the reduction in AA-induced rescue of wei2wei7 root gravitropic growth by the introduction of pin1 or pin3 mutations. Taken together, our results suggested that the endogenous compound AA played a role in root gravitropism by regulating the polarity and gravity-induced relocalisation of specific PIN proteins in the provascular and columella cells. Furthermore, this role of AA is distinct from its well known function in auxin biosynthesis, which we suggested is more important for root elongation than gravitropic growth.

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Author contributions

SMD, AR and SR designed the research; SMD, AR, PG, MKarady, DKB, MM, BP, MKampelias, MZ and AP performed the research under the supervision of FA, KL, ON and SR; SMD, AR, PG, ON and SR interpreted the data; SMD wrote the manuscript with input from AR, PG and SR; all authors gave feedback on the final manuscript version; SMD and AR contributed equally to this work; PG and MKarady contributed equally to this work.

ORCID

Deepak K. Barange https://orcid.org/0000-0003-1279-1068
Siamsa M. Doyle https://orcid.org/0000-0003-4889-3496
Peter Grones https://orcid.org/0000-0003-4132-4151
Michal Karady https://orcid.org/0000-0002-5603-706X
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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 AA and other IAA precursors are deficient in wei2wei7.

Fig. S2 Effects of AA and ES8 on root gravitropism growth and length in the wild-type.

Fig. S3 AA but not Trp analogues rescue root gravitropic growth but not length in wei2wei7.
Fig. S4 The ES8 compounds are not degraded/metabolised to release AA or Trp.

Fig. S5 Expression levels of ASA1 (WEI2) and PAT1 in AxP lines.

Fig. S6 IAA metabolite levels and root phenotypes of AxP lines.

Fig. S7 Expression pattern of the auxin-responsive promoter DR5 is altered in the root using ES8 treatment or AA deficiency.

Fig. S8 Expression levels of PIN genes in Col-0 and wei2wei7.

Fig. S9 Expression patterns of ASA1 (WEI2) and ASB1 (WEI7) in the root and immunolocalisation of PIN3, PIN4 and PIN7 in the columella.

Fig. S10 Gravitropic relocalisation of PIN3- and PIN7–GFP in root columella cells.

Fig. S11 Root gravitropic growth in pin and wei2wei7pin mutants.

Fig. S12 Root phenotypes of double and single mutants of wei2 and wei7.

Methods S1 Selection of homozygous crossed mutants, generation of 35S::ASA1 (35S::WEI2) and XVE::amiRNA-PAT1 lines and root growth measurements.

Methods S2 Chemical synthesis and IAA metabolite analysis.

Methods S3 Compound degradation analysis.

Methods S4 qPCR, GUS staining and generation of PIN7 antibody.

Methods S5 Microscopy image quantifications.

Table S1 Arabidopsis thaliana mutants and transformed lines used in this study.

Table S2 Genotyping, cloning and qPCR primers used in this study.

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