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The *Populus* homeobox gene *ARBORKNOX2* regulates cell differentiation during secondary growth

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SUMMARY

The stem cells of the vascular cambium divide to produce daughter cells, which in turn divide before undergoing differentiation during the radial growth of woody stems. The genetic regulation of these developmental events is poorly understood, however. We report here the cloning and functional characterization of a *Populus* class-I KNOX homeobox gene, *ARBORKNOX2* (*ARK2*), which we show influences terminal cell differentiation and cell wall properties during secondary growth. In the early stages of secondary growth, *ARK2* is expressed broadly in the cambial zone and in terminally differentiating cell types, before becoming progressively restricted to the cambium. *ARK2* overexpression and synthetic miRNA-suppression transgenics reveal positive correlations between *ARK2* expression level and the timing of cambium formation, the width of the cambial zone and inhibition of cambial daughter cell differentiation. These phenotypes in turn correlate with changes in the expression of genes affecting transcription, cell division, auxin and cell wall synthesis. Notably, wood properties associated with secondary cell wall synthesis are negatively associated with *ARK2* expression, including lignin and cellulose content. Together, our results suggest that *ARK2* functions primarily to regulate a complex suite of genes that together influence cell differentiation during secondary growth. We propose that *ARK2* may represent a co-evolved transcriptional module that influences complex, adaptive wood properties.

Keywords: cell wall synthesis, class I KNOX, lignification, forest trees, wood development.

INTRODUCTION

Secondary growth is the developmental process driving the radial expansion of woody stems, and is supported by a lateral meristem: the vascular cambium. The stem cells of the cambium are maintained in a dynamic stem cell niche, the cambium initials. The fusiform cambial initials are oriented longitudinally relative to the stem, and undergo periclinal divisions that produce two types of daughter cells, phloem mother cells centrifugally and xylem mother cells centripetally. Phloem and xylem mother cells undergo additional rounds of periclinal division to form transit cell populations that ultimately undergo terminal differentiation within secondary xylem (wood) or secondary phloem (bark) (Larson, 1994).

The regulation of cell division in the cambial zone and cell differentiation in secondary vascular tissues is dynamic, responsive to environmental conditions and tightly regulated. The conspicuous developmental gradient radiating from the dividing cells of the cambial zone into terminally-differentiated lignified cell types of secondary xylem illustrates the tight regulation of cell division and differentiation. At the same time, variation in the width of the cambial zone and the differentiation of daughter cells can vary dramatically during the growing season, and with environmental conditions. For example, in some angiosperm species, favorable spring conditions with ample water leads to a wide cambial zone and the formation of 'earlywood'. Earlywood is characterized by vessel elements with wide lumens, that are efficient water conductors, but are more prone to drought-induced cavitation than the narrow lumens of 'latewood' vessel elements (Larson, 1994). Leaning woody stems of angiosperms change rates of cell division and the differentiation of cambial daughter cells to produce 'tension wood' on the upper surface of the stem, which is enriched in highly crystalline cellulose (Jourez *et al.*, 2001). The regulation of cell division and cell differentiation during secondary growth is thus dynamic, adaptive, and vital to proper growth and development. However, the underlying developmental mechanisms responsible are poorly understood.

Compared with the cambium, the regulation of the shoot apical meristem (SAM) is increasingly well understood (Williams and Fletcher, 2005; Scheres, 2007). Class-I KNOX transcription factors are well characterized for their roles in regulating the SAM. The Arabidopsis class-I KNOX homeobox gene *SHOOTMERISTEMLESS* (*STM*) is expressed broadly in the SAM, and is downregulated in organ primordia (Lincoln *et al.*, 1994). Loss-of-function *stm* mutants lack a functional SAM as a result of differentiation of stem cells during embryogenesis (Long *et al.*, 1996). *BREVIPEDICELLUS* (*BP*, also known as *KNAT1*) is expressed in the peripheral zone of the SAM, and is also expressed in cortical tissue, vascular tissue and in phloem cells adjacent to the inflorescence stem cortex (Lincoln *et al.*, 1994; Venglat *et al.*, 2002; Douglas and Riggs, 2005). Loss-of-function *bp* mutants exhibit short internodes and pedicels, downward-pointing siliques and stripes of chlorenchyma-deficient tissues in inflorescence stems (Douglas *et al.*, 2002; Venglat *et al.*, 2002). Overexpression of *BP* and *BP* orthologs can cause leaf lobing, meristem formation on the adaxial leaf surface and increased cytokinin levels in several species (Lincoln *et al.*, 1994; Chuck *et al.*, 1996; Ori *et al.*, 2000; Frugis *et al.*, 2001). In Arabidopsis, *BP* overexpression plants have impaired lignin deposition in inflorescence stems during primary growth, whereas *bp* plants show ectopic lignification in the interfascicular region of the inflorescence stem (Mele *et al.*, 2003). Importantly, the expression of key cell wall synthesis-related genes show negative correlation with *BP* expression levels, and BP protein binds to the promoters of some of these genes *in vitro* (Mele *et al.*, 2003). Partially redundant roles of *STM* and *BP* are revealed by *as1 stm* double mutants, which form a functional SAM that is lost in *as1 stm bp* triple mutants (Byrne *et al.*, 2002).

We previously showed that an ortholog of *STM*, *ARBORKNOX1* (*ARK1*), is expressed not only in the SAM but also in the cambial zone of *Populus* (Groover *et al.*, 2006). Detailed analysis of *ARK1* transgenics suggests that *ARK1* regulates specific aspects of cambial functions and cell differentiation during secondary growth in *Populus*, including regulation of cell wall biosynthesis (Groover *et al.*, 2006). That *ARK1* has important function in both the shoot apical meristem and the cambium illustrates that at least some mechanisms regulating the SAM have been co-opted during the evolution of the vascular cambium and secondary growth (Groover, 2005).

Although a presumed ortholog of *BP* has been shown to be expressed in the cambial zone of *Populus* (Schrader *et al.*, 2004), there have been no functional studies of the role of *BP* in secondary growth. Arabidopsis inflorescence stems do not form a vascular cambium or undergo secondary growth and thus defining aspects of *bp* mutants may or may not be directly relevant in woody species (e.g. the naming feature of foreshortened pedicles). It is thus unclear whether or not conclusions about *BP* function in the shoot apical meristem and primary growth in Arabidopsis are good predictors of *BP* function during woody growth.

Here, we describe the cloning and functional characterization of a *Populus BP* ortholog, *ARBORKNOX2* (*ARK2*). We show that *ARK2* displays an unexpectedly complex expression pattern that changes during the course of stem growth, and can include not only the cambial zone, but also actively lignifying cells. Transgenic *Populus* overexpressing *ARK2* or expressing a synthetic miRNA that targets *ARK2* transcripts present anatomical, gene expression and wood biochemical phenotypes consistent with a role for *ARK2* in regulating cell differentiation and wood properties. Our results suggest that *ARK2* affects wood phenotypes by regulating complex suites of genes with diverse functions.

RESULTS

ARK2 is a *Populus BP* ortholog

A putative ortholog of Arabidopsis *BP* was cloned from hybrid aspen clone INRA 717-IB4 (*Populus alba* × *Populus tremula*) and named *ARK2*. The full-length transcript (GenBank Accession bankit 1195038) includes 1107 bp of protein coding sequence, 571 bp of 5′ untranslated region (UTR) and 633 bp of 3′-UTR. As shown in Figure 1(a), the ARK2 protein contains conserved sequences found in the class-I *KNOX* gene family members encoding HOMEODOMAIN, KNOX and ELK domains. ARK2 is 69% identical and 83% similar at the amino acid level to Arabidopsis BP (Figure 1a). Phylogenetic analysis of *Populus* and Arabidopsis class-I KNOX proteins shows that ARK2 is more similar to BP than to any other Arabidopsis homeodomain protein, strongly suggesting that *ARK2* is a *Populus* ortholog of *BP* (Figure 1b). Analysis of the complete *Populus trichocarpa* genome revealed a single *ARK2* ortholog, estExt_Genewise1_v1.C_LG_II1820 (Figure 1b).

ARK2 has a dynamic expression pattern during primary and secondary growth

ARK2 expression is detectable in shoot apices, stems (both nodes and internodes) and roots, but not in leaves (Figure 1c). Whole-mount *in situ* hybridization (see Experimental procedures for details) of *Populus* stem internodes at different developmental stages reveals a complex expression pattern that changes through development. *ARK2* transcripts are detected in procambium tissue in the first elongating internode during primary growth (Figure 2a,b). Progressing down the stem and into transitional stages to secondary growth, *ARK2* is expressed broadly in the cambial zone and phloem of internode 3 (Figure 2g,h) and internode 5 (Figure 2m,n). Unexpectedly, *ARK2* expression is also found in differentiating phloem fibers that are well separated from the cambium, and also in secondary xylem that includes terminally differentiating tracheary elements and

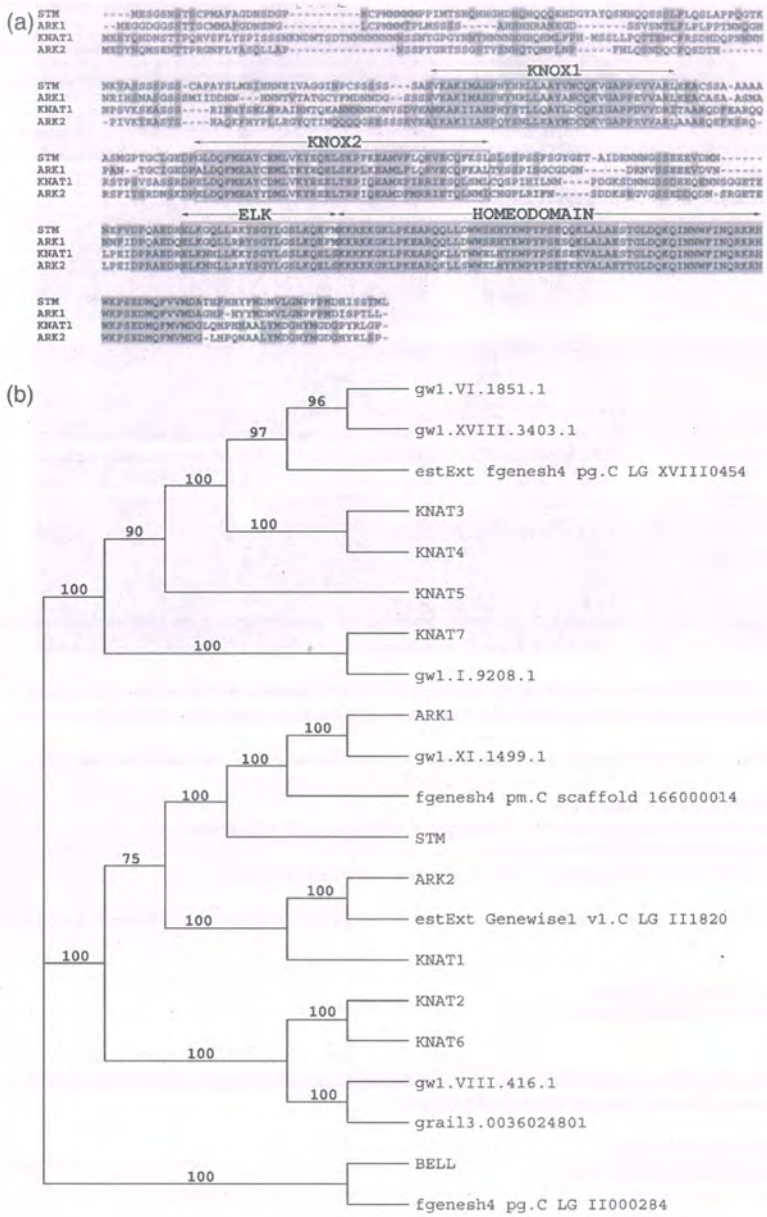

Figure 1. Sequence and phylogenetic comparison of *ARBORKNOX2* (*ARK2*) with Arabidopsis and *Populus* homologs.

(a) Amino acid sequence alignment of *ARK1* and *ARK2* from *Populus tremula* × *Populus alba*, and STM and KNAT1 from Arabidopsis.

(b) Neighbor-joining tree of class-I and -II KNOX proteins from Arabidopsis and *Populus*. ARK1 and ARK2 are from *P. tremula* × *P. alba*. Arabidopsis proteins are named using the convention KNAT (knotted-like *Arabidopsis thaliana*), with the exception of STM. The other *Populus* KNOX proteins shown are from the complete genome sequence of *P. trichocarpa*, using gene names from the Joint Genome Institute (JGI Populus genome release version 1.1). ARK2 is a member of the class-1 KNOX family, which is composed of *STM*, *KNAT1*, *KNAT2* and *KNAT6* in Arabidopsis. KNAT1 (BREVIPEDICELLUS) is more similar to ARK2 than to any other Arabidopsis protein. Numbers at nodes represent bootstrap values.

(c) Expression of ARK2 in organs, assayed by quantitative real-time PCR (qRT-PCR). Relative expression of ARK2 in apices, leaves, roots and stem was determined using qRT-PCR of 2-month-old tissue culture grown *P. tremula* × *P. alba*. ARK2 is expressed in all tissues assayed except leaves, and is highly expressed in stem tissue with active cambium. Stem tissue samples were confirmed to have a vascular cambium by phloroglucinol staining of secondary xylem. Relative expression (mean ± SE) was calculated from triplicate qRT-PCR reactions of independent RNA samples prepared from different trees.

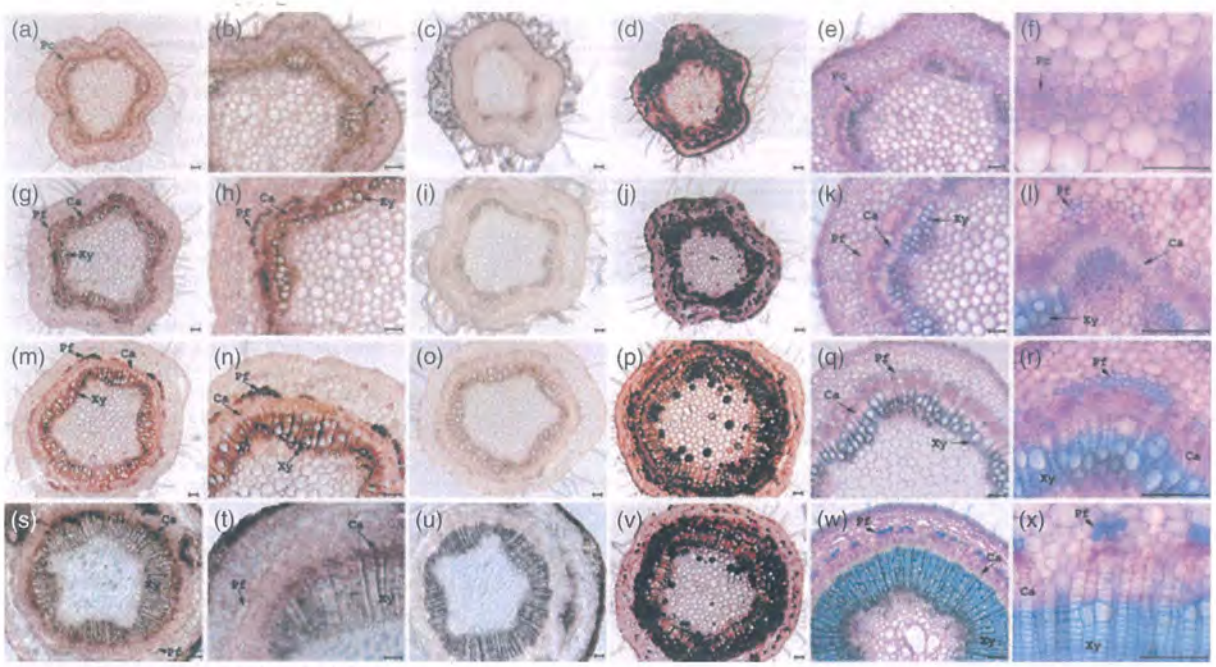

Figure 2. Expression of *ARBORKNOX2* (*ARK2*) during *Populus* stem development revealed by whole-mount *in situ* hybridization. Antisense (first and second column) and sense negative control (third column) *ARK2* probes were hybridized to stem sections from 2-month-old tissue culture-grown trees. The fourth and fifth columns are matched sections stained with toluidine blue.
(a) Section from first elongating internode hybridized with antisense *ARK2* probe. *ARK2* is expressed broadly during primary growth, with strongest expression associated with procambium.
(b) Higher magnification of first elongating internode hybridized with antisense *ARK2* probe.
(c) Section from first elongating internode hybridized with sense *ARK2* probe (negative control), showing minimal background hybridization.
(d) Section from first elongating internode hybridized with antisense pop50S probe (positive control).
(e) Section from the first elongating internode and stained with toluidine blue (see Experimental procedures) for anatomical reference.
(f) Higher magnification of (d).
(g) Section from the third internode, hybridized with antisense *ARK2* probe. *ARK2* is expressed broadly in the cambial zone, differentiating phloem fibers and lignifying xylem.
(h) Higher magnification of (f).
(i) Section from the third internode hybridized with negative control sense *ARK2* probe.
(j) Section from third internode hybridized with positive control antisense pop50S probe.
(k) Section from the third internode stained with toluidine blue.
(l) Higher magnification of (i).
(m) Section from fifth internode hybridized with antisense *ARK2* probe. The strongest *ARK2* expression is associated with differentiating secondary xylem and secondary phloem fibers, which are undergoing lignification. Lower expression is detected in the cambial zone.
(n) Higher magnification of (k).
(o) Section from fifth internode hybridized with sense *ARK2* probe (negative control).
(p) Section from fifth internode hybridized with positive control antisense pop50S probe.
(q) Section from fifth internode and stained with toluidine blue.
(r) Higher magnification of (n).
(s) Section from the base internode hybridized with antisense *ARK2* probe. *ARK2* expression is largely limited to the cambial zone.
(t) Higher magnification of (p).
(u) Section from the base internode hybridized with sense *ARK2* probe (negative control).
(v) Section from the base internode hybridized with positive control antisense pop50S probe.
(w) Section from the base internode stained with toluidine blue.
(x) Higher magnification of (s).
Ca, cambial zone; Pf, phloem fiber; Ph, phloem; Xy, xylem. Scale bar: 100 μm.

fibers. However, in the basal internode, *ARK2* expression becomes restricted to the cambial zone (Figure 2s,t). The secondary xylem almost certainly contains living parenchyma at this stage (although these cell types are limited in number), suggesting that earlier expression was primarily associated with early terminal differentiation of lignified cell types.

ARK2 expression affects secondary growth and stem anatomy

To understand the function of *ARK2* during secondary growth, hybrid poplar clone INRA 717-IB4 was transformed with constructs to either overexpress *ARK2* (*35S::ARK2*) or downregulate *ARK2* using an artificial microRNA

(*amiRNA::ark2*) or RNA interference (*RNAi::ark2*). *ARK2* was upregulated by threefold or greater in each of the three independently transformed *35S::ARK2* lines selected for in-depth characterization (see Figure S1a on line), whereas *ARK2* transcript was downregulated approximately fivefold or greater in the three independently transformed *miRNA:ark2* lines selected for further analysis (Figure S1b). Initial histological analysis of *RNAi::ark2* trees showed similar phenotypes to *amiRNA::ark2* trees (Figure S2), but in extended analysis the *RNAi::ark2* stem phenotype decreased in severity over time, and had a correspondingly modest decrease in *ARK2* transcript abundance (Figure S1c). These results suggest that *RNAi* knock-down was not stable, possibly because of transgene silencing. For this reason, additional analysis was limited to the *amiRNA::ark2* transgenics.

Altering *ARK2* expression results in changes in stem and leaf development. Compared with developmentally staged wild-type controls, *35S::ARK2* trees have darker green leaves (Figure 3b) and shorter internode lengths (Figure 3g). In contrast, *miRNA:ark2* trees have elongated internodes (Figure 3c,g), but no other obvious phenotypic changes. Leaf development is similar in wild-type (Figure 3d) and *miRNA:ark2* trees (Figure 3f). In contrast, *35S::ARK2* leaves have short petioles, a shorter blade that is more deltate in shape, with minor lobing, bulging of mesophyll between veins, and secondary veins that intersect the midvein shortly before it enters the petiole (Figure 3e).

ARK2 expression also correlates with changes to the cambial zone and the development of lignified tissues. Transverse sections of the first elongating internode of *35S::ARK2* trees have a nascent continuous vascular cambium with some secondary phloem (Figure 4b,d), in contrast to matched wild-type controls that do not have evidence of an interfascicular cambium (cambium between vascular bundles), and still have procambial strands (Figure 4a,c). Sections of the fourth internode of *35S::ARK2* stems have a more obvious cambium and a relatively wide cambial zone that has produced substantial secondary phloem (Figure 4f,h), whereas matched wild-type control sections are at the incipient stages of cambium formation (Figure 4e,g). The cambial zone and secondary phloem in the basal internode of *35S::ARK2* stems are increased in width (Figure 4j,l). At the same time, there are few phloem fibers and reduced lignified secondary xylem tissue (Figure 4j,l), in comparison with the wild type (Figure 4i,k). Thus, there is an overall decrease in the differentiation of lignified cell types in response to *ARK2* overexpression. Also, the wider cambial zone does not ultimately reflect a higher number of cell divisions leading to secondary xylem, as *35S::ARK2* trees have an average of 11.78 (SE 0.55) combined cell layers of cambium and xylem in the basal internode, versus 17.5 (SE 0.92) in wild-type trees.

Conversely, the stem anatomy of *amiRNA::ark2* trees reveal precocious differentiation of lignified cell types in secondary xylem, and of phloem fibers. The fourth internode of *amiRNA::ark2* transgenics already has obvious lignified phloem fibers (Figure 5b) that are lacking in matched wild-type trees at the same developmental stage (Figure 5a). There is no distinct difference in the timing of cambium formation, however. The seventh internode of *amiRNA::ark2* has increased lignified phloem fibers and a mature cylinder of continuous lignified xylem (Figure 5d,f), whereas wild-type controls do not have lignified fibers or a continuous ring of secondary xylem (Figure 5c,e). No strong anatomical differences were detected in the basal internodes of wild-type (Figure 5g) versus *amiRNA::ark2* trees (Figure 5h).

Expression of genes underlying secondary growth are altered in *ARK2* transgenics

Gene expression profiles of defoliated stems from *35S::ARK2*, *amiRNA::ark2* and matched wild-type control trees were determined using an Affymetrix *Populus* array containing probes for most of the predicted genes in the *Populus* genome (see Experimental procedures). Statistical analysis (see Experimental procedures) found that 869 genes show both statistically significant ($P \leq 0.05$) and twofold or greater expression differences in the *35S::ARK2* transgenic trees relative to the corresponding wild-type controls (Table S6). A similar analysis found only 63 genes that are 1.5-fold or greater misregulated in the *amiRNA::ark2* transgenic trees, relative to the corresponding wild-type controls (Table S7). The smaller number of genes misregulated in *amiRNA::ark2* transgenics is consistent with the less severe phenotypes of these trees, which could reflect partial redundancy of *ARK2* and *ARK1* (a *Populus* ortholog of *STM*), as has been shown for *BP* and *STM* in Arabidopsis (Byrne *et al.*, 2002; Magnani and Hake, 2008).

Genes misexpressed in the stems of *ARK2* transgenic trees reflect functional classes associated with these developmental gradients found extending across the cambial zone and into secondary vascular tissues, and include transcriptional regulators, hormonal regulation, cell division, cell expansion and cell differentiation-related genes, as discussed below.

ARK2 transgenics have altered expression of key transcriptional regulators

ARK transgenics (Table S1) misexpress several transcription factors previously described as having basal meristem functions and roles in the regulation of tissue polarity. Members of the class-III HD ZIP gene family act antagonistically with *YABBY* genes in regulating meristem functions and polarity, including polarity of vascular bundles (McConnell *et al.*, 2001; Emery *et al.*, 2003). Arabidopsis *YABBY1* (*FIL*) and *YAB3* genes downregulate meristematic genes during lateral organ development and promote abaxial cell fates, and *yab1 yab3* double mutants ectopically

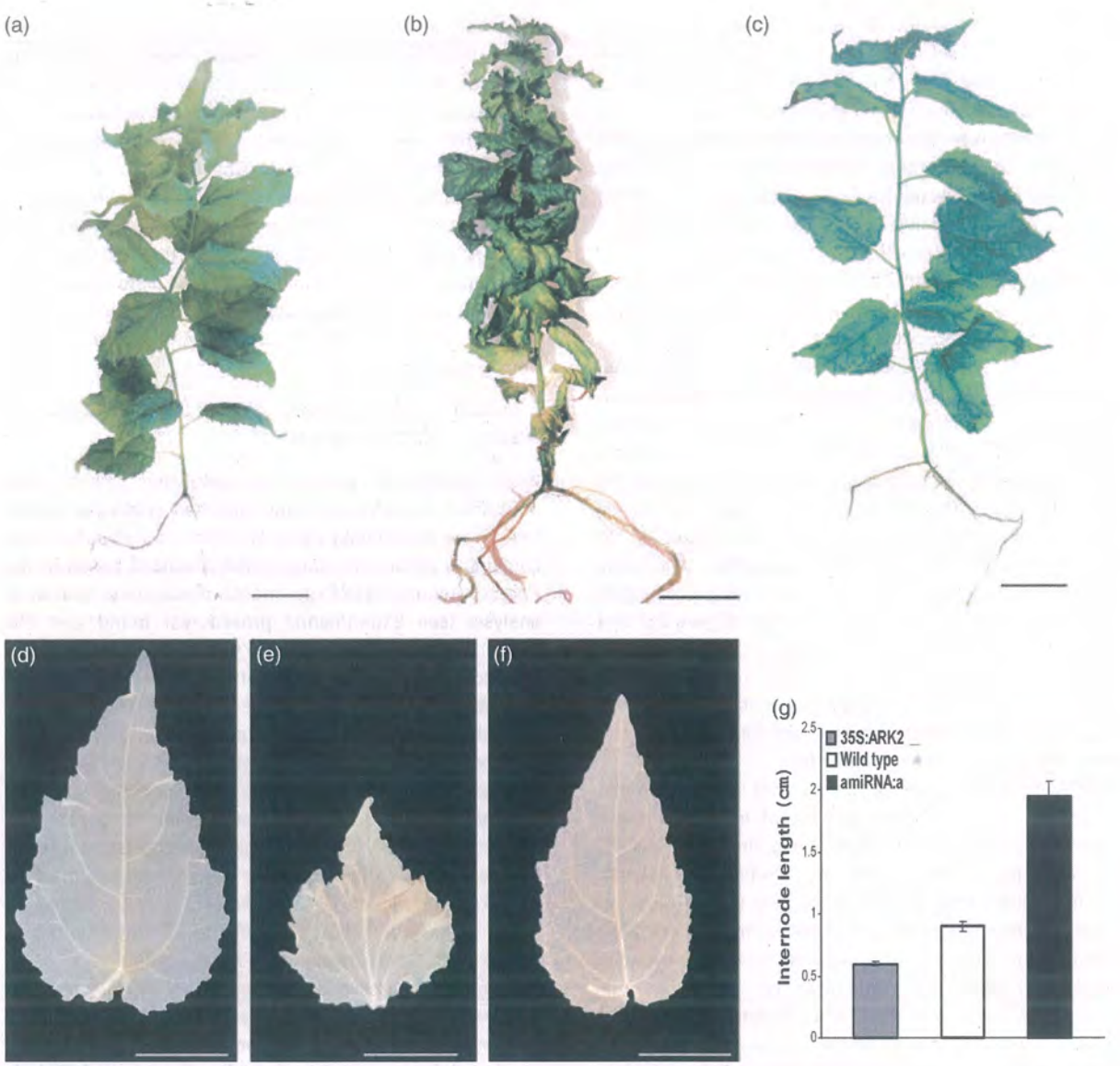

Figure 3. Phenotypes of *ARBORKNOX2* (*ARK2*) overexpression and knock-down plants compared with wild-type controls.
(a) Wild-type plant (2 months old).
(b) *35S:ARK2* overexpression (2 months old) plants have changes to plant architecture, including shorter internodes and altered leaf shapes.
(c) *amiRNA:ark2* knock-down plants (2 months old) have longer internodes.
(d) Leaf of a wild-type plant from the fifth internode.
(e) Leaf of a *35S:ARK2* plant from the fifth internode with deltate shape, altered venation pattern and foreshortened petiole.
(f) Leaf of an *amiRNA:ark2* plant from the fifth internode, showing no strong differences from the wild type.
(g) Inverse relationship of ARK2 expression and internode length shown for wild type, *35S:ARK2*, *amiRNA:ark2* (from internode 2 to internode 7 of 2-month-old plants). Values are reported as means ± SEs ($n = 3$), Student's *t*-test $P < 0.05$, compared with the wild type. Scale bar = 2.5 cm.

express *BP*, and form meristems on the adaxial surfaces of the cotyledons and leaf blades (Kumaran *et al.*, 2002). Three putative *Populus* orthologs of *YABBY1* (grail3.0035001101, grail3.0033028501, and grail3.0018017701) are downregulated in the *35S::ARK2* trees, and are upregulated in *amiRNA::ark2* trees, consistent with increased meristematic potential. Class-III HD ZIPs play partially redundant, overlapping roles in regulating the shoot apical meristem, polarity and vascular development. Triple *revoluta* (*rev*) *phavoluta* (*phv*) *phabulosa* (*phb*) mutants have abaxialized vascular bundles that have phloem surrounding xylem, whereas gain-of-function alleles of *PHAB* or *PHV* have adaxialized bundles with xylem surrounding phloem (McConnell and Barton, 1998; McConnell *et al.*, 2001; Emery *et al.*, 2003). *35S::ARK2* trees downregulate putative orthologs of *REV* (estExt_fgenesh4_pm.C_LG_VI0713) and two putative ortho-

logs of *PHAB/PHAV* (estExt_fgenesh4_pg.C_LG_I2905, estExt_fgenesh4_pg.C_2360002), whereas the *amiRNA::ark2* trees upregulate *Populus REV* by 1.4-fold. These changes in *YABBY* and class-III HD ZIP gene expression could be causative or a consequence of the delayed differentiation of secondary xylem in the *35S::ARK2* stems, and the observed enhancement of secondary xylem differentiation in *amiRNA::ark2* trees.

The Arabidopsis homeobox gene *WUSCHEL* (*WUS*) is required for stem cell maintenance in the shoot apical meristem (Mayer *et al.*, 1998). The putative *Populus* ortholog of *WUS* (estExt_fgenesh4_pg.C_570090) is downregulated in *35S::ARK2* stem tissue (Table S1). This stands in conflict with previous reports that a *Populus WUS* ortholog is not expressed in *Populus* stems (Schrader *et al.*, 2004). However, quantitative real-time PCR (qRT-PCR) using gene-specific primers for *estExt_fgenesh4_pg.C_570090* confirmed expression in the stem (data not shown). Phylogenetic analysis of *WUS*-like genes in *Populus* revealed that *estExt_fgenesh4_pg.C_570090* is one of three duplicated *WUS* paralogs (not shown). It is thus possible that Schrader *et al.* (2004) assayed a different *WUS* ortholog, and that *estExt_fgenesh4_pg.C_570090* acquired a new expression pattern to function during secondary growth.

The *SHORT-ROOT* (*SHR*) and *SCARECROW* (*SCR*) transcription factors are involved in specifying tissue identity in Arabidopsis roots (Helariutta *et al.*, 2000). In the cambial zone, two close homologs of *SHR*, *PttSHR1* and *PttSHR2*, have increased expression towards the phloem (Schrader *et al.*, 2004). A putative *Populus* ortholog of *SHR*, gw1.VII.712.1, is downregulated in *35S::ARK2* transgenics (Table S1). The MADS-box gene *AGL12* (*XAL1*) has been identified as a modulator of cell proliferation versus differentiation, and *xal1* mutants have short roots with an altered cell proliferation rate, meristem size and cell-cycle

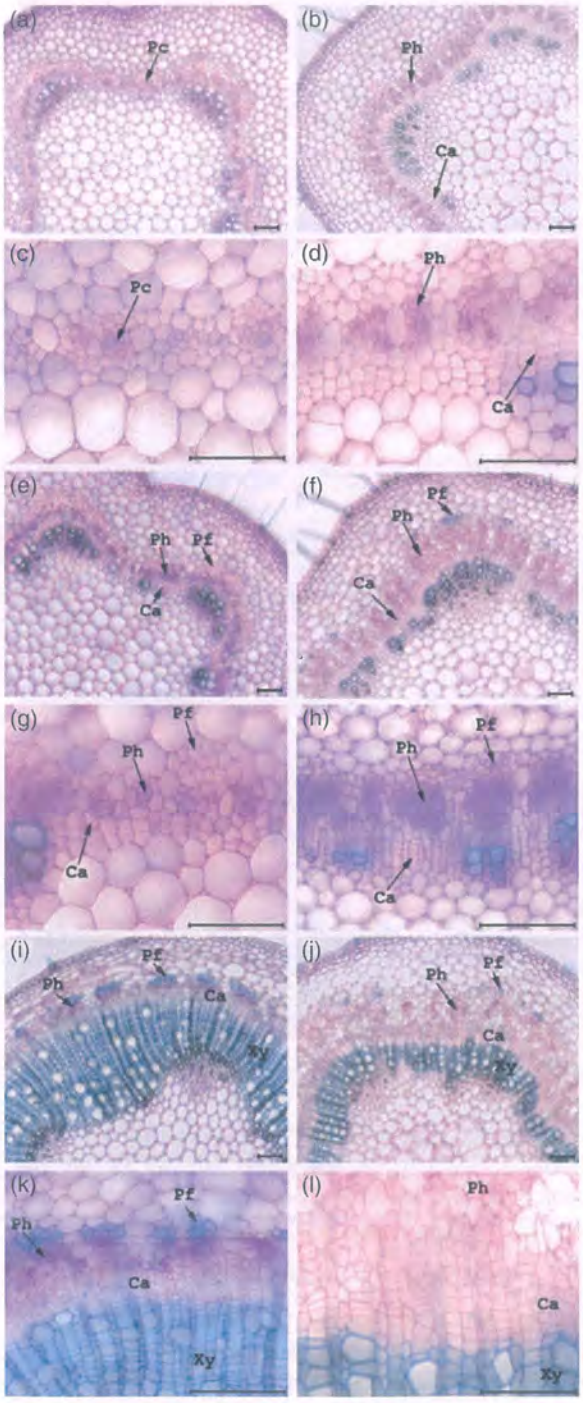

Figure 4. Transverse sections of stems from 2-month-old wild-type and *35S:ARK2 Populus* stained with toluidine blue.
(a) Section from first elongating internode of a wild-type *Populus* stem during primary growth.
(b) Section from first elongating internode of a *35S:ARK2 Populus* stem showing early formed cambium and early secondary xylem and phloem.
(c) Higher magnification of a section from the first elongating internode of the wild type. Arrows indicate the location of procambium, which is composed of small, dark stained cells surrounded by ground tissue.
(d) Higher magnification of section from first elongating internode of *35S:ARK2 Populus*, showing early cambium and phloem.
(e) Section from fourth elongating internode of a wild-type *Populus* stem.
(f) Section from the fourth elongating internode of a *35S:ARK2 Populus* stem showing a widened cambial zone.
(g) Higher magnification of a section from the fourth elongating internode of the wild type, showing initiation of cambial activity.
(h) Higher magnification of a section from the fourth elongating internode of *35S:ARK2 Populus*, showing obvious cambial activity and copious phloem.
(i) Section from the bottom internode of a wild-type *Populus* stem, with a continuous cylinder of cambium, secondary xylem and secondary phloem fibers, with lignified secondary cell walls.
(j) Section from the bottom internode of *35S:ARK2* transgenic *Populus* showing altered secondary growth. The cambial zone is increased in width, and differentiation of cambial daughter cells within secondary xylem tissue is inhibited. Phloem fibers are absent from their normal positions and/or are inhibited in their differentiation.
(k) Higher magnification of the section from a wild-type bottom internode.
(l) Higher magnification of the section from a bottom internode of *35S:ARK2 Populus* with a wide cambial zone.
Ca, cambial zone; Pc, procambium; Pf, phloem fiber; Ph, phloem; Xy, xylem. Scale bar: 100 μm.

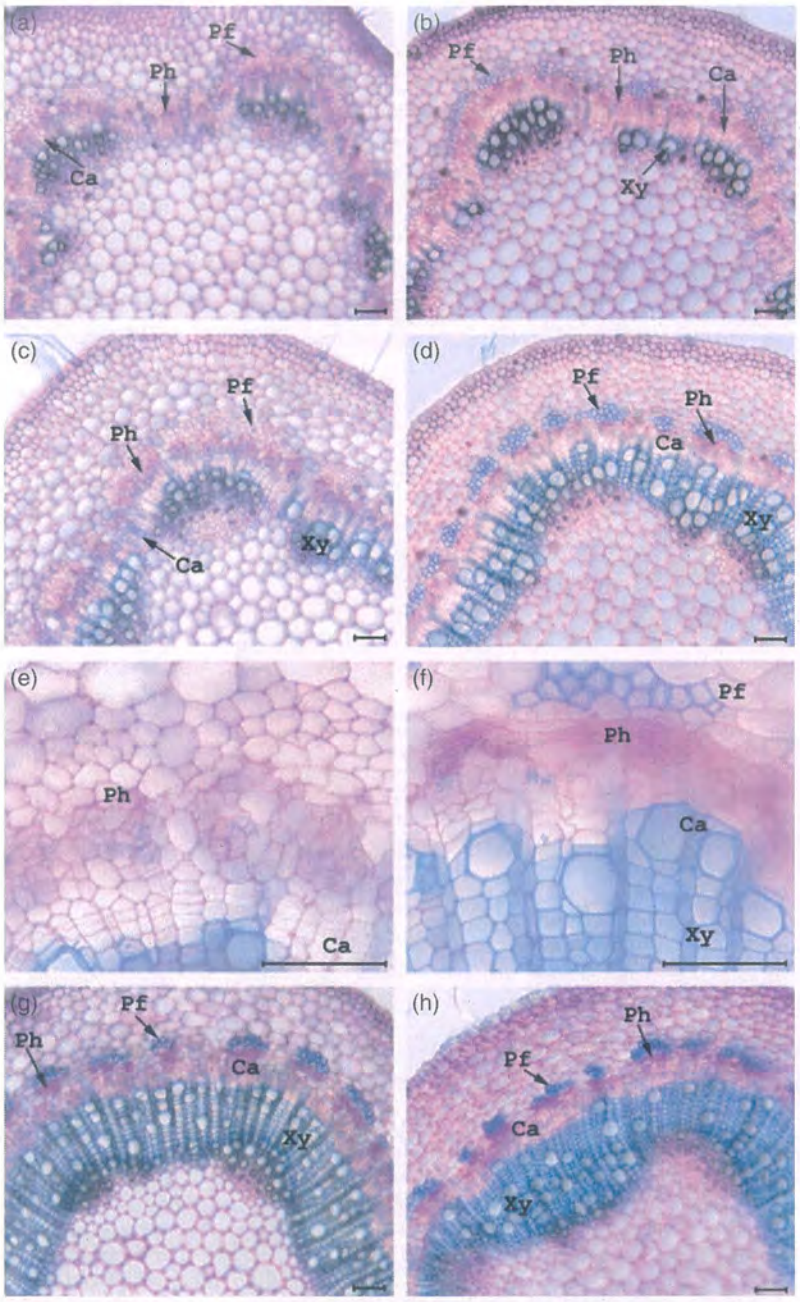

Figure 5. Transverse sections of 2-month-old wild type and *amiRNA:ark2 Populus* stained with toluidine blue.
(a) Section from the fourth internode of a wild-type *Populus* stem showing procambium.
(b) Section from the fourth internode of an *amiRNA:ark2* transgenic stem, showing early transition to secondary growth, including cambium and early secondary phloem fibers and xylem cells.
(c) Section from the seventh internode of a wild-type *Populus* stem, showing secondary growth.
(d) Section from the seventh internode of an *amiRNA:ark2* transgenic stem, showing *ark2* with precocious secondary phloem fibers and secondary xylem.
(e) Higher magnification of the section from the seventh internode of a wild-type *Populus* stem.
(f) Higher magnification of a cross section from the seventh internode of *amiRNA:ark2*.
(g) Section from the base internode of a wild-type *Populus* stem.
(h) Section from the base internode of an *amiRNA:ark2* stem.
Ca, cambial zone; Pc, procambium; Pf, phloem fiber; Ph, phloem; Xy, xylem. Scale bar: 100 μm.

duration (Tapia-Lopez *et al.*, 2008). A putative *Populus* ortholog of *AGL12* (grail3.0041013401) is downregulated in *35S::ARK2* trees, consistent with the inhibition of cell differentiation.

KNOX proteins are known to form heterodimers with BEL-like homeodomain transcription factors (Bellaoui *et al.*, 2001; Muller *et al.*, 2001; Byrne *et al.*, 2003; Smith and Hake, 2003). A BEL1-like protein (fgenesh4_pg.C_LG_II000285) is downregulated in *35S::ARK2* stems and upregulated in *amiRNA::ark2* stems (Table S1). Previous studies found no evidence of physical interaction between BP and the Arabidopsis ortholog of fgenesh4_pg.C_LG_II000285, however, leaving the significance of this misregulation unclear.

ARK2 and cell division in the cambial zone

Cell division is a defining process of the cambial zone. Cell cycle related genes *Cyclin A1* and *Cyclin D3*, cyclin-dependent kinase CDKB2, *Cyclin A2* (*PttCYCA2*) and a *DP-E2F-like*

(*DEL*) gene show similar profiles across the cambial zone, with a steep increase in expression towards the xylem (Schrader *et al.*, 2004). Histone H4 expression is associated with DNA replication (Chaubet *et al.*, 1996), and a *Populus* histone H4-encoding gene is expressed maximally in the cambium proliferation zone, and declines in developing xylem and phloem. A histone H4-encoding gene (*grail3.0121000801*), orthologs of *DEL 1* (*fgenesh4_pm.C_LG_I001083*), *Cyclin A2* (*eugene3.00061782*) and Cyclin D3 (*estExt_Genewise1_v1.C_LG_IX2293*) genes are all downregulated in *35S::ARK2* stems. These observations aid in explaining the apparent wider cambial zone but fewer total number of cambial plus secondary xylem layers observed in the *35S::ARK2* stems, and are likely to reflect delayed division of xylem mother cells.

Cell expansion genes are positively correlated with *ARK2* expression

After dividing, precursors for cell types in secondary xylem, such as vessels, undergo significant expansion before terminal differentiation (Larson, 1994). Several genes thought to play a role in cell expansion are expressed in the cambium zone (Schrader *et al.*, 2004). *35S::ARK2* trees show an upregulation of several of these genes, including genes encoding xyloglucan endotransglycosylase (*fgenesh4_pg.C_LG_VI0-00528*), pectin methylesterase (*estExt_Genewise1_v1.C_LG_VII1401*), three expansins (*gw1.118.151.1*, *estExt_fgenesh4_pg.C_LG_VI1270* and *grail3.0020020701*) and an aquaporin (*grail3.0065000501*). The cells of the cambial zone of *35S::ARK2* appear extended in the radial dimension compared with wild-type trees (Figure 4k,l), which could be a consequence of misregulation of the aforementioned genes.

ARK2 misregulation changes hormone-related gene expression

Hormones, including auxin, have long been known to affect vascular development (Jacobs, 1952). Several genes are misexpressed in the *35S::ARK2* transgenics that are associated with specific hormones known to influence vascular development or xylem formation. Two *Populus* orthologs (*estExt_Genewise1_v1.C_LG_XI072* and *estExt_fgenesh4_pg.C_LG_XV0366*) of the auxin efflux carrier *PIN-FORMED* (*PIN1*) are downregulated in *35S::ARK2*. BP is thought to antagonize PIN1 activity during leaf initiation (Hay *et al.*, 2006). Our results suggest a similar relationship may exist in the cambial zone, with BP negatively regulating *PIN1* at the level of transcription. Various auxin-responsive *AUX/IAA* family members are also downregulated in *35S::ARK2*, including *IAA3*, which has been shown to negatively influence cell division in the cambial region in *Populus* (Nilsson *et al.*, 2008). In addition, putative *Populus* orthologs of auxin modification *IAA-amino acid hydrolase 1* (*ILR1*) (*gw1.XVI.2349.1*) is upregulated in response to *ARK2* overexpression. Cut stems of *35S::ARK2* plants respond to exogenous IAA by callusing, whereas wild-type trees respond by rooting (data not shown), indicating that ARK misexpression also has auxin-related physiological consequences.

GA20 oxidase expression is excluded from the shoot apical meristem, but is expressed in the zones where initial differentiation occurs (Nagasaki *et al.*, 2001). Importantly, *GA20 oxidase* expression is negatively regulated directly by class-I KNOX transcription factors (Sakamoto *et al.*, 2001). Also, GA20 oxidase-overexpressing aspen (Israelsson *et al.*, 2003) and tobacco (Biemelt *et al.*, 2004) have significantly increased biomass accumulation and increased xylem formation. Two genes encoding putative GA20 oxidases (*estExt_fgenesh4_pm.C_LG_V0384* and *fgenesh4_pm.C_LG_XIV000079*) are downregulated in *35S::ARK2* and are upregulated in the *amiRNA::ark2* stems, consistent with the negative regulation of GA20 oxidase by *ARK2*, and the negative relationship between ARK expression and secondary xylem production.

ARK2 influences expression of genes associated with terminal cell differentiation

Expression of genes coding for enzymes involved in carbohydrate biosynthesis and secondary cell wall formation are negatively correlated with *ARK2* expression in *ARK2* transgenics (Table S2). Cellulose and lignin content in the secondary cell walls of woody tissues are crucial to adaptive and industrial wood properties. Consistent with wood properties in *ARK2* transgenics (see below), *35S::ARK2* stems downregulate the *cellulose synthase-like* (*CSL*) gene (*estExt_fgenesh4_pm.C_LG_VIII0087*), three xylem-specific *CesA* genes (*PttCesA1*, *eugene3.00002636*; *PttCesA3-1*, eugene3.00040363; *PttCesA3-2*, *gw1.XI.3218.1*) and two phloem-specific *CesAs* (*PttCesA2*, *gw1.XVIII.3152.1*; *estExt_Genewise1_v1.C_LG_VI2188*). Strikingly, genes encoding key enzymes involved in lignin biosynthesis, including *ferulate-5-hydroxylase* (*F5H*, *grail3.0057011701*, *eugene3.00071182*), *caffeoyl-CoA 3-O-methyltransferase* (*COMT*, *grail3.0001059501*), *p-coumarate 3-hydroxylase* (*C3'H*, *estExt_fgenesh4_pm.C_LG_VI0096*), *4-coumarate-CoA ligase 1* (*4CL*, *grail3.0100002702*), *cinnamic acid 4-hydroxylase* (*C4H*, *estExt_fgenesh4_pg.C_LG_XIII0519*), *cinnamyl-alcohol dehydrogenase* (*CAD*, *estExt_Genewise1_v1.C_LG_IX2359*) and *laccase* (*estExt_fgenesh4_pm.C_LG_VI0293*, *estExt_fgenesh4_pg.C_LG_VIII0541*) are all downregulated in *35S::ARK2* trees and upregulated in the *amiRNA::ark2* trees (Table S3). At the level of transcriptional regulation, *MYB4* (*AT5G26660*) encodes an Arabidopsis MYB transcription factor that positively regulates the lignin synthesis pathway (Patzlaff *et al.*, 2003), and a *Populus* ortholog of *MYB4* (*gw1.XII.1714.1*) is downregulated in *35S::ARK2* and upregulated in *amiRNA::ark2* trees (Table S3). The modulation of lignin-related genes with

ARK2 expression is reflected in the wood properties of *35S::ARK2* and *amiRNA::ark2* transgenics (see below).

Cell wall chemistry of *ARK2* transgenics reflects changes in the expression of cell wall-associated genes

Wood chemistry analysis (see Experimental procedures) reveals altered cellulose and lignin content in *ARK2* transgenics in comparison with wild-type controls. Notably, total structural xylem carbohydrates are altered in *ARK2* transgenic stems. In *35S::ARK2* stems both cellulose content, as represented by glucose moieties, and hemicellulose-derived carbohydrates are slightly reduced when compared with the corresponding wild-type stems (Table 1). In contrast, the *amiRNA::ark2* stems show significantly elevated levels of secondary cell wall carbohydrates, including significant increases in glucose (a partial surrogate for cellulosic polymers) content, as well as in arabinoxylan and galactomannan levels (Table 1). Transcript abundance of key genes involved in cellulose and xylan biosynthesis (Table S2) correspond to these biochemical characters of the *35S::ARK2* trees, although there does not appear to be any notable increase in these same genes in the *amiRNA::ark2* trees.

More strikingly, total lignin content (Table 2) is reduced in the *35S::ARK2* stems and increased in *amiRNA::ark2* by as much as a 10%. The syringyl:guaicyl (S:G) lignin monomer ratio is significantly increased in *amiRNA::ark2* stems, and is decreased in *35S::ARK2* stems (Table 3). These altered lignin properties are consistent with the misregulation of key lignin biosynthetic genes in *35S::ARK2* and *amiRNA::ark2* transgenics (Table S3).

DISCUSSION

The vascular cambium is responsible for the radial growth of woody stems, and is thus central to key innovations in the evolution of land plants. We report here a functional characterization of a class-I KNOX transcription factor, *ARBORKNOX2* (*ARK2*), which is expressed in both the shoot apical meristem and the vascular cambium. This observation is consistent with the co-option of important genes and mechanisms from the shoot apical meristem during the evolution of the vascular cambium (Groover, 2005; Groover *et al.*, 2006). We used detailed characterization of transgenic *Populus* overexpressing or downregulating *ARK2* to provide basic insights into the regulation of the cambium and secondary growth, including the regulation of cell division and cell differentiation. In addition, our analysis of *ARK2* suggests how complex developmental mechanisms can be coordinated to produce adaptive wood phenotypes, through transcriptional regulation of complexes of genes with diverse functions.

Analysis of *ARK2* transgenics suggests that a primary function of *ARK2* is to regulate the differentiation of cambial daughter cells and lignified cell types. *ARK2* overexpression results in greatly reduced phloem fibers and less secondary xylem in stems. Conversely, downregulation of *ARK2* results in more pronounced phloem fibers with thicker cell walls and more secondary xylem. In addition, cell division-promoting genes are downregulated in the stems of *ARK2*-overexpressing trees. The width of the cambial zone is typically thought to reflect the rate of cell division, yet the cambial zone is wider in *ARK2* overexpressing stems, which seems contradictory to the downregulation of cell division genes. However, although the cambial zone is wider in *ARK2*-overexpressing trees, the total number of cell layers of cambium and secondary xylem is reduced. It would thus appear that xylem mother cells (daughters of the cambial initials) arrest cell division in the cambial zone, and are slow to differentiate. Together, these observations are consistent with the activity of a compensatory mechanism within cambial cell files, where cell divisions in the cambial zone are delayed until differentiation proceeds in older cells within secondary xylem. Such a mechanism could also explain the observed coordination of cell division and differentiation during secondary growth under various growth and environmental conditions.

Interestingly, whole-mount *in situ* hybridization showed that *ARK2* expression is broad in the cambial zone, and is not limited to the cambial initials or even immediate daughter cells (phloem and xylem mother cells). In younger stems, *ARK2* expression extends well into the lignifying tissues of secondary xylem (wood). Even more strikingly, *ARK2* is expressed in differentiating phloem fibers. These cells are well separated from the cambial zone, and *ARK2* expression in these cells is not contiguous with the cambial zone. These observations suggest that ARK2 may function not only in influencing fates for cells directly associated with or derived from meristems, but may play a more general role in regulating the differentiation of cells that undergo lignification or differentiate within lignified tissues.

Table 1 Monomeric carbohydrate composition (mg/100 mg) in developing stem of wild type, *35S::ARK2* and *amiRNA::ark2* stems

	Arabinose	Rhamnose	Galactose	Glucose	Xylose	Mannose	Total
Wild type	0.37 ± 0.01	0.25 ± 0.01	1.54 ± 0.05	44.50 ± 0.68	17.42 ± 0.45	1.83 ± 0.04	65.91 ± 0.41
35S::*ARK2*	0.35 ± 0.008	0.28 ± 0.03	**2.47 ± 0.36**	42.72 ± 0.73	**15.67 ± 0.26**	**0.63 ± 0.08**	**62.14 ± 0.03**
amiRNA:*ark2*	**0.42 ± 0.01**	0.30 ± 0.03	**2.13 ± 0.16**	**48.30 ± 0.39**	**19.04 ± 0.54**	**1.43 ± 0.05**	**71.61 ± 0.84**

Bold denotes significant difference from control values at $P < 0.05$.

Table 2 Total lignin composition of wild type, *35S::ARK2* and *amiRNA::ark2* stems

	Total lignin (mg/100 mg)	Acid-insoluble lignin (mg/100 mg)	Acid-soluble lignin (mg/100 mg)
Wild type	20.10 ± 0.24*	17.59 ± 0.28	2.52 ± 0.11
35S::*ARK2*	19.92 ± 0.17	17.44 ± 0.07	2.47 ± 0.34
amiRNA:*ark2*	**21.54 ± 0.17**	**18.42 ± 0.14**	**3.12 ± 0.07**

Bold denotes significant difference from control values at $P < 0.05$.

Table 3 Syringyl, guaiacyl and *p*-hydroxyphenyl monomer contents (moles) of transgenic and wild-type trees as determined by thioacidolysis

	Total monomer Yield (μmol/g lignin)	*p*-Hydroxyphenyl	Guaiacyl	Syringyl	S:G monomer ratio
Wild type	669.93 ± 4.32	0.53 ± 0.02	32.14 ± 0.51	67.33 ± 0.51	2.10 ± 0.05
35S::*ARK2*	547.33 ± 119.70	0.60 ± 0.16	**35.44 ± 2.41**	**63.96 ± 2.58**	**1.82 ± 0.20**
amiRNA:*ark2*	**741. 25 ± 18.91**	0.46 ± 0.03	**29.04 ± 1.02**	**70.50 ± 1.04**	**2.45 ± 0.12**

Bold denotes significant difference from control values at $P < 0.05$.

Microarray analysis of *ARK2* transgenics revealed the misregulation of genes from various functional classes, including transcriptional regulators, hormone-related and, most notably, cell wall biosynthesis-related genes. One possibility is that ARK2 directly regulates many of the genes that are misregulated in these various classes. This is supported by previous analysis of the Arabidopsis ortholog of *ARK2, BP*, which directly binds the promoters of genes encoding cell wall biosynthetic enzymes (Mele *et al.*, 2003), and by the observation that KNOX proteins can directly bind the promoter of the gibberellin biosynthetic gene *GA 20-oxidase* (Sakamoto *et al.*, 2001). We see misregulation of homologous genes in *ARK2* transgenics, suggesting our analysis was successful in the identification of genes directly regulated by ARK2. If our hypothesis is correct, it would suggest how complex wood phenotypes can be coordinated in response to environmental change: by changing the expression of one or a few transcriptional regulators that control suites of genes of diverse functions required to affect complex phenotypes.

We previously characterized a related KNOX gene, *ARBORKNOX1*, a poplar ortholog of *STM* (Groover *et al.*, 2006). Just as *STM* and *BP* play unique roles in regulating the shoot apical meristem, we find significant differences between *ARK1* and *ARK2* in regulating secondary growth. ARK1 expression is limited to the cambial zone, which is distinct from the dynamic expression of *ARK2*. *ARK1* overexpression phenotypes are severe, and include the formation of ectopic meristems on the adaxial surface of leaves (Groover *et al.*, 2006). In addition, expression profiling of *ARK1* and *ARK2* mutants show that although both transcription factors affect the same general classes of genes (including cell wall-related genes), there is little overlap in the putative target gene sets (see Table S8). Thus, although we cannot dismiss the possibility for some overlap in function, *ARK1* and *ARK2* appear to play distinct roles in regulating secondary growth.

Importantly, our analysis suggests that *ARK2* plays a major role in determining the fundamental wood properties of stems. In general, changes in global gene expression in response to *ARK2* misexpression were well-correlated with related changes in wood chemistry, suggesting that transcriptional regulation is central to wood formation. For example, the *Populus FLA 12* and *FLA 11* genes encode members of the fasciclin-like arabinogalactan proteins (FLAs) (Johnson *et al.*, 2003; Seifert and Roberts, 2007). Homologous genes have been shown to be highly coregulated with cellulose synthesis in Arabidopsis stems, especially with the onset of secondary cell wall cellulose synthesis (Brown *et al.*, 2005; Persson *et al.*, 2005). At-*fla11* mutants (Brown *et al.*, 2005; Ito *et al.*, 2005; Persson *et al.*, 2005) have a moderate collapsed-vessel phenotype and a reduction in stem cellulose content (Persson *et al.*, 2005). Similarly, differential *FLA* gene expression is correlated with differences in wood properties in the xylem fibers of *Populus* during tension wood formation (Lafarguette *et al.*, 2004). We found strong downregulation of *Populus FLA11, FLA12* and three secondary cell wall cellulose synthase genes in ARK2 overexpressing stems. These stems also have reduced cellulose content, whereas stems of trees expressing a synthetic miroRNA targeting *ARK2* transcripts have increased cellulose content. These results suggest that *ARK2* impacts cell wall-related carbohydrate biosynthesis, potentially in part through regulation of cellulose synthase-related and *FLA*-related genes.

More strikingly, *ARK2* also appears to repress the expression of several key lignin biosynthetic pathway genes. Genes encoding lignin biosynthetic enzymes 4CL, C4H, C3'H, F5H and CAD are all downregulated in stems of *ARK2*-overexpressing trees. In addition, the *Populus* ortholog of

AtMYB4, which influences sinapate ester synthesis and regulates the transcription of C4H, is also downregulated. In contrast, stems of trees with synthetic miRNA reduction of ARK2 transcripts upregulate these same genes. Importantly, these changes in gene expression are clearly manifested by dramatic changes in the lignin chemistry of the stems.

Our findings are generally consistent with previous studies of gene regulation by the Arabidopsis *ARK2* ortholog, *BP*. Mele *et al.* (2003) demonstrated that *BP* negatively regulates 13 genes involved in cell wall synthesis and lignification in Arabidopsis seedlings during primary growth. These same authors also demonstrated that BP protein directly binds to the promoters of some of these genes. Our microarray analysis of *ARK2* transgenics showed misregulation of a more extensive suite of cell wall-related genes. Although similar classes of genes are misregulated in *bp* seedlings and in the stems of *ARK2* transgenic trees, only some of the assumed orthologous genes are similarly misregulated. These differences in gene expression between *ARK2* and *BP* are likely to represent technical differences between experimental approaches, biological differences between Arabidopsis and *Populus*, and differences between the tissues profiled. We profiled gene expression in *Populus* stems that included true secondary growth, and compared both overexpression and downregulated *ARK2* transgenics with wild-type trees. In contrast, gene expression was compared for entire, 2-week-old *bp* and wild-type Arabidopsis seedlings with no secondary growth. Although the misregulated genes in the current *ARK2* transgenic stems are likely to include genes that are not direct targets of ARK2, it is also highly unlikely that the small number of misregulated genes reported by Mele *et al.* (2003) represent all of the direct targets of BP. More comprehensive analysis of ARK2 and BP target genes (e.g. through ChIP-chip) would be one approach for further defining the function of these important plant regulators. In addition, follow-up studies will be required to determine the distinct roles that *ARK2* might play in primary versus secondary growth, and in the diverse tissues and cell types where it is expressed.

ARK2 transgenic phenotypes have intriguing similarities to the variation seen in nature for cambial activity and woody growth. Cell division in the cambium and differentiation of cambial daughters vary to produce adaptive wood phenotypes in response to environmental cues (e.g. drought stress affects on vessel element diameter), seasonal changes (e.g. early wood and late wood) and across years (e.g. heartwood formation). As a specific example, *Populus* stems challenged by gravitational force form tension wood on the upper surfaces of stems that realigns the stem. Tension wood is characterized by a widening of the cambial zone, overexpression of specific FLAs, changes in auxin-related gene expression, and wood containing low lignin and copious, highly crystalline cellulose. *ARK2*-overexpressing trees show coordinated misregulation of these same processes. Although we are not suggesting that *ARK2* is a regulator of tension wood, we do present these findings as an example of how changes in the expression of a transcription factor can make coordinated changes required to alter complex wood phenotypes. Thus, we hypothesize that *ARK2* could identify a regulatory module composed of a co-evolved suite of genes of diverse function, which together produce complex stem phenotypes with adaptive significance.

The manipulation of wood properties is crucial to forest industry and to the optimization of wood feedstock properties for industrial applications, carbon sequestration, ecosystem biodiversity and, more recently, as a feedstock for biofuel production. Traditional tree breeding has been successful in making incremental changes to wood properties, but is limited because it is relatively slow, expensive and indirect. Transgenics have been used to change the expression of key genes encoding enzymes involved in cell wall synthesis and lignification (Li *et al.*, 2003). However, the ability to manipulate complex wood phenotypes by altering one or a few biosynthetic enzymes is limited, and can result in undesired side effects. *ARK2* illustrates another strategy for manipulating complex wood phenotypes, through the selection or manipulation of transcriptional regulators that control suites of genes, which together influence wood phenotypes with ecological and industrial relevance.

EXPERIMENTAL PROCEDURES

Plant cultivation and transformation

Hybrid aspen clone INRA 717-IB4 (*P. alba* × *P. tremula*) was used for all experiments and gene cloning. Plants were propagated and transformed using previously published methods (Han *et al.*, 2000). Three independently transformed lines were used for overexpression analysis (35S37-1, 35S37-2 and 35S37-5) and downregulation analysis (amiRNA2-1, amiRNA2-2 and amiRNA2-5). All experiments were repeated at least twice using each of the above transformed lines and matched wild-type controls with similar results, unless otherwise stated.

Nucleic acid isolations, amplifications and analysis

Genomic DNA was isolated with Qiagen Dneasy (http://www.qiagen.com); RNA was isolated using Qiagen RNeasy, following the manufacturer's protocols. cDNA was generated using iScript cDNA Synthesis Kit (Bio-Rad, http://www.bio-rad.com), following the manufacture's protocols. *ARK2* was cloned from cDNA using degenerate primers popKNAT1-A 5′-GCYTACWTGGAYTGYCAAARGRKGG-3′ and popKNAT1-B 5′-CCRTCCATHACCATRAAYTGCATGTC-3′ (where Y = C or T; R = A or G; H = A, C or T; W = A or T). Primers to amplify the 5′ and 3′ ends were designed based on the sequence of the amplified fragment (TCCTCACGGTACTTCACTAGCATGTCG for 5′ RACE and GAAGCCAGGCAGAAGCTACTCAGTTGG for 3′ RACE), and were used for SMART RACE (Clontech, http://www.clontech.com) according to the manufacturer's protocol.

Primary products were diluted 1:50, and a second round of PCR was performed with primers GTTCTGGGTCTTTCGAATTGTCCCTTG for 5′ RACE and CCTTCAGAGACGGAGAAGGTGGCATTA for 3′ RACE. The coding sequence was re-amplified from cDNA using gene-specific primers and then re-sequenced (Genebank Accession bankit 1195038). Alignment of ARK2, BP (KNAT1), ARK1 and STM proteins was performed with CLUSTALX 2.0. (Thompson *et al.*, 1997; Larkin *et al.*, 2007) using default settings. Phylogenetic analysis was performed using the full amino acid sequence of class-I KNOX proteins from Arabidopsis and *P. trichocarpa*; *ARK1* and *ARK2* are from *P. tremula* × *P. alba*. Phyologenic trees were estimated using the neighbor-joining method within PAUP (Wilgenbusch and Swofford, 2003).

Relative expression of *ARK2* in apices, leaves, roots and stem of 2-month-old tissue culture-grown *P. tremula* × *P. alba* was determined using qRT-PCR, using a tubulin-encoding gene (JGI accession *estExt_fgenesh4_pm.C_LG_III0736*) as a reference gene.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as previously described (Groover *et al.*, 2006). A 220-bp fragment from the 5′ end of the *ARK2* coding region and a 292-bp fragment from the 5′ end of the *Pop50S* coding region were selected to design primers to generate the template of probes using gene-specific primers: ARK2-F, 5′-CTTCTTGCACCCAATTCCTC-3′; ARK2-R, 5′-GCTGCTGGTTAATTGTTTGG-3′; Pop50S-F, 5′-CCTAGTGTTCCTGTAACTCGCATTGG-3′; Pop50S-R, 5′-CTCCCACCACCATGTTGTCCGTAAGTG-3′. The T7 promoter sequence 5′-TAATACGACTCACTATAGGG-3′ was added to the 5′ end of the ARK2-R primer and Pop50S-R primer sequences to generate templates of antisense probes for ARK2 and pop50S. The T7 promoter sequence was added to the 5′ end of ARK2-F primer sequence and Pop50S-F primer sequence to generate templates of sense probes for ARK2 and pop50S.

Recombinant DNA constructs

The coding sequence of *ARK2* was amplified with PCR amplified with primers ARK2_F 5′-GAGATGGAGGACTACAATCAAATG-3′ and ARK2_R 5′-TGGATCCAAGCTTGACGCTCATGGACT-3′ and sub-cloned into gateway vector Pk2GW7 to make the entry clone pTAG-ARK2. The insert was recombined into Pk2GW7 to generate *35S::ARK2*. For the *RNAi::ark2* construct, 114 bp of the 5′ end of the ARK2 coding sequence was PCR amplified with the following primers: ARK2-F, 5′-GAGCAATCATCAGACCCAGA-3′, and ARK2-R, 5′-CACCGCATGTGAAGTGCTGG-3′. The PCR product was cloned into directional TOPO pENTER vector (pENTR/D-TOPO), and recombined into the gateway vector Pk7gwiwg2(ii).

The *amiRNA::ark2* construct was assembled to drive expression of a synthetic miRNA by the 35S promoter as follows. A 21-nt fragment of the ARK2-specific sequence 5′-GAAAGACCCAGAACTAGATCA-3′ was targeted, based on published targeting parameters (Mallory *et al.*, 2004; Schwab *et al.*, 2005, 2006) and uniqueness to *ARK2*. The exact complementary sequence (miRNA) is 5′-TGATCTAGTTCTGGGTCTTTC-3′. Mismatches were introduced at positions 4, 9 and 10, and in positions 20 and 21 in the complementary strand, to produce the following miRNA and miRNA* sequences: miRNA, 5′-TGATCTAGTTCTGGGTCTTTC-3′; miRNA*, 5′-CTAAGACCCAGTCCTAGTTCA-3′.

A DNA strand was synthesized with these sequences replacing the normal miRNA and miRNA* sequences within MIR164b (Schwab *et al.*, 2005) to produce the following sequence containing *Xho* and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively: Xho-Bam MIR-KNAT1, 5′-CTCGAGGAGAATGATGAAGGTGTGTGATGAGCAAGATGATCTAGTTCTGGGTCTTTCTTACTAGCTCATATATACACTCTCACCACAAATGCGTGTATATATGCGGAATTTTGTGATATAGATGTGTGTGTGTTGAGTGTGATGATATGGATGAGTTAGTTCCTAAGACCCAGTCCTAGTTCATCATGACCACTCCACCTTGGTGACGATGACGACGAGGGTTCAAGTGTTACGCACGTGGGAATATACTTATATCGATAAACACACACGTGCGGGATCC-3′.

Microarray analysis

For each genotype, bulks of three plants were combined for RNA isolation from shoot apices, leaves, stem and root tissue of 2-month-old tissue culture trees for qRT-PCR. Affymetrix GeneChip® Poplar Genome Array oligonucleotide microarrays were used for all microarray hybridizations. For microarray hybridizations, total RNA was isolated from entire, defoliated stems of 2-month-old tissue-culture grown plants. Three independent biological replicate RNAs were isolated for each of three overexpression and miRNA lines, and four independent biological replicates of matched wild-type controls. Total RNA quantity and quality was determined using an Agilent Bioanalyzer (Agilent Technologies, http://www.agilent.com). Biotin labeling of target RNAs was performed with the one-cycle target kit (One-Cycle Target Labeling and Control Reagents, P/N 900493; Affymetrix, http://www.affymetrix.com), and then hybridized according to the manufacturer's protocol.

Analysis of microarray data was performed with dChip. Data was normalized using median probe intensity of the baseline array. The model-based expression data was filtered by removing genes for which representative probes did not exceed ≥40% (presence call %) on a given array, and ≥50% among arrays. Filtered genes were then compared based on -fold expression difference and Student's *t*-test (*P* value of 0.05). Preliminary analysis established appropriate filtering and statistical cut-off thresholds, using the false discovery rate, the identification of biologically meaningful genes and the inclusion of genes confirmed as being misexpressed by qRT-PCR as primary criteria. The false discovery rate was tested by 20 permutations, and was <0.4% in the final analysis. Microarray data and further details of the samples are available through NCBI GEO (GEO Submission GSE15595).

Gene expression differences estimated by microarray analysis were confirmed using qRT-PCR. Gene-specific PCR primers were designed to target genes showing differential expression in the microarray comparison of *35S::ARK2, amiRNA::ark2* and wild-type trees. Primers with melting temperature (T_m) of >59°C were designed to produce a product of 200–300 bp. A tubulin-encoding gene (JGI accession *estExt_fgenesh4_pm.C_LG_III0736*) was used as a reference gene for qRT-PCR The primers sequences used for qRT-PCR are listed in Table S5. qRT-PCR was performed with an MJ Mini Opticon (Bio-Rad) following the manufacturer's protocols. The-fold change from qRT-PCR are shown in Figure S3 and Tables S1–S4.

Cell wall chemistry analysis

Three corresponding control (wild type) trees and three independently transformed lines were selected from *35S::ARK2* and *amiRNA::ark2* lines. Wild-type control trees and each transgenic line, represented by a minimum of 10 individual trees, were transferred into 7.5-L pots (50% peat, 25% fine bark and 25% pumice soil mixture) in the glasshouse, and were allowed to acclimate under 500-ml clear plastic cups for 1 week. Trees were grown under 16-h days supplemented with overhead lighting with a radiant flux density of 300 W m^{-2}. Trees were harvested after 4 months growth in the glasshouse. The tissue developmental stage was standardized using a plastichron index, where *PI* = 0 was defined as the first leaf greater than 5 cm in length, and *PI* = 1 is the leaf immediately below *PI* = 0. Stem segments spanning *PI* = 5–15 were retained for

wood cell wall and chemical analysis. The chemical composition of stems was determined as previously described (Groover *et al.*, 2006).

Glasshouse-grown poplar xylem tissue of control and transgenic trees were ground using a Wiley mill to pass through a 40-mesh screen (40 μm), and were then Soxhlet extracted overnight with hot acetone. Lignin and carbohydrate content of the extractive-free material was determined by a modified Klason technique, treating extracted ground stem tissue (0.2 g) with 3 ml of 72% H_2SO_4, as described by Coleman *et al.* (2006). Carbohydrate concentrations in the acid hydrolyzate were determined using anion-exchange HPLC (Dionex DX-600; Dionex, http://www.dionex.com) equipped with an ion-exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode and a Spectra AS 3500 auto-injector (Spectra-Physics, now part of Newport, http://www.newport.com). Acid-insoluble lignin was determined gravimetrically, whereas acid-soluble lignin was determined using spectrophotometric analysis at 205 nm according to the TAPPI Useful Method UM-250.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. *ARBORKNOX2* (*ARK2*) expression levels in *35S:ARK2* and *amiRNA:ark2* transgenic plants relative to wild-type controls.

Figure S2. Transverse sections of stems from 2-month-old wild type and *RNAi:ark2* stained with toluidine blue.

Figure S3. Real-time PCR data confirmation of microarray data.

Table S1. Transcription factors mis-regulated in *ARBORKNOX2* (*ARK2*) transgenics.

Table S2. Genes up- or downregulated in *ARBORKNOX2* (*ARK2*) transgenics involved in cell wall synthesis.

Table S3. Genes up- or downregulated in *ARBORKNOX2* (*ARK2*) transgenics involved in lignin biosynthesis genes.

Table S4. Genes up- or downregulated in *ARBORKNOX2* (*ARK2*) transgenics involved in hormone-related processes.

Table S5. Primers for quantitative real-time PCR (qRTPCR).

Table S6. Genes that show a twofold change in *35S::ARK2* transgenics.

Table S7. Genes that show a twofold change in *amiRNA::ark2* transgenics.

Table S8. Genes misregulated in both *ARK1* and *ARK2* transgenics.

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