**Title: PRE3 and WOX11 transcription factors are involved in the formation of new lateral roots from secondary growth taproot in *A. thaliana***

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**Abstract**

* The spatial deployment of lateral roots determines the ability of a plant to interact with the surrounding environment for nutrition and anchorage. This paper shows that besides the pericycle the vascular cambium, becoming active in *Arabidopsis thaliana* taproot at a later stage of development, is also able to form new lateral roots.
* Demonstration of this event required the implementation of a two-step approach in which the first leads to development of a secondary structure in *A. thaliana* taproot, and the second applies a mechanical stress on the vascular cambium to initiate the formation of a new lateral root primordium.
* GUS staining shows PRE3, DR5 and WOX11 signals in the cambial zone of the root during new lateral root formation. An advanced level of wood construction characterized by the presence of medullar ray was achieved.
* Preliminary investigations suggest the involvement of auxin and two transcription factors (PRE3/ATBS1/bHLH135/TMO7 and WOX11) in the transition of some vascular cambium initials from a role as producers of xylem/phloem mother cells to founder cells of a new lateral root primordium.

**Introduction**

The formation of an optimal root system in any plant is fundamental to ensure its growth, development, and productivity (Khan et al., 2016). Besides nutrient uptake and water acquisition from the soil (Montagnoli et al. 2014), it also plays a vital role in the mechanical anchorage of the plant (Scippa et al. 2006). Root system architecture (RSA), i.e. the overall shape and structure of the root system, as well as the number of lateral roots and their 3-D distribution along the root axis, is determined by various environmental factors and has a direct influence on plant yield. In particular, the production of a lateral root (LR) in a specific direction represents a response to both abiotic and biotic stimuli from the environment (Szymanowska-Pułka, 2013).

*Arabidopsis thaliana* (L.) is the most studied model plant species today (Koorneef and Meinke, 2010). Among its several different uses, it has also been adopted for studying the origin of post-embryonic lateral roots from founder cells in the pericycle (Beeckman and De Smet, 2014; Möller et al., 2017). In particular, it has been demonstrated that these roots may originate from competent cells (founder cells; FCs) situated in front of the xylem poles (Beeckman and De Smet, 2014; Möller et al., 2017). Moreover, *A. thaliana* is known to form a secondary structure in proximity to the hypocotyl enabling the investigation of lateral root formation from a secondary structure (Dolan et al. 1993). *A. thaliana* lacks the construction of an extensive secondary structure with annual rings and medullar rays. Nevertheless, it has been known for a long time (Lev-Yadun, 1994) that at the end of its life cycle a thickening appears at the base of the floral stalk and in the hypocotyl (Ragni et al. 2014;) due to the onset of vascular cambium activity. Sibout et al. (2008) suggested that this might represent a response to mechanical stress induced by the inflorescence. Other reports stated that the thickening of the *A. thaliana* taproot remains incomplete in the tissue differentiation describing only the development of secondary xylem and phloem (Dolan et al., 1993; Zhang et al., 2011; Nieminen et al., 2015). Indeed, independently from the organ considered, no report published so far indicates that the thickeningof *A. thaliana* taproot shows annual rings and medullar rays like those observed in woody perennial plants. The above considerations explain the still ongoing debate as to whether *A. thaliana* is a suitable model plant to investigate the molecular control of wood formation.

The priming of competent pericycle cells to become FCs involves a network of coordinated events under the control of several factors. Plant hormones (auxin, cytokinins, ABA, GA, and ethylene) have been shown to play a key role in signal transduction during this process (De Smet et al., 2003; De Smet et al., 2007; Ivanchenko et al., 2008; Peret et al., 2013). Other regulatory mechanisms are based upon the crosstalk between a) migratory molecules (miRNAs, transcription factors, and small peptides) able to move among tissues through plasmodesmata (for short distances) or through apoplasts (for long distances) (Marin et al., 2010; Meng et al., 2012; Rossi et al., 2015; Li and Zhang, 2016); b) membrane receptors (with kinase activity) regulating the movement of migratory molecules (Yue and Beeckman T., 2014); and c) transcription factors regulating specific target genes (Fernandez et al., 2015; Ramirez-Parra et al., 2016). It is surprising that the same type of modules (despite the differences in nature of the molecules involved in the various roles) seems to regulate homeostasis of the initial cell niche in the shoot apex meristem (SAM) and root apex meristem (RAM) (Tucker and Laux, 2007; Negin et al., 2017).

The basic helix-loop-helix (bHLH) transcription factor (TF) PRE3/ATBS1/bHLH135/TMO7 (hereafter named PRE3) has been shown to be expressed in RAM and to function as a downstream target of AUXIN RESPONSE FACTOR 5 (ARF5)/MONOPTEROS (MP), a key regulator of root initiation (Schlereth et al., 2010). In addition, Castelain et al. (2012) found PRE3 expression in very young lateral roots and in the vascular region spanning the primordia. Moreover, several studies have uncovered the importance of WUSCHEL-RELATED HOMEOBOX (WOX) TFs in meristem initiation and maintenance (Haecker et al., 2004; van der Graaff et al. 2009). In particular, WOX11 seems to promote the activation of WOX5/7 during the transition from root founder cells to root primordium (Hu et al., 2016) and to regulate the transition from procambium stem cells into root founder cells during both callus and adventitious root development from wounded leaf explants (Liu et al., 2014). WOX11 expression is directly regulated by the auxin signaling pathway which is also involved in lateral root initiation as shown by several authors using the auxin-response reporter DR5 (De Smet et al., 2007; Xuan et al., 2015).

During the formation of secondary tissues in the root, all primary tissues (including the pericycle) external to the vascular cylinder are shed, and thus abandoned (Esau, 1965; Dolan, 1993). Contemporaneously, root girth starts to increase due to the onset of the activities of both the vascular and cork cambia, according to Esau, 1965 and Mauseth, 1988. It has been reported that a secondary root is also able to produce new lateral roots in sectors where primary tissues are no longer present. This event occurs in response to different environmental stimuli such as water stress (Bao et al., 2014; Montagnoli et al. 2012), fire (Di Iorio et al. 2011; Montagnoli et al., 2016), anoxia or pruning (Paolillo, 2006), specific nutrients distribution in the soil (Rella-Alvarez et al., 2016), and mechanical stress (De Zio et al. 2016; Lombardi et al. 2017). In previous studies on *Fraxinus ornus* (L.) and *Populus nigra* (L.) was demonstrated that a new lateral root primordium, in a woody parental, origin from the vascular cambium initials (Chiatante et al. 2007b; 2010). Same findings were also hypothesized by Paolillo (2006) and demonstrated, later on, by Rigal et al. (2012). However, to the best of our knowledge, despite these anatomical demonstrations, no molecular and biochemical investigation has been conducted so far.

Therefore, the aim of the present study was to investigate at the molecular level whether the vascular cambium might replace pericycle competences during the emergence of new lateral roots from a secondary structure taproot by the use of three different *A. thaliana* lines. Our hypothesis was that if the vascular cambium of a root with secondary growth provides the FCs necessary to produce a new lateral primordium, then this activity could be regulated by the same modules observed in SAM and RAM or during the emission of a LR from the pericycle. To test our hypothesis, we induced the formation of new lateral roots from the vascular cambium by the application of bending to the taproot of *A. thaliana* seedlings.

**Materials and methods**

*Plant materials and growth conditions*

Seeds of three different lines, PRE3pro:GUS, DR5rev:GUS and WOX11pro:GUS, of *A. thaliana* Columbia (Col-0) background were used. PRE3pro:GUS and WOX11pro:GUS are transformed lines described in Castelain et al. (2012) and Liu et al. (2014) respectively. DR5rev:GUS was generated by recombining the 1R4-DR5rev, 221a-GUS and 2R3a-3AT entry clones with destination vector pCAM-hyg-R4R3 in a MultiSite Gateway LR reaction (Siligato et al., 2016). Seeds of each line were sterilized in 0.1% (v/v) Triton X-100, washed with 100% (v/v) ethanol and placed on square plates containing ½ Murashige and Skoog (MS), supplemented with 0.08 μM benzylaminopurine (6BA) to avoid lateral root emissions (Li et al., 2006). To synchronize germination, plated seeds were first cold treated for two days at 4°C in darkness and then incubated in vertical position under long day conditions (16 h light/8 h dark cycles) with a medium light intensity of 150 μmol m−2 s−1 at 23 °C. After 10 days, seedlings were transferred in ½ MS medium without 6BA to interrupt its inhibiting effect (Figure 1A). Subsequently, seedlings of the PRE3pro:GUS line were left to grow for 15 days, while seedlings of the DR5rev:GUS and WOX11pro:GUS lines were left to grow for 35 days. In this way, the taproot axis could be divided in two sectors: a) a proximal sector (corresponding to the taproot axis developed in the presence of 6BA) with a much reduced number of lateral roots (indicated by the black line in Figure 1B); and b) a distal sector (corresponding to the taproot axis developed in the absence of 6BA) which presents a considerable number of lateral roots (Figure 1B).

To increase the amount of secondary structure formed in taproots, the floral stalks were regularly removed at their appearance using a surgical scalpel (Zhao et al., 2000; Oh et al., 2003; Lev-Yadun, 1994). All seedlings were anatomically investigated for the presence of secondary growth along the root axis. Once secondary growth was detected (i.e. presence of vascular cambium and secondary xylem tissues) at the proximal sector without lateral roots, seedlings were transferred to 1/5 MS medium with 15 mg/L indole-3-butyric acid (IBA) (according to Welander et al., 2014) and their taproot bent at an angle range of 120°-130° at 15 mm (PRE3pro:GUS) and 20 mm (WOX11pro:GUS and DR5rev:GUS) distance below the hypocotyl, while the distal sector of the taproot with lateral roots was removed (Figure 1 C). Control seedlings were also transferred to 1/5 MS medium with IBA and subject to the taproot removal at the distal sector, with the only difference that the taproot was not bent. After these treatments, seedlings were sampled at different sampling points as indicated in Table 1. Finally, in order to anatomically investigate exclusively the formation of secondary tissues, other 40 not-treated seedlings for DR5rev:GUS line and 40 not-treated seedlings for WOX11pro:GUS line were left to grow up to three months. Also in this case floral stalks were regularly removed.

*Anatomy and histology*

The formation of secondary growth along the taproot axis was investigated by sampling 10 seedlings for each line. Microsections of the taproot were fixed overnight in 25% (v/v) glutaraldehyde and 37% (v/v) formaldehyde in 0.05 mol/L sodium phosphate, dehydrated in a graded series of ethanol (30% - 50% - 70% - 90% - 100% twice) and embedded in glycol methacrylate resin (Technoivit 7100, Bio Optica, Kulzer, Germany; see Terzaghi et al., 2016). Embedded samples were sectioned (10 μm thick) with a sliding microtome (Leica 2400) and stained with toluidine blue or ruthenium red. Finally, microsections were observed with an optical microscope (OLYMPUS BX63) and images acquired by an embedded digital camera (OLYMPUS DP72). For each treatment, GUS staining was performed as described (Siligato et al., 2016) on 15 seedling taproots at each sampling point (Table 1). Samples were clarified (Malamy and Benfey, 1997), fixed and embedded as described above. Images were processed through open source ImageJ software (www.imaj.org).

**Results**

*Induction of lateral root formation from a sector of taproot secondary structure*

Analysis of transverse sections collected from the proximal taproot sector at different distances from the hypocotyl showed that already at 5 mm below the hypocotyl (Figure 2 A) the vascular cambium was active, producing a considerable amount of wood. At a higher distance, i.e. 5-10 mm (Figure 2 B), 15-20 mm (Figure 2 C), and 30 mm (Figure 2 D) from the hypocotyl, the amount of secondary structure decreased as these distal sections were younger than the proximal ones. Moreover, our data show in the oldest wood (i.e. most proximal) the presence of medullar rays in correspondence to the former xylem poles in the primary vascular cylinder (arrows in Figure 2 A).

The new stress-induced lateral roots had been produced by the vascular cambium as confirmed by the analysis of their trace. In fact, when old lateral roots were anatomically examined (Figure 3), their origin from the pericycle could be traced to the xylem pole (Figure 3 A). On the contrary, newly-induced lateral roots that originated from the vascular cambium can be traced to the secondary xylem at the point where the vascular cambium initials were located at the time of their initiation (Figure 3 B).

*Involvement of transcription factors (PRE3pro and WOX11) and auxin in lateral root formation from A. thaliana secondary structure taproot*

To verify the involvement of transcription factors in the production of new lateral roots in a taproot that had developed a secondary structure, we used the *A. thaliana* PRE3pro:GUS line (Figure 4). Our data show that already 1 day after the beginning of the bending treatment GUS staining becomes visible on the convex side of the root (Figure 4 A). The GUS expression is localized in an area corresponding to the vascular cambium initials (Figure 4 B). After 5 days of treatment, the protrusion of lateral root primordia could be observed also externally to the taproot axis (Figure 4 C, D).

Similar results were obtained when analysing the WOX11pro:GUS line (Figure 5). Furthermore, Figure 5 shows that our bending treatment is a repeatable method to elicit the formation of new lateral roots from the secondary structure of an *A. thaliana* taproot. The GUS signal could not be detected 6 hours after the beginning of bending treatment (data not shown) but started to become visible 1 day after bending treatment, limited to the convex side of the bending zone (Figure 5 A). Transverse sections showed that the GUS staining was diffused in the area of the vascular cambium and xylem parenchyma (Figure 5 B). After 2 and 3 days of treatment, the GUS signal was still visible externally on the convex side of the bending zone (Figure 5 C, E), but the transverse section showed that internally the staining was concentrated in a specific zone of the vascular cambium (Figure 5 D, F). Protrusion of lateral roots from the taproot tissue became externally visible only after 7 days of treatment (Figure 5 G), whereas the transverse sections showed that WOX11 expression, unlike PRE3, was more localized to the vascular cambium zone directly below the newly formed root primordium (Figure 5 D, F, H).

When the DR5 auxin reporter was used, a GUS signal in the bending zone could already be observed at 6 hours after the application of bending (Figure 6 A). The GUS signal was scarcely visible in transverse sections (Figure 6 B) but gradually increased in size and intensity after 15, 24, and 48 hours (Figure 6 C-H). In all cases, the staining was localized to the vascular cambium zone, which showed to be characterized by the highest mitotic activity on the convex side (Figure 6 G-H). The initial phase of a new root primordium formation was visible after 3 days (arrows in Figure 6 G, J). In the case of the control seedlings (no bending application), no new lateral roots emerged along the taproot axis.

**Discussion**

Here we describe the application of the bending method to induce lateral root formation in *A. thaliana* taproots after first having achieved the formation of a secondary structure. In previous experiments the bending treatment was devised for poplar woody root (see description of methods in Scippa et al. (2008) and De Zio et al. (2016)) to mimic the effect of mechanical stress, which in nature is known to induce the formation of laterals from a woody parental root (Stoke et al., 2007). In particular, the emission of new lateral roots from a woody parental has been interpreted as the way to mechanically reinforce plant anchorage (Chiatante et al., 2003; Chiatante et al., 2007a; Scippa et al. 2008; Trupiano et al., 2012; De Zio et al., 2016). Similar results were obtained when the bending treatment was applied to the taproot of *A. thaliana* characterized by a primary structure (i.e., the presence of a pericycle) (Ditengou et al., 2008; Richter et al., 2009). These results are in agreement with the model developed by Trupiano et al. (2012) indicating that when a root is bent, tension and compression forces act on the tissues with a maximal strain on the bending sector of both convex and the concave side, respectively. Moreover, the similarity of response detected in different plant species, with either the pericycle (Ditengou et al., 2008; Richter et al., 2009) or the vascular cambium (Chiatante et al. 2007a, b) as tissue source of the founder cells, let us assume that the formation of new lateral root primordia might be controlled by similar molecular mechanism. In the present work, the similar localization of PRE3, WOX11, DR5 expression detected on the convex side of the bent *A. thaliana* taproot, suggests that these three factors could interplay in lateral root initiation. This is supported by previous work that found PRE3 expression in meristems and elongation zones of primary and lateral roots, in very young primordia during early lateral root development, and in the vascular region spanning the primordium (Castelain et al., 2012). Moreover, our results support the hypothesis that lateral root formation is regulated by the same modules as those regulating embryonic root initiation in the RAM. Indeed, we also detected PRE3 expression in RAM that is related to MONOPTEROS (MP) TF activity, as PRE3 is a downstream target of ARF5/MP during embryonic root initiation (Schlereth et al. 2010). In our experiment, the auxin accumulation observed on the convex side, as a response to mechanical stress, could be due to cell-length increase that boosts auxin flux through the cell membranes (Laskowski et al. 2008). Auxin accumulation directly induces the expression of transcription factor WOX11 (Zhao et al., 2009; Liu et al, 2014; Chen et al., 2016; Hu et al., 2016), which is believed to be responsible for the priming of competent cells to become founder cells of a root primordium (Liu et al., 2014; Chen et al., 2016; Hu et al., 2016). Therefore, based on our results, it seems reasonable to assume that auxin plays a similar role in the formation of new lateral roots from vascular cambium initials in the secondary structure of *A.thaliana* taproot.

Our hypothesis is further supported by other works that demonstrated the expression of WOX11 and DR5 in adventitious roots initiation of *Arabidopsis* (Liu et al. 2014) and Rice (Zhao et al. 2009). Moreover, it was demonstrated that WOX11 is involved in founder cell establishment, activating both LATERAL ORGAN BOUNDARIES DOMAIN 16/19 (LBD16/19; Liu et al., 2014; Sheng et al., 2017) and WOX5/7 (Hu et al., 2016) before adventitious root primordium initiation. Most recently, Sheng et al. (2017) observed in *Arabidopsis* plants vertically grown on a medium, that WOX11 is required for adventitious but not for lateral root initiation. Furthermore, the same authors observed the expression of WOX11 in primary roots of plants grown in soil as a response to environmental signals, such as wounding or stress (Sheng et al. 2017). This result highlights that in our experiment the application of bending (i.e. mechanical stress) may induce such complex environmental conditions that require the expression of WOX11 to optimize root growth with the production of new lateral roots.

Reassuming, the localization of gene expression observed suggests that auxin accumulation on the convex side induces PRE3 and WOX11 expression during the transition of some vascular cambium initials from a role as producers of xylem/phloem mother cells to the role of founder cells of a new lateral root primordium from a secondary structure. Moreover, when exogenous auxin was added to the growth medium the number of lateral roots emerging from the taproots increased considerably (data not shown). This finding further confirms the role of auxin in lateral root formation as previously observed by Blakely et al. (1988) and Welander et al. (2014) who provided examples of adventitious lateral root formation by adding IBA.

In conclusion, there are at least two important considerations emerging from our work both having a large outstanding impact on this type of investigations. First, even though *A. thaliana* taproot with a secondary structure did not develop a cork cambium, our experimental setting showed that this model plant is suitable for studying the signaling network and regulatory mechanisms controlling the vascular cambium during wood formation. Furthermore, for the first time our data show that in the oldest wood of *A. thaliana* taproot it is possible to obtain a secondary structure with a more complete tissue differentiation characterized by the presence of medullar rays. Thus, these findings highlight the necessity to achieve an advanced level of wood construction (3 months in our experiment) to obtain wood with a high resemblance to the one normally produced by woody dicots. Moreover, our experimental approach is the first method reported to date that enables to sample with a high precision the specific tissue upon which mechanical forces have been applied (i.e. the convex side of a bending treatment). Samples collected with this approach could be used for any kind of “omics” analysis concerning lateral root formation from a woody parental or the response of tissues to mechanical stress. Second, the knowledge that the vascular cambium can replace the pericycle tissue giving origin to the formation of new lateral roots, even at a later stage of development of a woody plant, could be used to alter the root architecture of woody plants enlarging the horizons of agricultural and forestry practices. Finally, the use of specific *A. thaliana* lines has enabled us to collect the first evidence of a possible involvement of two transcription factors, PRE3 and WOX11, and auxin in lateral root initiation from the vascular cambium, albeit their precise role remains to be determined.

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**References**

Bao Y., Aggarwal P., Robbins N.E., Sturrock C.J., Thompson M.C., Qi Tan H., Tham C., Duan L., Rodriguez P.L., Vernoux T., Nooney S.J., Bennet M.J., Dinneny J.R. (2014) Plant roots use a patterning mechanism to position lateral root branches toward available water. PNAS, 111, 9319-9324.

Beeckman T., De Smet I. (2014) Pericycle. *Current Biology*, 24, R378.

Blakely L.M., Blakely R.M., Colowit P.M., Elliott D.S. (1988) Experimental Studies on Lateral Root Formation in Radish Seedling Roots II. Analysis of the dose-response to exogenous auxin. *Plant Physiology*, 87, 414-419.

Castelain M., Le Hira R., Bellini C. (2012) The non-DNA-binding bHLH transcription factor PRE3/bHLH135/ATBS1/TMO7 is involved in the regulation of light signaling pathway in *A. thaliana*. *Physiologia Plantarum*, 145, 450-460.

Chen L., Tong J., Xiao L., Ruan Y., Liu J., Zeng M., Huang H., Wang J. W., Xu L. (2016) YUCCA-mediated auxin biogenesis is required for cell fate transition occurring during *de novo* root organogenesis in *Arabidopsis*. *Journal of Experimental Botany*, 67, 4273-4284.

Chiatante D., Beltotto M., Onelli E., Di Iorio A., Montagnoli A., Scippa S.G. (2010) New branch roots produced by vascular cambium derivatives in woody taproots of *Populus nigra* L. *Plant Biosystems*, 144, 420-433.

Chiatante D., Di Iorio A., Scippa G.S (2007a) Modification of root architecture in woody plants is possible for the presence of two different mechanisms of lateral root production: The effect of slope in *Spartium junceum* L. seedlings. *Plant Biosystems*, 141, 502-511.

Chiatante D., Scippa G.S., Di Iorio A., De Micco V., Sarnataro M. (2007b) Lateral root emission in woody taproots of *Fraxinus ornus* L. *Plant Biosystems*, 141, 204-213.

De Smet I., Signora L., Beckman T., Inze D., Foyer C.H., Zhang H. (2003) An abscisic acid-sensitive checkpoint in lateral root development of *A. thaliana*. The Plant Journal, 33, 543–555.

De Smet I., Tetsumura T., De Rybel B., Frey dit Frey N., Laplaze L., Casimiro I., Swarup R., Naudts M., Vanneste S., Audenaert D., Inzè D., Bennet M.J., Beeckman T. (2007) Auxin-dependent regulation of lateral root positioning in the basal meristem of *A. thaliana*. *Development*, 134, 681-690.

De Zio E., Trupiano D., Montagnoli A., Terzaghi M., Chiatante D., Gross A., Marra M., Scaloni A., Scippa G.S. (2016) Poplar woody taproot under bending stress: the asymmetric response of the convex and concave sides. *Annals of Botany*, 118, 865-883.

Ditengou F.A., Teale W.D., Kochersperger P., Flittner K.A., Kneuper I., van der Graaff E., Nziengui H., Pinosa F., Li X. G., Nitschke R., Laux T., Palme K. (2008) Mechanical induction of lateral root initiation in *A. thaliana*. PNAS, 105, 18818-18823.

Di Iorio A., Montagnoli A., Scippa G.S., Chiatante D. (2011) Fine root growth of *Quercus pubescens* seedlings after drought stress and fire disturbance. *Environmental and Experimental Botany*, 74, 272-279.

Dolan L., Janmaat K., Willemsen V., Linstead P., Poethig S., Roberts K., Scheres, B. (1993). Cellular organisation of the *A. thaliana* root. Development, 119, 71-84.

Esau K. (1965) Plant anatomy, second edition. New York, USA: John Wiley & Sons Inc.

Fernandez A., Drozdzecki A., Hoogewijs K., Vassileva V., Madder A., Beeckman T., Hilson P. (2015) The GLV6/RGF8/CLEL2 peptide regulates early pericycle divisions during lateral root initiation. *Journal of Experimental Botany*, 66, 5245-5256.

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

Hu X., Xu L. (2016) Transcription factors WOX11/12 Directly Activate WOX5/7 to Promote Root Primordia Initiation and Organogenesis. *Plant Physiology*, 172, 2363-2373.

Khan M.A., Gemenet D.C., Villordon A. (2016) Root System Architecture and Abiotic Stress Tolerance: Current Knowledge in Root and Tuber Crops. *Frontiers in Plant Science*, 7, 1-13.

Koorneef M., Meinke D. (2010) The development of Arabidopsis as a model plant. The Plant Journal, 61, 909–921.

Ivanchenko M.G., Muday G.K., Dubrovsky J.G. (2008) Ethylene–auxin interactions regulate lateral root initiation and emergence in *A. thaliana*. The Plant Journal, 55, 335–347.

Laskowski M., Grieneisen V. A, Hofhuis H., Hove C. A., Hogeweg P., Maree A. F., Scheres B. (2008) Root system architecture from coupling cell shape to auxin transport. PLoS Biology 6, e307.

Lev-Yadun S. (1994) Induction of sclereid differentiation in the pith of *A. thaliana* (L) Heynh. *Journal Experimental Botany*, 45, 1845-1849.

Li C., Zhang B. (2016) MicroRNAs in Control of Plant Development. *Journal of Cellular Physiology*, 231, 3013-3013.

Li X., Mo X., Shou H., Wu P. (2006) Cytokinin-Mediated Cell Cycling Arrest of Pericycle Founder Cells in Lateral Root Initiation of *A. thaliana*. *Plant Cell Physiology*, 47, 1112 - 1123.

Liu J., Sheng L., Xu Y., Li J., Yang Z., Huang H., Xu L. (2014) WOX11 and 12 Are Involved in the First-Step Cell Fate Transition during de Novo Root Organogenesis in *A. thaliana*. *The Plant Cell*, 26, 1081-1093.

Lombardi F., Scippa G.S., Lasserre B., Montagnoli A., Tognetti R., Marchetti M., Chiatante D. (2017) The influence of slope on *Spartium junceum* root system: morphological, anatomical and biomechanical adaptation. *Journal Plant Research*, 130, 515-525.

Malamy J.E., Benfey P.N. (1997) Organization and cell differentiation in lateral roots of *A. thaliana*. *Development*, 124, 33-44.

Marin E., Jouannet V., Herz A., Lokerse A.S., Weijers D., Vaucheret H., Nussaume L., Crespi M.D., Maizel A. (2010) miR390, *Arabisopsis* TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR Targets define an autoregulatory network quantitatively regulating lateral root growth. The Plant Cell, 22, 1104–1117.

Mauseth J.D. (1988) Plant Anatomy. Menlo Park, USA: The Benjamin/Cummings Publishing Company, Inc.

Meng L., Buchanan B.B., Feldman L.J., Luan S. (2012) CLE-like (CLEL) peptides control the pattern of root growth and lateral root development in *A. thaliana*. PNAS, 109, 1760-1765.

Möller B.K.., Xuan W., Beeckman T. (2017) Dynamic control of lateral root positioning. *Current Opinion in Plant Biology*,35, 1-7.

Montagnoli A., Terzaghi M., Di Iorio A., Scippa G.S., Chiatante D. (2012) Fine-root morphological and growth traits in a Turkey-oak stand in relation to seasonal changes in soil moisture in the Southern Apennines, Italy. *Ecological Research*, 27, 1015-1025.

Montagnoli A., Di Iorio A., Terzaghi M., Trupiano D., Scippa G.S., Chiatante D. (2014) Influence of soil temperature and water content on fine-root seasonal growth of European beech natural forest in Southern Alps, Italy. *European Journal of Forest Research*, 133, 957-968.

Montagnoli A., Terzaghi M., Baesso B., Santamaria R., Scippa G.S., Chiatante D. (2016) Drought and fire stress influence seedling competition in oak forests: fine-root dynamics as indicator of adaptation strategies to climate change. *Reforesta*, 1: 86-105.

Negin B., Shemer O., Sorek Y., Williams L.E. (2017) Shoot stem cell specification in roots by the

WUSCHEL transcription factor. PLOS ONE, 12, e0176093.

Nieminen K., Blomster T., Helariutta Y., Mähönen A.P. (2015) Vascular cambium development. *The A. thaliana Book*: e0177.

Oh S., Park S., Han K-H. (2003) Transcriptional regulation of secondary growth in *A. thaliana* . *Journal Experimental Botany*, 54, 2709-2722.

Paolillo D.J. (2006) On the Structural Relationships of Branch Roots and Their Taproot Axes in Secondary Growth. *International Journal of Plant Sciences*, 167, 47-57.

Peret B., Middleton A.M., French A.P., Larrieu A., Bishopp A., Njo M., Wells D.M., Porco S., Mellor N., Band L.R., Casimiro I., Kleine-Vehn J., Vanneste S., Sairanen I., Mallet R., Sandber G., Ljung K., Beeckman T., Benkova E., Friml J., Kramer E., King J.R., De Smet I., Pridmore T., Owen M., Bennet M.J. (2013) Sequential induction of auxin efflux and influx carriers regulates lateral root emergence. *Molecular Systems Biology*, 9, 699.

Ragni L., Hardtke C.S. (2014). Small but thick enough - the A. thaliana hypocotyl as a model to study secondary growth. *Physiologia Plantarum*, 151, 164-171.

Ramirez-Parra E., Perianez-Rodriguez J., Navarro-Neila S., Gude I., Moreno-Risueno M.A., del Pozzo J.C. (2016) The transcription factor OBP4 controls root growth and promotes callus formation. *New Phytologist*, 213, 1787–1801

Richter G.L., Monshausen G.B., Krol A., Gilroy S. (2009) Mechanical stimuli modulate lateral root organogenesis. *Plant Physiology*, 151, 1855-1866.

Rigal A., Yordanov Y.S., Perrone I., Karlberg A., Tisserant E., Bellini C., Busov V.B., Martin F., Kohler A., Bhalerao R., Legué V. (2012) The AINTEGUMENTA LIKE1 Homeotic Transcription Factor PtAIL1 Controls the Formation of Adventitious Root Primordia in Poplar. *Plant Physiology*, 160, 1996-2006.

Rossi M., Trupiano D., Tamburro M., Ripabelli G., Montagnoli A., Chiatante D., Scippa G.S. (2015) MicroRNAs expression patterns in the response of poplar woody root to bending stress. Planta, 242, 339–351.

Schlereth A., Möller B., Liu W., Kientz M., Flipse J., Rademacher E.H., Schmid M., Jurgens G., Weijers D. (2010) MONOPTEROS controls embryonic root initiation by regulating a mobile transcription factor. *Nature*, 464, 913-917.

Sheng L., Hu X., Du Y., Zhang G., Huang H., Scheres B., Xu L. (2017) Non-canonical WOX11-mediated root branching contributes to plasticity in *Arabidopsis* root system architecture. *Development*, 144, 3126-3133.

Scippa G.S, Di Michele M., Di Iorio A., Costa A., Lasserre B., Chiatante D. (2006) The response of *Spartium junceum* roots to slope: anchorage and gene factors. *Annals of Botany*, 97, 857-866.

Scippa G.S., Trupiano D., Rocco M., Di Iorio A., Chiatante D. (2008) Unravelling the response of poplar (*Populus nigra*) roots to mechanical stress imposed by bending. *Plant Biosystems*, 142, 401-413.

Sibout R., Plantegenet S., Hardtke C.S. (2008) Flowering as a condition for xylem expansion in *A. thaliana* hypocotyl and root. *Current Biology*, 18, 458-463.

Siligato R., Wang X., Yadav S.R., Lehesranta S., Ma G., Ursache R., Sevilem I., Zhang J., Gorte M., Prasad K., Wrzaczek M., Heidstra R., Murphy A., Scheres B., Mahonen A.P. (2016) MultiSite Gateway-compatible cell type-specific gene-inducible system for plants. *Plant Physiology*, 170, 627-641.

Stokes A, Nicoll B.C., Coutts M.P., Fitter A.H. (1997) Responses of young Sitka spruce clones to mechanical perturbation and nutrition: Effects on biomass allocation, root development, and resistance to bending. Canadian Journal of Forest Research, 27, 1049-1057.

Szymanowska-Pułka J. (2013) Form matters: morphological aspects of lateral root development. *Annals of Botany*, 112, 1643-1654.

Terzaghi M., Di Iorio A., Montagnoli A., Baesso B., Scippa G.S., Chiatante D. (2016) Forest canopy reduction stimulates xylem production and lowers carbon concentration in fine roots of European beech. *Forest Ecology and Management*, 379: 81-90.

Trupiano D., Rocco M., Renzone G., Scaloni A., Viscosi V., Chiatante D., Scippa G.S. (2012) The proteome of *Populus nigra* woody root: response to bending. *Annals of Botany*,110: 415-432.

Tucker M.R., Laux T. (2007). Connecting the paths in plant stem cell regulation. TRENDS *in Cell Biology*, 17, 403-410.

Van der Graaff E., Laux T., Rensing.S.A. (2009) The WUS homeobox-containing (WOX) protein family. *Genome Biology*, 10:248.

Welander M., Geier T., Smolka A., Ahlman A., Fan J., Zhu L.H. (2014) Origin, timing, and gene expression profile of adventitious rooting in *A. thaliana* hypocotyls and stems. *American Journal of Botany*, 101, 255-266.

Xuan W., Audenaert D., Parizot B., Moller B.K., Njo M.F., De Rybel B., De Rop G., Van Isterdael G., Mähönen A.P., Vanneste S., Beeckman T. (2015) Root Cap-Derived Auxin Pre-patterns the Longitudinal Axis of the ArabidopsisRoot. *Current Biology*, 25, 1381-1388.

Yue K., Beeckman T. (2014) Cell-to-Cell Communication during Lateral Root Development. *Molecular Plant*, 7, 758–760.

Zhang J., Elo A., Helariutta Y. (2011) *A. thaliana* as a model for wood formation. *Current Opinion in Biotechnology*, 22: 293-299.

Zhao C., Johnson B.J., Kositsup B., Beers E.P. (2000) Exploiting secondary growth in *A. thaliana*. Construction of xylem and bark cDNA libraries and cloning of three xylem endopeptidases. *Plant Physiology*, 123, 1185-1196.

Zhao Y., Hu Y., Dai M., Huang L., Zhou D. (2009) The WUSCHEL-Related Homeobox Gene WOX11 Is Required to Activate Shoot-Borne Crown Root Development in Rice. *The Plant Cell*, 21, 736-748.

Figure 1: Seedlings at different experimental points. A) A 10-days-old *A. thaliana* seedling grown on 6BA-supplemented medium showing a taproot axis free of lateral roots. B) A 45-days-old *A. thaliana* plant with the taproot axis divided in a proximal sector, grown in the presence of 6BA, with no lateral root development (black line) and a distal sector, grown on ½ MS medium, with abundant lateral root growth. C) A bent taproot characterized by secondary growth. The selection indicates the sampled area where the GUS staining was detected for the three different *A. thaliana* lines. Bars = 1 cm.

Figure 2: Anatomical microsections of *A. thaliana* taproots that present secondary growth, at different distances from the hypocotyl. A) Fully developed secondary growth with visible medullar rays in 3-months-old seedlings, 5 mm below the hypocotyl. B-D) Secondary growth developed at 5-10 mm (B), 15-20 mm (C) and 30 mm (D), below the hypocotyl. sx = secondary xylem, vc = vascular cambium, sp = secondary phloem, mr = medullar rays. Bars: 50 microns.

Figure 3: Anatomical transverse sections of *A. thaliana* taproots at the point of lateral root emergence. A) Vascular traces (indicated by black lines) of an old lateral root developed from the xylem pole in the center of the taproot (indicated by circle) B) Vascular traces of a new lateral root which do not reach the center of the taproot but developed from the vascular cambium. sx = secondary xylem, vt = vascular traces. Bars = 50 μm.

Figure 4: PRE3pro: GUS expression pattern during lateral root formation. A) GUS staining at the convex side of the bending zone 1 day after bending application. B) Transverse section of the GUS-stained region of a taproot 1 day after bending application. GUS staining is concentrated to the vascular cambium region on the convex side. C) GUS staining in new primordia emerging at the convex side of the bending zone 5 days after bending application. D) Transverse section of a new root primordium with GUS staining 5 days after bending application. sx = secondary xylem, vc = vascular cambium, sp = secondary phloem, rp = root primordium. Bars = 1 mm (A, C, and D) and 50 μm (B).

Figure 5: Analysis of WOX11pro: GUS expression in bent taproots. GUS staining at the convex side of the bending zone, 1 day (A), 2 days (C), and 3 days (E) after bending application, and in the relative anatomical transverse sections (B, D, and F, respectively). GUS staining diffused in the vascular cambium zone and xylem parenchyma cells 1 day after bending application (B) but was concentrated to a few cells of the vascular cambium zone 2 days (D) and 3 days (E) after bending application. G) New lateral root emission at the convex side of the bending region 7 days after bending application. H) Transverse section of a new lateral root primordium 7 days after bending application. Note that GUS staining is concentrated in the vascular cambium zone directly below the newly formed root primordium. sx = secondary xylem, vc = vascular cambium, sp = secondary phloem, rp = root primordium. Bars = 1 mm (A, C, E, and G) and 50 μm (B, D, F and H).

Figure 6: Analysis of DR5rev: GUS expression in bent taproots. Left: GUS staining at the convex side of the bending zone, 6 hours (A), 15 hours (C), 1 day (E), 2 days (G) and 3 days (I) after bending application; and (right) in the relative anatomical transverse sections (B, D, F, H and J, respectively). No GUS staining is observable in the anatomical section 6 hours after bending application (B). GUS staining is found in the vascular cambium zone and xylem parenchyma 15 hours (D) and 1 day (F) after bending application, whereas it is mainly concentrated to the vascular cambium zone 2 days after bending application (H). J) Initiation of a root primordium showing concentrated GUS staining. sx = secondary xylem, vc = vascular cambium, sp = secondary phloem. Bars = 1 mm (A, C, E, G, and I) and 50 μm (B, D, F, H, and J).