Analysis of the full-length *WOX4* promoter activity in *Arabidopsis thaliana*

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I. SUMMARY

The understanding of vascular development in plants has been advanced rapidly in the last decades. Nevertheless, there are still many details to be elucidated about the early stages of vascular differentiation, which requires easily identifiable marker genes. The *WUSCHEL-related homeobox 4 (WOX4)* is a member of the *WOX* gene family (Eric van der Graaff, 2009) and it has been demonstrated that *WOX4* is involved in cambial stem cell maintenance (Suer *et al.*, 2011).

Existing *WOX4* expression analyses in *Arabidopsis thaliana* were conducted with a short *WOX4* promoter-reporter construct, which contains 2.9 Kb of the 5′ flanking region and 0.6 Kb of the 3′ flanking region (Ji *et al.*, 2010; Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011; Shi *et al.*, 2019). These studies describe a cambial cell specific expression pattern of *WOX4* promoter in the root, shoot, cotyledons and leaves (Y Hirakawa *et al.*, 2010). In upper part of the inflorescence stem, *WOX4* activity was confined only to fascicular cambium, but at the stem-base its activity extends into the interfascicular region and forms a circular expression domain and this pattern was implied to be responsible for the radial outgrowth of the stem (Suer *et al.*, 2011).

However, by detailed sequence analyses in this study we demonstrate that the *WOX4* sequence is spatially separated by long intergenic sequences, which contain several distal conserved regions. Moreover, by comparing phylogenetic shadowing results with published ATAC-seq data (Frerichs *et al.*, 2019) we show the positions of these conserved regions are in open chromatin configurations, suggesting a possible regulatory role of these areas in *WOX4* expression pattern. Hence, we have generated *WOX4* promoter-reporter fusions, which contains 9.2 Kb upstream and 1.7 Kb downstream sequences from the WOX4 coding sequence and transferred into *A. thaliana* with the aim to find a full spectrum of *WOX4* activity. Interestingly, the analyses of transgenic plants aligned with previously observed cambium cell specific *WOX4* activity but additionally it marked novel *WOX4* expression domains in the SAM, RAM, stem and leaves.

In the stem, the full-length *WOX4* promoter activity starts in groups of cells of the inflorescence meristem (IM), possibly marking the provascular cells of emerging primordia. Approximately 80-100 µm beneath the IM, the promoter activity was localized in a circular expression domain of subcortical region that prepatterns the vasculature of young stem. This circular *WOX4* expression domain was found to exist throughout the inflorescence stem and intrusions in the circle marks

fascicular cambium and interfascicular mark strands. Our findings suggest that the full-length *WOX4* promoter was active during the specification of provascular cells in the shoot apex, initiation of fascicular cambium in the young stem without losing competency in the interfascicular mark strands and then continuously active in the cambial ring of the matured stem. Additionally, its activity was also observed in the xylem parenchyma in different growth phases of the inflorescence stem. Similar to the shoot, the *WOX4* promoter activity was also found to start in the RAM marking the QC and its adjacent meristematic cells. Then its activity was found to confined to the vascular system of root. In the leaf, the *WOX4* promoter was active in the primary, secondary, tertiary and quaternary veins, marking the cambial cells of the complete leaf vascular system. Additionally, it was also active in the xylem parenchyma of leaf vascular bundles and the sub-epidermal cells in adaxial side of the leaf, marking the palisade parenchyma.

Taken together, our study indicates that the inclusion of distal conserved regions of the *WOX4* promoter is essential to show the picture of *WOX4* expression pattern in different organs of *A. thaliana*. Hence, the full-length *WOX4* promoter-reporter constructs analysed in this study could further be utilised to elucidate the gene regulatory networks that control vascular development. However, the complete upstream region is too large to be used as a standard promoter, therefore further promoter dissection studies are needed to identify the *cis*-regulatory elements, which could then be used for engineering approaches.

II. ZUSAMMENFASSUNG

Das molekularbiologische Verständnis der Gefäßentwicklung bei Pflanzen ist in den letzten Jahrzehnten weit vorangeschritten. Nichtsdestotrotz gibt es immernoch Details der frühen Entwicklungsstadien der vaskulären Differenzierung, die noch nicht vollständig beschrieben sind und von einfach detektierbaren Markergenen profitieren würden. *WUSCHEL-related homeobox 4 (WOX4)* gehört zur *WOX* Genfamilie (Eric van der Graaff, 2009). Es wurde gezeigt, dass *WOX4* an der Stammzell-Aufrechterhaltung im Kambium beteiligt ist (Suer *et al.*, 2011).

Existierende *WOX4* Genexpressionsanalysen in *Arabidopsis thaliana* wurden mit einem vergleichsweise kurzen *WOX4* Promoter-Reporter Konstrukt, welches 2.9 Kb der 5′ flankierenden Region und 0.6 Kb der 3′ flankierenden Region umfasst, durchgeführt (Yuki Hirakawa *et al.*, 2010; Shi *et al.*, 2019). Diese Studien berichten von einer Kambium-spezifischen Expression des *WOX4* Promoters in Wurzeln, Spross, Kotelydonen und Blättern (Y Hirakawa *et al.*, 2010). Im oberen Teil der Sprossachse der Infloreszenz beschränkte sich die *WOX4* Aktivität auf das faszikuläre Kambium, wohingegen sich die Aktivität in der Basis der Sprossachse auf die interfaszikülare Region ausgeweitete und eine zirkuläre Domäne formte. Dieses Expressionsmuster könnte das radiale Wachstum der Sprossachse einleiten (Suer *et al.*, 2011).

In einer detailierten Sequenzanalyse zeigen wir hier, dass die *WOX4* Sequenz räumlich von langen, intergenischen Sequenzen abgegrenzt ist, welche im distalen Bereich konservierte Regionen enthalten. Darüberhinaus zeigen wir durch den Vergleich von *phylogenetic shadowing*-Ergebnissen mit publizierten ATAC-seq Daten (Frerichs *et al.*, 2019), dass die Positionen der konservierten Regionen in offenen Chromatinbereichen liegen, was uns zu der Hypothese führt, dass sie eine wichtige regulatorische Rolle in der *WOX4* Expression spielen könnten. Aus diesem Grund wurden in dieser Studie *WOX4* Promoter-Reporter Fusionen generiert, die den 9.2 Kb upstream Bereich und den 1.7 Kb downstream Bereich der WOX4 kodierenden Sequenz umfassen. Die Promoter-Reporter Konstrukte wurden in *A. thaliana* transferiert mit dem Ziel das volle Spektrum der *WOX4* Aktivität zu erfassen. Interessanterweise stimmten die Analysen der transgenen Pflanzen mit der vorher beschriebenen Kambium-spezifischen *WOX4* Aktivität überein, jedoch konnten zusätzlich markierte, neue *WOX4* Expressionsdomänen im SAM, RAM, Sprossachse und Blätter identifiziert werden.

In der Sprossachse beginnt die Aktivität des *WOX4* Volllänge-Promoters in Zellgruppen des Inflorezenzmeristems (IM) was möglicherweise provaskuläre Zellen der sich entwickelnden Primordien markiert. Circa 80-100 µm unterhalb des IMs zeigte sich eine zirkuläre Expressionsdomäne der subcorticalen Region, welche das Muster der Vaskulatur der jungen Sprossachse anzeigt. Diese zirkuläre *WOX4* Expressionsdomäne zog sich durch die Sprossachse der gesamten Infloreszenz, wobei Einstülpungen des Kreisens das faszikuläre als auch Kambium markieren.

Unsere Ergebnisse deuten darauf hin, dass der *WOX4* Volllänge-Promoter während der Spezifizierung der provaskulären Zellen in der Sprosspitze aktiv ist, während der Initiierung des faszikulären Kambiums in der jungen Sprossachse ohne seine Kompetenz im interfaszikulären Markstrahl zu verlieren aktiv bleibt und seine Aktivität dann auch kontinuierlich im Kambiumring der reifen Sprossachse zu finden ist. Zusätzlich konnte seine Aktivität im Xylemparenchym in verschiedenen Wachstumsphasen der Sprossachse der Infloreszenz beobachtet werden. Wie im Spross zeigte sich die *WOX4* Promoteraktivität auch im RAM wo sie das QC und die angrenzenden meristematischen Zellen markierte. Außerdem beschränkte sich die Aktivität auf die Vaskulatur der Wurzel. Im Blatt befand sich die *WOX4* Promoteraktivität in den primären, sekundären, tertiären und quartären Venen, wo sie die Kambiumzellen der kompletten Blattvaskulatur markierten. Zusätzlich konnte seine Aktivität in Xylemparenchymzellen der Bündelscheidenzellen und der subepidermalen Zellen der adaxialen Seite des Blattes lokalisiert werden, wo sie das Palisadenparenchym markierte.

Zusammengefasst zeigt unsere Studie, dass der Einbezug der distalen, konservierten Promoterregionen essentiell für die volle Entfaltung der *WOX4* Expression in den verschiedenen Pflanzenorganen von *A. thaliana* ist. Die in dieser Studie analysierten Volllänge *WOX4* Promoter-Reporter Konstrukte könnten weiterhin der Erforschung der regulatorischen Netzwerke, welche die Gefäßentwicklung in Pflanzen steuern, dienen. Allerdings eignet sich die vollständige Upstream-Region aufgrund ihrer Größe nicht zum Einsatz als Standardpromoter. Stattdessen wäre es sehr interessant detailierte Promoterstudien an dieser Upstream-Region durchzuführen, um kleinere *cis*-regulatorische Elemente zu identifizieren, die in Zukunft in biotechnologischen Verfahren an Pflanzen eingesetzt werden können.

1. INTRODUCTION

1.1. The Plant vascular system – structure and function

The plant vascular system is a network of specialized conductive tissues that are spread throughout the entire plant body, connecting all the plant organs with each other in an organized manner and it facilitates the transport of water, minerals, and sugars (Lucas *et al.*, 2013). It is composed of two types of differentiated tissues, the xylem and the phloem; that are spatially separated by an undifferentiated tissue, the cambium (Nieminen *et al.*, 2015). The xylem tissue consists of xylem parenchyma, xylem fibers, tracheids and vessel elements and phloem tissue is composed of phloem parenchyma, phloem fibers, sieve tube elements and companion cells (Jouannet *et al.*, 2015).

The primary function of the cambium is to maintain its own stem cell population in the cambial zone, the differentiation of xylem and phloem tissues. The basic function of the xylem tissue is to transport water and mineral nutrients from roots to various parts of the shoot. The phloem tissue transports sugars that are produced in the photosynthetic organs to all other tissues of the plant body (Altamura *et al.*, 2001). The bulk flow of water through the xylem tissue is a passive transport mechanism, which is driven by the osmotic potential difference of the root surface and the tension created in the xylem tissue by transpiration of water through the stomata (Myburg and Sederoff, 2001). As a consequence, the turgor pressure gradient is established in the adjacent phloem tissue that directs the sugar translocation from shoot to root. However, the sugar transport functions via an active transport mechanism that requires direct energy inputs in order to perform the phloem loading and unloading processes (Hölttä *et al.*, 2009; Lucas *et al.*, 2013). Moreover, the plant vascular system plays a major role in long-distance communication, enabling the translocation of phytohormones, RNA molecules, and signal peptides within the shoot and between the shoot and root. For example, in response to water deficit conditions, roots produce the phytohormone abscisic acid, which is then transported to leaves through the xylem, where it reduces the water loss by stomatal closure (Hartung *et al.*, 2002). Another important long-distance communication is that of florigen, a protein encoded by the *FLOWERING LOCUS T* (*FT*) gene. It is expressed in the leaves and is then transported to the shoot apical meristem through the phloem, where it promotes the floral transition (Corbesier *et al.*, 2007). Interestingly, CLAVATA3/EMBRYO SURROUNDING REGION peptides (CLE peptides) secreted by roots in response to the plant-

microbe interaction are transported to the shoot via the xylem, which then activates a systemic responses that regulate root nodulation (Okamoto *et al.*, 2013; Notaguchi and Okamoto, 2015). These few examples demonstrate the important role of the plant vascular system in the exchange of signals between plant organs serving either internally as developmental regulators or enable the plant to react quickly to environmental conditions like biotic and abiotic stresses (Lucas *et al.*, 2013). In addition, the vascular system also provides mechanical support to the plant body and in tree species, it contributes to the development of wood through the secondary thickening growth (Ji *et al.*, 2010; Etchells and Turner, 2016).

1.2. Development of the vascular system in *Arabidopsis thaliana*

The vascular system of *Arabidopsis thaliana* begins with the specification of vascular initials in the embryo that gives rise to the vascular system of cotyledons, embryonic root and hypocotyl (Figure 1a). After seed germination, the vascular system of the root and shoot develops from the root and shoot apical meristems respectively, and grows into a continuous network by the action of cambium(Turner and Sieburth, 2003). However, the structural arrangement of cambium and vascular tissues varies from organ to organ (Figure 1b). For example, in the root, the vascular tissues are arranged in the diarch symmetry, whereas the stem exhibits the collateral arrangement (Dengler, 2006). Moreover, anatomical differences also exist within the same organ; for example, the anatomy of the inflorescence stem at the apex is generally different from its base (Bowman and Floyd, 2007). In the following sections, the vascular development in *Arabidopsis thaliana* is described in detail with an emphasis on both embryonic and post-embryonic differences that occur in different organs.

1.2.1. Initiation of provascular tissues during the embryogenesis

During the embryogenesis, the zygote undergoes an asymmetric division that produces a small apical cell with a dense cytoplasm and a larger basal cell with a big vacuole. The apical cell lineage develops into most of the embryo, whereas the basal cell lineage produces the extra-embryonic suspensor (Barton and Poethig, 1993). First, the apical cell goes through two rounds of periclinal divisions to produce the four celled pro-embryo and then an anticlinal division resulting in an octant embryo. The four cells in the upper tier of the octant embryo give rise to the shoot including the shoot meristem, whereas the four cells in the lower-tier give rise to the hypocotyl and

hypophysis (Jürgens *et al.*, 1995). Further periclinal divisions occurring in the octant result in an embryo with the specified cell fates, such as protoderm and ground meristem. The elongated cells in lower half of the early-globular embryo are specified as the vascular cells, which is the first sign of vascular tissue development (Jürgens, 1995; Scheres *et al.*, 1995). Further periclinal cell divisions in the globular embryo give rise to the cotyledon primordia and the subsequent divisions in the late heart stage develop the shoot apical meristem in between the developing cotyledons (Boscá *et al.*, 2011). Meanwhile, the simple anticlinal divisions that occurred in the basal cell give rise to the extra-embryonic suspensor. The upper cell of the suspensor develops into the hypophysis which forms the quiescent center of the root meristem and the root cap initials (Armenta-Medina and Gillmor, 2019). Numerous gene expression studies suggested that, although the provascular tissues were specified during the embryogenesis, the actual vascular differentiation marked by certain structural changes including the secondary cell wall formation occurs only after the seed germination (ten Hove *et al.*, 2015).

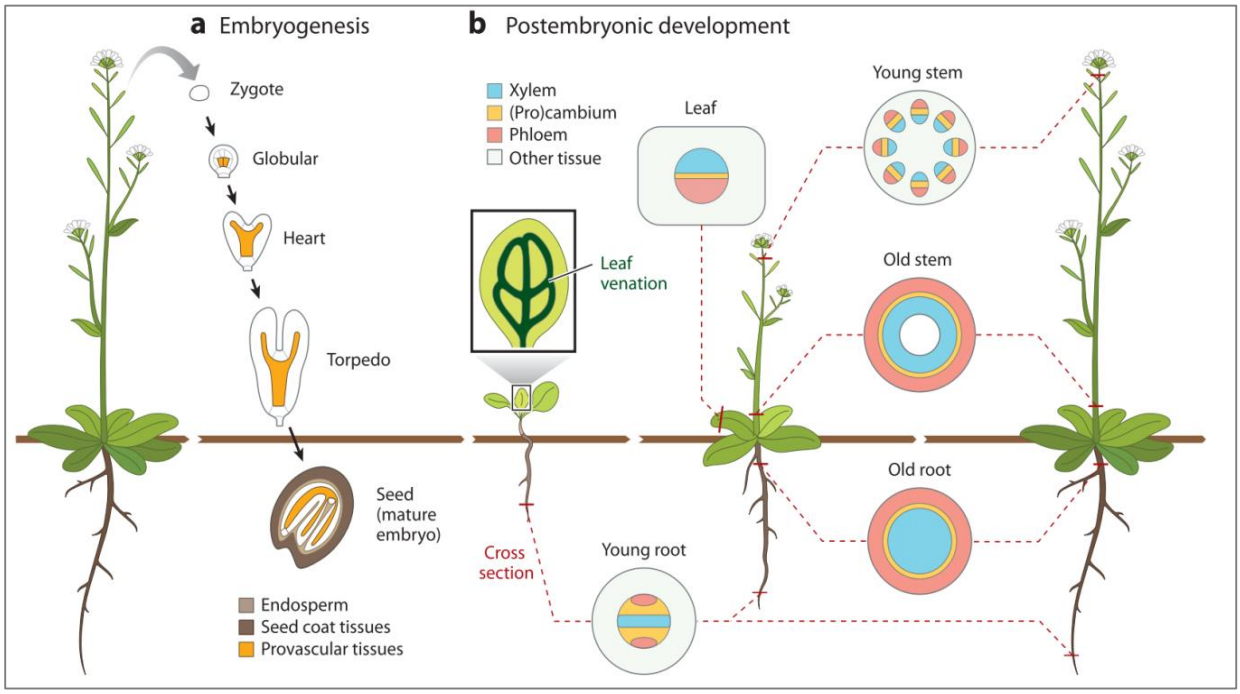

Figure 1. Schematic representation of the vascular tissue development and its organization in different parts of the *Arabidopsis thaliana* plant body during embryogenesis (a) and postembryonic development (b) (Ruonala *et al.*, 2017). Provascular tissues are marked in the orange colour, whereas the xylem and phloem tissues are marked in blue and red colours respectively.

1.2.2. Development of the vascular system in post-embryonic tissues

The post-embryonic development of the vascular system in organs such as the hypocotyl and cotyledons emerges from vascular precursors that are prepatterned during embryogenesis whereas the vascular system of shoot organs (leaves, side-shoots, inflorescence stems, flowers and pedicels) and roots originates from the specified vascular precursor cells of the shoot apical meristem and root apical meristem, respectively (Lucas *et al.*, 2013; De Rybel *et al.*, 2015).

1.2.2.1. Vasculature of the root

During early stages of seed germination, the embryonic root or radicle starts protruding from the seed by breaking the seed coat and later develops into the primary root. During root development, three different developmental zones such as meristematic, elongation, and differentiation zones can be observed. These zones are in turn distinguished by the following factors: the external root morphology, the internal root anatomy, the structural and functional differences in the vascular tissues (Turner and Sieburth, 2003).

The meristematic zone contains a four-celled quiescent center (QC), which is surrounded by actively dividing meristematic cells (Verbelen *et al.*, 2006). These cells are small in size, packed with cytoplasm, contain small or no vacuoles. They are enclosed by thin cell walls and their nuclei occupy a large area of the cell. The meristematic zone can be further divided into proximal and distal meristematic zones, based on the root anatomy. The proximal meristematic zone contains the stem cell niche formed by a centrally located QC and a layer of meristematic cells that are in direct contact with the QC (Bennett and Scheres, 2010). These meristematic cells are later specified as the mother cells that produce all other root tissues. The cells located below the QC serves as the columella-mother-cells, which differentiate into the columella cells. Similarly, the cells positioned above the QC are specified as the pericycle and vascular initials (Figure 2). The cells that are positioned peripherally to the QC, are specified as endodermis/cortex-mother cells and epidermal/root cap mother cells (Nieminen *et al.*, 2015). These mother cells give rise to different tissues, which forms the distal end of the meristematic zone. The root anatomy at this stage shows the following cell layers - the outer epidermal layer, the middle cortex tissue, a layer of endodermis, and then a single layer of pericycle that encloses the central stele. The central stele is composed of protoxylem tissue at the center, accompanied by two poles of the phloem cells that

are located in close proximity to the pericycle, and procambial cells that are positioned between xylem and phloem (Masubelele *et al.*, 2005).

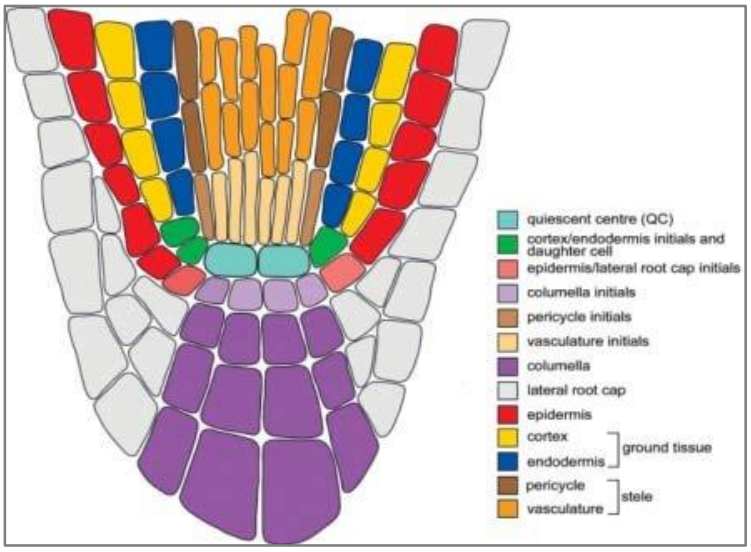

Figure 2. Graphical representation of the organization of different cell types in the root apical meristem (Pérez *et al.*, 2013)**.** The quiescent center (QC) and other meristematic cells of the root apex are shown in differentt colours.

The elongation zone is positioned at an approximately 300-850 µm away from the QC and in this region cell divisions do not occur frequently, but the rapid growth is achieved by cell expansion (Verbelen *et al.*, 2006). Several cytological changes occur in this zone such as enlargement of the vacuole that pushes the nucleus and cytoplasm towards the cell wall (Jing and Strader, 2019). The differentiation zone of the root starts in continuation with the elongation zone. Many anatomical changes occur in this zone with the rapid cell divisions gives rise to various vascular tissue types (De Rybel *et al.*, 2015). Based on the gene expression studies and the differences in cell morphology, the pericycle of the differentiation zone is divided into xylem-pole-pericycle and phloem-pole-pericycle (Parizot *et al.*, 2012). The lateral roots also originate from the specified cells of xylem pole associated pericycle (Jing and Strader, 2019). Inside the central stele of the differentiation root zone, the cambium tissue starts to differentiate into different types of metaxylem tissues such as the xylem vessels, xylem fibers, and the phloem tissues such as sieve elements and companion cells (Ruonala *et al.*, 2017). The root that is positioned between the differentiation zone and the hypocotyl exhibits secondary growth, in which the central part of the root is occupied by the concentric rings of secondary xylem tissue, the cambial cell layer and the secondary phloem tissues (Fukuda and Ohashi-Ito, 2019).

1.2.2.2. Vasculature of the Stem

The *Arabidopsis* stem originates from the shoot apical meristem, which is divided into central zone, peripheral zone, organizing center, and rib zone (Mayer *et al.*, 1998). The central zone contains slowly dividing stem cells that maintain the meristem integrity and also produces daughter cells to the sides and bottom. It is flanked by the peripheral zone, where actively dividing cells develop into lateral organs such as leaves and flowers. The stem cell population of SAM is maintained by the feedback mechanism operated between the central zone and the organizing center (Figure 3). The rib zone located below the organizing center takes part in the development of the vegetative/inflorescence stem (Bowman and Eshed, 2000). It is also believed that the rib zone is further divided into the peripheral-rib zone that produces the cortex and the central-rib zone that produce the pith parenchyma. The boundary cells between the peripheral-rib zone and central-rib zone produce the primary vasculature (Serrano-Mislata and Sablowski, 2018).

However, the continuation of vascular pattern formation in vegetative stem has been seen in two different views: according to the first view, the vasculature of emerging lateral organs are eventually connects to the existing vasculature of the stem, which is supported by the direction of auxin flow from tip of the leaf primordium towards the leaf base (Scarpella, Marcos, Jirí Friml, *et al.*, 2006). In the second view, the existing vasculature of the basal stem extends into the emerging leaf primordia and this view is supported by the fact that the plant growth occurs in an acropetal manner (Turner and Sieburth, 2003). The second hypothesis of the plant vascular development is extensively studied in *Arabidopsis* during the rosette growth (Kang *et al.*, 2003), in which the authors described the vasculature of the vegetative stem by combining the anatomical features with the expression pattern of provascular marker *HOMEOBOX GENE 8* (*ATHB8)*. According to the evidence presented in their report, the vasculature tissue of cotyledons and first four leaves are in direct contact with the central stele of the hypocotyl, suggesting that the primary vasculature develops as a continuation of the embryonic vascular pattern. Whereas, from the fifth leaf onwards the vasculature of each leaf is supplied by a single trace that is derived from two of the eight vascular bundles (or sympodia) of the vegetative stem. It was also found that each leaf vasculature can be traced back to another leaf; for example, the trace to leaf 5 derives from the base of leaf 2 trace (n+3 parastichy), while the traces of leaf number 6, 7 and 8 derives from their n+5 parastichy (Kang *et al.*, 2003). These studies demonstrate that the vascular system of vegetative rosette is

composed of approximately eight vascular bundles that form a reticulate pattern by the multiple connections among the different leaves and also with the vascular bundles of the stem (Dengler, 2006).

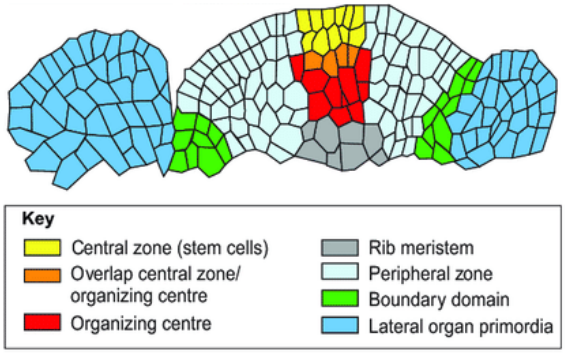

Figure 3. Schematic representation of the different zones in the shoot apical meristem (Gaillochet and Lohmann, 2015)**.** Different functional zones in the SAM are shown in different colours – Central zone in yellow, organizing center in red, overlapping zone in orange, lateral organs in blue, peripheral zone in the cyan, boundary domain in green and the rib meristem in green colours.

During the vegetative to reproductive transition, the vegetative shoot apical meristem transforms into an inflorescence meristem (IM), which later produces the cauline leaves, flower primordia, lateral branches, nodes and internodes of the inflorescence stem (Lucas *et al.*, 2013). Due to the compressed nature and the difficulties associated with imaging the vegetative stem, the inflorescence stem was used more frequently than the vegetative stem to study stem growth. The structural changes which occur during inflorescence stem growth in *Arabidopsis* and wood formation in tree species are found to be identical, which has driven researchers to use the *Arabidopsis* inflorescence stem to study radial outgrowth (Ragni and Greb, 2018).

In general, the development of the inflorescence stem has been divided into primary, intermediary and secondary growth depending on the anatomical variations occurring in the inflorescence stem over the period of plant growth (Tonn and Greb, 2017). The primary growth phase of an inflorescence stem is studied by using either the young inflorescence stem or the matured stem segment located beneath the shoot apical meristem. Both are composed of outer epidermis, middle cortex, vascular bundles, and central pith (Campbell *et al.*, 2016). Similar to the vegetative stem, the young inflorescence stem also possesses approximately eight to ten vascular bundles that are arranged in a circular manner (eustele). Each bundle contains the xylem tissue towards central pith,

the phloem tissue towards the cortex and the fascicular cambium positioned in between the xylem and phloem tissues (Figure 1b; Suer et al., 2011). The vascular bundles are separated by an interfascicular region. In the young inflorescence stem, the interfascicular region contains only parenchyma tissue, whereas in the mature stem it has lignified fibers (Altamura *et al.*, 2001). The molecular basis for the existence of a parenchymatic interfascicular region in the young *Arabidopsis* inflorescence stem is poorly understood so far, because the region is not marked by any well-known vascular or non-vascular markers, therefore it is also not clear whether this region develops from the cambium or cortex or pith.

The inflorescence stem that is located in between the shoot apex and the stem base has been attributed to study of the intermediate growth phase of the stem (Sanchez *et al.*, 2012). The anatomy of the stem undergoing the intermediate growth shows an outer epidermis, the cortex with multiple layers, the enlarged pith, and well-defined fascicular and interfascicular regions. The vascular bundles are highly differentiated and contain various vascular tissue cell types such as the xylem parenchyma, vessel elements, phloem parenchyma, and companion cells, whereas the interfascicular region contains xylem fibers with lignified cell walls. A small portion of the stem which shows the meristematic activity and appears at the junction of fascicular cambium, cortex, and interfascicular regions has been proposed to be the initiation site of stem secondary growth (Suer *et al.*, 2011). Similar to the interfascicular region in the young inflorescence stem, until now no vascular markers are reported to be active in the entire interfascicular region of the stem during the intermediate growth phase. However, one of the cambium associated markers, the *WOX4* promoter (2.9 Kb) activity has been shown in a few meristematic cells that are located at the merging point of fascicular and interfascicular regions (Miyashima *et al.*, 2013; Suer *et al.*, 2011). This expression pattern has been interpreted as the activation of interfascicular cambium that initiates the secondary growth of the inflorescence stem.

The radius of the inflorescence stem progressively becomes larger from the tip to the base which is described as the radial outgrowth or secondary thickening growth (Altamura *et al.*, 2001; Tonn and Greb, 2017). Hence the inflorescence stem-base has been used to describe the anatomical changes that are occurred in the secondary growth phase (Y Hirakawa *et al.*, 2010; Ji *et al.*, 2010; Barra-Jiménez and Ragni, 2017). In contrast to the anatomy of the apical part of the stem, the base of stem contains vascular tissues that are arranged in concentric rings (Suer *et al.*, 2011; Sanchez

et al., 2012). During the secondary growth, fascicular and inter-fascicular cambia merge to form a cambial ring. This cambial ring further produces the secondary xylem tissue towards the center of the stem and the secondary phloem tissue towards the cortex, increasing the number of vascular cell layers of the stem. Together with the subsequent secondary cell wall depositions, secondary growth contributes to the increase in the radius of the stem (Suer *et al.*, 2011; Ragni and Greb, 2018).

Overall, the initiation of meristematic cell divisions at the flank of fascicular cambium that is progressively extending into the interfascicular region to become the circular cambium and their further differentiation into the secondary vascular structures responsible for the radial growth of the stem has been supported by many independent studies (Ji *et al.*, 2010; Suer *et al.*, 2011; Shi *et al.*, 2017; Shi *et al.*, 2019). Interestingly, in all these studies the *WOX4* promoter (2.9 Kb) activity has been used to describe the changes that occur in the fascicular and interfascicular regions during the primary, intermediary and secondary phases of *Arabidopsis* inflorescence stem development.

1.2.2.3. Vasculature of the leaf

Arabidopsis leaf development begins with an initiation of leaf primordia at the periphery of vegetative shoot apical meristem (Kang *et al.*, 2003). The subsequent developmental phases such as the primordium outgrowth, abaxial-adaxial polarization, and leaf blade expansion take place in coordination with the specification and patterning of the leaf vascular system (Kalve *et al.*, 2014). Early leaf primordia contains L1, L2, and L3 layers, in which the L1 layer is responsible for the development of epidermis, stomata, and trichomes, whereas the L2 and L3 layers produce the ground tissue that is responsible for the development of all internal structures of the leaf including the vascular system (Bowman and Floyd, 2007).

The development of the leaf vascular system starts with the specification of provascular cells at the base of the primordium, where it connects with the stem. Initially, the provascular cells are morphologically indistinguishable from the surrounding cell pool, but the procambial markers like *ATHB8* start to express in these cells indicating the prepatterning of the vascular cambium (Nelson and Dengler, 1997; Dengler, 2006). As the leaf primordium expands further, the formation of provascular cells from the ground tissue occurs continuously to form the procambium (Scarpella, Marcos, Jiří Friml, *et al.*, 2006). The procambial cells undergo certain physiological changes to

acquire the characteristic thin, slender cellular morphology and these cells are easily distinguishable from the surrounding leaf tissue (Sawchuk *et al.*, 2007). The abaxial-adaxial polarization of the leaf triggers further development in the procambium of the mid-vein (first-order vein) of the leaf vascular system. The procambium tissue located at the center of the vascular bundle differentiates to produce the xylem tissue towards the adaxial side and the phloem tissue towards the abaxial side of the leaf (Rolland-Lagan, 2008).

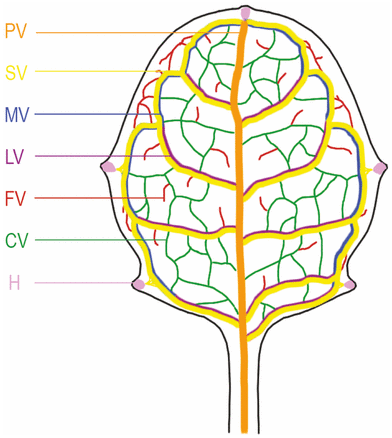

Figure 4. The *Arabidopsis* leaf shows different vein orders (Biedroń and Banasiak, 2018). The midrib comprised of the first order/primary vein (PV-orange) that extends into the secondary veins forming the loops (SV-Yellow). LV-lateral veins and MV-marginal veins. The secondary veins extend into the tertiary veins (green), which are inter-connected (CV) or free ending (FV).

After the specification of the apical-basal, dorsal-ventral body plan of the leaf, the development is dominated by the leaf blade expansion. In the meantime, second-order veins develop from the mid-vein and merge again with the mid-vein to form secondary vascular loops (Dengler, 2006; Scarpella et al., 2006b). Then third-order or tertiary veins are formed from the second-order veins. Small loops are formed either and by making connections with themselves or by looping back into the secondary veins. The reticulate venation of the leaf is completed by the formation of quaternary veins from the tertiary veins (Figure 4). The quaternary veins either merge back to the tertiary veins or end up freely making direct contact with the mesophyll cells (Scarpella, Marcos, Jirí Friml, *et al.*, 2006; Sawchuk *et al.*, 2007).

1.3. Regulation of vascular development

The development and patterning of plant vascular system is controlled by the gene regulatory networks that involve multiple interactions between phytohormones, transcription factors,

peptides and microRNAs (Ohtani *et al.*, 2017). Although the phytohormone Auxin is known to play a central role in plant vascular development (Little, 2002; Wenzel *et al.*, 2007), several studies revealed as well a significant involvement of other phytohormones such as cytokinins (Dettmer *et al.*, 2009), and gibberellins (Miyashima *et al.*, 2013). An auxin dependent transcription factor *MONOPTEROS* or *AUXIN RESPONSIVE FACTOR5* (*MP/ARF5*) plays an important regulatory role in specification and patterning of the vasculature during embryogenesis and post embryonic development and loss of function of the MP leads to severe defects in the vascular development (Hardtke and Berleth, 1998; Berleth *et al.*, 2004). The available evidences suggest that the *MP* acts as a positive regulator of the *PIN-FORMED1* (*PIN1*) that exports auxin from cells and provides the feedback on the auxin status in the future provascular cells (Wenzel *et al.*, 2007). The direct target of *MP* is *TARGET OF MONOPTEROS 5* (*TMO5*), which forms a heterodimer with *LONESOMEHIGHWAY (LHW)* and induces the expression of the *LONELYGUY (LOG)* to produce the bioactive cytokinins that regulate periclinal cell divisions specifying the provascular cells (De Rybel *et al.*, 2014). Interestingly, *MP* was also reported to upregulate the expression of *ATHB8,* which has a crucial role in leaf vascular patterning (Scarpella, Marcos, Jirí Friml, *et al.*, 2006).

Another regulatory pathway that influences several aspects of plant vascular development including the cambial cell divisions, vascular patterning, and differentiation of vascular cell types is the peptide signaling module (Fukuda and Ohashi-Ito, 2019). It is composed of the ligand *TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF)* and the receptor *PHLOEM INTERCALATED WITH XYLEM (PXY)* pair (Etchells and Turner, 2010). The ligand – TDIF peptide is secreted in the phloem tissue, then pairs with the PXY present in the cambium and decides the fate of xylem differentiation (Y Hirakawa *et al.*, 2010). The *TDIF-PXY* signaling module regulates the vascular development in coordination with the *WOX4* (Y Hirakawa *et al.*, 2010; Etchells *et al.*, 2016) which will be described in detail in the following sections.

1.3.1. *WOX4* as a regulator of plant vascular system

The *WUSCHEL-related homeobox 4 (WOX4)* is one of the members of *WOX* gene family, a plant-specific subclade of homeobox transcription factor superfamily (Ji *et al.*, 2010). In *Arabidopsis thaliana*, the *WOX* gene family is comprises of fifteen members, including the founding member, the transcription factor *WUSCHEL (WUS)* (van der Graaff *et al.*, 2009). Based on the phylogenetic relationship, the *WOX* gene family is divided into three clades and they are *WOX13*, *WOX9,* and

WUS clades (Nardmann *et al.*, 2009; Costanzo *et al.*, 2014). The *WOX13*-clade is comprised of *WOX13*, *10* and *14* genes, which can be found in vascular and nonvascular plants, including mosses and green algae. The *WOX9*-clade includes *WOX8, 9, 11* and *12* that can be found in vascular plants including the lycophytes. The *WUS*-clade contains the founding member *WUSCHEL* and the remaining seven *WOX* genes from *WOX1* to *WOX7* (Nardmann and Werr, 2012).

The *WOX* genes are essential for several plant developmental processes including the embryonic patterning, and the lateral organ development. Some of these *WOX* genes also show functional similarities such as the maintenance of stem cells. For instance, in the WUS-clade, the founding member *WUS* regulates the maintenance of stem cells in the shoot apical meristem (SAM) (Mayer *et al.*, 1998). The *WUS* gene encodes a homeodomain transcription factor in the organizing center and then the protein moves to upper layers of the central zone, where it activates the *CLV3* expression. The CLV3, a small extracellular peptide, negatively regulates the *WUS* expression by activating the CLV1/CLV2 receptor kinase complex. This feedback loop mechanism between CLV3 and WUS is important to maintain the stem cell homeostasis in SAM (Sarkar *et al.*, 2007; Yadav *et al.*, 2011). Similarly, another WUS-clade member *WOX5* exclusively is expressed in the quiescent center (QC) of the root apical meristem (RAM) and regulates the stem cell maintenance of RAM (Sarkar *et al.*, 2007). Here, the CLE40 peptide that is encoded by *CLAVATA3/EMBRYO SURROUNDING REGION-related* (CLE) family member, regulates the *WOX5* expression domain by activating the ARABIDOPSIS CRINKLY4 (ACR4) receptor kinase signaling mechanism (Stahl and Simon, 2009). Moreover, the recent studies indicates that another member of WUS-clade, the *WOX4* is preferentially expressed in the cambial cells and promote the proliferation of stem cells in the vascular cambium (Ji *et al.*, 2010; Suer *et al.*, 2011; Shi *et al.*, 2019). The TDIF peptides that are encoded by *CLE41* and *CLE44* in the phloem regulates the *WOX4* expression through the PXY leucine-rich receptor-like kinase (Y Hirakawa *et al.*, 2010). The *PXY*, similar to *WOX4* is expressed in the cambium and required for the normal organization of xylem, phloem and the cambium tissues in the vascular bundles (Etchells and Turner, 2010). Furthermore, the functional *WOX4* is necessary for *PXY* function (Y Hirakawa *et al.*, 2010). Thus, the *TDIF-PXY* signaling module regulates the vascular development in coordination with the *WOX4* (Y Hirakawa *et al.*, 2010; Etchells *et al.*, 2016). The *wox4-1* mutant showed a thin stem phenotype, in which the number of vascular tissues were decreased, but the acquisition of fascicular cambium was not much affected (Ji *et al.*, 2010). However, overexpression studies indicated that the *WOX4* can

promote the cell divisions in both young and matured stems and increase the area of secondary vascular tissue (Ji *et al.*, 2010).

In addition, the *WOX3* and *WOX4* genes appear to function together during the leaf development. In *Arabidopsis*, the *WOX4* participates in the development of leaf vascular system (Ji *et al.*, 2010) while the *WOX3* play a role in the expansion of the leaf blade by regulating the marginal and plate meristems identity (Matsumoto and Okada, 2001). Whereas, the *WOX3* orthologs in maize, the *NARROW SHEATH 1* and *2* (*NS1* and *NS2*) regulate both the leaf blade expansion and the number of leaf vascular bundles (Nardmann *et al.*, 2004). Similarly, *WOX3* orthologs in rice, the *NARROWLEAF 2 & 3* genes also involved in the regulation of both leaf blade expansion and the vascular patterning (Cho *et al.*, 2013). However, it has been shown in rice that *AtWOX4* ortholog, *OsWOX4* is active in SAM (Ohmori *et al.*, 2013). Interestingly, these studies have shown that *OsWOX4* functions in maintaining the stem cell identity in the SAM of rice, replacing the *WUS* function. Moreover, the *OsWOX4* expression was found to extend from the SAM into the early leaf primordia and taking part in the leaf development including the vascular patterning suggesting that the *OsWOX4* specifies vascular identity in the SAM in rice (Yasui *et al.*, 2018). In *Arabidopsis*, the available studies have been shown that *WOX4* is expressed in the vasculature of root, shoot, cotyledons and leaves, but its activity was only confined to the cambial cells of all these organs (Y Hirakawa *et al.*, 2010). The *WOX4* promoter in *Arabidopsis* was extensively used to mark the cambial cells during the different developmental phases of the inflorescence stem (Suer *et al.*, 2011). It has been shown that *WOX4* promoter was active only in the fascicular cambium of the young stem referring it as the primary growth phase. Then the expression was shown to extend from the fascicular cambium into few cells that are positioned in between the fascicular and interfascicular regions of the stem. This extension was stated as an indication of the initiation of secondary growth. Then the circular *WOX4* expression domain was observed in matured stem (Suer *et al.*, 2011). However, the published studies of *WOX4* expression pattern (Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011) were based on using 2.9 Kb upstream and 0.6 Kb downstream regions, although long intergenic sequences flank the *WOX4* gene.

2. AIM OF THE THESIS

The main aim of this thesis work is to find out new information about spatio-temporal expression patterns of the *WUSCHEL-related homeobox4 (WOX4)* gene in different organs of *Arabidopsis thaliana*.

Although the published *WOX4* promoter has shown cambial specific activity, it contains only 2.9 Kb upstream and 0.6 Kb downstream sequences, which results in weak overall activity. Hence, we analysed the large flanking area, including 9.2 Kb upstream and 1.7 kb downstream from the *WOX4* gene sequence to identify the distal conserved elements. Furthermore, full-length *WOX4* promoter-reporter constructs, targeting either the endoplasmic reticulum (*flWOX4::erGFP* and *flWOX4::erCERULEAN*) or the nucleus (*flWOX4::H3-GFP*) were generated and transformed into *Arabidopsis thaliana*. The consequences of addition of all conserved distal elements were studied in detail with respect to the *WOX4* expression pattern in the shoot, roots and leaves.

3. MATERIALS AND METHODS

3.1. List of chemicals

Name	Abbreviation	Manufacturer
Acetic acid		Roth
Acetone		Roth
Agar		Roth, Duchefa
Agarose		Bio-Budget
Bromophenol blue		Merck
Calcium chloride	$CaCl_2$	Merck
Chloroform		Roth
Dexamethasone	Dex	Roth
Disodium phosphate	Na_2HPO_4	Roth
Ethanol		Roth
Ethidium bromide	EtBr	Sigma Aldrich
Ethylinediamine tetra acetic acid	EDTA	Sigma Aldrich
Glacial acetic acid		Sigma Aldrich
Glufosinate-ammonium (Basta®)		Bayer
Glycerol		Sigma Aldrich
Hydrochloric acid	HCl	Roth
Isoamyl alcohol		Roth
Isopropanol		Roth
Magnesium chloride	$MgCl_2$	Roth
MES salt		Sigma Aldrich
Monopotassium phosphate	KH_2PO_4	Sigma Aldrich
Phenol		Sigma Aldrich
Phloroglucinol		Sigma Aldrich
Potassium chloride	KCl	Roth
Potassium hydroxide	KOH	Roth
Propidium iodide	PI	Sigma Aldrich
Silwet®		Sigma Aldrich
Sodium acetate		Merck
Sodium chloride	NaCl	Roth
Sodium dodecyl sulphate	SDS	Merck
Sodium hydroxide	NaOH	Merck
Sodium hypochlorite	NaClO	Roth
Tris base		Roth
Tryptone		Roth
Yeast-extract		Roth

3.2. Buffers

TAE Buffer (50X): 242 g Tris base, 57.1 ml Glacial acetic acid and 100 ml of 0.5M EDTA (pH 8.0) per 1L. The 1X working solution is 40 mM Tris-acetate/1 mM EDTA.

TBE Buffer (5X): 54 g Tris base, 27.5 g Boric acid and 20 ml of 0.5M EDTA (pH 8.0) per 1L. The 0.5X working solution is 45 mM Tris-borate/1 mM EDTA.

PBS Buffer: 25.6 g Disodium Phosphate, 80 g NaCl, 2 g KCl and 2 g Monopotassium phosphate per 1L.

3.3. Kits

Name	**Purpose**	**Manufacturer**
Nucleospin-plasmid	Plasmid Purification	Macherey-Nagel
Nucleospin- Gel and PCR clean-up	PCR clean-up & Gel purification	Macherey-Nagel
Invitrap spin	RNA extraction	Startec. molecular
Superscript II Reverse transcriptase	cDNA Synthesis	Thermo Scientific
SMARTer RACE 5′/3	5′- RACE	Clonetech
My-budget 5X Taq PCR master mix	Colony PCR	Bio-budget Tech

3.4. Growth Media

LB medium:	Tryptone – 1 %, Yeast Extract – 0.5 %, NaCl – 1 % **,** Agar – 15 % , pH: 7.0
SOC-medium:	Tryptone – 2 %, Yeast Extract – 0.5 %, NaCl – 1 %, $MgCl_2$ – 10 mM, $MgSO_4$ – 10 mM, KCl – 2.5 mM, Glucose – 20 mM, and Agar – 15 % , pH: 7.0
Infiltration medium:	50 g of Sucrose and 200 µl of Silwet in 1L of distilled water
MS medium:	2.3 g of MS salts with B5 vitamins (Duchefa Biochemie), 10 g Sucrose, 0.5 g MES salt, and 8 g Agar in 1L of distilled water. Total concentration of micro & macro elements including vitamins: 4414.09 mg/l, pH 5.7

3.5. Biological materials

3.5.1. Enzymes

Name	Purpose	Manufacturer
Phusion DNA polymerase	DNA amplification	Sigma Aldrich
Taq DNA polymerase	DNA amplification	Sigma Aldrich
Restriction endonucleases	To cut the DNA specifically	New England Biolabs
Alkaline Phosphatase, Calf Intestinal phosphatase (CIP)	Dephosphorylation of 5´ and 3´ ends of DNA	New England Biolabs
Klenow fragment	Blunting the DNA ends, Second strand cDNA synthesis	New England Biolabs
T4 DNA ligase	Ligation	New England Biolabs

3.5.2. Primers

Oligo Name	Sequence 5' to 3'	Purpose
Up region1-FP_Asc1	GGCGCGCCAGCAAACATACCCACACAAAAG	Cloning
Up region1-RP_Sac1	GAGCTCGGGGGTATTTTAAAAAAATCTGATG	Cloning
Up region2-FP_Sac1	GAGCTCTCTAAATGCCTTGTCACCAAATC	Cloning
Up region2-RP_NcoI	CCATGGCTGCTATATGTTAAAACTAGCAAATGC	Cloning
Downstream-FP_Apa1	GGGCCCAGTCATGAAGGTGAGG	Cloning
Downstream-RP_Asc1	GGCGCGCCTCTTCTCATGGATTCT	Cloning
erCerulean-RP_BspHI	TCATGAAGACTAATCTTTTTCTCTTTCTCATCTTTTC	Cloning
erCerulean-RP_Apa1	GGGCCCTTAGAGTTCGTCGTGCTTGTACAG	Cloning
erGFP-FP_BspH1	GTCATGAAGACTAATCTTTTTCTC	Cloning
erGFP-RP_Apa1	TTAGAGTTCGTCGTGTTTGTATAG	Cloning
H3-FP_Nco1	CCATGGCTCGTACCAAGCAGAC	Cloning
H3-RP_Sal1	GTCGACAGCTCGTTCTCCTCTGATTC	Cloning
GFP-FP_Sal1	GTCGACAGTAAAGGAGAAGAAC	Cloning
GFP-RP_Apa1	GGGCCCTTAGAGTTCGTCGTGTTTG	Cloning
WOX4_cDNA_RP1	AAGACACCAGTGGTCGTGAAGC	RACE

WOX4_cDNA_RP2	GGTTGTTCCTCTTCTGCTTCTGTCTCTCCG	RACE
SMARTer-oligoIIA	AAGCAGTGGTATCAACGCAGAGTACXXXXX	RACE
RACE-UPM-long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATC AACGCAGAGT	RACE
RACE-UPM-short	CTAATACGACTCACTATAGGGC	RACE
Nested 1_N1	CAATCTGTTGAGCATTAGGAGTACG	RACE
Nested 2_N2	CTGTTCTTGAGTCGGGTTCCAC	RACE

3.5.3. Antibiotics

Name	**Working concentration (µg/ml)**	**Manufacturer**
Kanamycin	50	Duchefa
Ampicillin / Carbenicillin	100	Duchefa
Gentamycin	25	Duchefa
Rifampicin	50	Duchefa
Hygromycin	100	Duchefa

3.5.4. Cloning vectors

Name	**Source**	**Resistance**	**Function**
pJET 1.2/blunt	Thermo Scientific™	Ampicillin	Cloning
pBluescript KS (+)	(M.A.Alting-Mees and J.M.Short, 1989)	Ampicillin	Cloning
pGPTV - Kan	(Überlacker and Werr, 1996)	Kanamycin	Binary
pGPTV - Bar	(Überlacker and Werr, 1996)	Kanamycin, Phosphinothricin	Binary

3.5.5. Bacterial strains

Organism	**Strain**	**Function**
E. coli	DH5α (Hanahan, 1983)	Cloning
A. tumefaciens	GV3101 (pMP90) GentR, RifR (Koncz and Schell, 1986)	Plant transformation

3.5.6. Plant material

All the phenotypic and molecular biology studies were carried out in *Arabidopsis thaliana* Col-0 as the wild-type control. Same plant background was used to generate the transgenic lines.

Transgenic line	Ecotype	Selection	Purpose	Source
pWOX4:: YFP	Col-0	Phosphinothricin	Cambium	Thomas Greb
pATHB8:: YFP	Col-0	Phosphinothricin	Pro-cambium	E. Scarpella
flWOX4::erCER	Col-0	Phosphinothricin	Full-length *WOX4* promoter reporter	Current study
flWOX4::erGFP	Col-0	Phosphinothricin	Full-length *WOX4* promoter reporter	Current study
flWOX4:: H3-GFP	Col-0	Phosphinothricin	Full-length *WOX4* promoter reporter	Current study
flWOX4:gWOX4-GFP	Col-0	Phosphinothricin	Translational fusion	Current study
pWOX4-Ω::erCER	Col-0	Phosphinothricin	5′-modification in the *WOX4* promoter	Current study
pWOX4::erCER-PAS	Col-0	Phosphinothricin	3′-modification in the *WOX4* promoter	Current study

3.6. Methods

3.6.1. Phylogenetic Shadowing

Phylogenetic shadowing was used to identify the conserved sequences in the *WOX4* promoter region. First, the *WOX4* orthologs were identified by using *AtWOX4* coding sequence to BLAST against the genomes of *Brassicaceae* members' viz. *Arabidopsis thaliana, Arabidopsis lyrata, Arabidopsis halleri, Boechera stricta, Brassica rapa, Brassica oleracea, Capsella rubella,* and *Capsella grandiflora.* Using the locus information of these orthologs, the flanking genes were identified and then, the complete upstream and downstream sequences from the *WOX4* coding region were retrieved by using *Phytozome 10* and *Vector NTI*. These sequences were aligned with the *mVISTA* (http://genome.lbl.gov/vista/mvista/submit.shtml), at the default settings of LAGAN (Brudno *et al.*, 2003) alignment tool. Based on the sequence similarity obtained in vista plots, the conserved elements in the *WOX4* promoter are recognized.

3.6.2. Nucleic acid extraction & purification

Total genomic DNA was isolated from the leaf tissue according to the protocol described previously (K. Edwards, 1991) and purified by phenol-chloroform-isoamyl alcohol method.

Total RNA was isolated from young seedlings by Invitrap spin plant RNA mini kit, following the manufacturer protocol. An additional DNase treatment was performed and purified by phenol-chloroform-isoamyl alcohol method.

3.6.3. RACE (Rapid amplification of cDNA ends)

SMARTer® RACE 5′/3′ kit was used to identify the 5′ end of *WOX4* mRNA. 1µg of total RNA (DNA free) was used to synthesize the cDNA. Then the nested PCRs were performed by using N1, N2 primers that binds in the Exon 2 of *WOX4*. Subsequently, the RACE-PCR products were cloned into pJET1.2 blunt vector (Thermo Scientific) and sequenced with *WOX4* specific primers. The resulting sequences were aligned with the *WOX4* genomic DNA sequence to identify the transcription start site.

3.6.4. Promoter constructs

Following constructs were generated in the current study-

flWOX4::erCER	9183 bp upstream > *erCERULEAN* > 1704 bp downstream
flWOX4::erGFP	9183 bp upstream > *erGFP* > 1704 bp downstream
pWOX4::erCER:PAS	9183 bp upstream > *erCERULEAN*> poly-adenylation sequence
pWOX4::Ω:erCER	237 bp *WOX4* upstream region was replaced by TMV-Ω leader
flWOX4::H3-GFP	9183 bp upstream > *H3-GFP* > 1704 bp downstream
flWOX4:gWOX4-GFP	9183 bp upstream > *gWOX4>mGFP5*> 1704 bp downstream

3.6.5. Primer design

All primers were designed using the Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and then validated with Sigma-Oligoevaluator™ (https://www.sigmaaldrich.com/technical-documents/articles/biology/oligo-evaluator.html).

3.6.6. PCR Amplification

Total genomic DNA was used as the template for PCR amplification of upstream and downstream sequences from the *AtWOX4* coding region. The downstream sequence of 1704 bp was amplified in a single PCR event, whereas the upstream sequence of 9183 bp was amplified as an upstream region-I (6375 bp) and upstream region-II (3285 bp). Amplification with the *Phusion* DNA polymerase:

Reaction mixture		*PCR-program*	
5X HF buffer	10.00 µl	1. Denaturing	95°C, 3 min
10 mM dNTPs	1.00 µl	2. Denaturing	95°C, 30 sec.
10 µM Forward primer	2.50 µl	3. Annealing	58°C, 30 sec.
10 µM Reverse primer	2.50 µl	4. Elongation	72°C, 1 min per Kb
Phusion polymerase	0.50 µl	5. Amplification	2 to 4, 30 cycles
gDNA template	1.00 µl	6. Extension	72°C, 5 min
Water	32.50 µl	7. Stop	4°C, forever
Total	50.00 µl		

Amplification with the Taq-DNA polymerase (PCR Mastermix from bio-budget):

Reaction mixture		*PCR-program*	
5X Mastermix	10.00 µl	1. Denaturing	94°C, 3 min
10 µM Forward primer	2.50 µl	2. Denaturing	94°C, 30 sec.
10 µM Reverse primer	2.50 µl	3. Annealing	56°C, 30 sec.
gDNA template	1.00 µl	4. Elongation	72°C, 1 min per Kb
Water	34.00 µl	5. Amplification	2 to 4, 30 cycles
Total	50.00 µl	6. Extension	72°C, 5 min
		7. Stop	4°C, forever

3.6.7. Cloning

The amplified DNA fragments were first cloned into *pJET1.2* blunt vector, transformed into *E. coli DH5α* competent cells, selected against the antibiotic resistance, confirmed with the restriction digestion and with the Sanger sequencing. Then the fragments were cloned into *pBluescript* vector

using appropriate restriction sites to generate various constructs. Finally, these constructs were cloned into *pGPTV* binary vector using *AscI* site, transformed into *Agrobacterium tumefaciens GV3101* strain.

Ligation

NEB T4 DNA ligase was used in each ligation reaction following the manufacturer protocol. In all the ligation reactions, insert to vector ratio was 1:5 and their concentrations were calculated according to the formula described below.

([ng of vector] x [kb size of insert]) / (kb size of vector) x (insert: vector ratio) = ng of insert required

Reaction:		**Conditions:**
5X ligation buffer	4 µl	3 - 5 minutes at room temperature
Vector	1 µl	Or overnight at 4^{o}C
Insert	X µl	
Ligase	1 µl	

3.7. Transformation

3.7.1. Transformation of *Escherichia coli DH5α*

E. coli competent cells were prepared using $CaCl_2$ and stored in -80^{o}C. These competent cells were carefully thawed on ice prior to the transformation, 1-2 µl of plasmid or ligation mixture was added and mixed gently. Then the transformation was performed by the heat shock at 42^{o}C for 30 seconds, followed by the addition of 200 µl of SOC medium and incubation at 37^{o}C for an hour. The transformed cells were selected on the LB agar medium with an appropriate antibiotic.

3.7.2. Transformation of *Agrobacterium tumefaciens*

Agrobacterium cells were transformed by electroporation. The competent cells were carefully thawed on ice prior to the transformation, and then 1 µl of the plasmid was added and mixed gently. Electroporation was performed with the Gene Pulser (Bio-rad) at 1.8 kV. These cells were

incubated with YEB medium at 28^{o}C for an hour and transformed cells were selected on the LB agar medium with Kanamycin, Rifampicin, and Gentamycin.

3.7.3. Transformation of *Arabidopsis thaliana* (floral-dip method)

Plant transformation was performed with the floral-dip method (Clough and Bent, 1998). *Agrobacterium tumefaciens* GV3101 with binary vector *pGPTV* harboring a construct of interest was grown overnight in the LB medium with Kanamycin, Rifampicin, and Gentamycin at 28^{o}C. Then the cells were harvested by centrifugation at 5000 rpm and then suspended gently in the infiltration medium. The *Arabidopsis* inflorescences were dipped in the cell-suspension for about 3-4 minutes and incubated overnight in the dark covering with a dome-shaped cover. Plants were then transferred to the greenhouse and the seeds were harvested after 30-35 days.

3.8. Selection of the transformants

Seeds collected after the floral-dip were considered as T_1, they were germinated on large trays and 5mg/L BASTA (Glufosinate) solution was sprayed on the young seedlings to select the T_1 transgenic lines. Transgenic plants carrying *NptII* or *Hpt* resistance were selected on the ½ MS medium with 50mg/L Kanamycin or 100mg/L Hygromycin. At least six independent T1 transgenic lines were used for the microscopic analysis.

3.9. Confocal Imaging

Confocal imaging was performed with the confocal laser scanning microscopes (CLSM) - Zeiss LSM 700 and Leica TCS SP8. The *GFP* chromophore was excited at 488 nm and the emission signal was collected between 502-520 nm, whereas the *CERULEAN* chromophore was excited at 442 nm and the emission signal was collected in between 470-520 nm. YFP chromophore was excited at 514 nm and the emission signal was collected in between 590-680 nm. Propidium Iodide was excited with 488 nm and emission signal was collected in between 620-680 nm.

3.10. Image Processing

Processing of the CLSM 2D images and conversion of the Z-stacks into 3D images were performed with the software-Imaris (Bitplane, Switzerland).

4. RESULTS

4.1. Phylogenetic shadowing revealed conserved regions within the *WOX4* promoter

The function of the *WOX4* gene is conserved in plant vascular tissue development and the promoter elements that regulate the *WOX4* expression pattern might be located either in the upstream and/or downstream of the *WOX4* coding region. Therefore, phylogenetic shadowing was performed to identify the evolutionarily conserved regions in the *Arabidopsis WOX4* promoter by comparing the orthologous *WOX4* sequences from the seven *Brassicaceae* species including *Arabidopsis halleri, Arabidopsis lyrata, Brochera stricta, Brassica rapa, Brassica oleracea, Capsella rubella,* and *Capsella grandiflora.*

The orthologous *WOX4* genes from the above mentioned seven species were identified by using the *WOX4* coding sequence from *Arabidopsis thaliana (AT1G46480.1)* as a query in the NCBI-BLAST analysis. The genes that flank the *WOX4* were also identified and used to define the length of intergenic regions. Interestingly, in all the eight species that were examined in this study, *WOX4* coding sequence is separated by long intergenic sequences. This information was used to retrieve the sequences of *WOX4* orthologs along with the complete upstream and downstream regions from Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html) and National Center for Biological Information (NCBI, https://www.ncbi.nlm.nih.gov/genome/) databases. In the next step, phylogenetic shadowing was performed using the *WOX4* sequences along with the complete upstream and downstream intergenic regions by using the mVISTA. The mVISTA is an online toolkit with a set of computational programs generally used for studying the comparative genomics (http://genome.lbl.gov/vista/mvista/submit.shtml). At the default settings, the mVISTA browser uses the computational program LAGAN (Brudno *et al.*, 2003), which detects the conserved regions by conducting the progressive pairwise alignments of the nucleotide sequences. The *AtWOX4* gene sequence along with the complete upstream and downstream intergenic regions were used as a reference sequence (Figure 5C) for the pairwise alignment of orthologous *WOX4* genomic sequences obtained from the other seven *Brassicaceae* species. The annotation details of the *AtWOX4* gene from the TAIR database (TAIR10) were used in the LAGAN program to define the exon, intron and 5′, 3′ untranslated regions. The resulting VISTA plot was visualized in the VISTA browser with default settings that calculate 70 % consensus identity at the calculation

window of 100 base pairs. This analysis revealed that the *WOX4* orthologs are highly conserved within the three exons while the conservation is slightly lower within the two introns (Figure 5B). Moreover, several conserved blocks in the *WOX4* promoter with varying percentages of sequence homology were identified. Highly conserved sequences with at least 75 % homology were located both in the upstream and in the downstream regions from the *WOX4* coding region (Figure 5B). In the upstream region, three conserved blocks were identified between -1 to -1800 bp (Block 1), -4000 to -5000 bp (Block 2), and -6500 to -8500 bp (Block 3). In the downstream region, the conserved blocks were located in between 1 to 1700 bp after the STOP codon. The striking exception was *C. grandiflora*, in which only two conserved blocks in the upstream and one block in the downstream were identified (Figure 5B).

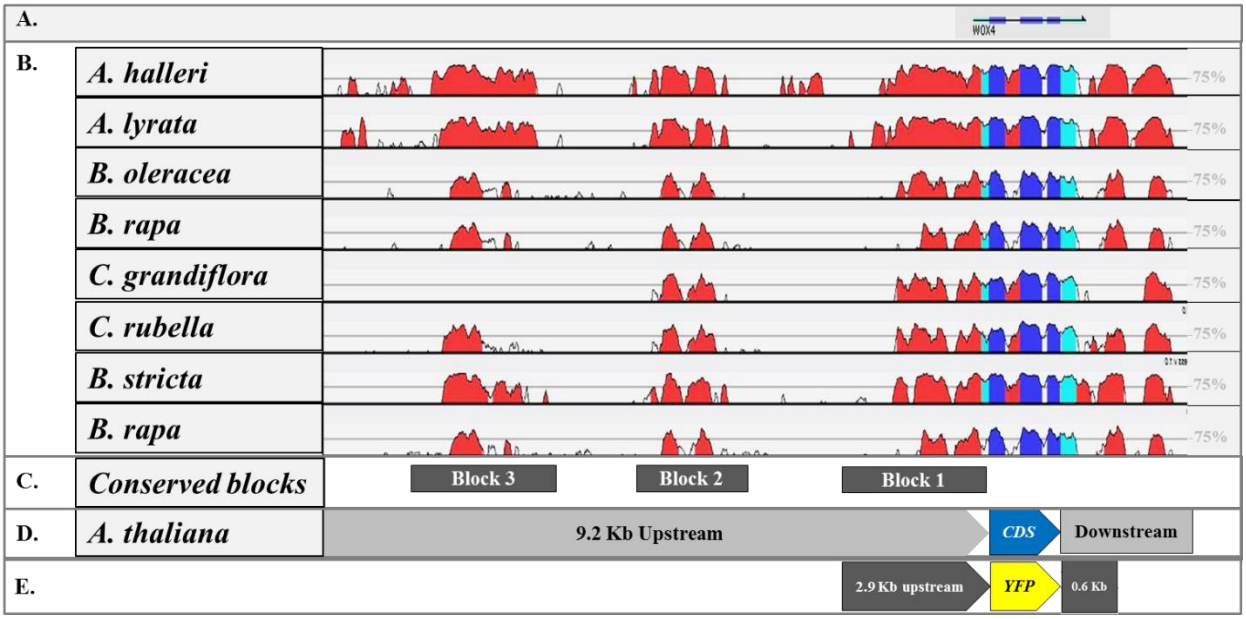

Figure 5. Phylogenetic shadowing of *WOX4* gene sequences, upstream and downstream regions.

A. Representation of the *Arabidopsis WOX4* gene model, based on the TAIR10 database. Three exons were shown in blue colour, two introns (colourless) and the 5′ & 3′ untranslated regions were shown in pale-blue colour. **B.** The mVISTA plot illustrating the conserved regions based on the pairwise nucleotide sequence alignment of orthologous *WOX4* sequences. The peaks in blue, pale-blue and red colours represent at least 70 % sequence conservation in the coding regions, 5′ & 3′ untranslated regions (5′ and 3′ UTR) and in the intronic, further upstream and downstream regions, respectively. **C.** Three identified conserved blocks in the upstream region **D.** The graphical representation of complete upstream (9,183 bp) and downstream (1,704 bp) regions from the *WOX4* coding region of *Arabidopsis thaliana*. **E.** The graphical representation of the short *WOX4* promoter construct (*WOX4::YFP*) that was used in the study by (Suer *et al.*, 2011).

The conserved elements that were identified by phylogenetic shadowing show that the previously published *AtWOX4* expression study (*WOX4::YFP)* by (Suer *et al.*, 2011) in which 2.9 Kb upstream and 0.6 Kb downstream region of *WOX4* coding sequence were used and it covers proximal conserved blocks but excludes the distal conserved elements (Figure 5C & E).

We were interested to know the chromatin accessibility of the *AtWOX4* promoter, and for this purpose we used the ATAC-seq (Assay for Transposase-Accessible Chromatin using high-throughput sequencing) data that was recently published from our group (Frerichs *et al.*, 2019). In this study, the *DORNRÖSCHEN-LIKE (DRNL)* promoter-reporter was used to mark the lateral organ founder cells in the peripheral zone of the inflorescence meristems in *Arabidopsis thaliana apetala1-1 cauliflower-1* double mutant background (Frerichs *et al.*, 2019). The lateral organ founder cells that express the reporter gene were separated by the Fluorescence-activated cell sorting (FACS) and then subjected to the ATAC-seq analysis. The ATAC-seq is a powerful technique that is used to assess the chromatin accessibility in the plant genome (Tsompana and Buck, 2014). It uses the Tn5 transposase activity for cleaving the open genomic DNA sequences, revealing the open chromatin configurations. By utilizing the data, we recognized that the peaks referring to the open chromatin are clearly correlating with the locations of the conserved regions identified in the phylogenetic shadowing of the *WOX4* promoter (Figure 6). One distal peak (indicated by star mark) was found to be specifically associated with a distal conserved region appeared only within the closely related species, *A. thalina, A. halleri and A. lyrata* (Figure 6A).

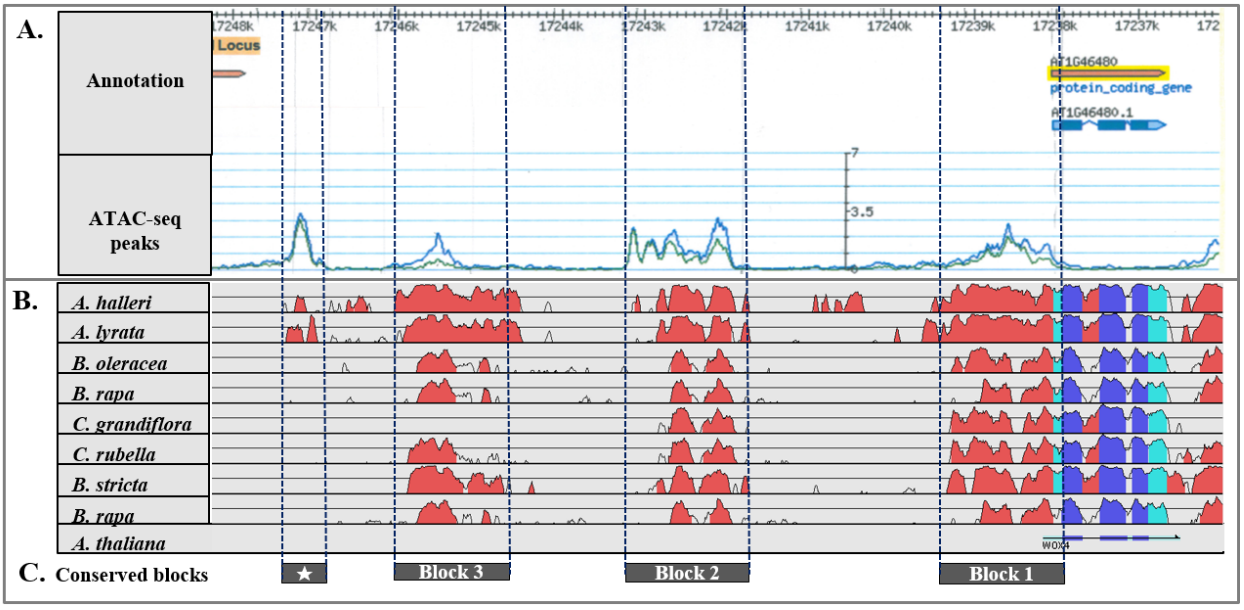

Figure 6. **ATAC-seq analysis of the *WOX4* promoter region**

A. Representation of the identified open chromatin peaks within the *WOX4* promoter region by ATAC-seq analysis. **B & C.** Comparison of the positions of the open chromatin peaks to the 9.2 Kb *WOX4* promoter region, indicating the exact overlap of the positions of the open chromatin peaks to the upstream conserved regions that were identified by phylogenetic shadowing. Star mark (★) indicates the ATAC-seq peak that belongs to the conserved region appeared only within the *A. halleri* and *A. lyrata*.

4.2. The proximal region of the *WOX4* promoter contains a conserved open reading frame

In the investigated seven *Brassicaceae* members, the immediate proximal promoter region (Block 1; -1 to -1800 bp) upstream to the *WOX4* translational start codon is relatively highly conserved (Figure 5B). Subjecting the proximal promoter region of *AtWOX4* for the detailed analysis revealed an AT-rich sequence and specifically the upstream 550 bp sequence comprised of several ATG triplets.

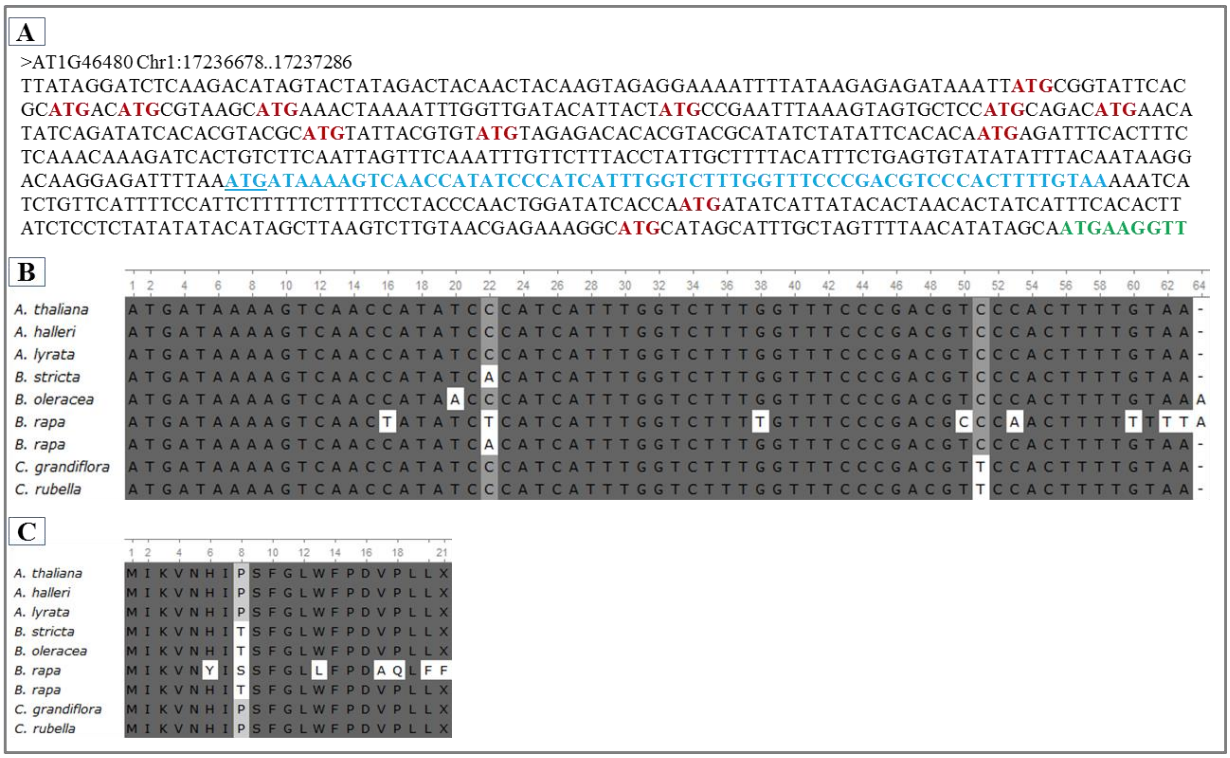

Figure 7. Analysis of the *WOX4* proximal promoter region

A. Analysis of the *A. thaliana WOX4* proximal promoter region revealed several conserved ATGs (red) and a 63 bp short open reading frame (blue) within the immediate upstream 550 bp region of the *WOX4* proximal promoter. **B and C**: Representing the nucleotide and amino acid sequence conservation of the 63 bp upstream ORF, respectively.

Additionally, the multiple sequence alignment of this region (-550 bp) also confirmed the conservation of upstream ATG triplets in the *WOX4* orthologous promoter sequences of the studied *Brassicaceae* members. A total of thirteen ATGs were observed within this 550 bp upstream region, out of which six ATGs were located in-frame with the *WOX4* translational start codon ATG_{+1} (Figure 7A). Moreover, a highly conserved 63 bp long upstream open reading frame (ORF) that started at -237 and terminated at -174 bp with a stop codon TAA was also identified (Figure 7B). Translation of this 63 bp ORF into amino acid sequence displayed 100 % identity within the five *Brassicaceae* members - *A. thaliana, A. halleri, A. lyrata, C. grandiflora, and C. rubella*. Whereas, in *B. stricta, B. oleracea and B. rapa* there is only one amino acid substitution (Proline to Threonine) at the position eight. However, in one paralogue of *B. rapa* this region showed only 66 % sequence identity (Figure 7C).

4.3. The transcriptional start site of the *WOX4* is located further upstream

The 5′ RACE (Rapid Amplification of cDNA Ends) was performed using the SMARTer RACE kit (Clonetech) in order to find out the *WOX4* transcriptional start site and to confirm whether the 63 bp upstream ORF is a part of the *WOX4* transcript.

Initially, the cDNA was synthesized by following the SMARTer-RACE user manual. The first round of nested PCR was performed using the purified cDNA as a template, the gene-specific reverse primer N1 and the forward primer UPM-long (Universal Primer A mix-long; Clonetech). The second round of nested PCR was performed using the tenfold diluted nested PCR1 product as a template, the forward primer UPM-short and gene-specific reverse primer N2 (Figure 8A). The primer sequences were listed in the materials and methods section (3.5.2). After two rounds of nested PCRs, the RACE-PCR fragments were purified, cloned into the pJET vector and transformed into the *E. coli DH5α*. The colony PCR was initially performed to select the clones with *WOX4* specific sequences and then the selected clones were confirmed with the restriction digestion followed by the Sanger-sequencing. In total, more than 50 clones were sequenced. All the sequences of RACE products were trimmed and aligned to the *WOX4* genomic sequence to identify the transcripts with desired splicing pattern and then the 5′ termini were examined. This analysis revealed that the cDNA sequences varied in their lengths with different 5′ termini and sequence alignment of the cDNA clones that contain long 5′ termini was shown in Figure 8B. Four

identical clones with longest 5′ RACE fragments were found to be terminated at -302 bp upstream of the *WOX4* coding sequence, which includes the conserved 63 bp upstream ORF (Figure 8C). No evidence was obtained for an additional intron or alternative splicing.

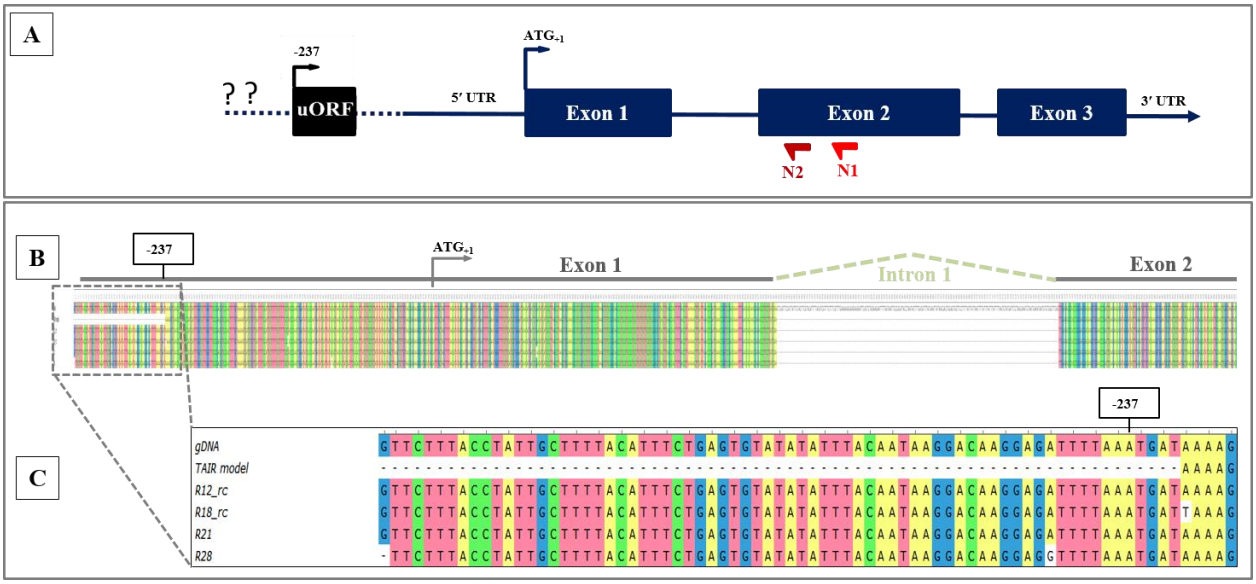

Figure 8. Identification of the *WOX4* transcriptional start site

A. Schematic representation of the *WOX4* gene model from TAIR database showing the exons, and UTRs. Approximate positions of the uORF, nested primers (N1, N2) used in the 5′ RACE-PCR are shown and the putative TSS is indicated by the question mark '?'. **B & C:** Multiple sequence alignment of the selected long 5′ RACE-PCR clones shows the intron splicing and transcripts starting from further upstream of the conserved upstream ORF.

4.4. The full-length *WOX4* promoter construct includes all the conserved regions

To examine whether the distal conserved sequences in the *WOX4* promoter region contribute to its expression pattern, the full-length *WOX4* promoter construct that includes all the conserved distal promoter elements were created, by combining the 9,183 bp upstream and 1,704 bp downstream sequences from the *WOX4* coding region.

The PCR amplification of the 1,704 bp downstream sequence was achieved in a single step, whereas the 9,183 bp upstream sequence was too large to be amplified in a single PCR reaction. Hence, upstream proximal 3.2 Kb and distal 6 Kb promoter regions were amplified separately and were fused together using restriction sites (Figure 9C). The three fragments (downstream and upstream proximal and distal promoter regions) were amplified using the primers listed in table 3.5.2 (materials and methods section) that adds the following restriction sites: *Asc*I at 5′ end &

*Nhe*I at 3′ end of the upstream distal promoter, the *Nhe*I at 5′ end & *Nco*I at 3′ end of the upstream proximal promoter and the *Sal*I, *Apa*I at 5′ end & *Asc*I, *Kpn*I at 3′ end of the downstream region. Here, two different restriction sites were added at the 5′ and 3′ end of the downstream region by keeping further cloning steps in mind. All three promoter fragments were initially cloned into the pJET 1.2/blunt cloning vector and confirmed by sequencing. The individual promoter fragments were then assembled in the *pBluescript* vector backbone by using the directional cloning approach. First, the distal and proximal promoter regions were combined using the *Nhe*I restriction site and then the downstream fragment was added by using the *Sal*I-*Kpn*I restriction sites. The resulting plasmid contains the multiple cloning site between the upstream and downstream promoter sequences, which was later used for creating different combinations of the promoter-reporter constructs.

The full-length *WOX4* promoter-reporter constructs with two different combinations of fluorophores, the *GFP* and *CERULEAN* that are either nuclear localized or targeted to the endoplasmic reticulum were generated to recognize the *WOX4* promoter activity in different tissues with variable morphology. The full-length *WOX4* promoter-reporter constructs ***flWOX4::erCERULEAN*** and ***flWOX4::erGFP*** were created by inserting endoplasmic reticulum (er) targeting *erCERULEAN* and *erGFP* fluorescent protein-coding sequences between the upstream and downstream promoter sequences by using the *Nco*I-*Apa*I restriction sites (Figure 9D & E). These constructs represent the *WOX4* transcriptional fusions as they contain the full-length promoter with only the reporter, but not the *WOX4* coding region.

The nuclear-localized full-length *WOX4* promoter-reporter construct ***flWOX4::H3-GFP*** (Figure 9F) was created by inserting the histone (*H3*) sequence and then the *GFP* coding sequence between the upstream and downstream promoter regions by using *Nco*I-*Sal*I and *Sal*I-*Apa*I sites, respectively. To examine whether the conserved upstream ORF affects the *WOX4* expression pattern or not, the *WOX4* promoter region from -1 to -237 bp was replaced by the *TMV*-Ω leader sequence (Töpfer *et al.*, 1993) and created the reporter construct ***WOX4-Ω::erCERULEAN*** (Figure 9G).

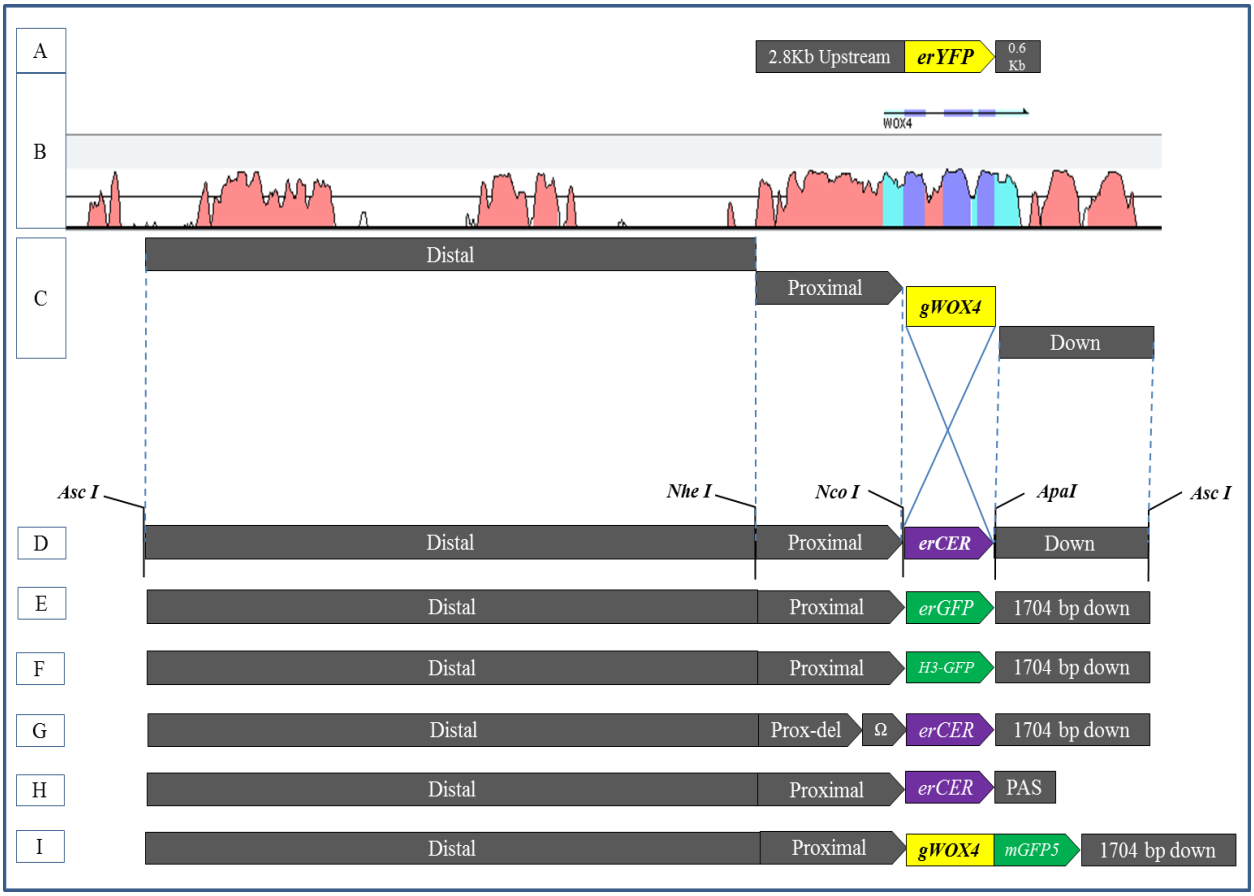

Figure 9. Graphical representation of the *WOX4* promoter region

A. Schematic representation of previously studied *WOX4* promoter construct (Suer *et al.*, 2011) **B.** Phylogenetic shadowing diagram illustrating the conserved areas within the full-length *WOX4* promoter and the gene sequences. The conserved coding regions, 5′ and 3′ UTRs and intronic, further upstream and downstream regions were shown in blue, pale-blue and red colour peaks, respectively. **C.** Schematic representation of the full-length *WOX4* promoter which includes – upstream distal, proximal and downstream regions. **D-F:** Schematic representation of different *WOX4* promoter-reporter constructs; **D.** *flWOX4::erCERULEAN*, **E.** *flWOX4::erGFP*, **F.** *flWOX4::H3GFP*. **G** and **H**: The truncated *WOX4* promoter-reporter constructs; **G.** *pWOX4::Ω:erCERULEAN,* in which the proximal promoter region (-237 bp) was replaced by TMV-**Ω** leader sequence, **H.** *pWOX4::erCERULEAN-PAS*, in which the downstream 1704 bp sequence was replaced with the downstream region of the *35S* gene including the polyadenylation sequence (*PAS*) and **I.** Schematic representation of the *WOX4* translational fusion construct *flWOX4::gWOX4-GFP*.

Likewise, the promoter construct ***WOX4::erCERULEAN-PAS*** (Figure 9H) was also created by replacing a 1.7 Kb downstream sequence of the *WOX4* coding region with the downstream region of the *35S* gene, which includes the polyadenylation sequence (*PAS*). This construct was used to study whether the downstream sequence is essential for the *WOX4* expression pattern or not. The

WOX4 translational fusion construct ***flWOX4:gWOX4-GFP*** (Figure 9I) was created by replacing the histone 3 sequence from ***flWOX4::H3-GFP*** construct with the *WOX4* gene sequence (*gWOX4*) by using *Pst*I and *Sal*I restriction sites. The comparison of *WOX4* expression patterns obtained by transcriptional and translational fusion constructs would be helpful to understand if the WOX4 protein moves from one cell to the other. The non-cell autonomous activity of the WUSCHEL protein was previously well defined by comparing expression patterns generated by transcriptional and translational fusion constructs (Yadav *et al.*, 2011). Therefore, we used the *WOX4* translational fusion and *WOX4* promoter-reporter constructs to study whether *WOX4* protein is mobile or not.

However, the respective *flWOX4* promoter-reporter cassettes were released from the *pBluescript* vector backbone using the restriction site *Asc*I, cloned into the destination vector *pGPTV-Bar* (Überlacker and Werr, 1996) and subsequently, transferred into the *Agrobacterium tumefacience* GV3101 (Koncz and Schell, 1986) by electroporation (Mersereau *et al.*, 1990) and the *Arabidopsis thaliana* Col-0 plants by the floral-dip method (Clough and Bent, 1998). The T1 generations were selected upon the *Basta* treatment and independent transgenic plants were used for the confocal microscopic studies.

4.5. Expression analysis of the full-length *WOX4* promoter-reporters in stem

All the three full-length *WOX4* promoter-reporters ***flWOX4::erCERULEAN, flWOX4::erGFP*** and ***flWOX4::H3-GFP*** showed a similar expression pattern throughout the development in different tissues, and the details are presented in the following sections. However, we noticed that the signal intensities varied depending on the tissue type and cell structure. For example, the endoplasmic reticulum-targeted *erCERULEAN* and *erGFP* reporters showed the superior signals in tissues such as the cambium and xylem parenchyma, whereas the nuclear-localized *H3-GFP* had shown excellent signals in the meristematic cells of the shoot apex. The meristematic cells of the shoot apex are small, hence mostly occupied by the nuclei; while the cells of the young/mature stem are large, hence the endoplasmic reticulum occupies more cellular space than the nucleus. Therefore, the nuclear-targeted ***flWOX4::H3-GFP*** (green signal) was used to visualize the *flWOX4* promoter activity in the apical meristems while the endoplasmic reticulum targeted ***flWOX4::erCERULEAN*** (blue signal) construct was used to visualize the *flWOX4* promoter activity in the stem and leaves.

4.5.1. *WOX4* promoter activity starts early during the shoot development

The shoots that comprised of the inflorescence meristem, flower primordia, young and mature stems were used to analyse the *WOX4* promoter activity by using the *flWOX4* promoter-reporter lines. The live imaging of the shoot apex was performed by placing the inflorescence on an agar block and carefully removing the flowers to expose the meristem. Whereas, the longitudinal sections of the shoot apex were obtained by embedding the young inflorescence in low-melting agar, then making thin sections using the vibratome. The stem sections were also obtained by hand sectioning wherever it was possible. All the sections were counterstained with the 1µg/ml propidium iodide solution prior to scanning for the reporter expression. The confocal laser scanning microscopy was used for the live imaging with the wavelengths 440 nm & 490 nm from argon laser to excite the *CERULEAN* & *GFP* fluorescent proteins, respectively. The emitted signals were collected in the range of 468-510 nm for *CERULEAN*, 510-545 nm for *GFP* and 610-630 nm for propidium iodide.

A top view at the shoot apex of *flWOX4-H3::GFP* reporter line revealed the early activity of the *WOX4* promoter and it was observed in the meristematic cells of the inflorescence meristem (IM) (Figure 10A). The fluorescent signal was found in the group of cells that are arranged like a spiral pattern (Figure 10A), which might correspond to the position of emerging flower primordia. However, the Z-stack images obtained from the top of the epidermis to few sub-epidermal cell layers revealed that the fluorescent signal was prominent in the cells of the sub-epidermal layer than in the epidermis (Figure 10B). On the other hand, there was no detectable signal in confocal image obtained from the top view of shoot apex from *flWOX4::erCERULEAN* reporter line (Figure 10C). However, the longitudinal section of the same inflorescence showed a weak *WOX4* promoter activity in the inflorescence meristem (IM), but stronger signal was detected in the stem tissues subtending to the IM (Figure 10D).

There is a discrepancy in visualizing *flWOX4-H3::GFP* and *flWOX4::erCERULEAN* reporter signals, due to the morphology of cells and the position of nucleus. The meristematic cells in the flower primordia were better visualized by *flWOX4-H3::GFP*, and the cells of the young/mature stem by *flWOX4::erCERULEAN* reporter. Therefore, combining the confocal images obtained by both the reporters showed a better overview of the full-length *WOX4* promoter activity during the

shoot development. For this reason, in the subsequent sections, we used *flWOX4::erCERULEAN* reporter lines to depict the *WOX4* promoter activity in young and mature stems.

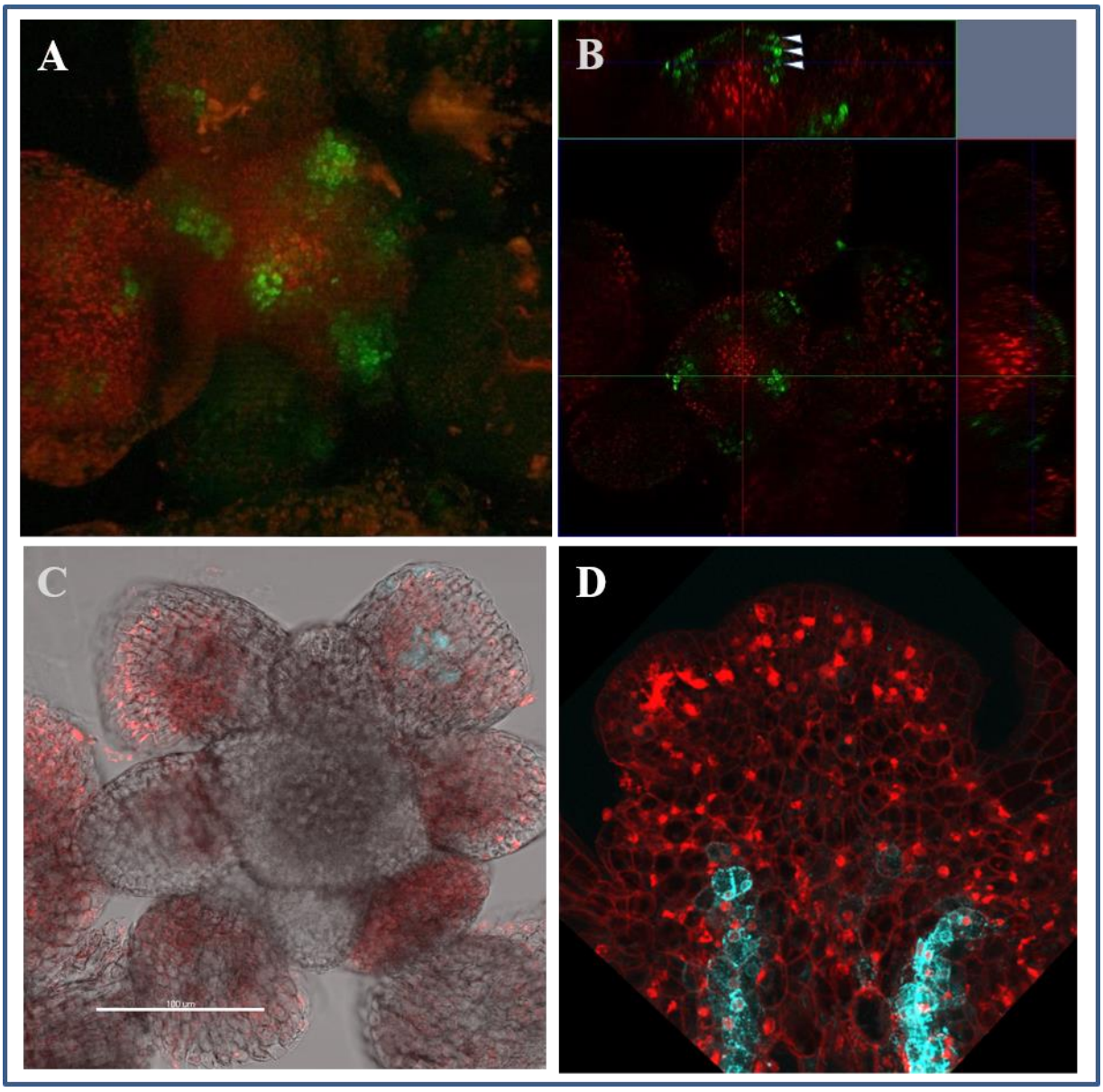

Figure 10. Full-length *WOX4* promoter activity at the shoot apex

A. The *WOX4* promoter activity depicted by the ***flWOX4::H3-GFP*** reporter line. The confocal laser scanning microscopy (CSLM) image of the inflorescence tip shows the GFP signal in the group of cells of IM. **B.** The 3D image of the shoot apex shows that the GFP signal in the sub-epidermal cells (marked by the arrowheads). **C and D:** The *WOX4* promoter activity depicted in the shoot apex by the ***flWOX4::erCERULEAN*** reporter line. The CLSM images indicate the lack of *CERULEAN* signal at the shoot apex in a top view **(C)** and the appearance of a high-intensity *CERULEAN* signal within the longitudinal section marking the stem region subtending to the IM **(D)**.

4.5.2. *WOX4* promoter is active in a circular domain of subcortical cells beneath the IM

A medial plane longitudinal section through the inflorescence meristem showed that the full-length *WOX4* promoter was active in the developing stem (Figure 11B). In order to depict in which cell-types it is active, a series of transverse sections (40 µm thick) were made, starting from the tip of the inflorescence to the subtending nodes. Two of the representative transverse sections (Figure 11C & D) were used to visualize the cells in which the *WOX4* promoter was active.

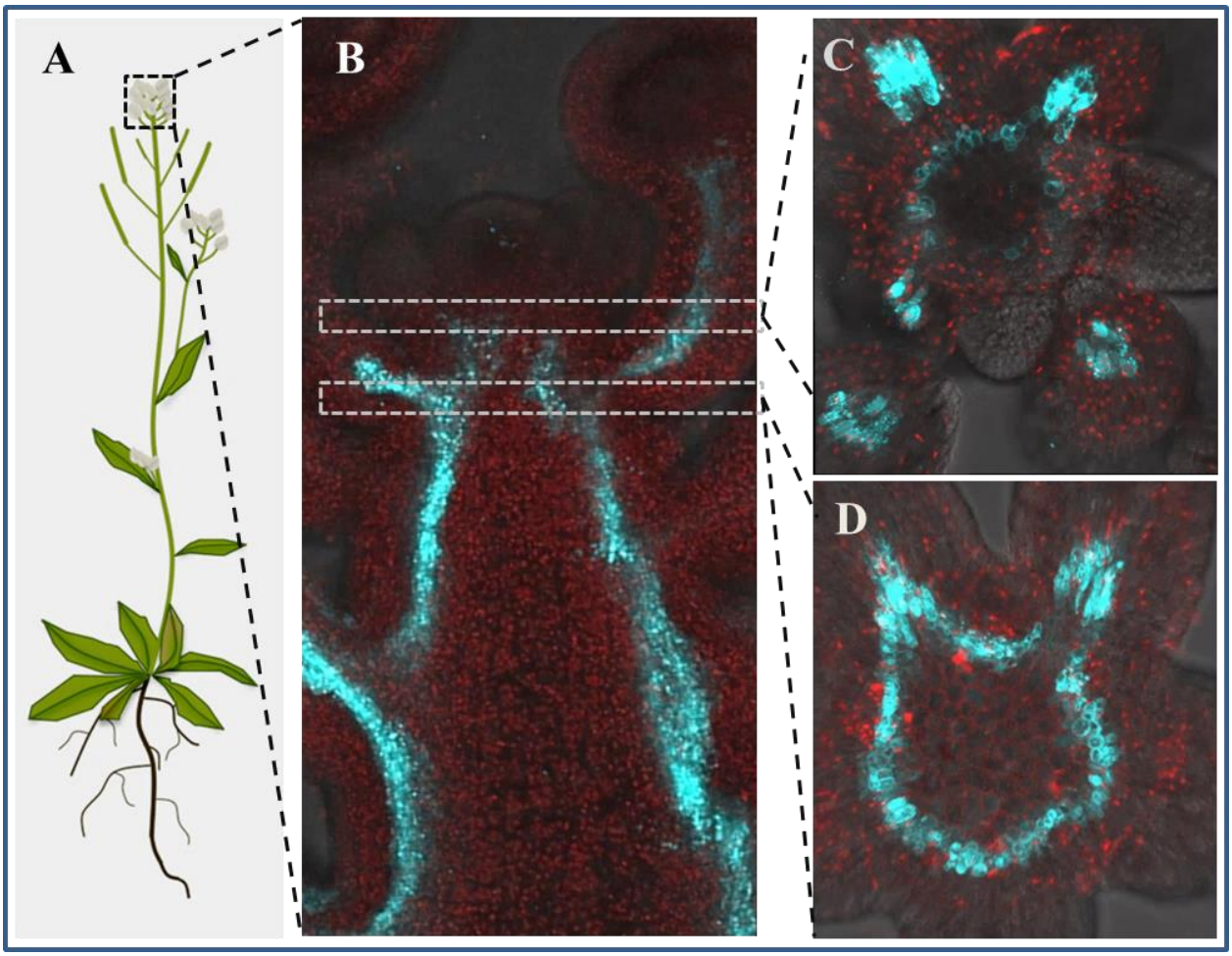

Figure 11. *flWOX4* promoter activity in the sub-cortical cells of the inflorescence meristem.

A. Graphical representation of the inflorescence region that was chosen for the confocal imaging **B.** Longitudinal section through the inflorescence tip of a *flWOX4::erCERULEAN* reporter line **C.** Transverse section of the shoot apex 80-100 µm below the IM showing a cylindrical domain of *WOX4* promoter activity in the sub-cortical area. **D.** Transverse section of the subtending node, showing a connection between the *WOX4* expression domain of the stem and flower pedicle.

The transverse section obtained from approximately 80-100 µm below the inflorescence meristem illustrates that the *WOX4*-expressing cells were positioned in the sub-cortical region of the stem (Figure 11C). The *WOX4* promoter activity was confined only to the region with one or two cell layers that were located between the cortex and pith regions creating a circular domain of the sub-cortical cells. Moreover, prominent fluorescence signal was also observed in flower pedicles (Figure 11C).

A series of transverse sections of inflorescence stem further below the shoot apex revealed the similar *WOX4*-expression domain and it was illustrated by one of the transverse sections (Figure 11D). It means that the *flWOX4* promoter activity was continued in a circular domain of sub-cortical cells from the tip of the inflorescence to the subtending young nodes and altogether makes a hollow cylindrical expression domain. The series of transverse sections of the stem also illustrated the connections of the *WOX4*-expression domain in the stem with the vascular bundles of surrounding flower pedicles (Figure 11C & D). All the connections of *WOX4*-expression domain in the stem with the flower pedicels seem to interrupt the circular shape (Figure 11D).

4.5.3. *WOX4* promoter activity continues into the subtending nodes of the stem

In subsequent nodes, the *WOX4* promoter also showed a continuous activity in a similar circular domain of sub-cortical cells. A transverse section obtained from the inflorescence stem approximately 300 µm beneath the IM showed the *WOX4* expressing domain was circular both in the stem and flower pedicels (Figure 12A). This expression pattern was similar to that of the pattern found with the transverse section 80 µm below the IM.

Interestingly, the transverse section obtained from the node far away from the IM also showed the *WOX4* promoter activity in the sub-cortical tissue of the main stem and its lateral branch (Figure 12C). Although the lateral branch contains a perfect circular domain of the *WOX4* expressing cells, the main stem showed a wavy circle. The wavy appearance of *WOX4* expression domain possibly indicate the developing vascular bundles and the anatomy also shows the emerging xylem and phloem tissues from the newly forming fascicular cambium (Figure 12C).

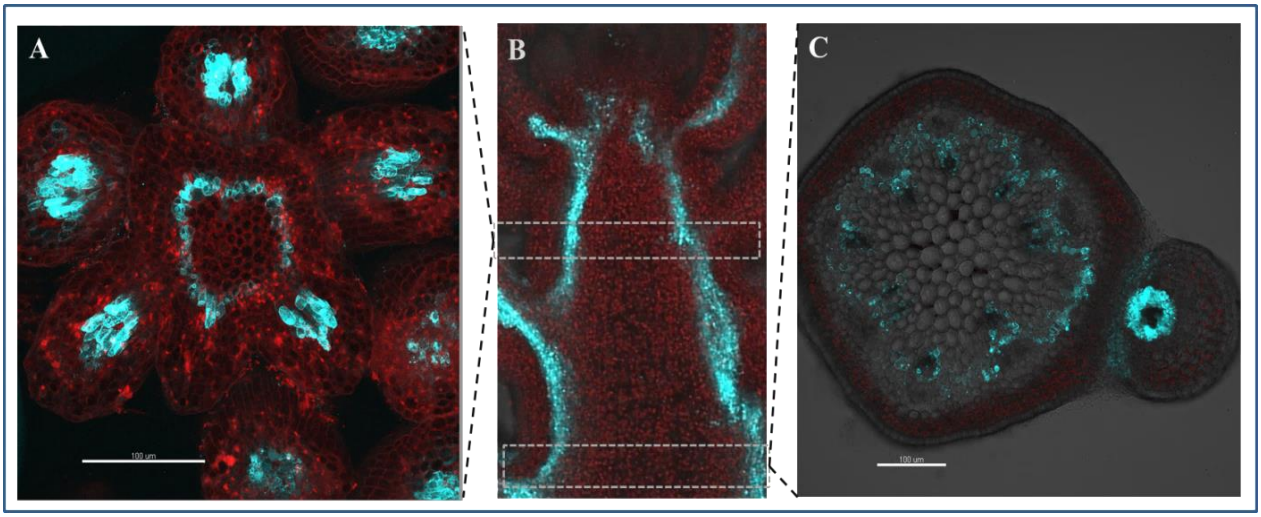

Figure 12. The *flWOX4* promoter activity in the subtending nodes of IM

A. The transverse section obtained from the inflorescence stem region approximately 300 µm below the IM and circular patterns of *WOX4* expression in the sub-cortical cells of both in the stem and its surrounding pedicels. **B.** The same image from Figure 12B is used to represent the approximate position of the transverse sections. **C.** *WOX4* promoter activity in the subcortical tissue of the main stem and its lateral branch when transverse sections obtained further away from the IM, the node that is below the flower cluster.

4.5.4. *WOX4* expression domain continued to exist throughout the developing stem

A transverse section acquired from the stem node further down the shoot apex also showed the *WOX4* promoter activity in a similar circular domain of subcortical cells (Figure 13B). The circle at this stage also contains intrusions, which indicate the fascicular cambium, xylem and phloem tissues (Figure 13C). Interestingly, the inner side of the expression domain at the intrusions marked a major portion of the developing xylem tissue (Figure 13C). These emerging vascular bundles are in direct connection with the rest of the subcortical tissue, specifying that the *WOX4* promoter was continuously active throughout the primary stem. According to the previously published reports with the short promoter construct (Suer *et al.*, 2011), the *WOX4* promoter was shown to be intermittently active only in the fascicular cambium at this developmental stage, but not in a complete subcortical circular domain as shown with the full-length *WOX4* promoter-reporter.

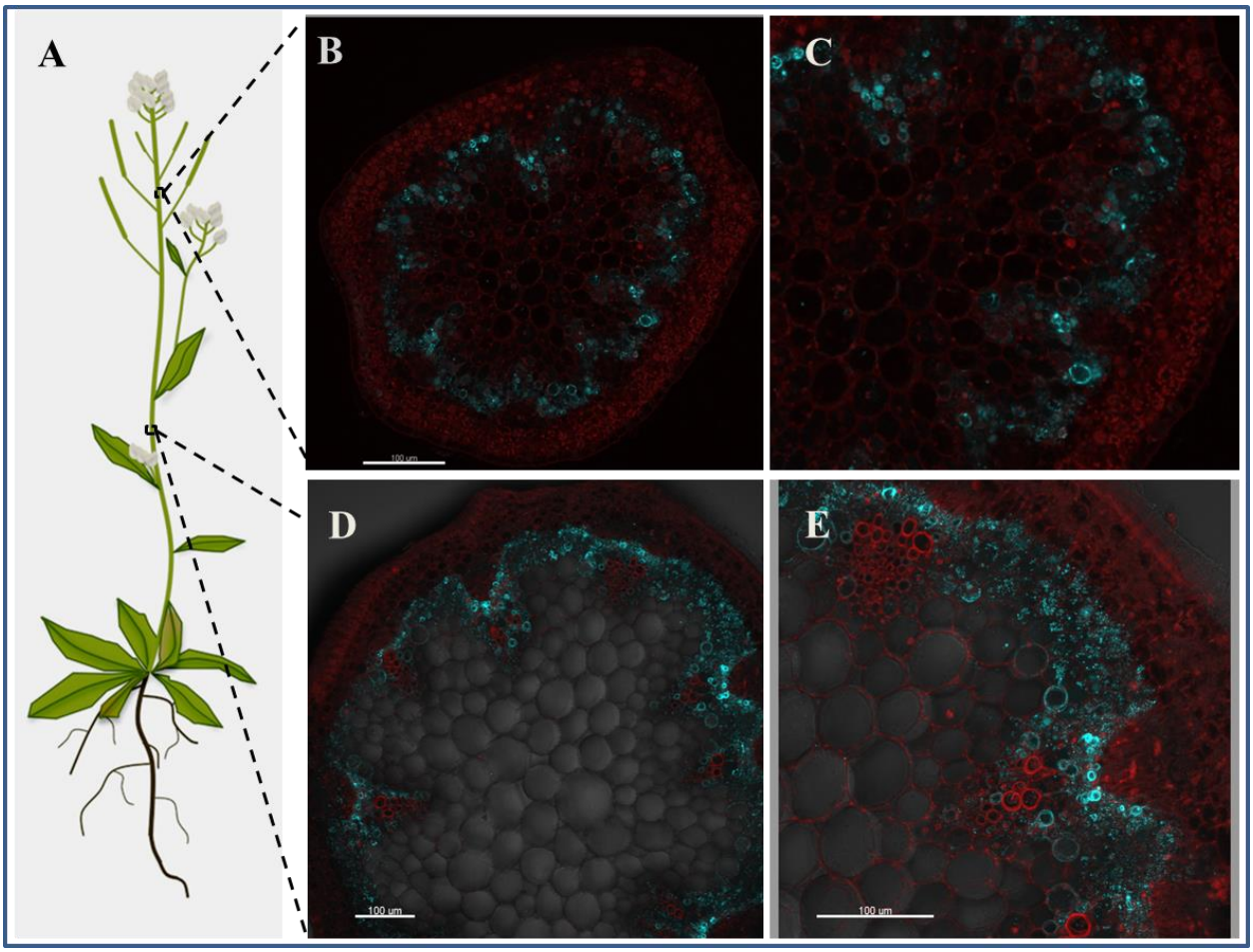

Figure 13. The *flWOX4* promoter activity continued as the circular domain of sub-cortical cells

A. The graphical representation of the stem section that was chosen for the confocal imaging. **B & D:** Transverse sections showing the circular expression domain of *WOX4* in the subcortical cells of the developing stem internode. **C & E:** The magnified views indicate the *WOX4* expression in different cell types.

The *WOX4* promoter activity further continued in a similar circular domain of subcortical cells even in the subtending nodes (Figure 13D). The transverse sections of these nodes showed that the stem has different anatomical features compared to the sections obtained from the top portion of the inflorescence. The characteristic features include large pith at the center with enlarged cells, a multi-layered cortex, the fully developed vascular bundles containing fascicular cambium with small dividing cells and the xylem & phloem tissues with different cell-types like xylem parenchyma, fibers, tracheary elements, phloem parenchyma, and companion cells. The stem also contains multiple layers of the interfascicular region positioned between two vascular bundles (Figure 13D & E). The previously published *WOX4* expression pattern (Suer *et al.*, 2011) at this

developmental stage, which was obtained using the short *WOX4* promoter, marked only the fascicular cambium and few cells in the interfascicular cambium. However, the transverse section obtained using the *flWOX4* promoter-reporter prominently illustrated that the *WOX4* promoter is consistently active in the circular domain including the cells of fascicular cambium that are in connection with the cell layers of interfascicular region, completes the circle.

4.5.5. The circular *WOX4* expression domain persists in the stem during secondary growth

The full-length *WOX4* promoter-reporter showed that the *WOX4* promoter is also active in the circular domain of sub-cortical cells of the matured stem, both in the internode and the base of the stem (Figure 14). The fascicular cambium gradually produces the phloem tissue towards the cortex and the xylem tissue towards the pith, which appears to stretch the area of fascicular cambium to form the intrusions in the circular expression domain (Figure 14A & B). The intrusions are more prominent in the sections of the internode than at the base of the stem.

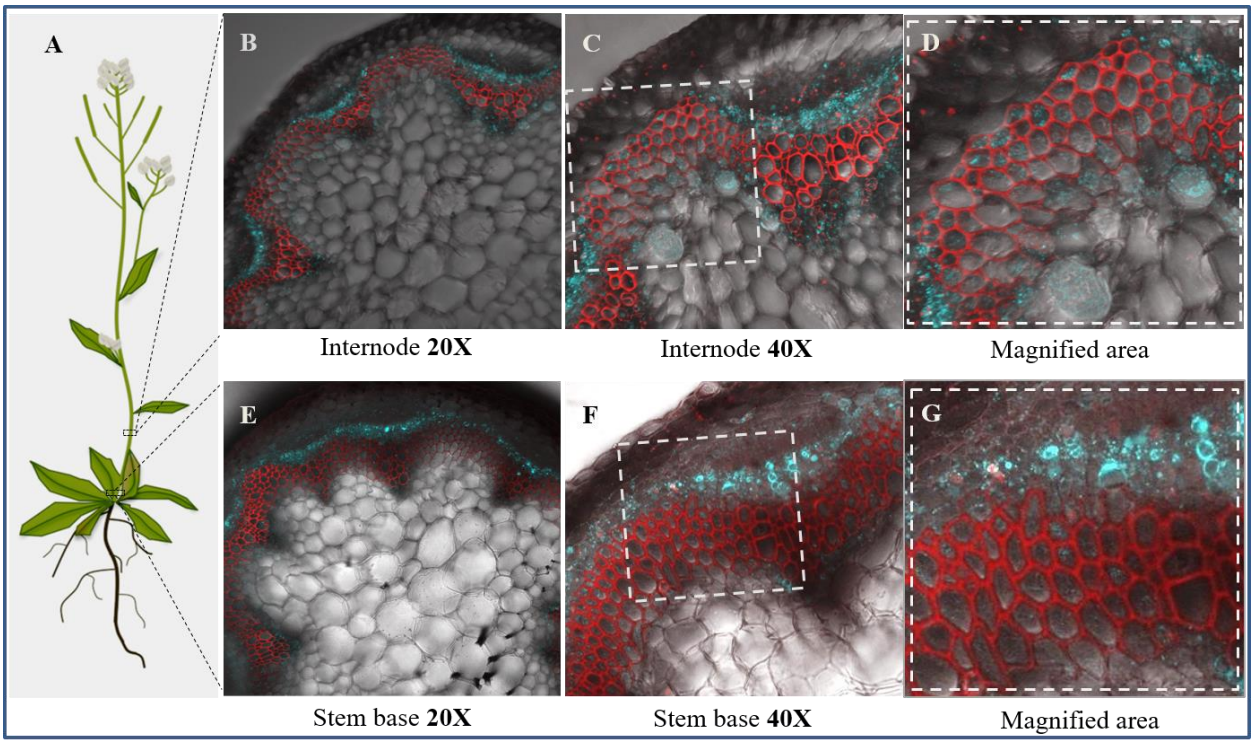

Figure 14: The *flWOX4* promoter activity during the radial growth of the stem

A. Graphical representation of the position of stem sections used for confocal imaging. **B, C & D:** Transverse section obtained from bottom-internode of the stem, **B.** 10X magnification of the stem showing the *WOX4* promoter activity,

C. 40X magnification of the same image clearly showing the signal in the fascicular, inter-fascicular regions**,** arrow pointing the reporter gene expression in the xylem tissue. **D:** The magnified view of the selected region in Figure 10C, indicating the signal in xylem parenchyma of the interfascicular region**,** arrows pointing the reporter gene expression towards in the cells of interfascicular region. **E, F & G.** Transverse section obtained at the base of the stem, **E.** 10X magnification showing the circular expression of *WOX* promoter-reporter, **F.** 40X magnification of the stem section shows a continuous activity in the fascicular and inter-fascicular cambium. **G.** The magnified view of the selected region from Figure 10F.

The signal intensity was higher in the fascicular cambium than the interfascicular region of the internode, which gives the impression that the circular expression domain looks discontinuous, however by visualizing the same sample at higher magnification clearly illustrate the *WOX4* expression in the interfascicular region (for example Figure 14B vs. 14C). The difference in these signal intensities might occur due to the different cellular structures; for example, the fascicular cambium is made up of two types of cells - smaller & angular shaped ray-initials and slender & cylindrical-shaped fusiform-cells. A close clustering of these cells possibly intensifies the signal in the fascicular cambium. Whereas, the interfascicular region is made up of tubular xylem fibers and larger xylem parenchyma cells (Figure 14D), in which some of the cells are lignified to transport water and other cells have big vacuoles, pushing the cytosol towards the cell walls, as a result, the weaker fluorescence signals were observed (Figure 14D).

At the base of the stem, fascicular and interfascicular regions are combined together to form a near-perfect-circular *WOX4* expression domain, that has no deeper intrusions (Figure 14E). Even at this stage, the *WOX4* promoter activity is not only restricted to the cambium, but it can also be seen in the xylem parenchyma (Figure 14E).

4.6. Expression analysis of the full-length *WOX4* promoter-reporter in the root

According to the expression patterns obtained by the short *WOX4* promoter-reporter, *WOX4* activity was confined only to the vascular cambium in the mature root (Y Hirakawa *et al.*, 2010) and there are no reports for the *WOX4* expression in the root apical meristem. However, when the pattern of the full-length *WOX4* promoter analysed using the construct *flWOX4::H3-GFP* we discovered the expression in the root meristem. As depicted in the Figure 15A, the *flWOX4::H3-GFP* construct the *WOX4* promoter is active in the quiescent center (QC). Subsequently, the expression appears to extend from the QC downwards into the columella-initials and upwards into

the vascular/pericycle-initials. However, the *GFP* signal is absent in other neighbouring cells of the QC, including the cortex-initials, endodermis-initials, and the root cap-initials.

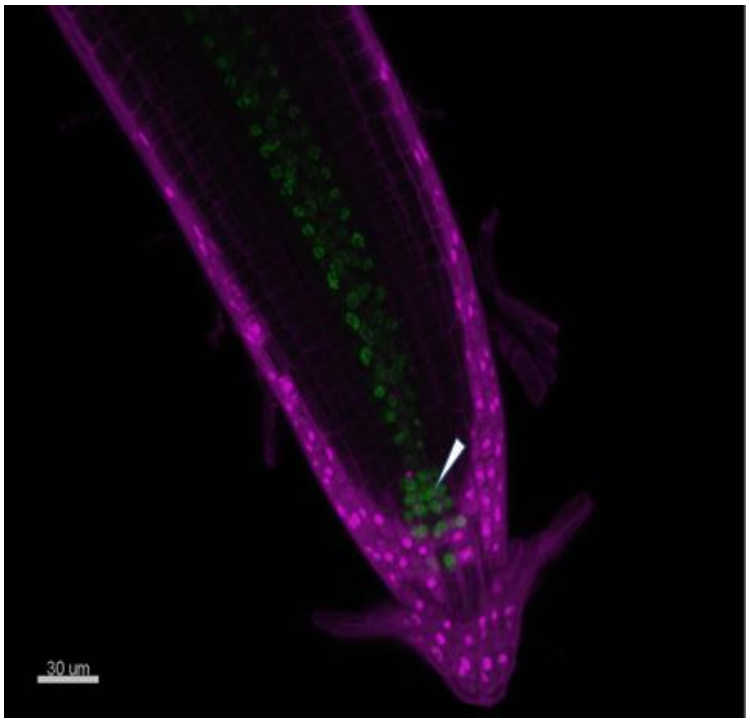

Figure 15: Activity of full-length *WOX4* promoter in the root tip.

CLSM image of the *flWOX4::H3-GFP* reporter indicates the full-length *WOX4* promoter activity in terms of GFP fluorescence in the root apical meristem marking the quiescent center (white arrowhead) and its neighbouring cells. The propidium iodide cell walls were false coloured (purple).

The *WOX4* promoter expression appears to extend further downwards from the columella-initials into the immediate descendant columella cells, however, the expression is absent in the cells of both root cap and epidermis. In the upper portion, the *WOX4* promoter activity appears to continue from the vascular/pericycle mother cells into their descendent cell files. Although this expression pattern appears to mark most of the cells in the central stele, it is not clear whether these cells belong to the pericycle or the other vascular tissues, at least with this resolution (Figure 15). Further, the *WOX4* promoter activity in the central stele extends from the meristematic zone to the cell elongation zone (upper portions of the root in Figure 15). A transverse section through the upper portion of the root would have provided the details of cell types in which the *WOX4* promoter is active, but due to the tender nature of this root zone, we were unable to get the transverse sections even after several attempts with hand sectioning and with the vibratome or cryotome.

The elongation zone contains thin and actively elongating cells, whereas the maturation zone contains large cells that are mostly occupied with the vacuole pushing the nucleus towards the cell wall. Such variation in the anatomy of cells makes it difficult to illustrate them by using the nuclear localized *WOX4* promoter construct. Hence, we used the endoplasmic reticulum targeted promoter construct *flWOX4::erCERULEAN* for further study. Interestingly, the patterns obtained by *flWOX4::erCERULEAN* also showed that the *WOX4* promoter is active in the central stele of the root (Figure 16). The expression patterns obtained by the *flWOX4::H3-GFP* (Figure 15) and the *flWOX4::erCERULEAN* (Figure 16) in two different zones but restricted to the central part of the root reveals that the *WOX4* promoter is continuously active from the elongation zone into the maturation zone.

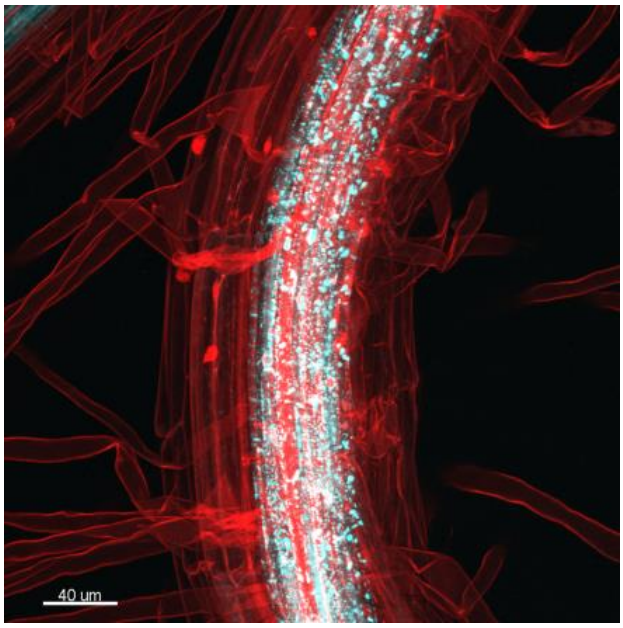

Figure 16: *WOX4* promoter activity in the root maturation zone.

CLSM image of the root depicts the *WOX4* promoter activity in the maturation zone of the root. Blue colour indicates the fluorescence of *flWOX4::erCERULEAN* construct and Propidium iodide stains the cell walls in red colour.

It is interesting to know the *WOX4* expression in different cell types of the mature root, but we encountered the same difficulties that are associated with the transverse sectioning of the root. As an alternative approach, we used the optical sectioning of a 3D image constructed from the Z-stack-images which were obtained from different focal planes of the matured root part that also contains the lateral root (Figure17A). The optical section shows the *WOX4* promoter activity in

the central parts of both main and lateral roots (Figure 17B). However, the optical sections also did not show the details of the vascular cell types in which the *WOX4* promoter is active, but at least it shows the strong cerulean signal marking all the cells in central stele of the lateral root (marked by the yellow arrow 17B), whereas the only few cells in the central stele of main root (marked by red arrow in Figure 17B). Such discrepancy in the signal indicates that the *WOX4* promoter is active not in the centrally located xylem tissue of the main root.

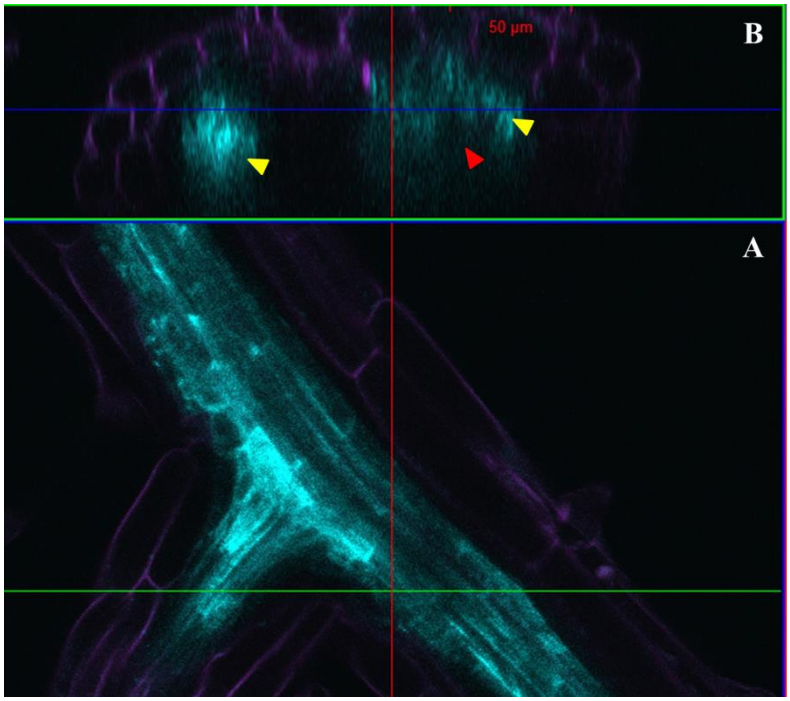

Figure 17: The *WOX4* promoter activity in the mature root along with the lateral root

A. CLSM image depicts the *WOX4* promoter activity in the central parts of both main and lateral roots in *flWOX4::erCERULEAN* lines. Pale-blue colour indicates the CERULEAN fluorescence signal and propidium iodide stained cell walls were false-coloured (purple). **B:** The optical section of the root at the junction of the lateral root emerging from the main root shows the expression of the *WOX4* promoter (yellow arrow). A weak CERULEAN signal in the center of the main root is indicated by red arrow.

4.7. Full-length *WOX4* promoter is active during the leaf development

Although, the short *WOX4* promoter constructs *pWOX4::GUS* (Y Hirakawa *et al.*, 2010) and *pWOX4::YFP* (Suer *et al.*, 2011) showed that the *WOX4* promoter activity was confined to the vascular cambium in the mature leaf, the activity was obscure in the young primordia. However, with the expression analysis of the *flWOX4::erCERULEAN* reporter, we could observe the early

activity of the *WOX4* promoter during the leaf development. To depict the young leaf primordia, the vegetative shoot apex was dissected by using the vibratome and the serial transverse sections were used for the confocal imaging. As shown in Figure 18A, the *WOX4* promoter is active in the group of cells (indicated with the star marks). The location of these cell clusters indicates the position of the leaf primordia and the signal is predominantly localized in the developing midrib, which is possibly marking procambial cells (Figure 18A). The subsequent developmental stages also show the *WOX4* expression located at the center of the leaf primordia, but the expression domain appears to expand in terms of the area which is also evident in the intensity of the enhanced signal (Figure 18A). Such an increase indicates that, as the primordium develops the *WOX4* expression expands from procambial cells into its descendants.

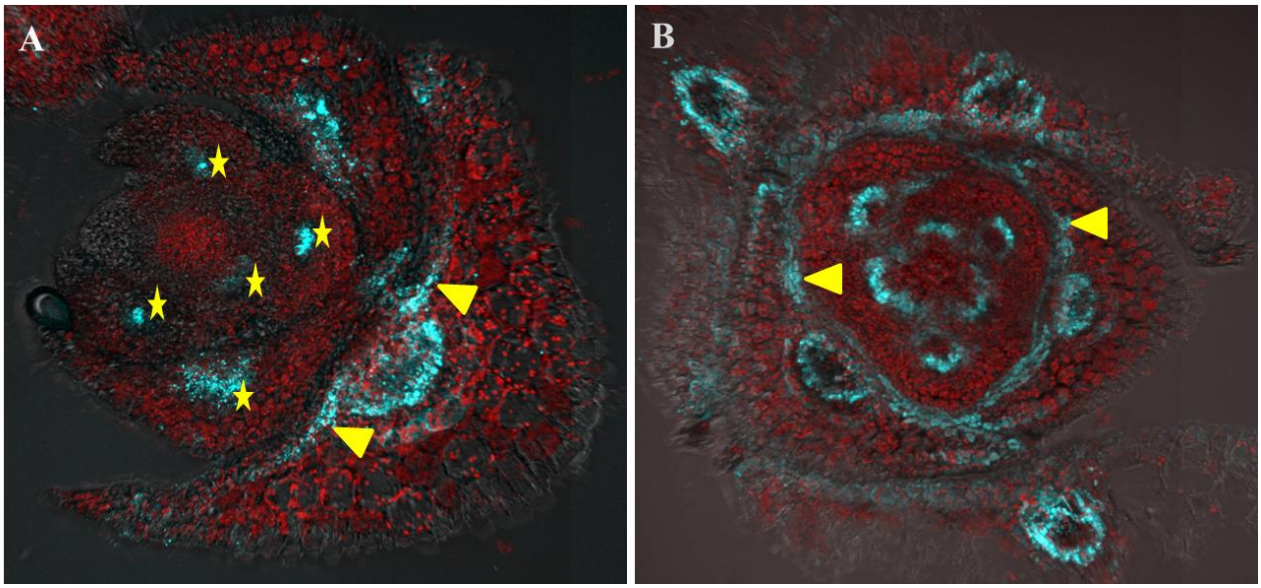

Figure 18: The *WOX4* promoter activity during the leaf development

A. CLSM image of the vegetative shoot apex depicting the *WOX4* promoter activity (blue colour) in the procambium of the young leaf primordia and the cambium of developing leaf. **B.** CLSM image of the transverse section obtained beneath vegetative shoot apical meristem illustrating the *WOX4* promoter activity in the cambial cells of developing leaves (at the center). The circular expression domain indicates the promoter activity in the petioles of the mature leaf. Arrow marks indicate the expansion of the *WOX4* promoter activity into the sub-epidermal layer of the mature leaf.

The transverse section obtained from the region beneath the vegetative shoot apical meristem further showed the *WOX4* promoter activity in the vegetative stem and developing leaves including the leaf blades and petioles (Figure 18B). As depicted in the image, in the vegetative stem the *WOX4* promoter showed enhanced activity in the fascicular cambium than the interfascicular

region, but they are well connected to each other forming an irregular circular expression domain which is similar to the young inflorescence stem. The matured leaves that are positioned away from the SAM also show the circular expression domain (Figure 14B). According to their position and angle of the transverse section, these circular expression domains could be identified as the vasculature of the leaf petioles. This expression pattern is similar to that of the flower pedicles seen on the inflorescence shoot.

Additionally, the matured leaf also exhibits the *WOX4* promoter activity and is appeared to be active in the sub-epidermal tissue of the leaf, however, this activity is confined only to the adaxial side (arrow marks in Figures 18A & B). This specific expression pattern was also observed in the transgenic leaves of the *flWOX4::H3-GFP* lines (Figure 19A & B). These expression patterns indicate that the *WOX4* promoter activity is not only restricted to the cambial cells of the leaves, but it also extends into the other tissues and marks the palisade parenchyma of the leaf adaxial portion.

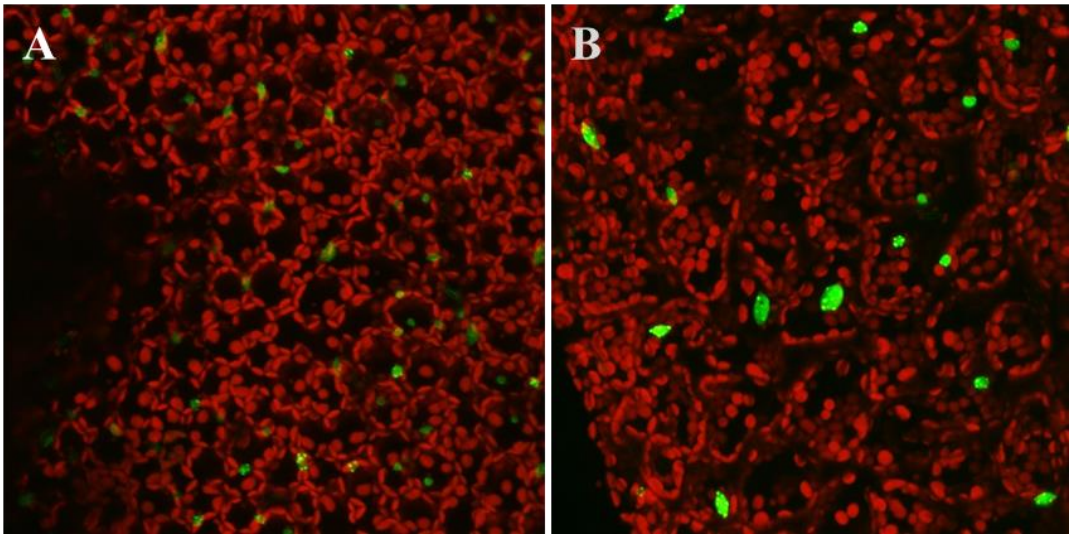

Figure 19: The *WOX4* promoter activity in the adaxial side of the matured leaf

A: CLSM image showing the full-length *WOX4* promoter activity in the sub-epidermal layer of the adaxial side of the leaf. **B:** CLSM image of the same leaf visualized with a 40X magnification lens. Green fluorescence represents *WOX4* promoter activity in the nuclei of the sub-epidermal layer; Red colour indicates the autofluorescence of chloroplasts.

The full-length *WOX4* promoter was active in all the parts of the vascular network of the matured leaf (Figure 20A). It includes the expression in a first-order vein or the mid-vein that connects the

leaf with the main stem, the second-order veins that emerge from the mid-vein and the third-order veins completing the vascular network (Figure 20A). It implies the expression of *WOX4* promoter throughout the development of vascular system of the leaf. The cerulean signal intensity in the mid-vein is higher than in the secondary veins, whereas the secondary veins show higher signal intensity than the tertiary veins. These signal-intensity differences seem to occur due to the size and anatomy of the different orders of veins. The tertiary veins are made up of only procambial cell files that occupy the smaller area, whereas the secondary veins contain the well-defined cambium and its descendant's which occupy a larger area than the tertiary veins, hence the secondary veins possess higher signal than the tertiary veins. The primary veins show highest cerulean signal intensity among all the veins, because the *WOX4* expression in the primary vein is not only restricted to the cambial cells (CC) but it also extends into the adjacent the xylem parenchyma. This expression pattern is clearly visible in the transverse section of the matured leaf through the mid-vein (Figure 20B).

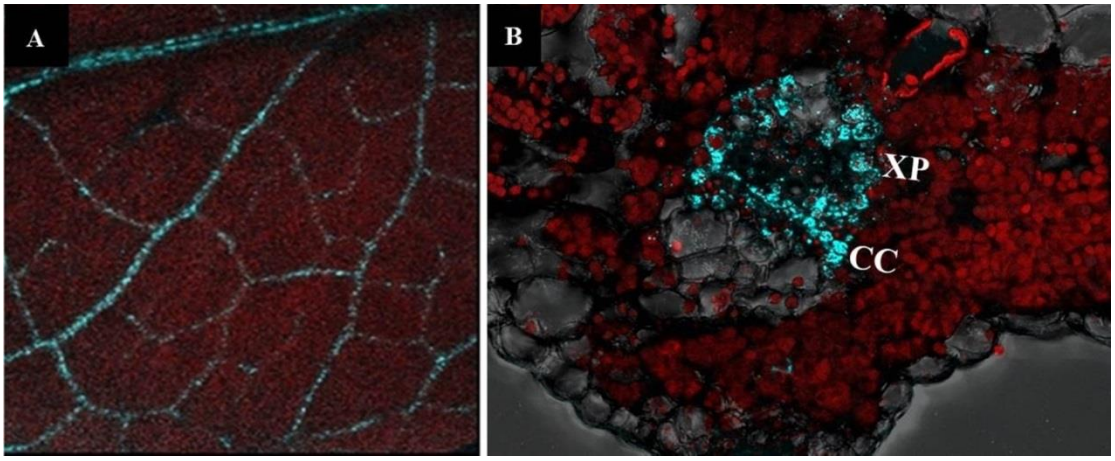

Figure 20: *WOX4* promoter activity in the matured leaf

A: CLSM image of the matured leaf depicting the *WOX4* promoter activity (blue colour) in the vasculature of the mid-vein, the second and third-order veins. **B:** CLSM image of the transverse section obtained through the mid-vein illustrating the *WOX4* promoter activity in the cambial cells (CC) and the xylem parenchyma (XP).

5. DISCUSSION

5.1. The conserved distal elements are essential for *WOX4* promoter activity

Phylogenetic shadowing of the *AtWOX4* upstream (9.2 Kb) and downstream (1.7 Kb) regions with the gene flanking areas of seven orthologous regions from *Brassicaceae* species revealed three conserved blocks in the upstream and one conserved block in the downstream region of the *WOX4* coding sequence (Figure 5). The importance of upstream and downstream conserved sequences in regulating gene expression patterns has already been reported in several studies. For instance, phylogenetic shadowing of the *DRNL* promoter revealed four conserved upstream regions which influence the *DRNL* expression pattern in lateral organ founder cells at the periphery of the inflorescence meristem (Comelli *et al.*, 2016). Similarly, an evolutionarily conserved 57 bp segment that was located -550 bp upstream of the *WUSCHEL* coding region was found to be needed for its spatio-temporal expression pattern in the shoot apical meristem (Bäurle and Laux, 2005). Furthermore, a small conserved sequence located in the 3′ flanking region of the *CLV3* was found to be essential for the control of both, the activation and the repression of *CLV3* transcription by regulating its binding affinity to the *WUSCHEL* homeodomain (Perales *et al.*, 2016). In another example, phylogenetic shadowing of *GIGANTIA* revealed three conserved regions in their promoter regions, in which a *cis*-regulatory module CRM2 controls transcriptional regulation of *GIGANTIA.* Further analysis showed that one of the sub-fragments (CRM2-A) controls light inducibility whereas another fragment namely CRM2-B exhibits a diurnal response that was sufficient enough to recapitulate the complete function of its full-length promoter (Berns *et al.*, 2014).

Upstream and/or downstream conserved elements regulate gene expression patterns by serving as binding sites for transcription factors. The open chromatin configurations indicate that a specific genomic region is bound by transcription factors. Several techniques were developed o identify open chromatin configurations, which are based on the accessibility of the genome for endonucleases such as DNase I and MNase (Tsompana and Buck, 2014). ATAC-seq (assay for transposase-accessible chromatin with high-throughput sequencing) is another powerful technique that uses Tn5 transposase activity for cleaving of open genomic DNA sequences (Tsompana and Buck, 2014). For this purpose, we could make use of recently published ATAC-seq data (Frerichs

et al., 2019) to locate positions of open chromatin peaks within the *AtWOX4* promoter. We then compared ATAC-seq data to positions of conserved regions which were identified previously by phylogenetic shadowing. We identified a significant overlap between positions of open chromatin peaks and the conserved regions of the *WOX4* promoter (Figure 6). This suggests that the identified conserved regions of the *WOX4* promoter contain open chromatin configurations, which might be essential for the regulation of *WOX4* transcription, while probably functioning as binding sites for transcription factors.

Until now, the expression pattern of the *AtWOX4* promoter was carried out by using the 2.9 Kb upstream region and the 0.6 Kb downstream region (Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011). This promoter construct included the proximal conserved region (Block 1), but excluded the distal conserved elements (Block 2 and 3) that were identified in our phylogenetic analysis (Figure 5B). However, the ATAC-seq analysis showed that Block 2 and 3 are also in open chromatin state (Figure 2). Taken together, this suggests that these conserved distal regions are involved in directing the *WOX4* expression pattern. This was analysed by confocal microscopy in transgenic *Arabidopsis* plants carrying *WOX4* promoter-reporter constructs that include the 9.2 Kb upstream and the 1.7 Kb downstream sequences. As a general note, we used two different reporters, *GFP* and *CERULEAN*, to overcome the issue of signal overlap with autofluorescence from secondary cell wall depositions such as lignin. Moreover, nuclear-targeted reporters were used to visualize the signal in meristematic cells while endoplasmic reticulum-targeted reporters were used to visualize the signal in differentiated tissues. However, our confocal analyses recapitulated the cambium specific activity of the *WOX4* promoter, as reported also in the previous studies (Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011). Interestingly, the inclusion of all conserved regions marked novel spatio-temporal expression domains of the *WOX4* in the SAM, RAM, stem and leaf (Figure 10 – 20). Identified new expression patterns will be discussed in detail in the following up sections.

The conserved block1 of the *WOX4* promoter was unique in its structure, specifically the region of 550 bp located immediately upstream of the *WOX4* coding region was found to contain multiple conserved ATGs and a small 63 bp ORF, from -237 bp to -174 bp (Figure 7A). Upstream ORFs (uORFs) are known to reduce the translation efficiency of genes by ribosomal stalling (Jorgensen and Dorantes-Acosta, 2012). For example, translation efficiency of Arabidopsis *SUPRESSOR OF*

ACAULIS 51 (*SAC51*) that plays a role in stem internode elongation is regulated by its small uORF. A mutation in the uORF of *SAC51* in *sac51* mutant lines led to the premature stop codon, which resulted in the increased translational efficiency of *SAC51*. The increased *SAC51* expression rescues the *acaulis 5* (*acl5)* dwarf phenotype in *sac51 acl 5* double mutants (Imai *et al.*, 2006). Similarly, *WOX4* also contains an uORF, which is part of the longest *WOX4* transcripts that start at -302 bp (Figure 8B & C). In order to validate, whether this putative uORF functions as a potential uORF, we replaced the complete -237 bp upstream fragment that includes the uORF with the *TMV*-Ω transitional leader sequence (Töpfer *et al.*, 1993) and fused it to a reporter gene (Figure 9G). In case uORF regulates *WOX4* expression, transgenic plants containing the above named construct were expected to show a robust activity of the *WOX4* promoter-reporter. But surprisingly, the analysis of transgenic plants showed no detectable reporter signal. This indicates that the proximal -237 bp region contains sequences that are essential for *WOX4* transcription. Therefore, we hypothesized that future experiments with an exclusive deletion of uORF would unravel the role of *WOX4* uORF in its regulation.

We replaced the *WOX4* downstream region (1.7 Kb) with downstream region of the *CaMV35S* gene (Figure 9H) to investigate whether the *WOX4* downstream region contains any other important regulatory elements. However, confocal imaging of transgenic plants expressing the *pWOX4::erCERULEAN-PAS* construct revealed no significant change in the expression pattern of *WOX4* compared to that of the images obtained by the full-length promoter, suggesting that the conserved downstream regions might not regulate the *WOX4* transcription.

Plant developmental processes can be mediated by an inter-cellular movement of transcription factors such as *SHORTROOT (SHR)* (Nakajima *et al.*, 2001), *SHOOTMERISTEMLESS (STM)* (Kim *et al.*, 2003) and *WUSCHEL (WUS)* (Yadav *et al.*, 2011). The homeodomain transcription factor WUS that regulates the maintenance of the stem cell population in SAM, is produced in the organizing center and moves to the central zone (CZ) to activate the *CLV3* expression (Yadav *et al.*, 2011). Thus, the dynamic expression pattern of WUS transcription and its movement is necessary for the maintenance of the stem cell population at the SAM. It is also known that *WOX4* expression promotes the development of the procambium (Ji *et al.*, 2010) and maintains stem cell identity of fascicular and interfascicular cambia (Suer *et al.*, 2011). However, the cell-to-cell movement of WOX4 is not reported yet. Therefore, we examined the possibility of intercellular

movement of the WOX4 protein by transforming *A. thaliana* with a *WOX4* translational fusion construct (Figure 9I). However, confocal images obtained of the transcriptional as well as the translational fusion constructs showed no difference between the two expression patterns, suggesting that WOX4 might not be moving out of the cells.

5.2. *WOX4* promoter activity starts in the shoot apex and continues as a circular domain

Until now, *WOX4* promoter activity has not been reported to be active in SAM of *Arabidopsis,* while its activity remained confined to cambial cells. Most of these studies were done by using a short *WOX4* promoter (2.9 Kb) construct (Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011). However, in rice, the *AtWOX4* orthologous gene *OsWOX4* was found to be expressed in cells of the SAM (Ohmori *et al.*, 2013). It was further shown that the functional relevance of *OsWOX4* was associated with the differentiation of both vascular and parenchyma cells, during rice leaf development (Yasui *et al.*, 2018). A recent study also investigated the expression pattern of *WOX4* in the shoot apex comprising early leaf primordia of Populus (dicot tree species) using the orthologous *PttWOX4* promoter (Kucukoglu *et al.*, 2017). One important finding of current study is the expression pattern of the *WOX4* promoter that appeared to start at the shoot apex. This striking feature was obtained by using the full-length *WOX4* promoter that includes all the evolutionarily conserved elements (Figure 10). The discrepancy of the expression pattern of our full-length *WOX4* and the previously described short*WOX4* promoter in *Arabidopsis* indicates the importance of the distal promoter elements for a proper regulation of *WOX4* expression in the shoot apex.

To obtain a complete picture of *WOX4* expression pattern within the shoot apex, Z-stacks of images were generated from the tip of the IM to its subtending regions and combined to create 3D images of the inflorescences. The analysis of the inflorescence shoot apices of transgenic plants carrying the nuclear-targeted promoter-reporter construct *flWOX4::H3-GFP* showed fluorescence in a group of cells that are in close proximity to the central zone of inflorescence meristem. The arrangement of these cell clusters suggests that the *WOX4* expression domain marks founder cells of the emerging flower primordia (Figure 10). In general, stem cell identity is maintained in the central zone of the SAM while lateral organs are formed from the peripheral zone. Groups of lateral organ founder cells shortly loose stem cell identity and then gain competence to differentiate

into primordia. Different zones within the IM can generally be visualized by promoter activities of *MP, PIN1, DRNL, DOF5* (Wenzel et al., 2007; Chandler et al., 2011; Kang et al., 2003) or by using a synthetic auxin-responsive *DR5* promoter (Chen et al., 2013). The phyllotactic pattern of lateral organs and the initiation of the vascular system within these lateral organs are closely associated (Kang *et al.*, 2003; Dengler, 2006). Nevertheless, it is not very clear whether vascular development defines the phyllotaxy pattern or if the development of lateral primordia provides a positional clue for vascular initiation. The first concept is supported by the fact that developing organs need to be connected with the main stem. The second one is supported by the action of auxin accumulation (Dengler, 2006). However, full-length *WOX4* promoter activity was found in a group of cells that are located in close proximity to the central zone of the IM, suggesting that *WOX4* might be expressed in lateral organ founder cells and possibly prepatterns the vascular structures of developing flower primordia (Figure 10A & B). The longitudinal view of the inflorescence at the apex region shows fluorescence of *WOX4*-expressing cell clusters, which are found preferentially in the subtending cells of the L1 layer (Figure 10B). This sub-epidermal expression domain denotes the ground meristem, which is later responsible for the development of internal structures of the lateral organs including the vasculature. Our observation supports that vascular prepatterning is closely associated with phyllotaxy.

Although the 3D image analyses of the inflorescence apices provided interesting aspects about the *WOX4* promoter activity, it was impossible to track fluorescence signals much deeper into the stem. Therefore, a series of transverse sections of the inflorescence stem were obtained by using the vibratome and used for the confocal microscopy. CERULEAN fluorescence signal could be tracked in transverse sections obtained from approximately 80-100 µm below the organizing center of the IM of *flWOX4::erCERULEAN* transgenic plants. Analysis of the confocal images revealed that CERULEAN signal marked the *WOX4* activity in a circular expression domain of one or two sub-cortical cell layers (Figure 11B & C). Identified cells showed a spherical shape with thin cell walls, which is a characteristic feature of meristematic cells that are described to have different cell faiths during development including vascular tissue (Scanlon and Freeling, 1997). The circular expression domain suggests that the *WOX4* promoter activity initially marks the sub-cortical cell layers, that might be associated with the vascular system in the stem. Our finding suggests that the *WOX4* expression pattern in *Arabidopsis* resembles the *OsWOX4* promoter activity in rice which

marks both the meristematic cells of SAM and the provascular cells in the primordia (Ohmori *et al.*, 2013).

The analysis of transverse sections obtained from the subtending node of the inflorescence stem, which has already acquired the circular expression domain showed that the cells in this domain are in direct connection with the cells of the flower pedicles (Figure 11B & D). These connections create the protrusions in the circular expression domain; however, the anatomy showed no sign of differentiated tissues like xylem or phloem, suggesting that the meristematic cells marked by the full-length *WOX4* promoter connects the emerging lateral organs such as flower pedicles with the main stem. It also resembled the expression pattern of another vascular marker *ATHB8* that was extensively studied with respect to the vascular connection and the formation of new vascular bundles of lateral organs such as leaves from the existing vasculature of the vegetative stem (Dengler, 2006).

5.3. The circular *WOX4* expression domain prepatterns the vasculature

To examine the *WOX4* expression pattern throughout the development of the stem, we analysed the transversal sections obtained from the different positions of the inflorescence stem, including the nodes and internodes at different positions. By combining this data with the details of expression patterns obtained at the shoot apex, we found that the activity of the full-length *WOX4* promoter continuously exists in the cells of the subcortical layer while maintaining a nearly perfect circular shape (Figure 12-14). This continuous existence of the circular *WOX4*expression domain throughout the stem represents a novel aspect identified in this study with the help of the full-length *WOX4* promoter. Previous reports described the *WOX4* activity in the fascicular cambium and only in some cells of interfascicular cambia of young stem while a circular expression domain was reported only in the mature stem (Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011). Our findings suggest that the existence of circular expression domain might prepatterns the vascular system throughout the stem development.

Due to the compressed nature of the vegetative shoot, the *Arabidopsis* inflorescence stem has rather been chosen to study developmental processes that are associated with vascular patterning (Turner and Sieburth, 2003). Due to its anatomical similarities with the wood formation in trees

the inflorescence stem was also used to study processes involved in the radial outgrowth or secondary growth of the stem (Barra-Jiménez and Ragni, 2017).

Conceptually, the growth of the *Arabidopsis* stem has been divided into three developmental phases - the primary, the intermediate and the secondary growths using the expression patterns obtained by the short *WOX4* promoter (Suer *et al.*, 2011). While the molecular studies focussing on auxin accumulation showed that vascular pattering starts early in the periphery of IM (Biedroń and Banasiak, 2018), anatomical features of the vascular development are also evident in the stem in close proximity to the SAM (Dengler, 2006). However, the changes that occur during the primary growth phase were surprisingly attributed to the *WOX4* expression pattern within the stem region far below the IM (Suer *et al.*, 2011), but not from the IM. A reason could be a weak or lacking expression of the short *WOX4* promoter in the shoot apex. These descriptions mainly include the expression in the half-moon shaped fascicular cambium of the primary stem that separates xylem and phloem; though it was not exactly clear where and when these cells differentiate to form cambium, xylem or phloem tissues. Nevertheless, in our study, the transversal sections obtained from similar locations of the stem showed full-length *WOX4* promoter activity in the subcortical layer of the circular expression domain (Figure 12C). Interestingly, the circular *WOX4* expression domain showed the intrusions towards the center of the stem. These intrusions were formed because of the differentiation of the phloem tissue that pushes the *WOX4* expression domain towards the center. Based on the differentiation of xylem and phloem tissues at this position of inflorescence stem, it could be assumed that *WOX4* expression domain in the intrusions indicates the the position of fascicular cambium (Figure 12C). By comparing changes in the shape of the circular expression domain at shoot apex (Figure 11) and the wavy circle in the subtending stem sections (Figure 12), it is possible that a few cells within the circular expression domain are specified to be the fascicular cambia. Taken together, our results showed that the meristematic cells in the circular *WOX4* expression domain prepatterns the development of the fascicular cambium within the vascular bundles of the inflorescence stem during primary growth.

In addition to marking the specification of the fascicular cambium, the same transverse sections showed the activity of full-length *WOX4* promoter in a V-shaped layer adjacent to the fascicular cambium and positioned towards the pith (Figure 12C). Based on the morphological features and the position, cells in the V-shaped layer belong to xylem parenchyma. The association of *WOX4*

expression with xylem parenchyma in the matured stem was evident from previous reports using the short *WOX4* promoter (Bennett *et al.*, 2016; Gursanscky *et al.*, 2016). In our present study, with the full-length *WOX4* promoter, xylem parenchyma associated expression could be visualized in the primary stem itself. Interestingly, the V-shaped structure formed by *WOX4* expressing xylem parenchyma cells indicates that these cells are closely associated with the circular expression domain. Nevertheless, this expression pattern is not sufficient to tell whether the xylem parenchymal identity is provided by the fascicular cambium or these cells are direct descendants of the circular expression domain of sub-cortical layers. The first aspect could be more plausible, but the latter aspect also cannot be completely ruled out, as the xylem parenchyma cells keep the competency to express *WOX4* promoter throughout the stem.

The intermediate growth phase of the stem has been described in the previous studies by using the transverse sections of the internode regions positioned between the shoot apex and the base of the stem. The expression of the short *WOX4* promoter in these sections has been shown to be concentrated in the fascicular cambium but also in a few cells that share the boundary between fascicular and the interfascicular region (Suer *et al.*, 2011). However, *WOX4* expression patterns of the full-length promoter showed a continuous existence of *WOX4* expressing cells in the circular domain (Figure 13 & 14). Such expression pattern could support the radial outgrowth, but in a different perspective; instead of the reactivating *WOX4* expression in a few cells of the interfascicular region, the whole interfascicular region might provide the basis for radial growth in the intermediate growth phase of the stem.

To describe the secondary growth phase, the base of the inflorescence stem has been used extensively where the short *WOX4* promoter activity was shown to be restricted to the cambial ring that differentiates into secondary xylem towards the pith and the secondary phloem towards the periphery (Suer *et al.*, 2011; Gursanscky *et al.*, 2016). The full-length *WOX4* promoter also marked a similar expression domain specifying the vascular cambium ring (Figure 14B & E).

Overall, the detailed analysis of the full-length *WOX4* promoter activity in different parts of the stem gives a complete picture of the *WOX4* expression pattern over the entire period of stem development. This analysis provides a different perspective of radial outgrowth. *WOX4* expression starts at the IM periphery marking founder cells of the lateral organs and then becomes confined

to the circular domain of the subcortical cells. Then, this circular expression domain is continuously maintained throughout the inflorescence stem with some intrusions providing the basis for the prepatterning of the fascicular cambium without losing its competency in the interfascicular region and finally forms a ring of vascular cambium. The existence of such a continuous circular expression domain aligns with the radial growth of the stem. Moreover, the very young stem that is localised in close proximity to the IM also possesses some radius, which might be provided by the circular expression domain It is also possible that the radial expansion of the stem from the shoot tip to the base is a more gradual process, rather than the intermittently activated growth.

5.4. *WOX4* activity starts in the RAM and continues into the vascular system

The importance of the transcription factors *WUS*, *WOX5* and *WOX4* in the maintenance of their specific stem cell zones in the Arabidopsis is well known. The *WUS* plays an important role in the stem cell maintenance of the SAM (Schoof *et al.*, 2000). Similarly, It has been shown in earlier studies that the expression of *WOX5* was exclusively confined to the QC where it functions in the stem cell maintenance of the root (Haecker *et al.*, 2004; Sarkar *et al.*, 2007). The role of *WOX4* in maintaining cambial cell identity has also been reported (Y Hirakawa *et al.*, 2010), but no studies have shown *WOX4* expression in RAM so far. However, we could show that *WOX4* is active in the QC of RAM with full-length *WOX4* promoter (Figure 15). In addition to the QC, full-length *WOX4* promoter activity also marked cells adjacent to the QC, including the columella mother cells and the descendant columella cells towards the root cap. Further, the *WOX4* promoter activity marks only mother cells that give rise to the stele, but not the mother cells of endodermis, cortex, and epidermis (Figure 15). Although *WOX4* promoter activity was found in the stele, marking the vascular initials, it is not clear whether it marks the pericycle layer at this resolution. Subsequently, the *WOX4* promoter activity continued throughout the root vasculature (Figure 16 & 17). Although, a decent signal intensity was found in the vasculature, details of *WOX4* expressing vascular cell types could not be distinguished. Analysis of root transverse sections might provide more insights into cell types. However, due to the tender nature of the roots, we could not succeed in obtaining good cross-sections. To overcome this, optical dissection in the form of 3D images were obtained at the junction between the main and the lateral roots. We observed that the *WOX4* promoter was active in all cell types of the stele in lateral roots while the fluorescence signal in the

main root was not detected at the very center of the stele (Figure 17). This could be explained by the fact that in mature roots, the centrally located xylem vessels are dead in nature. Taking together, we could show the full-length *WOX4* promoter-reporter activity that starts in the QC and its adjacent mother cells, marking the vascular initials and further continue into the vascular system of root. However, we could not study the complete details of *WOX4* expression in different vascular cell types, due to the difficulties in obtaining transverse sections of root. Possibly, this problem could be resolved further by using advanced techniques such as growing the roots on slides with micro chambers.

5.5. The *WOX4* promoter marks novel expression domains in the leaf

Leaf development is a complex process that involves many molecular regulators. Leaf vascular initiation and pattern formation were extensively studied using the *ATHB8* promoter (Kang *et al.*, 2003). The leaf primordium is specified at the periphery of the SAM, in which the specification of epidermis is controlled by the L1 layer and L2, L3 layers which contribute to the development of mesophyll and vascular tissues (Kalve *et al.*, 2014). The initiation of first-order veins which are specified within the ground meristem and prepatterns of the cambium can be visualized by the *ATHB8* promoter (Scarpella, Marcos, Jirí Friml, *et al.*, 2006).

The leaf specific expression pattern of *WOX4* has already been reported using the short *WOX4* promoter (Suer et al., 2011); but its activity was not observed in the leaf primordia. However, similar to the early expression pattern observed in flower primordia of the inflorescence shoot using the full-length *WOX4* promoter, we also observed that the *WOX4* promoter is active in leaf primordia, close to vegetative SAM (Figure 18), which is comparable to *ATHB8* expression pattern (Dengler, 2006; Scarpella, Marcos, Jiří Friml, *et al.*, 2006). A transverse section below the SAM showed the *WOX4* expression pattern in the first and second-order veins depending on the developmental stage of the leaf. The analysis on whole leaf samples further showed a *WOX4* activity in the tertiary and quaternary veins (Figure 20A). However, the signal intensity of the reporter gradually reduced from the first-order veins to the fourth-order veins. This expression strength was apparently depending on the size of the veins, which can also be seen by observing the adaxial side of leaf. The midvein that develops earlier than the other veins apparently grow bigger in size and contains more cells. The transverse section of leaf at the position of the midvein

showed these anatomical differences in terms of a higher signal intensity (Figure 20B). The anatomy of the midvein showed the *WOX4* promoter activity in the cambial and the xylem parenchyma cells that are positioned on the adaxial side of the leaf (Figure 20B). In previous studies *WOX4* activity was confined to the undifferentiated cambial cells of the leaf, but not in the differentiated tissues such as the xylem parenchyma (Y Hirakawa *et al.*, 2010; Suer *et al.*, 2011). However, *SlWOX4*, a *WOX4* ortholog in tomato showed an enormous increase in the number of both xylem and phloem tissues when expressed under the control of the *35S* promoter (Ji *et al.*, 2010), suggesting a role of *WOX4* in vascular tissue differentiation.

Interestingly, the *WOX4* promoter activity was also found in the mesophyll of mature leaves in transgenic plants carrying the *flWOX4::H3-GFP* (Figure 19). Promoter activity was confined to the adaxial part of the leaf, representing the nuclear-localized signal in the palisade parenchyma, but not to the abaxial side of the leaf with spongy parenchyma. Thus, the parenchyma-specific expression pattern of the *WOX4* promoter partially overlaps with the *WOX3* expressing middle domain of the leaf (Matsumoto and Okada, 2001).WOX3 is an important transcription factor that controls marginal and plate meristem identity in Arabidopsis leaves (Nakata and Okada, 2012). The *WOX3* ortholog in maize regulates both, leaf blade growth and the number of vascular bundles (Nardmann *et al.*, 2004). However, it would be further interesting to understand how interactions between *WOX3* and *WOX4* expressing domains control the fate of vascular and mesophyll tissue identity.

Taken together, full-length *WOX4* promoter-reporter analyses showed that *WOX4* promoter activity starts early in young leaf primordia and marks the provascular cells within the midrib. Then expression extends from first order veins to the next order veins marking the complete vascular network of the leaf. Additionally, *WOX4* expression domain marked xylem parenchyma in vascular bundles and the palisade parenchyma of the mesophyll tissue, suggesting that *WOX4* promoter activity is not only restricted to the cambial cells, it is also active in non-vascular cells.

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IV. ERKLÄRUNG

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