Development 137, 767-774 (2010) doi:10.1242/dev.044941

The PXY-CLE41 receptor ligand pair defines a multifunctional pathway that controls the rate and orientation of vascular cell division

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SUMMARY

Controlling the orientation of cell division is fundamental to the development of complex body plans. This is particularly apparent in plants, where development is determined by differential growth that results solely from changes in cell expansion and orientation of the cell division plane. Despite the fundamental importance of cell division orientation to plant development, the mechanisms regulating this process remain almost completely unknown. During vascular development, the meristematic cambial cells divide down their long axis in a highly orientated manner to generate clear files of cells. The receptor kinase PXY has previously be shown to be essential for this orientation. Here, we demonstrate that the division plane is determined by the interactions of PXY and its peptide ligand, CLE41. PXY is expressed within dividing meristematic cells of the procambium, whereas CLE41 localises to the adjacent phloem cells. Altering the pattern of CLE41 expression leads to a loss of cell division orientation and a dramatic loss of ordered vascular tissue development. By contrast, increasing phloem-specific expression of CLE41 results in more cell divisions, but the orientation of cell division is retained, leading to both increased and well-ordered vascular development. We demonstrate that PXY signalling is down-regulated by CLE41. This feedback mechanism is crucial in integrating the different roles of PXY signalling in controlling xylem differentiation, regulating the rate of vascular cell division and determining the orientation of cell division. Parallels with animal systems indicate that localised signalling from adjacent cells is a general mechanism for defining the plane of cell division.

KEY WORDS: *Arabidopsis*, Vascular development, Cell division

INTRODUCTION

Orientated cell divisions are an essential part of development in a wide range of multicellular organisms (Müller et al., 2009; Siller and Doe, 2009). This is particularly evident in plants, where even complex and elaborate morphologies are the consequence solely of differential growth. The absence of cell migration in plants means that growth occurs as a consequence of cell expansion and orientation of the cell division plane. Despite the importance of the orientation of cell divisions in plant development, how this process is regulated remains unclear.

In the shoot apical meristem it is well established that signalling via the receptor-like kinase (RLK) CLAVATA 1 (CLV1) is essential to maintain the balance between stem cell division and differentiation (Clark et al., 1997; DeYoung et al., 2006; Fletcher et al., 1999; Schoof et al., 2000). More recently, it has been demonstrated that orientated cell divisions in the apical meristem are an essential part of organ formation (Reddy et al., 2004). Furthermore, the presence of ectopic CLAVATA3/ESR-RELATED (CLE) proteins in roots results in alterations in the normal pattern of highly orientated cell divisions (Fiers et al., 2005). Other signalling components such as POLTERGEIST (POL) and POLTERGEIST LIKE 1 (PLL1), which are phosphatases acting downstream of CLV1 (Song et al., 2006), also influence the position of cell divisions in *Arabidopsis* embryos (Song et al., 2008).

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Accepted 9 January 2010

More direct evidence for a role for RLKs in controlling cell division in plant meristems has come from studies of the vascular meristem where the highly ordered nature of vascular development makes it an excellent system for studying the mechanisms that control the orientation of cell division. In contrast to initials in apical meristems, vascular initials are long thin cells, yet they divide down the centre of their long axis and parallel to the outside of the plant (periclinally) in order to generate files of cells along the radial axis that are also aligned along the apical basal axis (Fig. 1A; Fig. 2E; Fig. 4E). This process is disrupted by mutations at the *PHLOEM INTERCALATED WITH XYLEM* (*PXY*) locus, which encodes an RLK that is essential for ordered, coordinated cell divisions in the *Arabidopsis* procambium (Fisher and Turner, 2007). A more general role for RLKs in the orientation of cell division is supported by the identification of RLKs as being essential for the asymmetric cell division that occurs prior to lateral root initiation (De Smet et al., 2008) and a polarised cell division in Maize stomatal mother cells (Cartwright et al., 2009). Although these studies all illustrate the importance of receptor-like kinases in orientating plant cell divisions, in the absence of information on a ligand, how these kinases function to impart the positional information required to orientate the cell division plane remains unclear.

A short peptide, CLV3/ESR1-LIKE 41 (CLE41) has been shown to repress xylem differentiation in cell culture (Ito et al., 2006). CLE41 has been recently shown to bind to PXY and is thought to act synergistically with CLE6 to regulate the rate of cell division in a PXY-dependent manner (Hirakawa et al., 2008; Whitford et al., 2008). None of these studies, however, addresses how PXY or CLE41 function to orientate the plane of cell division.

A model for PXY function predicts that localised expression of the PXY ligand is essential to generate the spatial information required for oriented cell divisions (Fisher and Turner, 2007) (Fig.

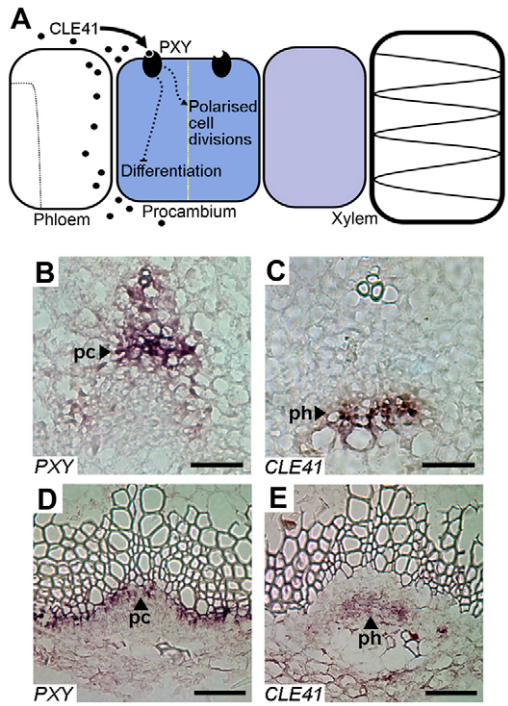

Fig. 1. A model to show how CLE41 and PXY determine the orientation of the cell division plane. (**A**) CLE41 is expressed in the phloem and signals to PXY in the procambium, thus providing positional information to the dividing cell, which sets the appropriate division plane (yellow line). (**B-E**) In situ hybridisation showing *PXY* expressed in the procambium (B,D) and *CLE41* expressed in the phloem (C,E) at the top (B,C) and the base (D,E) of inflorescence stems. pc, procambium; ph, phloem. Scale bars: 25 μm in B,C; 25 μm in D,E.

1A). Here, we demonstrate how CLE41 expression specifically localised in phloem cells is perceived by PXY and used to generate the spatial information essential for regulating the proper orientation of cell division.

PXY signalling clearly has multiple functions in vascular development that include orientating the plane of cell division, repression of xylem differentiation and regulation of the rate of cell division. We are also able to demonstrate that interactions between CLE41 and PXY involve a negative-feedback mechanism that is essential to integrate the different outputs of PXY signalling that are essential for plant vascular tissue development.

MATERIALS AND METHODS

Generation of plant stocks

All plant work was carried out in the Columbia ecotype. *Arabidopsis* DNA sequences were obtained from the TAIR database (Swarbreck et al., 2008). Oligonucleotides used are listed in Table S1 in the supplementary material. *35S* constructs for *Arabidopsis* transformation were generated by cloning *PXY*, *CLE41* and *CLE42* genomic DNA sequences into *pK2GW7,0* (Karimi et al., 2002) via *pENTR-D-TOPO* (Invitrogen). *IRX3::CLE41* was made similarly using *p3HSC* gateway destination vector (Atanassov et al., 2008). For *SUC2::CLE41*, we used overlapping PCR. The *SUC2* (*SUCROSE-PROTON SYMPORTER 2*) promoter and *CLE41* coding sequences were amplified separately with overlapping ends. These products were mixed, annealed and elongated prior to amplification with *SUC2* and *CLE41* forward and reverse oligos, respectively. The resulting PCR product was cloned into *pTF101.gw1* (Paz et al., 2004) via *pENTR-D-TOPO*. For tissue specific expression, promoters known to give xylem (*IRX3*)- (Gardiner et al., 2003) or phloem (*SUC2*)- (Truernit and Sauer, 1995) specific expression were used. Plasmids were sequenced, then transformed into *Arabidopsis* using floral dip (Clough and Bent, 1998). Phenotypes described are representative of 8 of 10, 11 of 12 and 14 of 15 independent events for plants transformed with *35S::CLE41*, *IRX3::CLE41* and *SUC2::CLE41*, respectively. *35S::CLE42* phenotypes were found in ten independent transgenic lines. The *pxy-3* allele (Fisher and Turner, 2007) was used for genetic analysis. Homozygous *pxy-3* plants were identified by PCR. *35S::CLE41 pxy*, *35S::CLE42 pxy*, *35S::CLE41 35S::PXY* and *35S::CLE42 35S::PXY* lines were generated by crossing and were identified in the F2 population. *IRX3::CLE41 35S::PXY* and *SUC2::CLE41 35S::PXY* lines were generated by directly transforming plants carrying the *35S::PXY* construct with *pIRX3::CLE41* or *pSUC2::CLE41*. *SUC2::CLE41* and *35S::CLE41* cell counts were carried out on ten independent T2s (2 bundles/plant) and six independent T1s (3 bundles/plant), respectively. Five-week-old plants were used.

Histology

Analysis of vasculature using thin sections cut from JB4 resin embedded material was carried out as previously described (Pinon et al., 2008). For hand cut sections, tissue was stained with either aqueous 0.02% Toluidine Blue or 0.05 M Aniline Blue in 100 mM phosphate buffer, pH7.2. Vascular tissue was considered to be ordered in stem vascular bundles if the xylem and phloem could be separated by a simple curved line and ordered in the hypocotyl if xylem could be incorporated within an elliptical shape that excluded the phloem.

Gene expression

qRT-PCR analysis was carried out using the gene-specific primers listed (see Table S1 in the supplementary material) using SYBR Green JumpStart Taq ReadyMix (Sigma) and an ABI Prism 7000 machine (Applied Biosystems) with the standard SYBR Green detection programme. A melting curve was produced at the end of every experiment to ensure that only single products were formed. Gene expression was determined using a version of the comparative threshold cycle (Ct) method. The average amplification efficiency of each target was determined using LinReg (Hardstedt et al., 2005) and samples were normalised to *18S* rRNA and *ACT2*. Results were similar, independent of the control used. All samples were measured in technical triplicates on biological triplicates and in all experiments, controls were within two Cts. Methods for in situ hybridisation analysis using digoxigenin-labelled mRNA probes were carried out as previously described (Narita et al., 2004). Oligos for making probe templates are listed in Table S1 in the supplementary material.

RESULTS

Disruption of CLE41 expression alters the plane of vascular cell divisions

Our previous model for PXY function is dependent on the secretion of PXY ligand from cells adjacent to those expressing *PXY* (Fisher and Turner, 2007) (Fig. 1A). Using in situ hybridisation, we found that *CLE41* is expressed throughout the phloem in a domain that is adjacent to dividing cells in the procambium where *PXY* is expressed in both young (Fig. 1B,C) and old (Fig. 1D,E) inflorescence stems. Thus, *PXY* and *CLE41* are expressed in adjacent, non-overlapping domains and, as such, secretion of CLE41 could impart positional information to adjacent cells expressing PXY (Fig. 1A).

CLE41 RNAi lines have previously been described as having no phenotype (Whitford et al., 2008). We reduced *CLE41* expression using RNA silencing by generating plants harbouring an artificial microRNA against *CLE41*. There are thought to be 83 CLE genes in *Arabidopsis*. These give rise to peptides that have been classified into 13 groups, based on the CLE motif, which corresponds to their known biological functions (Oelkers et al., 2008). CLE41 was identified as a member of the group 5 family of CLE peptides, of

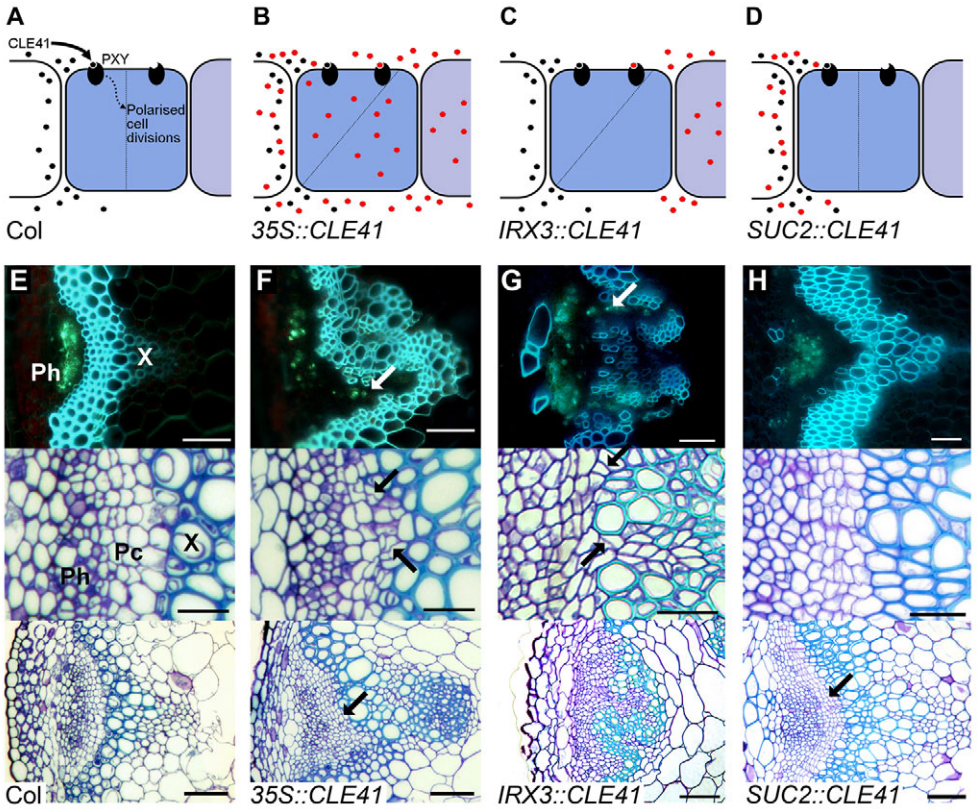

Fig. 2. Ectopic *CLE41* expression results in disrupted vascular bundles. (**A-D**) Diagrammatic representation of vascular cell divisions specified by CLE41 and PXY in wild-type and transgenic lines. Native CLE41 is represented by black dots, CLE41 from overexpression in red. In wild type (A), CLE41 is expressed in the phloem and signals to PXY in the procambium, thus providing positional information to the dividing cell, which sets the appropriate division plane (dotted line). In *35S::CLE41* (B) and *IRX3::CLE41* (C) lines, the polar nature of the CLE41 signal is disrupted, resulting in loss of oriented cell division. *SUC2::CLE41* (D) lines have more ligand than do wild-type lines, but it is expressed in a polar manner such that positional information is maintained and the cell division is oriented. (**E-H**) Col stems in transverse section (E) compared with *35S::CLE41* (F), *IRX3::CLE41* (G) and *SUC2::CLE41* (H) lines. Phloem sieve plates fluoresce green in Analine Blue stain (top panel) and are found in a discrete arc in wild-type (E) and *SUC2::CLE41* (H) lines, but are displaced towards the centre of the stem in *35S::CLE41* and *IRX3::CLE41* lines (F,G, white arrows). Procambial cells divide in a highly ordered process (middle panel) in wild type (E) and *SUC2::CLE41* (H) lines compared with *35S::CLE41* (F) and *IRX3::CLE41* (G) lines, where some cell divisions are present at altered orientations (black arrows). A large number of undifferentiated cells are present in *35S::CLE41* and *SUC2::CLE41* lines (F,H, bottom panel, black arrows) compared with wild-type lines (E). Pc, procambium; Ph, phloem; X, xylem. Scale bars: 50 μm in E-H top and bottom panels; 20 μm in E-H middle panels.

which CLE42 and CLE44 are the other members in *Arabidopsis* (Ito et al., 2006; Oelkers et al., 2008; Strabala et al., 2006). These three CLE genes have previously been identified as encoding tracheary element differentiation inhibitory factor (TDIF) on the basis of their ability to inhibit xylem differentiation (Ito et al., 2006). We crossed our strong *CLE41* knockdown lines to plants carrying a T-DNA insertion disrupting *CLE42*. In all cases, plants appeared as wild type (data not shown). It is probable that redundancy among family members explains the lack of phenotype. We analysed a large number of publicly available array experiments using the Genevestigator Digital Northern tool (Zimmermann et al., 2004). *CLE44* was expressed in array sets rich in vascular tissue, albeit at very low levels (not shown). CLE41 has been shown to demonstrate synergism with CLE6, a member of the group 2 CLE peptides, of which there are seven members in *Arabidopsis*. As a result, it might be the case that a knockdown/knockout of ten *CLE* genes (three from group 5 and seven from group 2) is required to observe a phenotype.

To test whether localised *CLE41* expression is important for determining the orientation of cell divisions in the procambium and cambium, we disrupted its highly localised pattern by expressing it ubiquitously using the *35S* promoter and in its polar opposite location using the well-characterised xylem-specific *IRX3* promoter (Gardiner et al., 2003; Mitsuda et al., 2007). *35S::CLE41* vascular bundles had significantly more cells than wild-type bundles (311.6±15.6 cells in wild type; 373.17±24.3 in *35S::CLE41*; $P<0.05$; Fig. 2A,B,E,F), consistent with previous reports that established that CLE41 overexpression results in increased vascular tissue in the root (Hirakawa et al., 2008). CLE41 clearly influences the orientation at which procambial cells divide because in contrast to the highly polarised periclinal divisions seen in the wild-type procambium, both *35S::CLE41* and *IRX3::CLE41* lines exhibited cell divisions in a range of different orientations (Fig. 2A-C,E-G). In wild-type stems, highly orientated cell division results in spatially separated xylem and phloem, in which the phloem is restricted to a discrete arc adjacent to the cortex. Disorganised cell divisions in *35S::CLE41* and *IRX3::CLE41* lines result in the phloem expanding towards the centre of the stem and developing in regions where xylem is normally localised (Fig. 2E,F).

Misaligned cell divisions and organisational defects were observed in newly generated vascular tissue at the top of *35S::CLE41* inflorescence stems (Fig. 3A,B), suggesting that polar

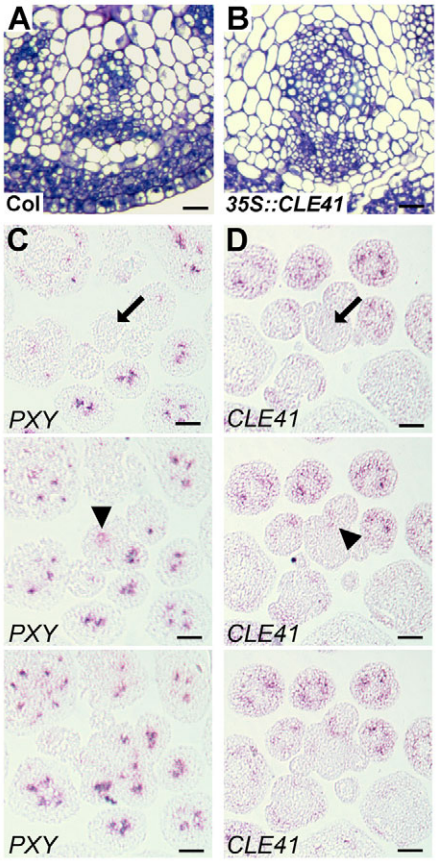

Fig. 3. PXY-CLE41 signalling acts early in vascular development. (**A**,**B**) Transverse sections through wild-type (A) and *35S::CLE41* (B) vascular tissue in recently initiated vascular bundles 5 mm below the shoot apex of 4-week inflorescence stems. (**C**,**D**) In situ hybridisation showing that both *PXY* (C) and *CLE41* (D) expression is absent from the apical meristem (top panels, black arrow), appearing just below it as vascular development is initiated (middle panel, arrowheads), and is apparent in several vascular bundles in older tissue (lower panel). Scale bars: 20 μm in A,B; 50 μm in C,D.

CLE41 expression is required early in vascular development. In situ hybridisation experiments demonstrated that no *PXY* or *CLE41* expression was observed in apical meristems. Expression was first observed below the apex where vascular development is initiated (Fig. 3C,D), suggesting that PXY/CLE signalling is required both to set up and to maintain organised vascular tissue.

We addressed whether *CLE42*, which is predicted to generate a similar peptide to *CLE41* (Kondo et al., 2006), demonstrated similar function. *35S::CLE42* lines had similar, albeit slightly weaker, phenotypes to *35S::CLE41* in stems (see Fig. S1 in the supplementary material), demonstrating that CLE41 and CLE42 have similar functions. For both *35S::CLE41* and *35S::CLE42*, these phenotypes were *PXY*-dependent. *pxy 35S::CLE41* and *pxy 35S::CLE42* plants had identical phenotypes to those of *pxy* single mutants (Fig. 5; see Fig. S1 in the supplementary material). As such, *CLE41* and *CLE42* are epistatic to *pxy*, indicating that CLE41 and CLE42 signal through PXY and are dependent on the presence of a functional PXY receptor. These observations are consistent with CLE41/42 and PXY acting as a ligand-receptor pair, as has been demonstrated previously by studies showing that CLE41 binds to PXY (Hirakawa et al., 2008).

CLE41 acts in secondary growth

To address the role of CLE41 in PXY signalling during secondary growth, we analysed hypocotyls in plants with altered *CLE41* expression (Fig. 4A-C,E-G). Phenotypes were even more dramatic than those observed in inflorescence stems. Altering the *CLE41* expression domain overrides the patterning set up across the whole hypocotyl as it completely lacks organisation. The highly ordered and predictable pattern of vascular tissue seen in the wild-type plants (Fig. 4A,E) is largely lost in *35S::CLE41* and *IRX3::CLE41* plants (Fig. 4B,C,F,G). Some of the cells within the hypocotyl do have a xylem or phloem fate, but these tissues are completely interspersed such that xylem and phloem are no longer separated into recognisable domains. Phloem cell differentiation occurs even in tissue surrounded by xylem and vice-versa, indicating that cambial cells retain the ability to generate xylem and phloem whatever their position within the tissue and that their fate is not reprogrammed dependent on mediolateral position in the stem. Not only is mediolateral patterning disrupted but apical-basal organisation of cells within these hypocotyls is also altered. In wild-type plants, all xylem cells are oriented in a similar manner and run either parallel to the plane of longitudinal sections or at right angles to it in transverse sections (Fig. 4E). This is in striking contrast to *35S::CLE41* and *IRX3::CLE41* plants where xylem vessels are present with dramatically differing orientations, such that cells exhibiting a normal orientation are located adjacent to cells in a perpendicular orientation (Fig. 4F,G). *35S::CLE42* lines had similar, albeit slightly weaker, phenotypes to *35S::CLE41* in hypocotyls (Fig. 5; see Fig. S1 in the supplementary material).

To confirm that the plane of cell division was disrupted by changes to the location of CLE41 expression and not simply by increasing CLE41 expression per se, we expressed CLE41 from the strong phloem-specific and widely used *SUC2* promoter (Truernit and Sauer, 1995) and found that vascular tissue was highly ordered (Fig. 2D; Fig. 4D). The *SUC2::CLE41* construct was clearly functional as, in common with *35S::CLE41* lines (see above), significant increases in vascular cell number were observed (358.8±15.1 cells per bundle in wild type; 557.0±27.6 in *SUC2::CLE41*; $P<0.0001$). Our data demonstrate that expression of CLE41 in or around cells expressing PXY is sufficient to drive vascular cell division but localised expression of CLE41 in the phloem is essential to maintain properly orientated cell divisions. To confirm that this was indeed the case, and that cell misarrangements were not the consequence of high-level *CLE41* expression for an extended period of time or the result of very different expression levels in different lines, we screened the progeny from five independent transformants for each of the *SUC2*, *IRX3* and *35S* promoter constructs and selected three lines that exhibited comparable *CLE41* expression but were relatively weak overexpressing lines (see Fig. S2A in the supplementary material). When *CLE41* was expressed from the phloem using the *SUC2* promoter, cell divisions were ordered. By contrast, when *CLE41* was expressed either ubiquitously using the *35S* promoter or from the xylem using the *IRX3* promoter, this was not the case and divisions were found that were misoriented (see Fig. S2, arrows, in the supplementary material). The result of the misoriented cell divisions is most clearly seen in the hypocotyl, where *SUC2:CLE41* retains the wild-type orientation, but this is lost in lines using either the *35S* and/or *IRX3* promoter to drive *CLE41* expression. As mentioned above, these lines are weak overexpressers of *CLE41* (see Fig. S2A in the supplementary material) and, consequently, the data clearly demonstrate that the orientation of cell division is dependent on

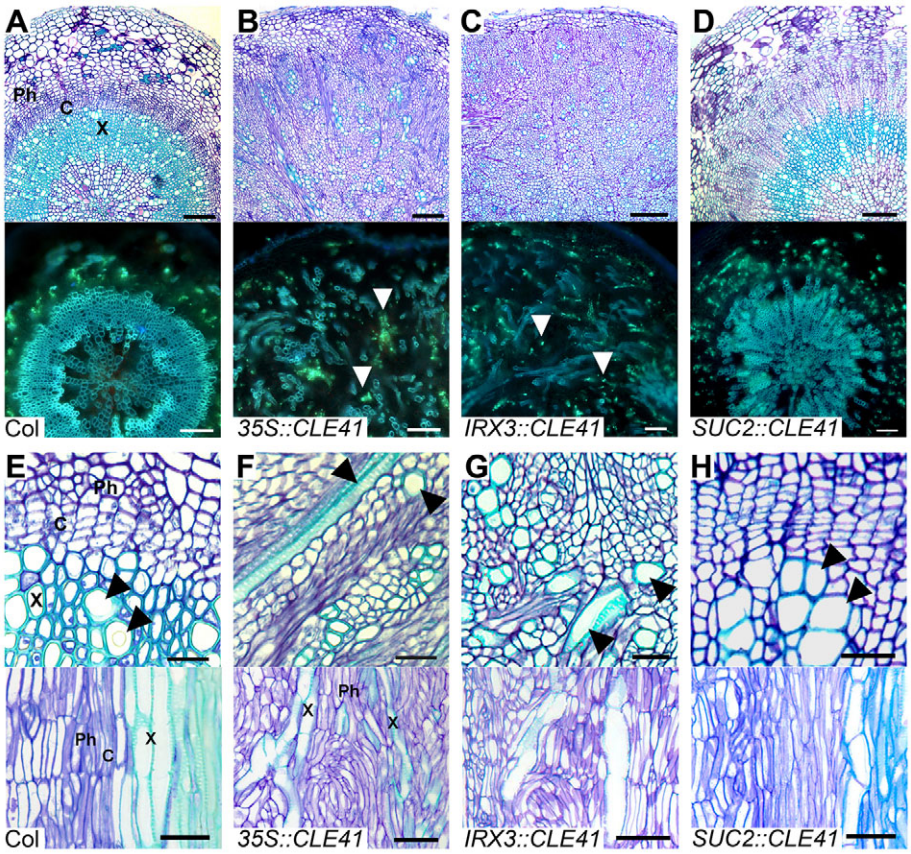

Fig. 4. Ectopic CLE41 expression results in intercalation of xylem and phloem in hypocotyls. (**A-D**) Transverse sections of Toluidine Blue-stained hypocotyls (upper panels) demonstrate that in wild-type (A) and *SUC2::CLE41* (D) hypocotyls, the vascular cylinder is highly ordered with a continuous ring of xylem. Conversely, *35S::CLE41* (B) and *IRX3::CLE41* (C) hypocotyls are disorganised, with cell types interspersed. In sections stained with Aniline Blue (lower panels), wild-type (A) and *SUC2::CLE41* (D) hypocotyls have phloem (fluorescing green) on the outside of the stem surrounding xylem on the inside. In *35S::CLE41* (B) and *IRX3::CLE41* (C) hypocotyls, phloem cells develop towards the centre of the stem (arrowheads) surrounded by xylem. (**E-G**) Toluidine Blue-stained close-up images show that in wild-type (E) and *SUC2::CLE41* (H) hypocotyls, all xylem cells are running parallel (arrowheads) into the plane of section; however, in *35S::CLE41* (F) and *IRX3::CLE41* (G) hypocotyls, xylem vessels are in a variety of orientations (arrowheads). Xylem cells running parallel to the plane of section demonstrate a lack of order along the apical-basal axis and this is clearly demonstrated in longitudinal section (lower panels). C, cambium; Ph, phloem; X, xylem. Scale bars: 100 μm in A-D; 20 μm in E-H upper panels; 50 μm in E-H lower panels.

CLE41 localisation and not due to high-level *CLE41* expression. Taken together, these results, for the first time, demonstrate that polarised ligand signalling to an adjacent receptor can set the cell division plane required for coordinated positioning of a cell division.

A negative-feedback mechanism regulates *PXY* expression

Previously, *PXY* expression has been shown to be higher in *pxy* mutants than wild type (Fisher and Turner, 2007). This suggests that a negative-feedback mechanism could be a feature of PXY signalling as PXY signalling represses *PXY* expression. To investigate this further, expression of *PXY* was assayed in *35S::CLE41* plants. A reduction in *PXY* expression was observed in the inflorescence stem and hypocotyl consistent with CLE41 acting to negatively regulate its receptor (Fig. 6), as has been observed with some ligand-receptor interactions in animal systems (Cadigan et al., 1998). We addressed the consequences of relieving *PXY* from negative regulation of CLE41 by using a *35S::PXY* construct in a *35S::CLE41/42* background. The stems of *35S::CLE41 35S::PXY* and *35S::CLE42 35S::PXY* plants were characterised by dramatic increases in cell number in both the vascular bundle and in the interfascicular region, such that a continuous ring of additional tissue was observed within the stem. New cells were generated between the xylem and phloem in vascular bundles and in the interfascicular region, making the phenotype characteristic of dramatically increased secondary growth (Fig. 7D-G). These results provide strong genetic evidence that CLE41/42 and PXY are a ligand-receptor pair and are sufficient to promote vascular cell division within the procambium and for the induction of secondary growth in the interfascicular region.

Interestingly, the majority of increased cell divisions occurring when both CLE41/42 and PXY were overexpressed were relatively ordered, although aberrant cells divisions were still present (Fig. 7F). How relative order is restored upon ectopic expression of both PXY and CLE41/42 is unclear; however, *35S::PXY* might act to prevent the native receptor from becoming saturated, thus allowing the receptor to perceive a small ligand gradient that might still exist. To test this hypothesis, we made lines harbouring both *IRX3::CLE41* and *35S::PXY* constructs. Consistent with our model (Fig. 1A), we found that vascular organisation was disrupted in *35S::PXY IRX3::CLE41* plants (Fig. 7G,H) but increased secondary growth was also observed, supporting the hypothesis that a signal gradient that is high on the phloem side of the cambium is required for vascular organisation and that overcoming *PXY* negative regulation by *CLE41* can initiate

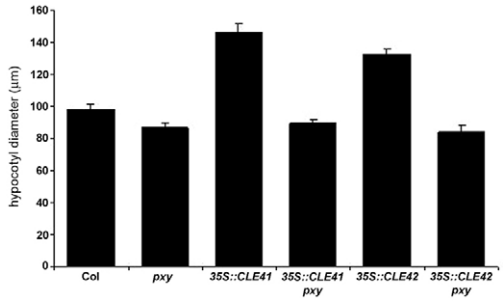

Fig. 5. The diameter of plant hypocotyls at senescence. Error bars are standard error. *t*-tests show that *pxy* is significantly smaller than Col ($P<0.005$) and that *35S::CLE41* and *35S::CLE42* are significantly larger than Col ($P<0.0001$). *35S::CLE41 pxy* and *35S::CLE42 pxy* were not significantly different from *pxy*.

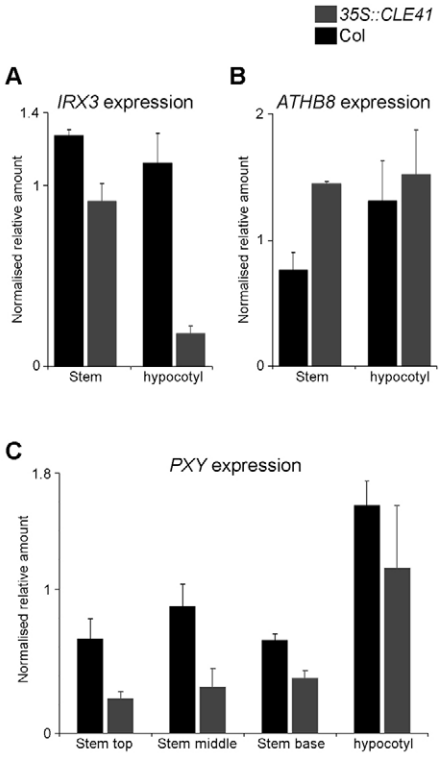

Fig. 6. Changes in gene expression in *35S::CLE41* compared with wild type, normalised to *18s* rRNA. (**A-C**) Quantitative RT-PCR was used to assay *IRX3* (A), *ATHB8* (B) and *PXY* (C) expression in stems and hypocotyls of wild-type plants compared with *35S::CLE41*. Error bars are standard error.

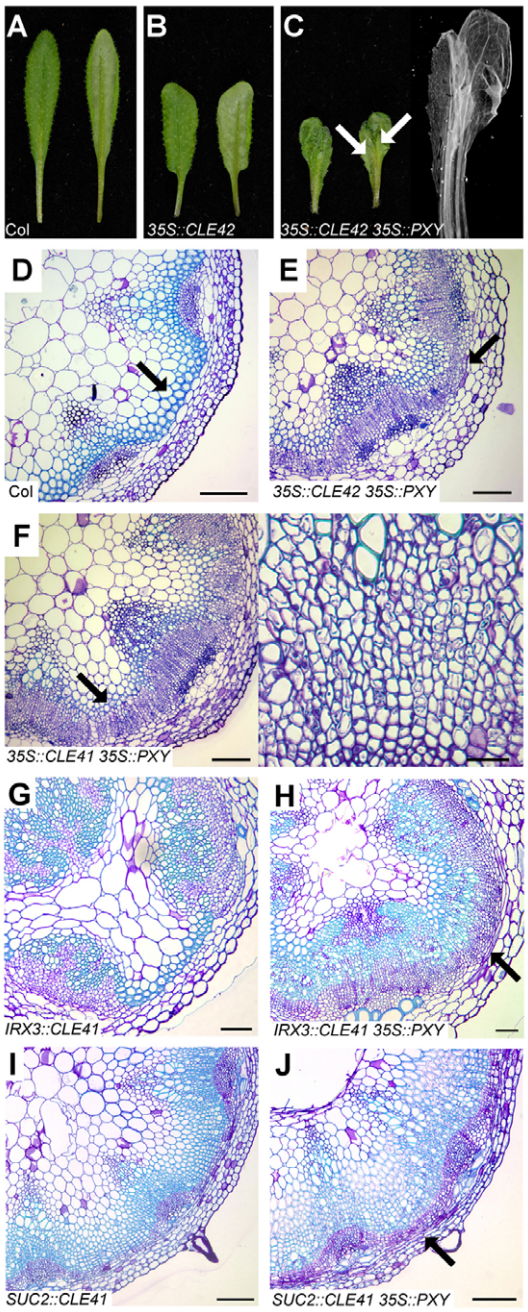

Fig. 7. Ectopic vascular tissue in *35S::PXY 35S::CLE41/42* plants. (**A-C**) Wild-type (A) and *35S::CLE42* (B) leaves have a single midvein, whereas leaves from some *35S::CLE42 35S::PXY* (C, arrows) plants have more. (**D-F**) Vascular and interfascicular tissue (arrow in D) from wild-type (D), *35SCLE42 35S::PXY* (E) and *35SCLE41 35S::PXY* (F) plants. *35SCLE42 35S::PXY* and *35SCLE41 35S::PXY* have more undifferentiated cells than do wild type. (**G-J**) *IRX3::CLE41* (G) and *IRX3::CLE41 35S::PXY* (H) have disorganised vascular bundles, but this is not the case in *SUC2::CLE41* (I) and *SUC2::CLE41 35S::PXY* (J), which have a large number of vascular cells that are highly ordered. Arrows in E, F, H and J point to areas of strong secondary growth. Scale bars: 100 μm, except 20 μm in F, right-hand panel.

secondary growth. *35S::PXY SUC2::CLE41* plants also demonstrated enhanced secondary growth (Fig. 7I,J) but, in contrast to *35S::PXY IRX3::CLE41*, vascular tissue was highly ordered.

An additional phenotype was observed in the leaves of *35S::CLE42 35S::PXY* plants. In Col, *35S::CLE42* (Fig. 7A,B) and *35S::PXY* (data not shown) plants, leaves have a single midvein; however, in a minority of *35S::CLE42 35S::PXY* plants, the leaves appeared to exhibit increased vascular development. This additional vascular tissue develops together with the associated lamina, suggesting development of ectopic midveins (Fig. 7C) reminiscent of plants overexpressing both CLE41 and CLE6 (Whitford et al., 2008). This leaf phenotype adds to the increasing evidence that co-expression of CLE41/42 and PXY is sufficient to drive vascular cell division.

A previously described function of CLE41-derived peptides is repression of tracheary element differentiation in Zinnia cell culture (Kondo et al., 2006). *ATHB8*, which is a provascular marker (Baima et al., 1995), has been found to be upregulated in plants treated with CLE41 peptide (Whitford et al., 2008) and, similarly, we observed high *ATHB8* expression in the stems of *35S::CLE41* plants (Fig. 6B). *ATHB8* does not mark cambial cells and, as such, its expression in the hypocotyl was not different from that in the wild type. We used *IRX3* expression as a marker for xylem development as IRX3 is a subunit of the cellulose synthase complex that acts in secondary cell wall formation and is strongly upregulated as xylem cells differentiate (Taylor et al., 1999). Consistent with the observations of Kondo (Kondo et al., 2006), *35S::CLE41* had reduced *IRX3* expression compared with that of wild type (Fig. 6A), suggesting that xylem differentiation was repressed. This is clearly reflected in *35S::PXY 35S::CLE41* and *35S::CLE42* plants (Fig. 7E,F), which had a reduction in xylem differentiation as the additional cells generated in this line appeared to be undifferentiated. However, in

35S::PXY SUC2::CLE41 lines, xylem was well-developed, suggesting that the range of CLE41 is limited as it is unable to repress xylem differentiation when expressed from the phloem. This suggests that phloem-expressed CLE41 does not cross the procambium to act on young developing xylem cells and is consistent with the range of the well-characterised and closely related CLAVATA3 peptide, which is expressed in layers 1 to 3 of the apical meristem (Fletcher et al., 1999) and binds its receptor, CLV1, in layer 3 and a further two cell layers below (Clark et al., 1997).

DISCUSSION

Genetic analysis presented in this manuscript, together with previous data showing that PXY binds to a dodecapeptide derived from *CLE41* and/or *CLE44* using photoaffinity labelling (Hirakawa et al., 2008), indicate that CLE41 and CLE42 are ligands for the PXY receptor kinase. Our results show that signalling through PXY/CLE regulates division of vascular cells and that specific localisation of CLE41 in the phloem adjacent to the dividing cells that express PXY is required for properly orientated vascular cell divisions. The disrupted tissue phenotype occurs only when the specific localisation of *CLE41* is disrupted (i.e. by using the *35S* or *IRX3* promoter to drive its expression) and is therefore the result of altering the *CLE41* expression domain. Disruption does not occur when expression is increased specifically in the phloem (using the *SUC2* promoter), where native *CLE41* is expressed. The lines used in this study are directly comparable as the increased cell division output of PXY-CLE41 signalling is similar in *35S::CLE41* lines (which have disorganised vasculature) and *SUC2::CLE41* lines (which have organised vasculature). As both lines demonstrate similar increases in cell division, there must be similar levels of signalling through PXY. The difference in the phenotypes is likely to be a consequence of the difference in expression domain. The 'disrupted' phenotype is unlikely to be due to a general increase in cambial or procambial cell division because lines that have previously been described with increases in vascular cell number, such as *acl5* (Hanzawa et al., 2000), *hca* (Pineau et al., 2005), *soc1 ful* (Melzer et al., 2008) or *hca2* (Guo et al., 2009) have highly ordered vascular tissue, clearly demonstrating that this phenotype is not a general symptom of high levels of vascular cell division. Our experiments show an essential role for localised expression of *CLE41* in the highly ordered nature of cell divisions in vascular meristems. We demonstrate for the first time in the plant kingdom that polar localisation of a ligand can confer positional information through a receptor, which sets a cell division plane. Our model of CLE41/PXY signalling is reminiscent of the ordered cell division that occurs in the *C. elegans* EMS cells. Orientation of the EMS division is dependent on the position of the neighbouring P2 cell, from which the MOM-2 ligand signals to the MOM-5 receptor in the EMS cell (Schlesinger et al., 1999). This suggests that orientation of cell division based upon signalling from an adjacent cell is a general mechanism that has arisen in a diverse array of multicellular organisms.

The signalling pathway involving CLE41/PXY is multifunctional as, in addition to its previously identified role in repressing xylem development, it plays a central role in regulating the number and orientation of cell divisions in vascular meristems. Just such a signalling mechanism was first proposed four decades ago in classical experiments carried out on castor bean hypocotyls where, prior to cambial development, a piece of interfascicular tissue was removed, rotated 180° and reinserted such that cells at the periphery were now positioned towards the centre of the stem. In successful grafts, the cambium developed in the expected position but orientation of xylem and phloem generated in the graft was consistent with the original orientation of the tissue, thus at 180° to surrounding tissue. Xylem was therefore on the periphery with phloem towards the centre of the stem (Siebers, 1971). In further experiments using crab apple trees, the signal which orients the cambium was shown to be maintained in tissue that was grafted at 90° to its original orientation in the third growing season (Thair and Steves, 1976). This suggests that the mechanism by which this organisation is set acts early – prior to formation of the cambium – and is set by a short range signal, i.e. from within the graft area (Siebers, 1971), as polarity is retained irrespective of the organisation of surrounding tissue and, therefore, in the absence of any additional positional information. Our data suggest that this polarity is set up by the expression of PXY in nascent vascular initials and CLE41 expression in nascent phloem cells. The grafting experiments described above suggest that once this spatial expression pattern is in place it is sufficient to propagate itself without any additional spatial cues.

Negative regulation of a receptor by a ligand is a mechanism for regulating the ligand-receptor dynamic. Our observation that high levels of *CLE41* expression results in suppression of *PXY* expression are consistent with previous data that show increased *PXY* expression in *pxy* mutants (Fisher and Turner, 2007). One such example of ligand-mediated repression of receptor expression occurs in early fly wing development, where the Wingless (Wn) ligand is highly expressed at the dorsoventral boundary of imaginal discs and thus forms a gradient that is high at the dorsoventral stripe and low in regions distal to it. Wn negatively regulates its receptor, Frizzled (Fz), where it occurs in high concentrations at the boundary. This negative regulation does not extend to Fz expression further from the boundary where Wn concentration is lower, thus allowing regulation of the Wn morphogen gradient (Cadigan et al., 1998).

We have shown that the PXY-CLE41/42 ligand-receptor pair is essential to regulate the rate of cell division, cell differentiation and the orientation of the cell division (Fig. 1A; see also Fig. S1 in the supplementary material). Given the importance of RLKs in cell division and orientation in other systems, it is possible that similar integration is essential in many other aspects of plant development.

Acknowledgements

We thank Ilian Atanassov for assistance with *pIRX3::CLE41* and especially Andrew Hudson for assistance with in situ hybridisation. This work was funded by BBSRC (grant BB/E00380X/1).

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.044941/-/DC1

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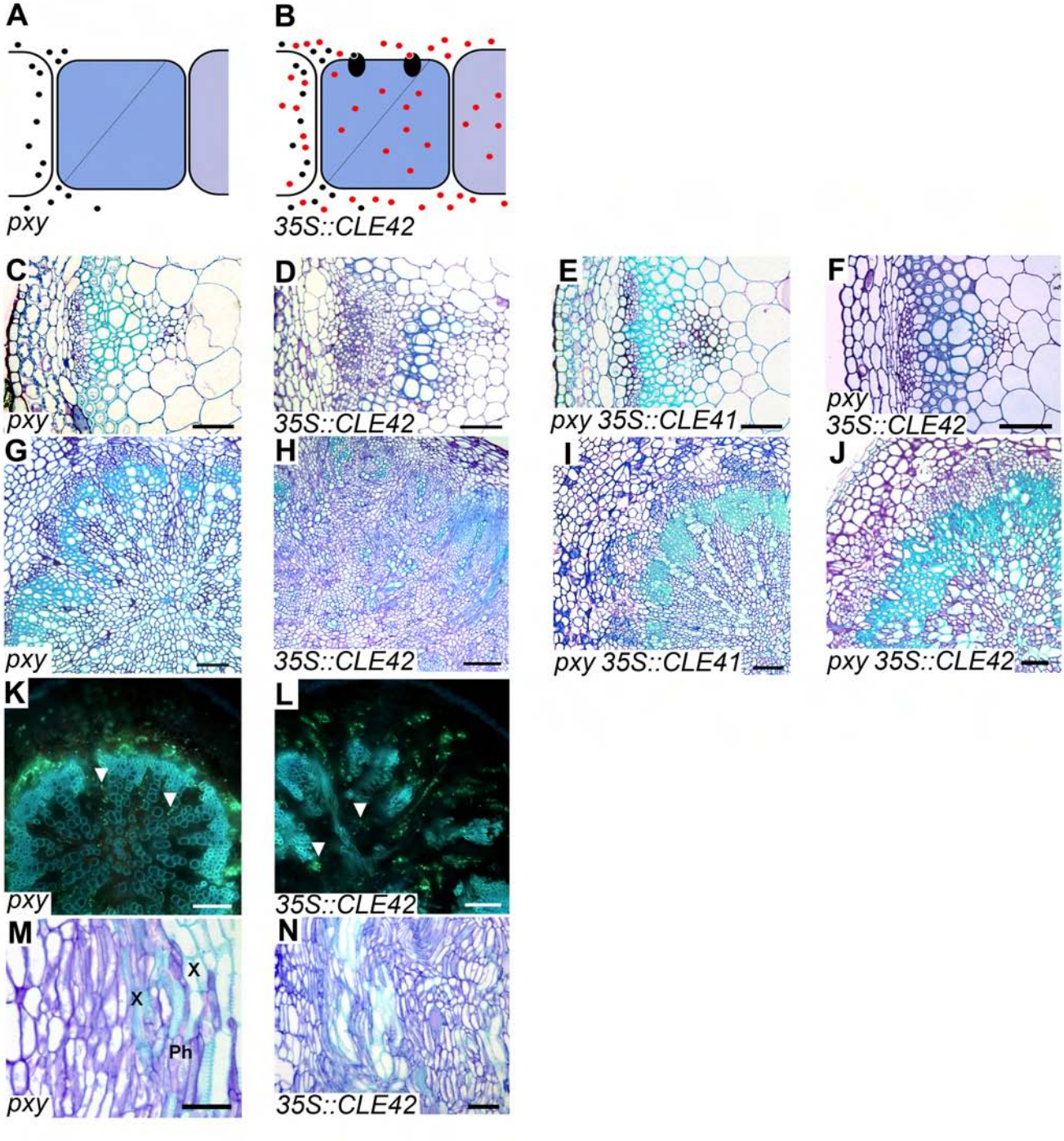
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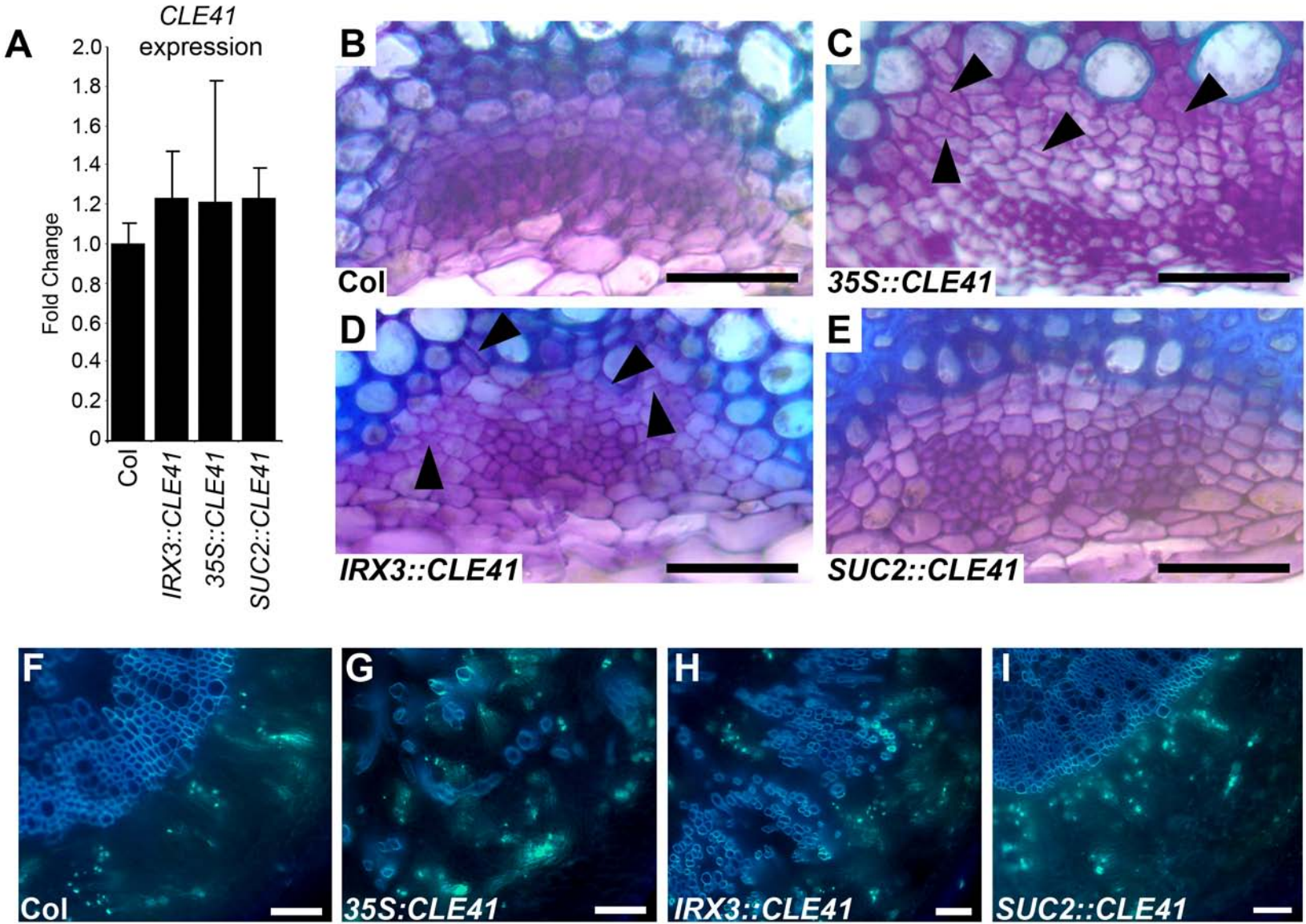
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Table S1. Oligonucleotides used in the generation of plant stocks

Oligo name	Sequence (5′-3′)	Function
CLE41F	CACCATGGCAACATCAAATGAC	CLE41 entry clone/*SUC2::CLE41* overlap PCR entry clone/in situ hybridization probe
CLE41R	aaaccagatgtgccaactca	*CLE41* entry clone/in situ hybridization probe/*CLE41* construct genotyping
CLE42F	caccatgagatctcctcacatc	*CLE41* entry clone/*35S::CLE42* genotyping
CLE42R	tgaatcaaacaagcaacataacaa	*CLE42* entry clone
PXY_ORF_f	CACCTTAAATCCACCATTGTCA	*35S::PXY* construct
PXY_ORF_r	CCAAGATAATGGACGCCAAC	*35S::PXY* construct
PXY_SEQ_1F	CGGAACCTCTCTGGTCGTAT *PXY*	in situ probe
PXY_SEQ_1R	TGATCGGTGAATCTGTTGTTG	*PXY* in situ probe
SUC2promFtopo	caccaacacatgttgccgagtca	*SUC2::CLE41* overlap PCR entry clone & genotyping
SUC2pro/CLE41(1)	GTCATTTGATGTTGCCATgaaatttctttgagagggtttttg	*SUC2::CLE41* overlap PCR entry clone
SUC2pro/CLE41(2)	caaaaaccctctcaaagaaatttcATGGCAACATCAAATGAC	*SUC2::CLE41* overlap PCR entry clone
CLE41_RTF	CCATGACTCGTCATCAGTCC	qRT-PCR
CLE41_RTR	TTTGGACCACTAGGAACCTCA	qRT-PCR
qATHB8f	ctccgaattccctcagatca	qRT-PCR
qATHB8r	tcgacatgcatattcctcca	qRT-PCR
qIRX3f	gcgtgtgcacccatatcc	qRT-PCR/*IRX3::CLE41* genotyping
qIRX3r	tcatccattctttcccgcc	qRT-PCR
18s rRNA F	CATCAGCTCGCGTTGACTAC	qRT-PCR control
18s rRNA R	GATCCTTCCGCAGGTTCAC	qRT-PCR control
35S promoter F	cgcacaatcccactatcctt	Genotyping
salk_LBa1	TGGTTCACGTAGTGGGCCATCG	Genotyping
pxy-3-f	TTCATCCCCTTCTCCTTCTTC	Genotyping
pxy-3-r	TTACCGTTTGATCCAAGCTTG	Genotyping