WOX4 and WOX14 act downstream of the PXY receptor kinase to regulate plant vascular proliferation independently of any role in vascular organisation

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SUMMARY
In plants, the cambium and procambium are meristems from which vascular tissue is derived. In contrast to most plant cells, stem cells within these tissues are thin and extremely long. They are particularly unusual as they divide down their long axis in a highly ordered manner, parallel to the tangential axis of the stem. CLAVATA3-LIKE/ESR-RELATED 41 (CLE41) and PHLOEM INTERCALATED WITH XYLEM (PXY) are a multifunctional ligand-receptor pair that regulate vascular cell division, vascular organisation and xylem differentiation in vascular tissue. A transcription factor gene, WUSCHEL HOMEOBOX RELATED 4 (WOX4) has been shown to act downstream of PXY. Here we show that WOX4 acts redundantly with WOX14 in the regulation of vascular cell division, but that these genes have no function in regulating vascular organisation. Furthermore, we identify an interaction between PXY and the receptor kinase ERECTA (ER) that affects the organisation of the vascular tissue but not the rate of cell division, suggesting that cell division and vascular organisation are genetically separable. Our observations also support a model whereby tissue organisation and cell division are integrated via PXY and ER signalling, which together coordinate development of different cell types that are essential for normal stem formation.

KEY WORDS: Arabidopsis, Cell division, Phloem, Procambium, Signalling, Vascular

INTRODUCTION
Differential growth is the basis of all plant morphology and is determined by cell proliferation, cell expansion and the orientation of cell division. In the cambium and procambium, which are meristems from which vascular tissue is derived, cells divide down their long axis in a highly oriented manner, perpendicular to the radial axis of the stem. These divisions result in highly distinctive cell files that are characteristic of the xylem in most dicots. The cell files that are generated are also essential for maintaining the spatial separation of xylem and phloem (Caño-Delgado et al., 2010). These properties make vascular tissue an ideal system for examining factors that regulate cell division and its effect on tissue organisation during differential growth.

CLE41 (CLAVATA3/ESR LIKE 41) and PXY (PHLOEM INTERCALATED WITH XYLEM) are a ligand-receptor pair, constituting a multifunctional pathway that regulates vascular cell division, vascular bundle organisation and xylem differentiation (Etchells and Turner, 2010; Fisher and Turner, 2007; Hirakawa et al., 2008). Vascular bundle organisation defects in pxy mutants result in both increased vascular cell division, and organisational defects in the vascular tissue. This is consistent with a role for localised CLE41 expression providing a positional signal to PXY that is required for correct orientation of the division plane in vascular stem cells (supplementary material Fig. S1A,B) (Etchells and Turner, 2010).

Although these experiments are all consistent with a role for PXY and CLE41 in regulating the orientation of vascular cell division, an alternative model for the intercalation of xylem and phloem in pxy mutants (Hirakawa et al., 2010) followed the identification of a downstream target of CLE41/PXY signalling as WOX4. WOX4 had previously been shown to promote vascular cell division (Ji et al., 2010), and its expression was found to be rapidly increased by exogenous application of TDIF (Hirakawa et al., 2010), the active peptide derived from CLE41 (CLE41, CLE42 and CLE44 (Ito et al., 2006)). WOX4 was also found to be expressed in a broader region in pxy mutants compared to wild type, indicative of a ‘cavity’ defect in the organisation of the vascular system. Therefore, WOX4 regulates the orientation of vascular cell division and spatial separation of xylem and phloem (Ito et al., 2006; Hirakawa et al., 2008). To determine whether there is a genetic interaction between CLE41/CLE42, TDIF and WOX4, the resulting tissue organisation was investigated in pxy mutants overexpressing CLE41, CLE42, CLE44 and TDIF. These experiments revealed a correlation between the degree of cavity defects and expression of WOX4, suggesting that WOX4 acts as an effector of CLE41 signalling to regulate vascular cell division and spatial separation of xylem and phloem (Ito et al., 2006). TDIF has also been shown to repress xylem differentiation in cell culture, and plants overexpressing both CLE41 and PXY result in more undifferentiated vascular cells (Etchells and Turner, 2010; Hirakawa et al., 2008; Ito et al., 2006), which was suggested that the ‘cavity’ is the result of the meristem differentiating into xylem and that vascular cell proliferation subsequently reinitiates via a compensatory mechanism between the phloem and cortex (Hirakawa et al., 2010). A compensatory mechanism has recently...
been described that involves ethylene maintaining vascular cell numbers by promoting vascular cell division in the absence of functional *pxy* or *wox4* (Etchells et al., 2012).

Although *WOX4* is reported to be downstream of *pxy*, no reduction in *WOX4* expression has been reported in *pxy* mutant seedling hypocotyls, demonstrating that the *pxy* phenotype is not a result of changes in *WOX4* expression (Hirakawa et al., 2010). Alternative transcriptional targets of *PXY*/*CLE41* signalling must therefore exist.

**ERE**CTA (*ER*) encodes a RLK involved in mediating cell-cell communication in several plant developmental contexts (van Zanten et al., 2009), including repression of xylem expansion in hypocotyls (Ragni et al., 2011). The importance of the role of *ER* in cell-cell communication is perhaps most clearly evident in stomatal spacing, a role in promoting cell division (Shpak et al., 2004), controlled by *ERf* receptors (Hara et al., 2007; Hara et al., 2009; Hunt and Gray, 2009; Lee et al., 2012). In the cortex, *ERf* receptors also have a role in promoting cell division (Shpak et al., 2004), controlled by *CHALLAH-LIKE1* (*CHL1*) and *CHL2* ligands (*EPFL6, EPFL5* and *EPFL4*, respectively). *CHAL* and *CHL2* are expressed in the endodermis and signal to *ER*, which is present in the phloem, promoting growth of the inflorescence stem (Abrash et al., 2011; Uchida et al., 2012).

Here, we demonstrate that *WOX14* acts redundantly with *WOX4* in promoting vascular cell division downstream of *PXY* signalling. *wox4* *wox14* double mutants demonstrate similar reductions in vascular cell division to those observed in *pxy wox4* lines, but do not have vascular organisation defects. By contrast, *er* dramatically enhances the effects of *pxy* on organisation of inflorescence stem vascular bundles, resulting in intercalation of xylem and phloem, but *er* has no effect on vascular cell number. Our results suggest that vascular organisation can be disrupted in the absence of reductions in vascular cell division, and disrupted rates of cell division do not lead to disrupted vascular organisation.

**MATERIALS AND METHODS**

**Generation of plant stocks**

*wox14-1* and *er-124* T-DNA insertion mutants were identified using the TAI database (Swarbreck et al., 2008) and confirmed by PCR with primers described in supplementary material Table S1. *pxy-1, pxy-3, pxy-5, pxy-3* *pxl1-2 pxl2-1, wox4-1, er-105 erl1-2 erl2-1, IRX3::CLE41* and *35S::CLE41* lines have been described previously (Etchells and Turner, 2010; Fisher and Turner, 2007; Shpak et al., 2004). *IRX3::CLE41* *wox4-1, pxy-5 er-124, pxy-3* *wox4-1, wox14-1* *wox4-1* and *psy-3 psy-3* *wox4-1* *wax4-1* were identified using standard PCR-based screening of segregating F2 populations using primers described in supplementary material Table S1. For *WOX4::GUS* lines, *2.5 kb of the *WOX4* upstream region was PCR amplified using primers described in supplementary material Table S1, and cloned into pKGFWST via pENTR-D-TOPO. Transgenic plants were generated using Agrobacterium infiltration (Clough and Bent, 1998). Images shown are representative of eight independent transgenic lines.

**TDIF/P9A synthetic peptide treatments**

Seeds were plated on 0.5×MS, 1% Agar and stratified for 4 days at 4°C prior to transfer to a Sanyo MLR-351H plant growth chamber set at 23°C with constant light. After 5 days, seedlings were transferred to 0.5×MS liquid media supplemented with either 5 mM TDIF (His-Glu-Val-Hyp-Ser-Gly-Hyp-Asn-Pro-Ile-Ser-Asn) or negative control P9A (His-Glu-Val-Hyp-Ser-Gly-Hyp-Asn-Ala-Ile-Ser-Asn; Bachem, Switzerland) and maintained on a rocking platform for either 1 or 16 hours. Following treatment, samples were snap frozen and RNA was extracted using Trizol (Life Technologies, USA). RNA was DNase treated prior to cDNA synthesis using BioScript reverse transcriptase (Bioline, UK).

**Gene expression analysis**

qRT-PCR primary inflorescence stem expression analysis, was performed on stems from 5-week old plants (~10 cm), stripped of side branches and cauline leaves and divided into three equal lengths. In all qRT-PCR analysis, cDNA was prepared as described above. qRT-PCR was carried out using SensiMix SYBR Hi-ROX reaction mix (Bioline), primers described in supplementary material Table S1, and an ABI Prism 7000 machine (Life Technologies) with the standard SYBR green detection programme. Melting curves were analysed to ensure single products were formed. Relative expression was determined using a version of the comparative threshold cycle (Ct) method using average amplification efficiency for each primer pair, determined using LinReg (Hårdstedt et al., 2005). Samples were normalised to 18S *rRNA* (not shown) and *ACT2* (shown). Results were similar independent of the control used. Samples were measured in technical triplicates on biological triplicates.

For determination of spatial gene expression patterns in *WOX4::GUS* lines, stain was prepared as described previously (Young et al., 2010). Plant tissue was stained overnight and destained in 70% ethanol. Methods for *in situ* hybridization using digoxigenin-labelled probes were carried out as described by Etchells et al. (Etchells et al., 2010). *WOX4* antisense probe was generated from a pT48 template (Suer et al., 2011). *WOX4* sense and antisense probes were made by cloning a *WOX4* PCR fragment, amplified using primers shown in supplementary material Table S1 into pGEM-T easy (Promega, USA), which was subsequently used in PCR with M13 primers to generate a probe template.

**Histology**

For thin sections, hypocotyls or tissue 1 cm from the base of inflorescence stems was fixed in FAA and dehydrated through an ethanol series prior to infiltration and subsequent embedding with JB4 embedding media. Sections (4 μm) were stained with 0.05% aqueous Toluidine Blue. Callose sieve plates marking the phloem were observed by staining hand sections with 500 mM Amline Blue in 100 mM phosphate buffer (pH 7.2) and visualising tissue on a Leica DMR microscope using the UV lamp. Tissue was considered to be ordered in vascular bundles when xylem and phloem could be separated by a simple arced line and in hypocotyls if xylem could be incorporated in an elliptical shape that excluded the phloem.

**RESULTS**

**Evidence for factors downstream of *PXY* signalling in addition to *WOX4***

*WOX4*, a downstream target of *PXY* signalling, is upregulated by application of CLE ligand in seedling hypocotyls; however, no reduction in *WOX4* expression was observed in *pxy* mutants (Hirakawa et al., 2010). Consequently, *pxy* phenotypes cannot solely be attributable to changes in *WOX4* expression and we examined this possibility by crossing *IRX3::CLE41* and *wox4*, and comparing *IRX3::CLE41* *wox4* with parental lines and wild-type counterparts. We reasoned that if other factors do act redundantly with *WOX4* then *wox4* mutants should only partially suppress a *CLE41* overexpression phenotype.

*wox4* mutants have been described with gross morphology that is similar to wild type but have been reported to contain fewer cells in vasculature undergoing secondary growth, either directly above the rosette leaves in inflorescence stems (Suer et al., 2011) or in seedling hypocotyls (Hirakawa et al., 2010). We measured vascular cell division in areas of primary growth in 6-week-old plants 1 cm above the rosette, avoiding the secondary growth region directly above rosette leaves. At this developmental stage, *wox4* mutants demonstrated vascular cell numbers that were not significantly different from wild-type counterparts (Fig. 1A). *IRX3::CLE41* plants are characterised with large vascular bundles...
containing many undifferentiated cells and also have phloem intercalated with xylem (Fig. 1A; supplementary material Fig. S2A,B) (Etchells and Turner, 2010). Analysis of vascular tissue in the stem revealed a significant modification of the IRX3::CLE41 phenotype by wox4, as wox4 IRX3::CLE41 vascular tissue demonstrated a 28.6% reduction in cell number when compared with IRX3::CLE41. However, wox4 did not completely abolish the increase in cell division in IRX3::CLE41 vascular tissue. wox4 IRX3::CLE41 lines still had a further 28.6% more cells than wild type and 53.3% more than wox4 single mutants (Fig. 1A), clearly demonstrating that factors exist that act redundantly with PXY signalling.

We tested whether evidence for WOX4-independent PXY targets was also present in hypocotyls, where vascular tissue undergoes secondary growth. wox4 IRX3::CLE41 hypocotyls were 19.8% smaller than those of IRX3::CLE41 lines, but as was the case in vascular bundles, IRX3::CLE41 wox4 were larger than those observed in wox4 single mutants (Fig. 1B; Fig. 2), demonstrating that WOX4 acts with additional factors. Our previous analysis suggested that increases in vascular cell division associated with CLE41 overexpression is entirely dependent on the presence of a PXY receptor as pxy 35S::CLE41 lines are identical to pxy single mutants in Arabidopsis hypocotyls (Etchells and Turner, 2010). As wox4 acts as only a partial suppressor of IRX3::CLE41, we hypothesised that WOX4 may act with other factors in mediating the promotion of cell division in the procambium in response to and downstream of PXY signalling.

**Both WOX4 and WOX14 act downstream of PXY signalling**

Our genetic analysis (Figs 1, 2; supplementary material Fig. S2) suggested that WOX4 is only partially responsible for promoting cell division downstream of the PXY signalling so must act redundantly with other factor(s). The CLE-RLK-WOX interaction in regulation of stem cell niches has been widely reported (Brand et al., 2000; Hirakawa et al., 2010; Sarkar et al., 2007; Schoof et al., 2000) so we analysed microarray data comparing expression in pxy-3 mutants and wild type (experiment E-MEXP-2639, http://www.ebi.ac.uk/arrayexpress) (Etchells et al., 2012), for changes in WOX expression. WOX4 demonstrated a 1.7-fold reduction in expression in pxy suggesting that it may be regulated by PXY signalling. WOX14 expression was compared with that of WOX4, a previously identified target of PXY signalling (Hirakawa et al., 2010) in 5-day-old wild-type seedlings subjected to a 1- or 16-hour exposure to exogenous application of TDIF, the peptide derived from CLE41 or negative control peptide P9A (Ito et al., 2006). Consistent with the observations of Hirakawa et al. (Hirakawa et al., 2010), WOX4 expression was increased following either a 1- or 16-hour treatment (Fig. 3). Although WOX14 expression appeared increased at 1 hour, differences in expression proved not significant (Fig. 3A); however, at 16 hours a small (1.6-fold) but significant increase in expression was observed (Fig. 3B) that occurred in a PXY-dependent manner, demonstrating that WOX14 acts downstream of PXY signalling. Increases in WOX14 expression were not dependent on functional WOX4, as a clear WOX4 response to TDIF was observed in wox4 mutant lines (Fig. 3), demonstrating that WOX14 is not a downstream target of WOX4.

Inflorescence stems represent a developmental series with most recently derived vascular tissue present in apical regions of the stem and the oldest tissue in basal regions. We compared WOX4 and WOX14 expression in apical, central and basal regions of inflorescence stems from 5-week-old plants using qRT-PCR and found that the dynamics of WOX14 expression differed from that of WOX4. WOX4 was expressed at a relatively consistent level along the length of the inflorescence stem (Fig. 4A); however, WOX14 expression was low at the top but expression increased along the apical-basal axis such that expression was 20-fold higher at the base than at the top (Fig. 4B).

WOX4 and WOX14 expression levels were also compared in pxy and 35S::CLE41 lines. WOX4 expression was maintained at the level of wild-type expression in apical regions of pxy inflorescence stems; however, in central and basal regions WOX4 expression was significantly reduced (Fig. 4A). Consequently, we have demonstrated that PXY signalling is required for normal levels of WOX4 expression. WOX14 expression was also dependent upon PXY as mRNA levels were similarly reduced in pxy mutant stems (Fig. 4B). In the apical third of the stem, WOX14 expression was reduced in pxy by a small (1.3-fold) but significant amount, and this reduction was more apparent in the middle of the stem (Fig. 4B). The trend of reduced WOX14 expression appeared similar in the stem base of pxy mutants, but these differences proved not to be statistically significant. In 35S::CLE41 lines, WOX4 expression was increased approximately twofold at all stages of stem development relative to wild type (Fig. 4A). WOX14 expression levels were also elevated in these lines, but in this case the largest increase in expression was
observed at the base of the stem (Fig. 4B). Wild-type expression levels of WOX4 and WOX14 are therefore dependent on the presence of a PXY receptor kinase and promoted by PXY ligand, demonstrating that both of these WOX genes act downstream of PXY signalling.

**WOX14 is expressed predominantly in vascular tissue**

We determined the spatial expression pattern of WOX14 by generating eight independent WOX14::GUS lines. A 2.5 kb region of WOX14 upstream regulatory sequence was cloned behind a GUS-GFP cassette in a binary vector and subsequently transformed into Arabidopsis. When subjected to staining, in all cases 14-day-old seedlings demonstrated no expression in leaves, cotyledons or in the shoot apex. Staining was observed where the leaves met the stem (supplementary material Fig. S3A, arrows, upper panel). Strong staining was observed in the vascular cylinder of the hypocotyl and root, and was also apparent in the root elongation zone but not the root tip (supplementary material Fig. S3A, arrows, upper panel). Strong staining was observed in the vascular cylinder of the hypocotyl and root, and was also apparent in the root elongation zone but not the root tip (supplementary material Fig. S3A, arrows, upper panel).

In rosette leaves of 5-week-old plants, GUS stain was not observed in expanding leaves (not shown) but was present in midveins (supplementary material Fig. S3B, arrowheads) but no other leaf veins of fully expanded leaves (supplementary material Fig. S3B; arrow in lower panel). These results suggest that WOX14 upstream regulatory sequences do not restrict expression in initiating vascular tissue but do promote WOX14 expression in established vasculature.

To better understand cell-type specific WOX14 expression in stained GUS lines, transverse inflorescence stem sections were taken to determine spatial expression within the vascular tissue. At 2 cm below the shoot apex, GUS staining was expressed broadly in vascular tissue, present in xylem, phloem and procambium; however, in vascular tissue at the base of stems 1 cm above the rosette, staining was restricted to the procambium and adjacent areas of xylem and phloem (supplementary material Fig. S3C). To confirm that the GUS pattern represented a true reflection of WOX14 expression, RNA in situ hybridization was performed on tissue from the same stage in development. In these experiments, WOX14 antisense probes broadly marked the vascular bundles in apical sections, but demonstrated predominantly procambium expression in basal sections (Fig. 5A), in common with the GUS lines (supplementary material Fig. S3C). By contrast, signal was not observed above background levels using a WOX14 sense control (Fig. 5C). To compare WOX4 and WOX14 expression, we also
carried out in situ hybridization using an antisense probe previously described for WOX4 (Suer et al., 2011). WOX4 expression was confined to the procambium in both apical and basal sections (Fig. 5B). Consequently, expression of both of these genes overlaps in the procambium.

**WOX14 acts redundantly with WOX4 in regulation of vascular cell division**

Expression analysis suggested that WOX14 acts downstream of the PXY receptor (Figs 3-5). Consequently, we identified a wox14 T-DNA insertion mutant (GabiKat_469C11), renamed it wox14-1 and used it to test for a role for WOX14 in Arabidopsis vascular tissue. These plants exhibited no obvious alteration in plant growth or morphology (not shown), and no alterations in vascular organisation or cell number were observed (Figs 6, 7). We reasoned that WOX4 and WOX14 may act redundantly, so generated wox4 wox14 double mutants. wox4 wox14 gross morphology was also indistinguishable from that of wild-type plants; however, when cell number was analysed in vascular bundles, fewer cells were present in wox4 wox14 than wild type or single mutants (Figs 6, 7), demonstrating that WOX4 and WOX14 act redundantly in promoting vascular cell division.

Strikingly, wox4 wox14 double mutant vascular bundles were smaller than those observed in pxy single mutants, and were a similar size to those observed in pxy wox4 lines (Fig. 6A). In contrast to pxy and pxy wox4 lines, which exhibit characteristic defects in vascular organisation, organisation of wox4 wox14 vascular bundles was similar to the wild type (Fig. 7). A similar situation was observed in hypocotyls, where cambium activity was assessed by measuring hypocotyl diameter. wox4 wox14 mutants demonstrated a similar diameter to that observed in pxy (Fig. 6B), but in contrast to pxy (Fig. 7B; arrowheads) no defects in vascular organisation were observed.

We further characterised the relationship between pxy and wox14 by analysing pxy wox14 and pxy wox4 wox14 mutant combinations.
pxy wox14 lines were identical to pxy single mutants for all parameters tested, i.e. for vascular proliferation measures (cell number in inflorescence stem vascular bundles and hypocotyl diameter; supplementary material Figs S4, S5) and for vascular organisation phenotypes (Analine Blue stained sections; supplementary material Fig. S5). This result is in contrast to that observed between pxy and wox4 as pxy wox4 lines demonstrate an enhancement (Hirakawa et al., 2010). Similarly, pxy wox4 wox14 triple mutants were identical to pxy wox4 lines (supplementary material Figs S4, S5). These observations are consistent with WOX14 acting in an epistatic manner to PXY, and suggest that the strong phenotype observed in pxy wox4 may in part be due to the loss of control of WOX14 in pxy mutants.

pxy interacts with er during vascular development

Our results with wox4 and wox14 suggested that the vascular organisation defect in pxy mutants may be genetically separable from proliferation defects. In our original description of pxy, the pxy-1 allele in Ler background demonstrated a stronger organisation defect than mutants in the Col background (Fisher and Turner, 2007) (supplementary material Fig. S6). pxy-1 has a premature stop codon in the kinase domain that is presumably essential for transmitting the CLE41/42 signal through PXY, but a variety of Col pxy alleles, including those that entirely lack the kinase domain do not exhibit similarly strong phenotypes (Fisher and Turner, 2007).

We explored the reasons behind the difference in the strength of Ler and Col pxy alleles to investigate further whether vascular organisation and proliferation are genetically separable. One prominent difference between Ler and Col ecotypes is the presence of a mutant er gene in Ler. ER acts redundantly with closely related RLK proteins ERL1 and ERL2 in promotion of cell division in Arabidopsis (Shpak et al., 2004), including in the vascular tissue (Fig. 8), but is also involved in intertissue communication between endodermis and the phloem, where both ER (Uchida et al., 2012) and CLE41 (Etchells and Turner, 2010) are expressed. We hypothesised that the severity of the pxy-1 Ler allele could be due to a genetic interaction between pxy and er. To test this, we isolated a novel er allele in the Col background which harboured a T-DNA insertion in the LLR region of the gene (GabiKat_364C05) (Rosso
et al., 2003), renamed er-124 following the allelic sequence described (Lease et al., 2001). er-124 was indistinguishable from other er alleles – siliques were short, blunt and wider than those of wild type, and inflorescence stems were shorter (supplementary material Figs S7, S8). er single mutants demonstrated no inflorescence stem vascular cell division defects (Fig. 8A) or secondary growth defects, as er hypocotyls were the same size as wild type (Fig. 8B). We nevertheless examined whether ER and PXY interacted by generating px y er double mutants. These plants accumulated anthocyanin in cotyledons (supplementary material Fig. S7D) in a manner reminiscent of the px y-1 Ler allele (Fisher and Turner, 2007). px y er mutants demonstrated a height defect but this was an additive phenotype as average plant height was a combination of the reductions observed in the single mutants (supplementary material Figs S7, S8). Similarly, no interaction was found between px y and er in silique length (px y mutants have the same pod length as wild type, er mutants are shorter and er px y double mutant pods are the same as those of er single mutants; supplementary material Fig. S8). Consequently, px y is not a general enhancer of er.

We assayed the average number of cells in inflorescence stem vascular bundles and found that px y er double mutants had similar numbers of vascular cells to px y single mutants (Fig. 8) and, therefore, er does not enhance the px y vascular cell number defect in inflorescence stems. However, vascular organisation has been quantitatively characterised by determining the width:length (tangential:radial) ratio of vascular bundles as a measure of changes in organisation in vascular bundles (Fisher and Turner, 2007). We applied this measure to examine px y w o x 4 mutants that have been reported to have more severe vascular bundle disruption than px y single mutants. Although px y w o x 4 did appear to show a small enhancement of the px y phenotype (Fig. 9A), much more striking changes in the shape of vascular bundles were observed in px y er double mutants, and this was much greater than that observed for either of the corresponding single mutants (Fig. 9B; Fig. 10A). The large increase in the tangential:radial ratio in px y er (Fig. 9B) was similar to that of the original px y-1 allele (Fisher and Turner, 2007). Consequently, px y er double mutants have the same number of cells as px y single mutants, but the shape of the vascular bundle is altered.
We further investigated vascular organisation in *pxy* *er* lines by observing Analine Blue-stained hand sections and found that xylem and phloem were clearly intercalated in *pxy* *er* to a greater extent than observed in *pxy* single mutants (Fig. 10A-D).

Finally, we analysed the *pxy* *er* interaction during secondary growth in hypocotyls. In contrast to our observations in the inflorescence stem, *pxy* lines has fewer vascular cell divisions than those of *pxy* and *er* single mutants, as determined by hypocotyl diameter measurements (Fig. 8B), suggesting that *ER* and *PXY* may interact to promote cell division during secondary growth; however, interpretation of this phenotype is not straightforward as *ER* is known to repress xylem expansion in this context (Ragni et al., 2011).

*ER* expression overlaps with that of *CLE41* (Etchells and Turner, 2010; Uchida et al., 2012) so we tested whether *er* mutants displayed differences in *CLE41, CLE42* or *CLE44* expression; however, none was observed (supplementary material Fig. S9). Thin sections were analysed for morphological differences between *er* and wild type in the phloem, but no clear differences were apparent (supplementary material Fig. S10). Similarly, vasculature of *er* *erl1* *erl2* triple mutants was analysed and these were found to retain wild-type organisation (Figs 9, 10), despite large reductions in vascular cell number (Fig. 8).

**DISCUSSION**

**WOX4** acts as a transcriptional target for *PXY* signalling controlling the rate of vascular cell division; however, vascular proliferation caused by overexpression of the *PXY* ligand in *IRX3::CLE41* lines was only partially abolished in *wox4 IRX3::CLE41* (Figs 1, 2; supplementary material Fig. S2), suggesting that other factors must also act downstream of *PXY* to promote vascular cell divisions. *PXY* could act redundantly with other RLK proteins such that *WOX4* levels are partially maintained in *pxy* owing to *CLE41* acting through redundant receptors and this model would be consistent with transmission of the CLV3 signal in the shoot apical meristem, which acts through the CLV1 receptor (Clark et al., 1997; Fletcher et al., 1999; Ogawa et al., 2008), RECEPTOR-LIKE PROTEIN KINASE2 (Kinoshita et al., 2010) and CLV2-CORYNE receptor heterodimers (Jeong et al., 1999; Müller et al., 2008; Zhu et al., 2010). There is some support for this hypothesis, as levels of *WOX4* expression were partially increased upon a 16-hour TDIF induction in *pxy* mutants (Fig. 3). However, although there is evidence of enhancement of the organisational defect in *pxy* by mutating related RLK genes *PXL1* and *PXL2* (Fisher and Turner, 2007), there is no evidence of enhancement of the *pxy* cell division phenotype (Fisher and Turner, 2007) (Fig. 9), suggesting that if further receptors do confer the TDIF signal promoting cell division they are not closely related to *PXY*. On the other hand, in our previous analysis, we observed that hypocotyl diameter increases in 35S::*CLE41* lines were suppressed in 35S::*CLE41* *pxy* apparently to the level of *pxy* mutants (Etchells and Turner, 2010), so although this analysis does not completely rule out receptor redundancy in promoting cell division, it suggests that any redundancy is likely to be limited. As *WOX4* levels are unchanged in *pxy* mutant seedlings, despite a clear phenotype (Hirakawa et al., 2010), we hypothesised that *WOX4*-redundant transcriptional targets of *PXY* signalling may be present. *WOX4* is related to *WOX4* (Haecker et al., 2004), downregulated in *pxy* mutants and upregulated in lines overexpressing *CLE41* (Fig. 4). Strikingly, when expression of *WOX4* and *WOX14* was compared in *pxy* mutants, *WOX4* was significantly reduced at the base of stems, in contrast to *WOX14*, the expression of which was reduced at the top of stems. These results suggest that *PXY*-mediated control of *WOX* expression is dependent on developmental stage (Fig. 4).

*wox14* strongly enhanced *wox4*, such that *wox4 wox14* mutants had comparable cell division defects to those observed in *pxy wox4* double mutants (Figs 6, 7). However, *pxy wox14* plants were indistinguishable from *pxy* single mutants (supplementary material Figs S4, S5). *pxy wox4 wox14* lines appeared identical to *pxy wox4* and had vascular cell numbers similar to those of *wox4 wox14* (supplementary material Figs S4, S5). These observations are consistent with *WOX14* acting downstream of *PX*Y and redundantly with *WOX4* (Fig. 11). Strikingly, *wox4 wox14* double mutants exhibited no defect in the organisation of vascular tissue in contrast to that observed in *pxy, wox4* wox14 and *wox4 wox4* lines (Figs 6, 7; supplementary material Figs S4, S5).

Partly based upon the phenotype of *pxy wox4* mutants, it has been proposed that the organisation defect in *pxy* is due to consumption of the vascular meristem in some cell files followed by re-initiation of a vascular developmental programme (cavity model; supplementary material Fig. S1C) (Hirakawa et al., 2010). At the shoot apex, *shoot meristemless* (Barton and Poethig, 1993), *wuschel* (Lau et al., 1996) and *pinhead* (McConnell and Barton, 1995) mutants all have defective apical meristems but in all instances, some level of meristem re-initiation occurs in adventitious positions on the flank of the original meristem. We have previously demonstrated that a mechanism exists whereby procambial cell divisions are promoted by ethylene in the absence of *PXY* signalling (Etchells et al., 2012). *wox4 wox14* double mutants have a more severe proliferation defect to that observed in *pxy* mutants but vascular organisation is maintained; consequently, our results...
suggest that if the cavity model can explain the loss of vascular organisation in *pxy* it must involve factors other than *WOX4* and *WOX14*.

For the ‘localised ligand’ model to be correct, the rate of cell division and vascular organisation must be genetically separable, independent outputs of *pxy* signalling. Evidence that this is the case comes from analysis of *pxy er* double mutants. Vascular cell number in *pxy er* lines was unchanged compared with that observed in *pxy* single mutants; however, defects in the arrangement of vascular tissue along the tangential and radial axes were observed (Fig. 8B) in addition to more prominent intercalation of xylem and phloem in *pxy er* compared with *pxy*. Our model, in which procambium-expressed *PXY* receives positional information from the phloem is analogous to a WNT signalling-controlled oriented cell division in *C. elegans* EMS cells (Goldstein, 1995; Schlesinger et al., 1999). ER protein interacts directly with the TOO MANY MOUTHS (TMM) receptor controlling asymmetric cell divisions during stomata development (Abrash et al., 2011; Lee et al., 2012); however, a distinct mechanism must work in the context of vascular tissue. *ER* is able to affect vascular organisation in the procambium but in stems it is expressed in the xylem, phloem and epidermis (Uchida et al., 2012). As such, it does not overlap with procambium-expressed *PXY* (Etchells and Turner, 2010), making a physical interaction between *PXY* and ER proteins unlikely. Although the PXY ligands CLE41/42/44 are also phloem expressed, their mRNA levels are unaffected in *er* mutants (supplementary material Fig. S9). Within the stem CHAL, CLL1 and CLL2 have been identified as the ligands for ER, yet they are expressed specifically in the endodermis (Abrash et al., 2011; Uchida et al., 2012). Neither *chal/cll* (Uchida et al., 2012) nor *ERf* mutants (Figs 9, 10) exhibit defects in the orientation of vascular cell division. Although it may be the case that the change in vascular organisation in *pxy er* is due to an unknown effect caused by the pleiotropic nature of *er* loss-of-function phenotypes, we speculate that the interaction between *PXY* and *ER* may represent a novel signalling pathway that coordinates differential growth over several cell layers in *Arabidopsis* via non-cell autonomous factors acting downstream of *ER* (Fig. 11).

In plants, there are a growing number of cell-surface receptors described as conferring positional information in proliferating cell lineages, possibly as a consequence of interpreting positional

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*Fig. 10. Xylem and phloem are intercalated in pxy er mutants.* (A–E) Toluidine Blue-stained thin sections (upper panels); Analine Blue-stained hand sections (lower panels) of Col (A), *pxy* (B), *er* (C), *pxy er* (D) and *er er1 er2* (E) 8-week-old inflorescence stem vascular tissue. Vascular organisation is perturbed in *pxy* and severely perturbed in *pxy er* lines (arrowheads show adjacent xylem and phloem, which are spatially separated in wild type). (F–J) Toluidine Blue (upper) and Analine Blue (lower) stained sections of Col (F), *pxy* (G), *er* (H), *pxy er* (I) and *er er1 er2* (J) hypocotyl vascular tissue. Arrowheads indicate intercalated xylem and phloem. Scale bars: 50 μm.
Fig. 11. The interaction between PX, WOX4, WOX14 and ER. PX and ER signalling modules interacting in the Arabidopsis stem. Procambium expressed PXY controls cell division number via WOX4 and WOX14 as a separate outcome from vascular organisation. ER, which in stems is expressed in the epidermis, phloem and xylem, is required non-cell autonomously with PXY to control vascular organisation.

information derived from excreted peptide ligands. In the epidermis, stomatal spacing is enforced through EPF ligands signalling to TMM/ERL receptors (Abrash et al., 2011; Hara et al., 2007; Lee et al., 2012) and similarly in anthers, the ligand TAPETUM DETERMINANT 1 (Yang et al., 2003) signals to the EXCESS MICROSPOROCYTES 1 receptor (Yang et al., 2005), resulting in oriented divisions that form the tapetum in a contiguous layer (Canales et al., 2002; Feng and Dickinson, 2010; Zhao et al., 2002). We suggest that this may be a general mechanism for maintaining tissue integrity during cell division and differentiation during differential growth.

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References

Abrasheva, E. B., Davies, K. A. and Bergmann, D. C. (2011). Generation of signaling specificity in Arabidopsis by spatially restricted buffering of ligand-receptor interactions. Plant Cell 23, 2864-2879.

Bartos, M. K. and Poethig, R. S. (1993). Formation of the shoot apical meristem in Arabidopsis: an analysis of development in the wild type and in the shoot meristemless mutant. Development 119, 823-831.

Brand, U., Fletcher, J. C., Hobe, M., Meyerowitz, E. M. and Simon, R. (2000). Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. Science 289, 617-619.

Canales, C., Bhatt, A. M., Scott, R. and Dickinson, H. (2002). EKS, a putative RLR receptor kinase, regulates male gametophyte cell number and tapetal identity and promotes seed development in Arabidopsis. Curr. Biol. 12, 1718-1727.

Cano-Delgado, A., Lee, J.-Y. and Demura, T. (2010). Regulatory mechanisms for specification and patterning of plant vascular tissues. Annu. Rev. Cell Dev. Biol. 26, 605-637.

Clark, S. E., Williams, R. W. and Meyerowitz, E. M. (1997). The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. Cell 89, 575-585.

Clough, S. J. and Bent, A. F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735-743.

Etchells, J. P. and Turner, S. R. (2010). The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division. Development 137, 767-774.

Etchells, J. P., Provost, C. M. and Turner, S. R. (2012). Plant vascular cell division is maintained by an interaction between PXY and ethylene signalling. PLoS Genet. 8, e1002997.

Feng, X. and Dickinson, H. G. (2010). Tapetal cell fate, lineage and proliferation in the Arabidopsis anther. Development 137, 2409-2416.

Fisher, K. and Turner, S. (2007). PX: a receptor-like kinase essential for maintaining polarity during plant vascular-tissue development. Curr. Biol. 17, 1061-1066.

Fletcher, J. C., Brand, U., Running, M. P., Simon, R. and Meyerowitz, E. M. (1999). Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. Science 283, 1911-1914.

Goldstein, B. (1995). Cell contacts orient some cell division axes in the Caenorhabditis elegans embryo. J. Cell Biol. 129, 1071-1100.

Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M. and Laux, T. (2004). Expression dynamics of WOX genes mark cell fate decisions during early embryonic patterning in Arabidopsis thaliana. Development 131, 657-668.

Hara, K., Kajita, R., Torii, K. U., Bergmann, D. C. and Kakimoto, T. (2007). The secretory peptide gene EPF1 enforces the stomatal one-cell-space rule. Genes Dev. 21, 1720-1725.

Hara, K., Yokoo, T., Kajita, R., Onishi, T., Yahata, S., Peterson, K. M., Torii, K. U. and Kakimoto, T. (2009). Epidermal cell density is autoregulated via a secretory peptide: EPIDERMAL PATTERNING FACTOR 2 in Arabidopsis leaves. Plant Cell Physiol. 50, 1019-1031.

Hårdstedt, M., Finnegan, C. P., Kirchoff, N., Hyland, K. A., Wijkstrom, M., Murtaugh, M. P. and Hering, B. J. (2005). Post-transplant upregulation of chelonine messenger RNA in non-human primate recipients of intraportal pig islet xenografts. Xenotransplantation 12, 293-302.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakano, Y., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y. and Fukuda, H. (2008). Non-cell-autonomous control of vascular stem cell fate by a CLF/peptide receptor system. Proc. Natl. Acad. Sci. USA 105, 15208-15213.

Hirakawa, Y., Kondo, Y. and Fukuda, H. (2010). TDF1 peptide signaling regulates vascular stem cell proliferation via the WOX4 homo box gene in Arabidopsis. Plant Cell 22, 2618-2629.

Hunt, L. and Gray, J. E. (2009). The signaling peptide EPF2 controls asymmetric cell divisions during stomatal development. Curr. Biol. 19, 864-869.

Ito, Y., Nakano, Y., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N. and Fukuda, H. (2006). Dodeca-CLE peptides as suppressors of plant stem cell differentiation. Science 313, 842-845.

Jeong, S., Trotochaud, A. E. and Clark, S. E. (1999). The Arabidopsis CLAVATA1 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. Plant Cell 11, 1925-1934.

Ji, S., Strable, J., Shimizu, R., Koenig, D., Sinha, N. and Scanlon, M. J. (2010). WOX4 promotes procambial development. Plant Physiol. 152, 1346-1356.

Kinoshi, A., Betsuyaku, S., Osakabe, Y., Misono, S., Nagawa, S., Stahl, Y., Simon, R., Yamaguchi-Shinozaki, K., Fukuda, H. and Sawa, S. (2010). NLP2 receptor-like kinase that transmits the CLV3 signal in Arabidopsis. Development 137, 3911-3920.

Laux, T., Mayer, K. F., Berger, J. and Jürgens, G. (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. Development 122, 87-96.

Lease, K. A., Lau, N. Y., Schuster, R. A., Torii, K. U. and Walker, J. C. (2001). Receptor serine/threonine protein kinases in signalling: analysis of the erecta receptor-like kinase of Arabidopsis thaliana. New Phytol. 151, 133-143.

Lee, J. S., Kurola, H., Hnilova, M., Khayat, L., Kanaoka, M. M., McAbee, J. M., Sankaya, M., Tamerler, C. and Torii, K. U. (2012). Direct interaction of ligand-receptor pairs specifying stomatal patterning. Genes Dev. 26, 126-136.

McConnell, J. R. and Barton, M. K. (1995). Effect of mutations in the PINHEAD gene of Arabidopsis on the formation of shoot apical meristems. Dev. Genet. 16, 358-366.

Müller, R., Bleckmann, A. and Simon, R. (2008). The receptor kinase CORYNE of Arabidopsis transmits the stem-cell-limiting signal CLAVATA3 independently of CLAVATA1. Plant Cell 20, 934-946.

Ogawa, M., Shinohara, H., Sakagami, Y. and Matsubayashi, Y. (2008). Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. Science 319, 294-297.

Pagni, L., Nieminen, K., Pacheco-Villalobos, D., Sibout, R., Schwechheimer, C. and Hardtke, C. S. (2011). Mobile gibberellin directly stimulates Arabidopsis hypocotyl xylem expansion. Plant Cell 23, 1322-1336.

Rosso, M. G., Li, Y., Strizhov, N., Reiss, B., Dekker, K. and Weisshaar, B. (2003). An Arabidopsis thaliana T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol. Biol. 53, 247-259.

Sarkar, A. K., Luijten, M., Miyashima, S., Lenhard, M., Hashimoto, T., Nakajima, K., Scheres, B., Heidstra, R. and Laux, T. (2007). Conserved factors regulate signalling in Arabidopsis thaliana shoot and root stem cell organizers. Nature 446, 811-814.

Schellenger, A., Shelton, C. A., Maloof, J. N., Meneghini, M. and Bowerman, B. (1999). Wnt pathway components orient a mitotic spindle in the early
Caenorhabditis elegans embryo without requiring gene transcription in the responding cell. Genes Dev. 13, 2028-2038.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K. F. X., Jürgens, G. and Laux, T. (2000). The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. Cell 100, 635-644.

Shpak, E. D., Berthiaume, C. T., Hill, E. J. and Torii, K. U. (2004). Synergistic interaction of three ERECTA-family receptor-like kinases controls Arabidopsis organ growth and flower development by promoting cell proliferation. Development 131, 1491-1501.

Shpak, E. D., McAbee, J. M., Pillitteri, L. J. and Torii, K. U. (2005). Stomatal patterning and differentiation by synergistic interactions of receptor kinases. Science 309, 290-293.

Suer, S., Agusti, J., Sanchez, P., Schwarz, M. and Greb, T. (2011). WOX4 imparts auxin responsiveness to cambium cells in Arabidopsis. Plant Cell 23, 3247-3259.

Swarbreck, D., Wilks, C., Lamesch, P., Berardini, T. Z., Garcia-Hernandez, M., Foerster, H., Li, D., Meyer, T., Muller, R., Ploetz, L. et al. (2008). The Arabidopsis Information Resource (TAIR) gene structure and function annotation. Nucleic Acids Res. 36, D1009-D1014.

Uchida, N., Lee, J. S., Horst, R. J., Lai, H.-H., Kajita, R., Kakimoto, T., Tasaka, M. and Torii, K. U. (2012). Regulation of inflorescence architecture by intertissue layer ligand-receptor communication between endodermis and phloem. Proc. Natl. Acad. Sci. USA 109, 6337-6342.

van Zanten, M., Snoek, L. B., Proveniers, M. C. G. and Peeters, A. J. M. (2009). The many functions of ERECTA. Trends Plant Sci. 14, 214-218.

Yang, S.-L., Xie, L.-F., Mao, H.-Z., Puah, C. S., Yang, W.-C., Jiang, L., Sundaresan, V. and Ye, D. (2003). Tapetum determinant1 is required for cell specialization in the Arabidopsis anther. Plant Cell 15, 2792-2804.

Yang, S.-L., Jiang, L., Puah, C. S., Xie, L.-F., Zhang, X.-Q., Chen, L.-Q., Yang, W.-C. and Ye, D. (2005). Overexpression of TAPETUM DETERMINANT1 alters the cell fates in the Arabidopsis carpel and tapetum via genetic interaction with excess microsporocytes1/extra sporogenous cells. Plant Physiol. 139, 186-191.

Young, B., Wightman, R., Blanvillain, R., Purcel, S. B. and Gallois, P. (2010). pH-sensitivity of YFP provides an intracellular indicator of programmed cell death. Plant Methods 6, 27.

Zhao, D.-Z., Wang, G.-F., Speal, B. and Ma, H. (2002). The excess microsporocytes1 gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the Arabidopsis anther. Genes Dev. 16, 2021-2031.

Zhu, Y., Wang, Y., Li, R., Song, X., Wang, Q., Huang, S., Jin, J. B., Liu, C.-M. and Lin, J. (2010). Analysis of interactions among the CLAVATA3 receptors reveals a direct interaction between CLAVATA2 and CORYNE in Arabidopsis. Plant J. 61, 223-233.