Plant embryogenesis requires AUX/LAX-mediated auxin influx

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ABSTRACT

The plant hormone auxin and its directional transport are known to play a crucial role in defining the embryonic axis and subsequent development of the body plan. Although the role of PIN auxin efflux transporters has been clearly assigned during embryonic shoot and root specification, the role of the auxin influx carriers AUX1 and LIKE-AUX1 (LAX) proteins is not well established. Here, we used chemical and genetic tools on Brassica napus microspore-derived embryos and Arabidopsis thaliana zygotic embryos, and demonstrate that AUX1, LAX1 and LAX2 are required for both shoot and root pole formation, in concert with PIN efflux carriers. Furthermore, we uncovered a positive-feedback loop between MONOPTEROS (ARF5)-dependent auxin signalling and auxin transport. This MONOPTEROS-dependent transcriptional regulation of auxin influx (AUX1, LAX1 and LAX2) and auxin efflux (PIN1 and PIN4) carriers by MONOPTEROS helps to maintain proper auxin transport to the root tip. These results indicate that auxin-dependent cell specification during embryonic development requires balanced auxin transport involving both influx and efflux mechanisms, and that this transport is maintained by a positive transcriptional feedback on auxin signalling.

KEY WORDS: Arabidopsis thaliana embryogenesis, Auxin transport, AUX1, LIKE-AUX1 (LAX), MONOPTEROS (ARF5), PIN, Brassica napus, Microspore

INTRODUCTION

Sexually reproductive organisms develop from a single-cell zygote, the product of fertilisation. Divisions of the zygote are precisely controlled in animals and plants and give rise to a population of cells that forms the embryo. In plants, embryos will develop a body plan along a shoot-root axis, containing one or two cotyledons, a shoot apical meristem, a hypocotyl and a root apical meristem. Notably, activation of transcriptional signalling pathways of the plant hormone auxin is pivotal for cellular patterning during embryogenesis (Rademacher et al., 2012; Schiereth et al., 2010; Weijers et al., 2006; Yoshida et al., 2014). However, auxins are not synthesized in all cells (Ljung et al., 2005; Petersson et al., 2009; Robert et al., 2013) and are therefore transported from source to sink tissues by specific influx and efflux proteins (Petrásek and Friml, 2009).

So far, it is assumed that the plasma membrane-localised PIN efflux proteins are responsible and rate-limiting for the directional auxin flow during embryogenesis (Friml et al., 2003; Weijers et al., 2006). Besides genetic and pharmacological evidence, this hypothesis is supported by the observation that auxin controls the direction of its own transport by regulating both the expression and localisation of PIN efflux transporters (Sauer et al., 2006a; Vieten et al., 2005). Recently, spatially and temporally defined foci of auxin production during embryogenesis were discovered to feed back on PIN proteins to regulate their polar localisation towards sink tissues, where auxin signalling triggers specific developmental programs (Robert et al., 2013; Wabnik et al., 2013). Also, indole-3-acetic acid (IAA), the major form of auxin, passively diffuses into the cytosol in its protonated form, suggesting that auxin efflux is the major mechanism for active auxin transport. However, in specific developmental situations, for example during root gravitropic responses (Swarup et al., 2001), lateral organ initiation and outgrowth (Kierzkowski et al., 2013; Swarup et al., 2008) and root hair development (Jones et al., 2008), passive auxin uptake needs to be supported by the amino acid permease-like proteins of the AUX1/LIKE-AUX1 (LAX) family (Bennett et al., 1996; Péret et al., 2012). A detailed analysis of these proteins during early embryogenesis has not been reported. So far, it was only demonstrated that members of the AUX1/LAX family are redundantly required for correct cell organisation in the radicle tip of mature embryos (Ugartechea-Chirino et al., 2009).

Here, we show that AUX1/LAX-dependent auxin influx is needed for cellular patterning from early embryogenesis onward and that the expression of AUX1 and LAX2 is controlled by the MP-BDL signalling pathway. We put forward a model in which auxin influx and efflux systems collaborate to regulate cell specification.

RESULTS

Microspore-derived embryos as a tool to study plant embryogenesis in high throughput

The study of molecular processes during plant embryogenesis is often limited by the relatively low sample numbers typically associated with laborious and technically challenging preparation methods. To overcome this limitation, we tested the potential of microspore-derived in vitro embryos of Brassica napus as an experimental system. Using heat-shock treatments together with specifically adjusted media, microspores isolated from early stage B. napus flowers can be induced to develop into suspensor-like structures, mimicking zygotic embryos (supplementary material Fig. S1) (Joosen et al., 2007; Supena et al., 2008). To test whether the microspore-derived Brassica embryos would respond in a similar way to zygotic Arabidopsis embryos, the effect of a collection of known chemicals on embryo development was investigated. The list consisted of the synthetic auxin analogues...
NAA and 2,4-D, the auxin antagonist PEO-IAA (Hayashi et al., 2008), the cytokinin BA, auxin transport inhibitors NPA and NOA, and chemicals affecting intracellular protein trafficking [brefeldin A (BFA), tyrphostin A23 and wortmannin].

The majority of the microspores cultivated in the presence of 0.1 µM 2,4-D developed into embryos with cotyledon and root pole specification defects (68/82; supplementary material Fig. S2B). In the presence of 1 µM 2,4-D there was an increase in the number of ball-shaped embryos (26/62; supplementary material Fig. S2C). Incubation of developing embryos in 0.1 or 1 µM NAA did not lead to obvious developmental defects, except for a mild increase in the number of ball-shaped embryos (5/67 or 6/71, respectively, as compared with 3/154 for the DMSO control; supplementary material Fig. S2L). In line with the reported role of auxin during Arabidopsis zygotic embryogenesis (Friml et al., 2003), treatments with the auxin antagonist PEO-IAA completely blocked Brassica microspore embryogenesis, even at low concentrations (supplementary material Fig. S2K). The same was observed by adding wortmannin, which is a strong inhibitor of intracellular protein trafficking, to the developing embryo cultures (data not shown). Also, tyrphostin A23, which is an inhibitor of endocytosis, strongly affected microspore embryogenesis (supplementary material Fig. S2L).

Blocking auxin transport by treating the Brassica microspore embryos with the auxin efflux inhibitor NPA induced severe patterning defects. At low concentrations (1 µM), NPA interfered with cotyledon initiation and development (31/62), whereas at higher concentrations (10 µM) it additionally affected apical-basal axis establishment, as demonstrated by the formation of ball-shaped embryos (12/55; supplementary material Fig. S2G,H). Very similar results were obtained with BFA (supplementary material Fig. S2D,F), a fungal toxin that affects intracellular trafficking of the PIN auxin transporters (Geldner et al., 2001). Interestingly, the phenotypes induced by 2,4-D, NPA and BFA resembled those observed in Arabidopsis auxin transport mutants or upon applying the same compounds to Arabidopsis in vitro ovule cultures (Friml et al., 2003). Also, for the cytokinin BA, which was previously shown to enhance the degradation of PIN transporters during Arabidopsis lateral root initiation (Marhavy et al., 2011, 2014) and to repress PIN expression in the Arabidopsis main root meristem (Růžicka et al., 2009), developmental defects were observed that can be related to a deficient auxin transport system. Incubation of developing microspores in 1 µM BA induced the formation of triangular embryos with a strong similarity to the 2,4-D-treated embryos (supplementary material Fig. S2I).

Thus, these observations show that B. napus microspore-derived embryos responded to the tested compounds in a very similar way to Arabidopsis zygotic embryos and suggest that the mechanisms that control auxin transport, which are intensively studied in Arabidopsis, are conserved in B. napus.

Pharmacological and genetic inhibition of auxin influx affects plant embryogenesis

Of all the compounds tested, the results obtained with the auxin influx inhibitor 1-naphthoxyacetic acid (1-NOA) (Parry et al., 2001) were not anticipated, as a role for auxin import during early embryogenesis has not been reported. After adding 1-NOA to microspore embryo cultures, an enhanced frequency of embryos with cotyledon specification and/or root specification defects was observed (60/153, as compared with 21/186 for the DMSO control; Fig. 1A-C; supplementary material Fig. S3). Since it is known that 1-NOA also partially reduces the activity of auxin efflux carriers (Lanková et al., 2010), we performed an NAA complementation treatment. Uptake of the synthetic auxin NAA is independent of auxin influx carriers (Delbarre et al., 1996) and should therefore rescue auxin influx inhibitor-related defects. Interestingly, the 1-NOA/NAA double treatment did not impact the root specification defects, but rather reduced the frequency of embryos of small size or with fused cotyledons (4/88 for NOA/NAA treatment as compared with 23/153 for NOA alone; supplementary material Fig. S3).

Alternatively, we used 2-NOA, which has been shown to inhibit auxin import more specifically (Lanková et al., 2010), and found that 2-NOA mainly affected cotyledon development (26/119; supplementary material Fig. S3). Similar experiments were performed in Arabidopsis ovules cultured on 2-NOA for 3 and 5 days. In contrast to Brassica microspore embryos, for which treatment started after microspore induction, Arabidopsis ovules were cultured starting from later embryonic stages, about 3-4 days after pollination, when the majority of the embryos were at the early globular stage. Arabidopsis ovules treated with 2-NOA produced embryos with weak cotyledon and root phenotypes (7/25=28% for 3-day and 25/60=41.7% for 5-day treatment, as compared with 13/33=3.0% and 5/47=10.6%, respectively, for the DMSO control; Fig. 1G-I). The defects resembled those of Brassica microspore embryos cultured in the presence of NOA, i.e. smaller or fused cotyledons or aberrations at the root pole. In these embryos, the expression of the DR5 auxin response marker (Friml et al., 2003; Ulmasov et al., 1997) was enhanced and more diffuse compared
with DMSO-treated embryos (Fig. 1J-L), possibly because disturbance of auxin influx by application of 2-NOA in post-globular embryos, in which the auxin response maximum is already established in the future root pole (Friml et al., 2003; Wabnik et al., 2013), may lead to a more diffuse auxin distribution (Bainbridge et al., 2008).

Taken together, these experiments show that pharmacological inhibition of auxin influx in *Brassica* microspore embryos and zygotic *Arabidopsis* embryos affects cotyledon development. Moreover, the defects in root development after NOA treatment, which were not rescued by NAA co-treatment, seemed indirectly linked to auxin influx defects and appeared to be a consequence of perturbed auxin distribution that affected auxin signalling. These data identify a novel role for auxin import in embryo patterning during plant embryogenesis, probably by ensuring the proper distribution of embryonic auxin.

**AUX1, LAX1 and LAX2 are redundantly required for *Arabidopsis* embryo development**

To study the putative role of auxin import during embryogenesis in more detail, we first determined which of the four members of the *Arabidopsis* AUX/LAX family of auxin influx carriers are expressed during embryogenesis. Expression analysis using a translational fusion reporter line (*pAUX1::AUX1-YFP*, Swarup et al., 2004), immunolocalisation (*pAUX1::AUX1-HA*; Swarup et al., 2001) and *in situ* mRNA hybridisation revealed a specific *AUX1* expression pattern in the inner cells at the 32-cell embryo stage and later in the provascular cells (Fig. 2A,B; supplementary material Fig. S4A).

A similar expression domain was observed for *LAX2*, using *LAX2* transcriptional (*pLAX2::GUS*, Bainbridge et al., 2008) and translational (*pLAX2::LAX2-Venus*, Pérét et al., 2012) reporter lines and immunolocalisation with a specific anti- *LAX2* antibody (Pérét et al., 2012). *LAX2* was expressed in provascular cells from the 32-cell stage onwards (Fig. 2I,J; supplementary material Fig. S4C,G-I). Additionally, *LAX2* expression was also detected in the hypophysis and the uppermost suspensor cells (Fig. 2I,J; supplementary material Fig. S4H,I). The earliest suspensor-specific *LAX2* expression was detected at the 16-cell stage (Fig. 2G,H; supplementary material Fig. S4B). In contrast to *AUX1* and *LAX2*, *LAX1* was expressed from the 1-cell stage onwards (*pLAX1::GUS*, *pLAX1::LAX1-Venus*; Bainbridge et al., 2008; Pérét et al., 2012). *LAX1* expression was specific to the apical cell and was restricted to the proembryo until the 16-cell stage (Fig. 2C,D; supplementary material Fig. S4D). From the 32-cell stage onwards, it gradually became more pronounced in the upper tier (Fig. 2E; supplementary material Fig. S4E), consistent with its expression in the upper half of heart stage embryos including the cotyledons (Fig. 2F; supplementary material Fig. S4F). No *LAX3* expression could be detected during any stage of embryogenesis (data not shown), consistent with available seed-specific microarray data (Belmonte et al., 2013; Le et al., 2010).

Next, embryo development in the aux1 lax1 Arabidopsis mutants was investigated. No obvious developmental defects were observed in the aux1, lax1, lax2 single mutants or in aux1 lax2 and lax1 lax2 double mutants. Patterning defects in the upper tier as well as in the future root pole could be detected in the aux1 lax1 double mutant, but with low penetrance (6/150=4%; Fig. 3C,M, Table 1). Interestingly, both the frequency (77/349=22.1%) and severity of the defects (Fig. 3) increased significantly in aux1 lax1 lax2 triple-mutant embryos, demonstrating a functional redundancy between members of the AUX/LAX family in mediating embryo development. aux1 lax2 embryos up to the globular stage showed defects in apical-basal axis establishment (11/125=8.8%; Fig. 3D) as manifested by aberrant division of the uppermost suspensor cells, giving the embryos an elongated shape and an unclear boundary between proembryo and suspensor. The most obvious defect observed from the early heart stage was the vertical symmetric instead of horizontal asymmetric division of the hypophysis (21/192=10.9%; Fig. 3E). Older embryos were much more affected and resembled *Brassica* microspore embryos grown in the presence of NOA (Fig. 1). Severe defects affecting both cotyledon and root development were observed (Fig. 3G-K,M-P). Consistently, these phenotypes were also reflected at the seedling stage, with a penetrance between 15 and 30% (Fig. 3Q,R). In line with the absence of *LAX3* expression, no additional effect was observed in aux1 lax1 lax2 lax3 quadruple mutants as compared with aux1 lax1 lax2 triple-mutant embryos, or in lax1 lax2 lax3 as compared with lax1 lax2 mutants (Fig. 3, Table 1). Taken together, these observations show that the differentially expressed auxin influx carriers are involved in embryo development.
Auxin influx and efflux as equivalent partners in the auxin flow towards the future root pole

Based on the expression patterns of \( \text{AUX1} \), \( \text{LAX1} \) and \( \text{LAX2} \) at the globular stage, we speculated that these proteins would contribute to the auxin flow from the future shoot meristem towards the hypophysis or future root pole. To investigate this, expression of the auxin response reporter \( \text{pDR5rev::GFP} \) (Friml et al., 2003) was examined in the \( \text{aux1 lax1 lax2 lax3} \) background. Whereas wild-type embryos accumulate a strong GFP signal at the hypophysis (Fig. 4A), 29.2% \((n=113)\) of \( \text{aux1 lax1 lax2 lax3} \) embryos showed a reduced \( \text{DR5} \) reporter activity (Fig. 4B). These results suggest that \( \text{AUX/LAX} \)-mediated auxin transport contributes to the auxin flow towards the future root pole.

Similar problems in building a strong and focussed auxin signalling maximum in the hypophysis have been seen in \( \text{pin4} \) embryos (Friml et al., 2002) and in embryos with a reversed \( \text{PIN1} \) polarity (Friml et al., 2004). Therefore, we tested the genetic interaction between the \( \text{AUX/LAX} \) and \( \text{PIN1/PIN4} \) genes. The embryonic phenotype was assayed at two locations: at PSB, Ghent, Belgium, and at CEITEC/MU, Brno, Czech Republic. We noticed that the phenotype penetrance was lower in Brno, where the multiple mutants were analysed. Possible reasons for these differences and incomplete penetrance are many, ranging from the quality of soil, water and light to the stability of growth temperature, pest control and watering frequency – especially in view of the susceptibility of auxin production to stress and growth conditions. In supplementary material Table S1, the phenotype percentages are detailed according to the growth location; those presented in the text below were obtained from plants grown in Brno.

In the progeny of \( \text{pin1-201/+} \) plants, 12.5% \((n=353)\) of the embryos showed defects during cotyledon development, whereas root pole defects were only occasionally observed (supplementary material Table S1). In the \( \text{aux1 lax1 lax2} \) background, 8.1% \((n=459)\) of the embryos were affected in cotyledon (1.1%) and/or root pole (7%) formation (supplementary material Table S1). The total frequency of embryo defects in \( \text{pin1-1/+} \) single, \( \text{aux/lax} \) triple and \( \text{pin1/+ aux1 lax1 lax2} \) quadruple mutants was comparable \( [12.5\% \,(n=353), \,8.1\% \,(n=459) \,\text{and} \,14\% \,(n=129), \text{respectively; supplementary material Table S1}] \). Interestingly, the majority of the defective \( \text{pin1 aux1 lax1 lax2} \) embryos had a \( \text{pin1} \)-like phenotype (12.4%), i.e. defects in cotyledon formation rather than root pole.

### Table 1. Summary of embryo phenotypes of \( \text{aux/lax} \) mutant combinations

<table>
<thead>
<tr>
<th>Line</th>
<th>Phenotype</th>
<th>( n )</th>
<th>Embryo defects from late globular stage on (%)</th>
<th>References for the line</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{aux1} )</td>
<td>None</td>
<td>465</td>
<td>0</td>
<td>Bennett et al., 1996</td>
</tr>
<tr>
<td>( \text{lax1} )</td>
<td>None</td>
<td>233</td>
<td>0</td>
<td>Bainbridge et al., 2008</td>
</tr>
<tr>
<td>( \text{lax2} )</td>
<td>None</td>
<td>165</td>
<td>0</td>
<td>Bainbridge et al., 2008</td>
</tr>
<tr>
<td>( \text{aux1 lax1} )</td>
<td>Low penetrance for shoot and root specification defect</td>
<td>150</td>
<td>4</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{aux1 lax2} )</td>
<td>None</td>
<td>360</td>
<td>0</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{aux1 lax2} )</td>
<td>None</td>
<td>187</td>
<td>0</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{aux1 lax2} )</td>
<td>None</td>
<td>205</td>
<td>0</td>
<td>This study</td>
</tr>
<tr>
<td>( \text{aux1 lax1 lax2} )</td>
<td>Enhanced penetrance and strength of ( \text{aux1 lax1} ) phenotypes</td>
<td>349</td>
<td>22.1</td>
<td>Bainbridge et al., 2008</td>
</tr>
<tr>
<td>( \text{aux1 lax1 lax2 lax3} )</td>
<td>No additive effects to ( \text{aux1 lax1 lax2} )</td>
<td>208</td>
<td>23.1</td>
<td>Bainbridge et al., 2008</td>
</tr>
</tbody>
</table>
defects. This suggests that the pin1 mutation rescues the root pole defects in aux1 lax1 lax2 mutant embryos. Similar observations were made using pin4. In pin4-2 single mutant embryos only subtle patterning defects in root and shoot were observed (2.1%, n=140), including premature divisions of the hypophysis daughter cells and vertical divisions of the uppermost suspensor cells (Fig. 4D). However, adding the pin4-2 mutation to the aux1 lax1 lax2 triple mutant rescued the developmental defects from 8.1% (n=459) to 4.9% (n=304) (Fig. 4E,F; supplementary material Table S1). Since PIN4 has been shown to partially compensate for the loss of PIN1 (Vieten et al., 2005), we also generated the quintuple mutant pin1-201/+ pin4-2 aux1 lax1 lax2 and analysed its embryo development. Of embryos from pin1-201/+ pin4 aux1 lax1 lax2 plants, 25.8% displayed defects in cotyledons but only 0.6% in roots (n=1424, 18 plants; Fig. 4H; supplementary material Table S1). In the quadruple pin4 aux1 lax1 lax2 plants, among progeny of the same quintuple mother plant segregating for the pin1-201 mutation, only 4.1% and 1% (n=432) of the embryos displayed cotyledon and root defects, respectively. Furthermore, pin1-201 pin4 aux1 lax1 lax2 seedlings did not have cotyledons and the first true leaves were fused (12.6%, n=1978, seeds from 15 plants; Fig. 4P). Genotype analysis of 34 of these seedlings indicated that this phenotype was present in homozygous pin1 pin4 aux1 lax1 lax2 quintuple mutants. In addition, aberrant cotyledon development was observed in 5.9% of the pin1/+ pin4 aux1 lax1 lax2 seedlings (Fig. 4O compared with pin1 in Fig. 4K,L), which is a similar proportion to that in the pin4 aux1 lax1 lax2 population (from plants segregating for the pin1-201 mutation) (4.4%, n=424, seeds from four plants; Fig. 4M,N; supplementary material Table S2). Notably, the root phenotype observed in aux1 lax1 lax2 seedlings (4.8%, n=289; Fig. 4J) was gradually rescued when the pin mutations were introduced: 0.6% (n=349) in pin4 aux1 lax1 lax2, 0.5% (n=554) in pin1/+ aux1 lax1 lax2 and 0.1% (n=1978) in pin1-201 pin4 aux1 lax1 lax2 (supplementary material Table S2).

Taken together, these genetic analyses show that the different aux/lax and pin mutations have additive effects on cotyledon formation and thus indicate that both auxin influx and efflux carriers play a role in this auxin transport-dependent developmental event. However, re-equilibrating auxin transport towards the root pole in aux/lax mutants by introducing mutations in efflux carriers rescued the root defects in these embryos, suggesting that proper auxin signalling for root pole specification might be restored in the quintuple mutant.

**AUX/LAX-mediated auxin import is controlled by the MP-BDL signalling pathway**

The defects in the aux/lax triple-mutant embryos strongly resembled those reported for the auxin response mutants monopteros (mp) and bodenlos (bdl) (Berleth and Jürgens, 1993; Hamann et al., 1999). MP (also known as ARF5) is an auxin-responsive transcription factor that regulates auxin-dependent gene expression, while BDL (also known as AUX1/IAA12) is an auxin-degradable repressor of MP activity. Using qRT-PCR, expression of the auxin influx carrier was analysed in seedlings expressing an inducible auxin-insensitive bdl mutant protein (Schlereth et al., 2010). In pRPS5A::bdl-GR seedlings, the expression of AUX1, LAX2 and PIN1 was less strongly induced than in the control (pRPS5A::BDL-GR, expressing the wild-type BDL gene) after 1 h DEX/NAA co-treatment (supplementary material Fig. S5A), whereas the expression of LAX1 was less affected by this treatment. In line with this, AUX1 and LAX2 expression levels were reduced in the mp mutant, while LAX1 expression was reduced but to a lesser extent (supplementary material Fig. S5A). To confirm the embryonic transcriptional regulation of AUX1, LAX1 and LAX2 by MP, transgenic (LAX1, LAX2) and translational (AUX1) reporters were introduced into the mp strong mutant allele background (mp3146). Whereas LAX1 expression was unaffected (Fig. 5D), that of AUX1 and LAX2 was reduced or absent in mp embryos (Fig. 5E,F).

Next, the genetic interaction between MP and the AUX/LAX genes was tested by generating multiple mutants. All mutants were screened for the frequency of mp-like defects during embryogenesis, as well as for the percentage of rootless seedlings. In both cases we observed that adding aux1, aux1 lax2 or the aux1 lax1 lax2 mutations to the incompletely penetrant mp allele mp3149 both qualitatively and quantitatively enhanced the mp phenotype (Fig. 5J; supplementary material Table S3). Based on these results we conclude that AUX1 and LAX2 act downstream of the MP-BDL signalling pathway.

Similar transcriptionsal regulation of the PIN1 auxin efflux carrier by the MP-BDL pathway has been demonstrated previously (Weijers et al., 2006). Since we showed that both auxin influx and efflux systems work together during embryo development, we examined the...
extent to which they contribute to MP-mediated embryonic root formation. Both the strong (mpB4149) and weak (mpS319) mp alleles were transformed with pMP::AUX1, pMP::LAX2 or pMP::PIN1 constructs. The mp+/T2 segregating lines were screened for the frequency of rootless seedlings (supplementary material Table S4). None of the constructs affected the percentage of rootless seedlings in the mpB4149 background: 25.7±3.7% in mp/+ pMP::AUX1 (28 lines), 25.5±4.8% in mp/+ pMP::LAX2 (31 lines) and 23.2±5% in mp/+ pMP::PIN1 (13 lines), as compared with 24.9±2.6% in mpB4149/+.

However, in the mpS319 background, MP promoter-driven expression of the auxin transporters enhanced the frequency of rootless seedlings: 9.2±3.3% in mp/+ pMP::AUX1 (33 lines), 13.7±6.5% in mp/+ pMP::LAX2 (29 lines) and 9.6±4.3% in mp/+ pMP::PIN1 (28 lines), as compared with 4.6±1.6% in mpS319/+.

Given the cooperative role of auxin influx and efflux carriers, we anticipated that adding one auxin transport component is insufficient to complement the mp mutation and would moreover disturb the affected system even more. To test this hypothesis, mp/+ pMP::AUX1 and mp/+ pMP::LAX2 were crossed to mp/+ pMP::PIN1 and F1 progenies were analysed (supplementary material Table S5 and S6). F1 progeny of the crosses mpB4149/+ pMP::AUX1×mpB4149/+ pMP::PIN1 and mpB4149/+ pMP::LAX2×mpB4149/+ pMP::PIN1 produced a similar proportion of rootless seedlings as the control cross (supplementary material Table S5). When the same experiment was performed in the mpS319 background, the same trend was observed in F1 crossed seedlings and individual lines, i.e. an enhancement of the penetrance of mpS319 rootless phenotypes (supplementary material Table S6). We conclude that reconstituting either AUX1 or LAX2 and PIN1 expression in the provascular expression domain of mp embryos is not sufficient to rescue the mpB4149 rootless phenotype, and even enhances mpS319 defects.

Altogether, these experiments showed that PIN1, AUX1 and LAX2 are under transcriptional control of the MP-BDL auxin-dependent signalling pathway, which is in line with earlier results for LAX2 (Schlereth et al., 2010). Genetic data suggest that the auxin transport machinery acts downstream of the MP-BDL signalling pathway for root pole formation. However, our attempts to rescue mp mutations by ectopic expression of auxin transport proteins indicated that restoring proper auxin transport machinery to the root pole is not sufficient to rescue impaired root development in these mutants.

**DISCUSSION**

While testing the utility of *B. napus* microspore embryos as a high-throughput assay system, we uncovered an unexpected role for auxin influx during early embryo development – unexpected, because auxin import during embryogenesis has been studied previously and only a role in mature embryos was reported (Ugartechea-Chirino et al., 2009). More precisely, it was demonstrated that aux1 lax mutants have a larger radicle root cap along with aberrant cellular organisation of the root tip. By investigating mature embryos only, the earlier role of AUX1/LAX family proteins has probably been overlooked. By performing transcriptional and genetic analyses in *A. thaliana* from fertilisation onwards, we demonstrated that all three auxin importers AUX1, LAX1 and LAX2 have specific expression patterns during early zygotic embryogenesis and that they act redundantly to specify embryonic root and shoot pole identity and development.

**Brassica microspore embryos as a model to study Arabidopsis embryogenesis**

Inhibiting auxin influx in *Brassica* embryos using 2-NOA affected cotyledon development. Because 1-NOA has been reported to affect efflux transport (Lanková et al., 2010) and since cellular uptake of NAA is independent of auxin influx carriers (Delbarre et al., 1996), a 1-NOA/NAA co-treatment was performed to dissect the actual effect of 1-NOA on auxin influx. This 1-NOA/NAA co-treatment confirmed the more specific action of 2-NOA and thus indicates that auxin influx in *Brassica* microspore embryos is mainly important for cotyledon development.

Switching from the *Brassica* system to *Arabidopsis* zygotic embryos, we were confronted with some phenotypic differences. Whereas *Brassica* microspore embryos treated with auxin import inhibitors were mainly affected in cotyledon development, *Arabidopsis* aux1/lax mutant embryos were mainly affected in root pole formation and to a lesser extent in cotyledon development. However, given the similarity of the phenotypes and the rescue of the 1-NOA-dependent cotyledon phenotype by NAA treatment, these observations suggest that the defects in cotyledon development are specifically related to disturbed auxin influx machinery. Several reasons for the differences in *Brassica* and *Arabidopsis* phenotypes can be proposed: (1) the different genetic backgrounds; (2) the differences between pharmacological and genetic disturbance of auxin import action; (3) differences in developmental context regarding zygotic embryo development in the presence of sporophytic ovule tissue and endosperm versus the *in vitro* microspore context from which the *Brassica* embryos developed. Despite the phenotypic differences, the *B. napus* microspore embryo system provided an important initial indication for a role for auxin import in *Arabidopsis* embryo development. Moreover, the fact that we observed the expected phenotypic output of treatments with a palette of other compounds affecting various cellular processes shows that the *B. napus* system can be used for the pharmacological study of embryo development.

**A cooperative role of auxin influx and efflux in embryogenesis**

In 16-cell-stage *Arabidopsis* embryos the PIN1 auxin transporter switches from an apolar to a polar localisation (Friml et al., 2003; Robert et al., 2013; Wabnik et al., 2013). Correspondingly, a directed auxin flow from the future shoot meristem towards the root...
pole is activated (Friml et al., 2003, 2004; Wabnik et al., 2013). Here, we showed that the expression of the auxin influx carrier AUX1 is first detected in the central cells of a 32-cell stage embryo, together with LAX2 expression from the 32-cell stage onwards. Given their spatial localisation pattern and their reported auxin import activity, we hypothesized that AUX1/LAX-mediated influx provides auxin to the provasculature for PIN1-mediated directional auxin flow towards the root pole (Fig. 6). Indeed, mutating AUX1 and LAX2 together with the functionally redundant LAX1 importer revealed clear defects in root pole formation and to a lesser extent in cotyledon development. Also, the DR5 auxin response marker was decreased in the root pole of aux1 lax1 lax2 lax3 mutant embryos, suggesting a role for auxin influx in shoot pole-to-root pole auxin transport, as previously speculated (Spitzer et al., 2009). Interestingly, mutations in the PIN1 auxin efflux carrier showed opposite developmental defects: mainly cotyledon defects and fewer defects at the root pole.

Combining mutations in both auxin efflux and influx carriers using quadruple and quintuple mutants would intuitively suggest stronger defects in both cotyledon and root pole developmental programs. However, whereas the pin1 aux1 lax1 lax2 quadruple mutant showed increased penetrance of the cotyledon phenotype, it unexpectedly showed fewer defects in root development compared with aux1 lax1 lax2. An explanation might be found in the tissue-specific expression of the auxin transporters. While PIN1, PIN4, AUX1, LAX2 are expressed in provascular cells, both LAX1 and PIN1 are also expressed in protoderm cells. In the protoderm layer, auxin is channeled from the suspensor to the cotyledon tips. Hence, in addition to a disturbed transport in the inner cells, upward auxin transport in the protoderm might also be affected in pin1/aux1/lax quadruple and quintuple mutants. We hypothesize that perturbing auxin transport in the protoderm might result in increased auxin accumulation in the suspensor and decreased auxin delivery to the apical regions, which would explain both the root phenotype rescue in pin1 aux1 lax1 lax2 embryos and the enhanced frequency of cotyledon defects.

**A positive transcriptional feedback of auxin signalling on auxin transport for root development**

The MP-BDL-mediated signalling pathway has repeatedly been shown to be crucial for hypocotyl specification and hence root pole formation. MP expression in the AUX1/LAX2/PIN1 expression domain drives the expression of both influx and efflux carriers, AUX1, LAX2 and PIN1, which are needed to establish the essential auxin response maximum in the root stem cell niche (Fig. 6). The close relationship between auxin transport and auxin signalling is illustrated by the similarities in the mp and aux1 lax1 lax2 mutant phenotypes. Therefore, we investigated the effect of restoring the expression of one or more auxin transport components in the mp mutant background. However, none of the combinations led to the reactivation of one of the auxin components in the mp background would intuitively lead to either a small rescue of the mp phenotype or no change in phenotype, the transformed mp<sup>319</sup> mutants in fact showed an enhanced frequency of the mp phenotype, suggesting that the constructs enhanced the mp-related defects. We hypothesize that fully operational and balanced auxin transport in the embryo requires both PIN and AUX/LAX components. This conclusion is consistent with recent findings in *Arabidopsis* root apical tissues (Band et al., 2014). In mp embryos, PIN1-dependent efflux and AUX1/LAX2-dependent influx machinery is not functional (Fig. 5) (Weijers et al., 2006). Adding only one component, i.e. PIN or AUX/LAX, would then disturb the defective system even more. This can be compared to a traffic jam: if an obstruction blocks the road, opening the entrances upstream of the obstruction would only make the traffic jam worse.

**Concluding remarks**

In this study, we showed the importance of auxin influx machinery for embryo development. In our attempts to uncouple the roles of the AUX/LAX proteins in cotyledon and in root development, we also identified a transcriptional feedback loop in which the MP-BDL auxin signalling pathway regulates *AUX1* and *LAX2* expression in the inner embryonic cells. Interfering with auxin transport from its source in the shoot apical meristem to the future root meristem resulted in aberrant root development. We also identified a cooperative role of auxin influx (LAX1) and efflux (PIN1) in cotyledon specification. Together, these results support the requirement of coordinated auxin influx and efflux for proper embryo development.

**MATERIALS AND METHODS**

**Plant material and growth conditions**

*A. thaliana* seeds were sterilised with chlorine gas, plated on half-strength Murashige and Skoog (MS) medium (pH 5.7) containing 1% sucrose, 0.01% myo-inositol, 0.05% MES and 0.8% agar, stored for 2 days at 4°C,
and grown vertically at 21°C under continuous light. Two weeks after germination, seedlings were transferred to soil and grown at 21°C under long-day conditions.

The following lines were described previously: aux1-21, lax1, lax2, aux1 lax1 lax2, aux1 lax1 lax2 lax3, pin1-201, pin4-2, pin4-3, mp3419, mp5319, pDR5rev::GFP, pLAX1:–LAX1-VENUS, pLAX2:–LAX2-VENUS, pLAX1::GUS, pLAX2::GUS, pLAX3::GUS, pAUX1::AUX1-IAA, pAUX1::AUX1-YFP, pRPS5A:–BDL-GR and pRPS5A:–bdl-GR (Bainbridge et al., 2008; Bennett et al., 1996; Cole et al., 2009; Friml et al., 2003; Furutani et al., 2004; Marchant et al., 1999; Péret et al., 2012; Sauer et al., 2006b; Swarup et al., 2004; Weijers et al., 2004; Marchant et al., 2004; Marchant et al., 1999; Péret et al., 2012; Swarup et al., 2004; Weijers et al., 2006). The following lines were generated by crossing: aux1 lax1, aux1 lax1 lax2, aux1 lax1 lax2 lax3, pDR5rev:GFP aux1 lax1 lax2 lax3 (generated by crossing pDR5rev::GFP aux1 lax1 lax2 and aux1 lax1 lax2 lax3 (Bainbridge et al., 2008)), pin1-201 aux1 lax1 lax2, pin4-2 aux1 lax1 lax2, pin4-3 aux1 lax1 lax2, pin4-3 aux1 lax1 lax2, pin1-201 pin4-2 aux1 lax1 lax2, mp3419 aux1 lax1 lax2.

### Generation and analysis of ectopic expression of PIN1, AUX1 and LAX2 in the mp background

The MP promoter from the pJet_MP-promoter (kindly provided by the Dolf Weijers lab) vector was cloned into pDONR2P41 (Karimi et al., 2007a) using primers attB4_MP_FOR and attB1R_MP_REV (see supplementary material Table S7). PIN1, AUX1 and LAX2 genomic DNA was amplified from Arabidopsis seedlings using primers (see supplementary material Table S7), and Gateway cloned in pDONR221 (Karimi et al., 2007b). Using a multisite Gateway reaction, PIN1, AUX1 and LAX2 entry clones were combined with the MP promoter into pHm24GW (Karimi et al., 2007a). Heterozygous mp5319 and mp5319 plants were transformed by the floral dip method. T1 transformants were selected for the presence of mp5319 and mp5319 mutations. Hemizygous T2 lines were analysed for mp-like phenotype penetrance. A selection of lines was tested for overexpression of the transgenes by qRT-PCR (see below), and grown to obtain T3 homozygous hemizygous lines. T3 lines were scored. Heterozygous mp5319 and mp5319 plants were transformed by the floral dip method. T1 transformants were selected for the presence of mp5319 and mp5319 mutations. Hemizygous T2 lines were analysed for mp-like phenotype penetrance. A selection of lines was tested for overexpression of the transgenes by qRT-PCR (see below), and grown to obtain T3 homozygous hemizygous lines. T3 lines were scored. Heterozygous mp5319 and mp5319 plants were transformed by the floral dip method. T1 transformants were selected for the presence of mp5319 and mp5319 mutations. Hemizygous T2 lines were analysed for mp-like phenotype penetrance. A selection of lines was tested for overexpression of the transgenes by qRT-PCR (see below), and grown to obtain T3 homozygous hemizygous lines. T3 lines were scored.

### Microspore-induced embryogenesis and chemical treatments

The generation of embryos using double-haploid B. napus cv Topas DH4079 microspores was performed according to Supena et al. (2008). After collecting the microspores, a heat shock of 22 h at 32°C in the dark was applied using a microscope density of 40,000 per ml. Microspores were distributed in 24-well plates (500 µl per well) and incubated at 25°C in the dark. Five days after the heat shock, when the microspores had a swollen appearance, compounds (or mock) were added in 500 µl medium, bringing the total volume to 1 ml per well. This stage was selected to overcome a putative negative effect of the compounds on the microspore embryo induction program. Microspore embryogenesis was evaluated daily using light microscopy and phenotypes were quantified 7 days after treatment. Embryos ranged from globular to torpedo stages. The compounds used were: NAA (α-naphthaleneacetic acid; Duchefa), 2,4-D (2,4-dichlorophenoxyacetic acid; Sigma), PEO-IAA (α-phenylethyl-2-one)-indole-3-acetic acid; a gift from Ken-Ichiro Hayashi, Okayama University of Science, Japan), BA (6-benzylaminopurine; Sigma), NPA (N-1-naphthylphthalamic acid; Sigma), 1-NOA (1-naphthoxyacetic acid; Sigma), 2-NOA (2-naphthoxyacetic acid; Sigma), brefeldin A (Sigma), wortmannin (Sigma), tyrphostin A23 (Sigma) and tyrphostin A51 (Sigma). Stock solutions were diluted in DMSO.

### In vitro culture of Arabidopsis embryos

Culture of embryos was carried out as previously described (Sauer and Friml, 2008). Culture medium was supplemented with 10 µM 2-NOA in DMSO, or the equivalent amount of DMSO as a solvent control. Seeds originating from siliques 3 and 4, counting from the first dehiscent flower, were cultured for 6 days. Embryos were extracted from the seeds for microscopy analysis. Only embryos from healthy seeds were analysed.

### Histological analyses, in situ hybridisation and microscopy

For GUS staining, embryos were dissected out of the seeds in 90% acetone. For embryos younger than the globular stage, seeds were opened but the embryos were not dissected. After dissection, seeds and embryos were transferred to sieves (BD Falcon, cell strainer 40 µm nylon) and were incubated under vacuum for 10 min in 90% acetone. Subsequently, three washing steps were performed under vacuum for 10 min each with 0.5 M phosphate buffer [Na2HPO4/NaH2PO4 (615/385), pH 7]. Sieves were then transferred to GUS staining solution [1 mM X-Glu dissolved in 0.5% (v/v) DMFO, 0.5% (v/v) Triton X-100, 1 mM EDTA, 0.5 mM K3Fe(CN)6, 0.5 mM K4Fe(CN)6, 0.5 M phosphate buffer pH 7] and incubated for 1 h under vacuum. After vacuum infiltration, samples were incubated at 37°C. The staining reaction was stopped by two washes with 0.5 M phosphate buffer under vacuum for 10 min each. Embryos were transferred to slides, mounted with 10% glycerol and analysed with a DIC fluorescence microscope (Olympus).

### Immunofluorescence analyses of Arabidopsis embryos

For confocal imaging, embryos were fixed in 3% PFA in PBS buffer (pH 7.4) and prepared on slides as described (Sauer et al., 2006b). Embryos were rehydrated in water, and when indicated stained for 2 h in SCRi Renaissance 2200 (Renaissance Chemicals; 2% in 4% DMSO/water solution), washed twice in water and mounted with an anti-fading solution.

For Arabidopsis in vitro-cultured embryos, GFP was visualised after dissection from the seed in a 5% glycerol/water solution.

For embryo phenotype analyses, embryos were cleared at the indicated stages in a chloral hydrate solution [chloral hydrate/water/glycerol (8/3/1, v/v/v)]. Whole-mount in situ hybridisation of embryos was carried out as previously described (Hejátko et al., 2006) using a full-length AUX1 RNA probe (see supplementary material Table S7 for primer sequences). Embryos were analysed by clearing seeds in chloral hydrate.

### RNA extraction, cDNA synthesis and qRT-PCR analysis

The experiment was set up according to Schlereth et al. (2010), pRPS5A::BDL-GR, pRPS5A::bdl-GR, mp3419 and Col seedlings were grown for 5 days, treated in liquid medium with 10 µM dexamethasone (DEX; Santa Cruz) for 1 h, then co-treated with 10 µM NAA and 10 µM DEX for 1 h, 2 h or 4 h. Presented data are from 1 h co-treatment. For RNA extraction, whole seedlings were ground in liquid nitrogen and total RNA was isolated with Trizol (Invitrogen) according to the manufacturer’s instructions. Poly(dT) cDNA was prepared from 2 µg total RNA with Superscript III reverse transcriptase (Invitrogen) and quantified on an LightCycler 480 apparatus (Roche Diagnostics) using the SYBR Green I Master Kit (Roche Diagnostics) according to the manufacturer’s instructions. All individual reactions were carried out in triplicate. Primers are listed in supplementary material Table S7. Data were analysed with gBase (Hellemans et al., 2007). Expression levels of AUX1, LAX1, LAX2, LAX3 and PIN1 were normalised to those of EEF1a4 and CDK4, which showed no clear systematic changes in Ct values. Data from BDL-GR were compared with bdl-GR and data from mp were compared with Col.

### Statistical analysis

Statistical analysis was performed by contingency table χ² tests. Details are provided in supplementary material Tables S1-S6.


