Investigating a feed-forward loop of transcription factors that acts in plant vascular development

SHAUNI MCGREGOR

Investigating a feed-forward loop of transcription factors that acts in plant vascular development

Shauni McGregor

Submitted for the qualification of Master of Science (MSc)
at the Department of Biosciences

Durham University

September 2017

Abstract

The plant vascular system facilitates the distribution of essential substances throughout the plant. Vascular tissue arises from highly organised cell division in the vascular meristem, known as the procambium. This displaces cells, which differentiate into phloem towards the outside of the stem or xylem towards the inside, resulting in a well-defined pattern of vascular tissue within the stem. The PHLOEM INTERCALATED WITH XYLEM (PXY) receptor and its peptide ligand, TRACHEARY DIFFERENTIATION INHIBITORY FACTOR (TDIF), regulate the development of this pattern by influencing multiple developmental processes.

Recent Y1H assays demonstrate that PXY sits within a complex transcriptional regulatory network (TRN). This thesis investigates a feed-forward loop motif of interest within this TRN that sits downstream of PXY and may act to co-ordinate vascular development. It is comprised of three transcription factors: WUSCHEL HOMEOBOX RELATED 14 (WOX14), TARGET OF MONOPTEROS 6 (TMO6) and LOB DOMAIN-CONTAINING PROTEIN 4 (LBD4).

Here, we show that lines of *Arabidopsis thaliana* expressing *LBD4* ectopically in the xylem demonstrate difficulty in establishing xylem identities. Meanwhile, expressing *LBD4* in the phloem results in increased phloem proliferation, suggesting that *LBD4* plays a role in defining the phloem-procambium boundary. Furthermore, *tmo6* mutants demonstrate decreased phloem proliferation, indicating that the loop as a whole may be important in phloem development.

Both the *TMO6* and *LBD4* transcription factors show *WOX14* dependent expression. However, *LBD4* does not show expression dependent on PXY signalling. As *LBD4* shows high connectivity in the TRN, this suggests that *LBD4* is maintained by other signalling pathways. Thus this feed-forward loop may act as a point of cross-talk between PXY signalling and other signalling pathways.

Contents

Declaration and statement of copyright

I declare that no part of this thesis has previously been submitted for a higher degree and that the work presented in this thesis is the result of my own work.

Acknowledgements

There are a number of people, without whom this research would not have been possible.

Firstly, I would like to thank my supervisor, Peter, for the opportunity to work on this research and all of his help and advice throughout this project. I'd also like to thank everyone in ICBL plant labs for all the support and guidance (and lab consumables!) that they've given me throughout the year. Finally, I'd like to thank my family for their constant support and encouragement of my studies.

1. Introduction

1.1 – Vascular tissue in plants

The plant vascular system is essential for a plant's growth and survival. It transports essential substances around the plant, such as photosynthate from the leaves and water from roots (Esau, 1977). It also presents significant economic importance. Plant vascular tissue comprises the majority of plant biomass, making it important in both biofuel production (Farell et al., 2006) and the lumber industry (Plomion et al., 2001). It also provides the plant with a large amount of mechanical strength. Thus, plant vascular tissue is important when considering the collapse of crop plants, a significant agricultural problem known as lodging (Kong et al., 2013).

Plant vascular tissue is comprised of three specialised tissue types. Phloem tissue, which facilitates the selective transport of nutrient solutes such as photosynthate, consists of highly specialised sieve elements and their associated companion cells. Meanwhile, xylem tissue transports water and is made up of dead, highly lignified cells which have a characteristic secondary cell wall. These two tissue types arise from the procambium, a bank of pluripotent stem cells that acts as the vascular meristem (Esau, 1977).

In the model system *Arabidopsis thaliana* these tissues are arranged in a highly organised pattern. At the base of the stem, vascular tissues are arranged in discrete vascular bundles around the circumference of the stem. In these bundles, phloem is positioned towards the outside of the stem and xylem towards the inside with the procambium running between the two tissues. In the hypocotyl, the procambium from separate vascular bundles has fused to form a single continuous ring known as the cambium. This marks the transition from primary

upwards growth to secondary growth, characterised by radial expansion (Sanchez et al., 2012). Therefore, above the ground, the *Arabidopsis thaliana* plant can be viewed as a developmental series, with vascular structures being least mature at the top of the plant and most mature at the bottom.

This pattern of vascular tissue develops from highly organised divisions of procambial cells. Procambial cells divide along the periclinal axis, displacing their daughter cells. Cells displaced towards the centre of the stem take on xylem identities while cells displaced towards the outside of the stem become phloem (Etchells and Turner, 2010a). This highly organised process is regulated by a number of signalling pathways (Miyashima et al., 2013)

1.2 – PXY signalling regulates vascular development

1.2.1 – PXY signalling

One of the signalling pathways that regulates vascular development is the PXY signalling pathway. PHLOEM INTERCALATED WITH XYLEM (PXY) is a leucine rich repeat (LRR) receptor like kinase (RLK) expressed on the surface of procambial cells. Vascular tissue in *pxy* mutants shows a loss of correct spatial patterning, characterised by formation of adjacent xylem and phloem cells (Fisher and Turner, 2007). *pxy* mutants also have lower numbers of procambial cells, suggesting changes in cell division as well as patterning (Hirakawa et al., 2008) and indicating PXY's importance in vascular development.

PXY binds a small peptide ligand called TRACHEARY DIFFERENTIATION INHIBITORY FACTOR (TDIF) (Hirakawa et al., 2008). TDIF is a dodecapeptide derived from three different *CLV3/ESR1-LIKE* (CLE) genes, *CLE41*, *CLE42* and

CLE44 (Ito et al., 2006) . Culturing *Zinnia elegans* mesophyll cells in a TDIF rich media prevents their spontaneous differentiation into tracheary elements, indicating that TDIF acts as a suppressor of xylem identity (Ito et al., 2006). Meanwhile, *Arabidopsis* seedlings grown in liquid media containing TDIF show increased numbers of procambial cells as well as inhibition of xylem vessel formation (Hirakawa et al., 2008).

TDIF is secreted by the phloem, creating a gradient of TDIF peptide across vascular tissue that acts as an important positional signal. This gradient can be disrupted via overexpression of the TDIF precursor, *CLE41*, or expression of *CLE41* under the xylem specific promoter of the *IRX3* gene. This results in a loss of co-ordinated cell division produced by the loss of the plant's ability to correctly position the plane of procambial cell division (Etchells and Turner, 2010b).

Therefore, TDIF activation of PXY leads to multiple developmental changes, including the repression of xylem identity and increased procambial activity. PXY signalling also acts as an important positional signal that defines the axis of procambial cell division. The discovery of a PXY ortholog in poplar also demonstrates the importance of PXY signalling in plant development, suggesting that PXY signalling is conserved across multiple plant families. This identifies PXY signalling as a research target of potential economic importance as transgenic poplar trees showing tissue specific over expression of *PXY* and *CLE41* show enhanced wood formation (Etchells et al. 2015).

The PXY receptor has two closely related receptor-like kinases, PXY-LIKE 1 (PXL1) and PXY-LIKE 2 (PXL2), which are thought to function redundantly with PXY (Fisher and Turner, 2007). Both PXL1 and PXL2 produce similar crystal structures to PXY. However, comparisons of these crystal structures reveals

substitutions of key TDIF interacting residues in PXL1 and PXL2 (Zhang et al. 2016). Additionally, both PXL1 and PXL2 show lower affinities for TDIF than PXY. Therefore, whether PXY signalling could also be mediated by PXL1 and PXL2 remains unclear.

1.2.2 –Transcription factors regulated by PXY signalling

A number of transcription factors have been implicated as PXY signalling regulated, presenting a possible mechanism by which PXY may co-ordinate multiple developmental processes. For example, PXY mediated xylem repression is thought to be mediated by the GSK3 activated BRI1-EMS SUPPRESSOR 1 (BES1) transcription factor. The PXY kinase domain interacts directly with GSK3 signalling components in a TDIF dependent manner. GSK3s go on to activate BES1 which facilitates the repression of xylem identities (Kondo et al., 2014).

Meanwhile, the WUSCHEL HOMEOBOX RELATED transcription factors, WOX4 and WOX14 have both been linked to PXY signalling regulation of procambial cell division. Not only do *WOX4* and *WOX14* show PXY signalling dependent expression but *wox4* mutants show defects in vascular cell division, characterised by decreased vascular bundle size (Etchells et al., 2013; Hirakawa et al., 2010). This phenotype is enhanced in the *wox4 wox14* double mutant suggesting that *WOX14* and *WOX4* function redundantly (Etchells et al., 2013). However, although *wox4 wox14* mutants show defects in vascular cell division they do not show any changes in vascular organisation. This presents a mechanism by which PXY signalling can regulate developmental outputs separately via separate transcription factors and suggests the existence of other factors outside of BES1, WOX4 and WOX14 that are involved in PXY signalling regulation of vascular development.

PXY signalling regulated transcription factors may also facilitate cross-talk between PXY signalling and other signalling pathways. For example, the WOX4 transcription factor is required for auxin stimulated cambial activity. This auxin regulated developmental response is thus dependent on PXY signalling which is required for sufficient *WOX4* transcription (Suer et al., 2011). Therefore, PXY signalling regulated transcription factors can make up a complex network facilitating the interaction of PXY signalling with other signalling pathways to fine tune developmental outputs.

1.3 – PXY signalling in a complex transcriptional regulatory network

Transcriptional regulatory networks (TRNs) specify interactions between transcription factors and their target genes. These can be compiled using data from high throughput Yeast-1-Hybrid (Y1H) assays, which screen for protein-DNA interactions. These are carried out using maintenance of an exogenous DNA sequence of interest in yeast. This DNA sequence contains a downstream promoter element and a reporter gene, all coded for on one plasmid. A second plasmid encodes a hybrid protein, consisting of the protein of interest fused to an activation domain. When these two plasmids are expressed in yeast, the protein of interest may bind to the DNA sequence of interest. If this occurs, the activation domain on the fusion protein is brought sufficiently close to allow it to bind to the promoter. This activates transcription of the reporter gene. Therefore, yeast strains showing expression of the chosen reporter gene demonstrate an interaction between the protein and DNA sequence of interest (Reece-Hoyes and Walhout, 2013). In this way, interactions between gene promoters and transcription factors of interest can be identified.

A recent Yeast-1-Hybrid (Y1H) assay screened 41 promoters of PXY, PXL, TDIF-encoding and PXY signalling target genes against a library of 812 transcription factors, representing 95% of the transcription factors expressed in root vascular tissue (Gaudinier et al., 2011). The results led to the construction of a large TRN consisting of 690 TF-promoter interactions, (Fig 1A, Etchells et al., personal communication). This TRN, which contains multiple PXY signalling components, is characterised by a series of feed-forward loops (Etchells et al., personal communication). These are specific network motifs where the expression of gene Z is regulated by both transcription factor Y and transcription factor X and the expression of transcription factor Y is also regulated by X (Mangan and Alon, 2003).

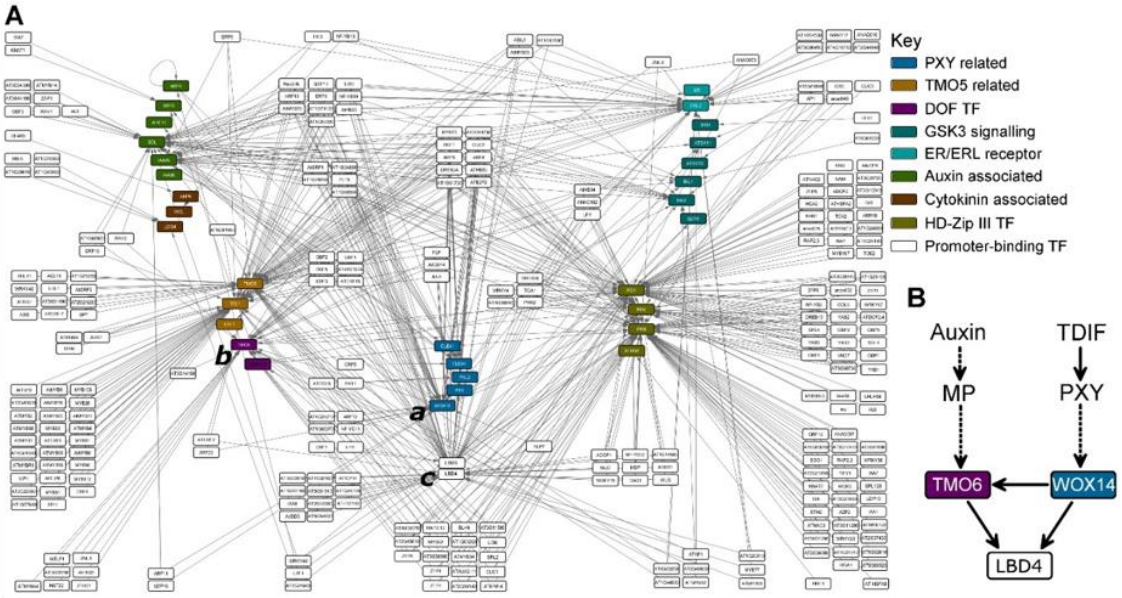


Figure 1. PXY signalling related and regulated genes are part of a complex TRN (Etchells et al., personal communication) **(A)** TRN generated from high throughput Y1H assay data. Coloured nodes indicate promoters. White nodes indicate binding transcription factors. Lines denote TF-promoter interactions. The position of three transcription factors of interest within the TRN are labelled: WOX14 (a), TMO6 (b) and *LBD4* (c), **(B)** Interactions between three transcription factors make up a feed-forward loop of interest in vascular development. Solid arrows mark shown and predicted direct interactions.

One particular feed-forward loop of interest was identified within the TRN. It consists of three transcription factors: WOX14, TARGET OF MONOPTEROS 6 (TMO6), and LOB DOMAIN-CONTAINING PROTEIN 4 (LBD4) (Fig. 1B). In the loop, the promoter of the *LBD4* gene is bound by the WOX14 and TMO6 transcription factors. Meanwhile WOX14 also binds to the promoter of the *TMO6* gene.

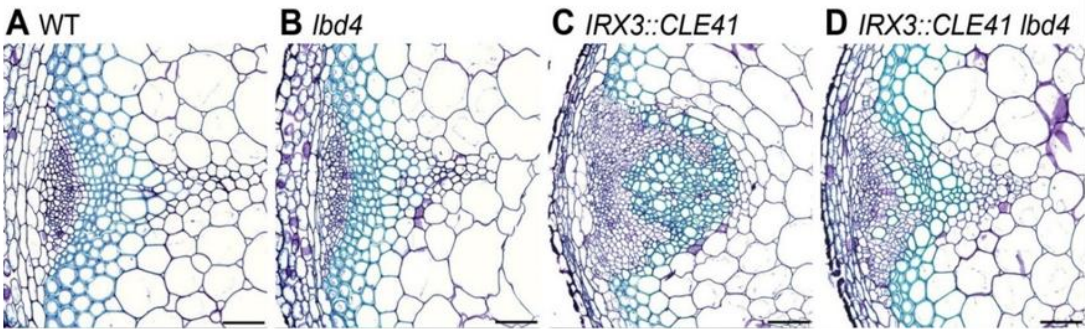
This loop is linked to the PXY signalling network by WOX14, which acts redundantly with WOX4 to regulate vascular cell proliferation in a PXY signalling dependent manner. Meanwhile, TMO6 shows expression dependent of the auxin response factor, MONOPTEROS (MP) (Schlereth et al., 2010). MP is a transcription factor that acts as the primary mediator of auxin response and is crucial for the formation of vascular tissue (Hardtke and Berleth, 1998). Therefore, like PXY signalling regulation of *WOX4*, WOX14 regulation of *TMO6* may act to integrate PXY and auxin signalling in vascular development.

Both WOX14 and TMO6 bind to the promoter of *LBD4*, making regulation of the *LBD4* gene the predicted primary output of the loop. Microarray analysis of gene expression in *35S::CLE41* mutants showed an 11-fold upregulation of *LBD4*, suggesting that expression of the *LBD4* is highly responsive to PXY signalling (Fig 2E, Etchells et al., personal communication). Meanwhile, defects in vascular organisation seen in *IRX3::CLE41* mutants can be partially rescued by *lbd4* (Fig. 2A-D, Etchells et al., personal communication). This suggests that the *IRX3::CLE41* phenotype can partially be attributed to the misregulation of *LBD4* and, together with microarray data, implicates *LBD4* as a key target of PXY signalling.

1.4 Current gaps in knowledge and project aims

1.4.1 – What are the functions of the feed-forward loop genes?

While the function of WOX14 in vascular development has already been studied, the specific roles of *TMO6* and *LBD4* are, as yet, unknown. TMO6 is a Dof transcription factor that shows expression localised to the vascular tissue (Gardiner et al., 2010). A number of functions have been attributed to Dof transcription factors, ranging from cambial activity to regulation of long-distance signalling (Le Hir and Bellini, 2013). However, the lack of an available *tmo6* knockout mutant has hindered the characterisation of TMO6 function.



E

Locus	Description	Fold change (35S::CLE41/WT)	q value
AT1G31320	LBD4 (LOB DOMAIN-CONTAINING PROTEIN 4)	11.15	1.63×10^{-5}
AT5G60200	TMO6 (TARGET OF MONOPTEROS 6)	4.86	9.45×10^{-6}
AT1G20700	WOX14 (WUSCHEL RELATED HOMEOBOX 14)	4.76	1.62×10^{-5}
AT3G24770	CLE41 (CLAVATA3/ESR-RELATED 41)	3.40	1.17×10^{-5}

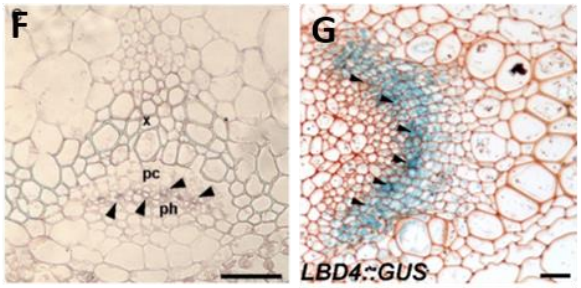


Figure 2. Preliminary data on *LBD4* and the other feed-forward loop genes. **(A-D)** *lbd4* partially rescues the *IRX3::CLE41* vascular phenotype. Scale bars represent 50um. Regions of phloem (ph), procambium (pc) and xylem (x) are marked in (A). **(E)** Microarray analysis demonstrating upregulation of the feed-forward loop genes in a *35S::CLE41* background. q values were obtained using empirical Bayes. (**F-G)** RNA in situ hybridisation (F) and *LBD4::GUS* (G) demonstrate that *LBD4* expression is localised to the phloem-procambium boundary. Scale bars represent 20um. Regions of phloem (ph), procambium (pc) and xylem (x) are indicated. Arrowheads indicate regions of probe-RNA hybridisation or GUS expression.

lbd4 knockout mutants do not demonstrate a visible vascular phenotype, suggesting that *LBD4* may function redundantly with other factors (Fig. 2B, Etchells et al., personal communication). Meanwhile, RNA in situ and GUS lines reveal that *LBD4* shows expression that is highly localised to the phloem-procambium boundary within vascular tissue (Fig. 2F-G, Etchells et al., personal communication). Other LOB-domain proteins show highly specific patterns of expression and have been attributed to defining organ boundaries (Husbands et al., 2007). Thus, the highly specific pattern of *LBD4* expression may be involved in defining the boundary between phloem and procambium tissues. In this case, the correct location of *LBD4* expression in space could be important for its function. Therefore, manipulation of *LBD4* expression in space could be a useful approach in elucidating its function. Meanwhile, although the region of *TMO6* expression within vascular tissue has not been as well characterised, considering there is currently no *tmo6* mutant available, a similar approach may be useful to investigate *TMO6* function.

Here, we have used a set of mutant plant lines expressing *LBD4* under promoters specific to particular vascular tissues. The *SUC2* promoter has been used to express *LBD4* in the phloem while the *IRX3* promoter has been used to express *LBD4* in the xylem. Meanwhile, a mutant line expressing *TMO6* in the xylem has also been used. These plant lines have been analysed histologically to investigate the effects of ectopic *LBD4* and *TMO6* on the structure of vascular bundles found at the base of *Arabidopsis thaliana* inflorescences. Any changes caused by ectopic *LBD4* or *TMO6* expression may provide useful insights into their function.

1.4.2 – How do *WOX4* and *WOX14* influence the feed-forward loop?

The proposed feed-forward loop presented here is based on interactions seen in a Y1H assay, an approach well known for producing false negatives and positives. Therefore, it is important that all interactions identified by Y1H are confirmed in planta. Here, we aim to verify genetic interactions between *WOX14* and the other two feed-forward loop genes. *WOX14* functions redundantly with *WOX4* however *WOX4* was not present in the transcription factor library used in Y1H assay. Therefore, genetic interactions between *WOX4, TMO6* and *LBD4* were also studied to elucidate any role that *WOX4* may have in the loop.

To examine genetic interactions between these factors, qRT-PCR analysis of gene expression was used to compare wildtype levels of *TMO6* and *LBD4* expression to those seen in *wox4, wox14* and *wox4 wox14* mutants. As well as verifying Y1H results, this study should enhance understanding of the regulatory roles of *WOX4* and *WOX14* within the feed-forward loop.

1.4.3 – How does PXY signalling influence the feed-forward loop?

Previous research suggests that this feed-forward loop may act as a point of cross-talk between auxin and PXY signalling via binding of the WOX14 transcription factor to the *TMO6* promoter. Thus, it is important to characterise the influence of these two signalling pathways on the feed-forward loop.

To investigate the influence of PXY signalling on the feed-forward loop at the genetic level, qRT-PCR analysis was used to measure *TMO6* and *LBD4* expression levels. Gene expression was measured in wild type tissue and in tissue from mutants deficient in PXY signalling. This examination will help to characterise the effect of PXY signalling on the loop and enhance our understanding of how this feed-forward loop fits into the PXY network as a whole.

1.4.4 - Improving genetic resources for loss of function analysis

The lack of a *tmo6* knockout places a significant limit on research that can be conducted on the feed-forward loop. Therefore, we generated a *tmo6* knockout mutant via CRISPR Cas9 genome editing. This knockout mutant was analysed histologically to identify whether a loss of TMO6 affects vascular development. This should reveal new information about the function of the *TMO6* gene.

The production of this mutant opens up several new possible avenues of research. One such avenue is the generation of a triple knockout of all three loop components, functionally knocking out the loop as a whole. This will enhance understanding of the role of the feed-forward loop as a functional unit. Consequently, a double *wox14 lbd4* mutant was also generated to ensure the raw materials would be available for production of this triple knockout in the future. This new mutant background was also analysed histologically to study the effects of a loss of both WOX14 and LBD4 on vascular development.

2. Materials and methods

2.1 – Plant materials

All *Arabidopsis thaliana* wildtype plants were of the Columbia ecotype (Col-0) and grown from seeds obtained from laboratory stocks. The *pxy pxl1 pxl2*, *wox4-1*, *wox14-1* and *wox4 wox14* knockout mutants (previously described in Fisher and Turner, 2007; Hirakawa et al., 2010 and Etchells et al., 2013 respectively) were also obtained from laboratory stocks. The *lbd4* mutant was acquired from a previously established SALK line (SALK_146141) and the *cle41 cle42 cle43 cle44* (hereafter referred to as *tdif*) knockout was obtained courtesy of Zachary Nimchuck (University of North Carolina). Segregating T2s of *IRX3::LBD4 lbd4*, *SUC2::LBD4 lbd4, IRX3::TMO6* and *wox14 lbd4* were acquired from laboratory stocks.

2.2 – Seed sterilisation

Seed sterilisation was carried out in sterile conditions in a laminar flow hood. Seeds were sterilised in Eppendorf tubes in 75% ethanol for two minutes. After removal of the ethanol, seeds were washed twice with 75% ethanol and then twice with sterile de-ionised water. Seeds were then suspended in 0.1% agarose and stored at 4°C.

2.3 – Plant growth conditions

2.3.1 – Seeds germinated on plates

All plants grown on plates were germinated on agar media containing 4.4g/l MS basal medium (Sigma Aldrich) and 10g/l agar (Formedium). The pH of the media was adjusted to 5.7 with 0.1M KOH before autoclaving at 120°C for 20 minutes. Sterile seeds suspended in 0.1% agarose were pipetted directly onto agar plates in sterile conditions under a laminar flow hood. Once dry, plates were sealed with Micropore tape and stratified at 4°C for a least 2 days before transfer to a growth cabinet (Panasonic MLR-352-PE Versatile Environmental Test Chamber, constant light, 22°C).

2.3.2 – Seeds germinated on soil

Seed was suspended in 0.1% agarose and pipetted directly onto soil made from a mix of 6 parts compost (Levington Advance Seed & Modular F2S Compost), 1 part perlite (Sinclair Pro Perlite Standard) and 1 part vermiculite (Sinclair Pro Vermiculite Medium). Pots were then watered with added fertiliser (Miraclegro Liquid All Purpose Plant Food, used according to manufacturer's instructions) before transfer to a growth chamber (Fitotron Growth Chamber, LD conditions, 22°C).

2.4 – Plant transformation

10ml cultures of *Agrobacterium tumefaciens* previously transformed with a CRISPR Cas9 plasmid targeted to the *TMO6* gene were obtained courtesy of Johan Kroon (Durham University). These 10ml cultures were used to inoculate 200ml of liquid LB media made with 11g 2x LB broth (Formedium) and

supplemented with 50ug/l of both kanamycin and gentamycin. The 200ml culture was then shaken at 30°C overnight. After overnight incubation, the 200ml culture was then centrifuged at 3000rpm for 30 minutes. The resulting supernatant was discarded and the pellet was re-suspended in 150ml infiltration media made with ½ MS basal medium, 5% sucrose and 300ul/l wetting agent (Silwet L-77).

Wildtype plants, with inflorescences measuring approximately 15cm, were dipped into the infiltration media so that flowers were completely submerged for 2 minutes. Plants were covered for the first two days after dipping and then left to set seed. Collected T1 seed was left to post-ripen for 2 weeks before screening (see below).

2.5 – Genotyping

2.5.1 – Screening mutant lines

Segregating mutant plant lines were screened for antibiotic resistance via germination and growth on MS agar plates supplemented with 50ug/l of the relevant antibiotic. *IRX3::LBD4 lbd4*, *SUC2::LBD4 lbd4* and *IRX3::TMO6* segregating T2 lines were screened with kanamycin. Segregating *wox14 lbd4* T2 lines were screened with kanamycin to isolate plants carrying the *lbd4* mutation. Meanwhile, *tmo6* T1 lines were screened using hygromycin to identify plants that had successfully incorporated the CRISPR Cas9 plasmid. After screening, resistant seedlings were transferred to soil for later identification of homozygous individuals via genotyping PCRs.

2.5.2 – DNA extraction

DNA was extracted from emerging leaf tissue of *Arabidopsis* plants. Tissue was ground in 400ul of CTAB extraction buffer containing d-Sorbitol (0.14M), TRIS-HCl pH8 (0.22M), EDTA pH8 (0.022M), NaCl (0.8M), CTAB (cetyltrimethylammonium bromide; 0.8%) and n-Lauroylsarcosine (1%), using a Tissue Lyser II (Qiagen). Ground tissue was then incubated at 65°C for 5 minutes before the addition of 400ul of chloroform. This mixture was centrifuged at 13000 RPM for 20 minutes and the resulting supernatant was added to 400ul of isopropanol. Tubes were then centrifuged at 13000 RPM for 20 minutes. After the resulting supernatant was discarded, the pellet was washed with 70% ethanol and re-suspended in 50ul TE buffer (10mM Tris-HCl, 1mM EDTA) plus 150ul deionised water. DNA samples were then stored at 4°C before use.

2.5.3 – Genotyping PCR

Standard PCR was used to genotype T-DNA insertion mutants and to detect CRISPR induced changes in product size in *tmo6* mutants. PCR reactions were run in a Bio Rad T100 Thermal Cycler, using the mixes and cycling conditions shown in Table 1. However, there were some exceptions to these standard conditions. For putative *tmo6* mutants, the total PCR reaction volume was increased from 10ul to 25ul to allow for purification and sequencing of the remaining PCR product after gel electrophoresis (Table 1A). Meanwhile, the extension temperature used to genotype putative *lbd4* mutants was lowered to 68°C to enable annealing of AT rich primers (Table 1B). Details of primer sets used for each mutant screen can be found in Table 2A.

PCR products were tested with gel electrophoresis. Gels were made with 1% agarose dissolved in TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) with

Table 1. Details of genotyping PCR **(A)** reaction mix components and **(B)** cycling conditions

(A) Genotyping PCR mixes			
	x1 Standard Reaction (ul)	**x1 *tmo6* Genotyping Reaction (ul)**	
2x PCRBIO Taq Mix Red (PCRBiosystems)	5	12.5	
Forward primer (10pmol/ul)	0.3	0.75	
Reverse primer (10pmol/ul)	0.3	0.75	
dH_2O	3.4	8.5	
Genomic DNA Template	1	2.5	
Total volume	**10ul**	**25ul**	
(B) Genotyping PCR cycling conditions			
	Temperature (°C)	**Duration (mm:ss)**	**Cycles**
Initial Denaturation	95	02:00	x1
Denaturation	95	00:15	x35
Annealing	55	00:20	
Extension	72 (68 when genotyping *lbd4* mutants)	00:45	
Final extension	72	05:00	x1

5ul of 10mg/ml ethidium bromide solution added per 100ml. 5ul samples of PCR products were loaded into gel lanes, with at least one lane per gel containing 7ul of Gene Ruler 1kb DNA ladder (ThermoFisher Scientific) for determination of fragment size. Gels were run at 120 volts for 25 minutes and visualised under UV light using an InGenius gel dock (Syngene). Meanwhile, PCR products produced from putative *tmo6* mutant DNA were run in 2% agarose gels at 110V for 30 minutes in order to better visualise any present band shifts.

2.5.4 – DNA sequencing and purification of PCR products

The presence and nature of induced mutations in the putative *tmo6* mutants was characterised by DNA sequencing. This was performed by DBS genomics (Durham University, www.dur.ac.uk/biosciences/services/dna/dnasequencing/), using the TMO6 F primer (Table 2A) as the forward primer.

Table 2. Details of primer sets used for **(A)** genotyping PCRs and **(B)** checking cDNA quality and qRT-PCR

(A) Genotyping Primers		
Oligo name	**Sequence**	**Function**
IRX3 LBD4 F	ACCGCGACGATGAAAGAAAG	Genotyping IRX3::LBD4 lines
IRX3 LBD4 R	GTTGCTAGCACCGAACACTC	Genotyping IRX3::LBD4 lines
SUC2 LBD4 F	GTCTTTGAGAATCGAACGTCCA	Genotyping SUC2::LBD4 and SUC2::CLE41 lines
SUC2 LBD4 R	TTGTTGACGTTGCTAGCACC	Genotyping SUC2::LBD4 lines
WOX14_screenF	CTCCTCCACAACTCCCTCTC	Genotyping wox14 mutants (wildtype screen)
WOX14_screenR	ATGAGAACTTGACGATGCCC	Genotyping wox14 mutants (wildtype screen)
GABI LB	ATATTGACCATCATACTCATTGC	Genotyping wox14 mutants (mutant screen)
LBD4 geno F	CTCGACCAGCAAAACGTAGG	Genotyping lbd4 mutants (wildtype screen)
LBD4 geno R	GCTGAGCTTGCAACACATCT	Genotyping lbd4 mutants (wildtype screen)
Salk LBb1.3	ATTTTGCCGATTTCGGAAC	Genotyping lbd4 mutants (mutant screen)
Salk LBD4 R	ATAATATAAACGGGTCGGCCC	Genotyping lbd4 mutants (mutant screen)
TMO6 F	CTCTTATGGATCATTTGTTACAACAC	Screening for CRISPR induced band shifts/sequencing
TMO6 R	GTTCAGCAAGGCAAAGGCTAAGC	Screening for CRISPR induced band shifts
(B) Gene Expression Analysis Primers		
Oligo name	**Sequence**	**Function**
ACT8-1	ATGAAGATTAAGGTCGTGGCA	Checking quality of cDNA
ACT8-2	CCGAGTTTGAAGAGGCTAC	Checking quality of cDNA
qACT2 F	GCCATCCAAGCTGTTCTCTC	qRT-PCR
qACT2 R	ACCCTCGTAGATTGGCACAG	qRT-PCR
18S rRNA F	CATCAGCTCGCGTTGACTAC	qRT-PCR
18S rRNA R	GATCCTTCCGCAGGTTCAC	qRT-PCR
qRT WOX4 F	TCACGACCACTGGTGTCTTT	qRT-PCR
qRT WOX4 R	CCCAGCTCCTACATGTCCTC	qRT-PCR
qRT WOX14 F	AAACGAAAGCAGCCTCAAAC	qRT-PCR
qRT WOX14 R	CCCTGAATCTCCACAACTCC	qRT-PCR
qRT TMO6 F	CATGCCGGAGATATTGGACT	qRT-PCR
qRT TMO6 R	GAGAAGACTTTCCCGTCGTG	qRT-PCR
qRT LBD4 F	ACGCCAGTCGACTAATTTCC	qRT-PCR
qRT LBD4 R	CCCTTGTTGTCCTGTGGACT	qRT-PCR

50 – 100ng DNA was sent for sequencing and was obtained from PCR products purified using magnetic Sera-mag SpeedBeads (ThermoFisher Scientific) prepared according to protocol specified in Rohland and Reich (2012). 36ul of magnetic beads was mixed with 20ul of PCR product in PCR tubes and left to stand at room temperature for 5 minutes. Tubes were then placed on a magnet and left to stand for 2 minutes before removal of the supernatant. Beads were then washed twice with 70% ethanol, with tubes being left to stand on the magnet for 2 minutes before each removal of ethanol. Tubes were then placed in a drying oven at 37°C for approximately 5 minutes, until the remaining ethanol had evaporated. 20ul of sterile deionised water was then mixed with beads before

standing tubes on the magnet for another 2 minutes. The resulting supernatant was then transferred to clean tubes and the DNA concentration measured using a Nanodrop ND100 Spectrophotometer (ThermoFisher Scientific).

2.6 – Gene expression analysis

2.6.1 – RNA extraction, DNase treatment and cDNA synthesis

For each qRT-PCR bioreplicate, RNA was extracted from the bottom third of two *Arabidopsis* inflorescences measuring 10-15cm. On collection, tissue was frozen using liquid N_2 and stored at 80°C.

To extract RNA, tissue was ground in a pestle and mortar with 1ml of TRIzol reagent (ThermoFisher Scientific) and the resulting mixture transferred to a 1.5ml Eppendorf tube. 200ul of chloroform was added before tubes were centrifuged at 13000 RPM for 20 minutes at 4°C. The resulting supernatant was transferred to 450ul isopropanol and the subsequent mixture stood on ice for 5 minutes. Tubes were centrifuged again at 13000 RPM for 20 minutes at 4°C. The supernatant was then discarded and the pellet washed with 70% ethanol made up with DEPC treated water. The pellet was then re-suspended in 15ul TE buffer plus 15ul DEPC treated water. The concentration of RNA was then checked using spectrophotometry.

4ug of RNA in a 10ul solution was DNase treated (Table 3A). Samples were incubated at 37°C for 45 minutes before the addition of 4ul RQ stop solution (Promega) per reaction. Samples were then incubated at 65°C for 10 minutes in order to fully stop the DNase reaction.

Table 3. Details of reaction mix components for **(A)** DNase treatment, **(B)** cDNA synthesis and **(C)** qRT-PCR

(A) DNase treatment mix	
	x1 reaction (ul)
5x RT Buffer (Bioline)	8
RQ1 RNase-free DNase (Promega)	4
DEPC treated H_2O	18
RNA template	4ug in 10ul DEPC treated water
	Total volume = 40
(B) cDNA synthesis mix	
	x1 reaction (ul)
Oligo dT primer (100pM)	1
dNTP mix (10mM)	1
DEPC treated H_2O	4
5x RT Buffer (Bioline)	2
RiboSafe RNase Inhibitor (Bioline)	1
Tetro RT Enzyme (Bioline)	1
DNase treated RNA	10
	Total volume = 20
(C) qRT-PCR reaction mix	
	x1 reaction (ul)
2x qPCRBIO Sygreen Mix Hi-ROX (PCR Biosystems)	10.0
Forward Primer (10pmol/ul)	0.8
Reverse Primer (10pmol/ul)	0.8
dH_2O	3.4
cDNA	5.0
	Total volume = 20

10ul of the DNase treated RNA was then used to make cDNA (Table 3B). Samples were incubated at 42°C for 1 hour before the temperature was raised to 70°C for 15 minutes. The synthesised cDNA was then checked with PCR amplification of *ACT8* (as in Table 1, primer set in Table 2B) using 0.5ul of cDNA as template DNA in a 10ul reaction. Once the quality of the cDNA was assured, the remaining cDNA was diluted with 120ul of deionised water.

2.6.2 – qRT-PCR

qRT-PCR reactions were carried out using SYBR Hi-ROX (PCR Biosystems) in a BioRad CFX Connect Real Time System. Three biological replicates were performed for each genetic background analysed and four technical replicates were carried out for each biological replicate. Reaction mixes were made up according to Table 3C using primers sets shown in Table 2B. Analysis was carried out over 55 PCR cycles.

Comparative quantitation was carried out using the comparative C_t method. PCR efficiencies were calculated with LinReg PCR and any changes in gene expression normalised to both *ACT2* and *18S rRNA* controls (Ramakers et al., 2003).

2.7 – Histological analysis

Basal inflorescence tissue for histological analysis was taken from plants measuring 20-25cm in height. Tissue was fixed using FAA containing 50% EtOH, 10% formaldehyde (37% solution) and 5% glacial acetic acid. Fixed tissue was embedded using a JB4 infiltration kit (Polysciences) according to manufacturer's instructions. Embedded tissue was glued to stubs and transverse sections, 4-6um thick, were taken using a Finesse ME+ microtome (ThermoFisher Scientific) fitted with a glass blade. Sections were floated on water and slides left to dry on a slide drying bench. When dry, slides were stained with a 0.05% Toludine Blue solution for 20 seconds. Coverslips were mounted onto slides using Histomount mounting medium (National Diagnostics).

All images of vascular bundles were taken using the 40x magnification lens of a Zeiss Axioscop microscope fitted with a QImaging Retiga 2000R camera. Phloem cell counting and measurement of the radial:tangential ratio of the vascular bundle was done in Image J.

3. Results

3.1 – What are the functions of the feed-forward loop genes?

WOX14 is already known to act as a regulator of cell division in vascular development (Etchells et al., 2013). However, the function of *TMO6* and *LBD4* in vascular development remains unknown. *lbd4* mutants show no apparent vascular phenotype suggesting potential functional redundancy (Etchells et al., personal communication). However, *LBD4* expression is highly localised to the phloem-procambium boundary (Etchells et al., personal communication). This highly specific region of expression may be important for *LBD4* function. Therefore, manipulating *LBD4* expression in space could provide new insight into its function.

The function of *TMO6* in vascular development is also unknown. However, at the start of this project there was no *tmo6* knockout mutant available for loss of function analysis. As TMO6 has been predicted to regulate *LBD4* expression by Y1H (Etchells et al., personal communication), the position of *TMO6* expression in vascular tissue might also be important for its function. Therefore, manipulating *TMO6* expression in space was also used to study the role of *TMO6* in vascular development.

3.1.1 - Ectopic expression of *LBD4* results in defects in vascular development

Two mutant genotypes were used to investigate the effect of altered *LBD4* expression in space: *IRX3::LBD4 lbd4* and *SUC2::LBD4 lbd4*. The *IRX3::LBD4 lbd4* mutant contains a construct where *LBD4* is expressed under the xylem specific promoter of the *IRX3* gene (Fig. 3C). Meanwhile, *SUC2::LBD4 lbd4*

contains a construct that expresses *LBD4* under the phloem specific promoter of the *SUC2* gene (Fig 3D). These two mutant backgrounds were compared to both wild type and *lbd4* controls.

Eight homozygous lines of each misexpression genotype were identified and screened for vascular phenotypes. Preliminary analysis of the T2 generation

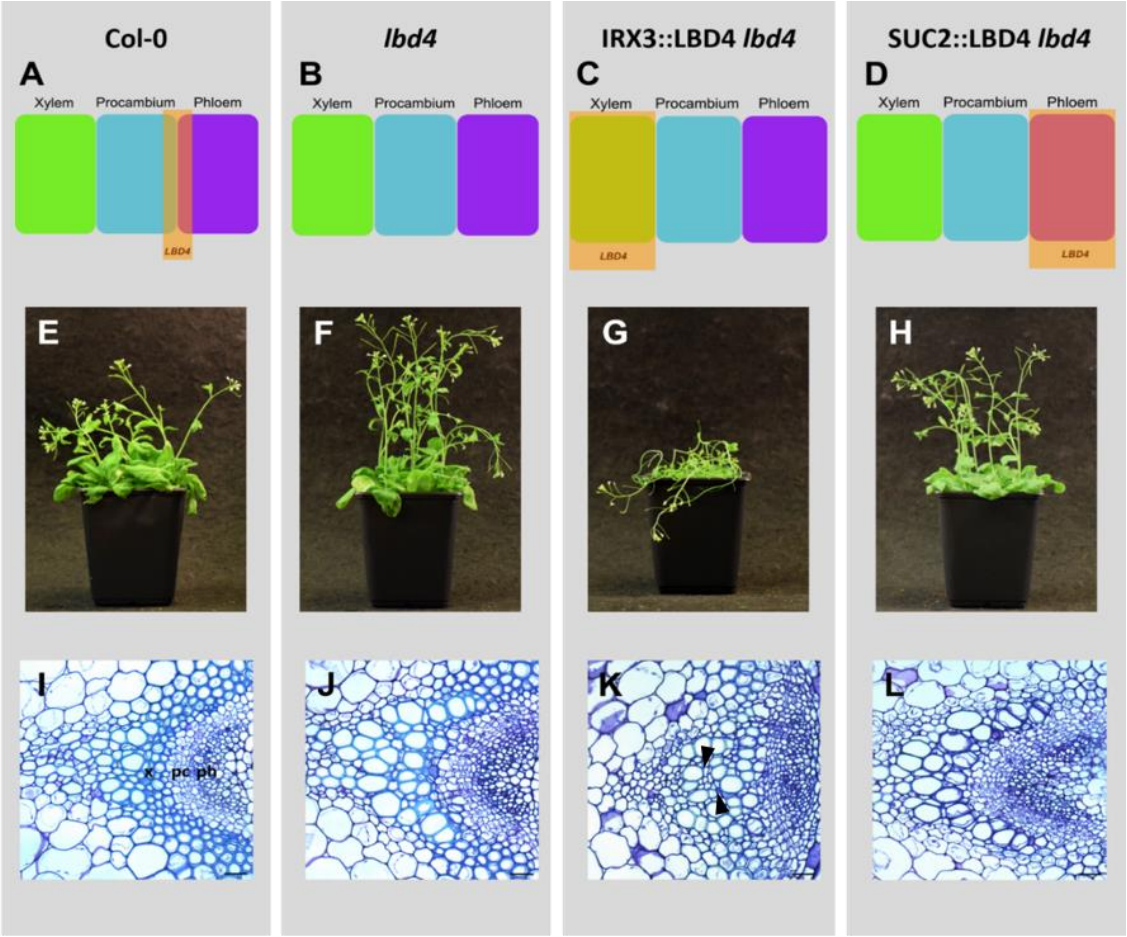


Figure 3. Comparison of *IRX3::LBD4 lbd4* and *SUC2::LBD4 lbd4* misexpression lines with Col-0 and *lbd4* controls. **(A-D)** Diagrams showing areas of *LBD4* expression, highlighted in orange, across vascular tissue regions. **(A)** Wild type expression of *LBD4* is highly localised to the phloem-procambium boundary. Misexpression lines express *LBD4* ectopically in the xylem **(C)** or in the phloem **(D). (E-H)** Photos of flowering *Arabidopsis thaliana* plants. *IRX3::LBD4 lbd4* **(G)** inflorescences are unable to support themselves. **(I-L)** Representative vascular bundles from basal inflorescence tissue of controls and transgenic lines. Regions of phloem (ph), procambium (pc), and xylem (x) are labelled on Col-0 **(I).** Arrows in **(K)** indicate xylem lacking secondary cell walls in *IRX3::LBD4 lbd4. SUC2::LBD4 lbd4* **(L)** vascular bundles appear larger than wild type or *lbd4* controls. Scale bars represent 20um.

suggested that both lines showed variable vascular phenotypes. These phenotypes were characterised by enlarged vascular bundles in *SUC2::LBD4 lbd4* lines and problems in secondary cell wall formation in the xylem fibre cells of *IRX3::LBD4 lbd4*. These observed phenotypes occurred in all lines screened for both genotypes, although variability across lines was seen. In *SUC2::LBD4 lbd4* the degree of enlargement of the vascular bundle varied across lines. Meanwhile, in *IRX3::LBD4 lbd4*, the most extreme phenotypes were characterised by the absence of secondary cell walls in most xylem fibre cells while defects were only seen in a few cells in the least extreme lines. In the homozygous T3 generation, three of the most extreme *IRX3::LBD4 lbd4* lines and two *SUC2::LBD4 lbd4* lines were taken forward for further analysis.

All three *IRX3::LBD4 lbd4* lines showed defects in xylem differentiation, with two of the three lines showing an absence of secondary cell walls in most xylem fibre cells (Fig. 3K). Secondary cell walls in xylem fibre cells are an important source of mechanical support and their absence in the two most extreme lines was associated with a whole plant phenotype, characterised by drooping inflorescences that were unable to support themselves (Fig. 3G).

The vascular bundles of *IRX3::LBD4 lbd4* lines also appeared to be flatter and wider than controls, suggesting changes in vascular patterning. To quantify this, the average tangential:radial ratio of vascular bundles was determined for each line. This was calculated by dividing the length of the bundle along the tangential axis by the length of the bundle along the radial axis (Fig. 4C). The resulting value can be used as a measure of vascular bundle shape, with higher values equating to bundles that are broader along the tangential axis and shorter along the radial axis (i.e. flatter and wider). *IRX3::LBD4 lbd4* lines gave higher values of this ratio than both controls and *SUC2::LBD4 lbd4* plants (Fig. 4B). This demonstrates that

vascular bundles in *IRX3::LBD4 lbd4* plants are wider and flatter than in controls and *SUC2::LBD4 lbd4.*

To address whether these changes in vascular organisation were associated with differences in the number of phloem cells, the average number of phloem cells per vascular bundle was calculated for misexpression lines and controls. The number of phloem cells per vascular bundle in *IRX3::LBD4 lbd4* was unchanged compared to wild type and *lbd4* controls (Fig. 4A). This suggests that the change in vascular bundle shape seen in *IRX3::LBD4 lbd4* was not associated with a change in the number of phloem cells per vascular bundle.

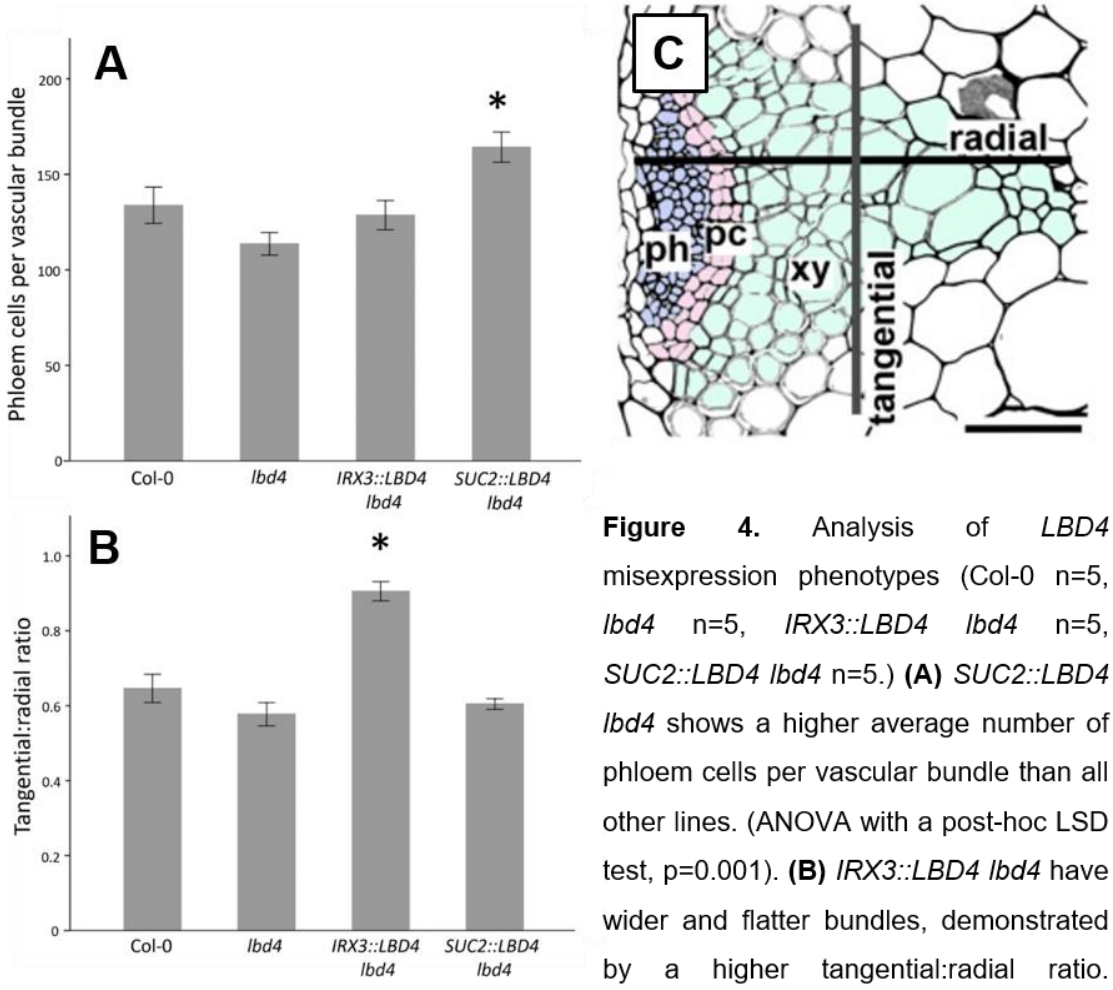


Figure 4. Analysis of *LBD4* misexpression phenotypes (Col-0 n=5, *lbd4* n=5, *IRX3::LBD4 lbd4* n=5, *SUC2::LBD4 lbd4* n=5.) **(A)** *SUC2::LBD4 lbd4* shows a higher average number of phloem cells per vascular bundle than all other lines. (ANOVA with a post-hoc LSD test, p=0.001). **(B)** *IRX3::LBD4 lbd4* have wider and flatter bundles, demonstrated by a higher tangential:radial ratio. (ANOVA with a post-hoc LSD test, p<0.001). Error bars indicate S.E.M. **(C)** Diagram illustrating measurements used to calculate tangential:radial ratio of the vascular bundle (personal communication, Peter Etchells). Regions of phloem (ph), procambium (pc) and xylem (xy) are marked.

Meanwhile, the two *SUC2::LBD4 lbd4* lines both appeared to have larger vascular bundles than controls (Fig. 3D). This increased vascular bundle size was associated with a significantly higher average number of phloem cells per vascular bundle than controls or *IRX3::LBD4 lbd4* lines (Fig. 4A). However, the average tangential:radial ratio of the vascular bundle remained similar to values seen in the controls (Fig. 4B), suggesting that the larger vascular bundles maintained normal proportions.

3.1.2 - Early flowering seen in *LBD4* misexpression mutants is common to several vascular defect mutants

Both *LBD4* misexpression lines and the *lbd4* knockout mutant showed earlier flowering times than wild type controls demonstrated by the production of significantly fewer rosette leaves before floral transition. Of these early flowering genotypes, *IRX3::LBD4 lbd4* flowered significantly earlier than both *SUC2::LBD4 lbd4* and the *lbd4* mutant (Fig. 5A.). This presents a flowering time phenotype not previously reported for *lbd4* and suggests that ectopic *LBD4* expression in the phloem or xylem is not sufficient to rescue this phenotype and, in the case of *IRX3::LBD4 lbd4*, can even act to enhance it.

To investigate whether this apparent regulation of flowering time by *LBD4* may itself be PXY regulated, flowering time was measured in two mutants defective in PXY signalling. The two backgrounds used were *pxy pxl1 pxl2,* which lacks the functional PXY receptor as well as its two redundant receptors PXL1 and PXL2 and *tdif,* which lacks functional TDIF peptide. Like *lbd4* and the *LBD4* misexpression lines, both PXY signalling defective mutants exhibited significantly earlier flowering times than the wild type control (Fig. 5B).

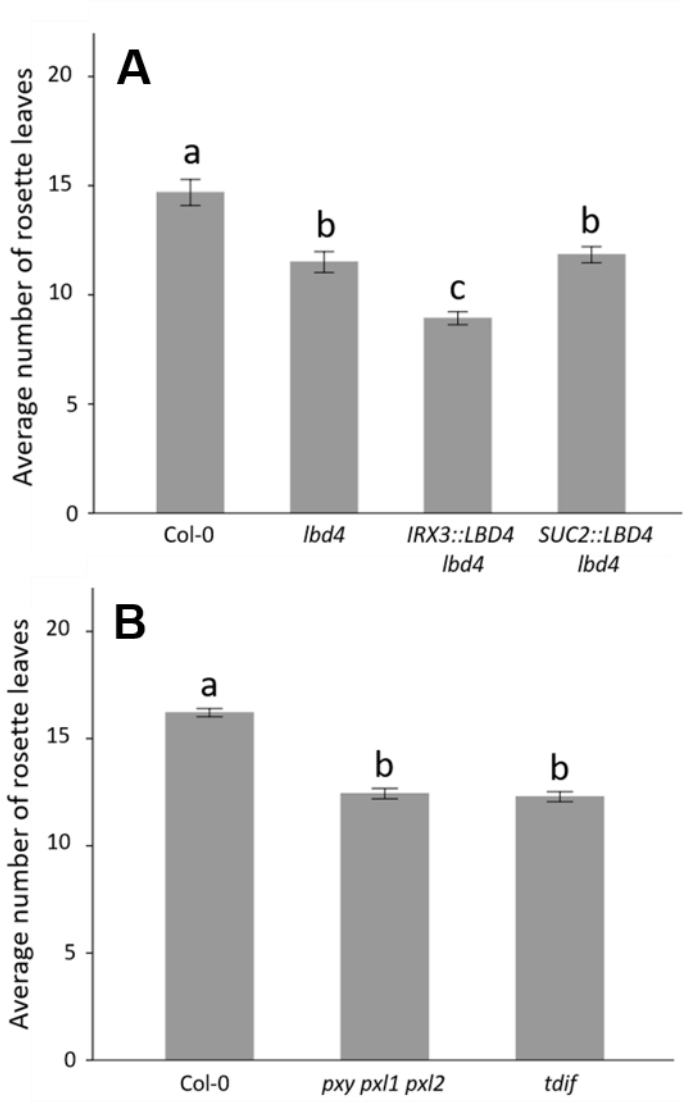


Figure 5. Comparison of flowering time measured via number of rosette leaves at floral transition. **(A)** Both *lbd4* (n=18) and the *LBD4* misexpression lines (*IRX3::LBD4 lbd4* n=26, *SUC2::LBD4 lbd4* n=19) show earlier flowering than wildtpye controls (n=16). **(B)** Mutants with defective PXY signalling (*pxy pxl1 pxl2* n=24, *tdif* n=24) show earlier flowering than wildtype (n=24). Error bars indicate S.E.M. Differing letters indicate p<0.001 (ANOVA with a post-hoc LSD test).

3.1.3 - One line of *IRX3::TMO6* shows increased phloem proliferation

The results in 3.1.1 suggest that the position of *LBD4* expression in space is important for its function. As TMO6 is a predicted regulator of *LBD4* and its role in vascular development is currently unknown, the effect of ectopic expression of

TMO6 on vascular development was also investigated. This was done using *IRX3::TMO6* lines, ectopically expressing *TMO6* in the xylem.

Eight T2 lines of *IRX3::TMO6* were screened for vascular phenotypes. In initial screening, there was no immediately apparent vascular phenotype and three lines were selected for further analysis in the T3 generation.

Unlike the *LBD4* misexpression lines, none of the three lines screened in detail demonstrated an apparent change in vascular development. Vascular bundle

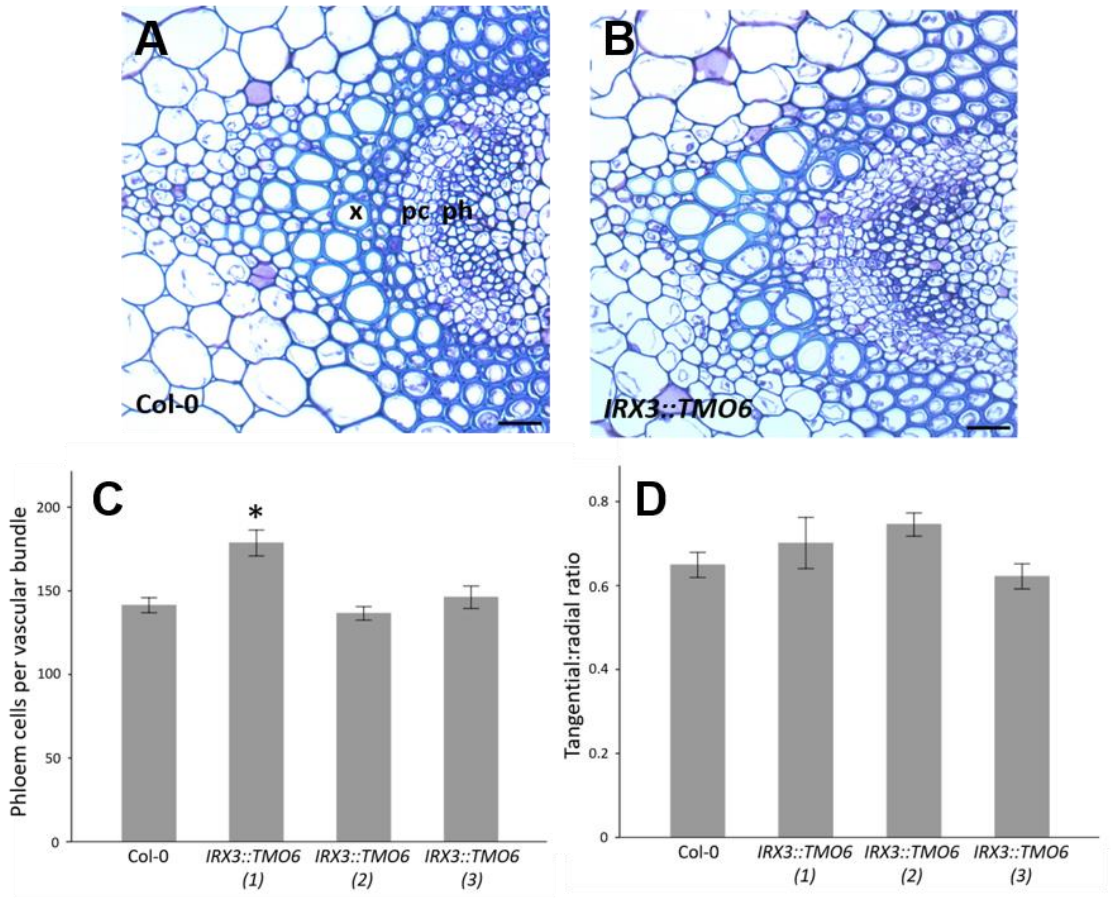


Figure 6. Analysis of *IRX3::TMO6* lines. **(A-B)** Representative vascular bundles of **(A)** Col-0 control, with regions of phloem (ph), procambium (pc) and xylem (x) labelled, and **(B)** *IRX3::TMO6 (1)* which appears similar to wild type. Scale bars indicate 20um. **(C)** *IRX3::TMO6 (1)* (n = 5) has a higher average number phloem cells per vascular bundle than the Col-0 control (n=5) and the other two *IRX3::TMO6* lines (n=6,6 respectively). (ANOVA with a post-hoc LSD test, $p<0.001$). **(D)** The average tangential:radial ratio of the vascular bundle is similar for Col-0 controls and all three *IRX3::TMO6* lines (ANOVA, $p=0.119$).Error bars indicate S.E.M.

morphology appeared similar to the wild type control (Fig. 6A-B). The average tangential:radial ratio of the vascular bundle was also similar across the wild type control and the *IRX3::TMO6* lines (Fig 6D), suggesting no quantifiable changes in vascular bundle shape. However, one of the three lines screened showed significantly higher numbers of phloem cells per vascular bundle than both the wild type control and the other two *IRX3::TMO6* lines (Fig. 6C). Whether this demonstrates an increase in phloem proliferation in the *IRX3::TMO6* phenotype cannot be concluded from results seen for only one line. Thus, screening of more *IRX3::TMO6* lines to investigate whether this represents an *IRX3::TMO6* phenotype or a positional effect of the insertion is still required.

3.2 – How is the feed-forward loop regulated?

The results from section 3.1 suggest that *LBD4* is important in vascular development. As regulation of the *LBD4* transcription factor is the main output of the feed-forward loop, understanding how the loop components interact to co-ordinate *LBD4* regulation is a key component of understanding the loop's role in vascular development.

Preliminary Y1H assay results show that WOX14 binds to the promoter of both the *TMO6* and *LBD4* genes. Here, we investigated whether WOX14 regulation of *TMO6* and *LBD4* is supported by genetic interactions in planta. Furthermore, WOX14 has been demonstrated to show PXY signalling dependent expression (Etchells et al., 2013). Therefore, we decided to investigate how this may translate into PXY signalling regulation of the feed-forward loop. To address these two questions, qRT-PCR gene expression analysis was used to measure

the expression of the feed-forward loop genes across a range of mutant backgrounds appropriate to each question.

3.2.1 - WOX4 and WOX14 are important for both *TMO6* and *LBD4* regulation

The WOX14 transcription factor binds to the promoters of both *TMO6* and *LBD4* genes in Y1H. WOX14 has also been shown to have importance in vascular development, acting redundantly with WOX4 to regulate vascular cell division. Therefore, how WOX14 may act to regulate the feed-forward loop components is of particular interest when considering the role of the feed-forward loop in vascular development. Additionally, WOX14's functional redundancy with WOX4 identifies WOX4 as a factor of interest. To investigate the influence of these two factors on the feed-forward loop components, qRT-PCR was used to measure the levels of *TMO6* and *LBD4* expression in a wild type control and *wox4, wox14* and *wox4 wox14* mutants.

All three of the mutant backgrounds, *wox4, wox14* and *wox4 wox14,* showed significantly lower levels of both *TMO6* and *LBD4* expression than the wild type when normalised to an *ACT2* control (Fig. 7A-B). There was also a significant decrease in *TMO6* expression in the *wox4 wox14* mutant compared to the other two mutant backgrounds (Fig. 7A). On the other hand, *LBD4* expression in the *wox4 wox14* mutant background was only significantly lower than the *wox4* mutant and did not differ significantly from the *wox14* mutant (Fig. 7B). Meanwhile, when normalised to *18S rRNA*, significant reductions in *TMO6* expression relative to wild type were only seen in the *wox4 wox14* double mutant (Fig. 7C). Together, these results suggest that *WOX14* is required for normal *TMO6* and *LBD4* expression levels. They are also consistent with the literature, where *WOX4* and *WOX14* are reported to act redundantly (Etchells et al., 2013).

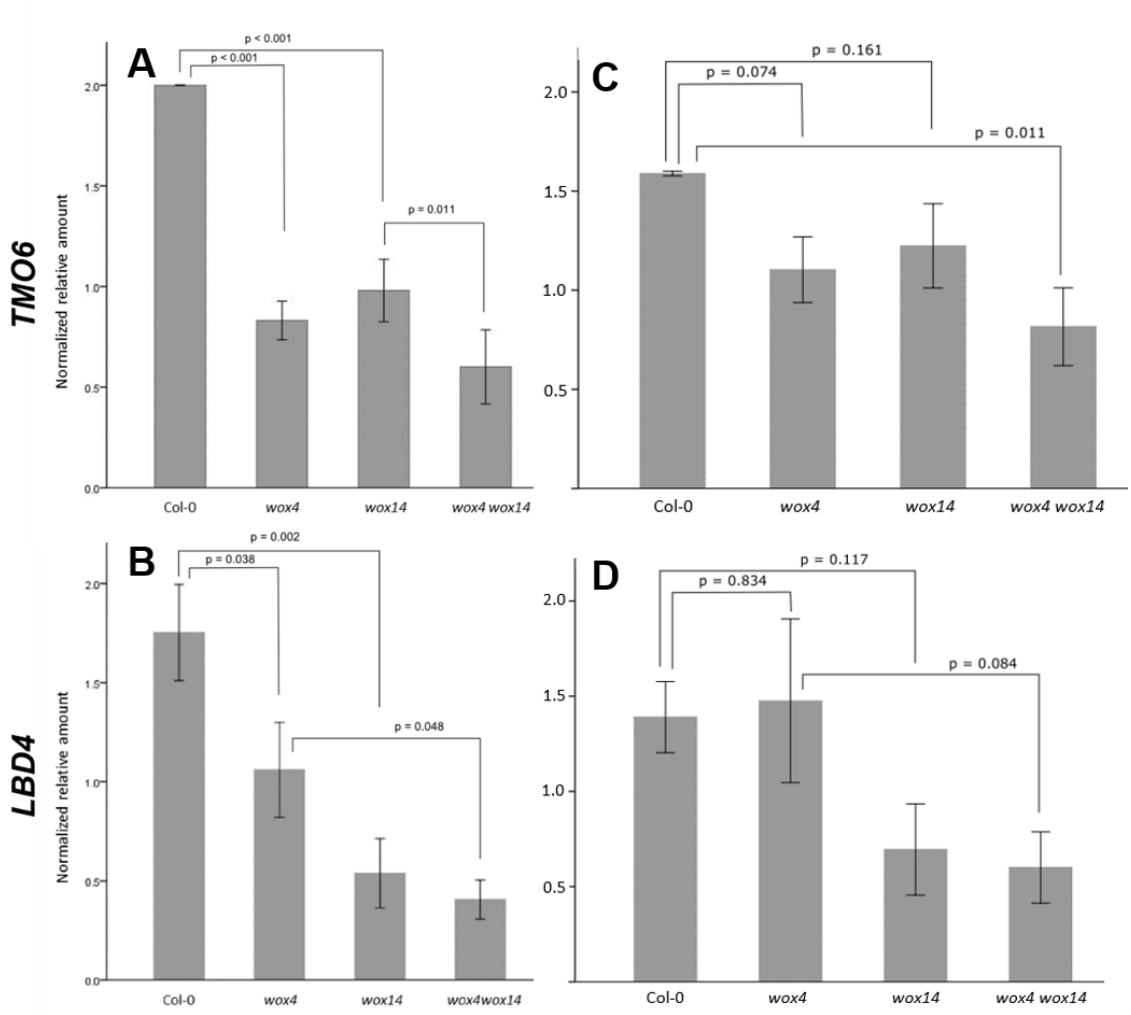


Figure 7. qRT-PCR analysis studying the effect of WOX4 and WOX14 on *TMO6* and *LBD4* expression. **(A-B)** *LBD4* and *TMO6* expression normalised to *ACT2*. **(A)** *TMO6* expression is reduced in *wox4, wox14* and *wox4 wox14* mutant backgrounds compared to wildtype. **(B)** *LBD4* expression is also decreased in all three mutant backgrounds compared to wildtype. **(C-D)** *LBD4 and TMO6* expression normalised to 18S rRNA. Error bars indicate S.E.M. P values calculated using ANOVA with a post-hoc LSD test.

The expression of the *WOX4* and *WOX14* genes were also measured during this experiment. Interestingly, *WOX4* expression levels were significantly lower than wild type levels in the *wox14* mutant when normalised to *ACT2* (Fig. 8A).

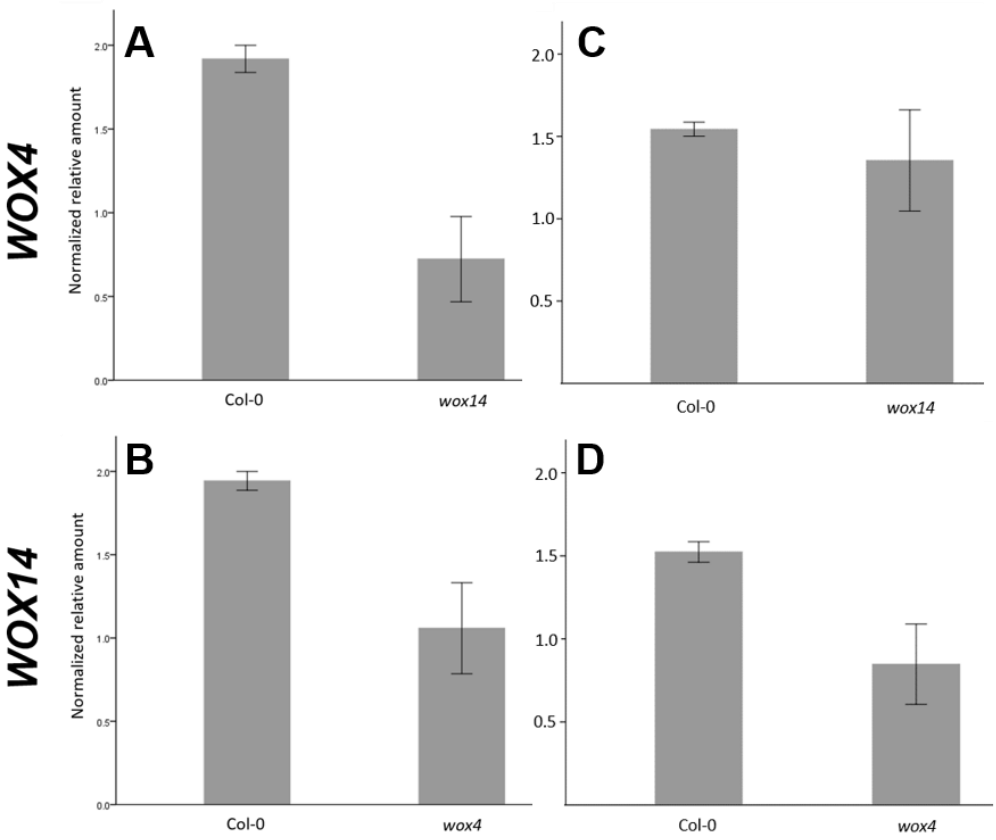


Figure 8. qRT-PCR analysis of *WOX4* and *WOX14* expression normalised to *ACT2* (A-B) and *18S rRNA* (C-D) **(A)** *WOX4* expression is reduced in the *wox14* mutant background compared to Col-0 (t-test, p=0.011). **(B)** *WOX14* expression is not significantly reduced in a *wox4* mutant (t-test, p=0.078). **(C)** *WOX4* expression is unchanged in the *wox14* mutant when normalised to *18S rRNA* (t-test, p=0.054). **(D)** *WOX14* expression in unchanged in the *wox4* background when normalised to 18S rRNA (t-test, p=0.574). Error bars indicate S.E.M.

Conversely, there was no statistically significant change in *WOX14* expression in the *wox4* background from wild type levels when normalised to both *ACT2* and *18S rRNA* (Fig. 8B,D).

3.2.2 - Regulation of *WOX14* and *TMO6* by PXY signalling does not translate to direct regulation of *LBD4* by PXY signalling

The results from section 3.2.1 suggest that both normal *TMO6* and *LBD4* expression levels are dependent on WOX14. As WOX14 functions in a PXY dependent manner (Etchells et al., 2013), the effect of PXY signalling on *TMO6* and *LBD4* was studied. qRT-PCR was used to measure *TMO6* and *LBD4*

expression in a wild type control and two mutant backgrounds defective in PXY signalling: *pxy pxl1 pxl2* and *tdif*.

TMO6 expression was significantly lower than wild type levels in both of the mutant backgrounds when normalised to *ACT2* (Fig. 9A) and in *pxy pxl1* pxl2 when normalised to 18S *rRNA* (Fig. 9C). However, *LBD4* expression did not differ significantly over the three genotypes when normalized to both control genes (Fig. 9B,D). As *LBD4* shows a high level of connectivity within the transcriptional

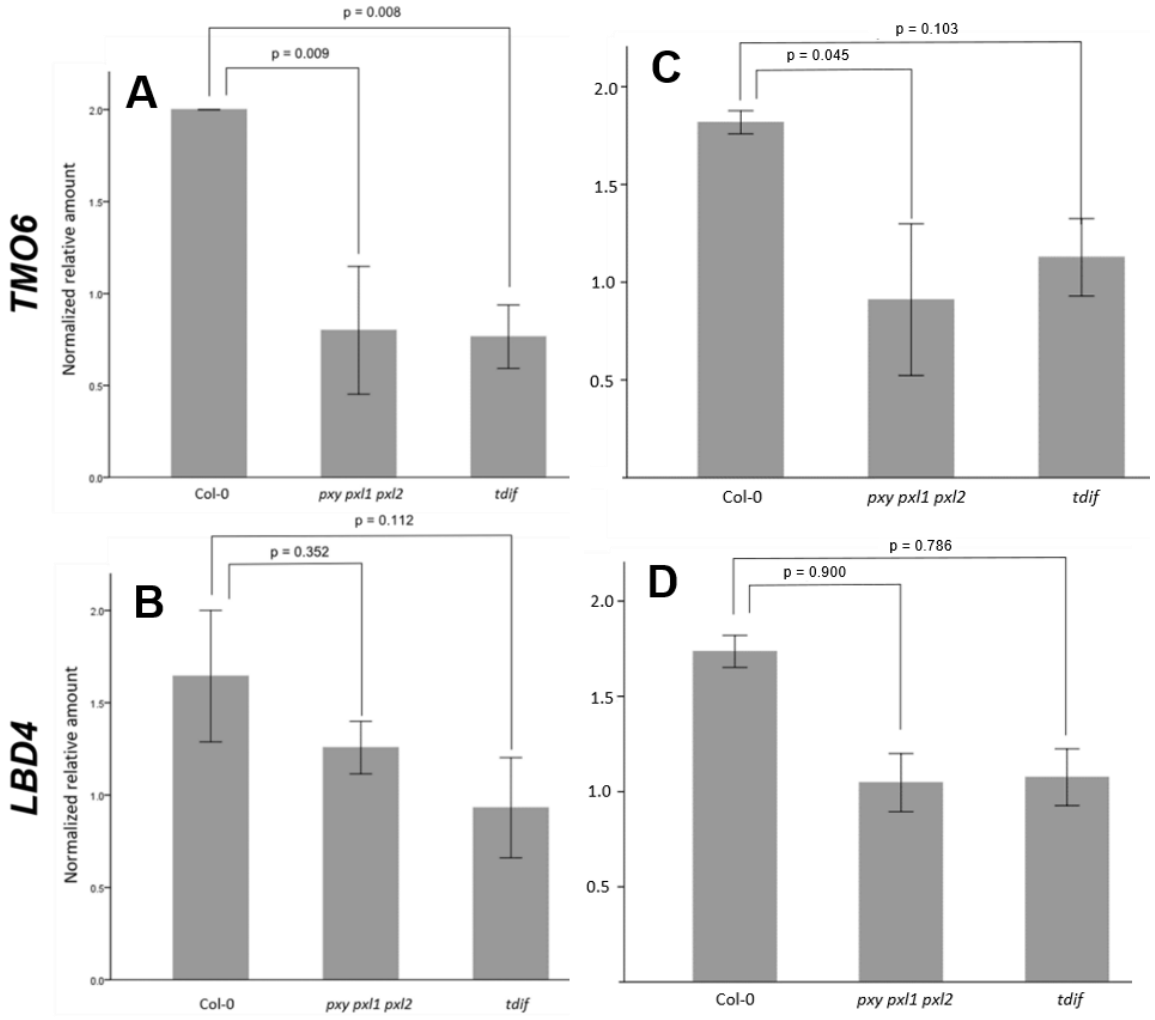


Figure 9. qRT-PCR analysis of *TMO6* and *LBD4* expression in PXY signalling defective backgrounds. **(A-B)** *TMO6* and *LBD4* expression normalised to *ACT2*. **(A)** *TMO6* expression is reduced in the both *pxy pxl1 pxl2* and *tdif* mutant backgrounds that are PXY signalling defective compared to wild type. **(B)** *LBD4* expression is not significantly reduced in the two PXY signalling defective mutants compared to wild type. **(C-D)** *TMO6* and *LBD4* expression normalised to *18S rRNA*. Error bars indicate S.E.M. P values calculated using ANOVA with a post-hoc LSD.

regulatory network, this may suggest that other factors act to maintain *LBD4* expression when PXY signalling is not present.

3.3 – Loss of function analysis

Current research investigating the feed-forward loop is limited by gaps in the bank of genetic resources available. Therefore, we endeavoured to increase the number of currently available genetic resources to facilitate future research and improve current understanding of the feed-forward loop.

Two novel loss of function mutants were generated: *tmo6-1* and *wox14 lbd4.* Vascular tissue from these mutants was analysed histologically in order to better understand the roles of these genes in vascular development.

3.3.1 – *tmo6-1* mutants show decreased phloem proliferation

At present, there is little understanding of the function of *TMO6* in vascular development. This is largely due to the lack of an available *tmo6* knockout mutant to enable loss of function analysis. Therefore, a *tmo6* knockout mutant was generated with CRISPR Cas9 genome editing.

The CRISPR Cas9 plasmid used was targeted to two sites within the *TMO6* gene, positioned approximately 300 base pairs apart (Fig 10A-B). On introduction of this plasmid, the resulting mutant, named *tmo6-1,* generated a PCR product that was visibly smaller than the wild type product after gel electrophoresis (Fig. 10C). This suggested that the entire 300 bp sequence between the two CRISPR target sites had been deleted. A BLAST search revealed that this deleted region fell within

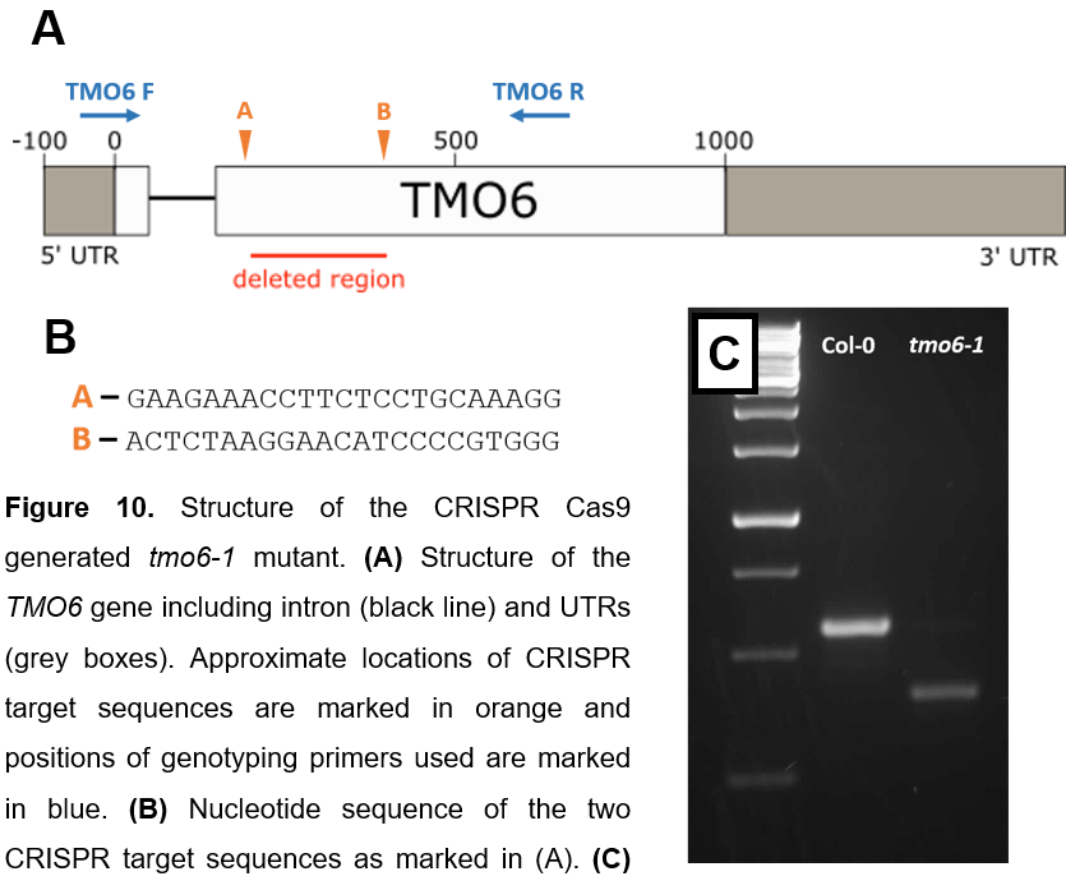


Figure 10. Structure of the CRISPR Cas9 generated *tmo6-1* mutant. **(A)** Structure of the *TMO6* gene including intron (black line) and UTRs (grey boxes). Approximate locations of CRISPR target sequences are marked in orange and positions of genotyping primers used are marked in blue. **(B)** Nucleotide sequence of the two CRISPR target sequences as marked in (A). **(C)** *tmo6-1* produces a smaller PCR product than the wild type control after gel electrophoresis.

the predicted coding sequence of the Dof DNA binding domain. Therefore, it is highly likely that this deletion equates to a functional knockout of the TMO6 transcription factor.

Further testing to ensure that *tmo6-1* is a true knockout is still required, as is removal of the active CRISPR Cas9 plasmid via backcrossing. However, preliminary histological analysis gave promising results. t*mo6-1* mutants appeared to have smaller vascular bundles than wild type controls (Fig. 11A-B). This was associated with significantly lower average numbers of phloem cells per vascular bundle. Conversely, the tangential:radial ratio of the vascular bundle remained similar to the wild type control, demonstrating that the smaller *tmo6-1* vascular bundles maintained a normal shape (Fig. 11C-D). This suggests that

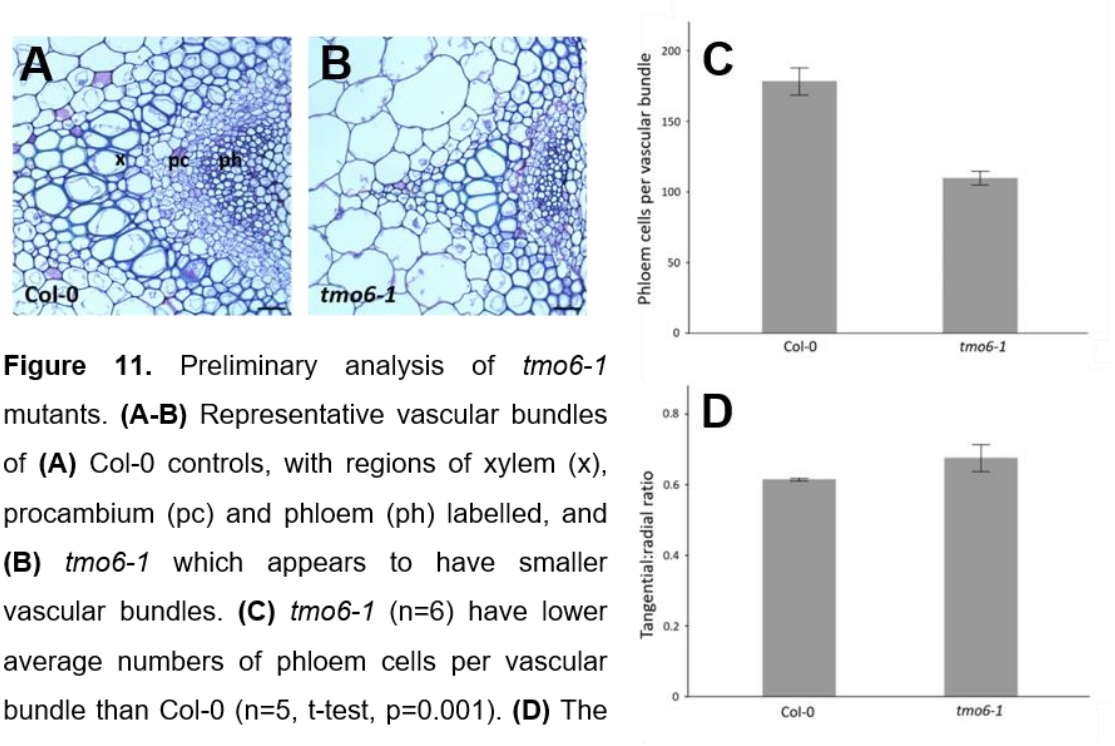


Figure 11. Preliminary analysis of *tmo6-1* mutants. **(A-B)** Representative vascular bundles of **(A)** Col-0 controls, with regions of xylem (x), procambium (pc) and phloem (ph) labelled, and **(B)** *tmo6-1* which appears to have smaller vascular bundles. **(C)** *tmo6-1* (n=6) have lower average numbers of phloem cells per vascular bundle than Col-0 (n=5, t-test, p=0.001). **(D)** The average tangential:radial ratio of the vascular bundle is similar for Col-0 (n=5) and *tmo6* (n=6, t-test, p=0.138). Error bars indicate S.E.M.

TMO6 is involved in cell proliferation in vascular tissue. As described in 3.1.1, *SUC2::LBD4 lbd4* mutants also showed changes in phloem proliferation. Therefore, this implies that both *TMO6* and *LBD4* play a role in the phloem and the feed-forward loop may act to co-ordinate phloem development.

3.3.2 - *wox14* and *wox14 lbd4* mutants both show similar vascular phenotypes

The generation of a *tmo6* knockout mutant opens up new possibilities for research into the feed-forward loop. One such possibility is a full knockout of the loop with a triple knockout of all three loop component genes. This could provide important insights into the function of the loop as a whole. In order to facilitate the generation of this triple mutant in the future, a *wox14 lbd4* mutant was generated for future crossing with *tmo6-1*. Furthermore, to better understand the function

and interaction between the feed-forward loop components, this novel double mutant was analysed histologically to determine any present vascular phenotype.

Homozygous *wox14 lbd4* double mutants were isolated from a population of segregating T2s and analysed alongside wild type, *lbd4* and *wox14* controls. Both *wox14* and *wox14 lbd4* appeared to have slightly larger vascular bundles than wild type and *lbd4* controls (Fig 12A-D). Further analysis demonstrated that this was associated with increased phloem proliferation in both *wox14* and *wox14 lbd4* mutants (Fig. 12E). This is a phenotype that has not previously been described for *wox14.* Meanwhile, measurements of the tangential:radial ratio of

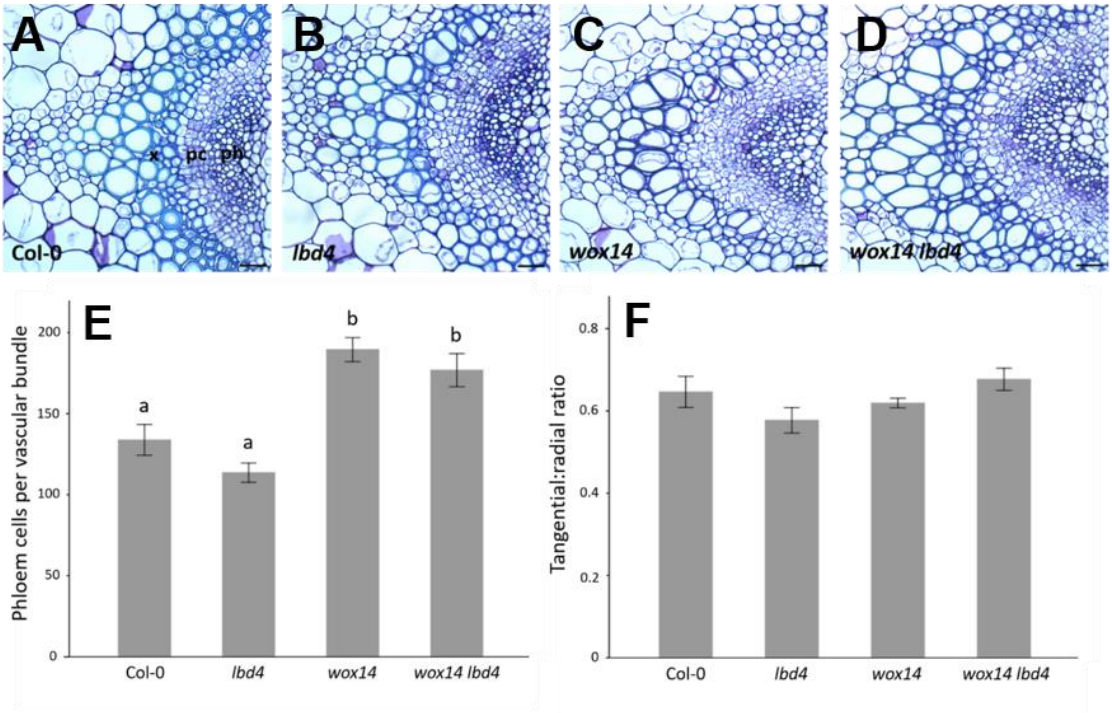


Figure 12. Analysis of *wox14 lbd4* mutants. **(A-D)** Representative vascular bundles from **(A)** Col-0 with (x) xylem, (pc) procambium and (ph) phloem labelled, **(B)** *lbd4*, **(C)** *wox14* and **(D)** *wox14 lbd4,* both of which appear to have larger vascular bundles than wild type and *lbd4* controls. **(E)** *wox14* (n=4) and *wox14 lbd4* (n=7) show higher average numbers of phloem cells per vascular bundle than Col-0 (n=5) and *lbd4* (n=5).. Differing letters indicate $p < 0.004$ (ANOVA and a post-hoc LSD test). **(F)** Average tangential:radial ratios are similar across control and mutant lines (ANOVA, p=0.127). Error bars indicate S.E.M

the vascular bundle remained similar across the four genotypes (Fig. 12F). Together, these results suggests that the *wox14* mutation acts epistatically to *lbd4* and the increased number of phloem cells seen in *wox14 lbd4* can likely be attributed to *wox14* alone.

4. Discussion

4.1 – What is the role of the feed-forward loop?

4.1.1 – What is the function of LBD4?

Ectopic expression of *LBD4* causes visible defects in vascular development. *SUC2::LBD4 lbd4* plants, which express *LBD4* in the phloem, show increased phloem proliferation (Fig. 3A). However, the shape of the vascular bundle remains similar to controls (Fig. 3B). Meanwhile, *IRX3::LBD4 lbd4* plants, which express *LBD4* in the xylem, show changes in vascular patterning, characterised by a flattening of the vascular bundle (Fig. 3B). However, the altered shape of *IRX3::LBD4 lbd4* vascular bundles is not associated with changes in phloem proliferation as phloem cell numbers were indistinguishable from wild type (Fig. 3B). These phenotypes demonstrate that correct expression of *LBD4* in space is essential for correct vascular development. Furthermore, *LBD4* appears to be involved in the regulation of both phloem proliferation and the organisation of the vascular bundle.

IRX3::LBD4 lbd4 plants also show a failure to form secondary cell walls in xylem fibre cells (Fig. 2K). Secondary cell walls are comprised of cellulose, hemicellulose and lignin and are thickened compared to the primary cell walls that are present in all plant cells (Esau, 1977). Secondary cell walls are characteristic to xylem cells and are important in supplying the extra mechanical support needed to withstand the negative pressure generated by water transport (Turner and Somerville, 1997). Therefore, a reduction in secondary cell wall material in the xylem could cause a significant reduction in the plant's mechanical strength. This could result in the plant's inability to support its own weight, leading to collapse of structures that require increased support, such as inflorescences.

Thus, the lack of secondary cell walls in *IRX3::LBD4 lbd4* xylem fibre cells likely accounts for the drooping inflorescence phenotype seen in the most extreme *IRX3::LBD4 lbd4* lines (Fig. 2G).

The mechanical strength of stems is a research topic of particular economic importance as good mechanical strength in stems is important for avoiding the agricultural problem of lodging (Kong et al., 2013). Lodging describes the permanent displacement of crop plants from a vertical position. This limits assimilate transport and decreases harvesting efficiency, leading to significant yield loss. For example, lodging in oilseed rape has been estimated to result in a 20-46% yield loss costing an estimated £47-120 million per year in the UK (Kendall et al., 2017).

There are multiple techniques that could be used to investigate whether the mechanical strength of *IRX3::LBD4 lbd4* is in fact decreased, such as a compression test or a three-point bending test (Hongo et al., 2012; Turner and Somerville, 1997). These techniques could also be applied to *SUC2::LBD4 lbd4* misexpression lines and *lbd4* knockouts to provide insight into the physical properties of these plants. This would allow for a more detailed comparison between the misexpression lines and identify whether *LBD4* has a more general role in maintaining a plant's mechanical strength.

The lack of secondary cell walls in *IRX3::LBD4 lbd4* xylem fibres also suggests that *LBD4* may have a role in determining cell identity. As secondary cell walls are a characteristic feature of xylem cells, the *IRX3::LBD4 lbd4* phenotype indicates that expression of *LBD4* in the xylem hinders the proper establishment of xylem identities.

Regulation of cell identity is a known output of PXY signalling. In *Zinnia elgans* xylogenic cultures, addition of the TDIF peptide inhibits undifferentiated cells from spontaneously differentiating into tracheary elements (Ito et al., 2006). This demonstrates that TDIF activation of PXY inhibits the establishment of xylem identities in differentiating cells. Therefore, like TDIF, *LBD4* may inhibit xylem identities. This may encourage the establishment of phloem identities in differentiating cells, as seen in the *SUC2::LBD4 lbd4* lines which show an over proliferation of phloem.

Although the number of phloem cells was increased in the *SUC2::LBD4 lbd4* lines, how this affected overall plant architecture could not be answered by the data collected in this study. In future, measurement of the stem thickness and plant biomass would provide insight into how these changes in cell proliferation may affect whole plant structure and physiology.

The importance of the correct location of *LBD4* expression could be attributed to a role in defining phloem identities at the phloem-procambium boundary. Thus, *LBD4,* like many other LOB domain proteins, may act to define an organ boundary (Husbands et al., 2007), in this case, the boundary between the phloem and procambium. As differentiating cells are displaced from the procambium region, the area of *LBD4* expression may act to exclude xylem identities from these cells, encouraging them to take on phloem identities as they leave the procambium and thus defining the boundary.

Although ectopic expression of *LBD4* results in vascular defects, *lbd4* knockout mutants show no obvious vascular phenotype. Therefore, it is likely that *LBD4* functions at the phloem-procambium boundary redundantly with other factors. In poplar, LBD genes functioning alongside *LBD1* in secondary growth have been

identified based on their specific region of gene expression (Yordanov et al., 2010). As the pattern of *LBD4* expression in *Arabidopsis thaliana* is highly specific to the phloem-procambium boundary, factors functioning redundantly with *LBD4* could possibly be isolated using a similar approach. The identification of these factors will improve understanding of the role of *LBD4* and the feed-forward loop in vascular development.

4.1.2 – What is the function of TMO6?

Unlike *LBD4,* expressing *TMO6* ectopically in the xylem does not result in a clearly visible vascular phenotype (Fig. 5A-B). However, one of the eight lines screened did show increased phloem proliferation when analysed quantitatively (Fig. 5C). This is particularly surprising as the *IRX3* promoter does not drive expression in phloem tissue. Thus, whether this is a positional effect of the insertion or an effect of ectopic *TMO6* expression cannot be concluded from analysis of just one line. Therefore, investigation of more *IRX3::TMO6* lines is required.

There are multiple reasons why *IRX3::TMO6* may not show a clear vascular phenotype. Firstly, although data from the *Arabidopsis* global phloem cells transcript database suggests that *TMO6* is expressed in the phloem (Le Hir and Bellini, 2013), the precise region of *TMO6* expression in vascular tissue is not well characterised. While *LBD4* shows highly localised expression characteristic of LOB domain genes (Husbands et al., 2007), it is possible that *TMO6* expression is more diffuse. Therefore, ectopic expression may not have as great an effect in *IRX3::TMO6.* This illustrates the need for *TMO6* expression in vascular tissue to be properly characterised before the nature of *IRX3::TMO6*

lines can be fully understood. This could be achieved by either GUS staining or RNA in situ hybridisation.

Additionally, the unavailability of a *tmo6* knockout mutant at the time that the *IRX3::TMO6* construct was produced means that the *IRX3::TMO6* construct was not introduced into a knockout background. Consequently, in *IRX3::TMO6* the wild type *TMO6* gene is still likely to be expressed at the same place and time.

Although *IRX3::TMO6* lines have revealed little about the function of *TMO6*, some insight has been provided by the production of a *tmo6* knockout by CRISPR Cas9 genome editing. *tmo6-1* mutants had smaller vascular bundles than wild type, a change that was associated with decreased phloem proliferation. This is consistent with previously produced lines containing an artificial microRNA targeted to *TMO6* which show reduced numbers of vascular cells (Etchells et al., personal communication). This suggests that *TMO6* is involved in cell proliferation in vascular tissue. This role is shared by *WOX14,* the putative regulator of *TMO6* in the feed-forward loop (Etchells et al., 2013). Meanwhile, *SUC2::LBD4 lbd4* shows increased numbers of phloem cells. Considered together, these results suggest that the feed-forward loop as a functional unit is involved in cell proliferation, possibly acting in phloem tissue to recruit procambium cells towards a phloem identity.

The creation of a *tmo6* knockout background also provides the opportunity to generate an *IRX3::TMO6 tmo6* line. In this mutant background, the wild type pattern of *TMO6* expression would not be maintained. Thus, any changes in vascular development in these lines will better reflect the effect of ectopic *TMO6* expression. Additionally, these lines will allow for more reliable comparisons between *TMO6* and *LBD4* misexpression lines. Therefore, *IRX3::TMO6 tmo6*

lines may in fact prove more useful than *IRX3::TMO6.* Thus, in future, the generation of *IRX3::TMO6 tmo6* lines should be prioritised over the analysis of more *IRX3::TMO6* lines. Additionally, the production of a *SUC2::TMO6 tmo6* transgenic line should also be considered. Comparison of this line with *IRX3::TMO6 tmo6* would allow for analysis of the effect of ectopic *TMO6* expression across differing vascular tissue types to investigate the importance of correct *TMO6* expression in space. However, the effect of ectopic *TMO6* expression on vascular development cannot be properly understood without first fully characterising the normal location of *TMO6* expression in space.

4.2 – What are the roles of WOX4 *and* WOX14*?*

4.2.1 Regulating the expression of multiple genes

WOX4 and *WOX14* have previously been shown to function redundantly in vascular cell division, with *wox14* enhancing the *wox4* phenotype that is characterised by decreased vascular cell division (Etchells et al., 2013). When normalised to *ACT2,* qRT-PCR results showed a decrease in *TMO6* and *LBD4* expression in both *wox14* and *wox4* mutants compared to wild type (Fig. 6). Meanwhile, in the *wox4 wox14* background, *TMO6* and *LBD4* expression was even lower than in the single mutants. When normalised to *18S rRNA*, *TMO6* and *LBD4* expression was only significantly lower than wild type in the *wox4 wox14* mutant background. Together, these results demonstrate redundancy between *WOX4* and *WOX14;* in this case, they function redundantly to promote *TMO6* and *LBD4* transcription. These results are also consistent with the proposed feed-forward loop, confirming that both *TMO6* and *LBD4* function downstream of *WOX14*.

The *wox4 wox14* double mutant has previously been characterised by decreased cell proliferation (Etchells et al., 2013). Here, both the *tmo6* knockout mutant and the *SUC2::LBD4 lbd4* mutant demonstrated changes in cell proliferation in the phloem (Figs. 10C & 3A respectively). Therefore, the feed-forward loop may represent part of how *WOX4* and *WOX14* work together to regulate the rate of vascular cell proliferation.

Interestingly, *WOX4* expression was significantly lower in the *wox14* mutant background than in wild type (Fig. 7A). *wox14* enhances the *wox4* phenotype but does not demonstrate a vascular phenotype itself (Etchells et al., 2013). This raises the question of how WOX14 may be influencing *WOX4* transcription without demonstrating a phenotype similar to that of *wox4*.

The WOX4 transcription factor was not included in previous Y1H. However, the *WOX4* promoter was subsequently screened against the library of transcription factors and the WOX14 transcription factor was not found to bind to the *WOX4* promoter (Etchells et al., personal communication). Y1H are well known to produce false negatives and positives but if these results can be *confirmed in planta*, this may demonstrate that *WOX4* is not influenced directly by WOX14.

The previously generated transcriptional regulatory network is characterised by feed-forward loops and as *WOX4* is not included in the network, it is entirely possible that *WOX4* and *WOX14* are members of another loop outside of or connected to the feed-forward loop investigated here. This demonstrates the difficulty in exploring such a large and complex network where the exclusion of even one factor can limit our understanding of the overall picture. It is likely that the reduced expression of *WOX4* in the *wox14* mutant does not represent a simple TF-gene regulatory relationship but instead illustrates a more complex

relationship by which *WOX4* is influenced by a series of intermediary factors downstream of *WOX14.*

4.2.2 – Re-assessing WOX14

The phenotype of the *wox14 lbd4* mutant (Fig. 11) produced within this project demonstrates that *wox14* is epistatic to *lbd4*. Therefore, the increased phloem proliferation seen in *wox14 lbd4* (Fig. 11E) can largely be attributed to *wox14.* This is consistent with the hypothesis that *WOX14* acts upstream of *LBD4.*

However, the phenotype described here for *wox14,* conflicts with previous findings in the literature. *wox14* has previously been reported to show no apparent phenotype while *wox4 wox14* shows decreased numbers of cells per vascular bundle (Etchells et al., 2013). This suggests that *WOX14* is a positive regulator of cell division. Conversely, here, *wox14* was characterised by larger vascular bundles and increased phloem proliferation, apparently contradicting the idea that *WOX14* acts as a positive regulator of cell division.

It is important to remember that *WOX14* and *WOX4* sit within a large and complex network and it is likely that only a small proportion of factors influencing their expression and action have been identified. Therefore, how the *wox14* phenotype manifests could be affected by any number of factors. For example, a recent study investigating the relationship between *WOX14* and gibberellin synthesis (Denis et al., 2017) used a *wox14* T-DNA insertion mutant different to the one used here and in previous research (Etchells et al., 2013). This mutant was also in a Nossen ecotype background of *Arabidopsis thaliana* as opposed to the Col-0 ecotype used here and in previous research. In this recent study, *wox14* mutants demonstrated defects in xylem lignification (Denis et al., 2017), a phenotype not previously reported. This may be an example genetic interactions

across the differing genetic backgrounds used affecting the manifestation of the *wox14* phenotype.

Furthermore, although environmental conditions are well known to have an effect on plant development, relatively little is known about the specific effects of certain environmental stimuli on vascular development. Therefore, it is possible that the growth conditions used here may differ enough from those used in previous studies as to profoundly affect how the *wox14* phenotype manifests. Thus, the findings here may demonstrate phenotypic plasticity in the *wox14* mutant.

As well as environmental conditions, the *wox14* phenotype will also likely vary across differing developmental stages. The structure of vascular tissue in the *Arabidopsis thaliana* inflorescence changes throughout development, with younger vascular tissue at the stem apex showing a different structure to that of older tissue at the base (Sanchez et al., 2012). Therefore, when comparing vascular tissue from different genotypes, it is important that the tissue used is all of an equivalent developmental stage. This is particularly difficult when studying the lines used for comparison here, as the *lbd4* mutant shows a significantly earlier transition to flowering than wild type (Fig 4A) while in *wox14* mutants, this transition is delayed (Deveaux et al., 2008; Denis et al., 2017). Therefore, vascular tissue taken from plants of the same age will likely differ in developmental stage as inflorescence tissue will be older in earlier flowering lines and younger in those where flowering is delayed. Here, tissue was collected based on height of the inflorescence instead of the age of plant, ensuring a similar developmental stage across the plants compared in this experiment. However, it is possible that the developmental stage of the *wox14* mutant studied here may not have been equivalent to that of *wox14* mutants studied in the previous literature, highlighting

the need for a more accurate way to ensure equivalence of developmental stage in vascular tissue across different plants.

The *wox14* phenotype reported here conflicts with previous findings in the literature. It also conflicts with other results from this project, for instance, the apparent regulatory role of *WOX14* over *WOX4* which has been shown to act as a positive regulator of cell division (Etchells et al., 2013; Hirakawa et al., 2010). This demonstrates that the current understanding of *WOX14* is still relatively small and suggests that the currently held model of functional redundancy between *WOX4* and *WOX14* may be overly simplistic. Equally, this previously uncharacterised *wox14* phenotype implies that *WOX14* regulation of vascular development may be influenced by certain environmental inputs that in turn affect how the *wox14* phenotype manifests.

Regrowth of plants and repetition of the *wox14* and *wox14 lbd4* analysis carried out here is still required to further investigate this newly reported phenotype. However, phenotypic plasticity within *wox14* mutants may identify *WOX14* as potential mediator between environmental stimuli and specific vascular developmental outputs. Thus, in future, the growth of *wox14* across a range of environmental conditions may provide insight into any interactions that may occur between *WOX14* and environmental cues.

Besides environmental stimuli, a large number of factors could affect the manifestation of the *wox14* phenotype. Hence, deciding the best approach to investigate the questions raised by our *wox14* mutant is difficult. However, further analysis of available Y1H data, using mathematical approaches, could reveal potential candidates influencing *WOX14* that may be promising targets for future research.

4.3 – What is the regulatory role of PXY signalling?

4.3.1 - The feed-forward loop

qRT-PCR results demonstrated that mutants defective in PXY signalling demonstrated significantly lower levels of *TMO6* expression than wild type (Fig 8A). This suggests that the *TMO6* gene is regulated by PXY signalling. This is consistent with previous microarray results which saw a 6 fold increase in *TMO6* expression in a *35S::CLE41* background, where PXY signalling is increased (Etchells et al., personal communication).

TMO6 expression was also reduced in *wox14* mutants compared to wild type (Fig. 6A). *WOX14* has previously been demonstrated to function downstream of PXY signalling (Etchells et al. 2013). Therefore, at the genetic level, these results considered together are consistent with the proposed feed-forward loop, whereby *TMO6* is regulated by PXY signalling via WOX14.

However, the PXY signalling defective backgrounds did not show a significant change in *LBD4* expression compared to wild type (Fig. 8B). This is unexpected as previous research suggests that *LBD4* may be an important PXY signalling output. Previous microarray analysis shows an 11 fold upregulation of *LBD4* in a *35S::CLE41* background (Etchells et al., personal communication). Meanwhile, *lbd4* acts to partially rescue the loss of vascular organisation seen in *IRX3::CLE41* plants, which show a loss of the normal TDIF gradient (Etchells et al., personal communication). Furthermore, the results here confirm that both of *LBD4's* regulatory factors in the loop, WOX14 and TMO6, are influenced by PXY signalling. Therefore, it is surprising that PXY signalling regulation of the other feed-forward loop genes does not translate into PXY signalling regulation of *LBD4*.

In poplar, the *LBD4* ortholog shows expression that is highly specific to secondary vascular tissues (Yordanov et al., 2010). In *Arabidopsis thaliana*, a large majority of the secondary vascular tissues are found in the hypocotyl and are only rarely seen at the base of the inflorescence. Thus, any changes in *LBD4* expression in basal inflorescence tissue may be less acute than in hypocotyl tissue due to lower basal *LBD4* levels. In this experiment, RNA for qRT-PCR analysis was extracted from the base of the inflorescence stem and not the hypocotyl. However, basal inflorescence tissue was also used to study *LBD4* expression in *wox14* mutants, where significant changes in *LBD4* expression were observed. This suggests that *LBD4* levels in basal inflorescence tissue are still sufficient to change significantly in mutant backgrounds compared to wild type. Therefore, although it would be more appropriate in future to analyse *LBD4* expression in both hypocotyl and basal inflorescence tissue, it is unlikely that the maintenance of *LBD4* expression in PXY signalling defective backgrounds can be attributed to low basal *LBD4* levels in basal inflorescence tissue.

LBD4 shows high connectivity within the transcriptional regulatory network (TRN) produced by previous Y1H work (Figure 1). Therefore, *LBD4* could be influenced by a number of factors outside of those in the predicted feed-forward loop. Thus, the qRT-PCR results here could suggest that there is a high level of redundancy between factors regulating *LBD4* expression, acting as a feedback compensatory mechanism. Consequently, removal of PXY signalling may only affect a small proportion of the factors involved in *LBD4* regulation, allowing *LBD4* expression to be maintained in the PXY signalling defective mutants.

4.3.2 - Flowering time

Aside from changes in gene expression, the mutant backgrounds defective in PXY signalling also demonstrated earlier flowering times than wild type controls (Fig. 4B). Earlier flowering time was also seen in *lbd4* knockout mutants as well as *LBD4* misexpression lines (Fig. 4A). This suggests that the early flowering seen in *lbd4* cannot be rescued by ectopic *LBD4* expression, further demonstrating the importance of correct *LBD4* expression in space.

A large amount of research has been carried out investigating the molecular mechanisms controlling flowering time. Many genes are involved, however, at the heart of the process are the *CONSTANS (CO)* and *FLOWERING LOCUS T (FT)* genes which regulate flowering in LD conditions (Kobayashi and Wiegel, 2007). Expression of the *CO* gene is regulated by the circadian clock, showing two strong peaks in expression in LD conditions but only one strong peak during SD conditions (Suárez-López et al., 2001). CO is then thought to regulate flowering time via activation of the *FT* gene (An et al., 2004; Kobayashi et al., 1999).

As day length is perceived in the leaves but not in the apical meristem, translocation of a mobile signal, commonly known as florigen, from the leaves to the apical meristem has long been identified as an important element in flowering time regulation (Wigge et al., 2005; Kobayashi and Weigel, 2007). Activation of FT by CO in the phloem is a key part of this mobile signal (An et al., 2004). The mobile FT protein then acts as a long distance signal, moving through the vascular tissue to induce flowering at shoot apex (Corbesier et al., 2007) Therefore, correct vascular development and the maintenance of a normal signalling pattern within vascular tissue is likely important in flowering time regulation.

The early flowering time of PXY signalling defective mutants suggests that the PXY signalling network may be involved in co-ordinating flowering time signals. This is supported by microarray data where *FT* is upregulated 3.5 fold in a *pxy* mutant compared to wild type levels (Etchells et al., 2012). However, *wox14* mutants, unlike PXY signalling defective mutants, show later flowering times (Deveax et al., 2008; Denis et al., 2017). As *WOX14* is known to function downstream of PXY (Etchells et al., 2013) it is possible that the earlier flowering time seen in *lbd4* might not be attributed to misregulation of PXY signalling outputs. Therefore, the interaction of the PXY signalling network with flowering time regulation is likely to be complex.

Therefore, although the vast majority of research focusing on PXY signalling places it within the context of vascular development it is important to remember that it sits within a complex signalling network. Hence, it is likely to affect other developmental processes, including flowering time and the transition to reproductive growth.

4.4 – Future questions and work

This work has highlighted several important targets for future research of the feed-forward loop. For example, identification of *LBD4*'s redundantly functioning factors, further analysis of the effect ectopic *TMO6* expression as well as reassessment of *WOX14* and its mutant phenotype. In addition to these aims, there are a number of other possible future studies that would provide new insight in the feed-forward loop and its role in vascular development.

4.4.1 – Knocking out the feed-forward loop as a functional unit

Our research suggests that the feed-forward loop may be important in phloem development. However, the work described here has investigated the function and regulation of the feed-forward loop genes individually. Thus, there is still much to be understood about how the loop functions as a unit.

Histological analysis of a triple knockout mutant of all three feed-forward loop genes could give insight into the function of the loop as whole. By removing all three transcription factors, the feed-forward loop can be considered as an entire functional unit rather than just as its component genes. Fortunately, the *wox14 lbd4* and *tmo6-1* mutants generated during this research mean that this mutant can now be easily generated via crossing of these two backgrounds.

4.4.2 – Confirming the interactions between the feed-forward loop components

This research has confirmed genetic interactions between *WOX14*, *TMO6* and *LBD4*, with both *TMO6* and *LBD4* requiring *WOX14* for normal expression, as predicted by Y1H results. Nevertheless, it is still unconfirmed as to whether these genetic interactions are a result of direct TF-promoter interactions. Furthermore, a genetic interaction was also identified between *WOX14* and *WOX4*, with *WOX4* requiring WOX14 for normal expression levels. However, Y1H results did not demonstrate WOX14 binding to the *WOX4* promoter. Therefore, screening for direct physical interactions between these factors and their target promoters *in planta* is required to fully understand the results here.

One way to screen for direct physical TF-promoter interactions *in planta* is chromatin immunoprecipitation (ChIP). ChIP can be used to confirm protein-DNA interactions by cross-linking proteins onto DNA and shearing the DNA into

fragments. Fragments of DNA associated with the protein of interest, in this case the feed-forward loop transcription factors, can then be isolated via tagging the protein with the appropriate antibody. The associated DNA can then be amplified using PCR to confirm if the TF is associated with a promoter of interest (Kuo and Allis, 1999).

4.4.3 Does the feed-forward loop facilitate cross-talk with other signalling pathways?

In the feed-forward loop, both *TMO6* and *WOX14* are influenced by PXY signalling. However, the influence of other signalling pathways on the feed-forward loop has not been addressed. Multiple signalling pathways may influence this loop but auxin signalling is a good candidate for preliminary investigations into signalling crosstalk.

Auxin is already well known as an important developmental hormone and *mp* mutants, lacking the key auxin response factor MP, show defects in vascular tissue formation (Hardtke and Berleth, 1998). *TMO6* shows expression dependent on MP (Schlereth et al., 2010), providing one avenue by which auxin may influence the feed-forward loop. Besides *TMO6*, *WOX4,* which regulates both *TMO6* and *LBD4* expression, also shows auxin responsiveness (Suer et al., 2011). Meanwhile, *wox14* knockout mutants have been reported to show inhibited lateral root formation (Devaux et al., 2008), a developmental processes in which auxin's role is well characterised (Lavenus et al., 2013). Inhibited lateral root formation is also seen in *pxy* mutants (Cho et al. 2013), suggesting an overlap of the developmental outputs regulated by PXY and auxin signalling pathways. Therefore, there is evidence suggesting that auxin may influence the feed-forward loop via multiple mechanisms.

The influence of auxin on the feed-forward loop genes could be investigated using qRT-PCR techniques similar to those used here. Tissue could be treated with exogenous auxin application before RNA extraction and subsequent qRT-PCR analysis of gene expression. Comparison of the levels of expression of the feed-forward loop genes across treated and untreated tissue will identify any effect that auxin may have on the transcription of the feed-forward loop components.

4.5 Conclusions

Using a range of approaches we have been able to enhance current understanding of this feed-forward loop in vascular development. Components of this loop, namely *TMO6* and *WOX14,* are dependent on PXY signalling. However, this does not directly translate into dependence of *LBD4* on PXY signalling, suggesting that there are other factors outside of the loop influencing its expression. Meanwhile, WOX14 promotes the transcription of *TMO6, LBD4* and *WOX4.*

Both *WOX14* and *TMO6* demonstrate roles in vascular cell proliferation while *LBD4* is important in specifying the phloem-procambium boundary. Thus, the feed-forward loop may act to fine-tune a range of developmental processes, enabling highly organised vascular structures to develop correctly.

Bibliography

An, H., Roussot, C., Suárez-López, P., Corbesier, L., Vincent, C., Piñeiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., Coupland, G. (2004) CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. *Development* **131**, 3615–3626.

Cho, H., Ryu, H., Rho, S., Hill, K., Smith, S., Audenaert, D., Park, J., Han, S., Beeckman, T., Bennett, M.J., Hwang, D., De Smet, I., Hwang, I. (2014) A secreted peptide acts on BIN2-mediated phosphorylation of ARFs to potentiate auxin response during lateral root development. *Nat Cell Biol* **16**, 66–76.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., Coupland, G. (2007) FT Protein Movement Contributes to Long-Distance Signaling in Floral Induction of Arabidopsis. *Science* **316**, 1030–1033.

Denis, E., Kbiri, N., Mary, V., Claisse, G., Conde e Silva, N., Kreis, M., Deveaux, Y. (2017) WOX14 promotes bioactive gibberellin synthesis and vascular cell differentiation in Arabidopsis. *Plant J* **90**, 560–572.

Deveaux, Y., Toffano-Nioche, C., Claisse, G., Thareau, V., Morin, H., Laufs, P., Moreau, H., Kreis, M., Lecharny, A. (2008) Genes of the most conserved WOX clade in plants affect root and flower development in Arabidopsis. *BMC Evolutionary Biology* **8**, 291.

Esau, K. (1977) Anatomy of Seed Plants. 2nd ed. New York: John Wiley & Sons.

Etchells, J.P., Mishra, L.S., Kumar, M., Campbell, L., Turner, S.R. (2015) Wood Formation in Trees Is Increased by Manipulating PXY-Regulated Cell Division. *Current Biology* **25**, 1050–1055.

Etchells, J.P., Provost, C.M., Mishra, L., Turner, S.R. (2013) WOX4 and WOX14 act downstream of the PXY receptor kinase to regulate plant vascular proliferation independently of any role in vascular organisation. *Development* **140**, 2224–2234.

Etchells, J.P., Provost, C.M., Turner, S.R. (2012) Plant Vascular Cell Division Is Maintained by an Interaction between PXY and Ethylene Signalling. *PLOS Genetics* **8**, e1002997.

Etchells, J.P., Turner, S.R. (2010a) 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., Turner, S.R. (2010b) Orientation of vascular cell divisions in Arabidopsis. *Plant Signal Behav* **5**, 730–732.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., Kammen, D.M. (2006). Ethanol can contribute to energy and environmental goals. *Science* **311**, 506–508.

Fisher, K., Turner, S. (2007). PXY, a Receptor-like Kinase Essential for Maintaining Polarity during Plant Vascular-Tissue Development. *Current Biology* **17**, 1061–1066.

Gardiner, J., Sherr, I., Scarpella, E. (2010) Expression of DOF genes identifies early stages of vascular development in Arabidopsis leaves. *Int. J. Dev. Biol.* **54**, 1389–1396.

Gaudinier, A., Zhang, L., Reece-Hoyes, J.S., Taylor-Teeples, M., Pu, L., Liu, Z., Breton, G., Pruneda-Paz, J.L., Kim, D., Kay, S.A., Walhout, A.J.M., Ware, D., Brady, S.M. (2011) Enhanced Y1H assays for Arabidopsis. *Nat Meth* **8**, 1053–1055.

Hardtke, C.S., Berleth, T. 1998. The Arabidopsis gene MONOPTEROS encodes a transcription factor mediating embryo axis formation and vascular development. *EMBO J.* **17**, 1405–1411.

Hirakawa, Y., Kondo, Y., Fukuda, H. (2010) TDIF Peptide Signaling Regulates Vascular Stem Cell Proliferation via the WOX4 Homeobox Gene in Arabidopsis. *Plant Cell* **22**, 2618–2629.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., Sawa, S., Ohashi-Ito, K., Matsubayashi, Y., Fukuda, H. (2008) Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *PNAS* **105**, 15208–15213.

Hongo, S., Sato, K., Yokoyama, R., Nishitani, K. (2012). Demethylesterification of the Primary Wall by PECTIN METHYLESTERASE35 Provides Mechanical Support to the Arabidopsis Stem. *The Plant Cell Online* **24**, 2624–2634.

Husbands, A., Bell, E.M., Shuai, B., Smith, H.M.S., Springer, P.S. (2007) LATERAL ORGAN BOUNDARIES defines a new family of DNA-binding transcription factors and can interact with specific bHLH proteins. *Nucl. Acids Res*. **35**, 6663–6671.

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N., Fukuda, H. (2006) Dodeca-CLE Peptides as Suppressors of Plant Stem Cell Differentiation. *Science* **313**, 842–845.

Kendall, S.L., Holmes, H., White, C.A., Clarke, S.M., Berry, P.M. (2017). Quantifying lodging-induced yield losses in oilseed rape. *Field Crops Research* **211**, 106–113.

Kobayashi, Y., Weigel, D. (2007) Move on up, it's time for change—mobile signals controlling photoperiod-dependent flowering. *Genes Dev*. **21**, 2371–2384.

Kondo, Y., Ito, T., Nakagami, H., Hirakawa, Y., Saito, M., Tamaki, T., Shirasu, K., Fukuda, H. (2014) Plant GSK3 proteins regulate xylem cell differentiation downstream of TDIF–TDR signalling. *Nature Communications* **5**,

Kong, E., Liu, D., Guo, X., Yang, W., Sun, J., Li, X., Zhan, K., Cui, D., Lin, J., Zhang, A. (2013) Anatomical and chemical characteristics associated with lodging resistance in wheat. *The Crop Journal* **1**, 43–49.

Kuo, M.-H., Allis, C.D. (1999) In Vivo Cross-Linking and Immunoprecipitation for Studying Dynamic Protein:DNA Associations in a Chromatin Environment*. Methods* **19**, 425–433.

Lavenus, J., Goh, T., Roberts, I., Guyomarc'h, S., Lucas, M., De Smet, I., Fukaki, H., Beeckman, T., Bennett, M., Laplaze, L. (2013) Lateral root development in Arabidopsis: fifty shades of auxin. *Trends in Plant Science* **18**, 450–458.

Le Hir, R., Bellini, C. (2013) The Plant-Specific Dof Transcription Factors Family: New Players Involved in Vascular System Development and Functioning in Arabidopsis. *Front. Plant Sci.* **4**.

Mangan, S., Alon, U. (2003) Structure and function of the feed-forward loop network motif. *PNAS* **100**, 11980–11985.

Miyashima, S., Sebastian, J., Lee, J.-Y., Helariutta, Y. (2013) Stem cell function during plant vascular development. *The EMBO Journal* **32**, 178–193.

Plomion, C., Leprovost, G., Stokes, A. (2001) Wood Formation in Trees. *Plant Physiol.* **127**, 1513–1523.

Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* **339**, 62–66.

Reece-Hoyes, J.S., Marian Walhout, A.J. (2012) Yeast one-hybrid assays: A historical and technical perspective. Methods, *Protein-Protein Interactions* **57**, 441–447.

Rohland, N., Reich, D. (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. *Genome Res*. **22**, 939–946.

Sanchez, P., Nehlin, L., Greb, T. (2012) From thin to thick: major transitions during stem development. *Trends in Plant Science* **17**, 113–121.

Schlereth, A., Möller, B., Liu, W., Kientz, M., Flipse, J., Rademacher, E.H., Schmid, M., Jürgens, G., Weijers, D. (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature* **464**, 913–916.

Suárez-López, P., Wheatley, K., Robson, F., Onouchi, H., Valverde, F., Coupland, G. (2001) CONSTANS mediates between the circadian clock and the control of flowering in Arabidopsis. *Nature* **410**, 1116–1120.

Suer, S., Agusti, J., Sanchez, P., Schwarz, M., Greb, T. (2011) WOX4 Imparts Auxin Responsiveness to Cambium Cells in Arabidopsis. *Plant Cell* **23**, 3247–3259.

Turner, S.R., Somerville, C.R. (1997) Collapsed xylem phenotype of Arabidopsis identifies mutants deficient in cellulose deposition in the secondary cell wall. *The Plant Cell Online* **9**, 689–701.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., Weigel, D. (2005) Integration of Spatial and Temporal Information During Floral Induction in Arabidopsis. *Science* **309**, 1056–1059.

Yordanov, Y.S., Regan, S., Busov, V. (2010) Members of the LATERAL ORGAN BOUNDARIES DOMAIN Transcription Factor Family Are Involved in the Regulation of Secondary Growth in Populus. *Plant Cell* **22**, 3662–3677.

Zhang, H., Lin, X., Han, Z., Qu, L.-J., Chai, J. (2016) Crystal structure of PXY-TDIF complex reveals a conserved recognition mechanism among CLE peptide-receptor pairs. *Cell Res* **26**, 543–555.