Plant embryogenesis requires AUX/LAX-mediated auxin influx

Hélène S. Robert1,2,*, Wim Grunewald1,*, Michael Sauer3,4, Bernard Cannoot1, Mercedes Soriano5, Ranjan Swarup5, Dolf Weijers7, Malcolm Bennett6, Kim Boutilier5 and Jiří Friml1,2,8,†

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 embryo 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 (Růžička 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 1/33=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

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Fig. 1. Auxin influx is required for proper embryogenesis. (A-C) Brassica napus microspore embryos treated with 1-NOA (B,C) displayed fused cotyledons (B) and aberrant root development (C) phenotypes. (D-F) Arabidopsis thaliana zygotic embryos of wild type (WT; D) and aux1 lax1 lax2 triple mutants (E,F) at heart stage. The mutant phenotypes resemble those of B. napus microspore embryos cultured in the presence of 1-NOA. (G-L) A. thaliana zygotic embryos cultured in the presence of 2-NOA (H,I,K,L) displayed fused cotyledons (H,K) and aberrant root development (I,L) phenotypes. Light (G-I) and fluorescence (J-L) images are shown of the pDR5rev::GFP signal in the same embryos. DMSO-treated controls are shown in A,G,J. Scale bars: 50 μm in A-C; 20 μm in D-L.
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 procambial 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; Péret et al., 2012) reporter lines and immunolocalisation with a specific anti-LAX2 antibody (Péret et al., 2012). LAX2 was expressed in procambial 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; Péret 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 aux/lax *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 lax1 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 AUX1, LAX1 and 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 pDR5rev::GFP (Friml et al., 2003) was examined in the aux1 lax1 lax2 lax3 background. Whereas wild-type embryos accumulate a strong GFP signal at the hypophysis (Fig. 4A), 29.2% (n=113) of aux1 lax1 lax2 lax3 embryos showed a reduced DR5 reporter activity (Fig. 4B). These results suggest that 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 pin4 embryos (Friml et al., 2002) and in embryos with a reversed PIN1 polarity (Friml et al., 2004). Therefore, we tested the genetic interaction between the AUX/LAX and 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 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 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 pin1/+ single, aux/lax triple and pin1/+ aux1 lax1 lax2 quadruple mutants was comparable (12.5% (n=353), 8.1% (n=459) and 14% (n=129), respectively; supplementary material Table S1). Interestingly, the majority of the defective pin1 aux1 lax1 lax2 embryos had a pin1-like phenotype (12.4%), i.e. defects in cotyledon formation rather than root pole formation.
quintuple mother plant segregating for the (Vieten et al., 2005), we also generated the quintuple mutant. PIN4 has been shown to partially compensate for the loss of PIN1. Of embryos from pin1/+ pin4 aux1 lax1 lax2 seedlings, the expression of PIN1 auxin efflux carrier was reduced 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, translational (AUX1, LAX2) and translatable reporters were introduced into the mp mutant allele background (mpB4149). 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 mpS319 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 transcriptional 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 \( mp^{B4149} \) and weak \( mp^{S319} \) \( mp \) 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 \( mp^{B4149} \) 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 \( mp^{B4149/+} \). However, in the \( mp^{S319} \) 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 \( mp^{S319/+} \).

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 \( F1 \) progeny were analysed (supplementary material Table S5 and S6). \( F1 \) progeny of the crosses \( mp^{B4149/+} \) \( pMP::AUX1 \times mp^{B4149/+} \) \( pMP::PIN1 \) and \( mp^{B4149/+} \) \( pMP::LAX2 \times mp^{B4149/+} \) \( pMP::PIN1 \) produced a similar proportion of rootless seedlings as the control cross (supplementary material Table S5). When the same experiment was performed in the \( mp^{S319/+} \) background, the same trend was observed in \( F1 \) crossed seedlings and individual lines, i.e. an enhancement of the penetrance of \( mp^{S319/+} \) 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 \( mp^{B4149/+} \) rootless phenotype, and even enhances \( mp^{S319/+} \) 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 provascular 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 channelled from the suspensor to the cotyledons. Hence, in addition to a disturbed transport in the inner cells, upward auxin transport in the protoderm might also be affected in pin/aux/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 hypophysis 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 a reduced frequency of mp-like defects or to a reduction in mutant phenotypes. The most plausible explanation is that reactivation of auxin transport is not able to activate the expression of the MP target genes TARGET OF MONOPTEROS 5 (TMO5) and TMO7, which have been shown to be involved in MP-controlled root pole development (Schlereth et al., 2010).

Interestingly, although 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

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**Fig. 6. Model for balanced and regulated auxin transport during embryo development.** Local auxin production occurs in the two opposite poles of the embryo: in the suspensor and the shoot apical meristem. Auxin is transported by PIN1 and LAX1 from the suspensor to the tips of the cotyledons via the protoderm. Auxin is transported by efflux (PIN1 and PIN4) and influx (AUX1 and LAX2) transporters from the shoot apical meristem to the root meristem, where it accumulates and triggers auxin signalling. From the root meristem, auxin is transported away from the embryo by PIN7. Transcriptional feedback involving MP (dashed arrow) regulates expression of PIN1, AUX1 and LAX2 in the inner embryonic cells.

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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, then cultured as described above.
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, pin-1, pin-2, pin-3, mpS319, mpS319, pDR5rev::GFP, pLAX1::LAX1-VENUS, pLAX2::LAX2-VENUS, pLAX1::GUS, pLAX2::GUS, pLAX3::GUS, pAUX1::AUX1-HA, pAUX1::AUX1-YFP, pRPS5A::BDL-GR and pRPS5A::BDL-GR (Bainbridge et al., 2008; Bennett et al., 1999; Cole et al., 2009; Friml et al., 2003; Furutani 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 lax2, lax1 lax2, lax1 lax2 lax3, pDR5rev::GFP aux1 lax1 lax2 [generated by crossing pDR5rev::GFP aux1 lax1 lax2 aux1 lax1 lax2 lax3 (Bainbridge et al., 2008)], pin-1 pin-2 aux1 lax1 lax2, pin-2 aux1 lax1 lax2 pin-4 aux1 lax1 lax2, pin-4 aux1 lax1 lax2 pin-4 aux1 lax1 lax2, mpS319 aux1 lax1 lax2.

**Generation and analysis of octopine expression of PIN1, AUX1 and LAX2 in the mp background**

The MP promoter from the pet MP-promoter (kindly provided by the Dolf Weijers lab) vector was cloned into pDONRP41PR (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 pH7m24GW (Karimi et al., 2007a). Heterozygous mpS319 and mpS319 plants were transformed by the floral dip method. T1 transformants were selected for the presence of mpS319 and mpS319 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 confirm T3 homozygous hemizygous lines. T3 lines were distributed in 24-well plates (500 µl per well) and incubated at 25°C for 6 days. Embryos were taken from the seeds for embryo induction program. Microspore embryogenesis was evaluated over a putative negative effect of the compounds on the microspore development. T1 transformants were selected for the presence of the transgenes by qRT-PCR (see below), and grown to obtain T3 lines. T3 lines were combined with the MP promoter into pH7m24GW (Karimi et al., 2007a). Heterozygous mpS319 and mpS319 plants were transformed by the floral dip method. T1 transformants were selected for the presence of mpS319 and mpS319 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 confirm T3 homozygous hemizygous lines. T3 lines were distributed in 24-well plates (500 µl per well) and incubated at 25°C for 6 days. Embryos were collected 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 (615/358), 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 were performed as previously described (Sauer et al., 2006b) using mouse anti-HA (1:600; 5B1D10, catalog number 32-6700, Life Technologies), rabbit anti-LAX2 (1:200; Péret et al., 2012), mouse anti-GFP (1:600; clone GFP-20, G6539, Sigma), Cy3-conjugated goat anti-mouse (1:600; polyclonal, C2181, Sigma) and anti-rabbit (1:600; polyclonal, C2306, Sigma), and Alexa488-conjugated goat anti-mouse (1:600; A-11001, Life Technologies). Nuclei were stained with DAPI (1 µg/l in water; Sigma).

For GFP visualisation, embryos were fixed in 4% 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 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 phenotyping, embryos were cleared at the indicated stages in a chloral hydrate solution [chloral hydrate/water/glycerol (8/3/1, w/v)].

Whole-mount in situ hybridisation of embryos was carried out as previously described (Hejátó 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.

Confocal imaging was performed on Zeiss Exciter 5, 710 and 780 confocal laser scanning microscopes using 405 nm (DAPI, Renaissance), 488 nm (YFP, GFP, Alexa488) and/or 543 nm (Cy3) excitation filters with 420-480 nm band pass (DAPI, Renaissance), 505-530 nm band pass (GFP, YFP, Alexa488) and 560 nm long-pass (Cy3) emission filters. Acquisition with multiple channels was performed by sequential scanning. Microscopy observations were performed on a DIC fluorescence microscope (Olympus). Images were processed in Adobe Photoshop CS and assembled in Adobe Illustrator CS.

**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, mpS319 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 a 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 qBase (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 Col-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.


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Competing interests
The authors declare no competing or financial interests.

Author contributions
W.G., H.S.R. and J.F. designed the project. W.G. performed the Brassica microprobe embryo related work, cloning and generation of the MP complementation lines. W.G. and H.S.R. performed expression analysis of the reporter lines. Mi.S. was funded by the Ramo and methods. W.G. and H.S.R. prepared the manuscript. Mi.S., M.B., D.W., Me.S., K.B. and J.F. edited the manuscript prior to submission.

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Supplementary material available online at CEITEC – Central European Institute of Technology.

References


