Title:

Overexpression of *ARBORKNOX1* delays the differentiation of Induced Somatic Sector Analysis (ISSA) derived xylem fibre cells in poplar stems.

Authors:

Emma Melder¹, Gerd Bossinger¹ and Antanas V. Spokevicius¹

Affiliations:

¹School of Ecosystem and Forest Sciences, University of Melbourne, Creswick, Victoria, Australia 3363

Short Title:

ARBORKNOX1 delays differentiation in ISSA poplar stems

Corresponding Author: A. Spokevicius

E-mail address: avjs@unimelb.edu.au

Phone: +61 3 5321 4153

Fax: +61 3 5321 4166

Key Words: Cambium, ARBORKNOX, Wood Formation, Cell Wall, Dormancy, Xylem Fibres.

Abbreviations: CK = cytokinin, GA = gibberellic acid

Abstract

ARBORKNOX1 (*ARK1*), a poplar functional homolog of the *Arabidopsis* Class-I KNOTTED- like homeobox (*KNOX1*) transcription factor *SHOOT MERISTEMLESS* (*STM*), has been shown to have a role in maintaining meristematic cells in an undifferentiated state. Studies in which this gene was ectopically overexpressed identified a delay in the differentiation of cambial derivatives. Over-expression of *ARK1* in poplar stem sectors, using the Induced Somatic Sector Analysis (ISSA) experimental system, was undertaken to further investigate its role in the differentiation of xylem fibre cells. ISSA involves the *in vivo* transformation of a small number of cambial cells in the stem with a gene of interest giving rise to a discreet transgenic tissue sector that can be directly compared to adjacent non-transgenic fibres to elucidate gene function. Phenotypes obtained were comparable with the proposed role of *ARK1*, where a delay in the development of the cell wall in differentiating fibre cells of active cambia was observed. Additional evidence suggests a role for *ARK1* in the positive regulation of cell viability in the cambium. The significance of dormancy signals in the context of differentiation was also highlighted, as some transgenic fibres failed to fully differentiate prior to the onset of dormancy. Our findings are discussed in the context of wood formation and the use of ISSA as an experimental system.

Introduction

Plant meristems are responsible for the proliferation of cells which in turn form all structures and organs that ultimately constitute the plant body. Shoot and root apical meristems (SAM and RAM) ensure continued growth of the plant while the vascular cambium (cambium) is responsible for the continued production of secondary tissue within the stem. While the SAM and RAM have been studied extensively in model species such as *Arabidopsis thaliana* (as reviewed by Bowman & Floyd, 2008, Clark, 2001, Williams & Fletcher, 2005), comparatively less research has been undertaken regarding the vascular cambium or the molecular control of its development. This lack of knowledge stands at odds with both the ecological and economic importance of plant stems.

The vascular cambium in perennial trees, both gymnosperms and dicotyledonous angiosperms, is comprised of semi differentiated meristematic tissue responsible for the production of secondary xylem (wood) and phloem (bark) in the stem. Initials divide anticlinally to give rise to new initials during stem expansion and periclinally to create xylem and phloem mother cells. These mother cells in turn undergo additional rounds of periclinal division to produce xylem (wood) cells to the inside of the stem (centripetally) and phloem (bark) cells to the outside (centrifugally). In angiosperms, secondary xylem is predominantly comprised of fibre cells, but also ray parenchyma and vessel cells. These cells act as conduits for water and dissolved minerals, as well as providing support and rigidity to the stem.

Secondary phloem includes a range of cell types including fibres, ray parenchyma, axial parenchyma, sieve tube and companion cells with a primary role in transporting photosynthetic products, plant growth regulators and other substances down the plant stem.

Preventing terminal differentiation of meristematic tissue is essential for the continued function of the SAM, RAM and the vascular cambium and is collectively known as meristem maintenance. Although studies have predominantly focussed on the SAM (e.g. Clark et al., 1996, Endrizzi et al., 1996, Laux et al., 1996, Mayer et al., 1998), some evidence suggests that the process of meristem maintenance, and its

molecular regulation, are similar between the SAM and the cambium (Schrader et al., 2004, Du et al., 2009, Bao et al., 2009, Groover et al., 2006).

One regulator of meristem maintenance is *SHOOTMERISTEMLESS* (*STM*) which encodes a *Class-I KNOTTED-like homeobox* (*KNOXI*) transcription factor. Investigations of this transcription factor focussed on *Arabidopsis* mutant plants in which reduced *STM* activity resulted in a disruption of SAM development, with the most severe phenotypes resulting in a complete lack of SAM development in embryonic plants (Barton & Poethig, 1993, Clark et al., 1996, Endrizzi et al., 1996, Long et al., 1996). In these studies it was observed that *STM* continued to be expressed after meristem formation and that in some cases these mutants could form but not maintain a SAM, suggesting a role in maintaining rather than just initiating a SAM. Subsequent studies have proposed that the mode of action for *STM* is *via* negative regulation of cell fate by preventing differentiation rather than the promotion of cells to remain in an undifferentiated state (Mayer et al., 1998, Schoof et al., 2000, Gallois et al., 2002, Lenhard et al., 2002). More recent studies have indicated that *STM* acts through the Cytokinin (CK) and CK-inducible cell cycle regulators linking its negative regulation to cell cycle control (Scofield et al., 2013).

Ensuing work in *Arabidopsis* and poplar demonstrated that *STM* plays a similar role during the formation and function of the cambium compared to the SAM (Schrader et al., 2004, Bao et al., 2009, Groover et al., 2006). In these studies, *STM* homologs were observed to be highly expressed within the cambium as well as its immediate centripetal (xylem) derivatives, hinting at a more specific role in controlling xylem formation in this meristem. Transgenic poplar trees overexpressing *STM* and the poplar *STM* functional homolog *ARBORKNOXI* (*ARK1*) resulted in phenotypes consistent with those observed in *Arabidopsis*, as plants with thin stems were produced. Closer examination of these stems identified a lag in differentiation of xylem tissue and a loss of synchronisation of cell differentiation between adjacent cell files of xylem cells creating a ‘wavy cambium’ (Groover et al., 2006). However, a recent study in *Arabidopsis* hypocotyls in mildly down-regulated *STM* mutants and double mutants with *KNAT1*, another

KNOX1 family member, showed phenotypes that were contrary to their proposed role in the SAM, including a reduction and/or loss of fibre differentiation, hinting different roles for this gene in the cambium (Liebsch et al., 2014).

From a mechanistic perspective, transcriptional network analysis using chromatin immunoprecipitation sequencing (ChIP-seq) studies in wood forming tissues of poplar have shown that *ARK1* binds to thousands of loci, preferentially near transcriptional start sites, with target genes being mostly evolutionary conserved and linked to a wide variety of biological and cellular processes. *ARK1* targets also share high similarity with binding targets of other closely related transcription factors *ARBORKNOX 2* and *BELLRINGER* (Liu et al., 2015a, Liu et al., 2015b). In addition, when overexpressed in transgenic plants only a small proportion of target genes are shown to be differentially expressed suggesting that on its own it is not able to significantly modulate target gene expression (Liu et al., 2015b, Liebsch et al., 2014). These findings highlight the complex way *ARK1* interacts at a molecular level to regulate cambial processes.

In an effort to further elucidate the role of *ARK1* during cambium and wood differentiation, we used Induced Somatic Sector Analysis (ISSA) to over-express *ARK1* specifically and exclusively in the cambium of poplar stems. With particular focus on fibre cells, we describe here the effects of *ARK1* overexpression on cambial cell differentiation, cell viability during the stem growth and on differentiation in the cambium during the transition to dormancy.

Materials and Methods

Plant material, vector construction, creation and harvesting of transgenic tissue sectors

The *ARK1* gene (Accession No AY755413, kindly provided by Dr. Andrew Groover, USDA Forest Service, Institute of Forest Genetics) was recombined into a GATEWAY™ enabled destination vector behind a CAMV35s promoter (35S::*ARK1*) using the pCR8/GW/TOPO TA™ Cloning Kit (Invitrogen, Carlsbad, USA). The GATEWAY™ enabled destination vector also contained GUS reporter gene under the regulation of the CAMV35s promoter to assist locating transgenic tissue at harvest. The 35S::*ARK1* and the blank GATEWAY™ vectors (GW-blank, containing the GUS reporter gene only) were electroporated into *Agrobacterium* strain AGL1 using standard protocols (Sambrook & Russell, 2001).

For ISSA transformation, *Agrobacterium* containing the 35S::*ARK1* and a GW-blank were prepared using protocols described by Van Beveren et al. (2006) and outlined briefly below. Thirty five 1 cm² cambial windows were created for each of the two vectors in stems of 14 *Populus alba* ‘*pyramidalis*’ cuttings, where each stem was inoculated with both the 35S::*ARK1* and GW-blank vectors in different locations. *P. alba* ‘*pyramidalis*’ trees were sourced from cuttings of a single clone and were six to eight months old at the time of inoculation. Window creation took place during the southern hemisphere mid-summer (January) to ensure the presence of a fast growing and active cambium for transformation. Plants containing transformed windows were then left to grow in a glasshouse (14°C to 17°C at night and 21°C to 25°C during the day, 16 hour photoperiod maintained with lighting) prior to harvesting. Harvesting of windows occurred at two points in time; with six plants (30 windows) harvested after three months whilst plants were still actively growing, and the remaining eight (40 windows) after eight months during the dormancy or inactive phase of the plants. Windows were dissected transversely into 1 cm discs, placed in X-gluc buffer and incubated overnight to identify transgenic tissue. After this histological assay, discs were placed in 70% ethanol and the number of cambial sectors counted prior to further analysis.

Sample preparation and imaging of samples using scanning electron microscopy

For morphological analysis of transgenic sectors, scanning electron microscopy (SEM) was used as this required minimal sample preparation and allowed for relatively high throughput. Sample preparation started with the removal of phloem from discs containing GUS positive sectors by peeling away bark tissue and exposing the cambium and the immature xylem daughter cells, referred to here as the ‘cambial surface’. Removal of the phloem was found to be necessary (through some initial trials) to accurately measure cell size, number and distance from the cambium. Peeled samples were cut transversely with a double-edged razor blade through the centre of the transgenic sector to expose the transverse surface of the sector and surrounding non transgenic tissue. To assist locating the sector during SEM imaging a cut was placed on the centripetal surface of the disc directly opposite the cambial surface of the sector (Figure 1) and a colour photo taken (Figure 3a). Prepared discs were then dehydrated overnight using a vacuum desiccator to minimise the water content of the sample and enable the best quality image to be taken under low vacuum conditions. Images were obtained using the Quanta Environmental Scanning Electron Microscope (FEI, Oregon, USA) at 2000X magnification using the low vacuum mode. For each sector, images of both transgenic sector and adjacent non transgenic tissue, taking in the cambial surface, were taken and analysed using the freeware program ImageJ (<http://rsb.info.nih.gov/ij/>). A total of 17 35S::ARK1 sectors were investigated (ten from the three month and seven from the eight month harvests) and 14 GW-blank sectors (seven each from both the three and eight month harvests) were assessed. Images were visually examined for any gross morphological changes and measurements of fibre based on position (up to the first six fibres in a radial file as measured from the cambial surface - see Figure 2c) were undertaken for the following: cell wall thickness (average of four measurements per fibre), cell width (both radially and tangentially), cell area and lumen area. Results were analysed using t-tests (paired) with significance set at $\alpha = 0.05$.

Results

Stable transformation efficiency

The average number of cambial sectors per cm² of tissue inoculated (ACS⁻²) observed in cambial windows for the 35S::ARK1 vector was 5.12 (128 cambial sectors in total) and was significantly higher than the ACS⁻² of 2.36 (59 cambial sectors in total) observed in windows transformed with the GW-blank ($p = 0.0095$). These results indicate that cambial cells inoculated with the 35S::ARK1 vector were more than twice as likely to be stably transformed when compared to the GW-blank.

Assessment of fibre cells in active and dormant cambia

Fibre cells from the ‘three months’ samples were morphologically assessed to determine if 35S::ARK1 had any influence on the differentiation of cambial derivatives. Gross morphological assessment of xylem tissue did not reveal any obvious differences between transgenic and non-transgenic tissue. However, more detailed assessment showed that cell wall thickness of transgenic fibre cells was significantly lower in the 1st, 2nd, 3rd, 5th and 6th cell position from the cambial surface compared to the adjacent non-transgenic tissue (Figure 2a, c). Other fibre dimensions did not show any significant differences (Supplementary Table 1, 2). In the case of the GW-blank vector, fibre cell walls were consistently thinner than in non-transgenic tissue (Figure 2b), however this difference was not significant. Taken together these results indicate that cambial cells transformed with 35S::ARK1 showed a slight delay in the development (thickening) of their secondary cell walls in active cambia.

Similarly, fibre cells from ‘eight-months’ samples were assessed morphologically to determine the influence of 35S::ARK1 on the differentiation of cells at dormancy. At harvest, onset of dormancy in plants was confirmed as they had formed autumn buds, leaf senescence had commenced and phloem and xylem could not be easily separated when trying to peel the bark. Gross visual assessment of cambial sectors transformed with 35S::ARK1 highlighted one distinct morphological difference that occurred in two of the seven sectors investigated. A small number of fibres contained within these sectors, which

were positioned closest to where the dormant cambium had been, were characterised by strikingly thin secondary cell walls (

Figure 3c) relative to the thicker secondary cell walls of adjacent cells outside the transgenic sector (

Figure 3d). Cell wall thickness of these fibre cells ranged from 0.368 μm to 0.438 μm compared to 1.000 μm to 1.743 μm in adjacent non transgenic fibre cells. GW-blank sectors harvested at the same time were also visually assessed and no thin cell walled fibres positioned closest to where the dormant cambium was observed. Detailed assessment of 35S::*ARKI* transgenic fibres did not show any statistically significant difference when compared to non-transgenic adjacent tissue for any of the measured morphological features (data not shown). Similarly, this was the case for the comparison between GW-blank transgenic fibres and their adjacent non-transgenic fibres (data not shown).

Discussion

ARK1 containing vector shows higher transformation efficiency

The transformation efficiency of the 35S::*ARK1* vector was shown to be more than double the efficiency of the GW-blank vector indicating a positive role for this vector on transformation efficiency. This difference is unlikely to be an artefact relating to the preparation of the *Agrobacterium* or T-DNA transfer as this was completed under similar conditions suggesting the mis-expression of the *ARK1* in cambial cells has a possible positive role on cell viability and/or fitness. While this study does not provide conclusive evidence to support this hypothesis, the Arabidopsis homologue of *ARK1* (*STM*) has been linked to the positive regulation of the cytokinin (CK) biosynthesis and cell proliferation in the shoot apical meristem (Jasinski et al., 2005, Yanai et al., 2005, Scofield et al., 2013) and similarly a positive link between CK and enhanced cell proliferation in the cambium has been demonstrated (Nieminen et al., 2008). Under such a scenario a localised increase and/or mis-expression of *ARK1* within a sector could impact on CK pathways which in turn promote cell proliferation potentially making cells less susceptible to turnover relative to their neighbours.

ARK1 delays cell wall thickening in active cambia

Fibre cell differentiation commences after division from the cambium starting with cell expansion followed by secondary cell wall formation and lignification (Fukuda, 1996, Larson, 1994). The observation that cell wall development was delayed in active cambia can therefore be reasonably interpreted as a delay in fibre differentiation and is consistent with previous observations of *ARK1* overexpression in cambial tissues where severe delays and asynchronous differentiation of xylem and phloem from the cambium were observed (Groover et al., 2006). However, fibre morphology was not assessed in that study and our results report a new role for *ARK1* in delaying fibre cell wall development.

Experimentally, increases in *KNOXI* proteins in the SAM can lead to increases in the expression of key CK biosynthesis genes, particularly in the case of *STM* (Yanai et al., 2005, Scofield et al., 2013, Jasinski

et al., 2005). In the SAM, CK is involved in maintaining cells in an undifferentiated state (Furutani et al., 2004), while in the cambium, CK plays a central role in regulating radial growth and cambial zone size linking it to the control of cell proliferation (Nieminen et al., 2008). In both the SAM and the cambium, the action of CK and the extent of its effects are influenced by interactions with other plant growth regulators. For example, *KNOX* proteins negatively regulate gibberellins (GA) through CK activity, stimulating GA catabolism pathways in the SAM that favour meristem maintenance (Jasinski et al., 2005) and similar interactions are likely to occur also in other meristems. In the cambial zone, GAs have roles in promoting xylem formation and more specifically in stimulating fibre elongation (Eriksson et al., 2000, Israelsson et al., 2003). It is therefore possible that, in our experiments, mis-expression of *ARK1* may have influenced CK signalling and/or biosynthesis within a cambial sector causing delays in fibre cell wall development from the cambium, perhaps through interactions with the GA and/or other plant growth regulator pathways. However, it is worth noting that some key CK pathway related genes were not significantly up-regulated in response to overexpression of *ARK1* or *STM* in the cambium of poplar (Groover et al., 2006) and recent ChIP studies have indicated complex transcriptional pathways involving *ARK1* (Liu et al., 2015b) suggesting other possible modes of action.

On the other hand none of the other fibre properties assessed as part of this study were affected significantly. A likely explanation for this relates to the way in which tissue was prepared and at what developmental stage these fibres were imaged. Mechanical removal of the phloem in this study led to the removal of the cambial zone, as well as many of the expanding xylem derivatives characterised by primary cell walls. This left only xylem fibres with secondary cell walls available for observation (see Figure 3b). Secondary cell wall development in these fibres commences when cellular expansion has ceased (Ye, 2002) and consequently the cells imaged as part of this study will have most likely reached their final cellular dimensions. In addition, it is worth noting that down-regulation of *STM* in *Arabidopsis* mutants lead to a decrease in fibre and vessel size relative to the wild type (Liebsch et al.,

2014) and, as a result, drawing definitive conclusion on the role of *ARK1* on the rate and extent of cellular expansion in this study would need to be approached with caution.

While a delay in cell wall development was observed in 35S::*ARK1* sectors, it was not as severe as phenotypes described by Groover et al. (2006) and Liebsch et al. (2014), and this is most likely a result of the experimental system used. Firstly, RNA expression levels cannot as yet be determined when using the ISSA system because tissue preparation for the identification of transgenic sectors requires an RNA degrading histological assay. Transgene expression between independent lines of whole transgenic plants has been shown to be highly variable (as reviewed by Spokevicius et al., 2007) and often individuals with large experimentally induced changes in target gene expression are chosen for further analysis. In contrast, ISSA cannot discriminate against individual sectors (independent transformation events) based on target gene expression changes and consequently, when phenotypic data from randomly selected sectors are combined for analytical purposes, only a minor overall average change in the phenotype of interest may be observed. Secondly, differentiation across the cambium is an intricately coordinated process (Catesson & Lachaud, 1993, Lachaud et al., 1999, Larson, 1994) and it is possible that, due to the close proximity of non-transgenic tissue and the relatively small size of a sector, intercellular signalling may have compensated for or reduced the impact of changes in *ARK1* expression at the local level. Numerous plasmodesmata create links between cambial cells and allow for short distance transport of signalling molecules preferentially across the radial surface providing symplastic connections between cells of similar developmental stage (Burch-Smith et al., 2011). Accordingly, signals from non-transgenic cells could have influenced the differentiation of adjacent transgenic cells in order to maintain coordinated xylogenesis. Conversely, 35S::*ARK1* is not likely to act cell autonomously and could have been transported outwards from the sector into non-transgenic tissue influencing fibre development in a decreasing gradient. Finally, it might also be likely that cells displaying more severe phenotypes may have been purged from the cambium through preferential turnover. These possibilities are not mutually

exclusive and it is conceivable that all or some of them are responsible for the moderate phenotypes observed when using ISSA.

35S::ARK1 fibres fail to mature prior to dormancy

Dormancy is a coordinated ‘arrest’ of plant growth in preparation for adverse environmental conditions and is characterised by two distinct stages, eco- and endo-dormancy (Lang et al., 1987). Eco-dormancy precedes endo-dormancy and is reversible in response to short term growth promoting signals, whereas endo-dormancy is not, and requires specific conditions, like for example periods of cold weather, for growth to be reinitiated. Cessation of cambial activity, autumn bud development and moderate cold tolerance are characteristic of eco-dormancy while leaf senescence and abscission in the case of deciduous plants and maximal cold tolerance is characteristic of endo-dormancy. At the cellular and molecular level, plasmodesmata connections between meristematic cells are blocked restricting cell to cell transport through symplastic pathways (Rinne et al., 2001) while significant down-regulation or complete lack of expression of key genes involved in meristem maintenance and cell division (Zheng et al., 2013, Espinosa-Ruiz et al., 2004) as well as insensitivity and/or negative regulation of cambial regulators auxin and GA (Schrader et al., 2003, Druart et al., 2007, Baba et al., 2011) are observed and thought to be involved in the process of cambial cessation. Movement through eco- and endo-dormancy has been demonstrated to be stage-specific and specific molecular responses have been described between these stages (Espinosa-Ruiz et al., 2004, Baba et al., 2011)

The presence of fibres with significantly thinner cell walls closest to the cambial surface in some 35S::ARK1 sectors suggests that these cells did not fully differentiate prior to the onset of dormancy and this is likely a result of dormancy mechanisms overriding cambial development and differentiation in a coordinated way. This finding highlights two possible regulatory control mechanisms relating to cambial cessation during dormancy. Firstly, that dormancy mechanisms act in a comprehensive fashion on a range of regulatory pathways that promote cambial activity, even counteracting the effects of key regulators

when intentionally mis-expressed. Secondly, that they act either post-transcriptionally or on downstream targets of *ARK1* as it is not likely that the 35S::*ARK1* transgene would be directly regulated by dormancy signals. Evidence of epigenetic control during dormancy in the cambial zone has pointed to potential chromatin remodelling which would support the latter mechanism (Karlberg et al., 2010, Conde et al., 2013). In addition, the large differences in cell wall thickness that we observed and the low frequency of this phenotype suggests a link between timing of dormancy initiation, the developmental state of fibre cells and *ARK1* expression. The exact mechanism for this remains unclear but a tipping point seems likely to exist, at which a cambial cell at a very early stage of development influenced by high levels of *ARK1* ceases differentiation immediately in response to dormancy signals or develops at a such a slow rate during the onset of eco-dormancy that its differentiation is retarded.

Acknowledgments

The authors would like to thank Hossein Valipour Kahrood for valuable and insightful feedback on the manuscript and Dr Andrew Groover for kindly providing the *ARK1* gene. E. Melder was funded through an Australian Postgraduate Award through the Australian Research Council.

Data Archiving Statement

Data created as part of this study relates to the characterisation of morphological traits in the stems of trees and has not been submitted to any public database. Those wishing to access this data please contact the corresponding author.

Compliance with Ethical Standards

E. Melder and this work were funded through an Australian Postgraduate Award through the Australian Research Council. The authors report no conflicts of interest relating to the work presented here.

References

- Baba K, Karlberg A, Schmidt J, *et al.*, (2011). Activity-dormancy transition in the cambial meristem involves stage-specific modulation of auxin response in hybrid aspen. *Proceedings of the National Academy of Sciences of the United States of America* 108:3418-23.
- Bao Y, Dharmawardhana P, Arias R, Allen MB, Ma C, Strauss SH, (2009). WUS and STM-based reporter genes for studying meristem development in poplar. *Plant Cell Reports* 28:947-62.
- Barton MK, Poethig RS, (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana* - an analysis of development in the wild-type and in the SHOOT MERISTEMLESS mutant. *Development* 119:823-31.
- Bowman JL, Floyd SK, (2008). Patterning and polarity in seed plant shoots. *Annual Review of Plant Biology* 59:67-88.
- Burch-Smith TM, Stonebloom S, Xu M, Zambryski PC, (2011). Plasmodesmata during development: re-examination of the importance of primary, secondary, and branched plasmodesmata structure versus function. *Protoplasma* 248:61-74.
- Catesson AM, Lachaud S, (1993). The Cambium, Structure, Function and Control of Its Seasonal Activity. *Acta Botanica Gallica* 140:337-50.
- Clark SE, (2001). Meristems: start your signaling. *Current Opinion in Plant Biology* 4:28-32.
- Clark SE, Jacobsen SE, Levin JZ, Meyerowitz EM, (1996). The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in Arabidopsis. *Development* 122:1567-75.
- Conde D, Gonzalez-Melendi P, Allona I, (2013). Poplar stems show opposite epigenetic patterns during winter dormancy and vegetative growth. *Trees-Structure and Function* 27:311-20.
- Druart N, Johansson A, Baba K, *et al.*, (2007). Environmental and hormonal regulation of the activity-dormancy cycle in the cambial meristem involves stage-specific modulation of transcriptional and metabolic networks. *Plant Journal* 50:557-73.
- Du J, Mansfield SD, Groover AT, (2009). The Populus homeobox gene ARBORKNOX2 regulates cell differentiation during secondary growth. *Plant Journal* 60:1000-14.

Endrizzi K, Moussian B, Haecker A, Levin JZ, Laux T, (1996). The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. *Plant Journal* 10:967-79.

Eriksson ME, Israelsson M, Olsson O, Moritz T, (2000). Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. *Nature Biotechnology* 18:784-8.

Espinosa-Ruiz A, Saxena S, Schmidt J, *et al.*, (2004). Differential stage-specific regulation of cyclin-dependent kinases during cambial dormancy in hybrid aspen. *Plant Journal* 38:603-15.

Fukuda H, (1996). Xylogenesis: Initiation, progression, and cell death. *Annual Review of Plant Physiology and Plant Molecular Biology* 47:299-325.

Furutani M, Vernoux T, Traas J, Kato T, Tasaka M, Aida M, (2004). PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. *Development* 131:5021-30.

Gallois JL, Woodward C, Reddy GV, Sablowski R, (2002). Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in Arabidopsis. *Development* 129:3207-17.

Groover AT, Mansfield SD, Difazio SP, *et al.*, (2006). The *Populus* homeobox gene *ARBORKNOX1* reveals overlapping mechanisms regulating the shoot apical meristem and the vascular cambium. *Plant Molecular Biology* 61:917-32.

Israelsson M, Eriksson ME, Hertzberg M, Aspeborg H, Nilsson P, Moritz T, (2003). Changes in gene expression in the wood-forming tissue of transgenic hybrid aspen with increased secondary growth. *Plant Molecular Biology* 52:893-903.

Jasinski S, Piazza P, Craft J, *et al.*, (2005). KNOX action in Arabidopsis is mediated by coordinate regulation of cytokinin and gibberellin activities. *Current Biology* 15:1560-5.

Karlberg A, Englund M, Petterle A, *et al.*, (2010). Analysis of global changes in gene expression during activity-dormancy cycle in hybrid aspen apex. *Plant Biotechnology* 27:1-16.

Lachaud S, Catesson AM, Bonnemain JL, (1999). Structure and functions of the vascular cambium. *Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences* 322:633-50.

Lang GA, Early JD, Martin GC, Darnell RL, (1987). Endodormancy, Paradormancy, and Ecodormancy - Physiological terminology and classification for dormancy research. *Hortscience* 22:371-7.

Larson PR, (1994). *The Vascular Cambium*. New York, Berlin, Heidelberg: Springer-Verlag.

Laux T, Mayer KFX, Berger J, Jurgens G, (1996). The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* 122:87-96.

Lenhard M, Jurgens G, Laux T, (2002). The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in Arabidopsis shoot meristem regulation. *Development* 129:3195-206.

Liebsch D, Sunaryo W, Holmlund M, *et al.*, (2014). Class I KNOX transcription factors promote differentiation of cambial derivatives into xylem fibers in the Arabidopsis hypocotyl. *Development* 141:4311-9.

Liu L, Ramsay T, Zinkgraf M, *et al.*, (2015a). A resource for characterizing genome-wide binding and putative target genes of transcription factors expressed during secondary growth and wood formation in Populus. *Plant Journal* 82:887-98.

Liu L, Zinkgraf M, Petzold HE, Beers EP, Filkov V, Groover A, (2015b). The Populus ARBORKNOX1 homeodomain transcription factor regulates woody growth through binding to evolutionarily conserved target genes of diverse function. *New Phytologist* 205:682-94.

Long JA, Moan EI, Medford JI, Barton MK, (1996). A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of Arabidopsis. *Nature* 379:66-9.

Mayer KFX, Schoof H, Haecker A, Lenhard M, Jurgens G, Laux T, (1998). Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* 95:805-15.

Nieminen K, Immanen J, Laxell M, *et al.*, (2008). Cytokinin signaling regulates cambial development in poplar. *Proceedings of the National Academy of Sciences of the United States of America* 105:20032-7.

Rinne PLH, Kaikuranta PM, Van Der Schoot C, (2001). The shoot apical meristem restores its symplasmic organization during chilling-induced release from dormancy. *Plant Journal* 26:249-64.

Sambrook J, Russell DW, (2001). *Molecular cloning: A laboratory manual*. New York: Cold Springs Harbour Press.

Schoof H, Lenhard M, Haecker A, Mayer KFX, Jurgens G, Laux T, (2000). The stem cell population of Arabidopsis shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 100:635-44.

Schrader J, Baba K, May ST, *et al.*, (2003). Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. *Proceedings of the National Academy of Sciences of the United States of America* 100:10096-101.

Schrader J, Nilsson J, Mellerowicz E, *et al.*, (2004). A high-resolution transcript profile across the wood-forming meristem of poplar identifies potential regulators of cambial stem cell identity. *Plant Cell* 16:2278-92.

Scofield S, Dewitte W, Nieuwland J, Murray JaH, (2013). The Arabidopsis homeobox gene SHOOT MERISTEMLESS has cellular and meristem-organisational roles with differential requirements for cytokinin and CYCD3 activity. *Plant Journal* 75:53-66.

Spokevicius AV, Tibbits JFG, Bossinger G, (2007). Whole plant and plant part transgenic approaches in the study of wood formation - benefits and limitations. *Transgenic Plant Journal* 1:49-59.

Van Beveren KS, Spokevicius AV, Tibbits J, Wang Q, Bossinger G, (2006). Transformation of cambial tissue *in vivo* provides efficient means for Induced Somatic Sector Analysis (ISSA) and gene testing in stems of woody plants species. *Functional Plant Biology* 33:629-38.

Williams L, Fletcher JC, (2005). Stem cell regulation in the Arabidopsis shoot apical meristem. *Current Opinion in Plant Biology* 8:582-6.

Yanai O, Shani E, Dolezal K, *et al.*, (2005). Arabidopsis KNOX1 proteins activate cytokinin biosynthesis. *Current Biology* 15:1566-71.

Ye ZH, (2002). Vascular tissue differentiation and pattern formation in plants. *Annual Review of Plant Biology* 53:183-202.

Zheng J, Xi ML, Lu YZ, Lu Y, Shi JS, (2013). Transcriptional Analysis Provides New Insights into Cold- and Dehydration-Tolerance Signaling Pathways and on Regulation of Stem Cell Activity in the Vascular Cambium of Poplar. *Plant Molecular Biology Reporter* 31:75-86.

Figure Legends

Figure 1: Sequence showing removal of phloem and cutting of sample in preparation for SEM. A 1 mm thick sample containing a transgenic sector is selected (a) and the phloem and cambial zone is peeled off (b), leaving only xylem tissue (c). This exposes the cambial surface and the longitudinal appearance of the transgenic sector (d) (3-dimensional view). The sample is then sliced transversely with a double-edged razor blade through the middle of the sector (e) leaving a smooth surface for imaging. A small cut is made into the centripetal surface to help identify the sector tissue when viewed under SEM (f). x = xylem; p = phloem; cs = cambial surface; blue = transgenic sector (GUS-stained); grey line = transverse cut; thick line = cambial zone

Figure 2: Average cell wall thickness of 35S::ARK1 and GW-control transgenic fibre cells compared to adjacent non transgenic control fibres based on position from the ‘cambial surface’ in samples harvested after three months. Cell wall of fibres transformed with the 35S::ARK1 vector (ARK1) resulted in reduced cell wall thickness in fibres at most positions investigated when compared to the non-transgenic adjacent cells (a) while no significant differences were observed between GW-control (GUS only) and adjacent non-transgenic fibres (b). Cell numbering starts with ‘Cell 1’ (1) being closest to the cambial surface (c). P-values were determined from paired t-tests comparing the transgenic and adjacent non-transgenic control fibre means with significance reported for values below $\alpha = 0.05$ denoted with *. cs = cambial surface.

Figure 3: Images showing how transgenic tissue is identified and scanning electron micrographs of both ARK 1 transgenic tissue and adjacent non transgenic tissue near a dormant cambium. Colour photograph of samples (a) where a cut is made (red arrows) on the centripetal surface is used to identify transgenic GUS positive tissue (blue arrows) in scanning electron micrographs (b) and compare this to adjacent non transgenic tissue (green arrows). An ARK1 sector (c) where immature fibre cells with relatively thinner cell walls were identified immediately next to the ‘cambial surface’ (white arrows)

when compared to adjacent non transgenic tissue (d) where cells containing thinner cell walls at the cambial surface were not observed (black arrows). V = vessel, F = fibres, cs = cambial surface, scale bar = 50 μ m (for both images).

Supplementary Materials

Supplementary Table 1 – Average cell and lumen width in both the tangential and radial plane (including standard errors) for both the 35::*ARK1* and GUS only vectors and adjacent non-transgenic tissue.

Supplementary Table 2 – Average cell, lumen and cell wall area (including standard errors) for both the 35::*ARK1* and GUS only vectors and adjacent non-transgenic tissue.

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:

Melder, E;Bossinger, G;Spokevicius, AV

Title:

Overexpression of ARBORKNOX1 delays the differentiation of induced somatic sector analysis (ISSA) derived xylem fibre cells in poplar stems

Date:

2015-10-01

Citation:

Melder, E., Bossinger, G. & Spokevicius, A. V. (2015). Overexpression of ARBORKNOX1 delays the differentiation of induced somatic sector analysis (ISSA) derived xylem fibre cells in poplar stems. TREE GENETICS & GENOMES, 11 (5), <https://doi.org/10.1007/s11295-015-0912-8>.

Persistent Link:

<http://hdl.handle.net/11343/282822>