From solving climate change to building space rockets, ERECTA - a multifaceted Receptor-Like Kinase.

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From solving climate change to building space rockets, ERECTA - a multifaceted Receptor-Like Kinase.

Identification and characterisation of ERECTA ligands, interacting proteins and transcription targets which regulate secondary growth.

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Abstract:

Wood and cork respectively originate from cell proliferation of the vascular cambium and the cork cambium. Both cambia consist of a single, bifacial, continuous ring of stem cells and share overlapping regulatory pathways. In Arabidopsis, the ERECTA (ER) receptor-like kinase is known to regulate several developmental processes including meristematic maintenance. Recent studies reported that mutation in the ER gene enhanced the vascular defects observed in the PHLOEM INTERCALADED WITH XYLEM (PXY) – also known as TDR – loss of function mutant. As PXY/TDR partially controls vascular cambium development, these results suggest that ER also regulates the activity of the vascular cambium. This raises the following questions: is ER also involved in cork cambium regulation? And what are the molecular mechanisms underpinning the ER-dependent cambium regulation? Using phylogenetic analyses and morphogenetic studies, we identified several EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) peptides as potential ER ligands which may redundantly regulate cambium activity. Interestingly, we also found that EPFL4-6, initially thought to be main cambium regulators, did not appear with the evolution of vascular cambium. Furthermore, loss-of-function experiments suggest that the ER family (ERf) receptors not only genetically interact with the PXY family (PXf) receptors to regulate vascular cambium activity but also to control the initiation and maintenance of the cork cambium. These findings indicated that the ERf-PXf signalling network ubiquitously regulates the initiation and maintenance of stem cells in post-embryonic tissues. Additionally, expression studies showed that ERf and PXf receptors had overlapping expression patterns in both the vascular and cork cambium as well as their neighbouring tissues. Interestingly, these results suggest that ERf and PXf could physically interact. For this reason, we prepared transgenic lines and genetic constructs for future protein-protein interaction assays. Finally, we found that auxin signalling played a key role downstream of the ERf-PXf network to control stem cell activity post-embryonically.

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List of abbreviations:

APAV	Adjusted Pairwise Attraction Values
CLANS	CLuster ANalysis of Sequences
CLE	CLAVATA3/ESR-RELATED
Co-IP	Co-Immunoprecipitation
EPF/EPFL	EPIDERMAL PATTERNING FACTOR (EPF)/ EPF-LIKE (EPFL)
ER	ERECTA
FRET	Förster Resonance Energy Transfer
GA	Gibberellic acid
HMM	Hidden Markov Model
MSA	Multiple Sequence Alignment
NAA	1-Naphthaleneacetic acid
ORF	Open Reading Frame
PAV	Pairwise Attraction Values
PSKR	Phytosulfokine Receptor
PSYR1	Tyrosine-sulfated glycopeptide receptor 1
PXY	PHLOEM INTERACALATED WITH XYLEM
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
SGS3	SUPPRESSOR OF GENE *SILENCING* 3
TDIF	TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR
WT	Wild Type

GENERAL INTRODUCTION:
Harder, Taller, Faster, Stronger - Secondary growth, an evolutionary strategy of land plants

Why is secondary growth a selective advantage for land plants?

With the colonisation of land, plants started to compete for light. As a result, growing taller and faster than their neighbours to reach the canopy became a key selective advantage for plants. However, to support the weight of their aerial organs, mechanical support became necessary and occurred through the radial growth of the plant's primary axes and the production of cells with thick walls (Fischer *et al.*, 2019). This evolutionary innovation called secondary growth evolved as early as 390 mya (mid-Devonian) with the apparition of seed plants, including gymnosperms and eudicots (Spicer and Groover, 2010). However, secondary growth may have originated even earlier (between 450-390 mya) as extinct lineages of ferns and lycophytes displayed features associated with the secondary growth of modern seed plants (Spicer and Groover, 2010; Gerrienne *et al.*, 2011; Hoffman and Tomescu, 2013; Strullu-Derrien *et al.*, 2014). This successful strategy was relatively well conserved through time and only few losses of secondary growth have been documented in land plants (Povilus *et al.*, 2020). Loss of secondary growth in lineages like monocots will be discussed further in Chapter I. From now on, we will refer to seed plants which undergo secondary growth as "plants".

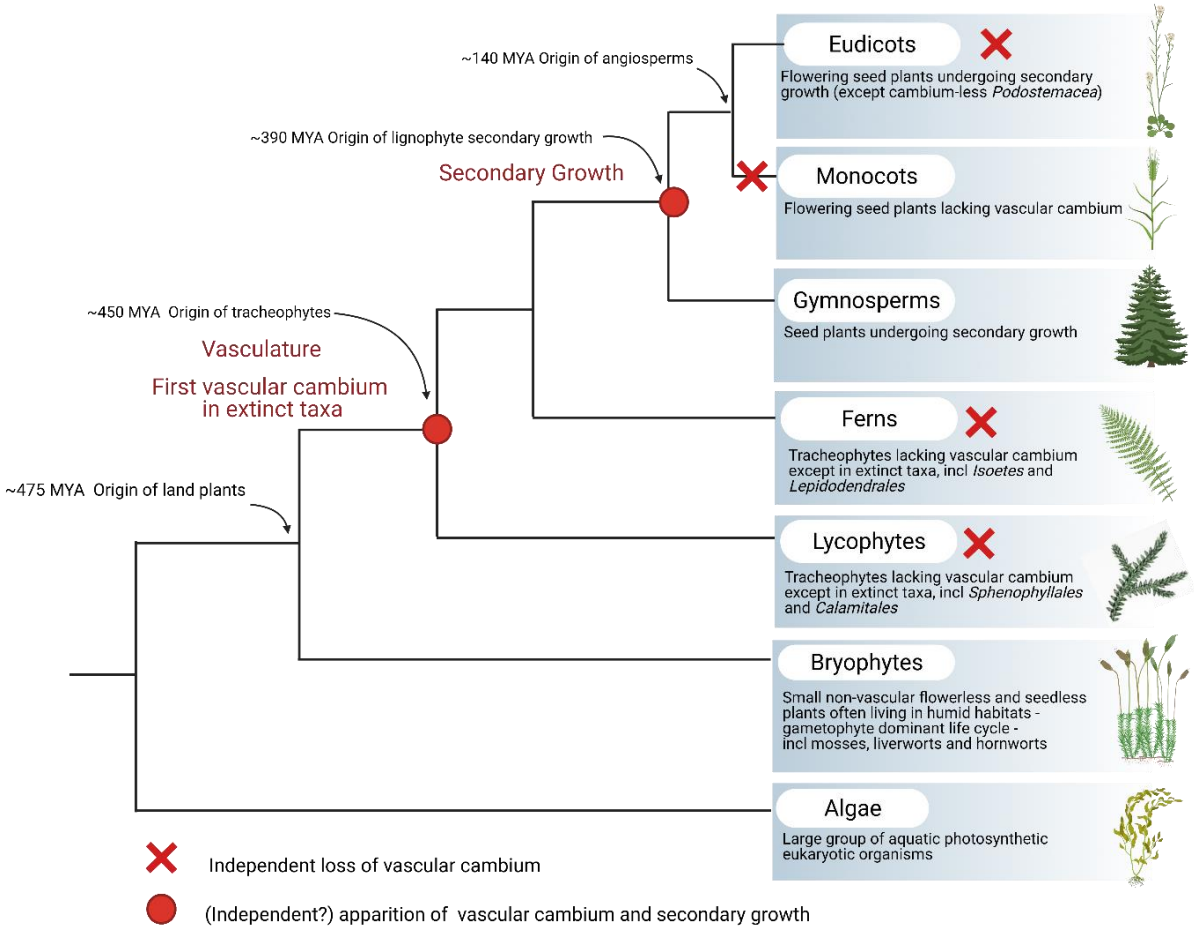


Fig.1. Phylogenetic relationships of major clades in the plant realm with notes on their secondary growth and vascular cambium. MYA, million years ago.

How does secondary growth occur?

After their embryo is formed, plants grow by synchronising their elongation (primary growth) and widening (secondary growth) via series of cell division and cell differentiation (Spicer and Groover, 2010) (Fig.2). While the stem cell niches responsible for the plant elongation - the shoot apical meristem (SAM) and the root apical meristem (RAM) - are formed at the embryonic stage after a few cell divisions, those responsible for the widening of the plant - the lateral meristems - are formed post-embryonically (Nieminen *et al.*, 2015). As a result, these two types of cell niches seem to be initiated and maintained by separate signalling pathways. Until now, plant developmental research focused on understanding the functioning of the stem cell niches responsible for the elongation of the plant. In comparison, the signalling pathways regulating stem cell niches responsible for the widening of the plant remain largely unknown. For this reason, this study bridged this gap in the literature by investigating the regulatory networks modulating stem cell niches responsible for the widening of the plant.

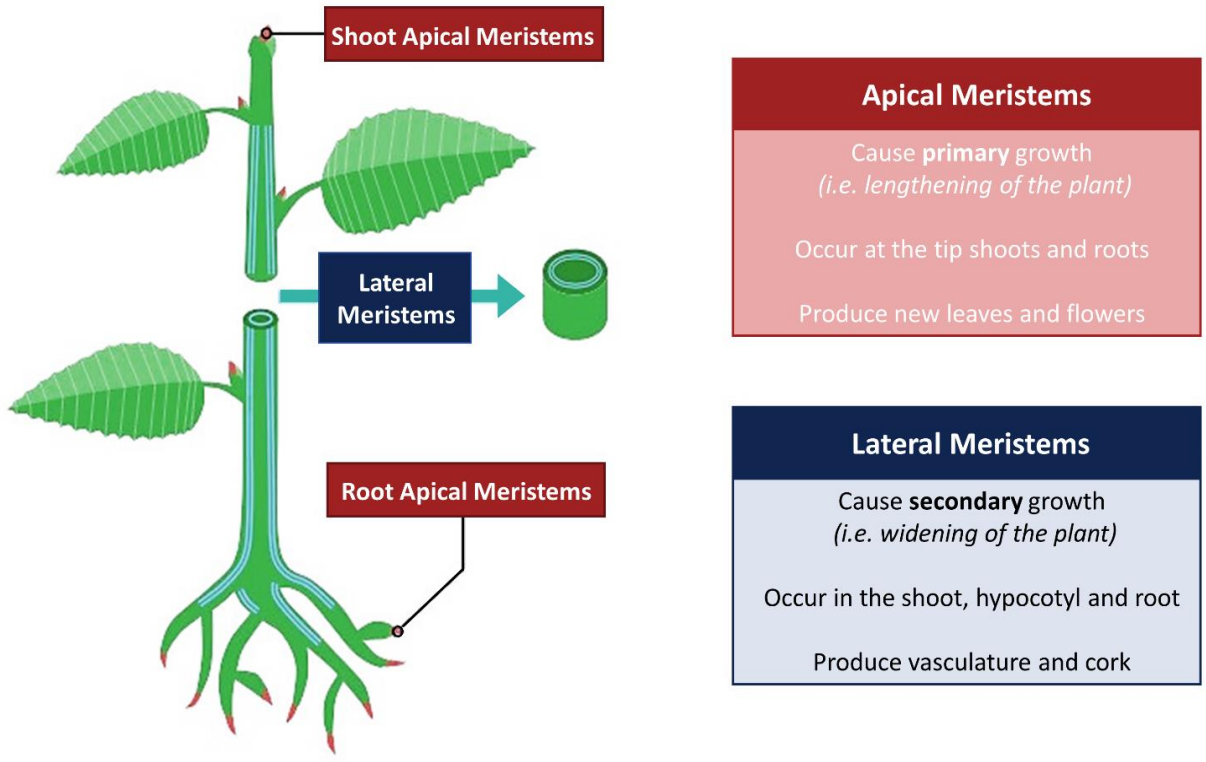


Fig.2. Lateral meristems, responsible for the widening of the plant, adopt a cylindrical shape along the stem, hypocotyl and root of the plant (cyan). Apical meristems are responsible for the plant elongation and can be found at the extremities of the shoot and the root of the plant (red). [adapted from website 1]

In plant biology, stem cell niches are called meristems and the ones involved in widening are known as lateral meristems because they regulate lateral growth. In plants undergoing extensive lateral growth, there exist two lateral meristems. Each lateral meristem forms a cylinder of stem cells along the apical-basal axis of the plant. As a result, these cylinders of stem cells can be found along organs like the stem, the hypocotyl and the roots (depicted in cyan in Fig.2).

Both lateral meristems function in a very similar manner to regulate lateral growth. The stem cells in the cylinder divide periclinally - parallel to the axis of the organ – on both sides of the cylinder. As stem cells divide, they push the newly formed cells toward the periphery and towards the centre of the organ where they differentiate into specialised tissues.

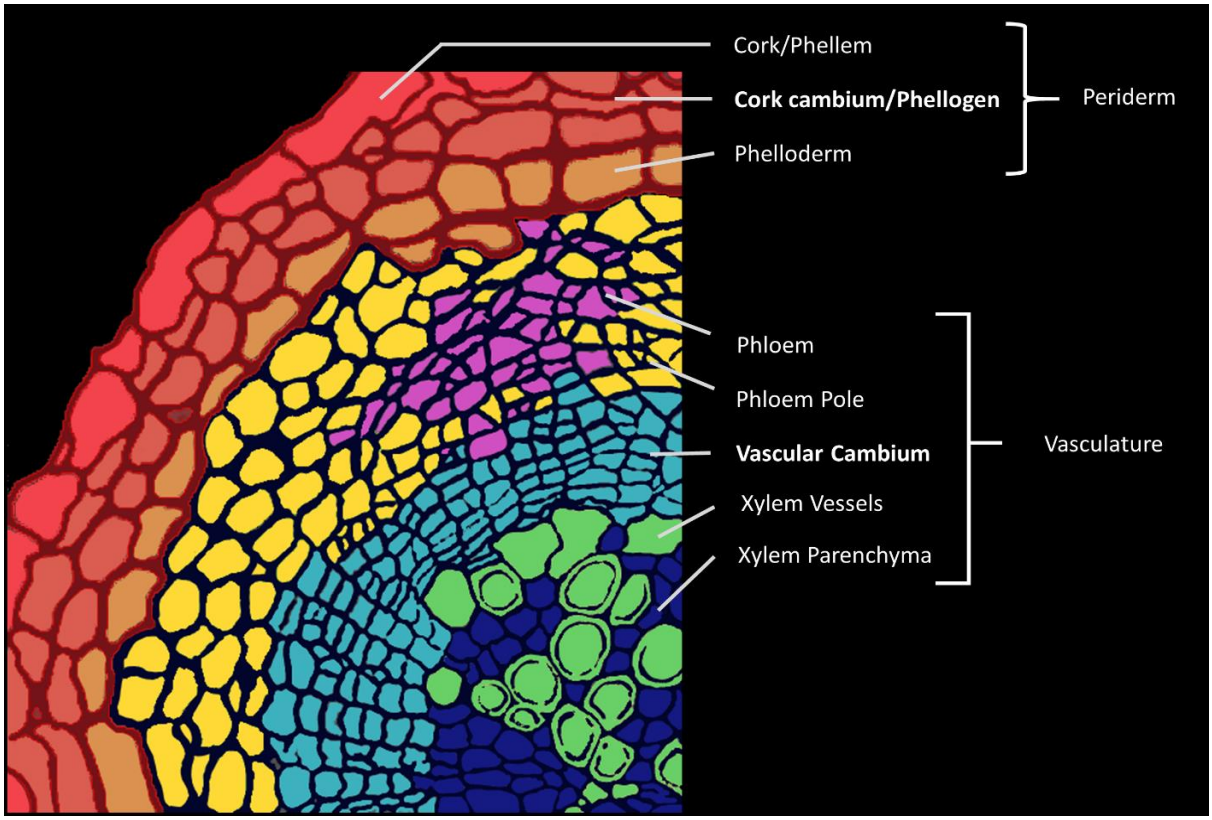


Fig.3. Transverse section of a 12-day-old Arabidopsis thaliana's hypocotyl where each tissue type is colour coded. The tissues produced by the vascular cambium are the phloem vessels on the outside and xylem vessels on the inside of the cambium while the tissues produced by the cork cambium are phellem on the outside and phelloderm on the inside.

The inner lateral meristem is called the vascular cambium. It generates xylem tissue on its inside and phloem on its outside, each of these tissues being crucial for the plant functioning (Fig.3). Xylem vessels transport water and minerals from the soil towards different plant organs and bring mechanical support to the plant through their lignified cell walls. On the other hand, phloem vessels transport photosynthetic carbohydrates and amino acids from photosynthetic cells towards other plant organs (Esau, 1977; Larson, 1994; Elo *et al.*, 2009). The specificity of this lateral meristem is that it is responsible for most of the lateral growth of the plant (Spicer and Groover, 2010).

The second and outer lateral meristem is called cork cambium (or phellogen) and generates cork (also known as phellem) on its outside and phelloderm on its inside (Fig.3). Together, the cork cambium and its derived secondary tissue are referred as periderm (Pereira, 2011). As the plant undergoes secondary growth, the periderm replaces the first protective layer called epidermis, as it is a more developmentally plastic and can adapt to the radial expansion of plant organs (Campilho, Nieminen and Ragni, 2020). Although the phelloderm does not have any known function, its formation increases the girth of plants primary axes and strengthens the mechanical strength of the plant. The other

secondary tissue produced by the cork cambium is the cork which protects the plant from environmental threats like mechanical deterioration, thermal shocks, water and pathogens (Campilho, Nieminen and Ragni, 2020).

Why should we study secondary growth and the mechanisms regulating the vascular and cork cambium?

In past decades, researchers have extensively studied stem cells niches specified at the embryonic stage and maintained post-embryonically, known as the shoot and root apical meristems (Steeves, 2006; Motte, Vanneste and Beeckman, 2019; Uchida and Torii, 2019). However, the vascular and cork cambium are unique stem cell populations which have the specificity of being initiated post-embryonically (Wunderling *et al.*, 2018; Smetana *et al.*, 2019). Discovering the regulatory mechanisms underpinning these post-embryonically induced stem cell populations is of high academic and agricultural importance (Fig.4). Studying these stem cells will deepen our understanding of key biological processes - such as naturally occurring cell de-differentiation and maintenance of a pluripotent state - and will allow us to control an important trait for agriculture, plant growth.

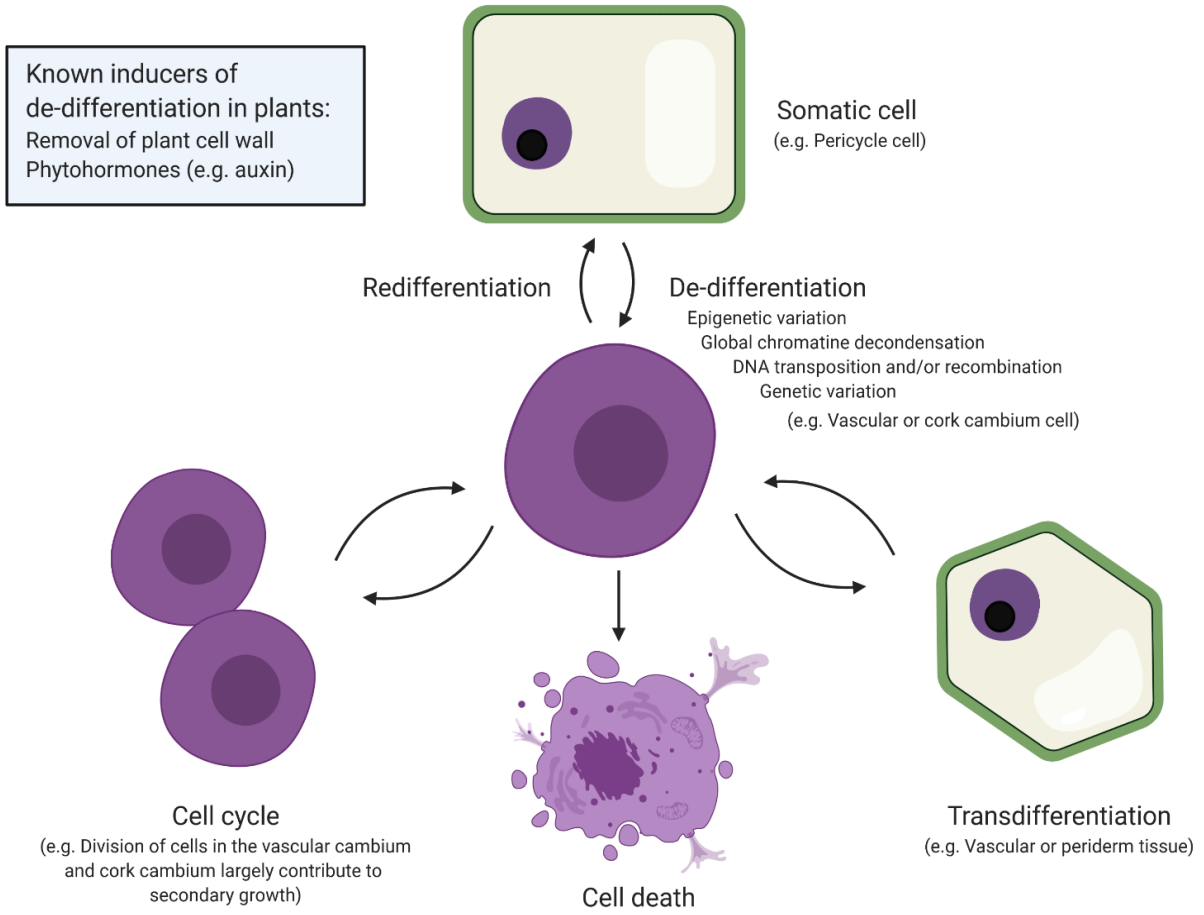


Fig.4. Cellular de-differentiation in plants. Differentiated cells, like pericycle cells, do not lose their developmental capacities and they can be induced to undertake a dedifferentiated stem-cell like state, similar to cells of the vascular cambium or cork cambium. Stem cell-like states have also been induced experimentally in plants using cell wall-degrading enzymes or/and phytohormones. The acquisition of this stem-cell like state is often accompanied by global chromatin decondensation, activation of transposable elements and DNA recombination. In the experimentally induced stem cell-like state, these events often lead to hazardous genetic variation and consequently to the formation of mutated cells or cell death (Grafi, 2009). However, in naturally occurring dedifferentiation this genetic variation is highly regulated and causes de-differentiated cells to acquire various fates such as re-differentiation, re-entry into the cell cycle or differentiation into a cell type different to the original (i.e. transdifferentiation of cambial cell into phloem, xylem, phelloderm or phellem). In the future, similar cell fates could be acquired experimentally when the appropriate stimuli regulating the vascular and cork cambium will be understood in more depth (figure adapted from Grafi, 2009).

Additionally, the secondary tissues produced by the vascular and cork cambium are highly valuable natural resources for industrial and ecological purposes. As cork produces high levels of suberin, this tissue exhibits unique and valuable properties: low density, impermeability, thermo-tolerance, biological and mechanical inertness (Leite and Pereira, 2017). As a result, cork has been used for a wide range of purposes from wine stopper production to insulating NASA rocket boosters (www.nasa.gov, 2018). Due to its defensive role, the periderm contains high value metabolites like alkanoids, terpenes and phenolics which could also be exploited in the future (Carranza *et al.*, 2015; Ferreira, Quilhó and Pereira, 2017).

Produced in large amount by the vascular cambium, the xylem tissue (wood) represents the principal form of terrestrial biomass through the production of lignin and cellulose in its cell walls (Stephenson *et al.*, 2014). Due to its mechanical strength and its inflammable properties, xylem tissue has been used for diverse applications including paper, fuel and construction materials (Somerville, 2006; Raunikar *et al.*, 2010). More importantly, xylem is an extremely important carbon sink which stabilised atmospheric CO2 levels for millennia (Stephenson *et al.*, 2014). Because of anthropogenic increase in CO2 levels, generating plants with enhanced secondary growth would not only protect them from extreme weather conditions to come (Etchells *et al.*, 2015) (e.g. plants would be stronger and more thermo-tolerant with increased xylem and cork respectively) but could also be crucial to re-establishing the atmospheric equilibrium of our planet.

Which organism and which tissue to use when studying secondary growth?

Most seed plants undergo secondary growth, even the herbaceous *Arabidopsis thaliana* forms a vascular and cork cambium at some point in its ontogeny (Donoghue, 2005; Feild and Arens, 2005; Rowe and Speck, 2005; Rothwell *et al.*, 2008). In *Arabidopsis*, secondary growth occurs in the stems, the hypocotyl and the roots (Ragni and Hardtke, 2014). However, the organisation and identity of its tissues greatly differs depending on the organ. The stem of *Arabidopsis* forms scattered bundles of vascular cambium (also known as procambium) and undergoes limited radial expansion (Eames and MacDaniels, 1947). Subsequently, Arabidopsis stem does not need a developmentally plastic protective layer and periderm does not grow. On the other hand, the root and the hypocotyl of Arabidopsis undergo more extensive secondary growth. The root and the hypocotyl form continuous rings of vascular cambium and cork cambium and have similar morphologies to trees (Fig.3).

However, as *Arabidopsis* root elongates while undergoing secondary growth, it displays an apical-basal developmental gradient of radial growth - with the largest part at the top, near the hypocotyl. By contrast, the hypocotyl stops elongating a few days after germination and its radial expansion is synchronised all along the hypocotyl (Ragni *et al.*, 2011). Additionally, key signalling components of secondary growth found in *Populus* trees are conserved in *Arabidopsis* (Zhong and Ye, 2001; Kubo *et al.*, 2005; Du *et al.*, 2011; Robischon *et al.*, 2011; Roberts, 2012) while its hypocotyl and roots undergo secondary growth in two phases, which is reminiscent of tree growth habits (Chaffey *et al.*, 2002; Nieminen, Kauppinen and Helariutta, 2004). At first, all secondary tissues derived from the vascular cambium grow proportionally while in the second phase, xylem fibres differentiate, and more xylem is produced than phloem. Altogether, the hypocotyl and the oldest part of the roots of *Arabidopsis* represent suitable model organs to investigate the basic regulations of secondary growth.

In this study, both hypocotyls and roots were used for morphogenetic and expression analyses in accordance with the already-developed protocols (de Reuille and Ragni, 2017; Wang *et al.*, 2019) and the available equipment (e.g. microtome or vibratome) in Etchells' and Ragni's laboratories.

Where do we start? Why should we study both the vascular cambium and the cork cambium?

Despite forming different tissues, the lateral meristems display similar structures and functions. Both lateral meristems regulate plant lateral growth, have a cylindrical structure and are formed post - embryonically. Subsequently, part of this study investigated whether the molecular networks initiating and maintaining stem cell activity is shared between the two lateral meristems.

What type of signalling network did this study focus on?

Since plant cells are unable to migrate, the initiation and elaboration of organs occurs via coordinated cell division and cell differentiation events (Anastasiou and Lenhard, 2007; Horiguchi and Tsukaya, 2011). In tissues organised in concentric rings - like in the vasculature and the periderm – the expansion of inner tissues is synchronised with the growth of outer layers to maintain organ integrity (Wang *et al.*, 2019). As a result, plants have evolved complex cell-cell communication systems within and between layers to coordinate the formation and expansion of specialised tissues.

Ligand-receptor-based signalling is one of the most important forms of intercellular communication in plants. Plants have evolved a unique set of membrane receptors that can sense diverse ligands to trigger signalling cascades controlling various developmental and physiological processes (Liu *et al.*, 2017). This transduction mechanism is coined "non-cell autonomous" (requiring the presence of other cells to function) as it requires surrounding cells to secrete the cognate ligands of RLKs (Hirakawa *et al.*, 2008; Rychel, Peterson and Torii, 2010). This elegant ligand-receptor-based signalling may have been selected throughout evolution to minimise the energy cost of the cell by producing fewer large protein receptors and diversifying their small cognate ligands. However, the processes they regulate occur in close spatiotemporal proximity and require tight control to prevent spill over of activation from one process to another (Abrash, Davies and Bergmann, 2011). As a result, these signalling cascades use ligand-receptor and receptor-coreceptor modules where components can be changed slightly depending on the physiological process to regulate. One of the receptor families involved in this ligand-receptor-based signalling is the LRR-RLK family.

Plants have evolved an expanded family of transmembrane receptors called leucine-rich repeat receptor-like kinases (LRR-RLKs) which controls key developmental and immune responses by forming complex intercellular signalling networks (Dievart and Clark, 2004; Burkart and Stahl, 2017). With about 225 members in *Arabidopsis thaliana*, these receptors not only synchronise the growth of different cell-layers within organs but they also coordinate the physiological responses of the plant according to its environment (Sun *et al.*, 2017). Due to their sessile nature, plants must show a high degree of developmental plasticity to coordinate their growth, their immune state and their reproductive stages with the environment. LRR-RLK signalling was one of the systems selected throughout evolution to simultaneously synchronise processes within the plant and responses of the plant to its environment by perceiving and processing both external and internal signals through the binding of ligands (Shiu and Bleecker, 2001).

In terms of protein structure, most LRR-RLKs possess a distinctive extracellular domain, a single membrane spanning region and a cytoplasmic serine/threonine kinase catalytic domain (Fig.6). The extracellular domain (ECD) of each LRR-RLK receptor is characterised by a unique leucine-rich repeats (LRR) domain, consisting of tandem repeats with conserved leucine amino acids. These LRR domains are found in a wide variety of eukaryotic proteins and are often implicated in protein-protein interactions (Smakowska-Luzan *et al.*, 2018).

The mechanism for LRR-RLK signalling involves three main steps (Fig.6). First, the extracellular domain of the RLK binds to the ligands secreted by neighbouring cells and tissues. Upon binding of the ligand, the receptor undergoes some conformational changes, triggering changes in the activity of the kinase domain. By modifying the activity of the kinase domain, downstream phosphorylation cascades are activated, leading to up or down regulation of transcription factors as well as a change in gene expression (Hirakawa *et al.*, 2008; Lehti-Shiu *et al.*, 2009; Liu *et al.*, 2017).

Most of the biological information processed by LRR-RLKs is encoded in the structure of their ligands as well as in the rate, the duration and the source of ligand production. The source of ligand production encompasses at least two positional cues: the proximity of the source directly influences the strength of the signal and the orientation of the source relative to the target cells provides a directionality to this signal (e.g. orientation of division plane in the vascular and cork cambium) (Abrash, Davies and Bergmann, 2011).

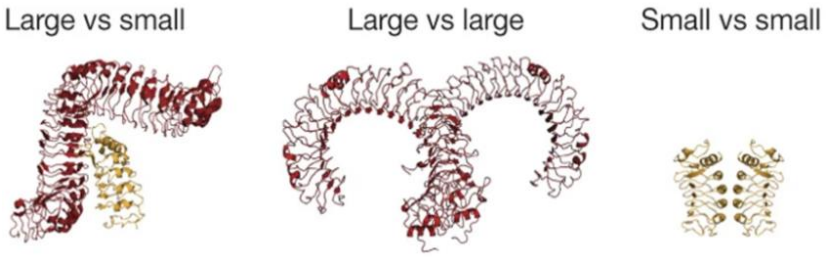


Fig.5. Structural representations of different LRR-RLK extracellular domains interacting (Smakowska-Luzan et al., 2018). The extracellular domain is considered large (depicted in red) when it has more than 12 Leucine-rich repeats (LRRs) and it is considered small when it has less than 12 LRRs.

Due to the pleiotropic nature of LRR-RLKs, additional regulation often takes place to prevent any spill-over from the activation of one process to another. Upon binding of the ligand, the extracellular

domain of the activated receptor can bind to a co-receptor LRR-RLK, leading to the formation of a dynamic receptor signalling complex (Fig.5) (Burkart and Stahl, 2017). The resulting physical interaction between LRR-RLKs tightly modulates the activity of the downstream signalling pathways which, in turn, controls the physiological responses of the plant (Fig.6). Typically the physical binding and coupling of LRR-RLKs enhances the overall stability of the signalling network and ensures the appropriate response modulation by feedback mechanisms (Smakowska-Luzan *et al.*, 2018).

What are the similarities and differences between the key genetic pathways of the SAM, the RAM and the lateral meristems?

The SAM, the RAM and the lateral meristems are characterised by their partially conserved regulatory circuits. In all four meristems, we find similar molecular players: a WUSCHEL-RELATED HOMEOBOX (WOX) transcription factor (TF) regulating stem cell fate and a peptide-receptor system modulating the activity of this TF via a feedback loop (Fig.6) (Greb and Lohmann, 2016).

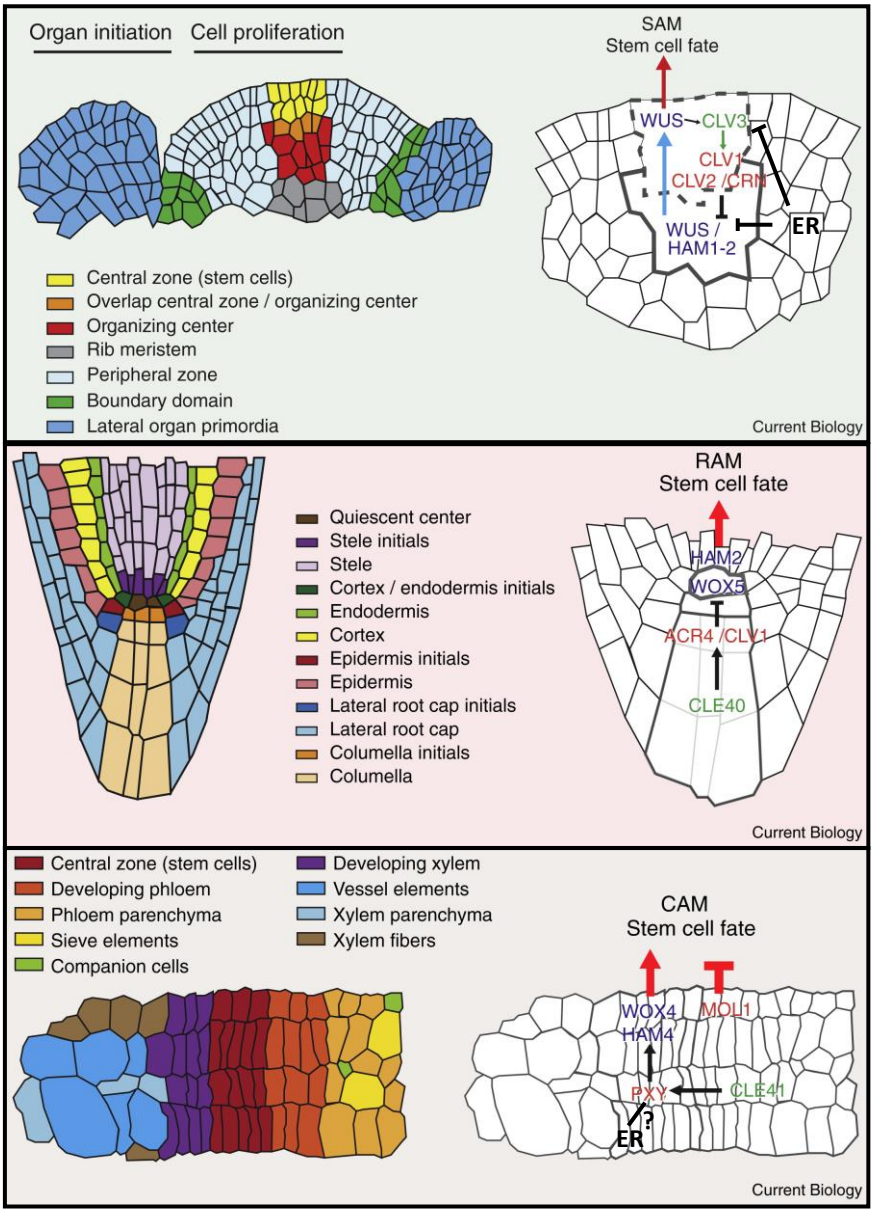


Fig.6. Signalling pathways in the shoot apical meristem (SAM), the root apical meristem (RAM) and the cambium (CAM). On the left, schematic representations of longitudinal sections through the SAM and RAM and a transversal section through the CAM highlight the different cell types found in each meristem. A central regulatory loop regulating each meristem is shown on the right. Arrows indicate a promotion of gene expression, protein activity or stem cell fate. Blunted arrows indicate a repression of gene expression or stem cell fate. Question marks indicate that the nature (positive or negative) of the interaction remains to be determined. Figure modified from (Greb and Lohmann, 2016).

Each meristem expresses a WOX TF which is necessary and sufficient to specify stem cell identity (Mayer *et al.*, 1998; Schuster *et al.*, 2014). The SAM and the RAM respectively express WUSCHEL (WUS) and WOX5 in the inductive niche of their stem cells – called the organising centre (OC) for the SAM and the quiescent centre (QC) for the RAM (Sarkar *et al.*, 2007; Schuster *et al.*, 2014) (Fig.6). The transcription factors then move from these inductive niches to the adjacent stem cells to instruct their fate (Yadav *et al.*, 2011; Daum *et al.*, 2014). Although the inductive niche of cambial stem cells is currently unknown, the expression of WOX4 is defined very locally in the vascular and cork cambium and promotes stem cell identity in these tissues (Fig.6)(Hirakawa, Kondo and Fukuda, 2010; Etchells *et al.*, 2013). However, it is currently unclear whether intercellular movement, as seen in WUS and WOX5 signalling, is required for the action of WOX4. To promote stem cell identity, the WOX TFs regulate the epigenetic landscape of the cell by interacting with TOPLESS (TPL) (Pi *et al.*, 2015; Dolzblasz *et al.*, 2016) and HISTONE DEACETYLASES (HDACs) family proteins (Liu and Karmarkar, 2008). Additionally, WOX factors regulate the gene expression of the cell by directly binding to the GRAS- transcription factors of the HAIRY MERISTEM family (Zhou *et al.*, 2015).

Except from the cork cambium where the WOX4 signalling cascade remains unknown, the other WOX regulatory modules are under the control of a peptide-receptor system. A short peptide from the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (CLE) family is secreted from stem cells (for the SAM) or their neighbouring cells (i.e., the cap cells for the RAM and the phloem cells for the vascular cambium) (Fig.6) (Fletcher *et al.*, 1999; Hirakawa *et al.*, 2008; Ohyama *et al.*, 2009; Etchells and Turner, 2010; Stahl *et al.*, 2013). The peptide then diffuses in the interstitial space and binds to their cognate transmembrane receptors (Ogawa *et al.*, 2008; Shinohara and Matsubayashi, 2015). Among the different receptors binding to the CLV3/CLE peptides, the leucine-rich-repeats receptor-like-kinases (LRR-RLK) from the CLAVATA1 (CLV1) family are common to the SAM, the RAM and the vascular cambium (Fig.6) (Schoof *et al.*, 2000; Hirakawa, Kondo and Fukuda, 2010; Stahl *et al.*, 2013).

These receptors are embedded in the plasma membrane in the cells of the OC of the SAM (Schoof *et al.*, 2000) and the stem cells of the RAM (Stahl *et al.*, 2009) while in the vascular cambium PHLOEM INTERCALATED WITH XYLEM (PXY)/TDR and MORE LATERAL GROWTH (MOL1), two CLV1-related LRR-RLKs, are respectively found in the cambium cells adjacent to the xylem and the cambium cells adjacent to the phloem (Fig.6) (Etchells and Turner, 2010; Gursanscky *et al.*, 2016).

Except from MOL1 whose signalling cascade remains to be elucidated, the other LRR-RLK receptors are known to be activated upon binding of their cognate CLV3/CLE ligand. In the SAM and the RAM, this ligand-receptor complex represses the expression of WUS and WOX5 (Fig.6) (Schoof *et al.*, 2000; Stahl *et al.*, 2009). Intriguingly, the expression of CLV3 in the stem cells of the SAM is promoted by WUS, thus creating a negative feedback loop (Fig.6) (Yadav *et al.*, 2011). However, this relationship between the WOX TF and the CLV3/CLE peptides has not yet been identified in the other meristems. In contrast to the SAM and the RAM, the actions of the two LRR-RLKs in the vascular cambium seem to be synchronised and spatially organised to balance the bidirectional tissue production. While MOL1 represses cambial activity on the phloem side of the cambium, PXY/TDR induces the expression of

WOX4, which then promotes stem cell identity on the xylem side of the cambium (Fig.6) (Gursanscky *et al.*, 2016).

Finally, the ERECTA (ER) family - another LRR-RLK family - are key regulators of plant meristems. These receptors coordinate stem cell behaviour between the different tissue layers of the SAM by restricting the expression zone of CLV3 and WUS to the centre of the SAM (Fig.6) (Kimura *et al.*, 2018; Zhang *et al.*, 2021). Similarly, the ER family receptors seem to be crucial for the development of the cambium (Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017) and genetically interact with the PXY receptor (see following section).

Altogether, an important regulatory module has been identified in the SAM, the RAM and the vascular cambium but the overall molecular makeup of their stem cells remains poorly characterised. This is particularly true for the lateral meristems whose existing regulatory cascades diversified during evolution and adapted to the bifacial mode of tissue production.

Which specific LRR-RLK signalling cascade regulating the lateral meristems should be studied?

The LRR-RLK family arising our interest is the ER family (ERf) - consisting of ER, ER-LIKE1 (ERL1) and ERL2 paralogues – as they redundantly regulate cell elongation, cell width and cell division (Torii *et al.*, 1996; Shpak, Lakeman and Torii, 2003; Shpak *et al.*, 2004) in the procambium of the stem (Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017) and in other developmental processes such as floral and stomatal patterning (Shpak et al., 2005; Bemis et al., 2013), shoot apical meristem fate (Uchida, Shimada and Tasaka, 2013; Kimura et al., 2018) and inflorescence architecture (Torii et al., 1996; Shpak et al., 2004). While ERf promotes vascular expansion in the stem (Uchida and Tasaka, 2013), the situation in organs undergoing extensive secondary growth – the hypocotyl and the roots - remains unclear. Removing ER and ERL1 leads to an increase in the hypocotyl radial expansion (and the premature development of fibre differentiation) but loss of all three ERf genes trigger a reduction in the hypocotyl diameter (Ikematsu *et al.*, 2017; Wang *et al.*, 2019). Although their function in secondary growth is obscure, ERf receptors are expressed in the vascular cambium, the xylem initials and the periderm of the hypocotyl, thus making them suitable candidates to regulate the initiation and/or activity of lateral meristems (Ikematsu *et al.*, 2017; Wang *et al.*, 2019).

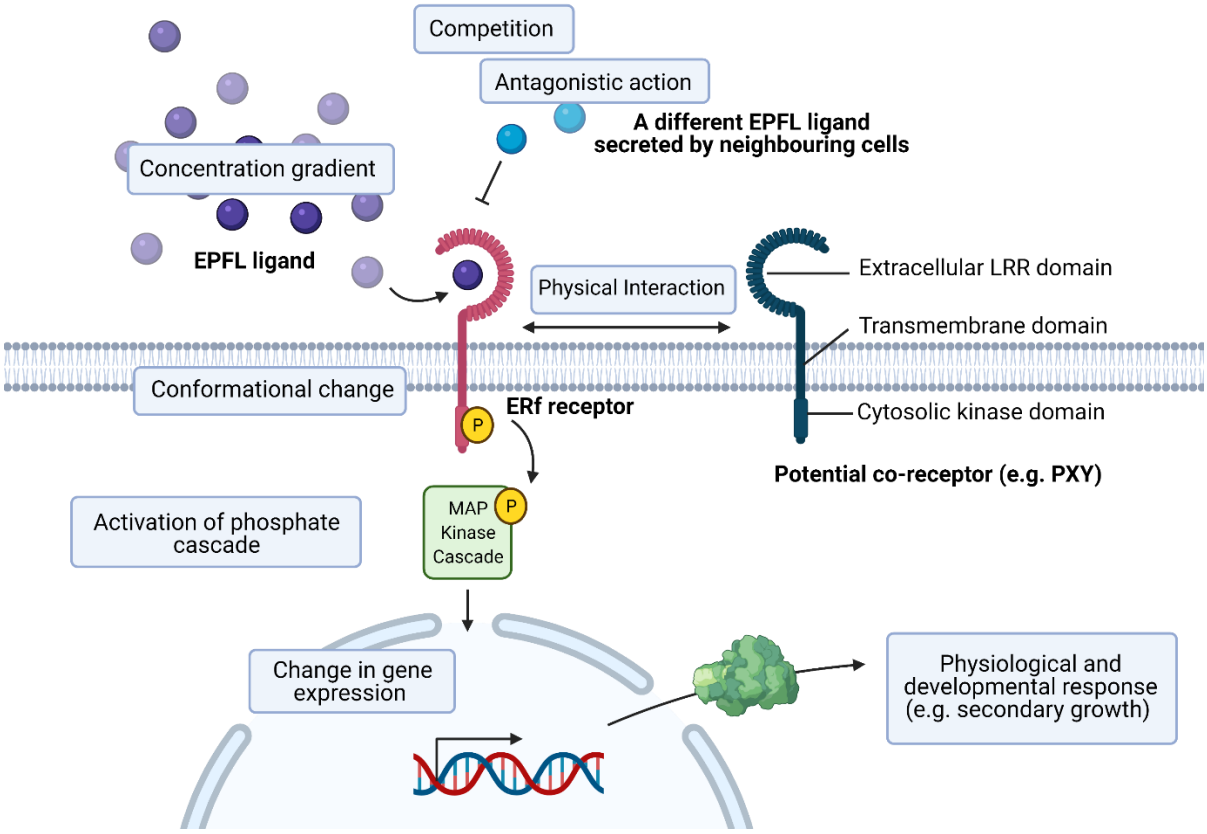


Fig.7. Signalling cascade of ER family LRR-RLK receptors.

For their intercellular communication, plants have acquired expanded families of genes encoding for short secretory peptides to act as ligands. One of these superfamilies consists of cysteine-rich secretory peptides which encode information in the complex knotted structures stabilised by intramolecular disulphide bonds. Among cysteine-rich secretory proteins, a subfamily called EPIDERMAL PATTERNING FACTOR and EPF-LIKE peptides (from now on referred as EPFLs) are the only known cognate ligands of the ERECTA family receptors (Rychel, Peterson and Torii, 2010). The specificity and complexity of EPFL-ER signalling is characterised by the competition, partial redundancy and antagonistic actions between EPFLs on the same ERf receptor complexes (Fig.7). With slight changes in EPFL-ERf modules, the ER signalling can thus regulate specific developmental and physiological processes in close spatiotemporal proximity (Shimada, Sugano and Hara-Nishimura, 2011). In this study, we examined and identified potential EPFL candidates regulating secondary growth (Chapter I).

EPFL peptides regulate the activity of the ERf receptors and control specific downstream signalling cascades. Upon ligand binding, the ERf undergo a conformational change which modulates the activity of their cytoplasmic Ser/Thr kinase domain and activates a phosphorylation cascade (Shpak, Lakeman and Torii, 2003; Kosentka *et al.*, 2017). Only the MAP phosphate cascade regulating stomatal patterning has been elucidated so far (Bergmann, Lukowitz and Somerville, 2004; Wang *et al.*, 2007; Meng *et al.*, 2013). However, the molecular mechanism of ERf and its downstream MAP signalling cascades change depending on pathway which needs to be regulated in specific organs and at specific developmental stage of the plant (Fig.7). As a result, the downstream signalling targets of ERf receptors which regulate secondary growth started to be investigated in this study (Chapter IV).

Although ligands encode most of the biological information, the transduced signal can be further regulated with the physical coupling of receptors (Fig.5,7). The ERf receptors not only form homo-

and heterodimers (Lee *et al.*, 2012) but they are also known to form complexes with the co-receptors SOMATIC EMBRYOGENESIS RECEPTOR KINASES (SERKs), TOO MANY MOUTHS (TMM) and SUPPRESSOR OF BIR-1 (SOBIR1) to regulate stomatal patterning (SERKs and TMM) or fibre differentiation during secondary growth (SOBIR1). Other co-receptors were hypothesised to bind ERf to regulate the activity and/or initiation of the lateral meristems.

PHLOEM INTERCALATED WITH XYLEM/TDIF-RECEPTOR (PXY/TDR) family LRR-RLKs were the first master regulators of lateral meristems to be identified. Although removing PXY and its paralogues – PXY-LIKE 1 (PXL1) and PXL2 – delays the formation of the vascular and cork cambium, the triple mutant *pxy pxl1 pxl2* still succeeds to transition to secondary growth (Etchells *et al.*, 2013; Wang *et al.*, 2019; Chapter IV). This suggests that complementary mechanisms are acting alongside the PXY family receptors to specify the identity and activity of the stem cells in the lateral cambia. As *pxy er* mutant showed greater vascular organisation defects in the stem and the hypocotyl than those of *pxy* single mutants, Etchells *et al.* (2013) proposed that the ERECTA (ER) family forms a genetic network with the PXY family (PXf) to regulate the vascular cambium. To strengthen this hypothesis, Wang *et al.* (2019) observed that removing all members of the PXY family and ER family prevents the hypocotyl from undergoing any lateral growth; thus raising the question of whether the cork cambium and the vascular cambium are simultaneously affected by the loss of ERf and PXf. In this study, we investigated the role of ERf and PXf genetic interactions in regulating the initiation and activity of both lateral meristems (Chapter II) and we examined whether ERf and PXf could physically interact by assessing their expression patterns (Chapter III).

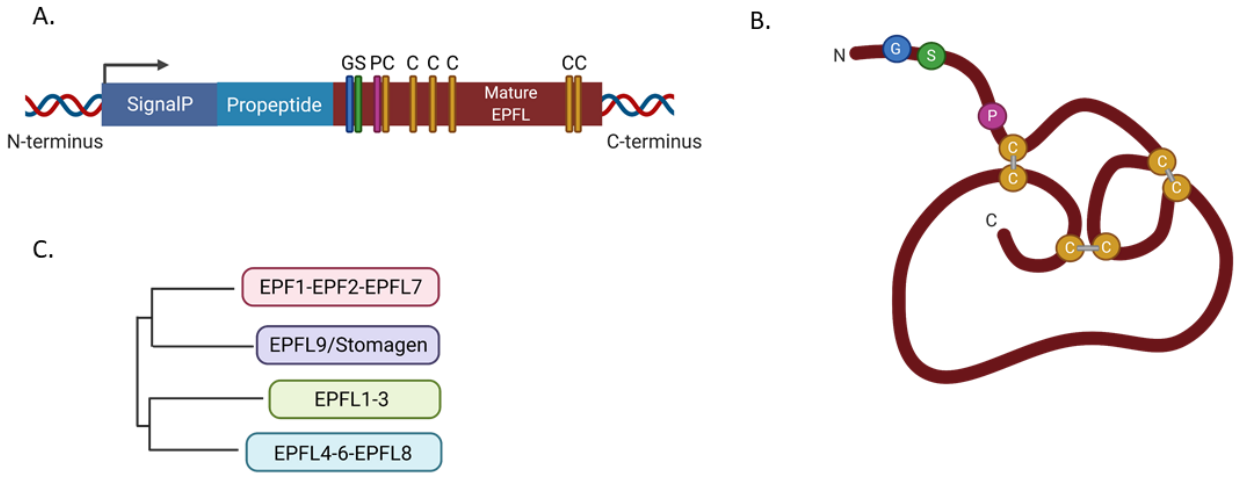
Notably, we used a top-down approach to identify and characterise the components of the ERf signalling cascade regulating secondary growth. We started from the top of the signalling cascade with the investigation of ERf ligands, then switched our focused to the identification of ERf co-receptors and finished with the examination of ERf downstream signalling targets involved in secondary growth.

Chapter I: Function and evolution of EPIDERMAL PATTERNING FACTOR peptides in the context of secondary growth.

Which EPFL peptides regulate the initiation and maintenance of cambium activity? Have EPFL genes evolved in association with secondary growth?

1.1 Introduction:

The EPIDERMAL PATTERNING FACTOR/EPF-LIKE peptides (referred to as EPFL hereafter) are the only known cognate ligands of ERECTA family receptors (from now on referred as EPFL). In the model organism *Arabidopsis thaliana*, the EPFL family consists of 11 members which share important sequence and structural features (Hara *et al.*, 2009; Shimada, Sugano and Hara-Nishimura, 2011). Each mature EPFL peptide possesses six or eight cysteine residues at conserved locations to stabilise their unique scaffold structure and a glycine-serine "GS" motif to interact with the leucine-rich repeat (LRR) domain of ERf receptors (Fig.1) (Kondo *et al.*, 2010; Sugano *et al.*, 2010; Ohki, Takeuchi and Mori, 2011; Lin *et al.*, 2017). Besides these conserved residues, the sequences of EPFL genes remain fairly distinct from one another and account for the functional diversity of the EPFL peptides. The signalling specificity of each EPFL peptide comes from the long loop between the fourth and fifth conserved cysteines which varies in sequence and length (4 to 31 amino acids) and additional sequence motifs conserved within subgroups of the EPFL family (Fig.1) (Ohki, Takeuchi and Mori, 2011). The members of the EPFL family are classified into four subgroups based on their amino acid sequences (Takata *et al.*, 2013): EPF1, EPF2 and EPFL7 are included in the same subgroup and are thought to be closely related to the EPFL9 subgroup, while EPFL1, EPFL2, EPFL3 form another subgroup sharing important residues - involved in ligand-receptor interaction - with the EPFL4, EPFL5, EPFL6, EPFL8 subgroup (although EPFL8 has also been suggested to constitute a subgroup of its own



(Bessho-Uehara *et al.*, 2016)) (Fig.1).

Fig.1. Structure and evolution of the EPFL family peptides. A) Primary structure shared between EPFL peptides. The conserved residues are glycine (G), serine (S), proline (P) and cysteine (C) residues. B) Secondary structure of EPFL peptides highlighting the intramolecular disulphide bonds and the resulting loops (adapted from Rychel, Peterson and Torii (2010)). C) Simplified phylogenetic tree of the EPFL family adapted from Takata et al. (2013).

Although partial redundancy between ligands is common, subgroups of EPFL peptides display strong ontogenic specialisation. EPFL peptides are involved in a wide range of patterning and growth processes such as stomatal patterning (EPF1, EPF2, EPFL9, EPFL6), leaf margin morphogenesis (EPFL2), ovule patterning and fruit growth (EPFL2, EPFL9), and elongation of rice awn (OsEPFL1) (Hara *et al.*, 2007, 2009; Hunt, Bailey and Gray, 2010; Bessho-Uehara *et al.*, 2016; Tameshige, Okamoto, *et al.*, 2016; Kawamoto *et al.*, 2020). Because ERf receptors play a pivotal role in secondary growth, EPFL peptides were hypothesised to be key regulators of radial expansion in plant organs. More specifically, two EPFLs - EPFL4/CHALLAH-LIKE2 and EPFL6/CHALLAH - were selected as potential candidates to regulate the development of the vascular cambium through ERf signalling based on protein interaction, loss-of-function and expression studies. First, EPFL4 and EPFL6 are known to act as ERf ligands in the inner tissues of the plant (Uchida *et al.*, 2012). Second, Uchida *et al.* 2012 showed that the *epfl4 epfl6* double mutant is significantly shorter than wild type plants and short plant phenotypes are often characteristic of an impaired vascular development (Hanzawa *et al.*, 2000; Parker *et al.*, 2003; Pineau *et al.*, 2005; Uchida and Tasaka, 2013). Finally, EPFL4 and 6 are expressed in the differentiating xylem cells, called xylem initials, which are adjacent to the cambium (Wang *et al.*, 2019). This local expression maximum overlaps with the expression of ER and ERL1, which are expressed both in the xylem initials and the vascular cambium in the hypocotyl (Ikematsu *et al.*, 2017; Wang *et al.*, 2019). Due to the known mobility of the EPFL peptides (Tameshige, Ikematsu, *et al.*, 2016), these ligands are likely to activate the ERf signalling cascade in the xylem initials and/or the vascular cambium and regulate the development of these tissues.

In addition to regulating the vascular cambium, EPFL4 and EPFL6 likely control the activity of the cork cambium as the vascular cambium and the cork cambium share similar ring-like organisation, bifacial stem cell populations and partially overlapping regulatory pathways (General Introduction). Altogether, EPFL4 and EPFL6 represent potential regulators of both lateral meristems and their roles in secondary growth was investigated in this chapter.

The morphogenetic results obtained in first part of this study highlighted the pleiotropy and genetic redundancy of EPFL-ER modules during secondary growth. The complexity of secondary growth and its regulation raised the question of how EPFL genes might have evolved in association with the appearance and loss of secondary growth throughout evolution.

The vascular cambium and the cork cambium as well as the production of their respective secondary tissues (e.g. secondary xylem and phloem as well as cork/phellem and phelloderm) are plesiomorphic of seed plants in extant taxa, meaning that these tissues are ancestral traits which are homologous within – but not necessarily unique to – seed plants. In fact, recent studies found that extinct lineages of ferns and lycophytes possessed a vascular cambium and secondary tissues comparable to modern seed plants (Spicer and Groover, 2010). These results suggest that secondary growth may have originated in early tracheophytes or may have been selected several times throughout evolution.

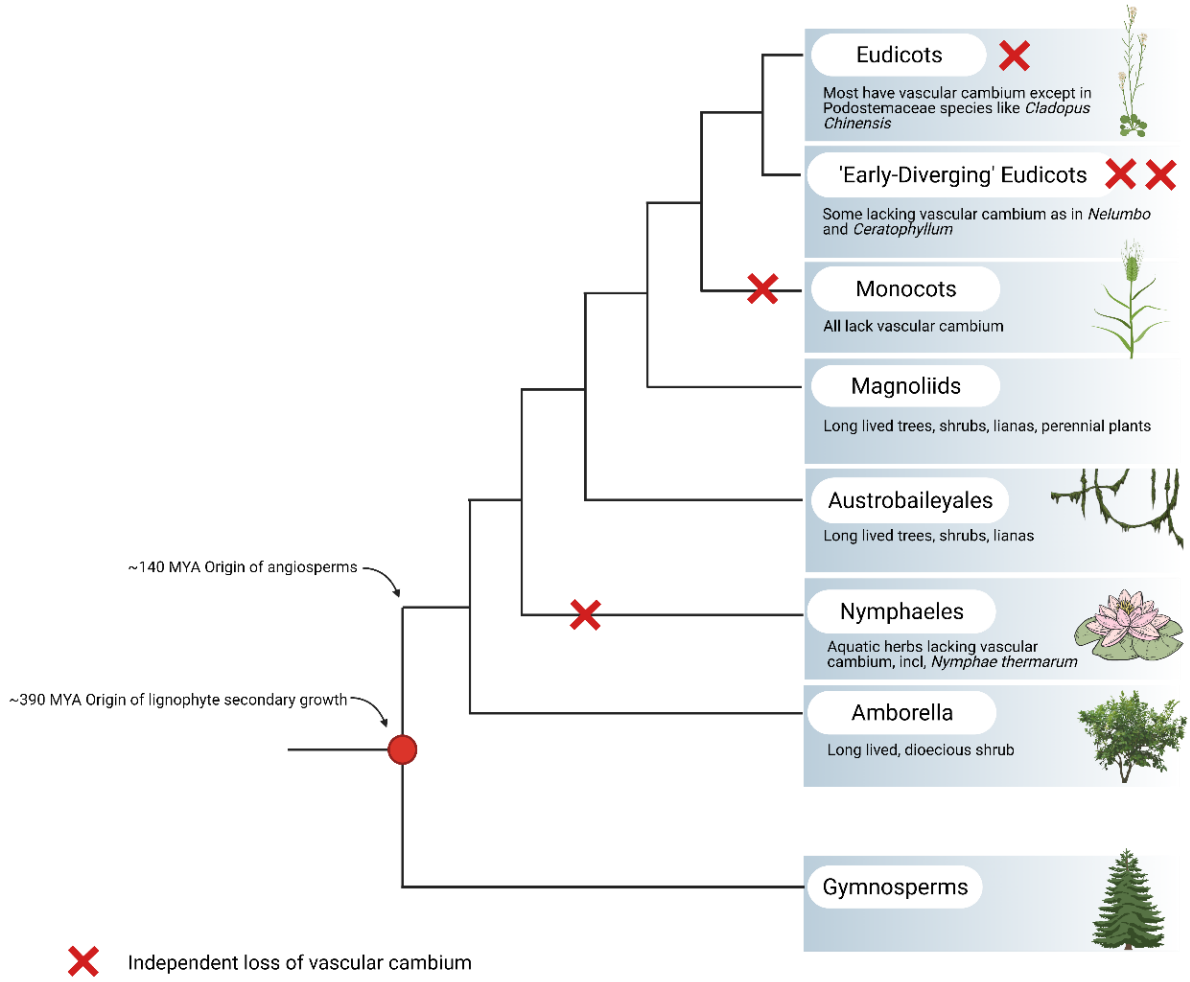


Fig.2. Vascular cambium and secondary growth evolution in major clades of modern seed plants. This close-up view of the previous phylogenetic tree (see General Introduction) highlights the five losses of vascular cambium across angiosperm evolution which have been well documented in the literature. MYA: million years ago. Adapted from (Povilus et al., 2020).

Only five losses of vascular cambium have been well-documented throughout the evolution of plants (Fig.2) (Spicer and Groover, 2010). All of these vascular cambium losses occurred over 140 MYA in angiosperms lineages (Spicer and Groover, 2010; Magallón *et al.*, 2015) (Nymphaeles, Certophyllum, monocots, Nelumbo and Podostemaceae) which have acquired diverse morphological adaptations as they (or their ancestors) transitioned from a terrestrial to a (semi-) aquatic environment (Cook, 1999; Givnish *et al.*, 2018). Although cork cambium losses may have occurred during evolution, only vascular cambium losses have been thoroughly documented in the literature. However, as periderm only forms in species with an active vascular cambium, this study assumes that plants lacking a vascular cambium also lack a cork cambium. Importantly, species lacking a vascular cambium often retain the vasculature originating from their primary growth and so remain categorised as vascular plants (except for bryophytes which do not possess a vascular cambium and are non-vascular plants, see General Introduction).

Because convergent loss or divergence of relevant genes has consistently been observed in convergent trait loss (Chan *et al.*, 2010; Zhen *et al.*, 2012; Sackton *et al.*, 2018; Povilus *et al.*, 2020), we used an evolutionary approach to identify which of the EPFL peptides could have evolved in

association with the loss of secondary growth. Although highly interconnected genes like the EPFL genes are less likely to be lost or modified with the loss of a specific trait (Lammers *et al.*, 2019), Povilus *et al.* (2020) showed that this methodology could reliably identify convergent loss and/or divergence of relevant genes in association with loss of traits such as secondary growth. In particular, Povilus *et al.* (2020) showed that modifications of pleiotropic genes from the CLE and HD-ZIP III families correlated with the loss of vascular cambium. While Povilus *et al.* (2020) tested their approach on CLE and HD-ZIP III genes already known to control the vascular cambium, we used their technique to identify - for the first-time - potential regulators of secondary growth within the EPFL gene family.

Altogether, EPFL genes - and more specifically EPFL4 and EPFL6 - were hypothesised to be key regulators of secondary growth. To test our hypothesis, we used morphogenetic analyses complemented by in-depth phylogenetic studies. These phylogenetic studies consisted of utilising the repeated loss of the vascular cambium during evolution to examine the importance of the EPFL family in secondary growth and identify the EPFL whose sequences changed in association with the loss of vascular cambium.

1.2 Materials & Methods:

1.2.1 Materials:

Resource Table:

Reagent or Resource	Source	Identifier
Technovit 7100	Heraeus Kulzer	Cat# 64709003
Histomount mounting solution	ThemoFischer Scientific	Cat# 008030
Experimental models: Organisms/Strains		
Arabidopsis: Col-0	Widely distributed	N/A
Arabidopsis: epfl4 in Col-0	Nottingham Arabidopsis Stock Centre (NASC)	http://arabidopsis.info/
Arabidopsis: epfl5 in Col-0	NASC	http://arabidopsis.info/
Arabidopsis: epfl6 in Col-0	NASC	http://arabidopsis.info/
Arabidopsis: epfl4 epfl5 in Col-0	NASC	http://arabidopsis.info/
Arabidopsis: epfl4 epfl6 in Col-0	NASC	http://arabidopsis.info/
Arabidopsis: epfl5 epfl6 in Col-0	NASC	http://arabidopsis.info/
Arabidopsis: epfl4 epfl5 epfl6 in Col-0	NASC	http://arabidopsis.info/
Software		
ZEN Black (Zen 2.3 SP1)	Zeiss	https://www.zeiss.de/corporate/home.html
ImageJ		https://imagej.net/Welcome
Jalview	(Waterhouse *et al.*, 2009)	https://www.jalview.org/
R version 4.0.2	The R foundation	https://www.r-project.org/
SWISS-MODEL	Swiss Institute of Bioinformatics	https://swissmodel.expasy.org/
PyMol 2.4	Schrodinger	https://pymol.org/2/

MUSCLE 3.8.31	EMBL-EBI	https://www.ebi.ac.uk/Tools/msa/muscle/
Remap 6.1.0	EMBOSS	http://emboss.bioinformatics.nl/cgi-bin/emboss/remap
Sixpack 6.6.0.0	EMBOSS	https://www.ebi.ac.uk/Tools/st/emboss_sixpack/
TimeTree	(Kumar *et al.*, 2017)	http://www.timetree.org/
HMMR.v3.3.2	(Finn, Clements and Eddy, 2011)	http://hmmer.org/

Experimental model:

Arabidopsis thaliana loss-of-function mutant lines were used to perform these experiments. The ecotype and background of each line is specified in the resource table. The plants were grown until 25 days post sowing (das) in soil under 16:8h light:dark cycles.

1.2.2 Method details:

Histology and Microscopy:

Hypocotyl samples of 1cm were sectioned directly at the base of the rosette and above the root. The samples were embedded in plastic using Technovit 7100 and sectioned with a Thermo Fisher Scientific Finesse ME 240 microtome following the protocol of de Reuille and Ragni (2017). The tissues were fixed in Formalin-Aceto-Alcohol (FAA) (50% Ethanol, 3.7% Formaldehyde, 5% acetic acid) for 30 minutes at room temperature. They were then dehydrated with a graded series of ethanol to 100% ethanol, infiltrated with JB4 medium and embedded and polymerised in Technovit 7100 (Heraeus Kulzer). The resulting 4 µm cross sections were stained with 0.05% toluidine blue and imaged using a Leica DM 2500 LED microscope. All sections were mounted in histomount.

Quantification and Statistical Analyses:

7-10 plants were analysed for each genotype. Using ImageJ, a single measurement of the stele radius, the xylem radius and the periderm width was taken per individual. Each measurement was consistently performed at the same angle of the transversal sections and this angle was chosen arbitrarily. Additionally, the number of periderm cell layers were counted using the CELL COUNTER plug-in of Fiji.

Statistical analyses were performed on the stele, xylem and periderm measurements using the R statistical packages. The normal distribution and homogeneity of variances of the datasets were respectively confirmed using a Shapiro-Wilk test and Levene's test. Significant differences between each dataset were calculated with a One-way ANOVA test followed by a Tukey's post hoc test (equal variances tested). The threshold for significance was indicated with P values (difference between samples is significant when P value < 0.05).

Modelling:

Tertiary structures of EPFL peptides were modelled with the homology modelling server SWISS-MODEL (see Resource Table). The structures of AtEPF1, AtEPF2 and AtEPFL4 solved by X-ray crystallography (Lin *et al.*, 2017) as well as the one of AtEPFL9 determined by NMR (Ohki, Takeuchi

and Mori, 2011) were the main structures which were employed as templates by the server. The peptides were then visualised using the PyMol version 2.4 software (see Resource Table).

Phylogenetic Analysis:

Gene list used for orthologue search:

Gene Abbreviation	Gene Name	Gene ID
EPF1	EPIDERMAL PATTERNING FACTOR 1	AT2G20875
EPF2	EPIDERMAL PATTERNING FACTOR 2	AT1G34245
EPFL1	EPIDERMAL PATTERNING FACTOR-LIKE 1	AT5G10310
EPFL2	EPIDERMAL PATTERNING FACTOR-LIKE 2	AT4G37810
EPFL3	EPIDERMAL PATTERNING FACTOR-LIKE 3	AT3G13898
EPFL4	EPIDERMAL PATTERNING FACTOR-LIKE 4	AT4G14723
EPFL5	EPIDERMAL PATTERNING FACTOR-LIKE 5	AT3G22820
EPFL6	EPIDERMAL PATTERNING FACTOR-LIKE 6	AT2G30370
EPFL7	EPIDERMAL PATTERNING FACTOR-LIKE 7	AT1G71866
EPFL8	EPIDERMAL PATTERNING FACTOR-LIKE 8	AT1G80133
EPFL9	EPIDERMAL PATTERNING FACTOR-LIKE 9	AT4G12970

Template sequence collection and sequence alignment:

The nucleotide and peptide sequences of all *Arabidopsis thaliana* EPF/EPFL homologues (referred as AtEPFLs) were retrieved from TAIR10 (The Arabidopsis Information Resource). As the sequences of the signal peptide and the propeptide (together known as prepropeptide) is poorly conserved among AtEPFL genes, two phylogenetic analyses were conducted to compare the results obtained with or without the prepropeptide sequence and investigate the impact of genetic changes in the prepropeptide and the mature sequences of AtEPFL proteins.

Since the cleavage site between the propeptide and the mature protein is not known for all AtEPFLs homologues but the AtEPF1 and AtEPFL9 mature peptides sequences are known to start with X-GS (Kondo *et al.*, 2010; Sugano *et al.*, 2010), AtEPFL amino-acid sequences were trimmed using Jalview from the N-terminus up to the residue preceding the conserved "GS" motif. The nucleotide sequences of Arabidopsis EPFL homologues were also trimmed until the codon for "X-GS" was reached using remap v.6.1.0. The peptide and nucleotide sequences coding for the full-length AtEPFL peptides and the mature AtEPFL peptide were aligned separately with MUSCLE v3.8.31. The multiple sequence alignments (MSAs) were not trimmed further as the sequences encoding for mature peptides were highly conserved and the sequences for the propeptide were kept despite being highly varied among EPFL gene family.

Orthologue search:

Putative EPFL orthologues were identified in a broad range of land plants using a HMMR.v3 1b1 search with the full-length AtEPFLs MSA or the mature AtEPFLs MSA as template. HMMR identifies

sequences of protein families using a hidden Markov model (HMM) to describe the frequencies of different amino acids encountered in a protein domain. This protein domain essentially corresponds to the conserved region of the queried MSA.HMMR searches similar sequences to this domain across databases.

Putative EPFL peptide sequences were collected using the function hmmrsearch (e-value cut-off = 1e-10) with aligned peptide sequences of all Arabidopsis homologues queried against the annotated protein sequences of 29 Phytozome v.13 genomes and the annotated protein sequences of *Nelumbo nucifera* and *Nymphaea thermarum* genomes retrieved from NCBI (National Centre for Biotechnology Information). When the annotated protein genomes, transcriptomes and raw transcript reads were not available, the aligned nucleic acid sequences of all Arabidopsis homologues were queried against the full genomic assembly of species such as C-fern *Certopteris richardiWe* (from NCBI), *Pinus taeda* (from NCBI) and *Picea glauca* (from treegenes) (e-value cut-off = 0.1). For these putative EPFL nucleotide sequences, open reading frames (ORFs) were identified using Sixpack and a HMMR search - with the aligned peptide sequences of all Arabidopsis homologues queried against these ORFs - was used to identify the ORFs with sequence similarity to the EPFL peptides of *Arabidopsis thaliana* (e-value cut-off = 1e-10). The efficiency of this method to collect sequences from full genomic assembly was tested on *Nymphaea thermarum* by comparing the results obtained from its annotated protein genome and its full genome assembly. Six putative EPFLs homologues were obtained from the full-genome assembly and each of these six sequences corresponded to one of the 12 putative EPFL peptides found in the annotated protein genome of *Nymphaea thermarum*. Despite the number of identified EPFL sequences being reduced, putative EPFL sequences could still be accurately identified in full genome assemblies of species lacking an annotated protein/transcript genome. Once the homologous ORFs were identified and translated using Sixpack and HMMR, they were aligned with the putative EPFL peptide sequences from other species with annotated protein genomes using MUSCLE. The final MSA containing all EPFL orthologues was manually trimmed to exclude poorly aligned regions using Jalview. The resulting alignments of approximately 100 amino acids (for full-length EPFLs orthologues) and 20 amino acids (for mature EPFL orthologues) were used to estimate the phylogeny of the EPFL family.

Cluster analysis to retrace their evolutionary trajectory of the EPFL gene family:

Due to the high number of collected sequences (between 500 to 700 sequences) and the low conservation in certain regions of EPFL sequences (e.g. propeptide), a CLANS (Cluster Analysis of Sequences) approach was taken (BLOSUM scoring matrix, HSP cut-off 1e-1, run for > 10,000 iterations) to characterise the patterns of divergence within the sequences of this gene family – meaning that we mapped out the relationships between all the EPFL peptides collected based on their sequence similarity to retrace the evolutionary trajectory of the EPFL family. CLANS uses a version of the Frutcherman-Reingold layout algorithm to identify clusters of closely related sequences. The software calculates the sequence similarity between each sequence pair (also called attractive forces or Pairwise Attraction Values (PAV)) using the P values obtained from an all-against-all BLAST search. These attractive forces are then represented on a graph as edges connecting the sequences represented by vertices/dots (a minimum repulsion force is attributed to each vertex to prevent them from collapsing into one point). Clustering is then achieved by randomly seeding the sequences (vertices/dots) and moving them according to the force vectors resulting from all pairwise attraction values and iterating this process until convergence (Offermann *et al.*, 2016).

Normalisation for different evolutionary distances between PAV and visualisation of the evolution of EPFL peptide sequences in association with the loss of vascular cambium:

The pairwise attraction values (PAV) of each subgroup were extracted, rescaled between 0 and 1 and analysed following Povilus *et al.* (2020)'s methodology. As the EPFL genes govern some domestication traits (e.g. growth, fruit size, seed awn), the EPFL genes from different species could have evolved at different rates depending on whether the species was domesticated on not. Additionally, the selected species have different ploidies, and this might have affected the evolution of their genes. For these reasons, we normalised each pairwise attraction value - indicating the similarity between two sequences coming from different species - for the evolutionary distance between those two species. The estimated divergence time between each species of interest was collected with TimeTree (Kumar *et al.*, 2017). Using a linear regression model, the effect of evolutionary distance was modelled on the PAV. The slope of the linear model was then used to remove the dependency between the two variables (see supplementary figure 1). The adjusted pairwise attraction values (APAV) were categorised according to whether they compared sequences of species forming a vascular cambium (VC – VC) or whether they compared species which form and do form not a vascular cambium (VC-NVC) (VC = form a vascular cambium; NVC = do not form a vascular cambium). For each EPFL subgroup, the APAV of the VC-VC and VC-NVC categories were compared using a two-samples Wilcoxon test because the data was not normally distributed (Shapiro-Wilk test, p-value <0.05).

1.3 Results:

1.3.1 The EPFL peptides redundantly regulate secondary growth:

To understand the role of the subgroup EPFL4-6 during secondary growth, we started by investigating the morphology of the hypocotyl in EPFL loss-of-function mutants. Similar to the inflorescence stem (Uchida *et al.*, 2012), *epfl4* or *epfl6* single mutants exhibited no detectable phenotype in the hypocotyl (Fig.3). Although the *epfl4 epfl6* double mutants are characterised by the same stem phenotype than the *er* mutant (i.e. large and expanded cortex cells resulting in a compact inflorescence stem) (Uchida *et al.*, 2012), we did not observe any obvious morphological change in the hypocotyl of the *epfl4 epfl6* mutant (Fig.3). Additionally, removing EPFL5 (the last member of the EPFL4-6 clade) in the *epfl4 epfl6* mutant background did not lead to further vasculature and/or periderm defects (Fig.3.4). Our statistical analysis in Figure 4 confirms that no significant morphological changes could be observed in the single, double and triple mutants of EPFL4-6 as the measurements of the xylem radius, stele radius and periderm width conducted in these mutants were not significantly different from ones in the wild type plants. The conservation of vascular and periderm size and organisation in the tested genotypes suggested that a parallel signalling pathway might mask the absence of these ligands and the appearance of vascular defects (e.g. TDIF-PXY signalling) (Uchida and Tasaka, 2013) or that other EPFL ligands might act redundantly with EPFL4, EPFL5 and EPFL6.

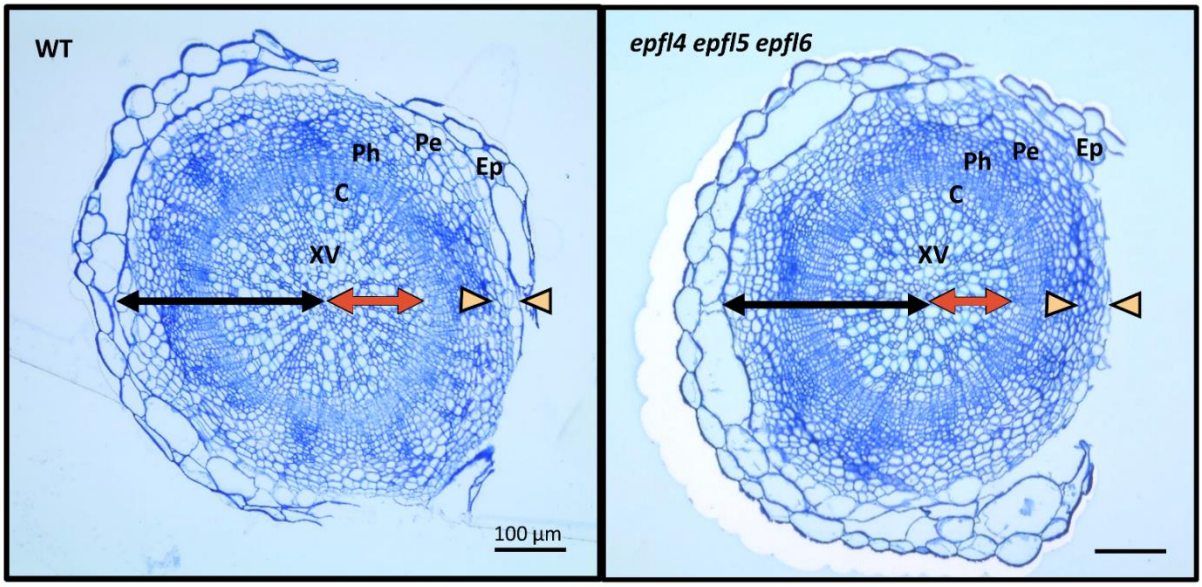


Fig.3. Transverse section of hypocotyl of the epfl4 epfl5 epfl6 loss-of-function mutant. The black arrows indicate the radius of the stele, the red arrows show the radius of the xylem tissue and the yellow pointers highlight the thickness of the periderm. XV; Xylem Vessels, C; vascular cambium, Ph; Phloem, Pe; Periderm, Ep; shedding epidermis.

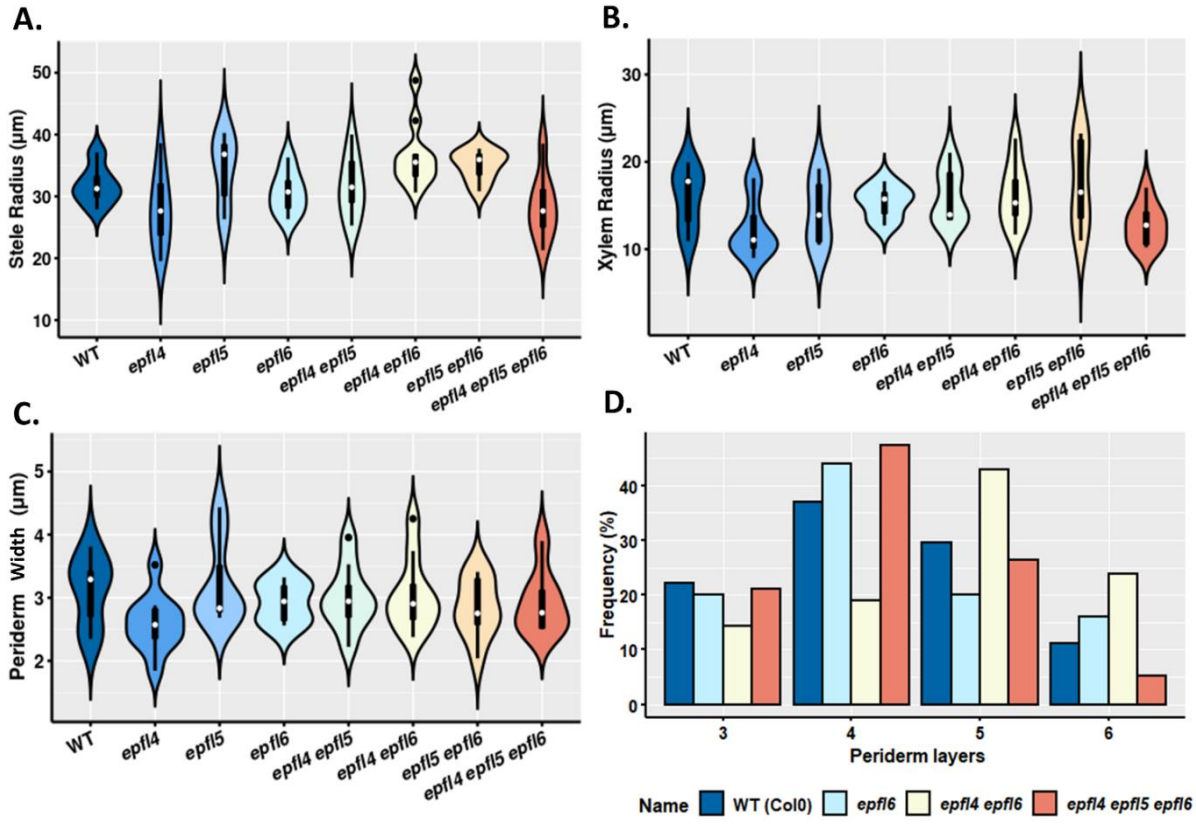


*Fig.4. Morphometric analyses of the overall radial expansion, vascular and periderm growth in EPFL4, EPFL5 and EPFL6 single, double and triple mutants. Stele radius (A), xylem cylinder radius (B), periderm width (C) and number of periderm layers (D) measured in the loss-of function-mutants. Boxes inside the violin plots represent the interquartile range (IQR= third quartile Q3 - first quartile Q1); the white dot marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper). ANOVA and a Tukey post hoc test showed no significant difference between the tested genotypes and the wild type (p-value>0.05)*

1.3.2 The evolution of the EPFL family peptide in the context of secondary growth:

Despite the size and complexity of the collected sequences, we created a network of relatedness between all EPFL amino acid sequences (Fig.5,6). The EPFL family was clustered into three major groups. Group 2 (EPF1-2, EPFL7) and Group 3 (EPFL9) are principally associated with stomatal patterning in the literature (Hara *et al.*, 2007, 2009; Hunt, Bailey and Gray, 2010). Interestingly, EPFL9 (group 3) was distant from the other groups, suggesting that the role of the EPFL9 peptide is fundamentally different from other EPFLs. In accordance to Takata *et al.* (2013), the group of EPFL9 solely consisted of sequences from spermatophytes (seed plants), indicating that EPFL9 evolved after the speciation of this lineage.

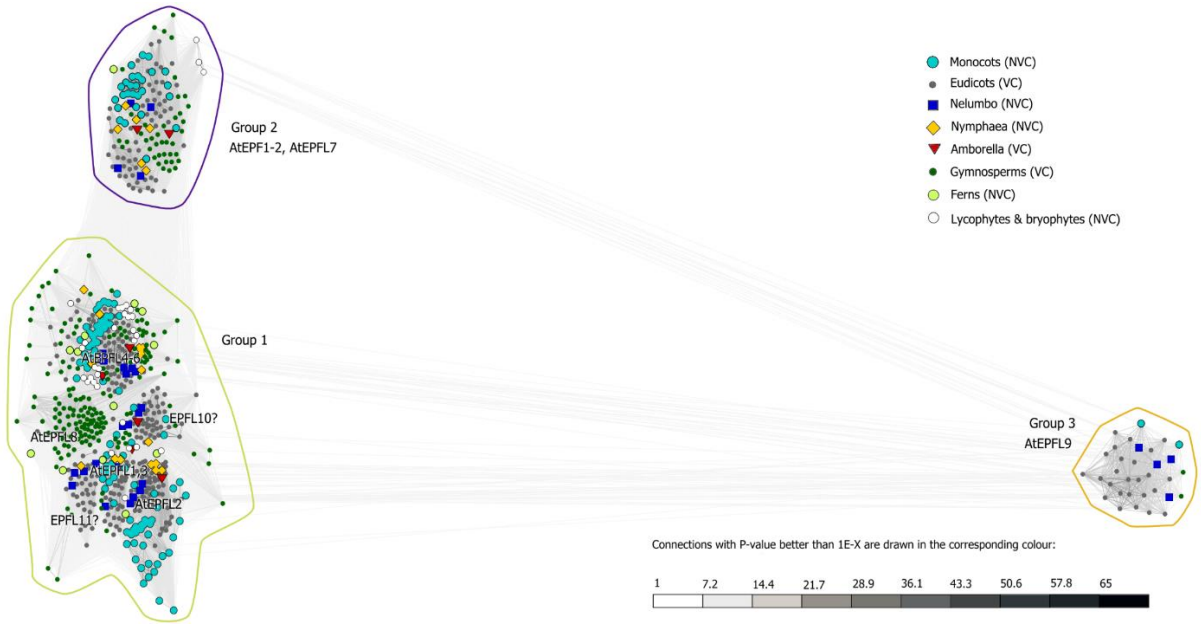


Fig.5. 2-dimensional CLANS clustering of mature EPFL peptide sequences in land plants. Dots/shapes represent individual sequences from species within clades which have vascular cambium (VC) or do not have vascular cambium (NVC). The distance between two dots corresponds to the similarity between the two compared sequences.

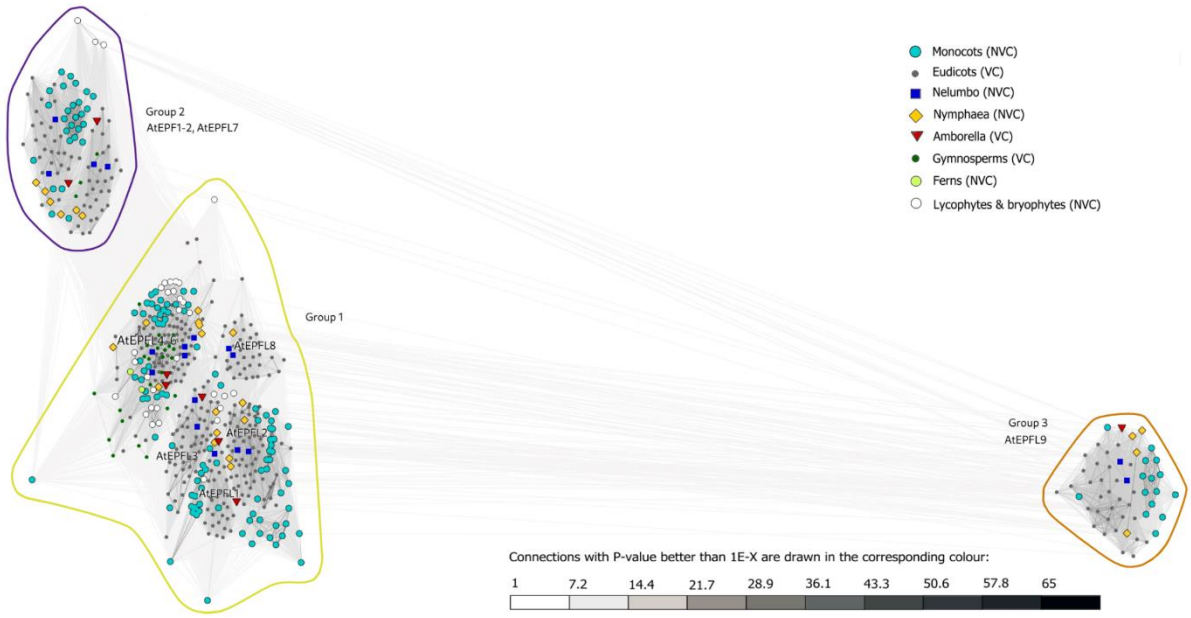


Fig.6. 2-dimensional CLANS clustering of full-length EPFL peptide sequences in land plants. Dots/shapes represent individual sequences from species within clades which have vascular cambium (VC) or do not have vascular cambium (NVC). The distance between two dots corresponds to the similarity between the two compared sequences.

Group 1 was the focus of our research as it contained EPFL4-6 peptides - which are potential regulators of secondary growth - as well as other closely related EPFL peptides. Any EPFL peptides which potentially regulate secondary growth alongside EPFL4-6 are likely to have similar sequences to EPFL4-6 and be part of Group 1. In contrast to other clusters, Group 1 was much more broadly defined and comprised several smaller subgroups (Fig.5,6). Most of the subgroups in Group 1 varied between the analysis with the full-length and the mature EPFL peptides, while the main clusters remain the same between the two analyses. More specifically, two previously unidentified subgroups were found in Group 1 of the mature EPFL amino acid sequences (Fig.5) but not in the full-length EPFL sequence analysis (Fig.6), highlighting the importance of comparing our results between the full-length and the mature EPFL amino acid sequences analyses. These two novel groups did not have any close homologue in *Arabidopsis* and their function remained to be discovered (referred as EPFL10 and EPFL11 in the Fig.5). Additionally, EPFL1 formed a subgroup with EPFL3 in the analysis of mature sequences (Fig.5) while being grouped with EPFL2 in the full-length analysis (Fig.6). Despite the function of EPFL3 being unknown, an orthologue of EPFL1 regulates awn formation in rice and EPFL2 regulates ovule and fruit patterning in *Arabidopsis* (Kawamoto *et al.*, 2020). In line with these results, the subgroup of EPFL2 principally comprised angiosperms (flowering plants) sequences, including many monocots sequences which started to diverge from the core of the EPFL2 subgroup. These results suggested that ovule and fruit formation in monocots is diverging from other angiosperms and could be potentially caused by domestication events. On the other hand, two of the subgroups in Group 1 were conserved in both analyses: EPFL8 and EPFL4-6 subgroups. These results confirmed that EPFL8 is a subgroup of its own (Bessho-Uehara *et al.*, 2016) and that its function needs to be investigated. Interestingly, the isoelectric point of EPFL8 differs from other EPFL peptides, suggesting

that EPFL8 is differently charged at physiological pH compared to other EPFL peptides. This indicates that the affinity of this peptide to the ER family receptors greatly differs from other EPFL peptides and that EPFL8 might have a unique function in plant development and/or physiology (see supplementary figure 3).

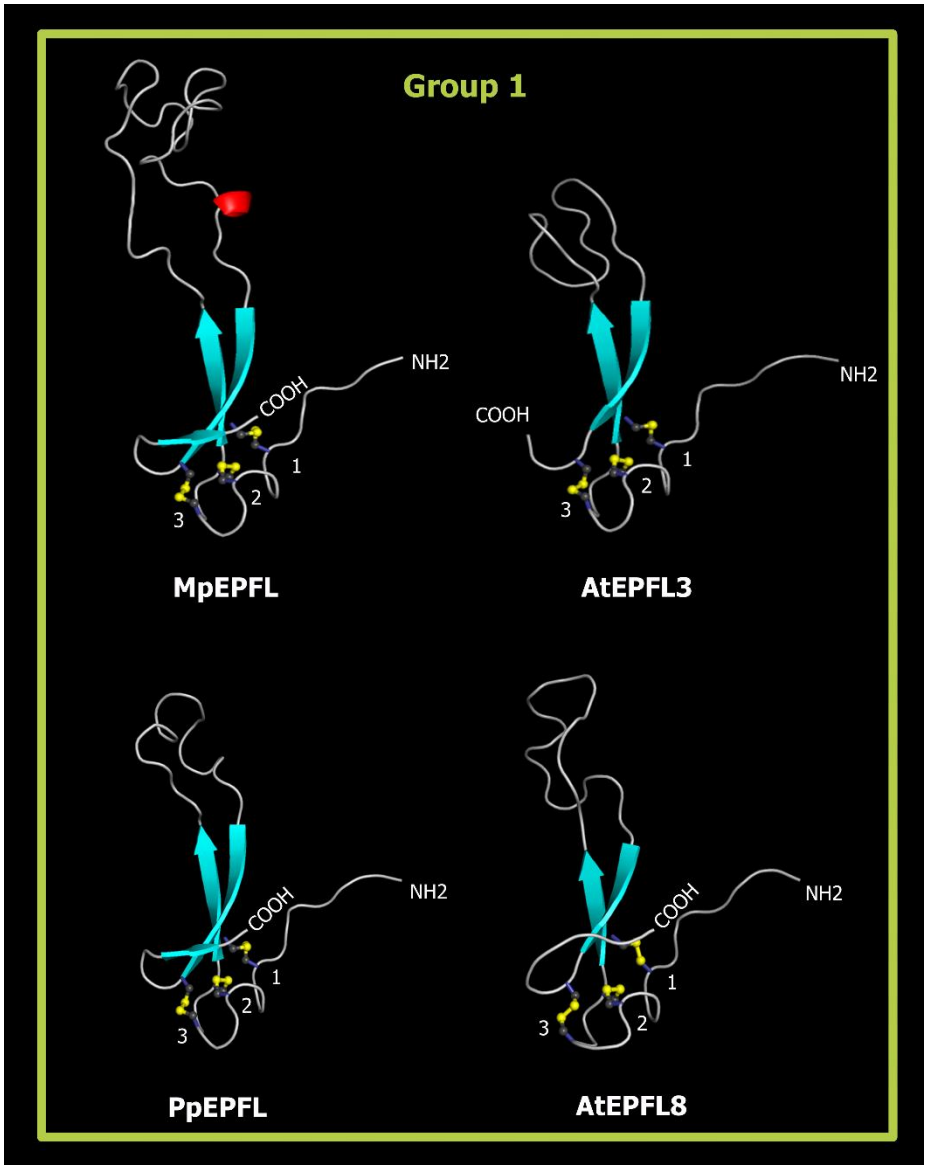


Fig.7. Predicted tertiary structure of bryophyte EPFL peptide homologues and Arabidopsis EPFL peptides from Group 1. Species used: Mp; Marchantia polymorpha (liverwort), Pp; Physcomitrella patens (moss), At; Arabidopsis thaliana. Structure colour-code: Cyan: antiparallel beta sheets, red: alpha helix, yellow: disulphide bonds.

Sequences from Group 1 seemed to be closely related to the first EPFL ancestor as most bryophyte and lycophyte sequences were found in this group. Notably, all the species lacking a vascular cambium (bryophytes, lycophytes, monocots, Nelumbo, Nymphaea) had EPFL homologues in the

largest subclusters of Group 1 – the EPFL4-6 subgroup – indicating that the EPFL4-6 peptides were not initially selected to regulate secondary growth but could have evolved for primary growth instead (Fig.5,6). Interestingly, clear structural similarity between bryophyte homologues and *Arabidopsis* EPFL peptides from Group 1 was observed using three-dimensional structure modelling (Fig.7). The EPFL peptides from *Arabidopsis* and bryophytes possessed six conserved cysteine residues forming disulphide bonds which stabilise the complex scaffold structure. The length of the loop and the number of beta sheets differed between the Group 1 EPFLs of the distantly related clades, suggesting that despite being in the same group these EPFL peptides could have slightly different functions.

To further understand the EPFL evolutionary trajectory, we compared the Adjusted Pairwise Attraction Values (APAV) between sequences of plants with a vascular cambium and plants without a vascular cambium for each subcluster. As the APAV corresponds to the similarity between a pair of sequences and the higher the APAV, the greater the similarity between the two sequences (Fig.8.9). This approach highlighted the EPFL genes which were modified in association with the loss of vascular cambium.

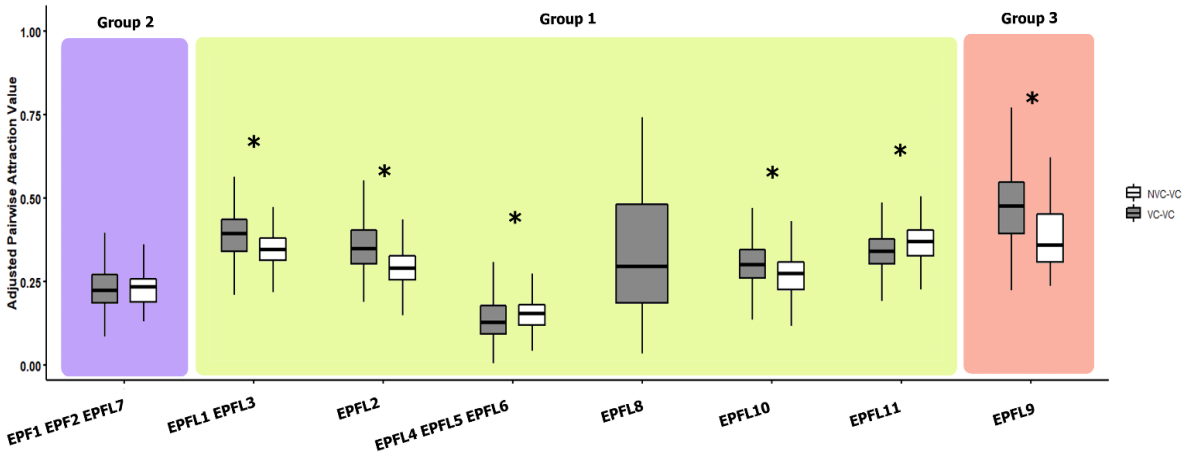


Fig.8. Pairwise comparisons of APAV from mature EPFL amino acid sequences belonging to vascular plants (VC-VC) or vascular and non-vascular plants (VC-NVC). The colour-code corresponds to the CLANS-2D graph and indicates the main groups from which APAV is comparing sequences. The asterisks indicate significantly different pairwise comparisons (p-value < 0.05)

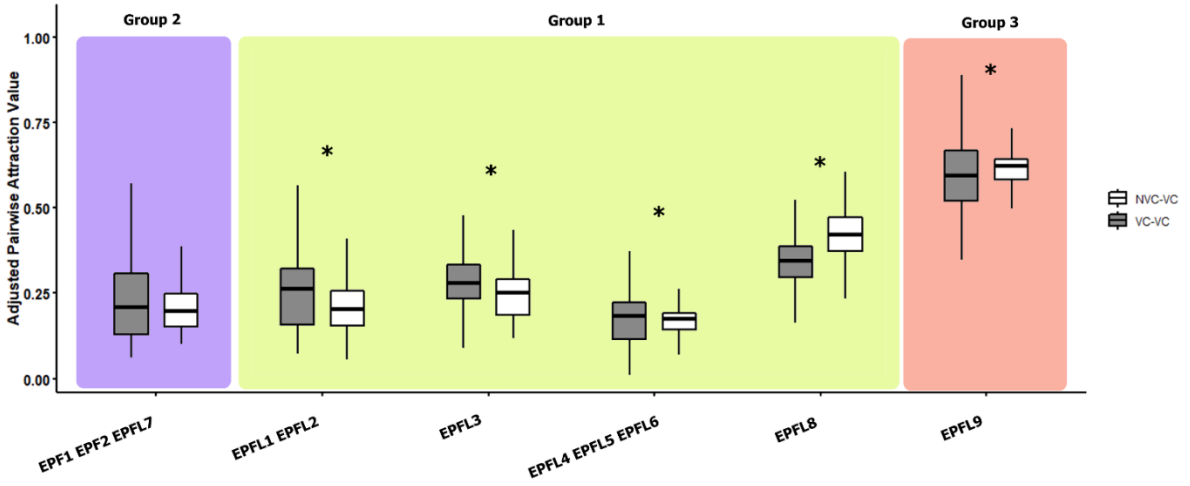


Fig.9. Pairwise comparisons of APAV from full-length EPFL amino acid sequences belonging to vascular plants (VC-VC) or vascular and non-vascular plants (VC-NVC). The colour-code corresponds to the CLANS-2D graph and indicates the main groups from which APAV is comparing sequences. The asterisks indicate significantly different pairwise comparisons (p-value < 0.05)

The sequences of EPFL4-6 subgroup from species without a vascular cambium (NVC) and species with a vascular cambium (VC) significantly differed from sequences from any two species that retained a vascular cambium (VC-VC) (Fig.8,9). This relationship indicated that EPFL4-6 sequences significantly vary between plants with a vascular cambium and plants without a vascular cambium, which confirmed that EPFL4-6 peptides might regulate vascular cambium initiation and/or maintenance. Similarly, the sequences of EPFL1,2,3,8,9 orthologues (and EPFL10,11 for the mature analysis) were significantly different between plants with a vascular cambium and plants without a vascular cambium suggesting that these EPFL peptides may play a key role during vascular cambium development (Fig.8,9). Interestingly, plants without a vascular cambium did not possess homologues for the mature sequence of EPFL8 (Fig.8). This renders EPFL8 a suitable candidate for regulation of vascular cambium development. By contrast sequences from EPF1, EPF2 and EPFL7 peptides (Group 2) did not significantly differ between plants with or without a vascular cambium, suggesting that this group does not regulate secondary growth (Fig.8,9). Altogether, these results indicated that members of EPFL family such as EPFL1,2,3,4,5,6,8,9 were modified in association with the evolution of the vascular cambium and that some of these peptides might redundantly act with EPFL4,6 during secondary growth.

1.4 Discussion:

Although EPFL4-6 were clearly expressed in the xylem initials (Wang *et al.*, 2019), no significant vascular or periderm defects could be detected. The organisation, overall size and the number of cell layers in the vasculature and the periderm of the single, double and triple mutants remained comparable to the wild type. While EPFL4-6 appeared before the evolution of vascular cambium, the sequences of EPFL4-6 orthologues from vascular and non-vascular plants significantly differed. Altogether, these results suggested that EPFL4-6 ligands might redundantly regulate cambial activity

with other EPFL peptides or that another signalling pathway, such as TDIF-PXY, may act in parallel with EPFL-ER to regulate secondary growth.

To identify other EPFL peptides as potential regulators of secondary growth, we investigated the patterns of divergence between EPFL sequences of vascular and non-vascular plants. Previous phylogenetic reconstructions clustered the mature EPFL peptides into four or five subgroups (Takata *et al.*, 2013; Bessho-Uehara *et al.*, 2016). However, these analyses used classic phylogenetic approaches which are restricted to a limited number of relatively well conserved sequences. For this reason, previous studies focused on the conserved region of the mature EPFL peptides of a limited number of species. Classic phylogenetic analyses are based on a multiple sequence alignment (MSA) of all collected sequences, meaning that the more sequences are collected and the more variability there is between these sequences, the less informative sites there are in the MSA. As we collected a large number of EPFL orthologue sequences using HMMR (between 500 and 700 sequences) and these sequences are highly variable in their propeptide and loop regions, we used CLANS clustering instead of a classic phylogenetic approach to analyse our data. With CLANS, we visualised the sequence similarities between EPFL orthologues from a wider range of species compared to previous phylogenetic analyses (Takata *et al.*, 2013). In particular, we highlighted the relatedness of EPFL sequences found in plants with a vascular cambium (VC) and plants without a vascular cambium (NVC) to visualise the evolution of EPFL peptides in association with the loss of vascular cambium. Additionally, we compared the clustering analyses of mature EPFL sequences and full-length EPFL sequences. The evolution of the full-length EPFL ligands was interesting to analyse in parallel to the mature EPFL peptides because the full-length sequences contain the propeptide sequence which has also been under selective pressure and regulates the activity of the EPFL ligands. Overall, a clustering approach allowed us to analyse a larger number of EPFL peptides orthologues with their poorly conserved regions in a novel and more detailed manner compared to previous papers.

Intriguingly, the presence of bryophyte and lycophyte orthologous sequences in the phylogenetic subcluster of our candidates EPFL4-6 indicates that these peptides evolved prior the emergence of vascular cambium and that these peptides might have other important roles in the development and/or physiology of land plants. These results raised the following questions: did EPFL4-6 specialise later in evolution to regulate the activity of the vascular cambium? Are other EPFL subgroups regulating secondary growth?

Several groups including EPFL1, EPFL2, EPFL3, EPFL8, EPFL9 (and the newly found groups EPFL10 and EPFL11) had sequences that diverged in association with the loss of vascular cambium. As a result, EPFL1, EPFL2, EPFL3, EPFL8, EPFL9 warrant further investigation as they seem to be more suitable candidates to regulate secondary growth than the EPFL4-6 peptides.

Due to the complex pleiotropic, redundant and antagonistic actions of EPFL peptides, the role of these secreted proteins has only been partially characterised. Although the functions of EPFL3 and EPFL8 remain to be elucidated, a recent study found that EPFL1 and EPFL2 act redundantly with EPFL4 and EPFL6 peptides during the development of the shoot apical meristem (SAM) (Kosentka *et al.*, 2019). Despite the *epfl4 epfl6* mutant exhibiting reduced apical growth, *er* remained significantly smaller than the *epfl4 epfl6* double mutant. However, the *epfl1 epfl2 epfl4 epfl6* mutant has the same stature as *er,* suggesting that EPFL1 and EPFL2 regulate plant elongation alongside EPFL4 and EPFL6 (Kosentka *et al.*, 2019). As shorter mutants often show impaired vascular development (Hanzawa *et al.*, 2000; Parker *et al.*, 2003; Pineau *et al.*, 2005; Uchida and Tasaka, 2013), primary and secondary growth might share important regulatory components, including EPFL1,2,4,6 peptides.

Importantly, the EPFL peptides might also synchronise secondary growth with other developmental processes through long-range signalling and competition on the same receptor complex. The hypocotyl of *Arabidopsis* undergoes secondary growth in two phases with phase II occurring after flowering and producing more secondary tissues (Ragni *et al.*, 2011). The phytohormones Gibberellins (GA) represent one of the long-distance signals synchronising flowering with secondary growth (Ben-targem *et al.*, 2020). However, recent studies have shown that an additional, but yet-unknown, long distance signal acts in parallel with GA (Ben-targem *et al.*, 2020). As EPFL2 and EPFL9 sequences significantly differ between vascular and non-vascular plants and these peptides are known to regulate the development of ovules and flowers (Kawamoto *et al.*, 2020), these peptides might regulate secondary growth by coordinating the radial expansion of organs with flowering. Other small-secreted peptides, such as FLOWERING TIME, are long distance signalling molecules which move through the phloem vessels (An *et al.*, 2004). Consequently, EPFL2 and EPFL9 could be transported from the ovules and flowers of the plant to the hypocotyl where they could bind to the ER family receptors. As EPFL2 and EPFL9 are strongly expressed during flowering, these peptides could act antagonistically with EPFL4 and EPFL6 by outcompeting these ligands and repressing secondary growth until flowering is over.

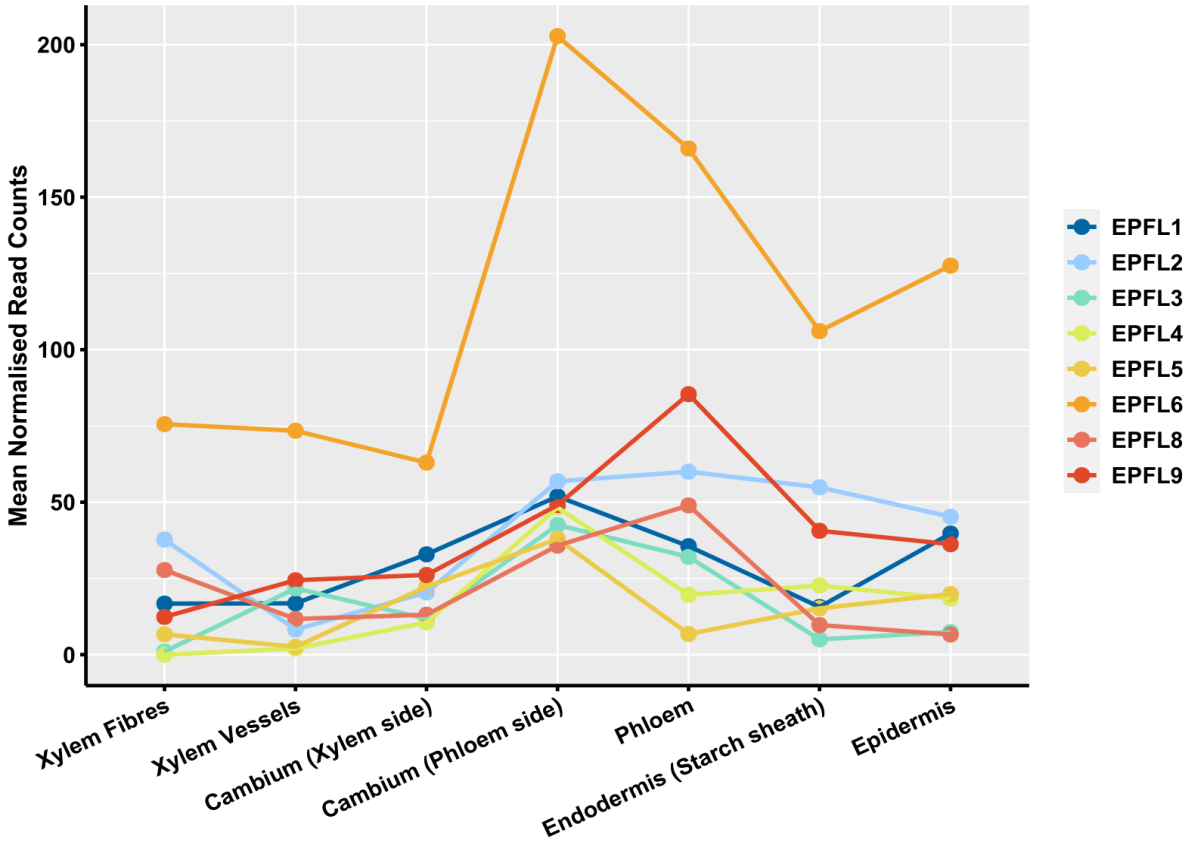


Fig.9. Expression patterns of EPFL1-9 peptides in the inflorescence stem of Arabidopsis from publicly available tissue-specific transcriptomic profiles (Shi et al., 2020). Shi et al. (2020) obtained these tissue-specific transcriptomes using Fluorescence-Activated Nucleus Sorting (FANS) to sort tissue-specific nuclei and extract their RNA for sequencing analysis. The graph omitted EPF1,2 and EPFL7 as they did not appear to be involved in vascular cambium development. The graph was generated from the available data using R v.4.0.2.

To better understand their role in secondary growth, the expression of the potential vascular cambium regulators - EPFL1, 2, 3, 4, 5, 6, 8, 9 - was examined using a recently published tissue-specific transcriptomic database of the inflorescence stem of *Arabidopsis* (Fig.9). EPFL1,2,3,4,5,6 exhibited similar expression patterns but EPFL6 was significantly more expressed compared to other ligands. By contrast, EPFL8 and EPFL9 expression was slightly shifted compared to other ligands (Fig.9). As the expression of the tested EPFL peptides peaked in the phloem side of the cambium or directly in the phloem tissue, these peptides could bind to ERf receptors in the phloem, thus mediating intercellular communication between the cambium and its secondary tissues.

1.5 Concluding remarks:

In this chapter, we showed that most EPFL sequences significantly differed with the loss of vascular cambium, suggesting that these EPFL ligands are relevant genetic components taking part in the complex regulation of secondary growth. In addition to clarifying the evolutionary relationship of the EPFL family peptides, we identified the EPFL peptides which could have evolved in association with the loss of secondary growth. These exciting results on the potential evolutionary trajectory and functions of the EPFL genes might be useful in the future to fully characterise the EPFL peptides regulating secondary growth

In the case of EPFL4 and EPFL6, several experiments would be interesting to carry out in the future such as examining their expression in the periderm, identifying changes in the expression of other EPFL ligands in *epfl4*, *epfl5* and *epfl6* double or triple mutants, and testing the genetic interaction between the EPFL4-6 peptides and PXY signalling by introducing *epfl4 epfl6* mutations in the *pxy* background.

Experiments on the entire EPFL gene family would also need to be conducted to complement our work, such as additional morphogenetic experiments as well as expression analyses in lateral meristems and secondary tissues. Although this study shows that the tested EPFL peptides have similar expression patterns in the stem, the expression analysis should be repeated in organs like the hypocotyl, as the morphology of the hypocotyl significantly differs from the stem and the stem of Arabidopsis lacks a cork cambium. Additionally, EPFL10 and EPFL11 do not have homologues in Arabidopsis and these peptides would need to be studied in another model organism.

Once all EPFL peptides regulating secondary growth will be experimentally characterised, it would be most beneficial to identify the exact modifications in the EPFL genes associated with the evolution of vascular cambium. Altogether, these experiments would help us understand the evolution of secondary growth and its underpinning genetic components.

The existence of several EPFL family peptides with different but partially redundant activities is essential to achieve the complicated and synchronised regulation of various physiological and developmental processes. To understand the functional harmonisation of these proteins, grafting studies and an analysis of the EPFL spatiotemporal expression profile would also represent an interesting research avenue.

Chapter II: Are PXY family and ERECTA family Receptor-Like-Kinases regulating stem cell activity in the root lateral meristems of *Arabidopsis thaliana*?

Quantitative genetics of PXf and ERf loss-of-function mutants.

2.1 Introduction:

Despite forming different tissues, the vascular cambium and the cork cambium display similar structures and functions. Both lateral meristems regulate plant lateral growth, have a cylindrical structure and are formed post - embryonically. Additionally, Xiao *et al.*, (2020) showed that the formation of the cork cambium relies on the development of the vascular cambium, suggesting that a complex inter-tissue communication might coordinate the growth of the vascular and the cork cambia. Due to their structural similarities and potential inter-tissue communication, we hypothesised that part of the molecular network initiating and maintaining stem cell activity is shared between the two lateral meristems. To test this hypothesis, we selected a genetic network known to regulate vascular cambium activity in the hypocotyl and investigated the role of this network in regulating the initiation and/or activity of both cork cambium and vascular cambium in the root.

The selected network consists of ERECTA (ER) family receptor-like-kinases (RLKs) and PHLOEM INTERCALATED WITH XYLEM (PXY) family RLKs. The PXY family (PXf) - composed of PXY, PXY-LIKE1 (PXL1) and PXL2 paralogues – regulates vascular cambium activity in the stem and the hypocotyl of *Arabidopsis thaliana.* Loss of PXY results in a reduction of cell division in the vascular cambium, premature xylem differentiation and a loss of boundaries separating the xylem, cambium and phloem tissues, leading to the disruption of vasculature organisation (Sieburth, 2007; Etchells and Turner, 2010; Etchells *et al.*, 2013). Despite regulating vascular cambium activity in both organs, the function of PXf varies slightly between the stem and the hypocotyl, highlighting that PXf activity is organ dependent (Etchells *et al.*, 2013; Wang *et al.*, 2019). Similarly, the ERECTA family (ERf) receptors – ER, ERL1, ERL2 - are positive regulators of procambium activity in the stem while their functions in the hypocotyl remain unclear (see General Introduction) (Ragni *et al.*, 2011; Etchells *et al.*, 2013; Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019). For this reason, we aimed to "kill two birds with one stone" by examining the roles and interactions of PXf and ERf receptors in *Arabidopsis* root.

The only member of PXY and ER families whose function has already been identified in *Arabidopsis* roots is PXY. PXY is expressed in the vasculature of the root and is a master regulator of stem cell activity in the vascular cambium (Smetana *et al.*, 2019; Xiao *et al.*, 2020). Other PXY paralogues and ER family receptors are expressed in the lateral meristems of the root and their derived secondary tissues (see Chapter III), making them particularly suitable candidates to regulate secondary growth in *Arabidopsis* root. Here, we investigated the individual functions of PXf and ERf receptors in the root.

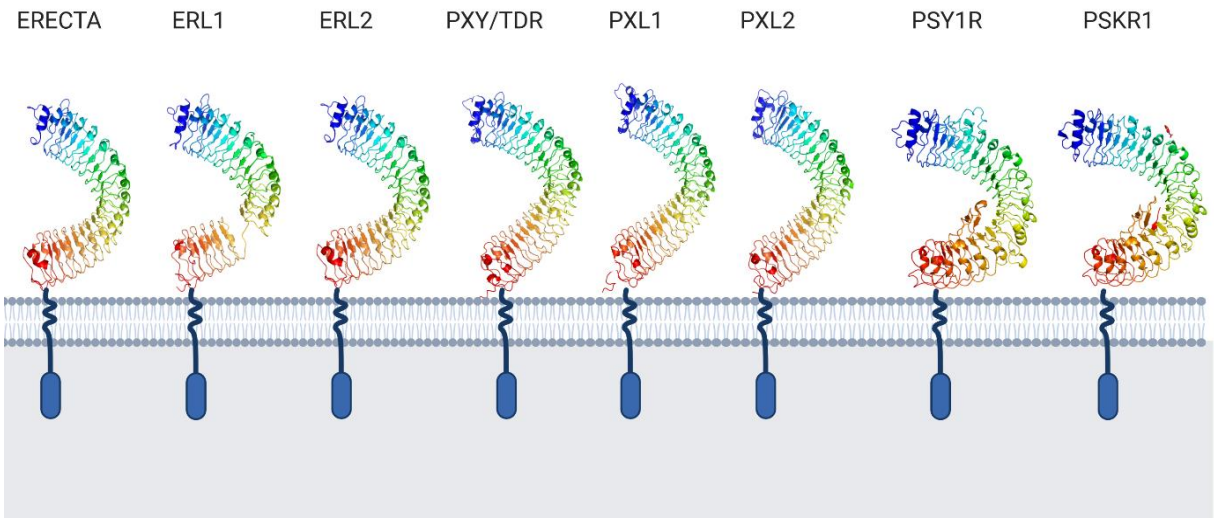


Fig.1. Tertiary structures of the leucine rich repeats (LRR) of receptors studied in this chapter. The ectodomains are represented with rainbow-coloured ribbons, the transmembrane domain is denoted with blue lines crossing the membrane while the kinase domain is displayed with blue ovals. The structure of LRR-RLK ectodomains varies slightly between receptors but the overall architecture of the receptors remains conserved. Each ectodomain displays between 20-29 LRR repeats and the interior concave surface of these LRR has been found responsible for the binding of ligands. Only PSKR1 possesses an island domain which also provides an interface for the binding of the ligand. An island domain is a spacer region between two tandemly arranged LRR repeats (Chakraborty et al., 2019). Additionally, the ectodomains of TDR/PXY, PSKR1, ERL1 and ERL2 already have their structures resolved with TDR (PDB ID: 5JFK) and PSKR1 (PDB ID: 4Z63) having their structures solved individually while ERL1 (PDB template ID: 5XJX), ERL2 (PDB template ID: 5XKN) having their structure elucidated as a receptor-ligand complex. The structure of other LRR-RLKs is modelled using structures of homologous proteins or proteins belonging to the same family such as PSYR1 (PDB template ID: 4z63.1.A), ER (PDB template ID: 5xkn.1.B), PXL1 (PDB template ID: 5gij.1.A) and PXL2 (PDB template ID: 5gij.1.A)

Additionally, we examined the network formed by PXf and ERf to control cell proliferation, cell size, and cell type organization in the lateral meristems. The literature suggests that PXf and ERf have overlapping signalling pathways as both interact with auxin signalling and members of the *HD-Zip III* family (Woodward *et al.*, 2005; Chen *et al.*, 2013). As previously mentioned, PXf and ERf genetically interact to regulate vascular cambium activity in the stem and the hypocotyl, with *pxy er* mutants showing greater vascular organisation defects in the stem and the hypocotyl than those of *pxy* single mutants (General Introduction) (Etchells *et al.*, 2013). Members of PXf and ERf are also known to regulate the expression of one another. In the stem, ER represses PXL1/2 expression and PXf and ER act as activators of ERL1/2 and their ERf ligand (CLL2/CHAL) expression. In the hypocotyl, PXf and ER downregulate ERL1/2 expression (Wang *et al.*, 2019). Altogether, PXf and ERf are likely to interact with each other to regulate stem cell activity in both the cork cambium and the vascular cambium of the root.

As lateral growth of the plant emerges from the division of stem cells in the lateral meristems, we decided to investigate the role of PXf and ERf following these steps: we first identified some candidates among the PXf and ERf which regulate the overall lateral growth of the plant (i.e.

secondary growth). To identify regulators of lateral growth, we measured the total area of the root of loss-of-function mutants and examined the morphological changes in these mutants (Fig.2.Step1).

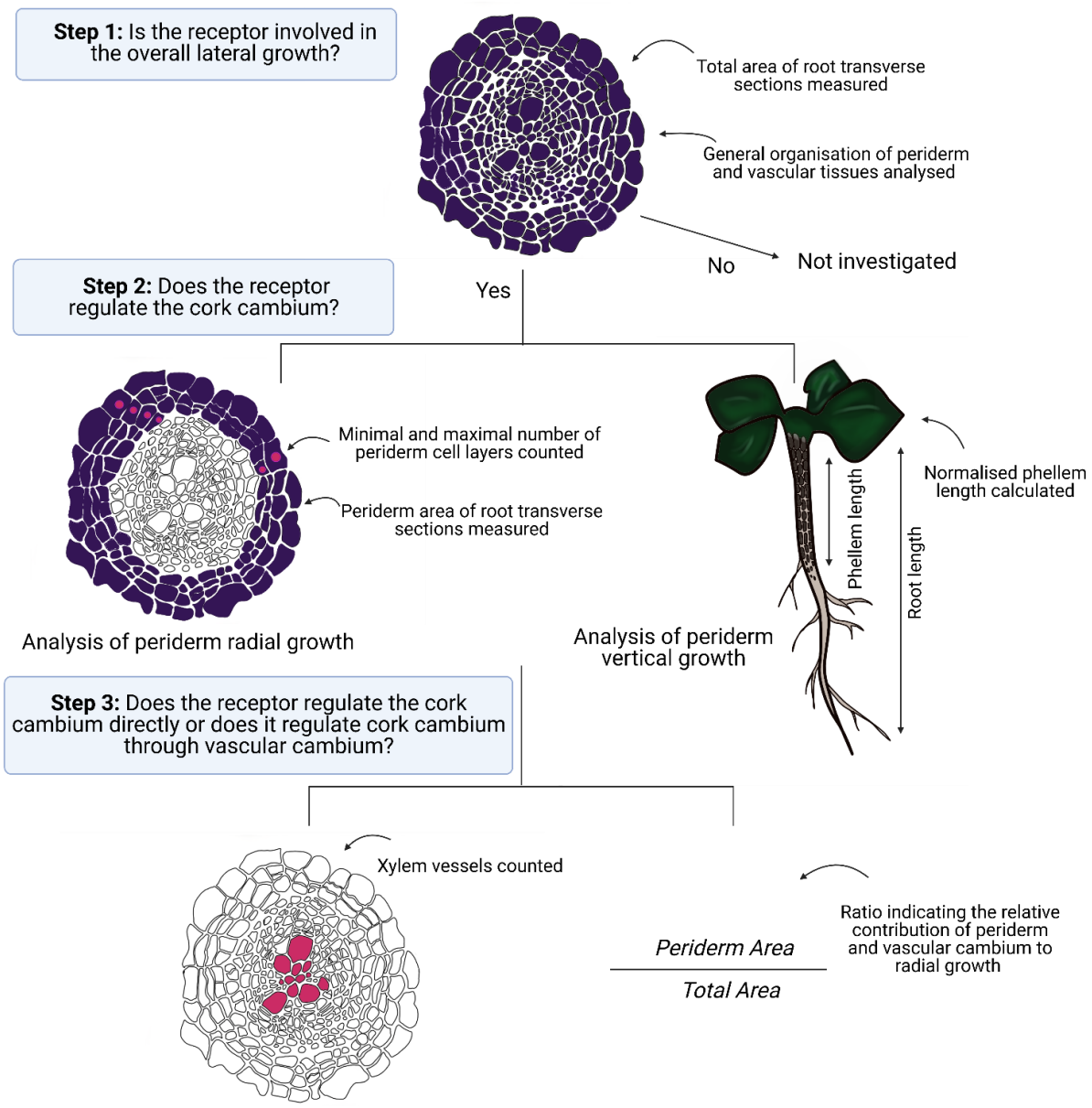


Fig.2. Methodology used to determine the roles and interactions of PXf and ERf receptors in the cork cambium. Loss-of-function mutants were used for transversal sections.

If the tested individual members of PXf and ERf were found to control the lateral growth of the plant, we considered them as candidates to regulate the activity of stem cells in one or both of the lateral meristems. As a result, our next step was to identify which of the lateral meristems between the vascular cambium and cork cambium, was controlled by the tested receptor. To check whether the PXf and ERf candidates regulate stem cell activity in the cork cambium, we quantified periderm growth along the vertical and radial axis by measuring the phellem length normalised to the total root length (vertical axis), the number of periderm cells (radial axis) and the periderm area (radial axis) in root transverse sections of loss-of-function mutants (Fig.2.Step2). Phellem is the external periderm

layer which was used to analyse periderm growth along the vertical axis of the root as it is the only periderm layer observable on the plant surface. The normalised phellem length is hereby also referred to as "phellem length".

After investigating their function in the periderm, we assessed the role of PXf and ERf in regulating the activity of the vascular cambium by counting the number of xylem vessels in root transverse sections of loss-of-function mutants and/or by identifying changes in vascular growth, using the ratio of periderm area over total area as the total area of root transverse section is the sum of vasculature area and the periderm area (e.g. the area of vasculature increases if the ratio of periderm/total area decrease and the total area of the root increases) (Fig.2.Step3).

To reconstruct the regulatory network underpinning the growth of lateral meristems, we first focused on the PXf family by investigating the roles of PXL1 and PXL2 and their interactions with PXY. Then, we extended our knowledge of the regulatory network by investigating the roles of ERf receptors and their interactions with PXf receptors to control the activity of stem cells in the cork and vascular cambium of *Arabidopsis* roots. Finally, the roles of other RLKs, such as PSYR1 and PSKR1, played during vascular and cork cambium development were investigated following the same methodology (Fig.1).

2.2 Materials and Methods:

2.2.1 Materials:

Resource Table:

Reagent or Resource	Source	Identifier
Murashige and Skoog Basal Medium (MS)	Duchefa	Cat# M0255
Plant Agar	Duchefa	Cat# P1001.1000
Technovit 7100	Heraeus Kulzer	Cat# 64709003
Chloral hydrate	Merck	Cat# 1.02425.1000
Glycerol	Roth	Cat# 6962.1
Experimental model: Organisms		
Arabidopsis: Col-0	Widely distributed	N/A
Arabidopsis: ERL2-YFP in *erl2*	(Wang *et al.*, 2019)	N/A
Arabidopsis: ERL2:GUS in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: pxy in Col-0	(Fisher and Turner, 2007)	N/A
Arabidopsis: pxl1 pxl2 in Col-0	(Fisher and Turner, 2007)	N/A
Arabidopsis: pxy pxl1 pxl2 (pxf) in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: pxl1 pxl2 er in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: er erl2 in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: pxf er in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: pxf er erl2 in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: pxy pxl1 pxl2 er erl2 erl1/+ in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: PXL1:GUS in Col-0	(Wang *et al.*, 2019)	N/A
Arabidopsis: PXL2:GUS in Col-0	(Wang *et al.*, 2019)	N/A

Arabidopsis: pskr1-2 psk2-1 in Col-0	(Ladwig *et al.*, 2015)	
Arabidopsis: 35S:PSKR1 in Col-0	(Ladwig *et al.*, 2015)	
Arabidopsis: pskr1-3 psy1R in Col-0	(Ladwig *et al.*, 2015)	
Arabidopsis: At3g47570-LRR receptor in Col-0	NASC	GK-155E07 1-12
Arabidopsis: At3g47570-LRR receptor in Col-0	NASC	GK-415H04 1-17
Arabidopsis: At1G07650-LRR receptor in Col-0	NASC	SALK-021171C
Software		
ZEN Black (Zen 2.3 SP1)	Zeiss	https://www.zeiss.de/corporate/home.html
Fiji	(Schindelin *et al.*, 2012)	https://fiji.sc/
R v.4.0.2	The R foundation	https://rstudio.com/

Experimental model:

Arabidopsis thaliana transgenic and mutant lines were used to perform experiments. The ecotype and background of each line is specified in the material table. The plants were grown in continuous light condition *in vitro* on ½ MS plates supplemented with 1% sucrose and 0.8 plant agar for 12 days.

2.2.2 Method Details:

Histology and Light Microscopy:

Root samples of 1 cm were sectioned 0.5mm under the hypocotyl and embedded following de Reuille and Ragni (2017)'s protocol. The tissues were fixed in a mixture of 4% paraformaldehyde, 0.1% Triton-X, 50mM Phosphate buffer pH7.2 for three days overnight at room temperature. They were then dehydrated with a graded series of ethanol to 100% ethanol, infiltrated with polymethacryl resin Technovit 7100 and embedded and polymerised in Technovit 7100 (Heraeus Kulzer). From these embedded root, thin plastic cross sections of 5-7 µm were obtained using the microtome and stained with 0.1% toluidine blue. All sections were mounted in in chloral hydrate solution (8:3:1; Chloral hydrate: Water: Glycerol).

All cross sections were imaged with a Zeiss Axiophot microscope coupled with a Zeiss Axiocam 512 color digital camera. The confocal images from whole-mount *erl2* ERL2:YFP samples were acquired with Dr. Ragni using a Zeiss LSM880 microscope. To identify the yellow fluorescent protein (YFP) in our images, the excitation wavelength was set at 490 nm and the emission wavelength was set at 510 nm. due to the absence of signal, no three-dimensional reconstructions and Ortho Views of a Z-stack were required.

Periderm and Vascular Quantification:

15 independent plants were analysed for each genotype unless stated otherwise. The phellem ratio was measured using ImageJ (Fiji). As described in Wunderling *et al.* (2018), the whole roots were mounted in 10% glycerol on a microscope slide. Using light microscopy, the closest point to the root tip where phellem can be identified as an outside tissue is marked (stage I periderm). The slides were

scanned and the root length and phellem length (from the base of the hypocotyl to phellem cells starting to emerge as an outside tissue) were measured with Fiji, allowing to calculate the ratio of phellem length : root length. To quantify the periclinal cell divisions in the periderm, the minimum and maximum number of periderm layers per cross-section per sample were counted (e.g. min = 2 and max = 5). This minimum and maximum number of layers was averaged (e.g. using the previous example, the average is 3.5) and according to this average, each individual was assigned a class (e.g. using the previous example, the class assigned is 3-4). The frequency of individuals belonging to each class was calculated and displayed as percentage. To measure the periderm area and the total area of root cross section we used the software Fiji while the number of xylem vessels was quantified using the CELL COUNTER plug-in of Fiji.

Quantification and statistical analyses:

Statistical analyses were performed using the statistical packages of RStudio. The datasets were first tested for normal distribution using a Shapiro-Wilk test and for homogeneity of variances using a Levene test. When the data was normally distributed, the significant differences between each dataset were calculated with a one-way ANOVA test followed by a Tukey's post hoc test (equal variances assumed) or a Tamhane's post hoc (equal variances not assumed). When the data was not normally distributed, the differences between each dataset were calculated with a non-parametric Kruskal-Wallis test followed by a Dunn's post hoc test. The threshold for significance was indicated with P values in the Table (the groups are significantly different when the P value < 0.05) and the sample groups which were significantly different from the wild-types were marked with asterisks in the graphs.

2.3 Results:

2.3.1 PXY family (PXf) regulates the overall lateral growth of the root as well as the organisation of the vasculature and the periderm tissues:

In this experiment, we investigated the role and interactions of PXY family members, PXY, PXL1 and PXL2 in root lateral growth by measuring the total area of root transverse sections of loss-of-function mutants (Fig.3A).

First, we sought to understand the interaction between PXY with the other members of the PXY family, PXL1 and PXL2. In accordance with previous studies (Xiao *et al.*, 2020), the root transverse sections of *pxy* loss-of-function mutants displayed a reduced area compared to the wild-type (Fig.3.A), confirming that PXY positively regulates the lateral growth of *Arabidopsis*' root (significant in Xiao *et al.*, 2020). Similarly to the results of Xiao *et al.* (2020), we observed fewer vascular cambium cells between the xylem and phloem cells (about 2 vascular cambium cell layers compared to 4 in the wild-type) and less periclinal division in the transverse sections of *pxy* mutant roots (Fig.3.B). These results confirmed that PXY is master regulator and enhancer of lateral growth regulating tissue organisation and cell division.

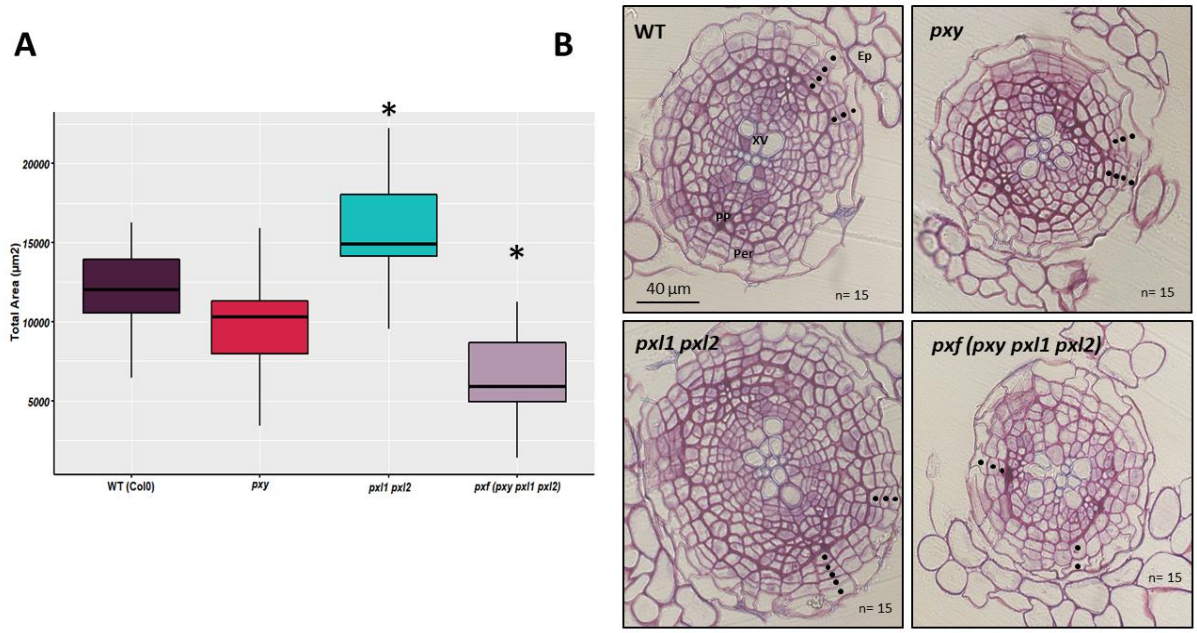


*Fig.3. Root lateral growth and organisation in Arabidopsis t. WT, pxy, pxl1 pxl2 and pxf (pxy pxl1 pxl2) mutants. A) Total Root Area of PXY family mutants cross section compared to the wild-type. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers. The asterisks indicate that the mean total area of the sample is significantly different from the wild-type (see table). B) Root transverse sections of the wild-type and PXY family loss-of-function mutants (Cell types: XV: Xylem Vessels, PP: Phloem Pole, Per: Periderm, Ep: Epidermis). The black dots indicate the minimum and maximum number of periderm cell layers per cross section. n specifies the number of individuals for each tested genotype.*

To understand the roles of PXL1 and PXL2 in the root, we analysed the lateral growth and general morphology of *pxf (pxy pxl1 pxl2)* and *pxl1 pxl2* mutants. Previous studies demonstrated that *pxl1* and *pxl2* enhance the *pxy* phenotype in the stem and the hypocotyl (Fisher and Turner, 2007; Etchells *et al.*, 2013; Wang *et al.*, 2019). By removing all members of the PXY family, the total area of *pxf* mutant cross sections, and therefore its lateral growth, was drastically reduced compared to the wild-type and the *pxy* single mutant (Fig.3.A.B). This strong phenotype might have resulted from the additive effects of PXY, PXL1 and PXL2 genes loss, suggesting that, together, PXL1 and PXL2 positively regulate the activity of one or both lateral meristems. However, the *pxl1 pxl2* double mutant surprisingly showed an opposite root phenotype to the one observed in the *pxy* mutant and *pxf* mutant. The total area of the cross sections of *pxl1pxl2* mutants' roots was strongly increased, contrasting with the reduced total area of the *pxy* and *pxf* mutant (Fig.3.A). Preliminary assessment of the regulatory function of PXL1 and PXL2 estimated the cell number and cell size in the transverse sections of *pxl1 pxl2* mutant. An apparent increase in cell number was observed in *pxl1 pxl2* transverse sections while the cell size of this mutant remained indistinguishable from wild-type. As previously reported in the hypocotyl and the stem (Fisher and Turner, 2007; Etchells *et al.*, 2013; Wang *et al.*, 2019), the *pxl1 pxl2* double mutant maintained the same root tissue organisation as the wild-type and formed simultaneously a vascular and cork cambium (Fig.3.B). These results showed that PXY and its paralogues maintain a complex relationship to regulate secondary growth (see discussion).

2.3.2 PXf receptors are involved in periderm development:

First, PXY was confirmed to regulate indirectly periderm growth by controlling the development of the vascular cambium - as vascular cambium is necessary and sufficient for periderm growth (Xiao *et al.*, 2020). In accordance with Xiao *et al.* (2020), loss of PXY led to a slightly decreased normalised phellem length along the vertical axis of the root (Fig.4.B). The distribution of periderm cell layers in *pxy* mutant ranged between 3-4 cells, like the wild-type (Fig.4A). Altogether, this data corresponded to previous studies (Xiao *et al.*, 2020) and the subtle defects observed in periderm vertical growth in the *pxy* mutants were indirectly caused by severe vascular cambium defects.

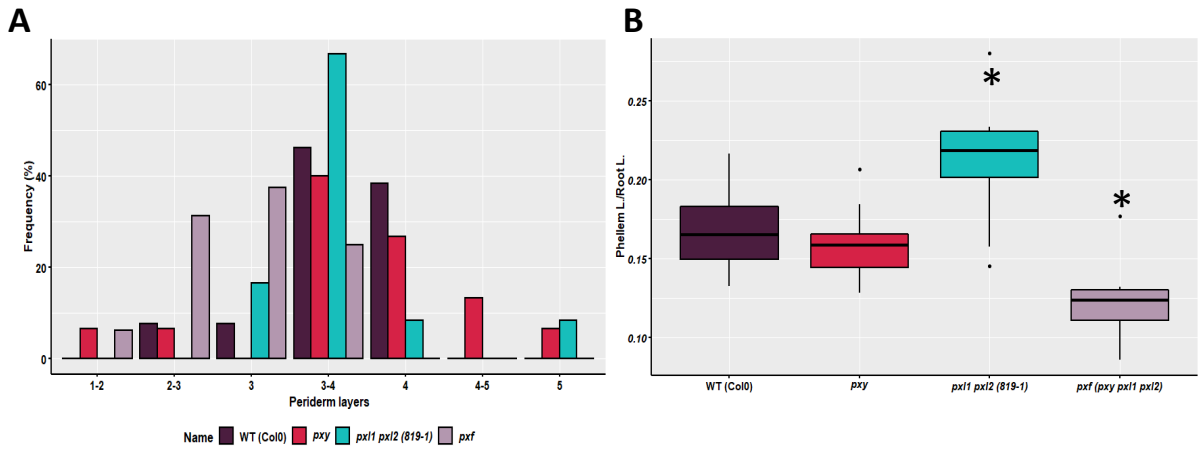


*Fig.4. Periderm study in the PXY family loss-of-function mutants. A) Histogram displaying the frequency of the tested genotypes for each range (minimum and maximum) of periderm cell layers around the circumference of root transverse sections. Each label on the x-axis represents a range of min. and max. periderm cell layers observed (e.g. the second label "2-3" corresponds to individuals possessing 2 to 3 layers of periderm while the third label "3" corresponds to individuals possessing 3 periderm layers all around the circumference of their root transverse sections with 3 layers being the min. and max. periderm cell layers in these individuals) B) Phellem length normalised to the total root length for each individual in the tested genotype. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers.The asterisks indicate that the mean phellem ratio of the sample is significantly different from the wild-type (see table).*

Due to their expression patterns in the periderm and its neighbouring tissues, PXL1 and PXL2 were interesting candidates to regulate periderm development (Chapter III). Both *pxl1 pxl2* and *pxf* double and triple mutants had periderm phenotypes corresponding to their respective lateral growth phenotypes. The *pxf* mutant phenotype displayed a severely reduced normalised phellem length as well as a clear reduction in the number of periderm layers with a distribution mean (= peak) at 3 periderm cell layers (mean = 2.8) compared to 3-4 periderm cell layers in the wild-type (mean = 3.5) (Fig.4). On the other hand, the normalised phellem length of the *pxl1 pxl2* mutant significantly increased and the distribution of *pxl1 pxl2* mutants across ranges of periderm cell layers slightly differed from wild-type (Fig.4). Some *pxl1 pxl2* mutants displayed up to 5 cell layers in their periderm while the wild-type plants only reached a maximum of 4 periderm cell layers, although the average number of periderm layers did not change (Fig.4.A). Overall, PXL1 and PXL2 appeared to regulate cork

cambium activity, particularly along the apical-basal axis of the plant. As Xiao *et al.*, 2020 found that proper vascular cambium development leads to the formation of cork cambium, this raised the question of whether PXL1 and PXL2 directly manipulate cork cambium activity or whether they control the development of the vascular cambium, which in turn affects periderm formation.

2.3.3 PXf specifically regulates the vascular cambium and/or the cork cambium:

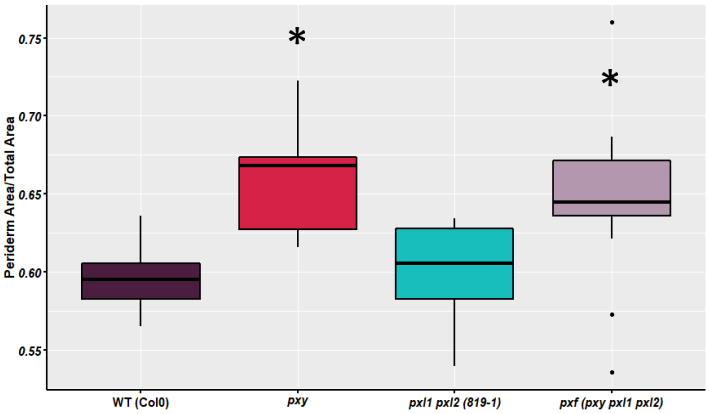


*Fig.5. Periderm area normalised to the total area of transverse sections of PXY family loss-of-function mutants. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers. The asterisks indicate that the mean periderm ratio of the sample is significantly different from the wild-type (see table).*

After investigating the role of PXf in the overall lateral growth, and the periderm growth of roots, an assessment of whether PXf regulate the vascular cambium and/or the cork cambium was made. The periderm area normalised to the total root area represents a quantitative measure of the relative contribution of periderm and vascular cambium to radial growth (as the total area is the sum of the vascular area and the periderm area). PXY family single, double and triple mutants, were tested to identify which of the vascular or cork cambium was more affected by the loss of PXY family members.

Measurements of the *pxy* mutant corresponded to the results of Xiao *et al.* (2020). The *pxy* mutant had an increased ratio of periderm area over the root total area, indicating that, in absence of PXY, the vasculature had a more severe defect than the periderm (Fig.5). These results confirmed that PXY mainly regulates vascular growth. On the other hand, the *pxl1 pxl2* mutants maintained the wild-type ratio of periderm and vasculature area (Fig.5), indicating that loss of PXL1 and PXL2 affect both the vascular and cork cambium activity. However, loss of PXY in a *pxl1 pxl2* mutant background - *pxf* mutant – led to an increased ratio of periderm area/total area, as was the case with the *pxy* single mutant (Fig.5). Altogether, these results indicated that PXL1 and PXL2 regulate both vascular and periderm growth while PXY specifically regulates the vascular cambium.

2.3.4 The roles of ERf receptors and their interactions with PXf regulating the radial expansion and the general morphology of the root:

Genotypes lacking both PXf and ERf members were studied to understand the genetic interactions between PXf and ERf and deduce the individual roles of ERECTA family members during secondary growth.

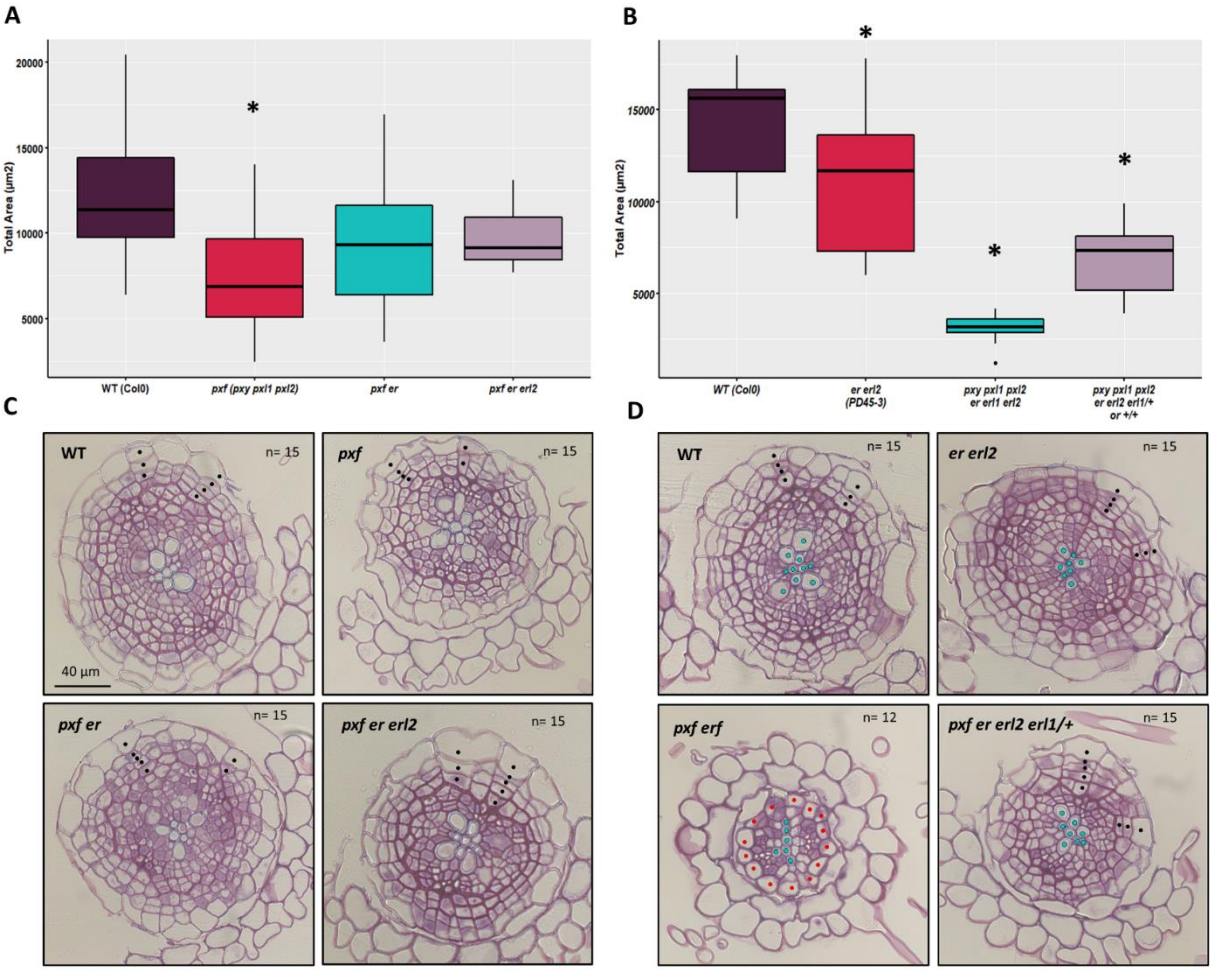


*Fig.6. Root lateral growth and root morphology in the PXY family and ERECTA family loss-of-function mutants. A) and B) Root total area of PXY and ER families mutants cross sections compared to the wild-type. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers. The asterisks indicate that the mean total area of the sample is significantly different from the wild-type (see table). C) and D) Root transverse sections of PXY and ER families loss-of-function mutants where the minimum and maximum number of periderm layers per cross section is indicated with black dots. The red dots mark the pericycle cells and the blue dots indicate the xylem vessels. "n" specifies the number of individuals for each tested genotype.*

The roles of ERf receptors in regulating lateral growth:

We examined the role of ERL2 in the lateral growth of roots and its interactions with ER and PXf. In earlier studies, ERL2 was found to be only expressed in the late developmental stage of hypocotyl and ERL2 was found to maintain the lateral growth of the hypocotyl in the absence of PXf and ER (Ikematsu *et al.*, 2017; Wang *et al.*, 2019). Here, no significant difference between the total areas or the tissue organisation of *pxf er* and *pxf er erl2* root transverse sections were observed (Fig.6.A.B). This data suggested that ERL2 is not involved in the lateral growth of the root at this developmental stage.

By contrast, in the *er erl2* root transverse sections, a decreased lateral growth phenotype was observed (Fig.6.B). This may be attributable to the *er* mutation alone as ERL2 seemed not involved in secondary growth (see above). The *er erl2* root maintained a near-wild-type organisation, as the root formed both a vascular cambium and a periderm (Fig.6.D). While previous measurements of *pxf er* showed that PXf and ER interact in the root, these results suggested that ER is a positive regulator of lateral growth in the root and that ER might not be required to initiate or maintain the activity of the vascular and/or cork cambium. Surprisingly, ER seems to have opposite roles in the root and the hypocotyl (at least compared to the results of Ikematsu *et al.*, (2017)), while sharing the same function in the root and the stem (Uchida *et al.*, 2012; Uchida and Tasaka, 2013). Interestingly, our results corresponded to what Etchells *et al.*, 2013 found in the hypocotyl. To verify these data, the phenotype of *er* single mutant would be interesting to analyse.

To understand the role of ERL1 in root lateral growth, we restored one or both ERL1 alleles in the *pxf erf* mutant background, giving rise to *pxf er erl2 erl1/+* and *pxf er erl2* mutants. ERL1 is known to act redundantly with ER in the stem and the hypocotyl, being respectively a stem enhancer and hypocotyl repressor of lateral growth (Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017). With wild-type ERL1 alleles, the root phenotype of *pxf er erl2 erl1/+* and *pxf er erl2* is partly rescued compared to the sextuple mutant *pxf erf*. Despite its reduced radial growth compared to the wild-type, the total area of *pxf er erl2 erl1/+* and *pxf er erl2* root transverse sections drastically increases when one ERL1 allele is restored in the *pxf erf* mutant, indicating that ERL1 might be a positive regulator of root lateral growth (Fig.6.B). Additionally, the periderm and a few vascular cambium cells were formed in the *pxf er erl2 erl1/+* and *pxf er erl2* while the *pxf erf* mutant did not develop any periderm tissue and formed a very limited number of vascular cambium cells (Fig.6.D). These results highlighted the importance of ERL1 to promote periderm and vascular formation in the absence of ER and suggest that one member of the ERf and PXf is sufficient to form vascular cambium and periderm, thus indicating that members of ERf and PXf have overlapping functions.

The interactions between ERf and PXf receptors to regulate lateral growth:

Previous studies on the inflorescence stem showed that PXY, PXL1 and PXL2 function redundantly with ER to regulate vascular proliferation, suggesting that ER and PXf constitute a mechanism to organise vascular cell layers in the stem (Etchells *et al.*, 2013;). However, these interactions are organ dependent as they were shown to differ between the stem and the hypocotyl. In the hypocotyl, PXY is the only member of the PXf to interact with ER and regulate its vascular organisation while PXL1 and PXL2 did not interact with PXY or ER receptors nor did they regulate hypocotyl lateral growth (Wang *et al.*, 2019). Due to this organ-dependency, this section focuses on the interactions of PXf and ERf in the third organ of Arabidopsis undergoing secondary growth, the root.

The interaction of ER and PXf in the root was investigated by quantifying the overall root lateral growth with the total area of the root in transverse sections and by assessing morphological changes in the vasculature and the periderm. Interestingly, loss of *er* partly rescued the lateral growth phenotype observed in *pxf*, although the lateral growth of *pxf er* remained reduced compared to the wild-type (Fig.6.A). In terms of morphology, no clear difference could be observed in the transverse sections of the *pxf* and the *pxf er* roots. Despite *pxf* and *pxf er* having fewer vascular cambium cells compared to the wild-type, the two mutants retained the capacity to form vasculature and periderm (Fig.6.C). Reduction of *pxf* phenotype by removing ER suggested that ER genetically interacts with PXf in the root to regulate general lateral growth. Additionally, no significant difference between the total areas or the tissue organisation of *pxf er* and *pxf er erl2* root transverse sections were observed, suggesting that ERL2 does not interact with PXf and ER to regulate root development.

By analysing *pxf erf* (*pxy pxl1 pxl2 er erl1 erl2*) sextuple mutants, we sought to determine the interactions between ERf and PXf and the roles of these two families in the overall lateral growth (radial expansion) of the root. Preliminary data showed that ERf and PXf genetically interact with one another to coordinate lateral growth in the hypocotyl and the inflorescence stem by controlling cell division and cell size (Etchells *et al.*, 2013; Wang *et al.*, 2019), but the relative contributions of cambium versus periderm was not known. To address this question, a more detailed study of the sextuple mutant was conducted. The total area of *pxf erf* root transverse sections was severely reduced in this experiment, indicating that the lateral growth of *pxf erf* mutant roots was nearly absent (Fig.6.B), in agreement with Wang et al. (2019). After complete removal of the PXf and the ERf, the root did not form any periderm and very few cell divisions occurred in the vascular cambium. Additionally, the few xylem vessels present remained extremely small (Fig.6.D). Altogether, these results suggested that PXf and ERf genetically interact in the root to initiate and maintain the cork cambium and to maintain the activity of the vascular cambium as well as regulating vascular cell size. Interestingly, PXf and ERf coordinated root lateral growth but not root elongation as the length of *pxf erf* roots appeared near-wild-type roots. An analysis of the periderm and vascular cambium was carried out at a cellular level in the following section.

Here, we showed that ER and ERL1 are both positive regulators of lateral growth at this developmental stage of the root while ERL2 is not. PXf and ERf have overlapping functions and coordinate root lateral growth but not root elongation. Additionally, PXf and ERf genetically interact in the root to initiate and maintain the cork cambium and to maintain the activity of the vascular cambium. To further our understanding of the specific roles and interactions occuring in the respective lateral meristems, the following section examined ERf receptors and their interaction with the PXf in the periderm and in the vasculature separately.

2.3.5 The roles of the ER family and its interactions with the PXY family to regulate cork cambium:

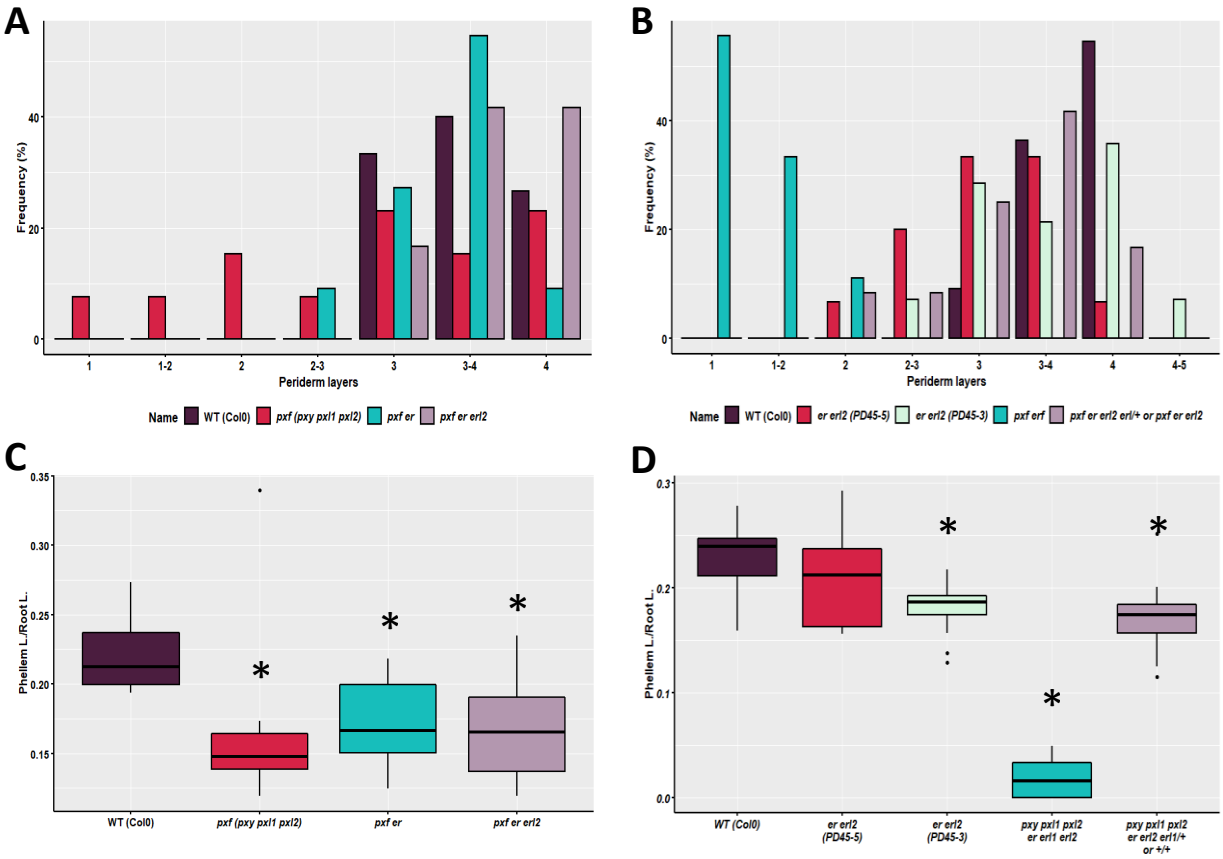


*Fig.7. Roles of PXf and ERf in regulating root periderm growth. A) and B) Histogram displaying the frequency of the tested genotypes for each range (minimum and maximum) of periderm cell layers around the circumference of root transverse sections for the tested genotypes. Each label on the x-axis represents a range of min and max periderm cell layers observed (e.g. the second label "1-2" corresponds to individuals possessing 1 to 2 layers of periderm while the third label "2" corresponds to individuals possessing 2 periderm layers around the circumference of their root transverse sections with 2 layers being the min and max periderm cell layers in these individuals). C) and D) Phellem length normalised to the root length for each tested genotype, pxf, pxf er, pxf er erl2, er erl2, pxf erf, pxf er erl2 erl1/+ or pxf er erl2. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers. The asterisks indicate that the mean of the phellem ratio of the sample is significantly different from the one of the wild-type (see table).*

Roles of ERf receptors in regulating the cork cambium:

We examined the phellem length and number of periderm cell layers of the *pxf er erl2* mutant to understand the role of ERL2 and its interactions with PXf and ER to regulate periderm growth and cork cambium activity. Previously, we showed that ERL2 does not regulate the lateral growth or the tissue organisation of roots. As expected from the lateral growth and morphological results, the number of periderm layers and the normalised phellem length of *pxf er erl2* remained similar to that of *pxf er*, indicating that ERL2 does not regulate cork cambium activity by itself (Fig.7.A.C).

As ERL2 did not seem to regulate periderm development, the phenotype of *er erl2* was used to identify the role of ER in periderm growth. The *er erl2* (PD45-5) mutants formed 3 periderm layers on average (mean = 3.1) while the wild-type had 4 periderm cell layers on average (mean = 3.7) (peak (Fig.7.B). Additionally, the normalised phellem length of the *er erl2* mutant was significantly reduced compared to the wild-type (Fig.7.D). These results correlated with the lateral growth experiment and suggested that ER might be a potential enhancer of periderm growth.

By restoring ERL1 alleles to the *pxf erf* mutant, the function of ERL1 in periderm formation could be assessed. In previous lateral growth experiments, the function of ERL1 was found to be redundant to ER, with ERL1 being a positive regulator of root radial expansion (Fig.6). In terms of periderm development, the *pxf er erl2 erl1/+* or *+/+* mutants had 3-4 periderm layers in average (mean = 3.25), while the wild-type had about 4 periderm layers. Similarly, the normalised phellem length in *pxf er erl2 erl1/+* or *+/+* mutants was reduced compared to the wild-type (mean = 3.7) (Fig.7.B.D). However, by restoring ERL1, the phellem length in *pxf er erl2 erl1/+* or *+/+* drastically increased compared to *pxf erf* sextuple mutant (Fig.7.D). The presence of one functional ERL1 allele in a *pxf erf* background partly rescued the *pxf erf* periderm phenotype, confirming the hypothesis that ERL1 is a positive regulator of periderm development.

PXf – ERf interactions regulating cork cambium activity:

The importance of the PXf - ER interaction in the vascular cambium had been described previously (Etchells *et al.*, 2013; Wang *et al.*, 2019), but not so in the initiation and maintainance of the cork cambium. In this section, the normalised phellem length of the *pxf* mutant and the number of periderm layers in the *pxf* mutants was found to be reduced, with 3 periderm cell layers on average (mean = 2.9) compared to 3-4 periderm cell layers in the wild-type (mean = 3.6). On the other hand, the *pxf er* mutant had about 3-4 periderm cell layers (mean = 3.4) like the wild-type (Fig.7.B). The normalised phellem length was partially rescued in the *pxf er* compared to the *pxf* phenotype (despite remaining significantly smaller than the wild-type) (Fig.7.D). Similar to the lateral growth experiment, *er* loss-of-function mutant in a *pxf* background partly rescued the periderm phenotype of *pxf* mutant. These results highlight the importance of ER interacting with PXf to regulate cork cambium/phellogen activity. Additionally, the number of periderm layers and the normalised phellem length of *pxf er erl2* remained similar to the one in *pxf er*, indicating that ERL2 does not interact with ER and PXf signalling pathways to regulate cork cambium activity (Fig.7.A.C).

To investigate PXf and ERf interactions and their importance in periderm formation, we quantified periderm growth in the root transverse sections of *pxf erf*. As previously seen in *pxf erf* transverse sections (Fig.6.B), *pxf erf* does not form any periderm but has a pericycle cell layer from which the periderm normally originates through anticlinal division. As a result, the single cell layer observed in most *pxf erf* mutant (Fig.6.B) and counted as periderm is in fact the pericycle layer (Fig.7.B). The pericycle just started to divide in a few *pxf erf* individuals, forming two cell layers which could become periderm given some time (Fig.7.B). This limited development of periderm correlated with the absence of phellem in Fig.6.B. The late formation of periderm or lack or periderm tissue suggested that PXf and ERf genetically interact to initiate and maintain cork cambium activity.

Interestingly, no significant difference could be observed between *pxf er erl2 erl1/+* or *+/+* and *er erl2* in terms of normalised phellem length and the number of periderm cell layer (Fig.7.B), highlighting the fact that *pxf* and *er erl2* do not have additive phenotypes despite PXf and ER being considered positive regulators of periderm growth.

After determining that PXf and ERf have crucial roles in periderm formation, the following section examined whether PXf and ERf regulate cork cambium initiation/activity through inter-tissue communication (as Xiao *et al.*, (2020) revealed inter-tissue dependency between the vascular and the cork cambium where the formation of the cork cambium relies on the proper development of the vascular cambium). We investigated the impact of ERf and PXF on both periderm or vascular tissue.

2.3.6 The roles and interactions between ERf and PXf regulating both vascular cambium and cork cambium activity:

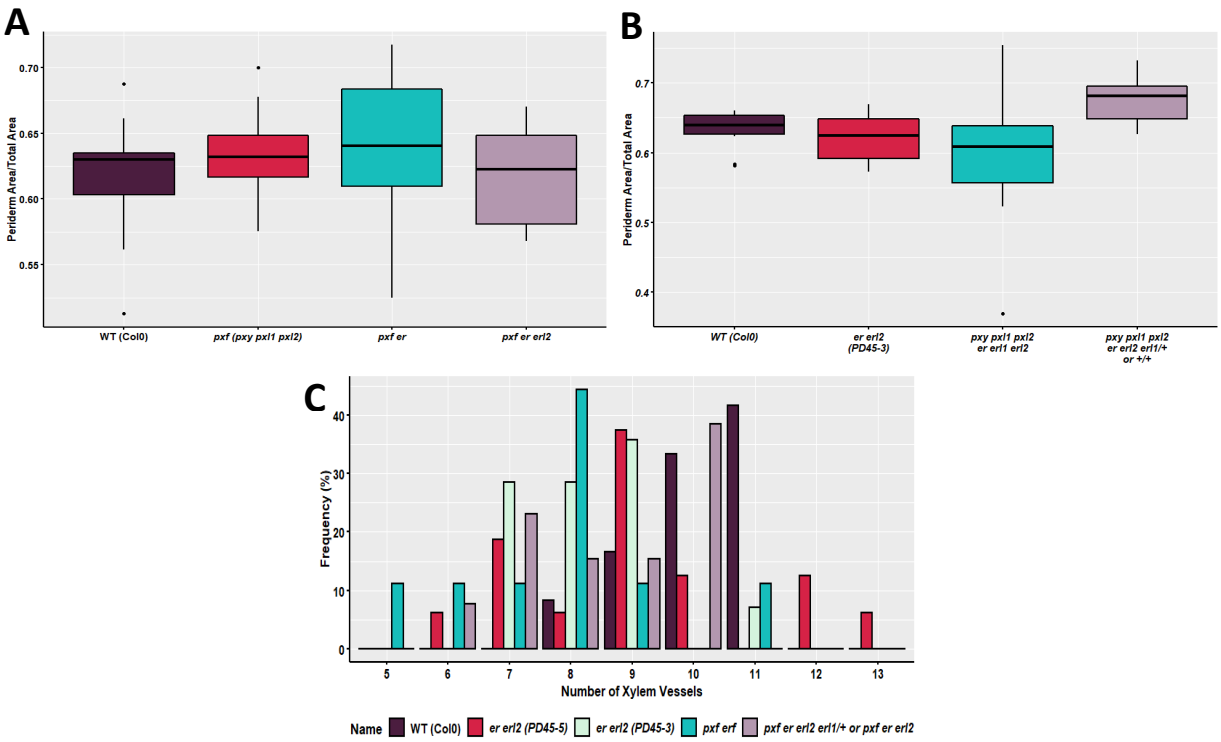


*Fig.8. Vascular cambium vs cork cambium activity in PXf ERf loss-of-function mutants. A) and B) Periderm area normalised to the total area of root transverse section in the tested genotypes pxf, pxf er, pxf er erl2, er erl2, pxf erf, pxf er erl2 erl1/+ or pxf er erl2. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers. The absence of asterisks indicates that the periderm ratios of the samples are not significantly different from the one of the wild-type. The ratio of periderm area/total area in the pxf erf mutant used pericycle cells instead of periderm cells as periderm tissue did not form in these mutants B) Distribution of the number of xylem vessels in the tested genotype er erl2, pxf erf and pxf er erl2 erl1/+ or pxf er erl2.*

As the total area of root transverse sections equals the sum of the periderm area and the vascular tissue area (Spicer and Groover, 2010), we used the periderm area normalised to the total root area of loss-of-function mutants to quantify which of the periderm or vascular tissue growth might be the most impacted by the loss of ERf and/or PXf members.

Role of ERf receptors in regulating the vascular cambium:

In the previous experiments, ERL2 was shown not to be involved in lateral growth or periderm development. Similarly, we used *pxf er erl2* to demonstrate that ERL2 is also not involved in vascular

cambium activity. In Fig.8.A, the ratio of periderm area over total area in *pxf er erl2, pxf er* and the wild-type remained the same, indicating that vascular growth is not affected by loss of ERL2. As a result, ERL2 does not regulate the vascular cambium nor the cork cambium. The function of other RLKs in root development was estimated by examining *er erl2, pxf er erl2, pxf er erl2 erl1/+* or *+/+* and *pxf erf* mutants and not taking into account ERL2 as potential regulator of vasculature or periderm formation.

While the overall lateral growth of *pxf*, *pxf er*, *er erl2* and *pxf er erl2* roots was shown to be drastically reduced compared to the wild-type (Fig.1), the ratio of periderm area over total area in these mutants did not differ from the wild-type. This suggested that both the vascular growth and periderm growth are affected by the loss of PXf and ER. The proportional decrease in both cork and vascular cambium activity could be explained by the vascular cambium and cork cambium co-dependency. As Xiao *et al.* (2020) previously showed, a defective vascular cambium activity leads to a defective cork cambium activity.

Although the overall lateral growth of *pxf er erl2 erl1/+* or *+/+* remained slightly reduced compared to the wild-type, restoring a functional ERL1 allele partly rescued the *pxf erf* lateral growth phenotype. In this section, the ratio of periderm area/total area of *pxf er erl2 erl1/+* or *+/+* was slightly above but not significantly different from the wild-type (p value = 0.224). These results suggested that ERL1 rescues the sextuple *pxf erf* mutant phenotype by enhancing both the vascular growth and the periderm growth and might have a slightly stronger enhancing action on cork cambium activity. Additionally, the number of xylem cells in the *pxf er erl2 erl1/+* or *+/+* mutant averaged 11 cells like the wild-type while *pxf erf* had about 8. Altogether, these results indicated that ERL1 might be an enhancer of both cork cambium (phellogen) and vascular cambium activity and that ERL1 might have a slightly stronger regulatory role in cork cambium activity.

Interactions between ERf – PXf regulating the vascular cambium:

Finally, we investigated the interactions between ERf and PXf families and their functions to regulate vascular cambium using the *pxf erf* sextuple mutant. As seen in previous sections, the *pxf erf* mutant did not form any periderm tissue (only pericycle tissue not yet divided nor differentiated into periderm), had a severely reduced number of vascular cambium cells and did not undergo any lateral growth (Fig.6.D). To complete these studies, we measured the changes in vascular cambium activity in the *pxf erf* mutant by counting the number of xylem vessels it formed. The *pxf erf* mutant formed about 8 xylem vessels (mean = 7.8), which was significantly lower compared to the 10 xylem vessels found in average in the wild-type. However, only 5 xylem vessels were usually generated during the embryonic stage (primary growth)(ten Hove, Lu and Weijers, 2015). These results indicated that *pxf erf* mutants formed 2 additional xylem vessels in average, as part of secondary growth. To conclude, PXf and ERf were sufficient and necessary for the maintenance of the vascular cambium and cork cambium activity. The extremely reduced cell divisions in vascular cambium and pericycle showed that PXf and ERf genetic network might be necessary to initiate cambial activity at the appropriate developmental stage.

2.3.7 Identification of other RLK as potential cork cambium regulators, the Phytosulfokine receptors (PSKRs) and PSY1R:

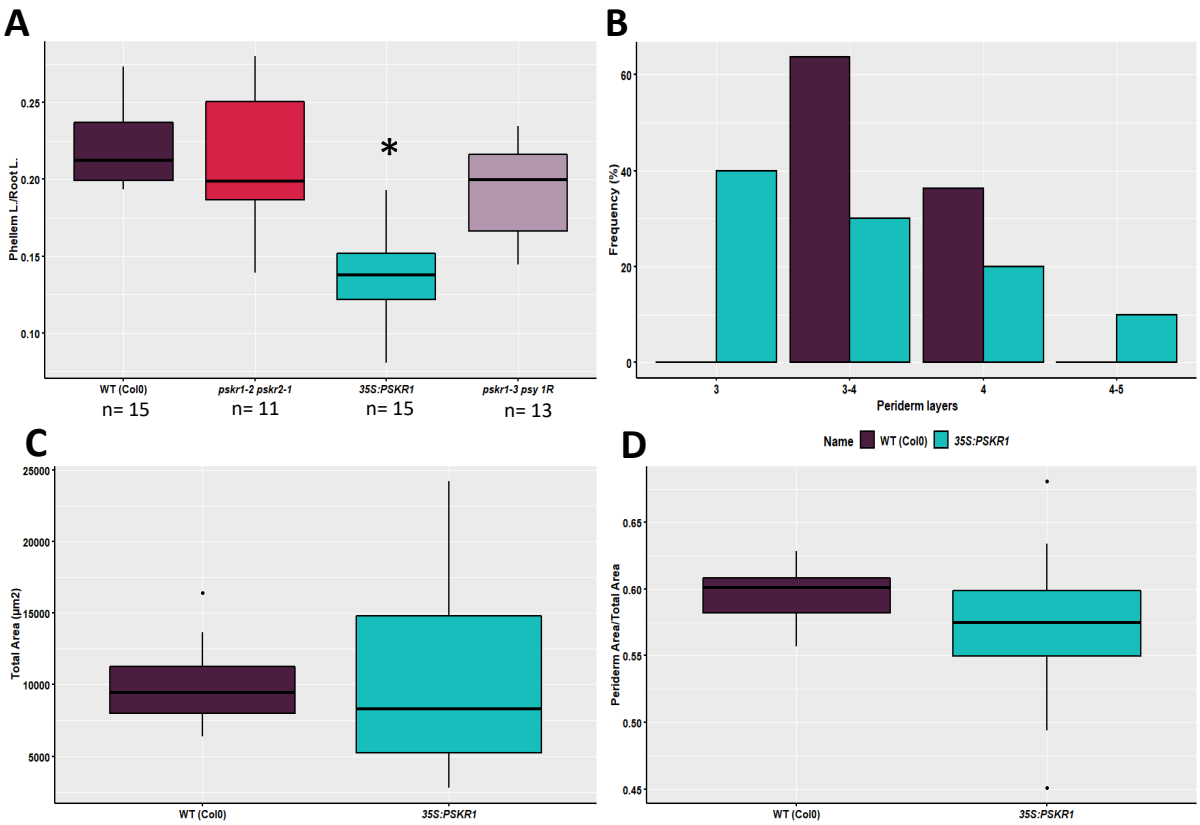


*Fig.9. Periderm and vascular analysis of the PSKR1 and PSY1R loss-of-function and/or overexpression mutants. A) Phellem length normalised to the root length in the tested genotypes pskr1-2 pskr2-1, 35S:PSKR1, pskr1-3 psy1R. The asterisks indicate that the mean phellem ratio of the sample is significantly different from the one of the wild-type (see table). B) Distribution of periderm cell layers range in 35S:PSKR1. C) and D) Total area and ratio of periderm area over total area of roots transverse sections for the tested genotype 35S:PSKR1. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers.The absence of asterisks indicate that the sample is not significantly different from the wild-type (see table).*

In this section we sought to determine whether phytosulfokine receptors (PSKRs) and Tyrosine-sulfated glycopeptide receptor 1 (PSY1R) - two Leucine-rich repeat receptor kinases (LRR-RLKs) - are involved in cork cambium or/and vascular cambium regulation. PSKR1 (phytosulfokine receptor 1), PSKR2 and PSKR3 are a family of membrane localised receptors controlling the procambial cell identity as well as primary growth and xylem differentiation (Reusche *et al.*, 2012; Kaufmann, Motzkus and Sauter, 2017; Holzwart *et al.*, 2018). Similarly, PSYR1 also regulates cellular proliferation and expansion (Amano *et al.*, 2007). As a result, both PSKR and PSY1R were potential candidates for regulating the stem cell activity in lateral meristems. While no significant difference can be observed between the tissue organisation and normalised phellem length of the loss-of-function mutants, *pskr1-2 pskr2-1, pskr1-3 psy1R*, and the wild-type, we noticed an interesting phenotype when

overexpressing PSKR1. In the 35S:PSKR1 line, the normalised phellem length was drastically reduced compared to the wild-type (Fig.9.A). With this potential candidate to regulate periderm growth, we measured the total area of 35S:PSKR1 transverse sections, as well as the periderm area normalised to the total area and the number of periderm layers, but no clear difference could be observed compared to the wild-type (Fig.9.B.D). Altogether, no significant difference was observed in terms of root morphology and tissue organisation in PSKRs and PSY1R (shown in supplementary figures). On the other hand, the phellem length, number of periderm layers, total area of transverse section and the periderm area normalised to the total root area remained the same as wild-type in the *psy1r* loss-of-function mutant, suggesting that PSY1R is not involved in radial growth or cork cambium activity.

2.3.8 Identification of other RLK as potential cork cambium regulators, the probable LRR receptor-like serine/threonine-protein kinases (At3g47570 and At1g07650):

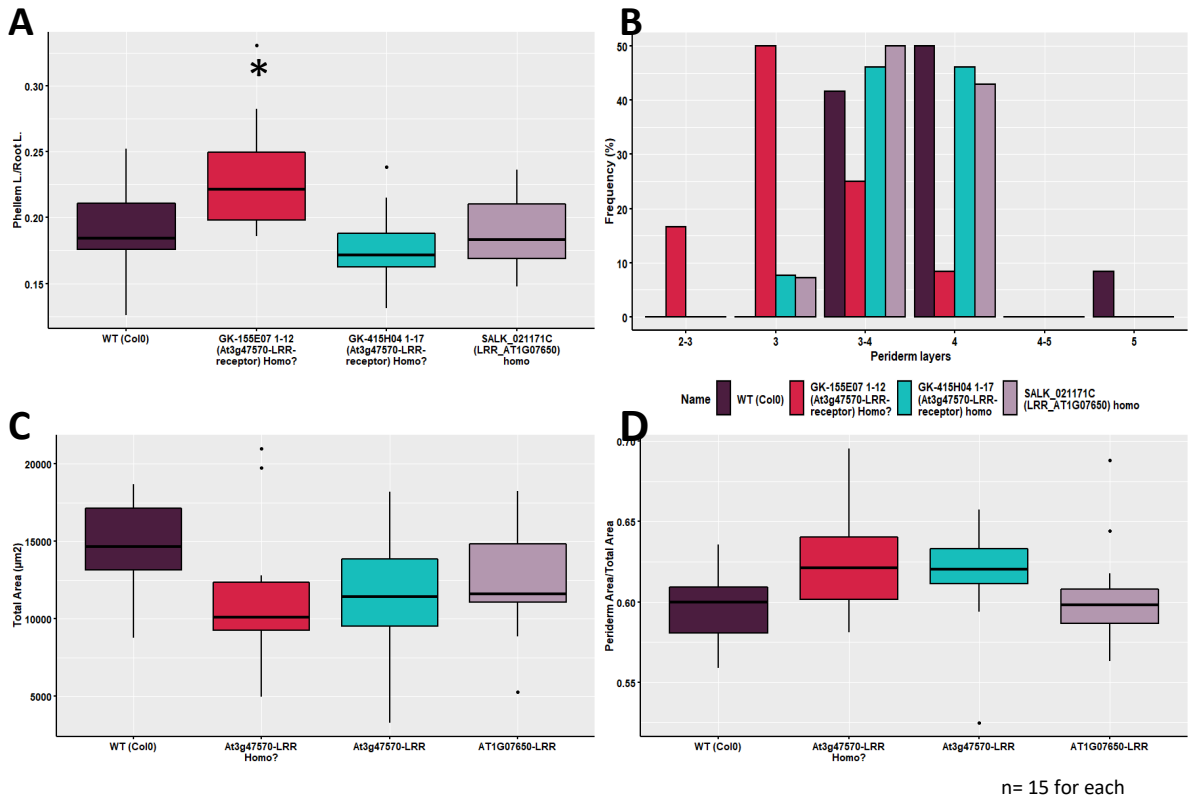


*Fig.10. Periderm and vascular analysis in the At3g47570 and At1g0765 loss-of-function mutants. A) Phellem length normalised to the root length in the tested genotypes At3g47570 and At1g0765 B) Distribution of periderm cell layers range per genotype At3g47570 and At1g07650. C) and D) Total area and ratio of periderm area over total area of roots transverse sections for the tested genotypes At3g47570 and At1g07650. Boxes show the interquartile range (IQR= third quartile Q3 - first quartile Q1); the middle line marks the median; the whisker's endpoints are the minimum and maximum values within the interval spanning Q1-1.5*IQR (lower) and Q3-1.5*IQR (upper); the dots represent the outliers.*

We investigated the role of these two-probable serine /threonine protein kinases - At3g47570 and At1g07650 – in lateral meristem regulation and general lateral growth. The *At1g07650* mutant had a similar phellem ratio, number of periderm layers, periderm area/total area and total area to the wild-type (Fig. 10) and therefore At1g07650 was not considered to regulate cork cambium activity or

vascular cambium activity or radial growth. On the other hand, *At3g47570* loss-of-function mutant had a significantly larger phellem ratio, a slightly reduced total area, a slightly increased periderm area/total area ratio and a significantly reduced number of periderm cell layers compared to the wild-type (Fig.10). These results suggested that At3g47570 is a regulator of periderm growth and further experiments would need to be conducted to confirm whether this receptor is a positive or negative regulate cork cambium activity.

Table: Significance of the difference between tested genotypes in terms of the normalised phellem length, total area or periderm area/total area. The colour gradient indicates whether the difference between the tested genotypes is significant based on p-values calculated as described in the method section. Green highlight indicates that the difference is significant with p value < 0.05 while yellow and pink indicate that the difference is not significant, p-value> 0.05. Grey cells mean that no measurements have been taken.

Statistical tests performed on the following pairs of genotypes:	Normalised Phellem length *p-value:*	Total Area *p-value:*	Periderm/Total Area *p-value:*
WT (Col0)-pxy	0.5513	0.1327	1.8e-4
WT (Col0)-pxl1 pxl2	0.0215	0.0315	0.9869
WT (Col0)-pxf (pxy pxl1 pxl2)	0.0013	4.03e-5	8.7e-4
pxy-pxl1 pxl2	0.0066	3.5 e-5	0.0011
pxy-pxf (pxy pxl1 pxl2)	0.0068	0.0299	0.9381
pxl1 pxl2-pxf (pxy pxl1 pxl2)	3.4e-09	0	0.0046
WT (Col0)-pxl1 pxl2	0.0564	0.0432	0.5297
WT (Col0)-pxf (pxy pxl1 pxl2)	9.5e-05	0.0065	0.7387
WT (Col0)-pxf er	0.0151	0.1726	0.6332
WT (Col0)-pxf er erl2	0.0016	0.2725	0.9999
pxf er-pxf (pxy pxl1 pxl2)	0.4653	0.6011	0.9961
pxf er erl2-pxf (pxy pxl1 pxl2)	0.9414	0.3970	0.7712
pxf er erl2-pxf er	0.9414	0.9907	0.6672
WT (Col0)-er erl2 (PD45-3)	5.929e-4	0.1918	0.9380
WT (Col0)-er erl2 (PD45-5)	0.3462		
WT (Col0)-pxy pxl1 pxl2 er erl1 erl2 *	<0.05	1.7e-07	0.8710
WT (Col0)-pxy pxl1 pxl2 er erl2 erl1/+ or +/+	3.08e-5	1.4e-05	0.0889
pxy pxl1 pxl2 er erl1 erl2*-er erl2 (PD45-3)	<0.05	0.0079	0.9380

pxy pxl1 pxl2 er erl2 erl1/+ or +/+-er erl2 (PD45-3)	0.9246	0.0017	0.0092
pxy pxl1 pxl2 er erl2 erl1/+ or +/+-pxy pxl1 pxl2 er erl1 erl2 *	<0.05	3.8e-05	0.0087
er erl2 (PD45-5)-er erl2 (PD45-3)	0.1272		
WT (Col0)-35S:PSKR1	<0.05	0.98	0.2471
WT (Col0)-pskr1-2 pskr2-1	0.9194		
WT (Col0)-pskr1-3 psy 1R	0.1666		
WT (Col0) vs GK-155E07 1-12 (At3g47570-LRR-receptor)	0.0146	0.1594	0.0950
WT (Col0) vs GK-415H04 1-17 (At3g47570-LRR-receptor)	0.7766	0.1929	0.0950
WT (Col0) vs SALK_021171C (LRR_AT1G07650)	0.8672	0.4039	1.0000

2.4 Discussion:

2.4.1 The members of the PXf and ERf are key regulators of the root secondary growth:

As previously suggested in the stem and the hypocotyl (Sieburth, 2007; Ragni *et al.*, 2011; Etchells *et al.*, 2013; Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019), each member of the PXY family and ERECTA family (except ERL2) is involved in the root secondary growth of *Arabidopsis thaliana*. However, not all investigated receptors regulate the secondary growth of Arabidopsis roots. For instance, phytosulfokine receptors and tyrosine-sulfated glycopeptide receptor 1 (PSY1R), which are known to control primary growth, vasculature development and procambial cell identity growth (Amano *et al.*, 2007; Reusche *et al.*, 2012; Kaufmann, Motzkus and Sauter, 2017; Holzwart *et al.*, 2018) did not seem to regulate the radial expansion of the root. Altogether, our results confirm that the elongation and radial expansion of the plant organs are distinct growth processes. In the following section, we discuss which of the vascular cambium or cork cambium stem cell niches are regulated by individual receptors.

2.4.2 PXf and ERf promote stem cell activity in the two lateral meristems:

In this chapter, we sought to understand the roles of PXY family and ER family members in stem cell division and differentiation of the vascular and cork cambium in *Arabidopsis* root. As previously shown, PXY is a master regulator of cell division and differentiation in the vascular cambium of the root (Xiao *et al.*, 2020) and PXL1 and PXL2 have overlapping functions with PXY in the stem and the hypocotyl (Sieburth, 2007; Etchells *et al.*, 2013; Wang *et al.*, 2019) This study confirms that PXY initiates and maintains the activity of the vascular cambium and show that PXL1/2 likely promote the activity of the vascular and cork cambium (see following section). While the loss of PXY triggers severe defects in the vascular cambium (Sieburth, 2007; Etchells *et al.*, 2013; Smetana et al., 2019; Xiao *et*

al., 2020; our own study), we demonstrated that PXL1 and PXL2 are not required to initiate and maintain stem cell division and differentiation in either lateral meristem. In summary, the roles of PXL1 and PXL2 are partly redundant with PXY, PXY being a stronger vascular cambium regulator compared to PXL1/2 while PXL1/2 seem to expand their regulation to both vascular and cork cambium.

One of the challenges encountered during the interpretation of these results is that the proper development of the inter-tissue dependency between lateral meristems where the vascular cambium is necessary and sufficient for the periderm to grow (Xiao *et al.*, 2020). As a result, PXL1 and PXL2 could indirectly control the activity of the cork cambium by regulating the development of the vascular cambium, in the same way that Xiao *et al.* (2020) have previously observed with the PXY mutant. Nevertheless, this hypothesis remains unlikely based on the expression patterns of PXL1 and PXL2 in the root (see the following chapter). PXL1 is expressed in the epidermis - the protective layer which is replaced by the periderm growing underneath during the root development - indicating that PXL1 potentially synchronises the initiation of the cork cambium and the shedding of the epidermis while PXL2, expressed in the periderm (and potentially the vasculature), might regulate the activity of stem cells in both lateral meristems (Chapter III). In these experiments, PXL1 and PXL2 have been studied together in double and triple loss-of-function mutants and the individual functions of PXL1 and PXL2 need to be investigated in the future with single mutants. Consequently, PXL1 and PXL2 likely directly regulate the activity of stem cells in the cork and vascular cambium but the individual function of PXL1 and PXL2 remain to be investigated using single mutants.

In the second part of this chapter, we investigated the roles of ER family members regulating stem cell activity in the lateral meristems as well as their genetic interactions with the PXY family. As expected from previous experiments in the stem and the hypocotyl, ERL2 does not regulate the root lateral growth or any lateral meristems while ER and ERL1 have overlapping functions in the root (Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017). Like in the hypocotyl, ER and ERL1 seem to be positive regulators of the root lateral growth, confirming that the functions of ER and ERL1 are organ dependent (as they are positive regulators of lateral growth in the root and the hypocotyl while being repressors of lateral growth of the stem)(Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019). Additionally, our results suggest that ER and ERL1 enhance root lateral growth by promoting the expansion of the periderm and the vasculature. ER and ERL1 also seem essential to initiate or maintain the activity of the cork cambium in the absence of PXf. Despite PXf being the main regulators of the vascular cambium (Fisher and Turner, 2007; Sieburth, 2007; Hirakawa *et al.*, 2008; Etchells and Turner, 2010; Etchells *et al.*, 2013; Kondo *et al.*, 2014), ERf seem to initiate and maintain the formation of a few vascular cambium cells in the absence of PXf. Altogether, ER and ERL1 seem to promote vascular and cork cambium activity in the root and despite failing to maintain the ring-like organisation of vascular cambium in the absence of PXf, ER and ERL1 still induce the formation of a few vascular cambium cells. To understand further the roles and interactions within the ER family, the root morphology of additional single, double and triple mutants should be investigated and the role of ERL2 should be studied in the absence of other ERf members. In the following section we assess the importance lateral meristems regulating each other's growth and the genetic interaction between PXf and ERf.

Interestingly, PXY and ER family members have overlapping functions and removing all members of PXY and ER family (sextuple mutant) hinders the initiation and maintenance of the vascular and cork cambium (our results). It is difficult to determine from our results whether the cork cambium fails to form due to the absence of the vascular cambium (Xiao *et al.*, 2020). However, the expression patterns of PXf and ERf suggest that the lateral meristems can also be regulated independently from one another as some members of PXf and ERf are expressed in the vascular and/or cork cambium

(Hirakawa *et al.*, 2008; Uchida *et al.*, 2012; Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017; Shi *et al.*, 2019; Smetana *et al.*, 2019; Wang *et al.*, 2019; Laura Ragni personal communication). Although minimal vascular cambium growth is necessary for cork cambium to form (Xiao *et al.*, 2020), the vascular cambium is drastically impacted by the loss of PXf while the cork cambium is impacted significantly less. These results indicate that, despite the vascular cambium being essential for cork cambium initiation, the stem cell activity in the cork cambium is also regulated by other signalling pathways (such as PXf and ERf genetically interacting with each other to regulate lateral meristem growth (Wang *et al.*, 2019; our results)). In terms of research avenue, it would be interesting to investigate whether PXf and ERf physically interact to control stem cell activity in the lateral meristems (see following chapter). Additionally, the directionality of the communication between the cork cambium and the vascular cambium, the intensity of this interaction as well as the signalling messengers between both cambiums remain to be characterised to complete our results. Following the investigation of the roles of PXf and ERf regulating stem cell activity, we assess the interactions between the two RLK families and the importance of these interactions in the root.

2.4.3 The complex epistatic relationship between PXY family and ER family members and the potential hypothesis to this relationship:

We observed a complex relationship between PXY family members in the *pxy*, *pxl1 pxl2* and *pxf (pxy pxl1 pxl2)* single, double and triple loss-of-function mutants. While loss of PXL1 and PXL2 was previously described as strengthening the PXY loss-of-function phenotype (Fisher and Turner, 2007; Sieburth, 2007; Etchells *et al.*, 2013), we observed a completely opposite phenotype between the root transverse sections of the *pxy* and the *pxl1 pxl2* mutants. The *pxy* single mutant had reduced lateral growth, fewer vascular cambium cells, a disorganised vasculature and reduced periderm growth. By contrast, *pxl1 pxl2* mutant displayed the opposite phenotype with enhanced lateral growth and an organised vasculature. These results suggest at first that PXY and PXL1/2 have opposite functions, with PXL1 and PXL2 being potential negative regulators of lateral meristem activity. However, the *pxf (pxy pxl1 pxl2)* mutant has an enhanced phenotype where the loss of PXL1 and PXL2 strengthens the loss of PXY (Fisher and Turner, 2007; Sieburth, 2007; Etchells et al., 2013; our results). This additive *pxf* phenotype contradicts the conclusion drawn from the *pxl1 pxl2* mutant and suggests that PXL1 and PXL2 might promote both vascular cambium and cork cambium activity.

If PXL1 and PXL2 are positive regulators of vascular and cork cambium activity, the difference between the *pxl1 pxl2* and the *pxf* loss-of-function mutant phenotypes can only be explained by the PXY family members having an epistatic relationship. In the *pxl1 pxl2* mutant, the loss of PXL1 and PXL2 might enhance PXY activity by triggering the overexpression of PXY or enlarging the PXY expression zone. Consequently, the increase of PXY activity might mask the loss of PXL1 and PXL2 in the *pxl1 pxl2* mutant, leading to the drastic increase of periderm and vascular growth observed in the *pxl1 pxl2* transverse sections. As a result, PXL1 and PXL2 might enhance both the vascular and cork growth while repressing the activity of PXY. To confirm this hypothesis, the expression of PXY should be investigated in a *pxl1 pxl2* loss-of-function mutant background.

Interestingly, at least one member of PXf and ERf (in our case ERL1) is necessary to initiate and maintain the secondary growth of plants as the *pxf erf* sextuple mutant barely form any periderm or vascular cambium (there is no periderm, but a few vascular cambium divisions still occur). These results indicate that PXf and ERf members have redundant functions in promoting the vascular and periderm growth (Wang *et al.*, 2019; our results). The redundancy between PXf and ERf remain to be

confirmed by assessing the secondary growth of various quintuple mutants, each expressing one single functional PXf or ERf member.

In terms of PXf and ERf interaction, removing ER rescues the PXf lateral growth phenotype and partly rescues its periderm growth phenotype. However, *er erl2* indicated that ER is an important enhancer of radial expansion of *Arabidopsis* root (as ERL2 is not being involved in secondary growth at this developmental stage (Wang *et al.*, 2019; our results). As a result, ER and PXf seem to all be positive regulators of vascular and cork cambium activity and the *pxf er* phenotype reveals the existence of a complex epistatic relationship between ERf and PXf members. Similar to the interaction between PXY and PXL1/2, removing ER could promote the activity of a positive regulator of periderm and vascular growth (this positive regulator might be ERL1 as ERL2 is not involved in root secondary growth(Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017; our results)). As a result, ER potentially downregulates ERL1 by repressing its expression levels or expression zone. This hypothesis would need to be confirmed in future overexpression and loss-of-function studies combined with expression analyses in the root.

2.5 Concluding Remarks:

The current analysis of stem cell activity could be improved by quantifying the elongation of periderm cells and vascular cells and by measuring the number of xylem vessels in all tested genotypes. In future experiments, the size of cells in the root transverse sections of PXf and ERf loss-of-function mutants could also be determined to further our understanding of RLKs-driven regulations of radial growth.

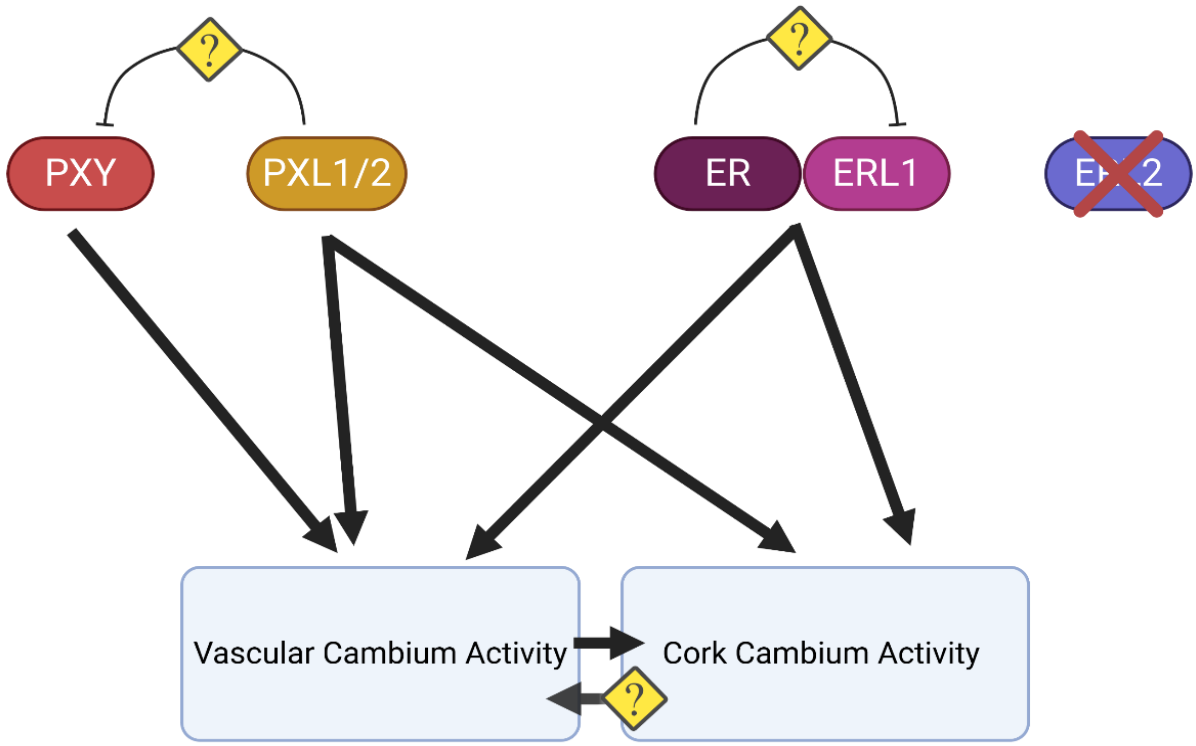


Fig.11. Hypothesis of PXY family and ER family roles and interactions in regulating the vascular and cork cambium. PXL1/2, ER and ERL1 positively regulate the stem cell activity in the vascular and the cork cambium. PXY mainly regulates the activity of the vascular cambium and ERL2 is not involved in lateral growth. The development of the vascular cambium is important for the activity of the cork cambium. The question marks highlight the signalling interactions to be confirmed. For instance, PXL1/2 and ER might respectively repress PXY and ERL1 expression and the cork cambium development might affect the vascular cambium activity.

This study showed that PXY family and ER family genetically interact not only to initiate and maintain stem cell activity in the vascular cambium but also in the cork cambium of *Arabidopsis* root. This suggests that a common regulatory mechanism is shared between the stem cells niches initiated post-embryonically. In terms of research avenue, it would be interesting to show that PXf and ERf not only genetically interact to regulate stem cell activity but also interact physically. For this purpose, expression analysis, FRET-FLIM and co-immunoprecipitation experiments will be conducted and prepared in chapter III. To understand the complex relationship between PXY and ER families, a transcriptional analysis of PXY and ER family mutants should be performed (chapter IV), and the expression patterns of different family members should be investigated in various PXf and ERf loss-of-function mutant backgrounds.

Chapter III: A shared molecular mechanism underpinning post-embryonic cambium regulation.

Are protein-protein interactions underpinning the previously identified genetic interactions between ER and PXY family receptors?

3.1 Introduction:

In the previous chapter, we partially unravelled a complex genetic network of LRR-RLKs regulating the initiation and activity of stems cells in the post-embryonic tissues of *Arabidopsis thaliana*. We conducted a detailed morphogenetic analysis of these PXf and ERf loss of function mutants in *Arabidopsis* root (Chapter II). While the literature mainly focuses on the formation of vascular cambium (Fig.1.A.B), we investigated the genetic interactions between PXf and ERf regulating both the vascular cambium and the cork cambium activity. Strikingly, the *pxf erf* mutant exhibited the most drastic secondary growth phenotype as very few cell divisions took place in the vascular cambium and the cork cambium was completely absent (Fig.1.C) (Chapter II). Consequently, we discovered that the interactions between PXf and ERf receptors are crucial in regulating the activity of different stem cell niches and these genetic interactions might form a common mechanism for plants to control stem cell initiation and/or maintenance post-embryonically. As a result, our subsequent goal was to understand the molecular mechanisms underpinning the genetic interactions between PXf and ERf receptors.

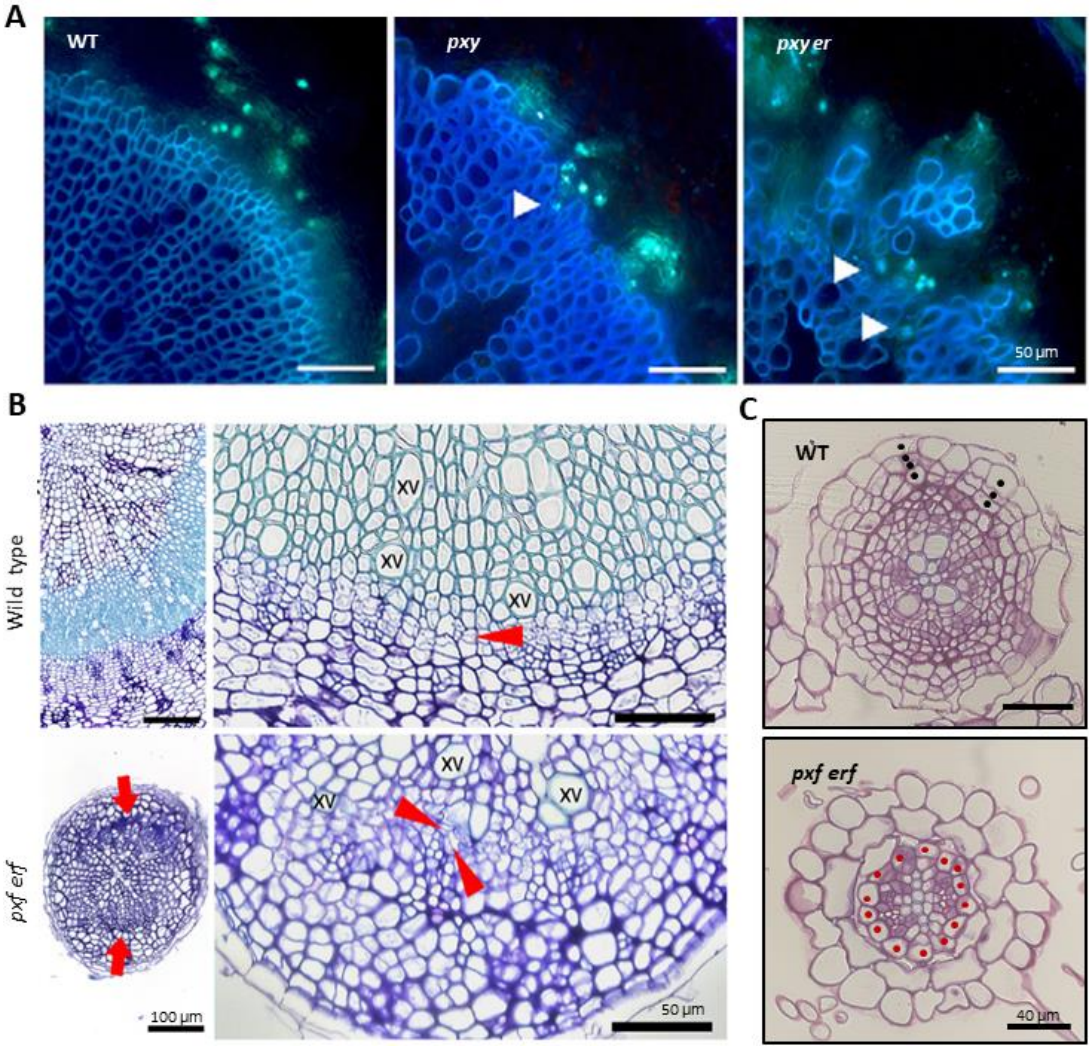


Fig.1. Transverse sections of pxf erf loss of function mutants. A) Analine Blue-stained hand sections of the hypocotyl show that the vascular organisation of pxy is perturbed and that this perturbation is enhanced in pxy er lines. The white arrowheads highlight intercalated xylem and phloem tissues which are spatially separated by the vascular cambium in the wild type (Etchells et al., 2013). B) Toluidine Blue-stained thin sections of the hypocotyl demonstrating that the radial growth and the cell division in the vascular cambium are both drastically reduced in the pxf erf (pxy pxl1 pxl2 er erl1 erl2) mutant. The red arrowheads show the phloem poles (left panel) and the cell divisions (right panel) (Wang et al., 2019). C) Toluidine Blue-stained thin sections of the root demonstrating that the radial growth and the cell division of both vascular and cork cambium are drastically reduced in the pxf erf mutant. The black dots indicate periderm cells while the red ones show pericycle cells (Chapter II).

Preliminary research, including expression studies and *in vitro* interactome assays, gave an insight into the molecular mechanisms underpinning these genetic interactions (Table.1). Some PXf and ERf members had their expression patterns identified in organs undergoing secondary growth, such as the inflorescence stem, the hypocotyl and/or the roots (Yokoyama *et al.*, 1998; Shpak *et al.*, 2004; Uchida *et al.*, 2012; Wang *et al.*, 2019). Across those different organs, PXY is expressed in the vascular cambium and the xylem initials (which are the stem cell organisers of the vascular cambium). (Hirakawa *et al.*, 2008; Shi *et al.*, 2019; Smetana *et al.*, 2019; Wang *et al.*, 2019). By contrast, PXL1 and PXL2 expression patterns remained obscure and were elucidated for the first time in this study.

Compared to PXY expression patterns, the expression of ER family receptors is significantly more complex (Table.1). The ER-family genes are expressed in a broad range of tissues (Yokoyama *et al.*, 1998; Shpak *et al.*, 2004; Uchida *et al.*, 2012; Ikematsu *et al.*, 2017) and their expression varies according to the organs and the developmental stage of the plant. Among the ER family members, ER and ERL1 often display overlapping expression patterns. In the inflorescence stem, ER and ERL1 are expressed in the phloem, xylem and epidermis tissues (Uchida *et al.*, 2012; Uchida and Tasaka, 2013). Similarly, in the hypocotyl, ER and ERL1 exhibit a first expression peak in the vascular cambium and the xylem initials while their second expression maxima is located in the secondary tissues emerging from the cork cambium, called periderm (Ikematsu *et al.*, 2017; Wang *et al.*, 2019). In the root, ER is expressed in the vasculature and the periderm, but ERL1 expression differs slightly by being restricted to the vasculature of the root (Dr. Ragni; personal communication). While previous studies thoroughly explored ER and ERL1 expression patterns, the expression of ERL2 remains largely unclear. ERL2 seems absent from the inflorescence stem and early developmental stages of the hypocotyl (in 9 day-old and 3 week-old hypocotyl) (Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017). By contrast, ERL2 is expressed in the late developmental stage of the hypocotyl and once expressed, ERL2 displays the same expression patterns as ER and ERL1. More specifically, one peak of ERL2 expression is localised in the vascular cambium and the xylem initials while a second peak is present in the periderm (Wang *et al.*, 2019). To further our understanding of the role played by ERL2 during secondary growth, the expression patterns of ERL2 in *Arabidopsis* root was investigated in this study.

Table 1. *Expression patterns of PXf and ERf members in organs of Arabidopsis undergoing secondary growth.*

RLK	Stem	Hypocotyl	Root	Ref
PXY		Vascular cambium; Xylem initials		Hirakawa *et al.*, 2008; Shi *et al.*, 2019; Smetana *et al.*, 2019; Wang *et al.*, 2019
PXL1		**Not described**		
PXL2		**Not described**		
ER	Phloem; Xylem; Epidermis	Vascular cambium; Xylem initials; Periderm	Vascular cambium; Periderm	Uchida *et al.*, 2012; Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019; Laura Ragni personal communication
ERL1	Phloem; Xylem; Epidermis	Vascular cambium; Xylem initials; Periderm	Vascular cambium	Uchida *et al.*, 2012; Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019; Laura Ragni personal communication
ERL2	Absent	Vascular cambium; Xylem initials; Periderm (at late developmental stage)	**Not described**	Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019

Altogether, the literature clearly shows the overlapping expression of PXY, ER and ERL1 (as well as ERL2 in the late developmental stage of the hypocotyl) in the vasculature (Table.1). However, we found that the genetic interactions between the PXf and ERf not only control the activity of the vascular cambium but also regulate the cork cambium (Chapter II). As a result, this study examined the expression patterns of PXL1, PXL2 and ERL2 to understand whether a shared mechanism regulates the initiation and activity of stem cells in both the vascular and cork cambium.

Interestingly, Smakowska-Luzan *et al.* (2018) predicted that 35.5% of LRR-RLKs with large extracellular domains physically interact. Since all members of the PXY family and ER family have large extracellular domains, these receptors are likely to interact when expressed in the same cells. To support this hypothesis, researchers observed strong *in vitro* interactions between the extracellular domains of PXf and ERf family receptors using an Extracellular Interactome Assay (ECIA). ER and PXY as well as ER and PXL1 were reported to interact *in vitro* in one direction (the first protein bind to the other but this interaction does not occur the other way around) while PXL1 and ERL2 interacted in both directions (mutual binding) (data available in BAR ePlant (Waese *et al.*, 2017)). Altogether, these results raise the question: if PXf and ERf receptors are expressed in both cambia and secondary tissues, do they physically interact to form a common mechanism controlling stem cell initiation and/or maintenance in post-embryonic tissues? To explore the molecular mechanisms underpinning PXf and ERf genetic interactions, *in vivo* protein-protein interaction assays will have to be performed in the near-future. Consequently, this study was the opportunity to prepare transgenic lines and genetic constructs for a co-immunoprecipitation assay and a Förster resonance energy transfer (FRET) experiment.

In summary, this chapter aimed to explore the molecular mechanisms underpinning the genetic interactions between PXf and ERf RLKs. Provided that these receptors interact as hypothesised, their expression should overlap in the stem cell tissues they regulate; namely the vascular cambium and the cork cambium. As PXf and ERf expression was already known to overlap in the vasculature, we examined the expression of these receptors in the periderm. In particular, we focused on PXL1 and PXL2 whose expression had never been examined before, as well as ERL2, which remained to be investigated in the root.

As ERL2 had never been studied in the roots and as a follow up of the genetic analysis from the previous chapter, these expression studies were conducted in the root of the model organism

Arabidopsis thaliana (Chapter II). GUS staining assays were used to localise the cells expressing PXL1/2 and ERL2 receptors by visualising the activity of their promoters. Since ERL2 expression fluctuates according to the organ and the developmental stage of the plant (Wang *et al.*, 2019), fluorescence microscopy, which has a better detecting sensitivity, was used as an additional method to study the expression of ERL2 tagged with YFP (yellow fluorescent protein).

Following these expression studies, we prepared transgenic lines and genetic constructs to test this hypothesis of protein-protein interaction through co-immunoprecipitation assay and FRET analysis. For the co-immunoprecipitation assay, we crossed *Arabidopsis* lines to obtain a transgenic line expressing both PXY tagged with HA (*hemagglutinin*) and one of the ER family members tagged with YFP (*yellow fluorescent protein*). The actual co-immunoprecipitation assay will be conducted in the future as depicted in Fig.2.

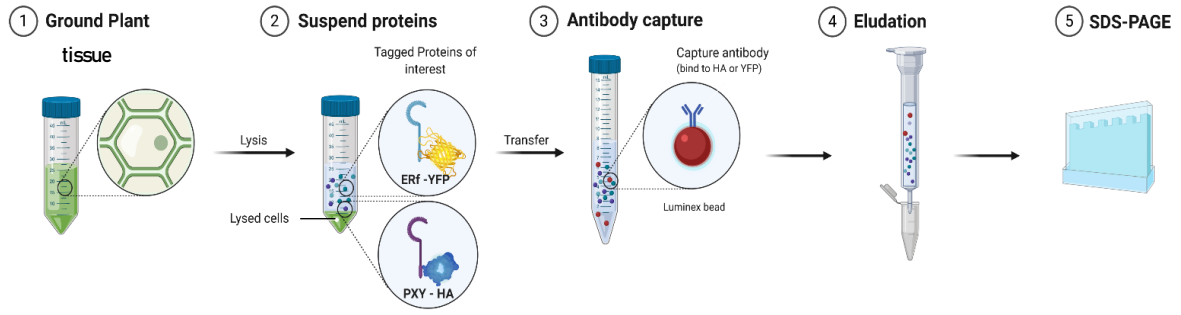


Fig.2. Co-immunoprecipitation assay designed to detect PXY-ERf protein complexes. Step 1: The tissue of the transgenic line expressing both PXY::PXY-HA and ERf::ERf-YFP is grounded. Step 2: The cell membranes are lysed, thus liberating proteins in the suspension buffer. Step 3: One of the tags (HA or YFP) is immobilised by antibodies on beaded support. Step 4: Any protein not precipitated on beads are washed away. The protein of interests or protein complexes are eluded from the support and collected. Step 5: The last eluded proteins or protein complexes are analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), often followed by western blot detection to verify the identity of the captured proteins.

However, the interactions between LRR-RLKs are usually transient and of low affinity, making the interaction poorly biochemically tractable using assays like co-immunoprecipitation (Mott *et al.*, 2019). Additionally, co-immunoprecipitation requires long separation procedures during which the LRR-RLKs are isolated from their native environment, where molecular crowding and high compartmentalisation are key for their binding (Margineanu *et al.*, 2016). To solve this issue, Förster resonance energy transfer **(**FRET) was the additional technique chosen to map and quantify PXf-ERf interactions in live cells (Fig.3). As PXf-CFP constructs were already provided by Dr. Yuki Kondo, we focused on generating 35S::ERf-YFP constructs in this chapter.

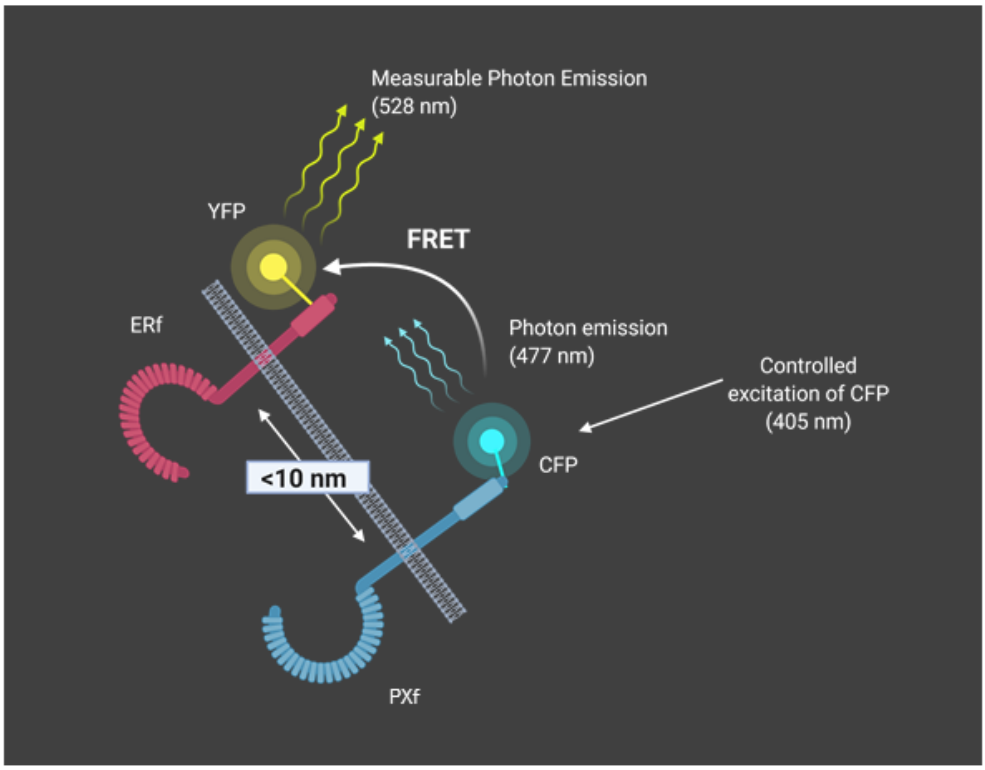


Fig.3. Schematics of FRET occurring between PXf-CFP and ERf-YFP. FRET utilises the transfer of energy between two fluorophore molecules with significant spectral overlap (i.e. the emission wavelength of the donor fluorophore overlap with the absorption wavelength of the acceptor)*. In close proximity (2-10 nm), the energy from one fluorophore (donor) is transferred to another fluorophore (acceptor). This implies that FRET solely occurs when two proteins tagged with a donor and an acceptor fluorophore are interacting. Such transfer of energy is radiationless and involves a dipole-dipole interaction (Shilpi et al., 2014; Margineanu et al., 2016). FRET can be read out using a wide range of techniques such as fluorescence lifetime imaging (FLIM) or spectral or polarisation ratiometric methods. FLIM only quantitates the exponential fluorescence decay of donor fluorophores while spectral and polarisation ratiometric techniques measure the fluorescence or polarisation ratio between the acceptor and donor fluorophores (Margineanu et al., 2016).*

3.2 Materials & Methods:

3.2.1 Materials:

Resource Table:

Reagent or Resource	Source	Identifier
Murashige and Skoog Basal Medium (MS)	Duchefa	Cat# M0255
Plant Agar	Duchefa	Cat# P1001.1000
Technovit 7100	Heraeus Kulzer	Cat# 64709003
X-Glux	Duchefa	Cat# X-1405
Chloral hydrate	Merck	Cat# 1.02425.1000
Glycerol	Roth	Cat# 6962.1
Kanamycin	Sigma-Aldrich	Cat# 70560-51-9
Chloramphenicol	Sigma-Aldrich	Cat# 56-75-7
GeneRuler 1kb, DNA ladder	Thermo Scientific	Cat# 11823963
6x DNA Loading Dye	Thermo Scientific	Cat# 11541575
2x PCRBIO Taq Mix Red Ready mix	PCR Biosystems	Cat# PB10.13-02
Commercial Assays		
QIAquick® Gel Extraction Kit	Qiagen	Cat# 28704
pENTR™/D-TOPO® vector	Invitrogen	Cat# K240020
GenElute™ Plasmid Miniprep Kit	Sigma	Cat# PLN70
Experimental models: Organisms/Strains		
Arabidopsis: Col-0	Widely distributed	N/A
Arabidopsis: ERL2::GUS in Col-0	(Shpak *et al.*, 2004)	N/A
Arabidopsis: PXL1::GUS in Col-0	Rebecca Doherty (2018) MBiol thesis, Durham University	N/A
Arabidopsis: PXL2::GUS in Col-0	Rebecca Doherty (2018) MBiol thesis, Durham University	N/A
Arabidopsis: PXY::PXY-HA in *pxy*	Laboratory stocks	N/A
Arabidopsis: ER::ER-YFP in *er*	(Ikematsu *et al.*, 2017)	N/A
Arabidopsis: ERL1::ERL1-YFP in *erl1*	(Ikematsu *et al.*, 2017)	N/A
Arabidopsis: ERL2::ERL2-YFP in *erl2*	(Ho *et al.*, 2016)	N/A
E.coli : DB3.1	Thermo Scientific	N/A
Oligonucleotides		
See Data S1	Sigma	N/A
Software		
ZEN Black (Zen 2.3 SP1)	Zeiss	https://www.zeiss.de/corporate/home.html
R v.4.0.2	The R foundation	https://rstudio.com/

Experimental model:

Arabidopsis thaliana transgenic and mutant lines were used to perform experiments. The ecotype and background of each line is specified in the resource table. For expression pattern experiments, five individual plants with each genotype were analysed to check reproducibility. The plants were grown

in continuous light conditions in vitro on ½ MS plates supplemented with 1% sucrose and 0.8 plant agar for 12 days. For ERL2::GUS lines, the plants were grown up to 12 days, 3 weeks and 4 weeks to be analysed at different time points. For crossing experiments, the parental lines were grown in 16:8h light: dark cycles in soil until they grew inflorescences and mature flowers.

3.2.2 Method Details:

Histology and GUS staining:

Root samples for expression studies were taken 0.5 mm underneath the hypocotyl. For GUS staining, the root samples were embedded in 6% agarose blocks and sectioned (50-80 µm) using a Leica VT - 1000 vibratome. GUS staining was performed as described by Jefferson (1987) and Belsson *et al.* (2007) by incubating the roots in a GUS staining solution (0.1 M NaH_2PO_4, pH 7.0, 10 mM Na_2-EDTA, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 0.1% Triton X-100 and 1.0 mM X-glucuronide freshly prepared each time in DMSO) at 37°C for 2 to 24 h (until staining was visible). Roots were mounted in chloral hydrate solution (8:3:1; Chloral hydrate: Water: Glycerol).

Light Microscopy and Confocal Microscopy:

All transverse sections were imaged with a Zeiss Axiophot microscope coupled with a Zeiss Axiocam 512 color digital camera. The confocal images from whole-mount *erl2 ERL2-YFP* samples were acquired with Dr. Ragni using a Zeiss LSM880 microscope. To identify the yellow fluorescent protein (YFP) in our images, the excitation wavelength was set at 490 nm and the emission wavelength was set at 510 nm. Due to the absence of a signal, no three-dimensional reconstructions nor orthographic views of a Z-stack were required.

Generation of transgenic lines:

The T3 generation of the *pxy PXY::PXY-HA* line was selected for homozygosity on 50 µg/ml kanamycin selective media. The antibiotic kanamycin selects for plants which have been transformed with a plasmid encoding for the gene of interest and a selection marker, which is a bacterial gene encoding the enzyme neomycin phosphotransferase (NPT). Previous members of the laboratory selected for the transgene (in heterozygotes and homozygotes) in the first and second generations of the line but after three self-reproduced generations, the transformants usually reach 100% of resistance to kanamycin and are then considered stable homozygous transformants. Once *pxy PXY::PXY-HA* was selected for homozygosity, this line was crossed with *er* ER::ER-YFP, *erl1 ERL1::ERL1-YFP* and *erl2 ERL2::ERL2-YFP* to generate three transgenic lines: *PXY::PXY-HA x ER::ER-YFP*, *PXY::PXY-HA x ERL1::ER-YFP* and *PXY::PXY-HA x ERL2::ER-YFP*. The seeds collected from these crosses remain to be selected for homozygosity.

Molecular Cloning:

All constructs were obtained using the Gateway cloning system (Invitrogen). Coding sequences of ER, ERL1 and ERL2 were amplified from cDNA with the primers listed in Data S1. The ER, ERL1 and ERL2 promoters were respectively 1072, 1846, 1963 bp in size and included the 5'UTR. The PCR products (25 µl) were run on an agarose gel and the bands corresponding to the correct size were excised from the gel under an open UV box using a sterile razor blade. The PCR products were purified from the agarose gel using a QIAquick® Gel Extraction Kit.

After purification, the amplified sequences were inserted into an entry plasmid using a topoisomerase reaction. The 4 µl of PCR product was mixed with 1 µl of salt solution (1.2 M NaCl, 0.06 $MgCl_2$) and 1 µl of pENTR™/D-TOPO®. The reaction mix was incubated overnight at ambient temperature for the plasmid circularisation reaction to happen. The resulting entry vectors were transformed into BIOLINE (A Meridian Life Science®) α-Select Bronze Efficiency Chemically Competent Cells. The transgenic bacteria were selected on kanamycin selective media at 37°C overnight.

The presence of ERf genes in the selected colonies was checked to confirm that the transformation was successful. Each colony was mixed in 5 µl sterile water and used as a template for a PCR. For each sample, the PCR was prepared by adding 7.5 µl of 2x PCR Ready mix to 0.5 µl of each forward and reverse primer, 4.5 µl of water and 1 µl of diluted colony. PCR was performed using a standard cycle with a 55 °C annealing temperature.

The transformed α-Select cells were grown overnight in Kanamycin selective media (LB agar plates followed by 5ml LB broth) at 37 °C. Part of the culture was used to make glycerol stocks by mixing the culture (750 µl) with 50% glycerol (750 µl) and store them at - 80°C. The entry vectors in the remaining culture were extracted with GenElute™ Plasmid Miniprep Kit. The amount of extracted plasmid was above 100 ng/ml for all samples, which will be sufficient to carry on the next Gateway reaction.

3.3 Results:

3.3.1 The expression patterns of ERL2, PXL1 and PXL2 in *Arabidopsis* root

With the role of ERECTA-LIKE 2 (ERL2) during secondary growth being obscure, we examined the expression patterns of ERL2 in the root of *Arabidopsis thaliana*. After verifying that ERL2-YFP was localised at the plasma membrane of stomatal cells (positive control), we found that ERL2 was absent in 12-day-old roots (Fig.4). Identical results were obtained with GUS staining in 3- and 4-week-old plants, confirming that ERL2 fails to express at these developmental stages of the roots (due to the absence of any signal only fluorescence microscopy pictures were taken). These results suggest that ERL2 is not involved in the initiation nor the activity of the vascular and cork cambium in *Arabidopsis* root, at least at the tested developmental stages. Following the study of ERL2, PXL1 and PXL2 were the only members of ER family and PXY family whose expression remained to be investigated.

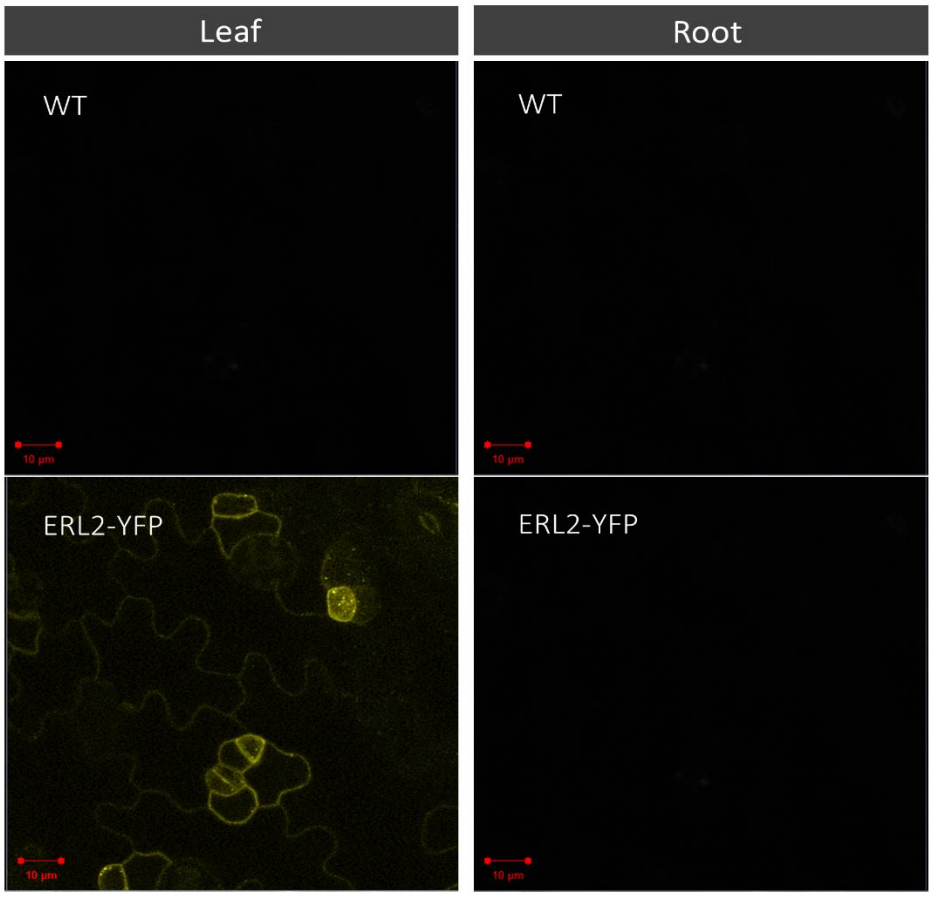


Fig.4. Expression of ERL2::ERL2-YFP in the root of 12-day-old Arabidopsis. ERL2-YFP expression under ERL2 promoter in the leaf is used as a positive control to confirm the proper expression of ERL2-YFP at the membrane of stomatal cells. The wild-type plant is used as a negative control.

To enhance our understanding of the PXY family signalling pathway, we examined the expression patterns of PXL1 and PXL2 in the root of *Arabidopsis*. Since PXY is known to be consistently expressed in the vascular cambium and the xylem initials of the stem, the hypocotyl and the root, PXL1 and PXL2 were the only members of the PXY family with their expression yet to be investigated (Hirakawa *et al.*, 2008; Shi *et al.*, 2019; Smetana *et al.*, 2019; Wang *et al.*, 2019). The spatial expression of PXL1 and PXL2 was determined using transverse sections of *Arabidopsis* root expressing GUS under PXL1 and PXL2 promoters. The promoter of PXL1 was active in the first protective layer developed by the plant, called the epidermal layer (Fig.5). As seen in Fig.5, the epidermis sheds in organs undergoing secondary growth and is replaced with a more plastic protective layer called periderm. As the epidermis is known to be occasionally involved in the formation of periderm (Crang *et al.,* 2018) and that PXY-LIKE receptors are positive regulators of cork cambium activity (Chapter II), PXL1 could potentially regulate cork cambium initiation from the epidermis via an unknown non-cell-autonomous signal.

However, the activity of PXL2 promoter was localised in the periderm and had a potential second peak of activity in the vasculature (Fig.5). This suggests that the more probable PXY-LIKE candidate to regulate cork cambium is PXL2. Altogether, PXL1 and PXL2 are – to varying extents - potential candidates to regulate the cork cambium and further loss-of-function and overexpression studies will need to be conducted to fully understand their roles in cambium development. Finally, PXL2 might also regulate the vascular cambium although its expression in the vasculature needs to be confirmed.

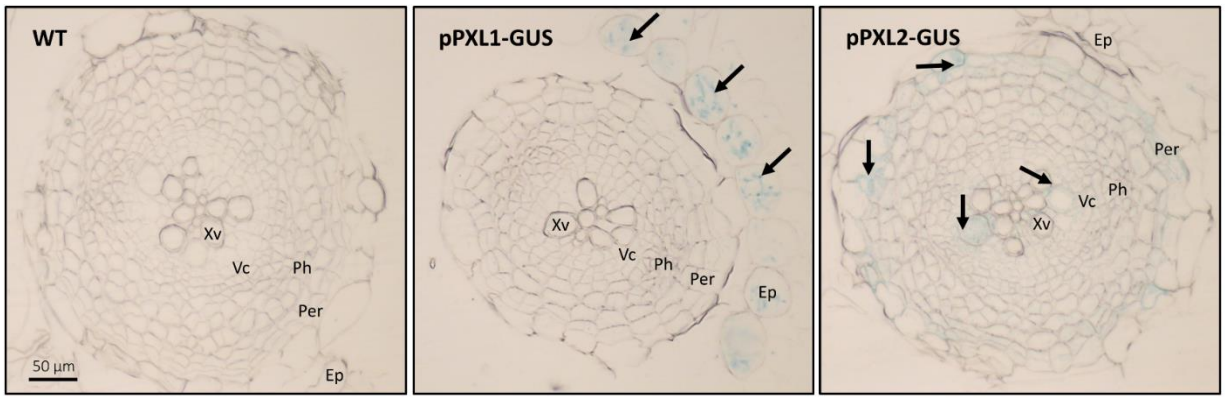


Fig.5. Expression zones of PXL1 and PXL2 in the root of Arabidopsis thaliana. GUS is expressed in the transversal sections of 12-day-old Arabidopsis roots under the promoters of PXL1 and PXL2 (pPXL1 and pPXL2). The arrows are pointing to the tissues expressing GUS. The annotated tissues consist of Xylem vessels (Xv), Vascular cambium (Vc), Phloem (Ph), Periderm (Per) and Epidermis (Ep).

3.3.2 Generation of lines to study the interactions between PXY family and ER family:

To examine the interaction between PXY and ER family members, we started to generate lines expressing both PXY and ERf respectively tagged with a *hemagglutinin* (HA) epitope or a *yellow fluorescent protein* (YFP). In previous studies, PXY::PXY-HA, ER::ER-YFP, ERL1::ERL1-YFP and ERL2::ERL2-YFP translational fusions were created and reported to complement the corresponding mutant phenotype. In this chapter, we successfully selected homozygous lines for PXY::PXY-HA in the second generation of plant F2. Once selected, we crossed PXY::PXY-HA with other parental lines ER::ER-YFP, ERL1::ERL1-YFP and ERL2::ERL2-YFP and we collected the seeds originating from these crosses. In the future, the generated lines will be tested for homozygosity and co-immunoprecipitation analysis will be conducted to test the interaction *in vivo* between PXY and different members of the ER family.

As LRR-RLK interactions are usually transient and of low affinity, an additional protein interaction experiment was prepared to strengthen Co-IP studies. Förster resonance energy transfer (FRET) was the technique chosen to visualise PXf and ERf interactions directly in plant living tissue. We generated the entry vectors for the ER coding sequence using the Gateway cloning system (Invitrogen). The validity of this entry vector was verified (Fig.6) and the vector was purified. This ER coding sequence remains to be transferred to a destination vector with a 35S promoter and *YFP* on either side of the attR recombination sequences. ERL1 and ERL2 coding sequences were poorly amplified from cDNA (and gDNA) and this was likely due to the low quality of the primers which had high melting temperatures (tm >70^{o}C). As a result, the entry plasmids for ERL1 and ERL2 would need to be generated in future experiments.

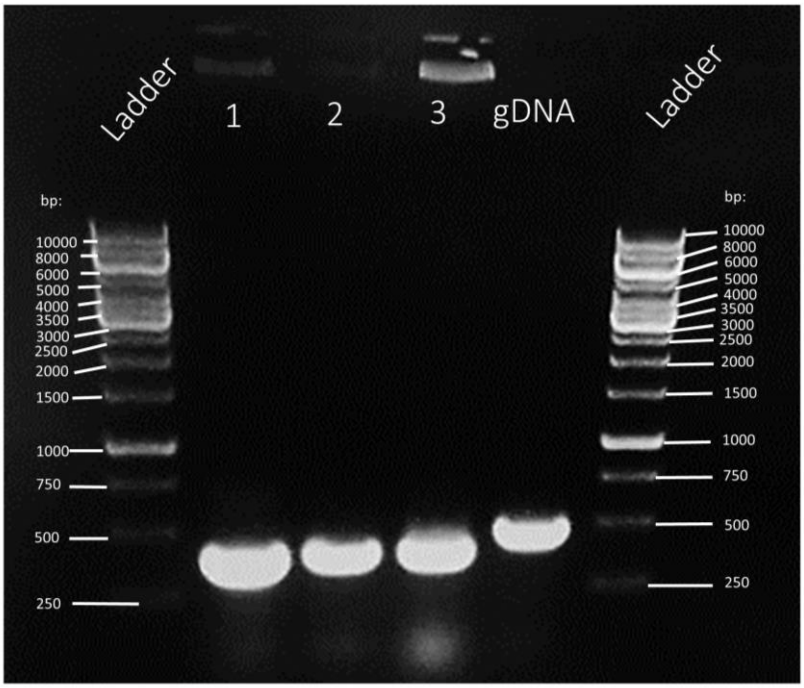


Fig.6. Verification by PCR for ER inserted in pENTR of transformed DB3.1 E. coli cells. Each number 1, 2 and 3 corresponded to a different bacterial colony. The primers used were the verification primers (see Data S1) and gDNA was used as a control. As expected, the bands corresponded to 406 base pairs (bp) for ER inserts in colony 1, 2 and 3 while corresponding to 490 bp for gDNA. The difference in sequence size was explained by the primers spanning an intron and the ER insert being amplified from cDNA.

3.4 Discussion:

3.4.1 ERf and PXf co-localise in the cambium area:

Preliminary studies found that (i) PXf and ERf genetically interact to regulate cambium activity (Chapter II); (ii) the extracellular domains of these receptors can physically interact *in vitro* (Waese *et al.*, 2017; Smakowska-Luzan *et al.*, 2018; Mott *et al.*, 2019); and (iii) the expression of these receptors overlaps in the vascular cambium (see Table.1). These three pieces of evidence strongly suggest that physical coupling between PXf and ERf is the molecular mechanism underpinning the observed genetic interactions. However, PXf and ERf expression had not been found to overlap in the second stem cell niche that they regulate - the cork cambium - nor its derived tissues - the periderm. As the expression patterns of PXY paralogues – PXL1 and PXL2 – had never been studied before and ERL2 expression remained unexplored in the root, we investigated their expression patterns in this chapter, with a particular focus on their expression in the periderm.

Similar to the stem and the early developmental stages of the hypocotyl, ERL2 seemed to be absent from the tissues of *Arabidopsis* root (Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017). These results correlated with the quantitative genetic analyses conducted in the previous chapter, suggesting that ERL2 might not play an important role in the regulation of the cambium (Chapter II). However, the oldest root investigated in this study were 4 weeks old and Wang et al. (2019) found that ERL2 was only expressed in hypocotyl after 5 weeks. As a result, ERL2 might only be expressed in late

developmental stages of the root. Despite its late expression in the hypocotyl, previous studies showed that ERL2 plays a crucial role in the vasculature of the hypocotyl as it compensates for the reduction in cell division of the *pxf er* mutant with an increase in cell size (Wang *et al.*, 2019). The tight regulation of ERL2 expression (and potential degradation) makes sense as growth regulators like EPFL2 act preferentially through ERL1/2 (Kosentka *et al.*, 2019; Kawamoto *et al.*, 2020). While ERL2 was absent from 5-week-old stems, online tissue-specific transcriptomic data suggested that ERL2 displays similar expression patterns to ER and ERL1 in 6 to 8-week-old stems (Fig.7). Despite being less expressed than ER and ERL1, ERL2 seems to be located in the cambium and the epidermis of older stems (Shi et al., 2020). Consequently, experimentally testing the expression and role of ERL2 in the late developmental stages of plant organs represents an interesting research avenue to further our understanding of cambial regulation.

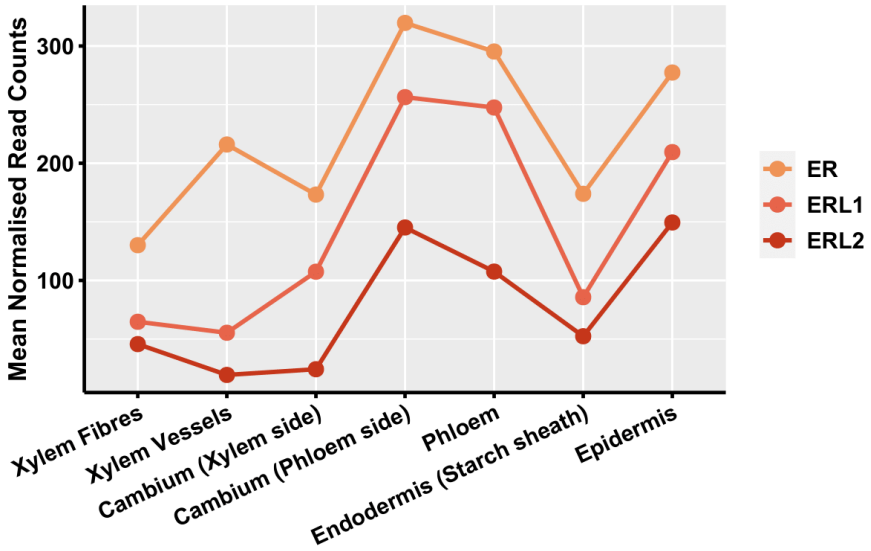


Fig.7. Expression patterns of ER family in the inflorescence stem of Arabidopsis from publicly available tissue-specific transcriptomic profiles (Shi et al., 2020). Shi et al. (2020) obtained these tissue-specific transcriptomes using Fluorescence-Activated Nucleus Sorting (FANS) to sort tissue-specific nuclei and extract their RNA for sequencing analysis. ER and ERL1 expressions correspond to the findings of Uchida and Tasaka (2013). The graph was generated from the available data using R v.4.0.2.

After investigating ERL2, this study focused on PXL1 and PXL2 expression patterns. While morphogenetic analyses from the previous chapter showed that PXY paralogues regulate both the vascular and cork cambium, the foregoing expression study clarified the individual functions of PXL1 and PXL2 receptors (Chapter II). For instance, PXL1 was expressed in the epidermis of the root which is the protective layer formed at the embryonic stage and is later replaced by the developmentally plastic periderm layer (Fig.5). This substitution of layer is specific to organs undergoing extensive secondary growth such as the root and the hypocotyl. While the epidermis expressed-PXL1 is unlikely to regulate the vascular cambium, this receptor might synchronise the shedding of the epidermis with the formation of periderm growing underneath. There are well studied examples of LRR-RLKs receptors regulating the homeostasis between the formation and detachment of cell layers. One of them is the receptor HAESA-LIKE2 (HSL2) which coordinates the dynamics of root cap detachment with the generation of new cap layers (Shi *et al.*, 2018). To understand how PXL1 could regulate the cork cambium while being expressed in the epidermis, PXL1 loss-of-function and overexpression mutants should be generated to analyse the activity of the cork cambium alongside the separation of the epidermis. Although the role of PXL1 remains inconspicuous in the context of secondary growth, the expression and subsequent role of PXL2 seems more straightforward.

The receptor PXL2 was clearly expressed in the periderm and seemed to display a second weaker peak of expression in the vasculature (Fig.5). This strengthened our conclusions from the previous chapter indicating that PXL2 is likely to regulate the initiation and maintenance of stem cells in the cork cambium (Chapter II). However, our previous morphogenetic analyses showed that PXY-LIKE receptors not only regulated the cork cambium, but they also controlled the vascular cambium (Chapter II). Although PXL2 is the most probable candidate to regulate the vascular cambium out of the two PXY paralogues (as it might be weakly expressed in the vasculature), further expression studies should be conducted to confirm the presence of PXL2 in the vasculature.

Interestingly, plants have evolved complex ligand-receptors systems such as TDIF-PXf and EPFL-ERf for inter-cell layer communication, suggesting that PXL2 does not have to be expressed in the vasculature to regulate it. In previous studies, Xiao *et al.*, (2020) found that the initiation and activity of the cork cambium strongly rely on proper vasculature development. Although this interaction has only been shown in one orientation, the cork cambium might also influence the formation of the vascular cambium. As a result, communication between the cork and vascular cambium might be essential for the plant to synchronise its radial expansion (mainly coming from vascular cambium division) with the formation of a developmentally plastic periderm layer which will adapt to the size of the organ. Although the non-cell-autonomous signal between the cork and the vascular cambium has not been identified yet, the hypothesis that PXL2 might indirectly regulate the vascular cambium while only being expressed in the periderm remains viable. In the same way, phloem-expressed ER and ERL1 regulate the activity of the procambium in the inflorescence stem by the means of an unknown non-cell-autonomous signal (Uchida and Tasaka, 2013). To strengthen this hypothesis, it would be interesting to express PXL2 under tissue specific promoters in PXf loss-of-function mutants and test whether the vascular cambium phenotype is restored.

This study shows that PXL2 has overlapping expression with ERf receptors in the root (whose expression patterns were already described). For instance, the expression of PXL2 overlaps with ER and, if it is expressed in the vasculature, its expression would overlap with both ER and ERL1 (Ragni; personal communication). As ERf members are found in the epidermis and periderm in the stem and the hypocotyl respectively, this raises the question of whether PXL1 and PXL2 expression remain conserved across organs. As the root and the hypocotyl roughly share the same layer organisation (xylem – vascular cambium – phloem – periderm from the inside to the outside layer), PXL1 and PXL2 might display similar expression patterns in these two organs. Although this hypothesis is viable, PXL1 and PXL2 expression remains to be experimentally tested in the hypocotyl (they are marked "?" in Table.2 and Fig.9). While root and hypocotyl could share similar expression patterns, the morphology of the stem drastically differs from the root, indicating that in the stem PXL1 and PXL2 are likely to exhibit different expression patterns in those organs. The stem possesses a regularly interspaced vascular cambium instead of a continuous ring like in the root and the hypocotyl, and due to its reduced secondary growth, the stem keeps its epidermal layer rather than growing a more developmentally plastic periderm (Eames and MacDaniels 1947). As a result, we searched for PXL1 and PXL2 expression in publicly available tissue-specific transcriptomics data in *Arabidopsis* inflorescence stem (Shi *et al.*, 2020). In this transcriptomic data, PXL1 expression peaks in the phloem and has a second weaker maximum in the xylem vessels of the stem (Fig.8). In contrast to PXL1, PXL2 expression peaks in the xylem vessels while displaying a second weaker maximum in the phloem side of the cambium (Fig.8). Surprisingly, the overall expression of PXL2 in the stem is significantly stronger than PXL1 (about 10 times stronger). Both PXL1 and PXL2 are expressed in the epidermis of the stem at reduced levels. This suggest that PXL1 and PXL2 might be co-localised with ER and ERL1 which are also expressed in the phloem, the xylem and the epidermis of *Arabidopsis* stem (Uchida *et al.*, 2012; Uchida and Tasaka, 2013). While the expressions of PXL1 and PXL2 remain to be experimentally

tested in organs like the stem and the hypocotyl, these transcriptomic profiles support the fact that PXL1 and PXL2 should have overlapping expression with ER family receptors in organs undergoing secondary growth (these receptors are also marked by "?" in Table.2 and Fig.9).

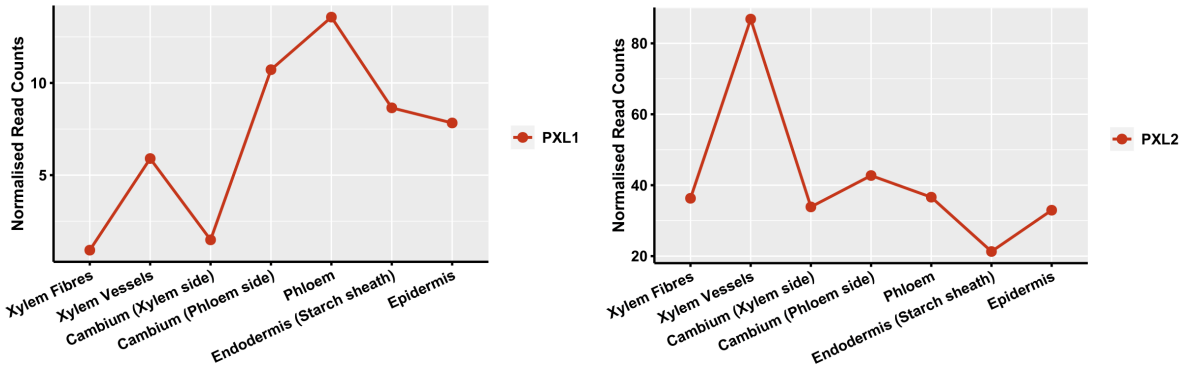


Fig.8. Expression patterns of PXL1 and PXL2 in the inflorescence stem of Arabidopsis from publicly available tissue-specific transcriptomic profiles (Shi et al., 2020). Shi et al. (2020) obtained these tissue-specific transcriptomes using Fluorescence-Activated Nucleus Sorting (FANS) to sort tissue-specific nuclei and extract their RNA for sequencing analysis. The graphs were generated from the available data using R v.4.0.2. Due to different levels of expression, the expression profiles of PXL1 and PXL2 were represented in separate graphs. PXY was not shown on the graph as its expression level was significantly higher than its paralogues by 2 orders of magnitude.

Altogether, this study successfully showed that PXY family and ER family are co-localised in the cambia and their derived secondary tissues. This close proximity between receptors of PXf and ERf in the vascular and cork cambium strengthens the hypothesis of physical interaction between ERf and PXf to regulate cambial activity. This suggests that the vascular cambium and the cork cambium share a PXf-ERf driven mechanism modulating the initiation and maintenance of stem cells in post-embryonic tissues.

Table.2: Overview of PXf and ERf expression patterns across the stem, the hypocotyl and the root of Arabidopsis thaliana. *Like PXY, PXL1 and PXL2 were assumed to be expressed in the same tissues across organs. The orange, red and yellow colours respectively highlight the vascular tissue, the periderm and the epidermis. The question marks indicate the expression patterns requiring further investigation.*

RLK	Stem	Hypocotyl	Root	Ref
PXY	Vascular cambium; Xylem initials			Hirakawa *et al.*, 2008; Shi *et al.*, 2019; Smetana *et al.*, 2019; Wang *et al.*, 2019
PXL1	Phloem; Xylem?	Epidermis?	Epidermis	This study
PXL2	Vascular cambium; Xylem?	Vascular cambium?	Vascular cambium?	This study
		Periderm?	Periderm	
ER	Phloem; Xylem;	Vascular cambium; Xylem initials;	Vascular cambium;	Uchida *et al.*, 2012; Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019; Laura Ragni personal communication
	Epidermis	Periderm	Periderm	
ERL1	Phloem; Xylem;	Vascular cambium; Xylem initials;	Vascular cambium	Uchida *et al.*, 2012; Uchida, Shimada and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019; Laura Ragni personal communication
	Epidermis	Periderm		
ERL2	Absent	Vascular cambium; Xylem initials; (at late developmental stage)	Absent	Uchida and Tasaka, 2013; Ikematsu *et al.*, 2017; Wang *et al.*, 2019; This study
		Periderm (at late developmental stage)		

Each organ exhibits unique growth habits and requires slightly different growth regulations. As a result, different ERf-PXf signalling modules might regulate distinct developmental aspects by slightly changing their components. Organs undergoing extensive secondary growth (e.g. the hypocotyl and the root) might differently express positive and negative regulators of radial expansion than organs like the stem. Consequently, PXf and ERf receptors co-localise in the vasculature and the periderm, but the number and identity of PXf and ERf members is specific to the tissue of each organ as well as its developmental stage.

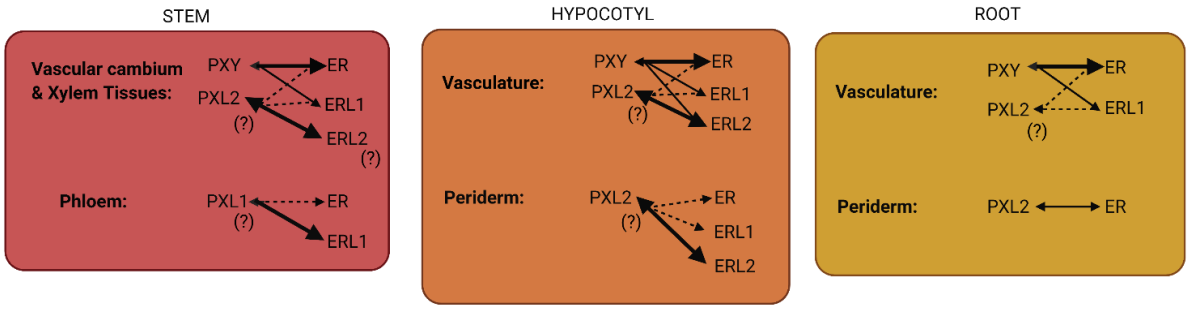


Fig.9. Network of hypothetical interactions between PXY and ER family receptors in the stem, the hypocotyl and the root of Arabidopsis thaliana. The network of interactions consists of three levels of confidence represented by three types of arrows. The bold arrows represent the interactions identified in vitro with the Extracellular Interactome Assay which remain to be identified in those specific tissues. The thin continuous arrows represent the possible interactions between receptors which have been experimentally shown to be expressed in the same tissues. The thin dashed arrows represent the hypothetical interactions between receptors marked with "?", whose expression has been hypothesised and needs to be confirmed experimentally. In the hypocotyl PXL2 expression was

hypothesised to resemble its expression in the root due to the morphological similarities between the two organs. In the stem, PXL1, PXL2 and ERL2 expression patterns were taken from Shi et al. (2020)'s transcriptomic profiles.

While ERf and PXf receptors are mainly localised in cambial tissues, ER, ERL1 and PXL1 are also found in non-cambial tissues like the epidermis, the phloem and xylem tissues. This suggests that the ERf and PXf receptors in the epidermis, the phloem or the xylem tissues might use an unknown non-cell-autonomous signal to regulate cambial cell activity. However, some of these LRR-RLK genes are highly pleiotropic and their expression in different tissues might account for the diversity of their functions. For instance, ER and ERL1 - expressed in the epidermis of the stem - regulate stomatal patterning (Shpak *et al.*, 2005; Abrash and Bergmann, 2010). These results suggest that the physical and genetic coupling of PXf-ERf in the lateral meristems and their secondary tissues likely regulate cambial activity but the receptors expressed in other tissues would require further investigation.

This study and the literature showed that PXY family and ER family have overlapping expressions, suggesting that potential interactions could exist between most PXf and ERf receptors (Fig.9 and Table.2). Previously, strong *in vitro* interactions were observed between ER and PXY, and ER and PXL1 in one orientation as well as PXL2 and ERL2 in both directions (Waese *et al.*, 2017). Further interactions could be discovered using the transgenic lines and genetic constructs we prepared in the following section.

3.4.2 Preparing protein-protein interaction experiments:

To investigate the protein-protein interactions between ER family and PXY family receptors, we generated transgenic lines for co-immunoprecipitation assays. Arabidopsis lines expressing PXY tagged with HA under PXY promoter were crossed to lines expressing one ER family member tagged with YFP under their native promoter. The generated lines remain to be tested for homozygosity. Once they will be selected, these transgenic lines will be used for co-immunoprecipitation.

As the interaction between LRR-RLKs is usually poorly biochemically tractable, we also prepared genetic constructs to investigate the binding of ERf-PXf extracellular domains directly in live cells using FRET analysis. In the future, the entry vector generated for 35S::ER-YFP will need to be transferred to a destination plasmid and the 35S::ERL1-YFP as well as 35S::ERL2-YFP constructs will have to be generated. Additionally, the promoter of the construct XVE::PXY-CFP provided by Dr. Yuki Kondo will have to be changed to a strong constitutive 35S promoter (Morita *et al.*, 2016). Although ER family receptors are natively expressed in the epidermis of *Nicotiana benthamiana*, the generation of 35S::ERf-YFP constructs is important for the consistent and strong expression of ERf-YFP, ensuring the success of FRET analysis. After finalising the constructs, they will be transiently expressed in the epidermis of *Nicotiana benthamiana* and ERf and PXf interactions will be investigated using fluorescence microscopy.

The main disadvantage of this method is that the interactions between PXf and ERf will be studied in the epidermis of *Nicotiana benthamiana* instead of being examined in the vascular and cork cambium of *Arabidopsis thaliana* where they genetically interact. However, the results of FRET analysis and co-immunoprecipitation assays combined with expression studies will already extensively explore the molecular mechanisms underpinning PXf-ERf genetic interactions and will represent a major breakthrough for plant development research.

3.5 Concluding remarks:

Repurposing large proteins - like ERf and PXf receptors - for different processes is a strategy commonly adopted by plants (and other multicellular organisms) throughout evolution to save energy. The ERf and PXf RLK possess a wide range of developmental and physiological functions, including growth, patterning, immunity and fertilisation (Tsukaya *et al.*, 1993; Torii *et al.*, 1996; Godiard *et al.*, 2003; Qi *et al.*, 2004; Shpak *et al.*, 2004; Hall *et al.*, 2007). However, these processes occur in close spatiotemporal proximity and require tight regulation of RLK signalling to prevent spill over of activation from one process to another (Abrash, Davies and Bergmann, 2011). As a result, RLKs have evolved to work by ligand-receptor and receptor-coreceptor modules. This modularity allows the signalling cascades to slightly change the key components of each module according to the regulated process. One well-studied example of modularity is the ERf receptors binding to the co-receptor TOO MANY MOUTHS (TMM) to modulate their affinity with different EPFLs (which act antagonistically) and control stomatal patterning (Abrash, Davies and Bergmann, 2011; Lee *et al.*, 2012). However, TMM expression is restricted to the epidermis, suggesting that ERf might associate with another co-receptor to regulate secondary growth. This study combined with the previous chapter indicate that ERf may bind to PXf to regulate the initiation and activity of the vascular and cork cambium (Chapter II). This physical coupling could modify the receptors' affinity for their respective ligands, thus preventing any ligand binding or selecting positive/negative growth regulators to bind their extracellular leucine-rich-repeats domains. To further our understanding of PXf and ERf relationship, spatiotemporal expression studies as well as structural analyses (e.g. protein-protein docking simulations and/or crystal structures) of the supposed PXf-ERf modules constitute interesting research avenues.

While ERf and PXf may physically interact, ERf and PXYf signalling pathways could also interact at other levels than at the membrane of cells. For instance, ERf and PXf are known to regulate each other's expression (Wang *et al.*, 2019). As a result, ER and PXY signalling pathways might regulate the same downstream transcription factors to tightly control the expression of the cell. To test this hypothesis, Dr. Etchells' laboratory is currently analysing the transcriptomes of PXY and ER family loss-of-function mutants.

Chapter IV: Which components regulate the cambial activity downstream of ERECTA signalling?

Time-course gene expression analysis under ERECTA family receptor altered activity & investigation of the interactions between the ERf-PXf genetic network and auxin signalling to regulate cork cambium formation.

4.1 Introduction:

In the previous chapters we investigated the signalling pathway(s) of ER family (ERf) receptors in the context of secondary growth. More specifically, we examined the protein-protein interactions occurring at the plasma membrane such as ligand-receptor and receptor-co-receptor interactions (Chapter I, II, III). However, the intracellular mechanisms regulating the vascular and the cork cambium activity downstream of ERf receptors remain largely unknown (Fig.1).

Upon binding of their ligands and/or co-receptors, the ERf receptors undergo important conformational changes which modulate the activity of their intracellular kinase domains. This change in kinase activity activates a mitogen-activated protein kinase (MAPK) cascade, leading to the up or down regulation of transcription factors as well as a change in gene expression (Fig.1) (Hirakawa *et al.*, 2008; Lehti-Shiu *et al.*, 2009; Liu *et al.*, 2017). Few transcription factors have been identified as part of the ERf signalling cascade (Shpak, 2013). Only transcription factors specific to stomatal development – such as the basic helix-loop-helix (bHLH) SPEECHLESS and MUTE transcription factors - are known to be regulated downstream of ERf signalling (Fig.1) (MacAlister, Ohashi-Ito and Bergmann, 2007; Pillitteri *et al.*, 2007). However, ERf receptors also interact with other transcription factors in the context of meristem maintenance and/or fibre differentiation. These transcription factors belong to families of the WUSCHEL-HOMEOBOX RELATED (WOX), CLASS III HOMEODOMAIN-LEUCIN ZIPPER (HD-ZIP III), AUXIN RESPONSE FACTORS (ARF), CLASS I KNOTTED1-LIKE HOMEOBOX (KNAT) and NAC SECONDARY WALL THICKENING PROMOTER FACTORs (NST). In *er erl1 erl2* shoot apical meristem, the expression of WUSCHEL (WOX) increased while the expression of AINTEGUMENTA (HD-ZIP III) and ARF5/MONOPTEROS was significantly reduced (Chen *et al.*, 2013). Similarly, in the hypocotyl, ER and ERL1 regulate the expression of NST1/3 in a BREVIPEDICELLUS/KNAT1-dependent manner (Ikematsu *et al.*, 2017). As these families of transcription factors have wide-ranging roles in vascular and periderm development (Berleth and Jurgens, 1993; Zhong and Ye, 1999; Baima *et al.*, 2001; Emery *et al.*, 2003; Mattsson, Ckurshumova and Berleth, 2003; Mitsuda *et al.*, 2007; Hirakawa, Kondo and Fukuda, 2010; Ragni *et al.*, 2011; Liebsch *et al.*, 2014; Woerlen *et al.*, 2017; Ramachandran *et al.*, 2017; Brackmann *et al.*, 2018; Xiao *et al.*, 2020), this study focuses on identifying which of the transcription factors involved in secondary growth are direct targets of ERf signalling.

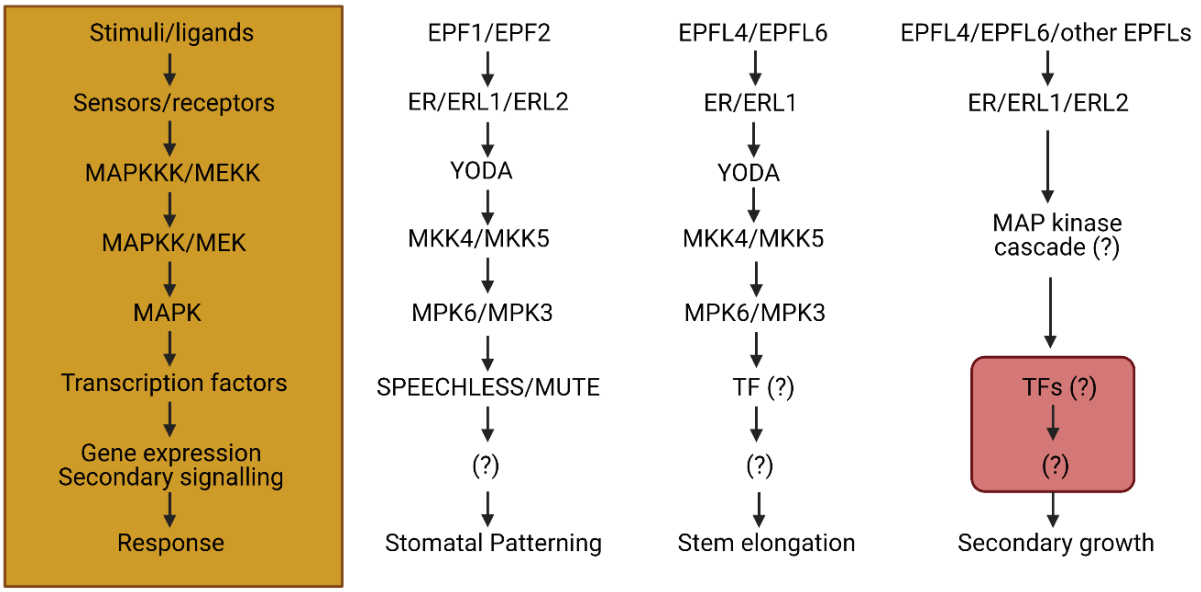


Fig.1. Signalling cascades downstream ER family receptors regulating stomatal patterning, stem elongation and secondary growth (adapted from Meng et al. (2013)). The red box indicates the signalling components investigated in this study.

Since a local maximum of the phytohormone auxin leads to the up regulation of HD-ZIP III and ARF transcription factors (Smetana *et al.*, 2019), we also examined the interaction between the ERf receptors and auxin. In particular, we investigated the relationship between auxin and the ERf-PXf genetic network (discovered in Chapter IV) as well as how this relationship regulates the activity of the cork cambium. Both ER family and PXY family interact with auxin signalling. PXY controls cell division in the vascular cylinder by co-regulating the activity of MONOPTEROS/ARF5 transcription factor alongside auxin (Han *et al.*, 2018). Additionally, PXY might regulate the production and perception of auxin as the HD-ZIP III paralogues of ATHB8 – one of PXY expression targets - modulate the expression of auxin biosynthesis and perception genes (Baima *et al.*, 2001; Müller *et al.*, 2016). On the other hand, ERf receptors regulate the transport and production of auxin by regulating the expression of the PIN1 auxin efflux protein in the vasculature of leaves (Chen *et al.*, 2013) and by controlling the expression of the YUCCA enzyme involved in auxin biosynthesis (Woodward *et al.*, 2005). While most researchers focused on the interaction between auxin and PXY or auxin and ER in the vasculature, a recent study found that periderm initiation was also regulated by auxin (Xiao *et al.*, 2020). Interestingly, this raises the question of how the genetic network formed by ERf and PXf receptors interacts with auxin signalling to control stem cell activity in the cork cambium.

To investigate the intracellular signalling pathway of the ERf receptors, a time-course gene expression analysis under ERf-altered activity was conducted in *Arabidopsis* hypocotyl (Fig.2). As EPFL6 is a ligand of ERf receptors which principally acts as a growth regulator (Abrash, Davies and Bergmann, 2011; Uchida *et al.*, 2012), we aimed to overexpress this gene using a β-Estradiol inducible system to alter the functions of ERf receptors during secondary growth (Fig.2) (Zuo, Niu and Chua, 2000). After ERf activity was altered, we aimed to examine changes to gene expression in the hypocotyls of induced lines by performing an RNA sequencing analysis at different points in time. As the application of β-Estradiol dictates the overexpression of EPFL6, the inducible lines should not display any of the severe morphological changes observed in the loss-of-function mutants. As a result, the changes in gene expression should come from the alterations in ERf signalling (and not morphological defects), thus making the inducible system a suitable approach to identify transcription targets of ERf signalling involved in secondary growth. Finally, we investigated the interactions between auxin and the PXf-ERf

signalling network by assessing changes in periderm development of auxin-treated *pxf erf* loss-of-function mutants.

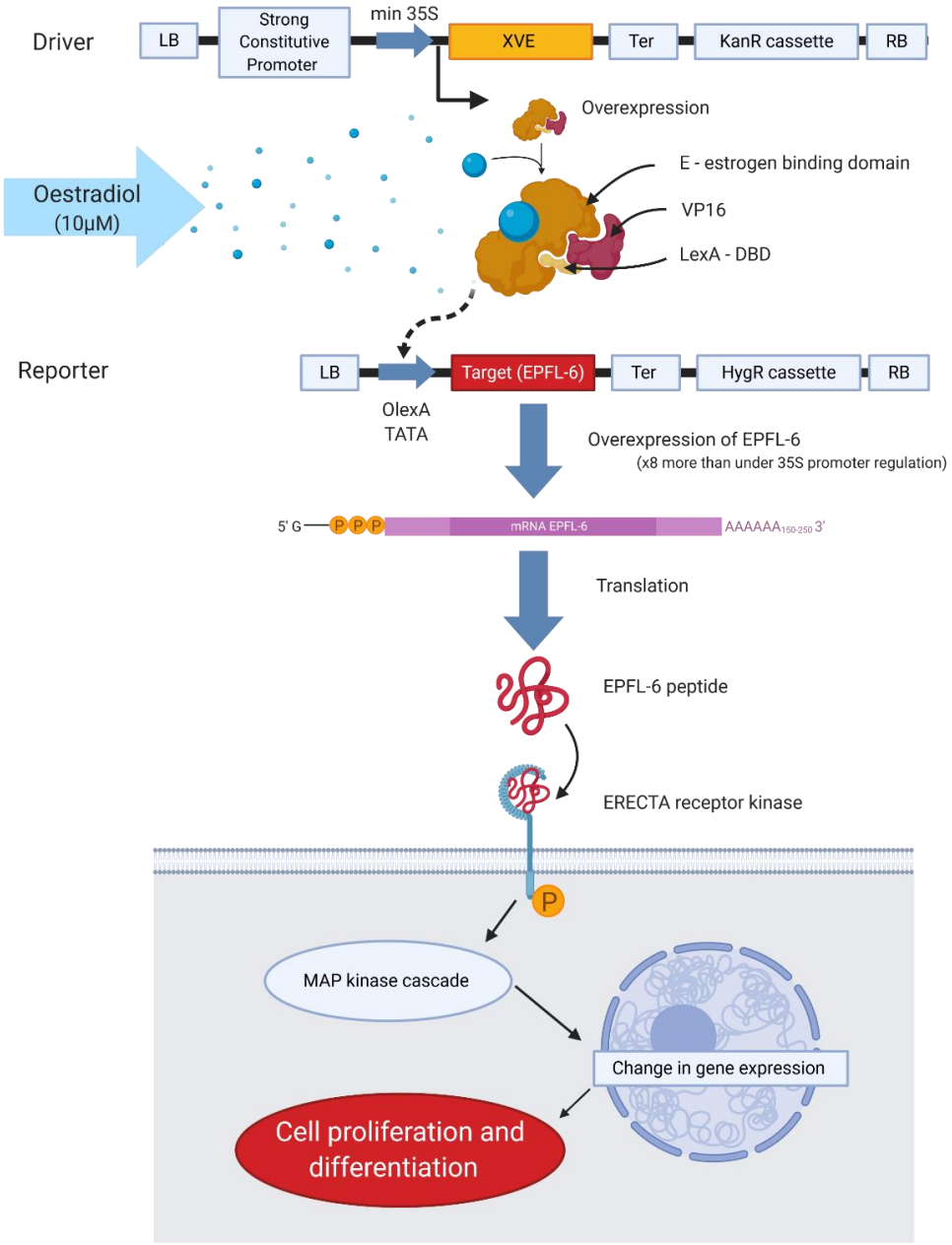


Fig.2. Overview of the β-estradiol induced EPFL6 expression system. Two cassettes are introduced in Arabidopsis (the 35S::XVE cassette and the OlexA::EPFL6 cassette) allowing the spatiotemporal regulation of EPFL6 overexpression under β-estradiol induction. ***Step 1:*** *A chimeric XVE transcription factor is constitutively overexpressed.* ***Step 2:*** *When β-estradiol is applied, XVE transcription factor binds to an β-estradiol molecule and becomes transcriptionally active.* ***Stage 3:*** *XVE strongly binds to OLexA promoter and leads to the overexpression of the target gene, EPFL6. This overexpression is eight-times higher than a routine overexpression system.* ***Stage 4:*** *EPFL6 peptide binds to ERECTA family receptors, activating the downstream signalling cascade which regulates secondary growth (e.g. cambial cell division and/or differentiation).*

4.2 Materials and Methods:

4.2.1 Materials:

Resource Table:

Reagent or Resource	Source	Identifier
1-Naphthaleneacetic acid (NAA)	Duchefa	Cat# N0926
β-Estradiol	Sigma	Cat# E8875
Murashige and Skoog Basal Medium (MS)	Duchefa	Cat# M0255
Plant Agar	Duchefa	Cat# P1001.1000
Chloral hydrate	Merck	Cat# 1.02425.1000
Glycerol	Roth	Cat# 6962.1
Zirconica/Silica beads	Biospec Products	Cat# 11079101z
SeraMag Oligo (dT) Coated Magnetic particles	Thermo Scientific	Cat# 3815-2103-011150
dNTP Mix	PCR Biosystems	Cat# PB10.71-05
qPCRBIO cDNA Synthesis Kit	PCR Biosystems	Cat# PB30.11-02
Commercial Assays:		
QuickClean II Plasmid Miniprep Kit	GenScript	
Experimental model: Organisms		
Arabidopsis: Col-0	Widely distributed	N/A
Arabidopsis: 35S::XVE-OlexA::EPFL6 in epfl4 epfl6	Laboratory stocks	N/A
Arabidopsis: sgs3 in Col-0	NASC	N/A
Arabidopsis: pxf er in Col-0	(Wang *et al.*, 2019)	N/A
Agrobacterium tumefaciens strain GV3101	Laboratory stocks	N/A
E. coli 35::XVE-OlexA::EPFL4 with Spectinomycin resistance	Laboratory stocks	N/A
E. coli 35::XVE-OlexA::EPFL6 with Spectinomycin resistance	Laboratory stocks	N/A
Oligonucleotides		
Data S2	Sigma	N/A
Software		
Fiji	(Schindelin *et al.*, 2012)	https://fiji.sc/
Rotorgene Q Series software v1.7	QIAGEN	
R v.4.0.2	The R foundation	https://rstudio.com/

Experimental model:

Arabidopsis thaliana transgenic and mutant lines were used to perform experiments. The ecotype and background of each line is specified in the resource table. For the gene expression experiment, plants were grown in soil in 16:8h light:dark cycles. At 26 das (days after sowing) when inflorescence stems were elongating, the plants were treated with 10 µM β-Estradiol. For the treatment with 1-

Naphthaleneacetic acid (NAA), a synthetic growth phytohormone in the auxin family, plants were grown on ½ MS and transferred after 5 days on ½ MS supplemented with 1µM NAA. After 12 days, the NAA-treated plants were collected for periderm analysis.

4.2.2 Method Details:

β-Estradiol induction:

The primary *35S::XVE-OlexA::EPFL6* transformants were generated in previous experiment (Rebecca Doherty, MBiol student). The progenies from these primary transformants were successfully selected by germination of seeds on hygromycin supplemented media. After selection, the plants were grown in soil. After 26 days post sowing (das), EPFL6 gene expression was induced in the transgenic line by watering the plants with β-Estradiol (10 µM). A mock solution of DMSO (the solvent of β-Estradiol) was used as a control. Hypocotyl tissues were collected and analysed for the wild type, the uninduced transgenic line, the transgenic line under 6 hours of induction and the transgenic line under 24 hours of induction. Three replicates were collected per line.

mRNA extraction and cDNA synthesis:

To test whether the β-Estradiol induction was successful, we measured the expression levels of EPFL6 in the collected hypocotyl tissues. The cells of the hypocotyl samples were mechanically lysed by immersing the tissues in liquid nitrogen and grounding them with a bead beater (Qiagen) using Zirconia/Silica beads and a lysis/binding buffer (100mM Tris-HCL, 1MLiCl, 10mM EDTA, 1% SDS, 5mM DTT, Antifoam A, RNAse Free H2O).

Poly(A)-mRNA was isolated from the resulting supernatant as follows: the supernatant containing the total RNA was heat denatured (65^{o}C for 4 minutes), incubated with SeraMag Oligo (dT) Coated Magnetic particles and placed on a magnet. The beads were washed with the lysis/binding buffer (first wash), a washing buffer A (2nd and 3rd washes), a washing buffer B (4th wash) and a low salt buffer (5th and 6th wash). The washing buffer A was a mixture of 10 mM Tris-HCL, 150 mM LiCl, 1mM EDTA, 0.1% SDS, RNAse Free H2O, the washing buffer B contains 10 mM Tris-HCL, 150 mM LiCl, 1mM EDTA, RNAse Free H2O and the low salt buffer was prepared with 20 mM Tris-HCL, 150 mM NaCl, 1mM EDTA, RNAse Free H2O.

The bead-poly(A) mRNA complexes were directly resuspended in cDNA reaction mix (dNTP Mix, 5xRT buffer, RNase Inhibitor, Thermo Scientific™ Maxima™ Reverse Transcriptase and RNase free H2O and separated by incubating the samples at 42 ^{o}C for 60 min, 70 ^{o}C for 15 min and 80^{o}C for 2min. For each experiment, three technical replicates of total volume of 20 ul were used for qRT-PCR. The qRT-PCR was performed using a Rotor-Gene Q Machine (QIAGEN®) with the following cycles (Table):

	Temperature (^{o}C)	Time
Hold	95	2min
40 x Cycles	95	5s
	58	10s
	72	10s
Melt curve	50-95	Increasing by 0.2 ^{o}C every 5s

Table: Program settings on Rotor-Gene Q Machine (QIAGEN®) to conduct qRT-PCRs.

The results from the qRT-PCR were analysed with a Rotorgene Q Series software v1.7 to identify changes in EPFL6 gene expression under β-Estradiol induction. The ACT2 gene (AT3G18780) was used

as the housekeeping gene for normalisation of the gene expression. The primer sequences used for amplifying the genes are presented in Data S2.

Statistical analysis of EPFL6 gene expression:

Statistical analyses were performed using the statistical package of RStudio. The datasets were first tested for normal distribution using a Shapiro-Wilk test and homogeneity of variances using a Levene test. The significant differences between EPFL6 expression of each dataset were calculated with a One-way ANOVA test followed by a Tukey's post hoc test.

Generation of transgenic plants:

As EPFL4 is a close paralogue of EPFL6, this peptide is also a potential regulator of secondary growth in *Arabidopsis* hypocotyl (Uchida and Tasaka, 2013; Wang *et al.*, 2019; see Chapter I). The inducible systems overexpressing EPFL4 and EPFL6 peptides, *35::XVE-OlexA::EPFL4* and *35::XVE-OlexA::EPFL6* constructs (resource table), were generated in previous experiments (Rebecca Doherty, MBiol student). The plasmids were extracted from the *E. coli* cultures (resource table) using QuickClean II Plasmid Miniprep Kit GenScript ®. The plasmids were then mobilised into *Agrobacterium tumefaciens* using electroporation with a Micropulser™ Electroporator. Out of the *35::XVE-OlexA::EPFL4* and *35::XVE-OlexA::EPFL6* constructs, only the latter was successfully transformed in *Agrobacterium*. As the overexpression of EPFL6 might be hindered by post transcriptional gene silencing (PTGS) (see results section), the *35::XVE-OlexA::EPFL6* construct was introduced by floral dip transformation into a SUPPRESSOR OF GENE SILENCING3 (SGS3) loss-of-function mutant, a mutant whose PTGS system is repressed (Clough and Bent, 1998; Vaucheret, Béclin and Fagard, 2001). Progeny from the primary transformants were identified by germination of seeds on ½ MS media containing 50 mg/ml of hygromycin.

Periderm quantification:

The phellem length of NAA-treated plants was measured and statically analysed using the methodology described in Chapter II.

4.3 Results:

4.3.1 Time-course EPFL6 gene expression analysis:

In this section, we aimed to overexpress the EPFL6 ligand to alter the activity of ERf receptors using a β-Estradiol inducible system (Zuo, Niu and Chua, 2000). Surprisingly, the induced lines did not show any significant change in EPFL6 expression compared to the wild type while the uninduced line expressed EPFL6 at greater levels than the wild type (Fig.3). One possible explanation for this phenotype could be that a post-transcriptional gene silencing (PTGS) system prevented the overexpression of the EPFL6 gene (Vaucheret, Béclin and Fagard, 2001). Consequently, RNA sequencing was not performed on these lines but this experiment was repeated on plants lacking a post- transcriptional gene silencing system (see following section).

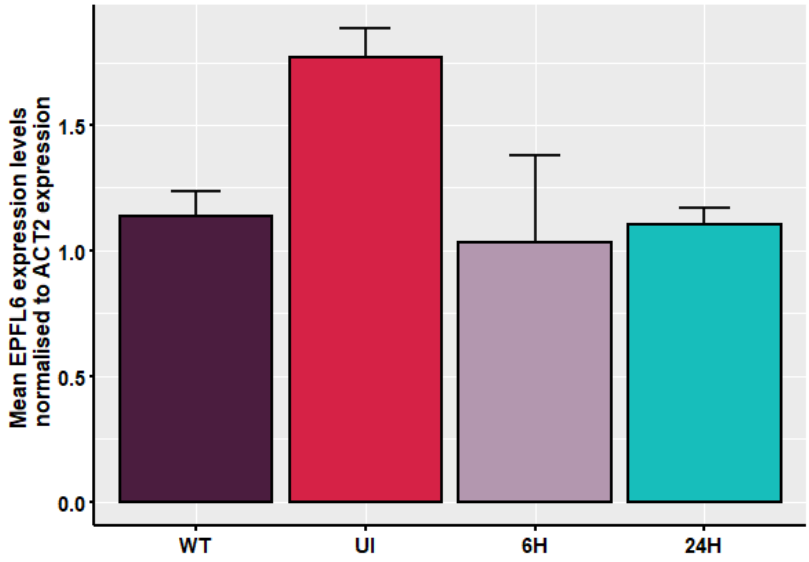


Fig.3. Expression levels of EPFL6 gene after induction of the 35S::XVE-OlexA::EPFL6 line. The expression EPFL6 was normalised to the expression of the housekeeping gene ACT2. UI corresponds to the uninduced transgenic line (control). The absence of asterisks indicates that none of the samples had significantly different EPFL6 expression levels compared to the wild-type (p-value >0.05).

Interestingly, some *35S::XVE-OlexA::EPFL6* transgenic plants displayed a short stature, a thick stem and shrivelled leaves, which are characteristic of the *er* loss-of-function phenotype. The resemblance with *er* phenotype suggested that the EPFL ligands might also compete and/or saturate the binding sites of ERf receptors and thus hinder ERf signalling.

4.3.2 Transforming *sgs3* lines with a β-Estradiol-inducible system:

Transformation of the 35::XVE-OlexA::EPFL6 construct into *sgs3 Arabidopsis* line was performed. However, none of the T1 seeds germinated on selective media, indicating that the transformation of *sgs3* loss-of-function mutants did not work. The lack of transformants was likely due to the poor quality of the plasmids and the low number of individuals to transform (n=18). As a result, this experiment would need to be repeated in the future with more *sgs3* loss-of-function individuals.

4.3.3 Interaction between auxin and PXf-ERf signalling pathways regulating the development of periderm:

We investigated the interactions between auxin and PXf-ERf and how these interactions might regulate the initiation and activity of the cork cambium. As described in Chapter II, the PXY family and ER family receptors form a genetic network regulating the initiation and activity of the cork cambium. Subsequently, removing all members of PXf and ERf led to a drastic reduction in phellem length along the vertical axis of the root (Fig.4). On the other hand, treating *erf pxf* sextuple mutant with auxin (1uM) entirely restored the wild-type periderm phenotype (Fig.4).

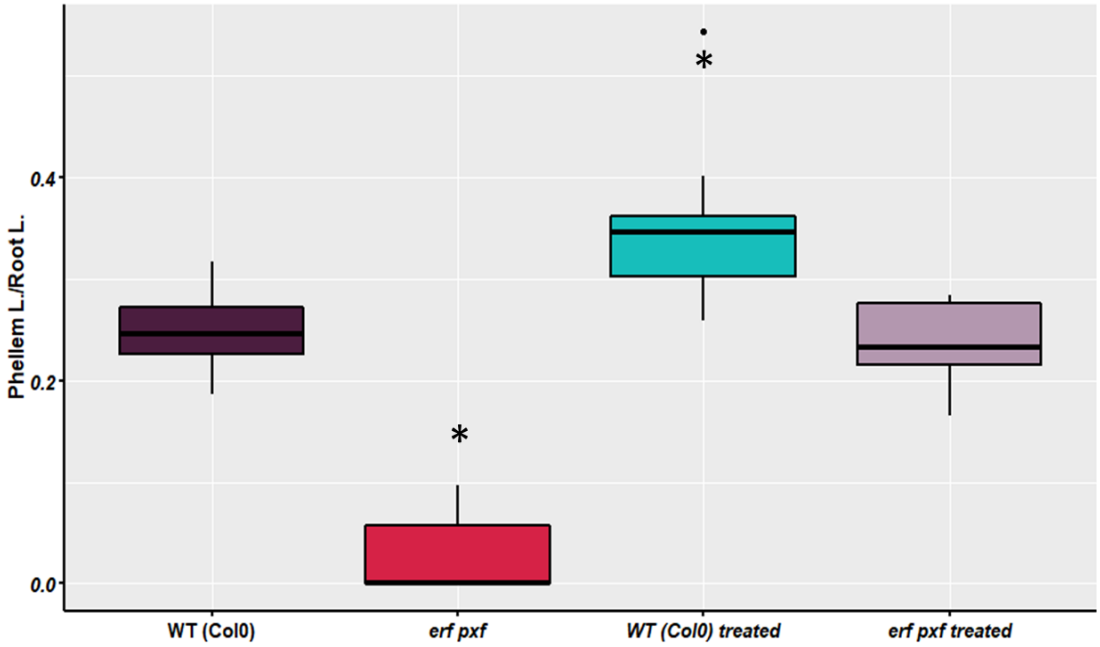


Fig.4. Phellem length normalised to the root length in auxin-treated erf pxf sextuple mutant. The phellem layer is the outer layer of periderm. The asterisks indicate the groups significantly different from the wild-type.

These results indicated that auxin is a key regulator of periderm development and that the PXf-ERf network might interact with auxin signalling (Fig.5). In future experiments, it would be interesting to test whether auxin acts in parallel or downstream of the PXf-ERf network by blocking the auxin signalling pathway and testing whether this enhances the *pxf erf* phenotype. Additionally, measuring the number of periderm cell layers and assessing the overall morphology of *erf pxf* plants treated with auxin would be an interesting research avenue to enhance our understanding of the interactions between PXf-ERf and auxin signalling pathways.

4.4 Discussion:

This study showed that auxin can rescue the initiation and maintenance of the cork cambium in the *pxf erf* mutant, suggesting that auxin might act downstream or in parallel to the previously discovered PXf-ERf signalling network. To further investigate this PXf-ERf-auxin interaction, it would be interesting to measure the concentration of auxin in the *pxf erf* mutant as well as examining the expression and the cellular localisation of auxin biosynthesis enzymes and auxin PIN transporters in ERf and PXf loss-of-function and/or overexpression plants.

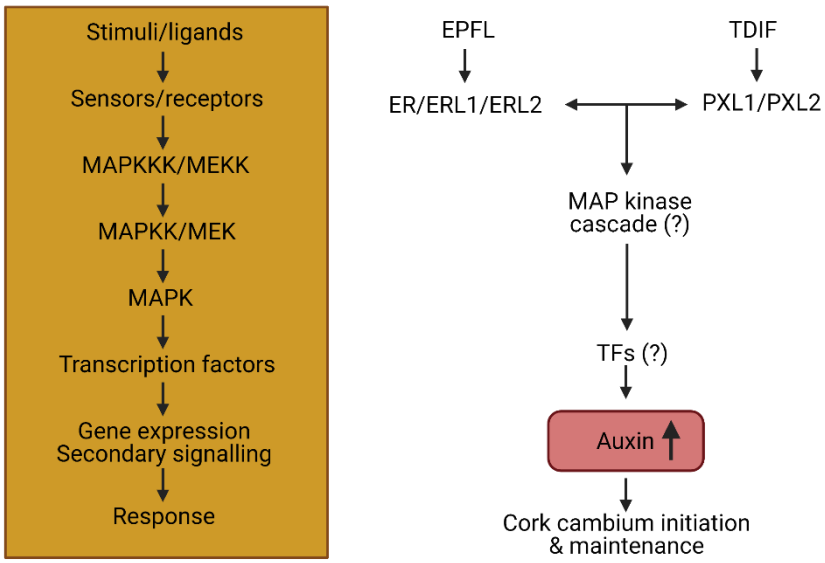


Fig.5. Hypothetical model of the PXf-ERf network interacting with auxin signalling to regulate periderm development. Despite not being displayed in this diagram, auxin signalling could also act in parallel of the ERf-PXf network.

Additionally, this study also showed that a post-transcriptional silencing system might prevent the overexpression of EPFL6 when using a β-Estradiol inducible system. As a result, this experiment would need to be repeated with *sgs3* mutants to identify the transcription targets of ERf signalling. Although *pxf erf* loss of function mutants display strong morphological defects, an RNA sequencing analysis could also be performed on those lines to identify the changes in gene expression caused by the loss of ERf and/or PXf receptors.

Finally, the ERf receptors activate the same mitogen-activated protein kinase (MAPK) cascade during stomatal patterning and inflorescence architecture. This MAPK cascade consists of YODA, MPKK4/5 and MPK6/3. However, the ERf-driven phosphate cascade regulating secondary growth remains to be discovered. To investigate which MAPK cascade is activated by ERf receptors during vascular and periderm development, loss-of-function and overexpression studies could represent interesting research avenues.

5. General conclusion:

5.1 Key points:

This study shines light on the potential EPFL candidates controlling the activity of lateral meristems. Our results indicate that several EPFL peptides redundantly regulate secondary growth. In addition to our first candidates EPFL4-6, the peptides EPFL1, EPFL2, EPFL8 and EPFL9 were identified as potential regulators of secondary growth and their function warrants further investigation. Interestingly, our study also suggests that the EPFL peptides might synchronise secondary growth with other developmental processes such as flowering.

After shifting our focus to the ERECTA family (ERf) receptors, we discovered that these receptors form a genetic network with PXY family (PXf) receptors not only to regulate the vascular cambium but also to control the initiation and maintenance of the cork cambium. These results suggest that the ERf-PXf genetic network ubiquitously regulates the initiation and/or maintenance of stem cells in post-embryonic tissues. Intriguingly, ERf and PXf receptors were also found to co-localise in the lateral meristems and their derived secondary tissues, suggesting that ERf and PXf might physically interact to modulate their intracellular signalling pathway. As a result, transgenic lines and genetic constructs were prepared for future protein-protein assays to investigate the physical coupling between the ERf and PXf receptors. Finally, a preliminary hormonal study showed that the ERf-PXf signalling network might regulate stem cell activity through the phytohormone auxin.

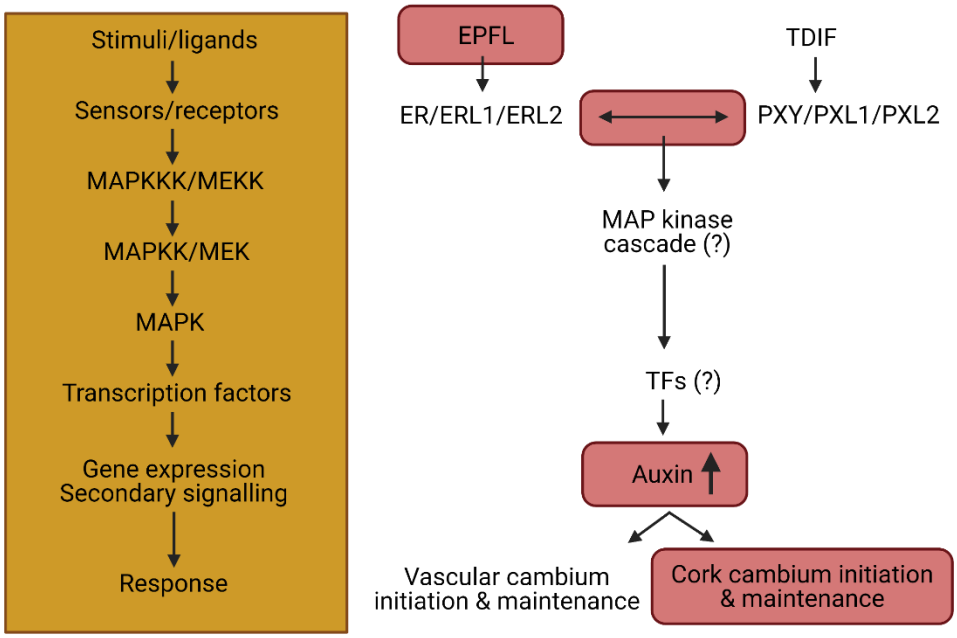


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Fig.1. Model of EPFL-ERf and TDIF-PXf signalling network regulating secondary growth. Key interactions and signalling mechanisms which have been investigated in this study are indicated with red boxes.

5.2 Outlook:

Following our investigation of the signalling pathways regulating the secondary growth of plants, we hope that the future will hold more discoveries about developmental plant biology as they could have an important impact on life on our planet. This knowledge could drastically improve crop yield, protect trees from climate change and enhance carbon capture of plants. Ultimately, it could even render futuristic visions possible such as achieving sustainable modern cities by growing our houses instead of building them (Fig.2).

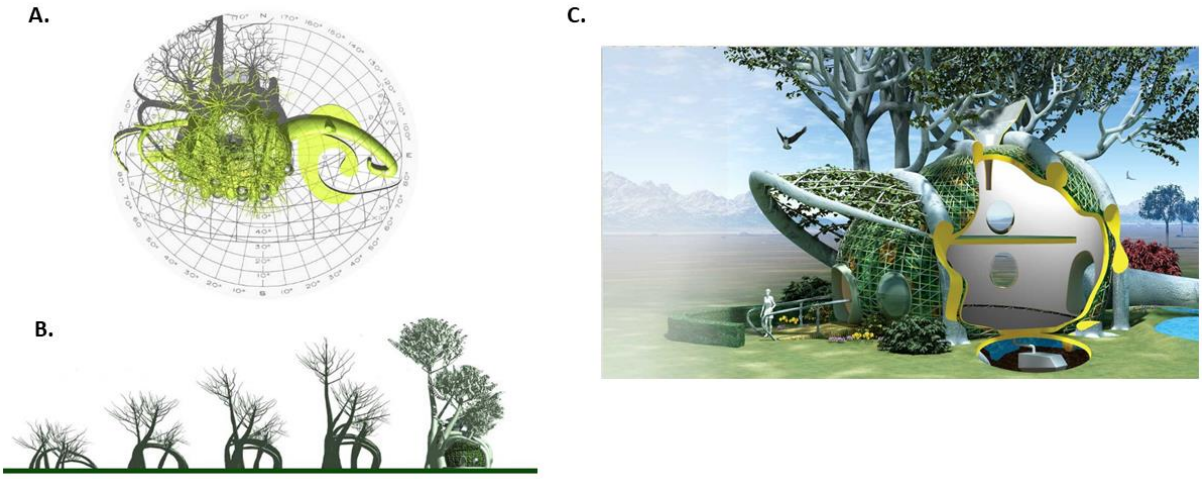

Fig.2. FAB TREE HAB, a house concept to grow homes from native trees and woody plant structures. Top view and geographical orientation (A), growth stages (B) and final view house (C) (https://www.archinode.com/fab-tree-hab)

6. Supplementary information:

Chapter I: The role of EPFL peptides in secondary growth

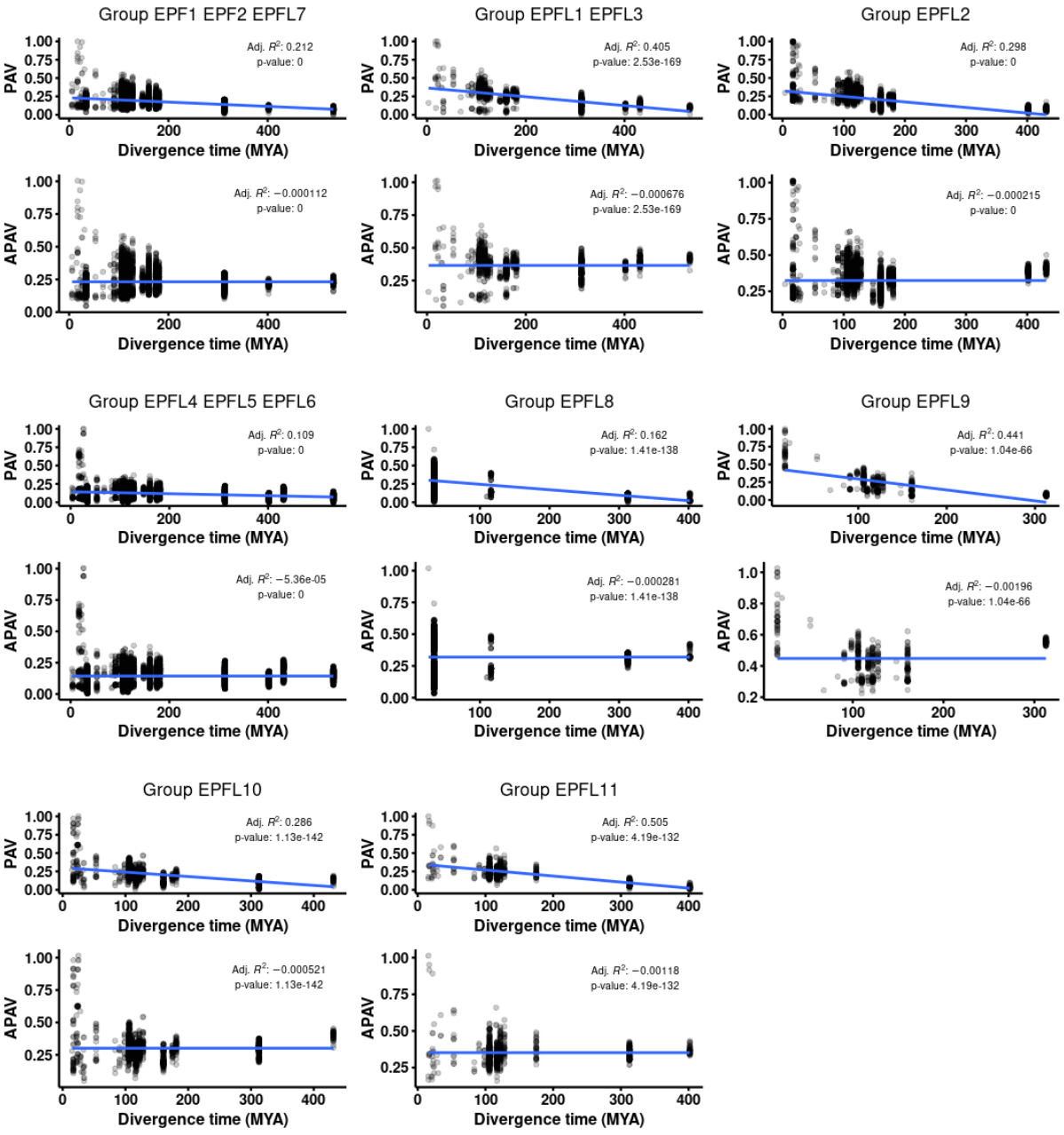

Fig.S1. Normalisation of the Pairwise Attraction Values (PAV) of mature EPFL peptide CLANS analysis for their evolutionary distance (divergence time in million years, MYA). Each PAV (point) represents the similarity between two mature EPFL sequences from different species collected with HMMR and CLANS. The linear model represents the effect of time on the PAV (similarity between two sequences) since divergence of the two species from which the two sequences have been collected. For each CLANS subgroup, the effect of time divergence on PAV was removed using the gradient of the slope of the linear model (top row). This gave the Adjusted Pairwise Attraction Values (APAV) which do not have any significant relationship with time (bottom row).

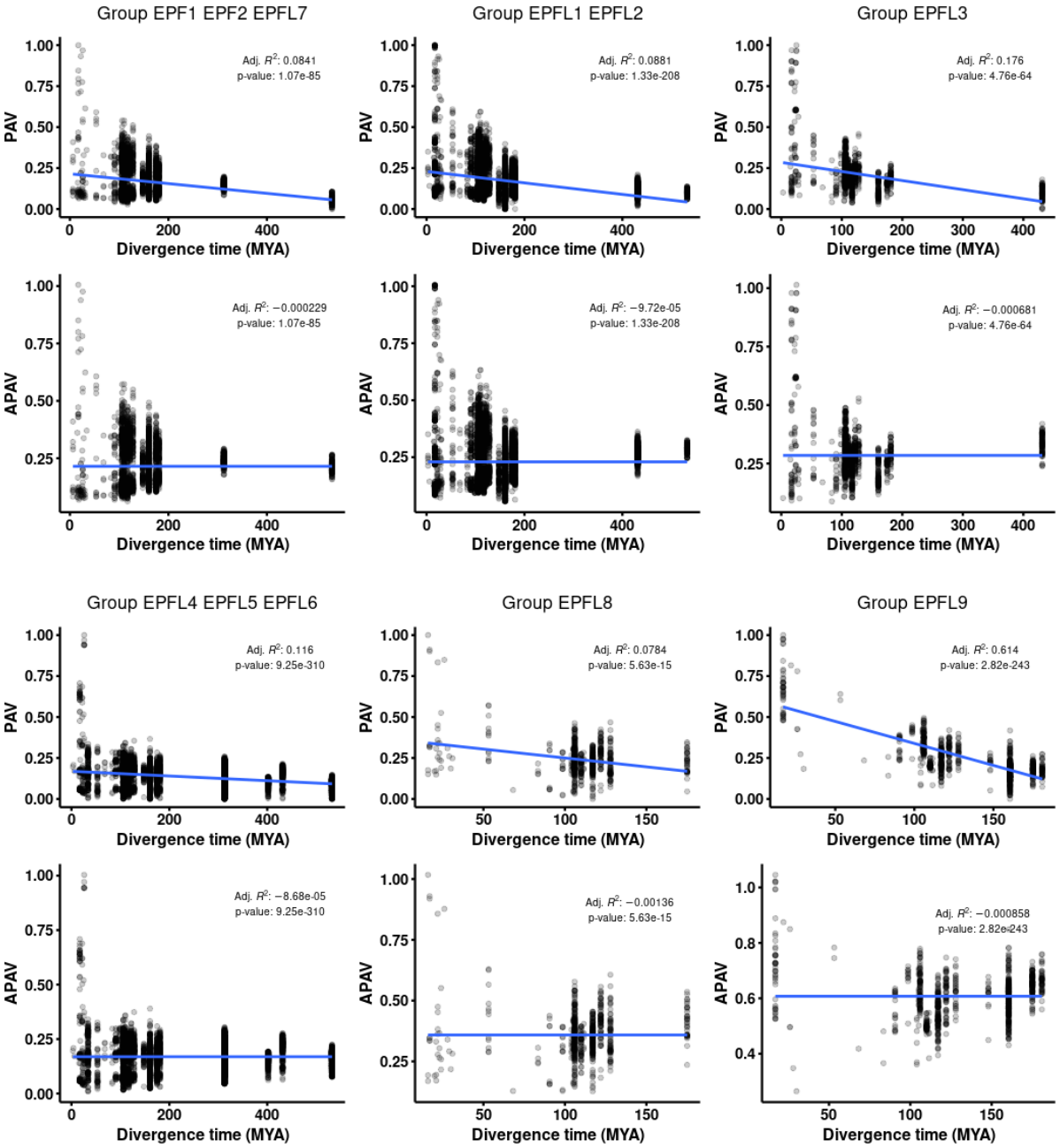

Fig.S2. Normalisation of the Pairwise Attraction Values (PAV) of full-length EPFL peptide CLANS analysis for their evolutionary distance (divergence time in million years, MYA). Each PAV (point) represents the similarity between two full-length EPFL sequences from different species collected with HMMR and CLANS. The linear model represents the effect of time on the PAV (similarity between two sequences) since divergence of the two species from which the two sequences have been collected. For each CLANS subgroup, the effect of time divergence on PAV was removed using the gradient of the slope of the linear model (top row). This gave the Adjusted Pairwise Attraction Values (APAV) which do not have any significant relationship with time (bottom row).

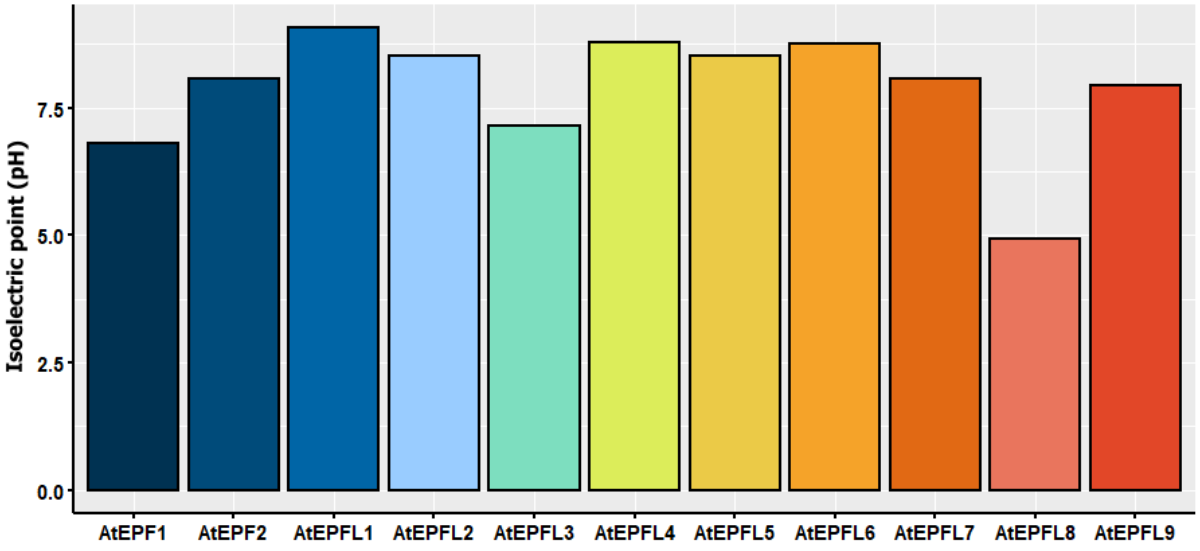

Fig.S3. Isoelectric points of the EPFL family peptides obtained with Iep EMBOSS. The data was plotted in R v.4.0.2.

While the functions of the EPFL3 and EPFL8 peptides remained unknown, we found that the isoelectric points of these peptides (as well as EPF1) are reduced compared to other EPFLs. This variation in isoelectric points and the resulting negative charges on the EPFL3 and EPFL8 peptides suggest that these peptides are likely to have different binding affinity and different functions to other EPFL proteins.

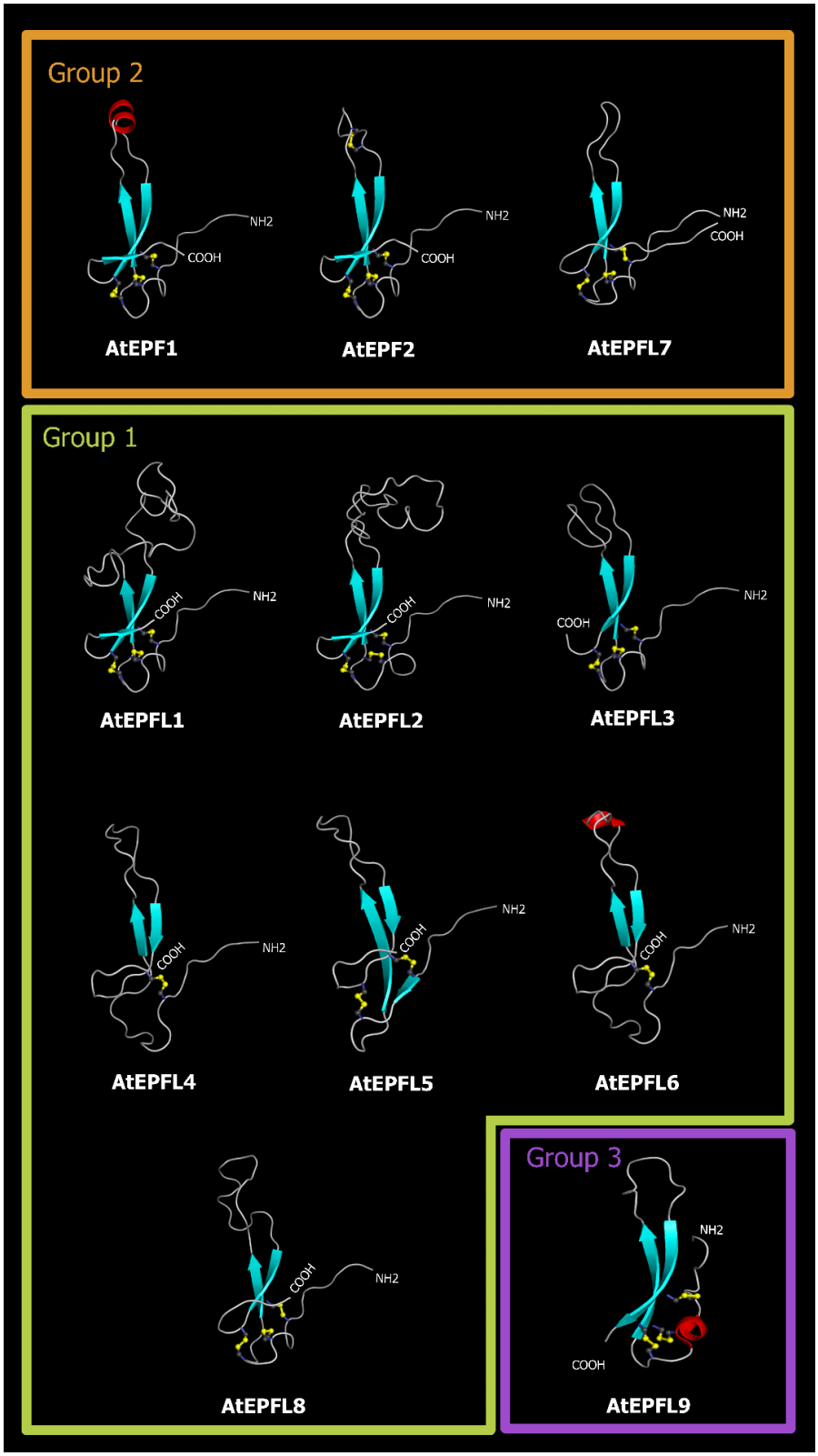

Fig.S4. Predicted tertiary structure of *Arabidopsis* EPFL peptides. The hypothetical 3D structure of these proteins was modelled using the homology modelling server SWISS-MODEL and visualised with PyMol software. The groups correspond to the three main CLANS groups. Species used: Mp; *Marchantia polymorpha* (liverwort), Pp; *Physcomitrella patens* (moss), At; *Arabidopsis thaliana*. Structure colour-code: Cyan: antiparallel beta sheets, red: alpha helix, yellow: disulphide bonds.

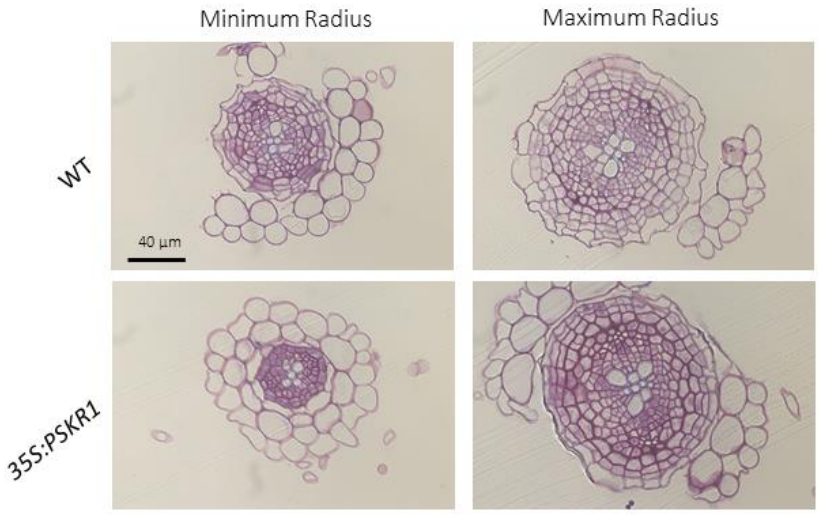

Fig.S5: Root transverse sections of the line 35S::PSKR1.

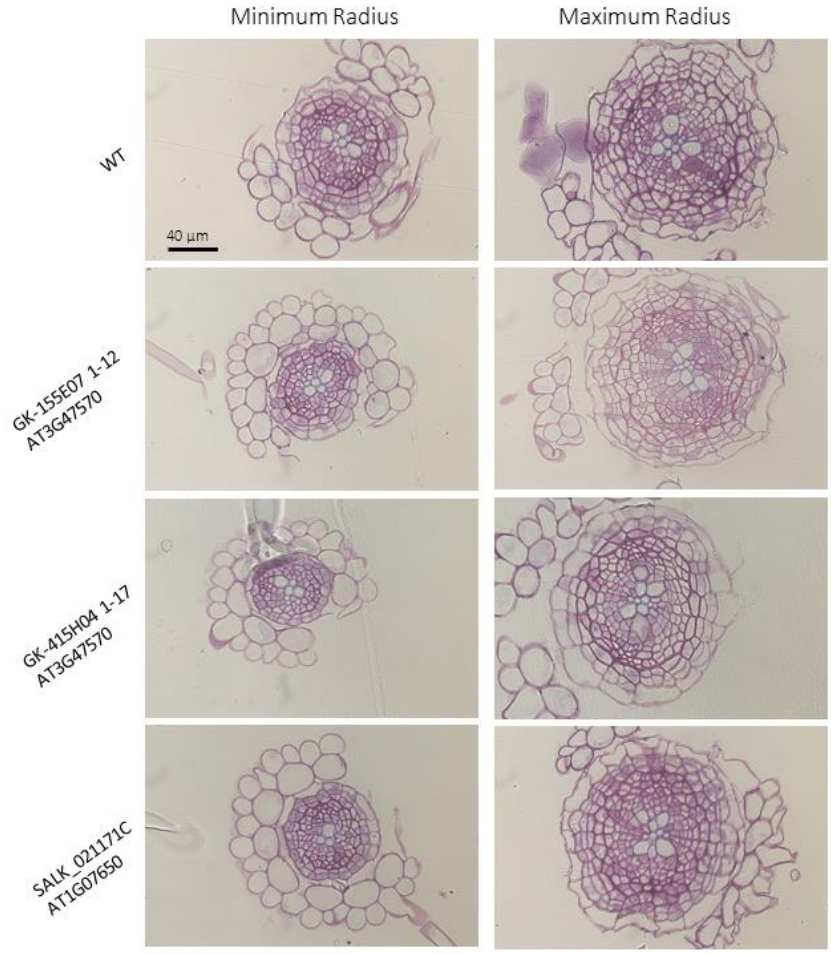

Fig.S6: Root transverse sections of *AT3G47570* and *AT1G07650*.

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