

AUX/LAX Genes Encode a Family of Auxin Influx Transporters That Perform Distinct Functions during *Arabidopsis* Development

Benjamin Péret,^{a,1} Kamal Swarup,^a Alison Ferguson,^a Malvika Seth,^a Yaodong Yang,^b Stijn Dhondt,^{c,d} Nicholas James,^a Ilda Casimiro,^e Paula Perry,^a Adnan Syed,^b Haibing Yang,^f Jesica Reemmer,^f Edward Venison,^a Caroline Howells,^a Miguel A. Perez-Amador,^{g,2} Jeonga Yun,^g Jose Alonso,^g Gerrit T.S. Beemster,^h Laurent Laplace,ⁱ Angus Murphy,^f Malcolm J. Bennett,^a Erik Nielsen,^b and Ranjan Swarup^{a,3}

^aSchool of Biosciences and Centre for Plant Integrative Biology, University of Nottingham, LE12 5RD Nottingham, United Kingdom

^bDepartment of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109

^cDepartment of Plant Systems Biology, Flanders Institute for Biotechnology, 9052 Ghent, Belgium

^dDepartment of Plant Biotechnology and Bioinformatics, Ghent University, 9052 Ghent, Belgium

^eUniversidad de Extremadura, Facultad de Ciencias, 06071 Badajoz, Spain

^fDepartment of Horticulture and Landscape Architecture, Purdue University, West Lafayette, Indiana 47906

^gDepartment of Genetics, North Carolina State University, Raleigh, North Carolina 27695

^hLaboratory for Molecular Plant Physiology and Biotechnology, University of Antwerpen, B-2020 Antwerp, Belgium

ⁱInstitut de Recherche pour le Développement, Unité Mixte de Recherche Diversité Adaptation et Développement des Plantes (Institut de Recherche pour le Développement/Université Montpellier 2), 34394 Montpellier, France

Auxin transport, which is mediated by specialized influx and efflux carriers, plays a major role in many aspects of plant growth and development. *AUXIN1* (*AUX1*) has been demonstrated to encode a high-affinity auxin influx carrier. In *Arabidopsis thaliana*, *AUX1* belongs to a small multigene family comprising four highly conserved genes (i.e., *AUX1* and *LIKE AUX1* [*LAX*] genes *LAX1*, *LAX2*, and *LAX3*). We report that all four members of this *AUX/LAX* family display auxin uptake functions. Despite the conservation of their biochemical function, *AUX1*, *LAX1*, and *LAX3* have been described to regulate distinct auxin-dependent developmental processes. Here, we report that *LAX2* regulates vascular patterning in cotyledons. We also describe how regulatory and coding sequences of *AUX/LAX* genes have undergone subfunctionalization based on their distinct patterns of spatial expression and the inability of *LAX* sequences to rescue *aux1* mutant phenotypes, respectively. Despite their high sequence similarity at the protein level, transgenic studies reveal that *LAX* proteins are not correctly targeted in the *AUX1* expression domain. Domain swapping studies suggest that the N-terminal half of *AUX1* is essential for correct *LAX* localization. We conclude that *Arabidopsis* *AUX/LAX* genes encode a family of auxin influx transporters that perform distinct developmental functions and have evolved distinct regulatory mechanisms.

INTRODUCTION

The phytohormone auxin indole-3-acetic acid (IAA) is a versatile trigger for plant development (Vanneste and Friml, 2009). Auxin regulates embryogenesis, organogenesis, vascular tissue formation,

and tropic responses in plants (Vieten et al., 2007; Petrásek and Friml, 2009). The polar transport of auxin from cell to cell is achieved through the coordinated process of efflux and influx transporters, encoded by *PIN-FORMED* (*PIN*) and *P-GLYCOPROTEIN* (*PGP*), respectively (Geisler et al., 2005; Petrásek et al., 2006; Cho et al., 2007) and *AUXIN1/LIKE AUX1* (*AUX/LAX*) genes (Bennett et al., 1996; Swarup et al., 2008). The *PIN* efflux transporters have a polar plasma membrane (PM) localization that regulates the direction of auxin flow (Wisniewska et al., 2006). Their mode of action during plant development shows strong redundancy and auxin-dependent cross-regulation of their expression (Vieten et al., 2005). Localization of *AUX1* has been described to be cell type-dependent and, together with *PIN* efflux transporters, it provides directionality of intercellular auxin flow (Swarup et al., 2001; Kleine-Vehn et al., 2006).

In *Arabidopsis thaliana*, the *AUX/LAX* family is represented by four highly conserved genes called *AUX1*, *LAX1*, *LAX2*, and *LAX3* (see Supplemental Figure 1A and Supplemental Data Set 1 online), which encode multimembrane-spanning transmembrane proteins and share similarities with amino acid transporters. This

¹ Current address: Laboratory of Plant Development Biology, Service de Biologie Végétale et de Microbiologie Environnementale/Institut for Biotechnology and Environmental Biology, Commissariat à l'Énergie Atomique et aux Énergies Alternatives Cadarache, 13108 St. Paul lez Durance, France.

² Current address: Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas–Universitat Politècnica de Valencia, 46022 Valencia, Spain.

³ Address correspondence to ranjan.swarup@nottingham.ac.uk. The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Ranjan Swarup (ranjan.swarup@nottingham.ac.uk).

Some figures in this article are displayed in color online but in black and white in the print edition.

Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.112.097766

protein family forms a plant-specific subclass within the amino acid/auxin permease super family (Young et al., 1999). Mutations in *AUX1* or *LAX3* result in auxin-related developmental defects. For example, *aux1* mutants are agravitropic and have a decreased number of lateral roots. By comparison, a loss-of-function mutation in *LAX3* results in delayed lateral root emergence, and together, *LAX3* and *AUX1* act concomitantly to regulate lateral root development by regulating the emergence (Swarup et al., 2008) and initiation (Marchant et al., 2002) steps, respectively. Auxin uptake experiments in heterologous expression systems have confirmed that *AUX1* and *LAX3* are high-affinity auxin transporters (Yang et al., 2006; Carrier et al., 2008; Swarup et al., 2008).

In contrast with *AUX1* and *LAX3*, the functional roles of the other two members of the *AUX/LAX* family are not well understood. Experimental observations suggest that both may also function as auxin influx carriers (Bainbridge et al., 2008), because mutating multiple members of the *AUX/LAX* family affects phyllotactic patterning—a process that is known to be regulated by auxin. This is supported by the fact that *AUX1* shares 82, 78, and 76% identity with *LAX1*, *LAX2*, and *LAX3*, respectively (see Supplemental Figure 1B online). Examination of their gene structure revealed well-conserved exon/intron boundaries for most of the sequence (see Supplemental Figure 1C online), indicating that all four members of the family have originated from a common ancestor through gene duplication. In this study, using a combination of genetic, molecular, and biochemical approaches, we provide experimental evidence that all members of the *AUX/LAX* family have auxin influx activity. Despite the conservation of biochemical function, we demonstrate that their regulatory and coding sequences have undergone subfunctionalization. We also show that the N-terminal domain of *AUX1* provides information for correct localization of *LAX* proteins in the *AUX1* expression domain.

RESULTS

AUX/LAX Genes Exhibit Nonredundant and Complementary Expression Patterns in Roots

To provide insight into the roles of *AUX/LAX* family members in plant growth and development, their expression was analyzed in detail using in situ immunolocalization and/or promoter: β -glucuronidase (*GUS*) fusions and genomic yellow fluorescent protein (YFP)/VENUS translational fusions. These studies revealed that the expression patterns of *AUX/LAX* genes are mostly nonredundant and complementary in the primary root apex. Previous studies have shown that *AUX1* is expressed in the columella, lateral root cap (LRC), epidermis, and stele tissues (Figure 1A; see Supplemental Figure 2A online) (Swarup et al., 2001; Swarup et al., 2005), whereas *LAX3* is expressed in the columella and stele (Figure 1D; see Supplemental Figure 2D online) (Swarup et al., 2008).

As part of this investigation, using two different approaches (promoter:*GUS* and genomic YFP/VENUS translational fusions), we report that *LAX1* is expressed in the mature regions of primary root vascular tissues (Figures 1E to 1I; see Supplemental Figures 2E to 2I online). Weak *LAX1* expression was also detected in the vascular tissues in the primary root apex in

ProLAX1:LAX1-VENUS lines (see Supplemental Figure 2B online) but was not detectable in the *ProLAX1:GUS* lines (Figure 1B) even after prolonged *GUS* staining. This discrepancy is likely to be caused by the much larger genomic region used in *ProLAX1-LAX1-VENUS* lines.

LAX2 expression is detected in young vascular tissues, the quiescent center, and columella cells (Figures 1C, 6A, and 6B; see Supplemental Figure 2C online). *LAX2* signal in the columella cells is most pronounced in the *ProLAX2:GUS* lines (Figure 1C), but is almost absent or very weak in the *ProLAX2:LAX2-VENUS* lines (see Supplemental Figure 2C online). Localization of *LAX2* by in situ immunolocalization using anti-*LAX2* antibody also showed a relatively weak expression of *LAX2* in the columella cells (Figures 6A and 6B), suggesting that the stronger signal of *LAX2* in *ProLAX2:GUS* lines is likely to be caused by the more stable nature of the *GUS* reporter.

The divergence in spatial expression patterns of *AUX/LAX* members is also clearly illustrated during lateral root development. As previously described, *LAX3* is expressed outside the emerging lateral root primordia (Swarup et al., 2008), whereas *AUX1* is localized within the lateral root primordia during all stages of development (Marchant et al., 2002). In comparison, *LAX1* expression is first detected in stage I primordia and then mainly persists at the primordium base throughout lateral root formation (Figures 1E to 1I; see Supplemental Figures 2E to 2I online). By contrast, *LAX2* expression is only detected in the central region of lateral root primordia (Figures 1J to 1N; see Supplemental Figures 2J to 2N online).

As previously reported, *LAX3* expression is auxin inducible (Swarup et al., 2008) (Figures 2G and 2H; see Supplemental Figure 4G and 4H online). We then tested whether the expression of other *AUX/LAX* genes can be regulated by auxin. A bioinformatic search for auxin-related transcription factor binding sites and the presence of canonical auxin response elements in a 2-kb upstream sequence from ATG of the *AUX/LAX* promoters revealed that *LAX3* and *LAX1* have the highest number of transcription factor binding sites (see Supplemental Figure 3 online). To test this directly, 7-d-old seedlings were treated for 16 h with 100 nM 2,4 dichlorophenoxyacetic acid (2,4-D). Under these conditions, both *LAX3-GUS* (Figures 2G and 2H) and *LAX3-YFP* (see Supplemental Figures 4G and 4H online) expression was strongly induced by auxin. Our results also revealed that *LAX1* transcript abundance was upregulated by auxin (Figures 2C and 2D; see Supplemental Figures 4C and 4D online). *LAX1* expression seems stronger in the presence of auxin and is detected much closer to the root apex compared with untreated controls (arrow in Figure 2D). However, unlike *LAX3*, *LAX1* is not induced in outer root tissues (compare Figure 2D with Figure 2H and Supplemental Figure 4D with Supplemental Figure 4H online). In contrast with *LAX3* and *LAX1*, neither *AUX1* (Figures 2A and 2B; see Supplemental Figures 4A and 4B online) nor *LAX2* (Figures 2E and 2F; see Supplemental Figures 4E and 4F online) expression seem to be altered in the presence of auxin.

These results indicate that the regulation of *AUX/LAX* gene expression has diverged during the course of evolution, suggesting that they have acquired distinct roles in different developmental/physiological processes, an evolutionary mechanism described as subfunctionalization.

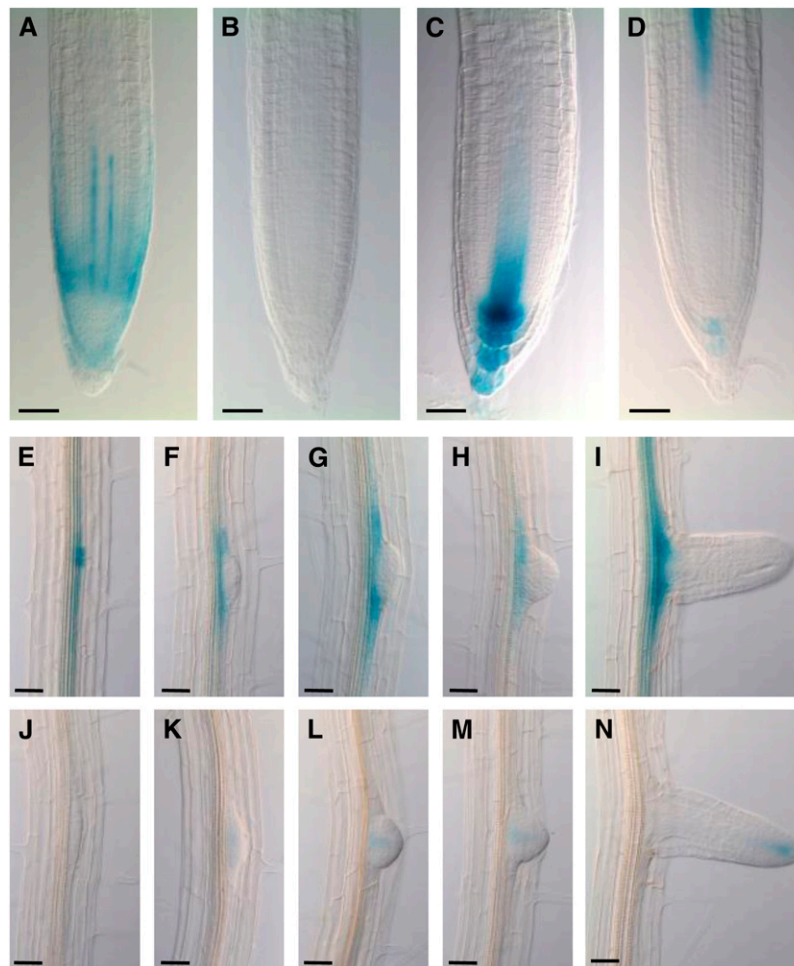


Figure 1. *Promoter:GUS* Studies Show That *AUX/LAX* Genes Exhibit Complementary Expression Patterns.

(A) to (D) Expression profile of *AUX1* (A), *LAX1* (B), *LAX2* (C), and *LAX3* (D) in the primary root apex.

(E) to (H) Expression profile of *LAX1* (E) to (I) and *LAX2* (J) to (N) during lateral root primordium development.

Bars in (A) to (D) = 35 μ m; bars in (E) to (N) = 40 μ m.

Members of the *AUX/LAX* Family Facilitate Distinct Auxin-Regulated Developmental Programs

To probe whether the *AUX/LAX* family of proteins exhibit subfunctionalization, a genetic approach was used to test the roles of these genes during *Arabidopsis* growth and development. *AUX1* has previously been reported to play an important role during the root gravitropic response (Swarup et al., 2001; Swarup et al., 2004; Swarup et al., 2005) as well as lateral root initiation (Marchant et al., 2002), whereas *LAX3* has recently been shown to be involved in lateral root emergence (Swarup et al., 2008). As part of this study, *lax1* and *lax2* mutants were analyzed for auxin-regulated developmental phenotypes. No root growth-related defects were obvious in either *lax1* or *lax2* mutants (see Supplemental Figures 5 to 7 online). Unlike *aux1*, mutations in *lax1* or *lax2* did not affect their root gravitropic responses (see Supplemental Figures 5A and 5B online) or sensitivity to synthetic auxin 2,4-D (see Supplemental Figure 6 online). Similarly, unlike *aux1* and *lax3*, no lateral root-related

defects were observed for either *lax1* or *lax2* mutant alleles (see Supplemental Figure 7 online).

To test the possibility of genetic redundancy between *AUX1*, *LAX1*, and *LAX2*, growth responses to synthetic auxin 2,4-D and lateral root development were investigated in double and triple mutants. The growth responses of double and triple mutant combinations to synthetic auxin 2,4-D were similar to *aux1*, suggesting that loss of *lax1* and/or *lax2* did not enhance the *aux1* phenotype (see Supplemental Figure 8A online). Similarly, the lateral root phenotypes of *aux1 lax1* and *aux1 lax2* double mutants or *aux1 lax1 lax2* triple mutants were not significantly different from single *aux1* mutants (see Supplemental Figures 8B and 8C online). Under the same conditions, the *aux1 lax3* double mutant showed a severe reduction in emerged lateral roots, in agreement with Swarup et al. (2008).

These results suggest that during the course of evolution, at least two members of the *AUX/LAX* family, *AUX1* and *LAX3*, have subfunctionalized, whereas *LAX1* and *LAX2* gene products do not

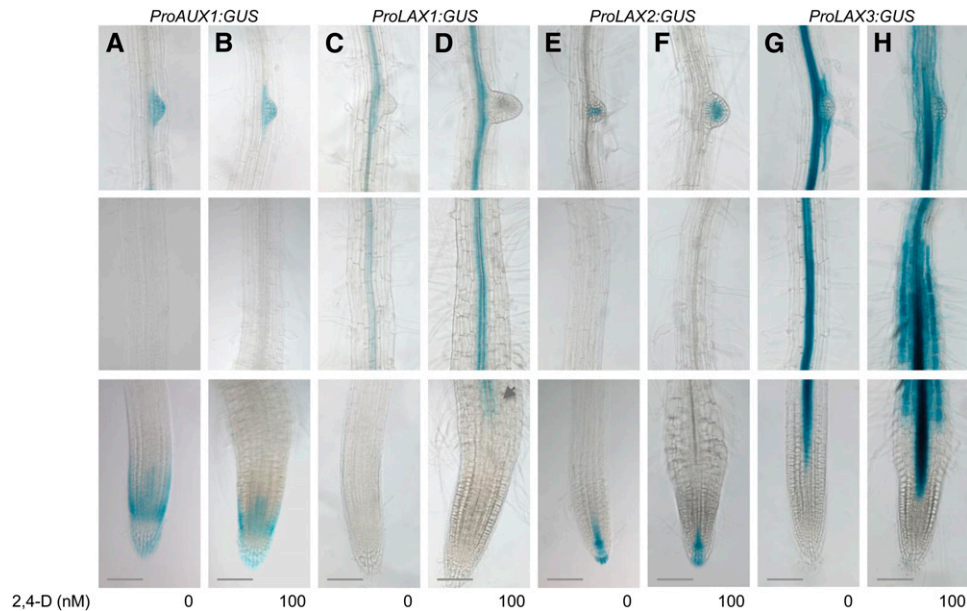


Figure 2. *LAX1* and *LAX3* Genes Are Induced by Auxin.

Expression profile of *AUX1* (**A**) and (**B**), *LAX1* (**C**) and (**D**), *LAX2* (**E**) and (**F**), and *LAX3* (**G**) and (**H**) in absence and presence of 100 nM 2,4-D. Note *LAX1* expression in the presence of auxin is detected much closer to the root apex (arrow in **D**). Bars = 50 μ m.

seem to influence root system architecture. However, it cannot be ruled out that *LAX1* and *LAX2* perform more subtle patterning functions, and specific conditions are required to uncover a root-related mutant defect. Alternatively, these genes may have acquired novel functions (neofunctionalization—no longer auxin influx carriers) or new roles (subfunctionalization—still auxin influx carriers) in other plant organs. The latter view is supported by the discovery that mutating all four members of the *AUX/LAX* family affects phyllotactic patterning (Bainbridge et al., 2008), and both *AUX1* and *LAX3* have also been implicated in apical hook development (Vandenbussche et al., 2010), processes that are known to be regulated by auxin.

Auxin is also known to regulate vascular development, and many auxin transport and response mutants have defects in vascular development (Reinhardt, 2003; Petrásek and Friml, 2009). *LAX2* promoter:*GUS* studies show that *ProLAX2:GUS* expression is associated with procambial and vascular tissues during embryogenesis (Figures 3A to 3C). In developing leaves, *ProLAX2:GUS* expression is detected very early at the sites of initiating veins, and starting from day 5, *LAX2* is expressed along the secondary loops, starting with the first loop followed by the second, third, and fourth (Figures 3D to 3I). By days 7 to 8, *LAX2* expression is also detected near the position of tertiary veins. Interestingly, *LAX2* is not expressed along the midvein (Figures 3D to 3I).

To assess the role of *LAX2* during vascular development, two different alleles of *LAX2* were analyzed (Figure 3J). The *lax2-1* allele represents an En element inserted into intron 2 (position 452 from ATG), whereas *lax2-2* has a T-DNA insertion in exon 6 (position 1239 from ATG). Both these alleles seem to be null alleles, because no *LAX2* cDNA is detected by RT-PCR (Figures

3K and 3L). Examination of vascular development in *lax2-1* and *lax2-2* cotyledons revealed that both alleles exhibit a significantly higher propensity of discontinuity in vascular strands, with almost 64% of *lax2-1* and 77% of *lax2-2* seedlings showing vascular breaks in their cotyledons (Figures 3M to 3Q) compared with only 20% of control seedlings. In contrast with cotyledons, no defect in vascular patterning was apparent in *lax2* leaves. This auxin-related developmental phenotype for *lax2* provides indirect evidence for a role for *LAX2* in facilitating auxin transport.

***AUX1*, *LAX1*, and *LAX3* Encode Functional Auxin Influx Carriers**

To directly test whether every *AUX/LAX* protein has auxin transport activity, experiments were performed in heterologous expression systems. Using an oocyte expression system, both *AUX1* (Yang et al., 2006) and *LAX3* (Swarup et al., 2008) were previously shown to be high-affinity auxin transporters. Similar experiments were performed for *LAX1* and *LAX2*. These experiments revealed that *LAX1* exhibited auxin uptake activity in oocytes (Figure 4A). Competition experiments with cold 2,4-D or IAA significantly reduced the uptake of radiolabeled IAA by oocytes injected with *LAX1* complementary RNA (cRNA), suggesting a carrier-mediated uptake (Figure 4B). By contrast, there was only a small reduction in tritium-labeled IAA (^3H IAA) uptake in the presence of the lipophilic auxin analog 1-naphthalene acetic acid or indole butyric acid (Figure 4B). Surprisingly, no auxin uptake activity was seen in *LAX2*-expressing oocytes (Figure 4A). Immunoblot experiments using specific anti-*LAX2* antibodies revealed that the protein was correctly expressed in these oocytes (Figure 4C, lane 1), thus ruling out defects in its translation. To test

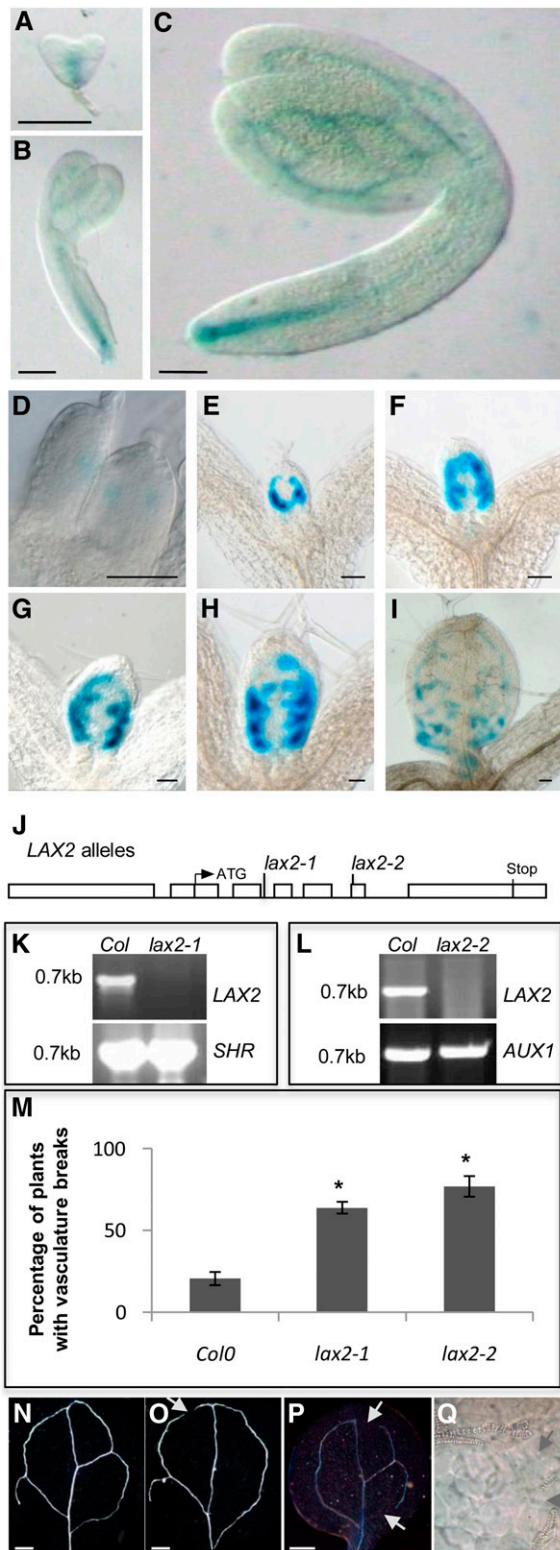


Figure 3. The *lax2* Mutant Exhibits Vascular Patterning Defects in the Cotyledons.

(A) to (C) *Promoter:GUS* analysis of *LAX2* expression in heart stage (A), torpedo (B), and mature (C) embryos.

whether *LAX2* is correctly targeted to the PM in oocytes, a YFP-tagged version of *LAX2* (*LAX2-YFP*) was expressed. Immunodetection again showed that *LAX2-YFP* was correctly expressed in these oocytes and was detected in the membrane and not the cytosolic fractions (Figure 4C, lanes 2 and 3). However, confocal analysis revealed no detectable *LAX2-YFP* on the PM (Figure 4D, panel III). In comparison, YFP fluorescence was clearly seen on the PM of oocytes expressing *AUX1-YFP* (Figure 4D, panel II). These results show that *LAX2-YFP*, unlike *AUX1-YFP*, is not properly targeted to the PM in *Xenopus laevis* oocytes and may suggest a requirement for some plant-specific accessory proteins/factors for its correct targeting that are lacking in *X. laevis*. As an alternative approach, *LAX2* transport activity was also assayed using a yeast-based heterologous expression system (Yang and Murphy, 2009) to determine the role of *LAX2* in IAA uptake. In this system, *LAX2*-expressing yeast cells displayed a weak but consistent IAA uptake activity compared with control cells (Figures 4E and 4F).

To further probe whether *LAX2* encodes an auxin influx transporter, we also used a genetic assay. We reasoned that if *LAX2* encodes a functional auxin transporter, an *AUX1* promoter-driven *LAX2* sequence would be expected to complement *aux1* mutants. The *aux1* mutant shows reduced sensitivity to auxins 2,4-D and IAA and has a strong agravitropic root phenotype (Bennett et al., 1996; Swarup et al., 2001, 2005) and a defect in lateral root initiation (Marchant et al., 2002). To test the ability of *LAX2* to complement the *aux1* mutant, a *ProAUX1:LAX2* construct was created to express *LAX2* under the control of the 1.7-kb *AUX1* promoter and was then introduced into an *aux1* mutant allele, *aux1-22* (Figure 5A). Homozygous T3 seedlings were then tested for the restoration of the *aux1* mutant phenotype (root gravitropic response and sensitivity to 2,4-D). As expected, *ProAUX1:AUX1* lines (*AUX1* promoter driving *AUX1* that was used as a positive control) fully rescued the 2,4-D-resistant root growth and agravitropic phenotypes of *aux1* seedlings (Figures 5B and 5C). By contrast, *ProAUX1:LAX2* lines failed to rescue the root agravitropic phenotypes of *aux1*

(D) to (I) *Promoter:GUS* analysis of expression of *LAX2* in developing leaf primordia.

(J) Structure of the *LAX2* with the positions of the *lax2* mutant alleles indicated. Boxes represent promoter, 5', and 3' untranslated regions and exons; lines represent introns.

(K) and (L) RT-PCR analysis of *lax2-1* (K) and *lax2-2* (L) alleles showing that *LAX2* cDNA is detectable in the wild type (Col-0) but not in *lax2-1* (K) and *lax2-2* (L). Positive controls *SHR* (K) and *AUX1* (L) are detected both in wild-type (Col-0) and *lax2* alleles ($n = 2$).

(M) Graph showing the frequency of vascular breaks in cotyledons of *lax2* mutant alleles compared with the wild type (Col-0). Error bars represent SE. * indicates statistically significant difference compared with the wild type (Col-0); $n = 30$; Student's *t* test, $P < 0.01$.

(N) to (P) Differential interference contrast images of wild-type (N), *lax2-1* (O), and *lax2-2* (P) cotyledons showing the vascular defect in *lax2*.

(Q) High-magnification differential interference contrast image pinpointing vascular break in a *lax2* cotyledon.

Bars in (A) to (C) = 40 μm ; bars in (D) to (I) = 100 μm ; bars in (N) to (P) = 200 μm .

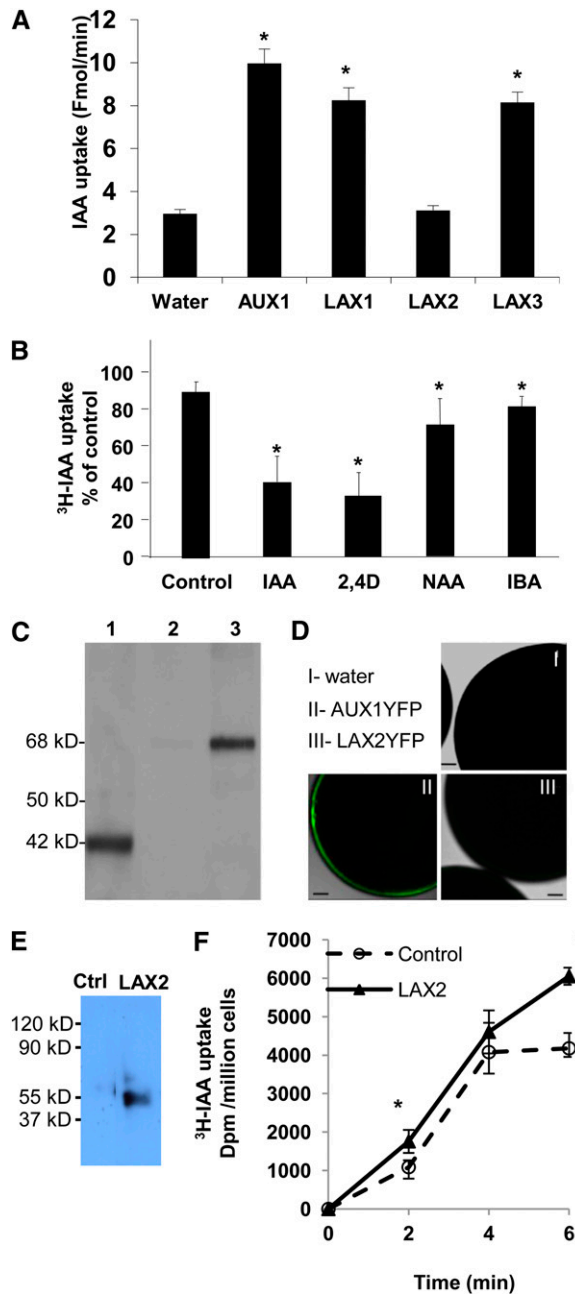


Figure 4. AUX/LAX Proteins Are Functional Auxin Influx Transporters.

(A) Uptake of [³H]IAA into *X. laevis* oocytes injected with water or *AUX1*, *LAX1*, *LAX2*, and *LAX3* cRNAs at pH 6.4. Oocytes injected with *AUX1*, *LAX1*, and *LAX3* cRNAs showed increased [³H]IAA uptake when compared with the water-injected control ($n = 8$).

(B) Uptake of [³H]IAA into oocytes injected with *LAX1* cRNA was examined in the presence of excess unlabeled IAA, the auxin analogs 2,4-D and 1-naphthalene acetic acid (NAA), and the naturally occurring auxin form indole butyric acid (IBA) ($n = 5$).

(C) Immunoblot analysis of oocytes injected with *LAX2* (lane 1) or *LAX2-YFP* (lanes 2 and 3) cRNAs. Total oocyte extract expressing *LAX2* (lane 1) or *LAX2-YFP* (cytosolic fraction, lane 2; microsomal fraction, lane 3) were separated by SDS-PAGE and immunodetected using anti-LAX2 antibodies

seedlings (Figure 5B) as well as 2,4-D-resistant root growth (Figure 5C).

LAX2 Is Mistargeted in AUX1-Expressing Cells

To determine why *LAX2* did not rescue the *aux1* phenotypes, a quantitative RT-PCR experiment was initially used to measure transgene expression levels of *ProAUX1:AUX1*, *ProAUX1:LAX2*, and *ProAUX1:N-terminal HA epitope-tagged AUX1 (NHA-AUX1)* (Swarup et al., 2001) lines compared with wild-type *AUX1* levels (see Methods). Quantitative RT-PCR revealed that *LAX2* transgene was consistently expressed at equivalent levels to those of *ProAUX1:AUX1* and *ProAUX1:NHA-AUX1* (see Supplemental Figure 9 online). Hence, transgene expression was not the basis for the lack of rescue of the *aux1* phenotypes. Next, we tested whether *LAX2* was either incorrectly translated or trafficked in *AUX1*-expressing cells in these lines by in situ immunolocalization using anti-LAX2 antibodies. Because of the high similarity between all AUX/LAX family members, the specificity of the antipeptide antibody was tested. In wild-type seedling roots, a strong signal was seen in vascular tissues, the S1 columella layer, and the quiescent center (Figures 6A and 6B), but no signal was detected in equivalent tissues of *lax2* seedlings (Figure 6C), confirming the high specificity of this antibody for *LAX2*. Furthermore, immunolocalization of *LAX2* exhibited a broadly similar spatial expression pattern to that obtained using *ProLAX2:GUS* (Figure 1C) and *ProLAX2:LAX2-VENUS* lines (see Supplemental Figure 2C online), suggesting the absence of posttranscriptional control of *LAX2* in *LAX2*-expressing cells (Figures 1C, 6A, and 6B; see Supplemental Figure 2C online). Slight differences in expression of *ProLAX2:GUS*, particularly in the columella cells, may be caused by differences in stability of *GUS* and *LAX2* proteins.

We then tested the localization of *LAX2* in *ProAUX1:LAX2* lines. As reported previously (Swarup et al., 2001; Swarup et al., 2005), *AUX1* is expressed in columella, LRC, epidermis, and proto-phloem cells (Figure 6D). In *ProAUX1:LAX2* lines, as expected, a strong *LAX2* signal was seen in *LAX2*-expressing cells (endogenous *LAX2*; Figures 6E and 6F); however, in cells that normally also express *AUX1*, the transgene-derived *LAX2* signal was either weak (LRC and columella; Figures 6F and 6G) or absent (epidermis; Figure 6H). Surprisingly, the *LAX2* signal in columella and LRC cells accumulated inside the cell and was only occasionally found

(dilution 1/1000). Note the size difference between native *LAX2* (42 kD) and *LAX2-YFP* (68 kD).

(D) Laser scanning confocal images of oocytes injected with water (I), *AUX1-YFP* cRNA (II), or *LAX2-YFP* cRNA (III).

(E) Immunoblot analysis of empty vector control or *LAX2* expressing *S. pombe* cells. Proteins were separated by SDS-PAGE and immunodetected using anti-LAX2 antibodies (dilution 1/1000).

(F) Uptake of [³H]IAA into empty vector control (dashed line) versus *LAX2*-expressing (solid line) *S. pombe* cells compared with zero time point.

Error bars represent sd. * indicates statistically significant difference. Student's *t* test $P < 0.05$.

Bar in **(D)** = 100 μ m.

[See online article for color version of this figure.]

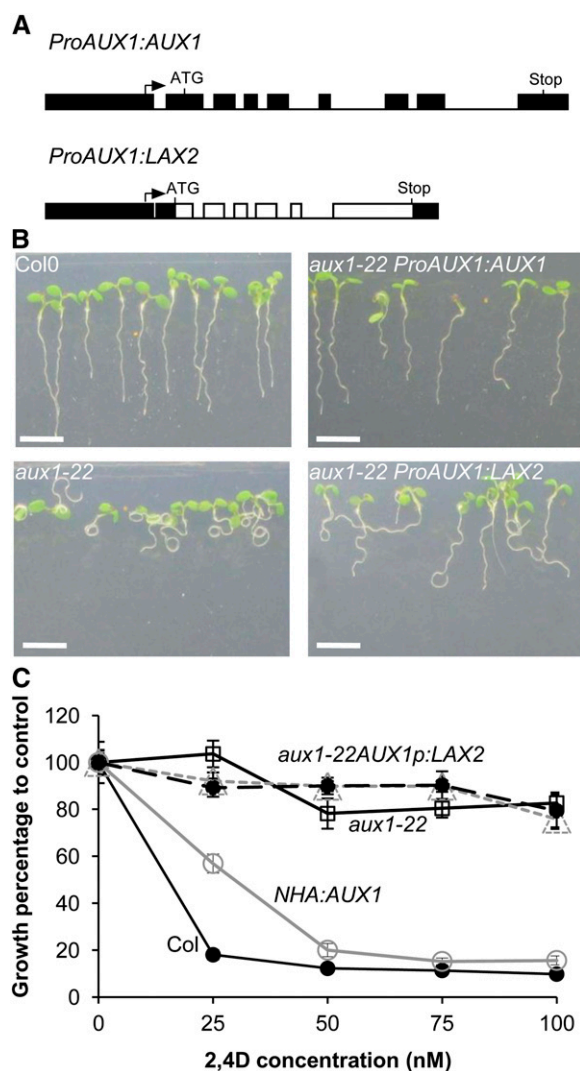


Figure 5. AUX/LAX Genes Are Not Fully Functionally Interchangeable.

(A) Gene constructs used for genetic complementation assays. *AUX1* (control) or *LAX2* genomic sequences were cloned between the *AUX1* promoter and terminator to create *ProAUX1:AUX1* and *ProAUX1:LAX2* (boxes represent promoter, 5', and 3' untranslated regions and exons; lines represent introns).

(B) Root gravitropic phenotypes of the wild type (Col-0), *aux1-22*, and *aux1-22* complemented by either *ProAUX1:AUX1* (control) or *ProAUX1:LAX2* transgenes ($n = 40$).

(C) Growth responses of the wild type (Col-0), *aux1-22*, and *aux1-22* complemented by either *ProAUX1:AUX1* (control) or *ProAUX1:LAX2* transgenes grown at various concentrations of 2,4-D and root growth expressed as percentage of zero control ($n = 40$). Error bars represent se . Bar in **(B)** = 5 cm.

[See online article for color version of this figure.]

at the PM (Figures 6G and 6H, compare with inset in Figure 6D). Altogether, our data demonstrate that misexpressing *LAX2* in *AUX1*-expressing cells results in targeting defects for the protein in these tissues. This was further supported by an analysis of *Pro35S:LAX2-YFP* lines. In these lines, YFP signal was clearly seen in

AUX1-expressing cells, including the epidermis, but most of the signal is localized inside the cell. By contrast, *LAX2*-YFP seems to be correctly localized to the PM in the *LAX2*-expressing cells (Figures 6I and 6J). These results suggest that the subcellular distribution of *LAX2* is distinct in different plant cells and tissues. To investigate whether other members of the family are also subject to such regulation, we expressed *LAX3* under the control of the *AUX1* promoter. The *AUX1* promoter-driven *LAX3* lines also failed to rescue *aux1* mutant phenotypes. In situ immunolocalization revealed reduced *LAX3* protein abundance and targeting defects that were similar in nature to those observed for *ProAUX1:LAX2* lines (Figures 6K and 6L). We conclude that although *AUX1/LAX* family members may share auxin transport characteristics, these transport activities seem to be dependent on their unique cell- or tissue-type expression patterns.

The AUX1 N Terminus Is Required for Correct Localization in the AUX1 Expression Domain

To further investigate the inability of *LAX2* to correctly localize in the *AUX1* expression domain, domain swap experiments were designed where either the N-terminal half of *LAX2* was fused to the C-terminal half of *AUX1* (*DS1*) or the N-terminal half of *AUX1* was fused to the C-terminal half of *LAX2* (*DS2*) to create chimeric genes driven by the *AUX1* promoter (Figure 7A). These constructs were then introduced into an *aux1* mutant allele, *aux1-22*. Homozygous T3 seedlings were then tested for the rescue of the *aux1* mutant phenotype (root gravitropic response and sensitivity to 2,4-D). The results revealed that, like *ProAUX1:LAX2* (Figures 7B, panels III and IV, and 7C), *DS1* lines also failed to rescue root agravitropic phenotypes of *aux1* seedlings (Figure 7B, panels V and VI) as well as 2,4-D-resistant root growth (Figure 7C). By contrast, *DS2* lines rescued both the 2,4-D-resistant root growth (Figure 7C) and agravitropic phenotypes of *aux1* seedlings (Figure 7B, panels VII and VIII).

To probe the molecular basis of rescue, in situ immunolocalization experiments were done using either anti-HA antibody (for *DS1*) or anti-*LAX2* antibody (for *DS2*). As shown in Figures 7F and 7G, *DS2* lines show strong signal in *AUX1* expression domains in the LRC and epidermal cells besides endogenous *LAX2* signal in the vascular tissues. By contrast, *DS1* lines show almost no signal in the LRC and the epidermal cell (Figures 7D and 7E), but a surprisingly strong signal is seen in the vascular tissues (Figure 7D). On the basis of these results, we conclude that the N-terminal half of *AUX1* is required for correct localization in the *AUX1* expression domain.

DISCUSSION

During evolution, gene family members acquire mutations that alter one or more subfunctions of the single gene progenitor. The fate of duplicated genes can encompass pseudogenization (loss of function), subfunctionalization, and neofunctionalization (Moore and Purugganan, 2005). This study shows that *AUX/LAX* family members in *Arabidopsis* have not undergone pseudogenization or neofunctionalization but have experienced subfunctionalization.

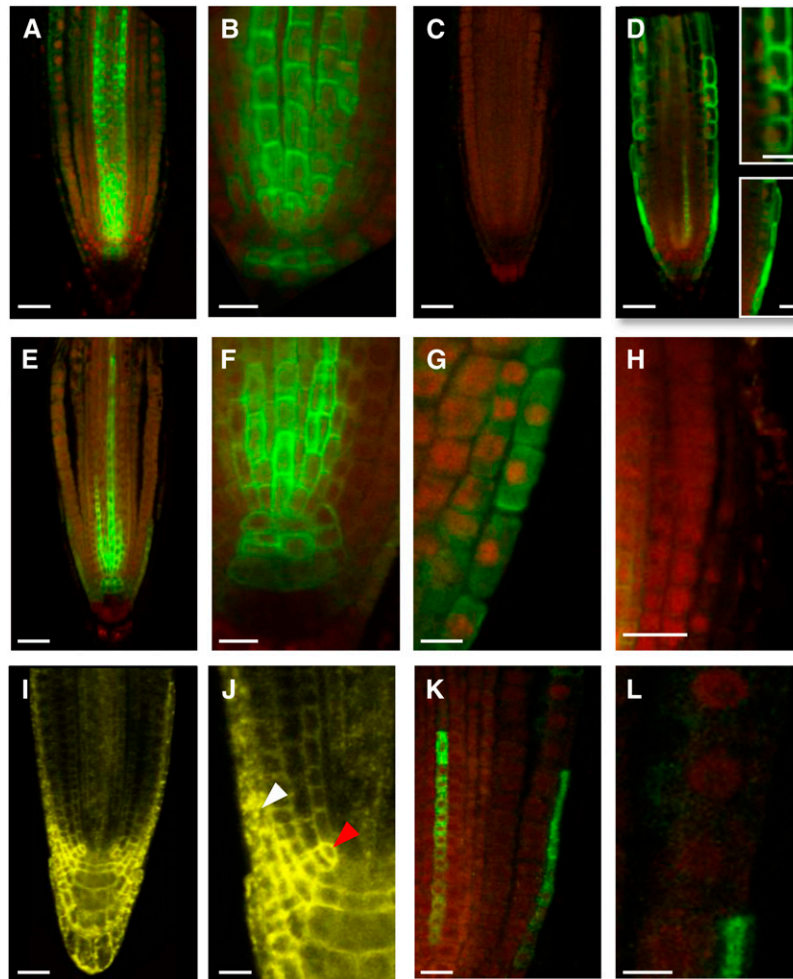


Figure 6. LAX2 and LAX3 Cannot Be Correctly Targeted in *AUX1*-Expressing Cells.

(A) to (C) In situ immunodetection of LAX2 (green) in the wild type [(A) to (B)] or *lax2* (C) primary roots counter stained with propidium iodide (red). (D) In situ immunodetection of NHA-AUX1 in root apex. Inset: Close-up of epidermal (Top) and LRC (Bottom) cells. (E) to (H) In situ immunodetection of LAX2 in *aux1-22 ProAUX1:LAX2* roots showing targeting defect of LAX2 in *AUX1*-expressing cells, including LRC (G) and epidermal cells (H). (I) and (J) Confocal imaging of seedlings expressing LAX2-YFP under the control of *CaMV35S* promoter showing correct targeting of LAX2 in LAX2-expressing cells (red arrowhead) but not in *AUX1*-expressing cells (white arrowhead). (K) and (L) In situ immunodetection of LAX3-FLAG in *aux1-22 ProAUX1>>LAX3* (Methods) roots, showing targeting defects of LAX3 in *AUX1* expression domains including LRC and epidermal cells (L). Bars in (A), (C) to (E), and (I) = 25 μm ; bars in (B), (F) to (H), (J), and (K) = 10 μm ; bars in (L) and insets = 5 μm .

Genetic evidence presented in this and other articles demonstrates that each *AUX/LAX* family member regulates an auxin-dependent developmental process. For example, several studies support a role for *AUX1* in auxin-mediated developmental programs, including root gravitropism (Swarup et al., 2001; Swarup et al., 2005; Dharmasiri et al., 2006), root hair development (Grebe et al., 2002; Jones et al., 2009), and leaf phyllotaxy (Reinhardt et al., 2003; Bainbridge et al., 2008), whereas both *AUX1* and *LAX3* are required for lateral root development (Swarup et al., 2008) and apical hook formation (Vandenbussche et al., 2010). Although a role for *LAX1* and *LAX2* in auxin-regulated root development is limited, evidence is growing that they are both required for *Arabidopsis* aerial development. In our study, we have

provided evidence that *LAX2* regulates vascular development, whereas *LAX1* and *LAX2* are required for leaf phyllotactic patterning (Bainbridge et al., 2008).

There is also no evidence to support neofunctionalization of *AUX/LAX* genes. Instead, all four *AUX/LAX* proteins retain an auxin influx carrier function. Using either heterologous oocyte or yeast expression systems or complementation of *aux1* mutant root phenotypes, we demonstrated that *AUX1* (Yang et al., 2006), *LAX3* (Swarup et al., 2008), and *LAX1* and *LAX2* (this study) encode a family of auxin uptake transporters.

Our study provides clear evidence for subfunctionalization of *AUX/LAX* sequences. As a result of divergence to their regulatory sequences, we observed that *AUX/LAX* spatial expressions

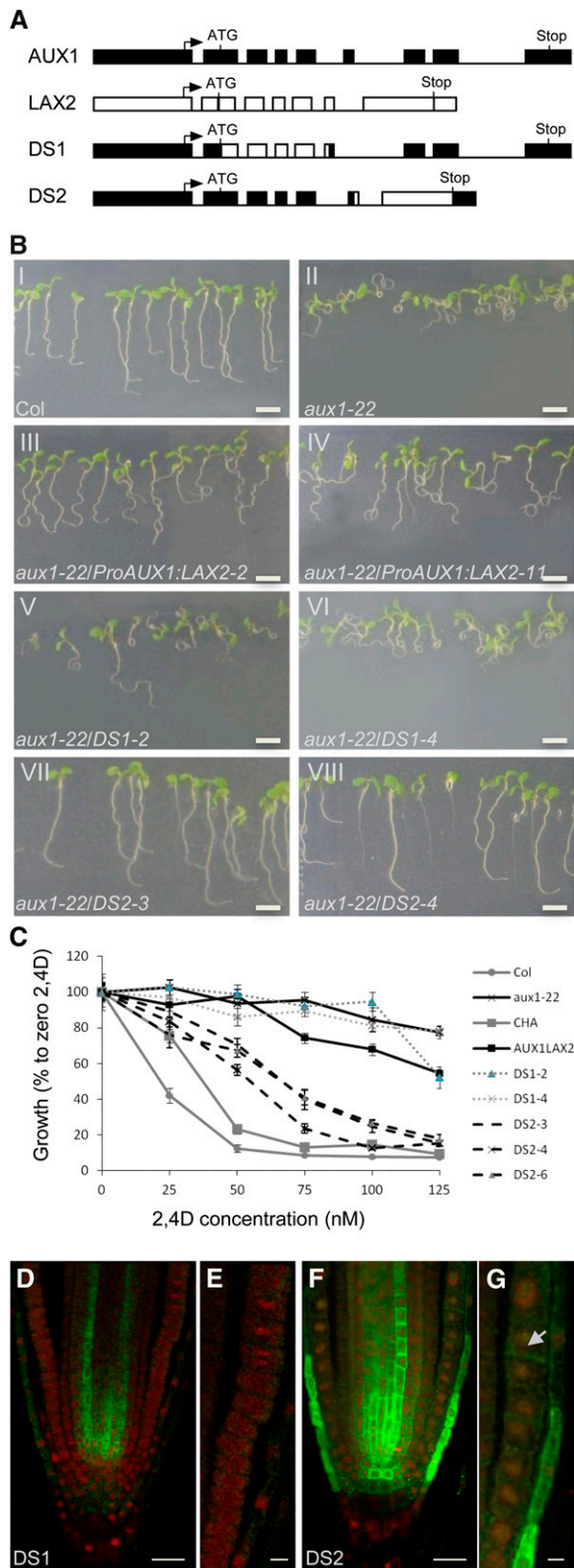


Figure 7. N-Terminal Half of AUX1 Is Required for Correct Localization in the AUX1 Expression Domain.

differ considerably within and between plant tissues (Figure 1; see Supplemental Figure 2 online) (Bainbridge et al., 2008; Swarup et al., 2008; Jones et al., 2009). We also report subfunctionalization of AUX/LAX coding sequences that regulate intracellular trafficking. When ectopically expressed, in situ immunolocalization revealed that LAX2 and LAX3 proteins were unable to be correctly targeted to the PMs of AUX1-expressing root cells. In wild-type roots, AUX1 is localized in cells that are involved in gravity perception (columella), signal transmission (LRC), and gravity response (epidermis) (Swarup et al., 2001; Swarup et al., 2005). The PM targeting defect of LAX2 and LAX3 is particularly severe in epidermal cells, where almost no LAX2 or LAX3 could be detected (Figures 6E to 6L). The simplest explanation for the observed tissue-specific intracellular targeting defect is the requirement of LAX2 and LAX3 for additional trafficking factors that are coexpressed in stele cells but absent in outer root tissues.

Domain swap experiments designed to test this support this notion and suggest that intramolecular trafficking signals are located in the N-terminal half of AUX1. Besides, the ability of DS2 to rescue the *aux1* mutant phenotype clearly suggests that the C-terminal half of LAX2 in the chimeric DS2 protein must play a key role in its overall function as auxin influx carrier, because several missense loss-of-function *aux1* alleles are located in the C-terminal half of AUX1 (Swarup et al., 2004).

All DS2 (ProAUX1:^NAUX1-^CLAX2 chimeric protein fusion) lines can rescue the root agravitropic defect and 2,4-D-resistant root growth of *aux1* seedlings plus show correct localization of chimeric DS2 protein in LRC and epidermal cells when probed using anti-LAX2 antibodies (Figure 7). By contrast, none of the DS1 (ProAUX1:^NLAX2-^CHA-AUX1 chimeric protein fusion) lines rescued *aux1* mutant phenotypes or showed much signal in LRC and epidermal cells (Figure 7). It has been previously shown that these expression domains of AUX1 are crucial for its function (Swarup et al., 2005), and the inability of DS1 but not DS2 to correctly localize in these expression domains provides strong

(A) Gene constructs used for domain swap experiments (boxes represent promoter, 5', and 3' untranslated regions and exons; lines represent introns).

(B) Root gravitropic responses of the wild type (Col-0), *aux1-22*, and *aux1-22* complemented by *ProAUX1:LAX2*, *DS1*, or *DS2* transgenes ($n = 40$).

(C) Growth responses of the wild type (Col-0), *aux1-22*, *CHA-AUX1* (*CHA*), and *aux1-22* complemented by *ProAUX1:LAX2*, *DS1*, or *DS2* transgenes grown at various concentrations of 2,4-D ($n = 40$). Error bars represent SE.

(D) In situ immunodetection of chimeric DS1 protein (green) by anti-HA antibody in primary roots counter stained with propidium iodide (red).

(E) Close-up of LRC and epidermal cells in DS1 roots.

(F) In situ immunodetection of chimeric DS2 protein (green) by anti-LAX2 antibody in primary roots counter stained with propidium iodide (red).

(G) Close up of LRC and epidermal cells in DS2 roots showing localization of DS2 protein (green) in epidermal (arrow) and LRC cells.

(E) to **(H)** Expression profile of *ProLAX1:LAX1-VENUS* [**(E)** to **(H)**] and *ProLAX2:LAX2-VENUS* [**(J)** to **(N)**] during lateral root primordium development.

Bar in **(B)** = 5 cm; bars in **(D)** and **(F)** = 20 μ m; bar in **(G)** = 5 μ m.

evidence that the N-terminal half of AUX1 is required for correct localization in these expression domains. Surprisingly, when probed using anti-HA antibodies, although almost no signal was detected in the LRC and epidermal cells, strong DS1 signal was detected in the vascular cells. As mentioned above, the DS1 protein is a translational fusion between the N-terminal half of LAX2 and C-terminal half of AUX1 (Figure 7A), and vascular tissues are the natural/endogenous expression domain of LAX2. Although we do not currently understand the molecular basis for this differential localization of DS1 protein, it is tempting to speculate that, because the N-terminal half of AUX1 is required for correct localization in the AUX1 expression domain, the N-terminal half of LAX2 contains molecular signals that are recognized by trafficking factors in those tissues. However, compared with endogenous LAX2, DS1 chimeric protein does not seem to be correctly targeted to the PM, suggesting that, in contrast with AUX1, in the case of LAX2, the N-terminal part is still not sufficient for proper membrane targeting. AUX1 intracellular targeting is known to be regulated by AXR4, which encodes a putative endoplasmic reticulum (ER) chaperone that has been proposed to facilitate the correct folding of AUX1 and its export from ER to golgi (Dharmasiri et al., 2006). Hence, the failure of LAX2 and LAX3 to be properly targeted in AUX1-expressing cells may simply reflect a need for their own specific ER chaperones. Future identification of such trafficking factors and of intramolecular trafficking signals within AUX/LAX coding sequences will help reveal how and why they have undergone subfunctionalization during evolution.

METHODS

Plant Materials and Growth Conditions

The *aux1-22* allele was used throughout this study (Swarup et al., 2004). *lax1*, *lax2*, and *lax3* insertion lines have been described previously (Bainbridge et al., 2008; Swarup et al., 2008). Plants were grown on vertical Murashige and Skoog plates at 23°C under continuous light at 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Gravitropic assays were performed as previously described (Swarup et al., 2005). Lateral root numbers were determined on 6-d-old plants using a stereomicroscope. Primary root length was measured using the NeuronJ plugin of ImageJ software.

Isolation of the LAX dSpm Insertion Lines and RT-PCR Analysis

Insertion lines for the *Arabidopsis thaliana* LAX1, LAX2, and LAX3 were identified in the Sainsbury Laboratory *Arabidopsis thaliana* population as described previously (Swarup et al. 2008). LAX2 RT-PCR analysis on RNA isolated from the wild type (ecotype Columbia [Col-0]) and dSpm *lax2-1* allele was performed using primers Lax2F2 (5'-GAGAACGGTGAGAAAGCAGC-3') and Lax2R4 (5'-CGCAGAAGGCAGCGTTAGCG-3').

Isolation of LAX2 GABI-Kat Allele (*lax2-2*) and RT-PCR Analysis

The *lax2-2* allele (line ID GK_345D11; Nottingham Arabidopsis Stock Centre ID N433071) of LAX2 was identified from the GABI-Kat T-DNA insertional population (Kleinboelting et al., 2012). T-DNA insertion was confirmed by PCR using primer pairs 0849 (left border primer 5'-ATATTGACCATCA-TACTCATTGC-3') and Lx2-25 (gene-specific primer 5'-CACAAAGTAGAGTGGCGTG-3'). The homozygous line was confirmed by the absence of a LAX2-specific band using primers Lx2-19 (5'-GGCACAAAGTGCTGTGAC-3') and Lx2-28 (5'-CAGACGCAGAAGGCAGCG-3'). LAX2 RT-PCR analysis of RNA isolated from the wild type (Col-0) and GABI-kat *lax2-2* allele was

performed using primers Lx2-19 (5'-GGCACAAAGTGCTGTGAC-3') and Lx2-28.

Generation of Transgenic Lines

The promoter GUS lines *ProAUX1:GUS* (Marchant et al., 2002), *ProLAX1:GUS*, *ProLAX2:GUS* (Bainbridge et al., 2008), and *ProLAX3:GUS* (Swarup et al., 2008) have been described before. Similarly, N- or C-terminal HA-AUX1 (NHA-AUX1 or CHA-AUX1) have been described before (Swarup et al., 2001). For genetic complementation of *aux1*, *AUX1* and *LAX2* genomic sequences were PCR amplified and fused with the *Arabidopsis AUX1* promoter (1.7 kb) and terminator (0.3 kb) in a pMOG402 binary vector (MOGEN International) as previously described (Péret et al., 2007). For creation of domain swap constructs (DS1 and DS2), CHA-AUX1 (Swarup et al., 2001) and LAX2 genomic sequences were cloned into Gateway entry vector pENTR11 (Invitrogen). Both these vectors were then cut with *SphI* (internal unique site at identical position in both CHA-AUX1 and LAX2) and *XhoI* (in the vector), and the resulting inserts were swapped to create DS1 and DS2. The resulting chimeric constructs DS1 and DS2 were cut out with *BamHI* and *XhoI* and fused with the *Arabidopsis AUX1* promoter (1.7 kb) and terminator (0.3 kb) in a pMOG402 binary vector (MOGEN International) as previously described (Péret et al., 2007). The LAX3-FLAG line was created by fusing the 2 \times FLAG epitope tag (MDYKDHIDYKDDDDK) to the C-terminal of LAX3. The upstream activating sequence was then fused upstream of LAX3-FLAG. This UAS:LAX3-FLAG line was then crossed to the *ProAUX1:GAL4* line (Swarup et al., 2005) to transactivate LAX3-FLAG in AUX1-expressing cells. The VENUS fluorescent protein (Tursun et al., 2009) fusions of LAX1 and LAX2 were generated by a recombineering approach (Zhou et al., 2011). VENUS was fused in frame after the codon 122 for *ProLAX1:LAX1-VENUS* and codon 110 for *ProLAX2:LAX2-VENUS*. Transformation of *Agrobacterium* (C58) and *Arabidopsis* was done as described before (Péret et al., 2007). Transgene-specific cDNA sequences of these lines were PCR-amplified and sequenced to ensure against rearrangements of the transgenes. All complementation experiments were performed on two independent homozygous T3 lines.

Histochemical GUS Staining

GUS staining was done as described previously (Péret et al., 2007). Plants were cleared for 24 h in 1 M chloral hydrate and 33% glycerol. Seedlings were mounted in 50% glycerol and observed with a Leica DMRB microscope.

Quantitative RT-PCR

Total RNA was extracted from roots using the Qiagen RNeasy Plant Mini Kit with on-column DNase treatment (RNase free DNase set, Qiagen). Poly (dT) cDNA was prepared from 3 μg total RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Quantitative PCR was performed using SYBR Green Sensimix (Quantace) on a Stratagene Mx3005P apparatus. PCR was performed in 96-well optical reaction plates heated for 5 min to 95°C, followed by 40 cycles of denaturation for 10 s at 95°C and annealing-extension for 30 s at 60°C. Target quantifications were performed with the following specific primer pairs: AtAUX1F 5'-tgctctgatcaaatcttctct-3' and AtAUX1R 5'-gaagagaagaaccagaaatgtg-3'. Expression levels were normalized to UBA using the following primers: UBAforward 5'-agtggagagctgcagaaga-3' and UBAreverse 5'-ctcggtagcagcagcttta-3'. All quantitative RT-PCR experiments were performed in triplicate, and the values presented represent means \pm SE.

Production of LAX2 Antibody

For generation of LAX2 antibody, a peptide containing the C-terminal 15 amino acids of LAX2 (PPPISHPHFNHHTGL) plus an added Cys (for

attachment to carrier protein KLH) was conjugated to KLH and was injected to rabbits in complete Freund's adjuvant. Boosters were given on days 14, 28, 42, 56, and 70. Immune serum was collected on days 49, 63, and 77. Affinity purification of the antiserum was done against the LAX2 peptide that was coupled to Pierce SulfoLink resin as per manufacturer's instruction. The column was washed twice with 10 mM Tris-Cl buffer (pH 7.5) containing 0.5 M NaCl, once with 100 mM Gly (pH 2.5), and finally with two more washes with 10 mM Tris-Cl buffer (pH 7.5). Crude LAX2 antiserum (10 mL) buffered in 100 μ L 1 M Tris-Cl (pH 7.5) was then applied on the column and rotated at 4°C overnight. Flow-through was passed twice on the column at room temperature followed by a wash each with 10 mM Tris-Cl buffer (pH 7.5) and 10 mM Tris-Cl buffer (pH 7.5) containing 0.5 M NaCl. Purified antibodies were eluted in 250- μ L fractions with 100 mM Gly (pH 2.5) and neutralized with 50 μ L of Tris-Cl buffer (pH 8.0).

Immunolocalization

Four-d-old seedlings were fixed, and immunolocalization experiments were performed as described previously (Swarup et al., 2005) using various primary and secondary antibodies. Localization was visualized using confocal microscopy. Primary antibodies anti-HA (Roche) and anti-FLAG (Sigma-Aldrich) were used at a dilution of 1:200, whereas anti-LAX2 was used at a dilution of 1:100. Oregon Green or Alexa Fluor-coupled secondary anti-rat, anti-mouse, or anti-rabbit antibodies (Invitrogen) were used at a dilution of 1:200. Background staining was performed with propidium iodide (Sigma-Aldrich).

Auxin Transport Assays

Auxin transport assays in oocytes were performed as previously described (Swarup et al., 2008). For uptake experiments in *Schizosaccharomyces pombe*, the LAX2 cDNA was amplified from pO02-LAX2 using primers ccacLAX5 (CACCATGGAGAACGGTGAGAA) and LAX2nonstop3 (AAGGCCGTGAGTGTGATTGA), cloned into Gateway cloning vector pENTR/TOPO (Invitrogen), and confirmed by sequencing. LAX2 cDNA was subsequently cloned into the *S. pombe* expression vector pREP41GWHA (Yang and Murphy, 2009). LAX2 expression in *S. pombe* vat3 cells (Yang and Murphy, 2009) was confirmed by immunoblot using anti-HA primary antibody (1:500 dilution; Santa Cruz Biotechnology).

Accession Numbers

Atg nomenclature gene accession numbers of The Arabidopsis Information Resource database (<http://www.Arabidopsis.org>) are: At2g38120 (*AUX1*), At5g01240 (*LAX1*), At2g21050 (*LAX2*), At1g77690 (*LAX3*), and UBA (At1g04850).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. The *AUX/LAX* Genes Represent a Highly Conserved Family of Auxin Influx Transporters.

Supplemental Figure 2. *AUX/LAX* Genes Exhibit Complementary Expression Patterns.

Supplemental Figure 3. Promoter Analysis of *AUX/LAX* Genes.

Supplemental Figure 4. *LAX1* and *LAX3* Genes Are Induced by Auxin.

Supplemental Figure 5. *lax1*, *lax2*, and *lax3* Mutants Exhibit Normal Gravitropic Response.

Supplemental Figure 6. *lax1* and *lax2* Mutants Exhibit a Normal Response to 2,4-D.

Supplemental Figure 7. *lax1* and *lax2* Mutants Exhibit Normal Root Growth and Lateral Root Growth.

Supplemental Figure 8. *lax1* and *lax2* Mutants Do Not Enhance *aux1* Mutant Phenotypes.

Supplemental Figure 9. *ProAUX1:AUX1* and *ProAUX1:LAX2* Seedlings Exhibit Comparable Transgene Expression Levels.

Supplemental Data Set 1. Alignment Used to Generate the Phylogeny Presented in Supplemental Figure 1A Online.

ACKNOWLEDGMENTS

This study was supported by a Marie Curie Intra-European Fellowship within the 7th European Community Framework Programme PIEF-GA-2008-220506 (B.P.), Biotechnology and Biological Science Research Council (M.J.B. and A.F.), U.S. Department of Energy grant DE-FG02-07ER15887 (E.N.), U.S. Department of Energy grant DE-FG02-06ER15804 (A.M.), a grant from the Bijzonder Onderzoeksfonds Methusalem project (BOF08/01M00408) of Ghent University, by Inter-university Attraction Poles grants 6/33 and 7/29 from the Belgian Science Policy Office (M.J.B., G.T.S.B., and S.D.), National Science Foundation Grant DBI0820755 (J.M.A.), and Ayudas de Movilidad de la Junta de Extremadura, Spain GRU09054 (I.C.).

AUTHOR CONTRIBUTIONS

R.S. and B.P. designed the research, performed research, analyzed data, wrote, and edited the article. K.S., A.F., M.S., Y.Y., S.D., N.J., I.C., P.P., A.S., H.Y., and J.R. performed research and analyzed data. E.V., C.H., M.A.P.-A., J.Y., and J.A. contributed new genetic tools (recombineering technology). L.L., A.M., G.T.S.B., and E.N. analyzed data. M.J.B. designed the research and edited the article.

Received March 4, 2012; revised May 4, 2012; accepted June 19, 2012; published July 5, 2012.

REFERENCES

- Bainbridge, K., Guyomarc'h, S., Bayer, E., Swarup, R., Bennett, M., Mandel, T., and Kuhlemeier, C. (2008). Auxin influx carriers stabilize phyllotactic patterning. *Genes Dev.* **22**: 810–823.
- Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996). *Arabidopsis* AUX1 gene: A permease-like regulator of root gravitropism. *Science* **273**: 948–950.
- Carrier, D.J., Bakar, N.T., Swarup, R., Callaghan, R., Napier, R.M., Bennett, M.J., and Kerr, I.D. (2008). The binding of auxin to the Arabidopsis auxin influx transporter AUX1. *Plant Physiol.* **148**: 529–535.
- Cho, M., Lee, S.H., and Cho, H.T. (2007). P-glycoprotein4 displays auxin efflux transporter-like action in *Arabidopsis* root hair cells and tobacco cells. *Plant Cell* **19**: 3930–3943.
- Dharmasiri, S., Swarup, R., Mockaitis, K., Dharmasiri, N., Singh, S.K., Kowalchuk, M., Marchant, A., Mills, S., Sandberg, G., Bennett, M.J., and Estelle, M. (2006). AXR4 is required for localization of the auxin influx facilitator AUX1. *Science* **312**: 1218–1220.
- Geisler, M., et al. (2005). Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1. *Plant J.* **44**: 179–194.
- Grebe, M., Friml, J., Swarup, R., Ljung, K., Sandberg, G., Terlou, M., Palme, K., Bennett, M.J., and Scheres, B. (2002). Cell polarity signaling in *Arabidopsis* involves a BFA-sensitive auxin influx pathway. *Curr. Biol.* **12**: 329–334.

- Jones, A.R., Kramer, E.M., Knox, K., Swarup, R., Bennett, M.J., Lazarus, C.M., Leyser, H.M., and Grierson, C.S. (2009). Auxin transport through non-hair cells sustains root-hair development. *Nat. Cell Biol.* **11**: 78–84.
- Kleinboelting, N., Huet, G., Kloetgen, A., Viehoveer, P., and Weisshaar, B. (2012). GABI-Kat SimpleSearch: New features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Res.* **40**: D1211–D1215.
- Kleine-Vehn, J., Dhonukshe, P., Swarup, R., Bennett, M., and Friml, J. (2006). Subcellular trafficking of the *Arabidopsis* auxin influx carrier AUX1 uses a novel pathway distinct from PIN1. *Plant Cell* **18**: 3171–3181.
- Marchant, A., Bhalerao, R., Casimiro, I., Eklöf, J., Casero, P.J., Bennett, M., and Sandberg, G. (2002). AUX1 promotes lateral root formation by facilitating indole-3-acetic acid distribution between sink and source tissues in the *Arabidopsis* seedling. *Plant Cell* **14**: 589–597.
- Moore, R.C., and Purugganan, M.D. (2005). The evolutionary dynamics of plant duplicate genes. *Curr. Opin. Plant Biol.* **8**: 122–128.
- Péret, B., Swarup, R., Jansen, L., Devos, G., Auguy, F., Collin, M., Santi, C., Hocher, V., Franche, C., Bogusz, D., Bennett, M., and Laplace, L. (2007). Auxin influx activity is associated with Frankia infection during actinorhizal nodule formation in *Casuarina glauca*. *Plant Physiol.* **144**: 1852–1862.
- Petrásek, J., and Friml, J. (2009). Auxin transport routes in plant development. *Development* **136**: 2675–2688.
- Petrásek, J. et al. (2006). PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**: 914–918.
- Reinhardt, D. (2003). Vascular patterning: More than just auxin? *Curr. Biol.* **13**: R485–R487.
- Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensperger, K., Bennett, M., Traas, J., Friml, J., and Kuhlemeier, C. (2003). Regulation of phyllotaxis by polar auxin transport. *Nature* **426**: 255–260.
- Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K., and Bennett, M. (2001). Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. *Genes Dev.* **15**: 2648–2653.
- Swarup, R., Kramer, E.M., Perry, P., Knox, K., Leyser, H.M., Haseloff, J., Beemster, G.T., Bhalerao, R., and Bennett, M.J. (2005). Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. *Nat. Cell Biol.* **7**: 1057–1065.
- Swarup, R., et al. (2004). Structure-function analysis of the presumptive *Arabidopsis* auxin permease AUX1. *Plant Cell* **16**: 3069–3083.
- Swarup, K., et al. (2008). The auxin influx carrier LAX3 promotes lateral root emergence. *Nat. Cell Biol.* **10**: 946–954.
- Tursun, B., Cochella, L., Carrera, I., and Hobert, O. (2009). A toolkit and robust pipeline for the generation of fosmid-based reporter genes in *C. elegans*. *PLoS One* **4**: e4625.
- Vandenbussche, F., Petrásek, J., Zádňíková, P., Hoyerová, K., Pesek, B., Raz, V., Swarup, R., Bennett, M., Zazimalová, E., Benková, E., and Van Der Straeten, D. (2010). The auxin influx carriers AUX1 and LAX3 are involved in auxin-ethylene interactions during apical hook development in *Arabidopsis thaliana* seedlings. *Development* **137**: 597–606.
- Vanneste, S., and Friml, J. (2009). Auxin: A trigger for change in plant development. *Cell* **136**: 1005–1016.
- Vieten, A., Sauer, M., Brewer, P.B., and Friml, J. (2007). Molecular and cellular aspects of auxin-transport-mediated development. *Trends Plant Sci.* **12**: 160–168.
- Vieten, A., Vanneste, S., Wisniewska, J., Benková, E., Benjamins, R., Beeckman, T., Luschnig, C., and Friml, J. (2005). Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* **132**: 4521–4531.
- Wisniewska, J., Xu, J., Seifertová, D., Brewer, P.B., Ruzicka, K., Blilou, I., Rouquié, D., Benková, E., Scheres, B., and Friml, J. (2006). Polar PIN localization directs auxin flow in plants. *Science* **312**: 883.
- Yang, H., and Murphy, A.S. (2009). Functional expression and characterization of *Arabidopsis* ABCB, AUX 1 and PIN auxin transporters in *Schizosaccharomyces pombe*. *Plant J.* **59**: 179–191.
- Yang, Y., Hammes, U.Z., Taylor, C.G., Schachtman, D.P., and Nielsen, E. (2006). High-affinity auxin transport by the AUX1 influx carrier protein. *Curr. Biol.* **16**: 1123–1127.
- Young, G.B., Jack, D.L., Smith, D.W., and Saier, M.H. Jr. (1999). The amino acid/auxin:proton symport permease family. *Biochim. Biophys. Acta* **1415**: 306–322.
- Zhou, R., Benavente, L.M., Stepanova, A.N., and Alonso, J.M. (2011). A recombineering-based gene tagging system for *Arabidopsis*. *Plant J.* **66**: 712–723.