Auxin controls petal initiation in Arabidopsis

Edwin R. Lampugnani*, Aydin Kilinc and David R. Smyth‡

SUMMARY
Floral organs are usually arranged in concentric whorls of sepals, petals, stamens and carpels. How founder cells of these organs are specified is unknown. In Arabidopsis, the PETAL LOSS (PTL) transcription factor functions in the sepal whorl, where it restricts the size of the inter-sepal zone. Genetic evidence suggests that PTL acts to support a petal initiation signal active in the adjacent whorl. Here we aimed to characterise the signal by identifying enhancers that disrupt initiation of the remaining petals in ptl mutants. One such enhancer encodes the auxin influx protein AUX1. We have established that auxin is a direct and mobile petal initiation signal by promoting its biosynthesis in the inter-sepal zone in ptl mutant plants and restoring nearby petal initiation. Consistent with this, loss of PTL function disrupts DR5 expression, an auxin-inducible indicator of petal-initiation sites. The signalling network was extended by demonstrating that: (1) loss of RABBIT EARS (RBE) function apparently disrupts the same auxin influx process as PTL; (2) the action of AUX1 is supported by AXR4, its upstream partner in auxin influx; (3) polar auxin transport, which is controlled by PINOID (PID) and PIN-FORMED1 (PIN1), functions downstream of PTL; and (4) the action of pmd-1d, a dominant modifier of the ptl mutant phenotype, is dependent on auxin transport. Thus, loss of PTL function disrupts auxin dynamics, allowing the role of auxin in promoting petal initiation to be revealed.

KEY WORDS: AUX1, Auxin, Petal development, PETAL LOSS, Polar auxin transport, Arabidopsis

INTRODUCTION
The floral blueprint is established early in flower development (Endress, 2011; Smyth, 2005). The number and location of floral organs depends on the specification of founder cells in the flower meristem (Chandler, 2011). These cells are first destined to grow into organ primordia, which later differentiate appropriately as sepals, petals, stamens or carpels. The identity of each is defined by combinations of well-known transcription factor genes (Krizek and Fletcher, 2005). However, the mechanism by which founder cells are specified is not known.

By contrast, signals involved in defining the sites of origin of leaves that arise from the shoot apical meristem, phyllotaxy, and of flowers from the inflorescence meristem, are beginning to be characterised. Auxin plays a key role in this process. It is sufficient to define the sites of leaf and flower primordia, as its exogenous application sets their development in train (Reinhardt et al., 2000). Disruption of its biosynthesis results in loss of most floral organs (Cheng et al., 2006; Stepanova et al., 2008; Tobeña-Santamaria et al., 2002). In Arabidopsis, auxin is apparently accumulated in the epidermal (L1) layer by the auxin influx protein AUX1 and transported in this layer towards the sites of organ initiation by the polar auxin transporter PIN-FORMED1 (PIN1) (Reinhardt et al., 2003). This process is positively regulated by auxin concentration, so that foci of increased auxin are reinforced (Heisler et al., 2005). Thus new primordia act as auxin sinks, and reduce the surrounding auxin concentration so that the next primordium arises at the furthest distance from pre-existing primordia where the auxin concentration remains relatively high. As a primordium continues to grow, auxin flow reverses and it moves away from the primordium (Heisler et al., 2005), and into the underlying provascularature (Reinhardt et al., 2003). Later it acts at the apex of the primordium itself (Benková et al., 2003). The cycle of auxin concentration and depletion radially around the meristem is sufficient to define the phyllotactic pattern of leaf and flower initiation (Jönsson et al., 2006; Smith et al., 2006).

Other factors are also involved. Loosening of cell walls by the application of expansin or pectin methyl esterase can promote primordium initiation from shoot and inflorescence meristems (Fleming et al., 1997; Peaucelle et al., 2008). Disruption of intrinsic physical forces within meristems by regional laser ablation also influences phyllotaxy (Hamant et al., 2008), a process linked with auxin dynamics (Heisler et al., 2010). Cytokinin signalling is also involved in maintaining phyllotaxy, again in association with polar auxin transport (Lee et al., 2009). In addition, mobile peptides, already known to be involved in meristem maintenance (Butenko et al., 2009), may also trigger organ initiation. Whether any of these factors are directly involved in specifying organ founder cells is not established.

One approach to identifying signals is to specifically disturb the signalling process by mutation. Disruption of petal initiation is frequent among floral mutants of Arabidopsis (Irish, 2008), suggesting that the threshold of response to signalling may be finely balanced. This could be a consequence of the relatively few founder cells involved (Bosssinger and Smyth, 1996).

Mutations of PETAL LOSS (PTL) specifically affect the initiation of petals (Griffith et al., 1999). There are naturally four petals in Arabidopsis flowers, arising at stage 5 internally and alternately with the four sepals (Smyth et al., 1990). In ptl mutants, petals arise sporadically, and the number falls to zero in later arising flowers. The few petals that do occur are initiated over a wider area and an extended time interval during flower development. It is likely that the petal initiation process itself is disrupted, as there is no indication of primordial cell divisions where petals fail to appear, and Griffith et al. (Griffith et al., 1999) have proposed that the signalling of petal initiation is disrupted. A dominant modifier of petal initiation, petal loss modifier-1d (pmd-1d), was also

School of Biological Sciences, Monash University, Melbourne, Victoria 3800, Australia.

*Present address: School of Botany, University of Melbourne, Victoria 3010, Australia

†Author for correspondence (david.smyth@monash.edu)

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uncovered specifically in the Landsberg erecta (Ler) background, resulting in a boost to petal numbers in ptl mutants, although they were still variably initiated. This allele may amplify the petal initiation signal, or the response to it. Finally, petal orientation is also disrupted in ptl mutant flowers although independently of petal initiation, and a second, independent signal may be involved (Griffith et al., 1999).

The PTL gene encodes a trihelix transcription factor (Kaplan-Levy et al., 2012) that is expressed in newly arising flower primordia, especially between sepals as they develop from stage 3 (Brewer et al., 2004). Expression occurs in the epidermis and underlying cell layers. Overexpression of PTL results in growth suppression wherever it is expressed (Brewer et al., 2004; Li et al., 2008). Conversely, loss of PTL function leads to overgrowth of the inter-sepal zone (Lampugnani et al., 2012). These findings indicate that the role of PTL is to help define the inter-sepal zone by keeping cell division there in check. PTL is not expressed in the second whorl, and so disruption of petal initiation in ptl mutants is apparently non-cell autonomous, perhaps by influencing the strength of a mobile founder cell signal (the movement of PTL protein itself has not been examined).

The aim of this study was to uncover such a petal initiation signal. To this end, we conducted a genetic screen to identify second site mutants that enhanced the loss of petals in ptl mutant flowers, where such a signal may already be partially disrupted. We revealed that the auxin influx gene AUX1 is directly associated with petal initiation, although detectable only when PTL function is lost. The signalling role of auxin was confirmed because petal initiation, although detectable only when PTL function is lost.

**RESULTS**

**no petals mutants enhance the petal loss phenotype**

To identify enhancers of the petal loss mutant phenotype, an EMS mutagenesis of ptl-3 pmd-1d seeds was screened. Two independent recessive mutations resulted in the loss of almost all petals (Fig. 1A-D,G; supplementary material Table S2). When these two mutant lines were inter-crossed, loss of petals still occurred, indicating their allelism. They were given the working names no petals-1 (nop-1) and nop-2. Plants homozygous for nop-1 or nop-2 but wild type for PTL almost always had the wild-type number of petals, although one was occasionally absent (Fig. 1E-G). Thus NOP is a modifier of petal initiation, and its function is only uncovered when PTL function is compromised.

**NO PETALS encodes the auxin influx carrier AUX1**

To clone the NOP gene from its map position, a cross was made between ptl-3 pmd-1d nop-1 er in Ler background and ptl-1 PMD NOP ER in Col background, and F2 plants were examined. Scoring of 866 unselected plants revealed that NOP is linked to the ERECTA (ER) locus on chromosome 2, separated by about 35-40 map units. Focusing on 990 homozygous nop-1 mutant plants from the same F2 family and designing appropriate DNA markers, the location of NOP was ultimately constrained to a 41 kbp region between 15,960 kbp and 16,001 kbp (supplementary material Fig. 2).
This includes seven annotated genes, of which \textit{AUX1} (At2g38120) was a candidate. \textit{NOP} was confirmed to be the same gene as \textit{AUX1} by five criteria. First, \textit{AUX1} expression driven by its own promoter was able to complement the severe loss of petal phenotype in \textit{ptl nop} double mutant plants (Fig. 1H). Second, \textit{nop-1} was shown to be allelic with \textit{aux1-7} in that petals were almost always lacking in progeny of crosses between \textit{ptl-3 nop-1} and \textit{ptl-1 aux1-7} plants. Third, \textit{nop-1} and \textit{nop-2} mutants each showed single amino acid changes in the deduced \textit{AUX1} sequence that are likely to disrupt function (G443D and W173*, respectively) (Fig. 1I). Fourth, the characteristic loss of petals in \textit{ptl-3 nop-1} plants was reproduced in \textit{ptl-1 aux1-7} and \textit{ptl-1 aux1-21} double mutant plants (Fig. 1J-P; supplementary material Table S3). These are unmodified \textit{PMD} lines in Columbia background, so the effect of loss of \textit{AUX1} function is not confined to the modified \textit{pmd-1d} genotype. Finally, \textit{aux1} mutants show agravitropic root growth (Bennett et al., 1996), and \textit{ptl-3 nop-1} seedlings were also agravitropic, although \textit{ptl-3} plants were not (Fig. 1Q-T). Thus the two mutants define alleles of \textit{aux1}, and they were named \textit{aux1-n1} and \textit{aux1-n2}.

\textit{AUX1} has three close relatives in \textit{Arabidopsis}, \textit{LIKE AUX1} (\textit{LAX}) genes, that are functionally redundant with \textit{AUX1} in maintaining leaf and floral bud phyllotaxis (Bainbridge et al., 2008). However, redundancy in the control of petal initiation was relatively weak, as the mean number of petals in \textit{aux1 lax1 lax2 lax3} quadruple mutant flowers was 3.65±0.09, close to the normal 4 (supplementary material Table S4).

\textit{aux1} mutations are semi-dominant in \textit{ptl} mutant homozygotes, and vice versa

To examine the effects of \textit{PTL} and \textit{AUX1} gene dosage on phenotype, plants from an F2 family derived from a cross between \textit{ptl-1} and \textit{aux1-7} were individually genotyped, and petal numbers were scored (Table 1, left). When \textit{PTL} function was lost (in \textit{ptl-1} homozygotes), the additional loss of one dose of \textit{AUX1} (\textit{aux1-7}+/ heterozygotes) resulted in a significant further drop in the mean

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**Fig. 1.** Phenotype of no petals (\textit{nop}) enhancers of the petal loss mutant phenotype, and demonstration of their allelism with \textit{aux1}. (A-F) Floral phenotypes of wild-type (\textit{Ler}) (A) and \textit{ptl-3} (B), \textit{ptl-3 nop-1} (C), \textit{ptl-3 nop-2} (D), \textit{nop-1} (E) and \textit{nop-2} (F) mutant plants. All plants carry the modifier \textit{pmd-1d}. Bars represent s.e.m. (G) Mean number of petals per flower in single and double mutant combinations of \textit{ptl-3} and \textit{nop-1} or \textit{nop-2}. N flowers were scored, the first six on each plant. All plants carry the modifier \textit{pmd-1d}. (H) Floral phenotype of \textit{ptl} double mutant flower complemented with p\textit{AUX1:AUX1-YFP116}. (I) Topological representation of the \textit{AUX1} protein, showing predicted amino acid substitutions of \textit{aux1-7}, \textit{aux1-21}, \textit{nop-1 (aux1-n1)} and \textit{nop-2 (aux1-n2)}. White circles, hydrophilic residues; black circles, predicted transmembrane domains. After Swarup et al. (Swarup et al., 2004). (J-O) Floral phenotypes of wild-type (\textit{Col}) (J) and \textit{ptl-1} (K), \textit{ptl-1 aux1-7} (L), \textit{ptl-1 aux1-21} (M), \textit{aux1-7} (N) and \textit{aux1-21} (O) mutant plants. (P) Mean number of petals per flower in single and double mutant combinations of \textit{ptl-1} and \textit{aux1-7} or \textit{aux1-21}. N flowers were scored, the first six on each plant. Bars represent s.e.m. (Q-T) Root phenotypes of wild-type (\textit{Ler}) (Q), \textit{ptl-3} (R), \textit{aux1-7} (S) and \textit{ptl-3 nop-1 (aux1-n1)} (T) seedlings. All flowers shown are the first one formed on the primary inflorescence. Scale bars: 0.5 mm.
number of petals per flower, from 1.35±0.08 to 0.86±0.11 (t135=3.50, P<0.001). Similarly, aux1-7 homozygotes had close to wild-type petal numbers (3.92±0.03) unless ptl-1 was heterozygous, in which case the mean number fell significantly to 2.72±0.09 (t198=12.77, P<0.001). Similar results were obtained from parallel studies of ptl-3 and aux1-n1 in pmd-1d background (Table 1, right), although differences were not as marked. Thus the two functions, PTL and AUX1, are sensitive to a reduction in the function of the other, as expected if they act additively to promote petal initiation.

**AUX1 is expressed in the epidermis of newly arising flower and floral organ primordia**

Expression of the fluorescent reporter pAUX1:AUX1-YFP116 (Swarup et al., 2004) was investigated by live imaging the developing inflorescences of Columbia wild-type plants (Fig. 2A-E). Expression was localised to the epidermis (L1) and was strong in inter-sepal zones (Fig. 2C-E, arrows), although not noticeably so in locations where petal primordia will arise.

To further define expression at stage 4, we also examined a new reporter construct, pAUX1:GUS with a longer upstream promoter region (3.67 kbp compared with 2.2 kbp). Serial transverse sections of stage 4 buds (supplementary material Fig. S2) confirmed that strong expression occurred in the inter-sepal zones (Fig. 2F), where it matched PTL expression (Fig. 2G), although the latter was also strong in the underlying cell layers (Lampugnani et al., 2012). The same construct was transformed into ptl-1 mutant plants, and the localisation of expression in early flowers of 12 independent transformants was not detectably different from that in nine wild-type transformants.

**Generation of auxin in the inter-sepal zone restores petal initiation in ptl mutant flowers**

To test if auxin deficiency is the primary cause of loss of petals in ptl mutant plants, we generated ectopic auxin in the inter-sepal zones using the bacterial auxin biosynthesis gene iaaH under the control of the PTL promoter. iaaH expression can restore petal initiation in Arabidopsis flowers doubly mutant for the auxin biosynthesis genes YUCCA1 (YUC1) and YUC4 (Cheng et al., 2006). The PTL promoter used, pPPTL(FI313), is sufficient to complement sepal and petal defects in ptl mutant plants when driving PTL expression (Lampugnani et al., 2012). When pPPTL(FI313):iaaH was transformed into wild-type plants, there was no effect on petal development. However, in seven transformed ptl-1 mutant plants petal numbers were boosted almost to wild-type (Col) levels (Fig. 3A; supplementary material Table S5). In many flowers four petals now occurred, one per position. By contrast, the mis-orientation defect in petals was not significantly ameliorated (69% were mis-oriented in the seven transformed ptl-1 plants compared with 71% in ten untransformed controls).

We next tested whether driving AUX1 expression using the same PTL regulatory sequence could restore petal initiation in ptl aux1 double mutants. There was no effect on petal numbers when the pPPTL(FI313):AUX1 construct was inserted into wild-type plants. However, in five out of nine ptl-1 aux1-7 transformants, the mean petal number per flower was raised from only 0.07±0.02 in untransformed controls to now match the ptl-1 single mutant control (1.45±0.11) (Fig. 3B; supplementary material Table S6). In 12 ptl-3 aux1-n1 transformants (carrying the pmd-1d modifier), the mean number was mostly increased although not matching the ptl-3 single mutant control (5.63±0.15) (Fig. 3C; supplementary material Table S7). Even so, four of the T1 lines had a mean of between three and four petals per flower, and some early flowers had five or six. Thus even the localised restoration of auxin influx to the inter-sepal zone in ptl aux1 mutants is able to promote significant petal initiation.

**Loss of PTL function disrupts DR5 expression in petal initiation zones**

The sites of organ initiation can be indicated by expression of the transcription factor DR5 (Arabidopsis) in the developing inflorescences of Arabidopsis (Griffith et al., 1999). In wild-type buds at stage 4, DR5 expression occurs at four sites where petal primordia will arise (Chandler et al., 2011; van Mourik et al., 2012). These lie immediately internal to the inter-sepal zones where PTL is expressed (Lampugnani et al., 2012), and each often involved two adjacent cells (Fig. 4A-I), as predicted by sector boundary analysis (Bossmann and Smyth, 1996). In ptl-1 mutants, these DR5-expressing sites were disrupted in four ways (Fig. 4J-R). First, their location was more variable, including occasionally occurring within the inter-sepal zone (Fig. 4P-R). Second, the number of cells in each was sometimes increased or decreased, and their orientation distorted. Third, their relative intensity was reduced. Finally, they could not be detected in some petal initiation zones. These changes are consistent with loss of PTL function causing disruption to the auxin signalling of petal initiation. Further, the petal phenotype of mature ptl mutant flowers (Griffith et al., 1999) reflects the

### Table 1. Effect of PTL and AUX1 gene dose on petal numbers

<table>
<thead>
<tr>
<th>Genotype (all PMD)</th>
<th>Number of plants (flowers)*</th>
<th>Mean number of petals±s.e.m.</th>
<th>Genotype (all pmd-1d)</th>
<th>Number of plants (flowers)*</th>
<th>Mean number of petals±s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygotes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type (Col)</td>
<td>18 (180)</td>
<td>4.00±0.01</td>
<td>Wild type (Ler)</td>
<td>18 (180)</td>
<td>4.02±0.01</td>
</tr>
<tr>
<td>ptl-1</td>
<td>24 (237)</td>
<td>1.35±0.08</td>
<td>ptl-3</td>
<td>21 (210)</td>
<td>5.44±0.09</td>
</tr>
<tr>
<td>ptl-1 aux1-7</td>
<td>24 (240)</td>
<td>0.02±0.01</td>
<td>ptl-3 aux1-n1</td>
<td>30 (300)</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>aux1-7</td>
<td>10 (100)</td>
<td>3.92±0.03</td>
<td>aux1-n1</td>
<td>10 (100)</td>
<td>3.95±0.02</td>
</tr>
</tbody>
</table>

Heterozygotes

| ptl-1 aux1-7/+     | 9 (90)                      | 0.86±0.11                    | ptl-3 aux1-n1/+        | 26 (260)                    | 3.99±0.06                    |
| ptl-1/+ aux1-7+    | 10 (100)                    | 2.72±0.09                    | ptl-3/+ aux1-n1       | 10 (99)                     | 3.82±0.04                    |
| ptl-1/+            | 10 (94)                     | 3.71±0.06                    | ptl-3/+ aux1-n1/+     | 10 (100)                    | 3.99±0.01                    |
| aux1-7/+           | 16 (160)                    | 3.90±0.05                    | ptl-3/+               | 18 (180)                    | 4.01±0.01                    |
| aux1-7/+           | 18 (180)                    | 4.00±0.00                    | aux1-n1/+             | 22 (220)                    | 4.00±0.01                    |

*pThe first 10 flowers on each plant were scored (with an occasional flower missed). All plants were F2 siblings within each series.*

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**Notes:**

- **Development 140 (1)**
- **YUCCA1:** YUCCA1 (YUC1) and YUC4 (Cheng et al., 2006). The PTL promoter used, pPPTL(FI313), is sufficient to complement sepal and petal defects in ptl mutant plants when driving PTL expression (Lampugnani et al., 2012). When pPPTL(FI313):iaaH was transformed into wild-type plants, there was no effect on petal development. However, in seven transformed ptl-1 mutant plants petal numbers were boosted almost to wild-type (Col) levels (Fig. 3A; supplementary material Table S5). In many flowers four petals now occurred, one per position. By contrast, the mis-orientation defect in petals was not significantly ameliorated (69% were mis-oriented in the seven transformed ptl-1 plants compared with 71% in ten untransformed controls).

We next tested whether driving AUX1 expression using the same PTL regulatory sequence could restore petal initiation in ptl aux1 double mutants. There was no effect on petal numbers when the pPPTL(FI313):AUX1 construct was inserted into wild-type plants. However, in five out of nine ptl-1 aux1-7 transformants, the mean petal number per flower was raised from only 0.07±0.02 in untransformed controls to now match the ptl-1 single mutant control (1.45±0.11) (Fig. 3B; supplementary material Table S6). In 12 ptl-3 aux1-n1 transformants (carrying the pmd-1d modifier), the mean number was mostly increased although not matching the ptl-3 single mutant control (5.63±0.15) (Fig. 3C; supplementary material Table S7). Even so, four of the T1 lines had a mean of between three and four petals per flower, and some early flowers had five or six. Thus even the localised restoration of auxin influx to the inter-sepal zone in ptl aux1 mutants is able to promote significant petal initiation.

**Loss of PTL function disrupts DR5 expression in petal initiation zones**

The sites of organ initiation can be indicated by expression of the auxin-inducible reporter DR5rev:GFP-ER (Friml et al., 2003). In wild-type buds at stage 4, DR5 expression occurs at four sites where petal primordia will arise (Chandler et al., 2011; van Mourik et al., 2012). These lie immediately internal to the inter-sepal zones where PTL is expressed (Lampugnani et al., 2012), and each often involved two adjacent cells (Fig. 4A-I), as predicted by sector boundary analysis (Bossmann and Smyth, 1996). In ptl-1 mutants, these DR5-expressing sites were disrupted in four ways (Fig. 4J-R). First, their location was more variable, including occasionally occurring within the inter-sepal zone (Fig. 4P-R). Second, the number of cells in each was sometimes increased or decreased, and their orientation distorted. Third, their relative intensity was reduced. Finally, they could not be detected in some petal initiation zones. These changes are consistent with loss of PTL function causing disruption to the auxin signalling of petal initiation. Further, the petal phenotype of mature ptl mutant flowers (Griffith et al., 1999) reflects the...
variability in size, orientation and location of these DR5-expressing cells at stage 4.

Petal initiation is also sensitive to the loss of AXR4 function in the auxin influx pathway
To test if other disruptions to auxin influx also abolish petal initiation in _ptl_ mutants, the consequences of loss of AUXIN RESISTANT4 (AXR4) function were examined. This acts upstream of AUX1 in the auxin influx pathway (Dharmasiri et al., 2006). Two mutant alleles, _axr4-1_ and _axr4-2_, in Col background were examined alone and in combination with _ptl-1_ (Fig. 5A-D). In each case, like _aux1_, they had a very minor effect on petal numbers when singly homozygous. However, double mutants of either with _ptl-1_ resulted in an almost complete loss of petals, much fewer than the 2.31±0.13 per flower in _ptl-1_ single mutants (Fig. 5E; supplementary material Table S7). Thus AXR4, just like AUX1, is required for petal initiation when PTL function is compromised.

RABBIT EARS and PTL share petal initiation function
RABBIT EARS (RBE) is a zinc finger regulatory protein that promotes petal initiation in many ways like PTL. Loss of RBE function also results in an acropetal decrease in petal initiation (Krizek et al., 2006; Takeda et al., 2004), although petal orientation is unaffected. It is reported to act downstream of PTL, as _RBE_ expression was not observed in stage 5 _ptl_ mutant flowers. Thus we were interested to examine whether loss of RBE function also revealed a role for auxin influx in petal initiation. Before doing this, however, we showed that _PTL_ is expressed normally in _rbe-2_ mutant flowers (Fig. 5F,G), and also that there is no further loss of petals in _ptl-1 rbe-2_ double mutants (Fig. 5H-L; supplementary material Table S8) (t112=0.68, P>0.05 comparing _ptl-1 rbe_ with _ptl-1_.

Fig. 2. Expression of AUX1 in wild-type floral meristems.
(A) Confocal image showing pAUX1:AUX1-YFP116 fluorescence in a virtual z-section through an inflorescence meristem (asterisk) and stage 1 and 2 buds. (B) A 3D reconstruction (vertical view) of a bud at stage 4 expressing the same construct. (C-E) Virtual z-sections through this bud taken transversely (C) or longitudinally in the medial (D) or diagonal planes (E) (indicated by lines in B). Arrows indicate stronger expression in inter-sepal zones. (F) GUS expression in a transverse section of a stage 4 bud carrying pAUX1(3.67kbp):GUS showing strong expression in the inter-sepal zones (arrows). See supplementary material Fig. S2 for further serial sections. (G) pPTL(1.3i):GUS expression in a transverse section of a stage 4 bud, showing localised expression in the inter-sepal zones. Scale bars: 20 μm.

Fig. 3. Restoration of petals in _ptl_ mutants by a transgene controlling auxin biosynthesis, and in _ptl aux1_ mutants by a transgene controlling auxin influx. The first 12 flowers of each plant were scored. Results for untransformed control plants are shown in the left and right columns. Bars represent s.e.m. (A) Mean number of petals per flower in seven _ptl-1_ T1 plants transformed with pPTL(FI313):iaaH. (B,C) Mean number of petals per flower in nine _ptl-1 aux1-7_ T1 plants (A), or in 12 _ptl-3 aux1-n1_ T1 plants (in _pmd-1d_ background) (B), transformed with pPTL(FI313):AUX1 in each case.
1), as expected if loss of PTL or RBE functions affect the same petal initiation pathway.

When the rbe-2 mutant was combined with aux1-7, a striking loss of petals was observed (Fig. 5L), closely similar to that in ptl-1 aux1-7 double mutants (Fig. 1P). A mean of only 0.22±0.04 petals per flower arose compared with the 2.02±0.10 observed in rbe-2 single mutants. Thus petal initiation in rbe mutants is now dependent on AUX1-controlled auxin influx function, just as it is in ptl mutants.

Loss of PTL function also disrupts petal initiation in pin1 and pid polar auxin transport mutants

Finally we assessed the contribution of polar auxin transport by generating multiple examples of ptl with aux1 and pin1 and pinoid (pid) (Figs 6, 7; supplementary material Table S9).

Flowers arose sporadically from inflorescence meristems of all pin1 mutants, and the mean number of petals is significantly increased (Bennett et al., 1995; Okada et al., 1991). This may occur through a defect in the later depletion of auxin from the second floral whorl, stimulating additional petal initiation. Strikingly, when PTL function was removed from strong pin1-3 mutant plants (in ptl-3 pin1-3 doubles) (Fig. 6A-D), the mean number of petals per flower was greatly reduced from over seven to around one (Fig. 7A, left) (t43=9.78, P<0.001). This also occurred when the weaker pin1-5 mutant was used, although to a lesser extent (Fig. 7A, right) (t30=10.8, P<0.001).

The PID gene acts upstream of PIN1 as the PID kinase activates it by phosphorylation (Christensen et al., 2000; Friml et al., 2004; Michniewicz et al., 2007). As in pin1 mutants, the number of petals in pid single mutant flowers is significantly increased (Bennett et al., 1995). Also as in pin1, we found that the additional loss of PTL function resulted in loss of many of these petals (Fig. 6E-J; Fig. 7B).

The pinoid mutant results also provide evidence that the pmd-1d modifier requires PID function. For the ptl-3 pid-1 mutant combination involving the modifier, the mean number of petals (1.28±0.15) was much reduced compared with the ptl-3 single mutant control (5.22±0.14) (Fig. 7B, left) (t116=18.2, P<0.001). However, for the ptl-1 pid-3 observations in unmodified background, the mean number per flower (2.20±0.31) was not reduced further than that seen in ptl-1 single mutants (2.60±0.20) (Fig. 7B, right) (t35=0.076, P>0.05).

Thus disruption of polar auxin transport generally boosts petal numbers, but this boost is dependant on PTL function, which presumably acts upstream of it. In light of this, we tested whether PTL function influences PIN1 expression. pPIN1:PIN1-GFP is expressed throughout the epidermis of wild-type buds up to at least stage 5, but there were no detectable differences in its relative intensity or tissue localisation in ptl-1 mutants, including in the inter-sepal and petal initiation zones at stage 4 (supplementary material Fig. S3; Movies 1-4).

Lastly we examined the consequences on petal initiation of the joint disruption of auxin influx and polar auxin efflux. For the strong pin1-3 allele, petal numbers in the aux1-n1 pin1-3 double mutant (Fig. 6K) were not significantly different from pin1-3 single mutants (Fig. 7C, left) (t35=0.076, P>0.05). However, when PTL function was also lost, in ptl aux1 pin1 triple mutants (Fig. 6L), petal numbers now fell to the low level seen in ptl aux1 double mutants (Fig. 7C, compared with Fig. 1G). Thus auxin influx...
regulated by AUX1 does not seem to be required for petal initiation in pin1 mutants unless PTL function is also compromised. This does not apply for the weaker allele pin1-5, because loss of AUX1 function in this case does result in a modest but significant reduction in the number of petals (from 6.43±0.26 in pin1-5 to 5.09±0.21 in pin1-5 aux1-n1 double mutants) (Fig. 7C, right) (t_{83}=3.50, P<0.001). Thus examination of this mutant allele with partially reduced PIN1 function (Bennett et al., 1995) has confirmed the role AUX1 plays in petal initiation, a role that is masked if PIN1 function is fully lost.

**DISCUSSION**

**Proposed genetic pathway controlling petal initiation**

Integrating our observations with predicted patterns of auxin transport (Heisler et al., 2005; Reinhardt et al., 2003), we propose the following pathway (Fig. 8A). Petal initiation (red) may require localised auxin accumulation (green). Auxin may be made available from two sources: one dependent on regional growth suppression jointly promoted by PTL and RBE (long green arrow), and another by a weaker AXR4-AUX1 influx pathway (dashed green arrow). If PTL or RBE function is lost, regional overgrowth may inhibit the first pathway, but some auxin may still be supplied by the AUX1 pathway for limited and variable petal initiation. If AXR4 or AUX1 function is lost, then sufficient auxin may be supplied by the alternative pathway for full petal initiation. However, if both pathway functions are lost, insufficient auxin may be provided and almost no petals would arise.

Polar transport of this available auxin is likely to be directed by the PID-PIN1 pathway. This may first promote auxin accumulation in the petal initiation zone from available sources, and its later depletion. When PID or PIN1 function is lost, additional petals arise, perhaps because auxin still accumulates independently but that its subsequent depletion is now compromised. When PTL function is also lost in pin1 or pid mutants, petal numbers are now low, indicating that only small amounts of auxin accumulate. However, when AUX1 and PIN1 functions are both lost, sufficient auxin may accumulate through the PTL-RBE promoted source to maintain high petal numbers. Finally, in ptl aux1 pin1 triple mutants, available auxin may be almost abolished so that polar auxin transport would now be irrelevant and, as in ptl aux1 double mutants, few if any petals would arise.

The role of the dominant allele of PETAL LOSS MODIFIER, pmld-1d, uniquely present in Ler background, apparently occurs after all these steps. The boost in petal numbers it provides is only apparent when PTL function is lost, suggesting that its function is normally repressed by PTL. However, its effect in ptl mutants is not significant when AUX1 or PID function is also absent (PIN1 was not tested), indicating that its action is dependent upon auxin accumulation. One possibility is that pmld-1d is a gain-of-function allele of an auxin response pathway gene that amplifies the response to the petal initiation signal.

**Loss of PTL function disrupts auxin availability**

We have shown that loss of PTL function uncovers a role for auxin influx in signalling petal initiation. Further, generation of auxin between developing sepals can restore nearby petal initiation in ptl mutant flowers. Finally, auxin-inducible DR5 expression in the petal initiation zones is disrupted when PTL function is lost. Thus loss of PTL function interferes with the normal action of auxin in promoting petal initiation.

How does PTL influence auxin dynamics? An established role of PTL is to repress cell division in the developing inter-sepal zone (Lampugnani et al., 2012). This region overgrows somewhat in ptl mutants buds, and this morphological distortion alone may be sufficient to incidentally disrupt auxin flow and thus nearby petal initiation (Fig. 8B).
Alternatively, PTL may play a more direct role. One possibility is that PTL normally supports auxin transport by another system, non-polar auxin efflux through the ABCB channels (Noh et al., 2001). In this case, auxin may be unable to escape from inter-sepal zone cells in ptl mutants, generating a sink that traps surrounding auxin, depleting it from the petal initiation zone. Such accumulation may also stimulate the additional cell divisions between sepals seen in ptl mutants (Lampugnani et al., 2012). However, we found no evidence for widespread DR5 expression in the inter-sepal zone in ptl mutant buds that might indicate such auxin activity.

Like PTL, loss of RBE function also sensitises petal initiation to reduced auxin influx. The defects associated with their loss of function are apparently shared as petal numbers are not further compromised in ptl rbe double mutants. As with PTL, RBE also dampens growth between developing sepals (Krizek et al., 2006). However, the mechanism of its growth suppression differs from that of PTL (Lampugnani et al., 2012) because RBE promotes the action of CUP-SHAPED COTYLEDON boundary genes through direct repression of their negative regulator EXTRA EARLY PETALS1 (Huang et al., 2012). Also, RBE normally represses AGAMOUS expression in the second whorl (Krizek et al., 2006), but PTL does not (Griffith et al., 1999). Even so, it may be that either form of mutant overgrowth in the inter-sepal zone incidentally disrupts auxin-induced petal initiation.

The location of sepals defines the sites of petal initiation
Generally, the sites of petal initiation are dependent on the location of earlier arising sepals (Endress, 2011; Smyth, 2005). If petal initiation sites are controlled by the same processes as leaves and flowers, they would arise where auxin levels are relatively high. Newly growing sepal primordia may be auxin sinks, depleting it in their vicinity but less so in the inter-sepal zone (Reinhardt et al., 2003). In addition, reversal of polar auxin transport may occur around newly arising sepal primordia (Heisler et al., 2005), driving auxin outward and generating an interference peak internal to the inter-sepal zone (Fig. 8B).

A role for sepal-controlled auxin dynamics in defining sites of petal initiation is suggested by their uncoupling in polar auxin transport mutants. In pin1 and pid flowers, extra sepals and extra petals both occur in variable numbers and sizes, but the sites of petals are now not strictly internal to inter-sepal zones (Bennett et al., 1995). PTL is also apparently involved in this auxin signalling process, either indirectly or directly, because loss of PTL function in pin1 or pid mutants greatly reduces petal initiation. However, there is no effect on the number of sepals (Brewer et al., 2004) (supplementary material Table S9).

**Fig. 6. Flower phenotypes in mutants of the polar auxin transport genes pin1 and pid showing the effects of loss of PTL and AUX1 function.** (A-D) Flowers of Ler wild type (A), ptl-3 (B), pin1-3 (C) and ptl-3 pin1-3 (D), showing that the increase in petal numbers in pin1-3 (C) is strongly disrupted in ptl-3 pin1-3 double mutants (D). All plants carry the modifier pmd-1d. (E, F) Flowers showing that the increase in petal numbers in pid-1 (E) is almost fully disrupted in ptl-3 pid-1 double mutants (F). Both plants carry pmd-1d. (G-J) Flowers of Col wild type (G), ptl-1 (H), pid-3 (I) and ptl-1 pid-3 (J), showing that the increase in petal numbers in pid-3 (I) is almost fully disrupted in ptl-1 pid-3 double mutants (J). All plants have the unmodified PMD background. (K, L) Flowers showing that the increase in petal numbers in pin1-3 (C) is unaffected in aux1-n1 pin1-3 double mutants (K), but almost fully disrupted in aux1-n1 pin1-3 pid-3 triple mutants (L). Both plants carry pmd-1d.

**Petal orientation is controlled by a different signal**
Loss of PTL function also disrupts petal orientation (Griffith et al., 1999), a process that precedes the imposition of dorsoventral polarity on developing organs (Siegfried et al., 1999). This was not affected by loss of other gene functions examined here, including RBE. Also, the restoration of petals in ptl mutants resulting from ectopic auxin was not associated with the amelioration of petal orientation. The only other mutants with a similar reported disruption to petal orientation are those of the ASYMMETRIC LEAVES1 (AS1) and AS2 organ growth genes (Xu et al., 2008). Double mutants of as1 and ptl result in a strengthening of petal mis-orientation, pointing to overlap in their orientation function (Xu et al., 2008). A similar strengthening was seen in double mutants of ptl and B function organ identity genes (Griffith et al., 1999). The nature of this possible signal remains unknown.

**Is auxin the primary signal for founder cell specification?**
Auxin may be universally involved in regulating organ initiation in plants (Petrášek and Friml, 2009). In addition to the primordia of petals (present study), leaves and flowers (Reinhardt et al., 2000), it is required for development of the hypophysis in the developing embryo (Friml et al., 2003), and for lateral root initiation (Dubrovsky et al., 2008).

Organ initiation requires the prior specification of founder cells that are competent to respond to a specific induction signal.
Thus induction occurs before any morphological evidence of organ initiation. Despite strong evidence in favour, it is not proved that auxin itself is the induction signal. In developing flowers, one complication is that relevant auxin events occur in the epidermis (Reinhardt et al., 2003), but the first signs of organogenesis are seen in the underlying layers. For Arabidopsis petals, this is a periclinal division in the L2 (Hill and Lord, 1989). Expression of the DR5 auxin response reporter consistently indicates the sites of future organogenesis, and its expression occurs in the L1 (Chandler et al., 2011; Heisler et al., 2005; Lampugnani et al., 2012; van Mourik et al., 2012). Whether the DR5-expressing epidermal cells receive the induction signal, or whether it occurs earlier, either in the epidermis or in underlying cells, remains to be established. Study of genes that are expressed earlier than DR5 where organ primordia will arise, such as DORNRÖSCHEN-LIKE (Chandler et al., 2011), may help uncover mechanisms of specification.

In conclusion, we have revealed that auxin is required for petal initiation and the possible pathways involved. Future studies are needed to determine whether auxin is the ultimate inducer of floral organ founder cells, how the cells have become competent to respond, and the molecular and cellular mechanisms of their specification.

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Competing interests statement
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