ORIGINAL PAPER

A *KNAT3*-like homeobox gene from *Juglans nigra* L., *JnKNAT3*-like, highly expressed during heartwood formation

Zhonglian Huang · Richard Meilan · Keith Woeste

Abstract The value of black walnut (*Juglans nigra* L.) is affected by the quality and quantity of darkly colored heartwood in its stem. We are exploring the regulation of heartwood production by identifying genes associated with the transition from sapwood to heartwood. Previous microarray data indicated that heartwood formation may be related to programmed cell death (PCD). To test this hypothesis, we analyzed the region of heartwood formation in walnut stems (i.e., the transition zone, TZ) for the expression of 80 ESTs putatively associated with PCD. Semi-quantitative RT-PCR and real-time PCR was performed to detect the expression changes in candidate genes in the TZ and sapwood of trees harvested in summer and fall. The results revealed that the transcript of a clone that encodes a presumed homeobox protein knotted-1-like 3 (KNAT3) was highly expressed in the TZ when compared with other tissues. Analysis of the full-length coding sequence revealed that the black walnut gene contains regions with 67% similarity to Knox1 and Knox2 domains from the *Arabidopsis thaliana KNAT3*, as well as a putative homeodomain known to be a transcription factor in other plants. *JnKNAT3*-like transcript was detected in the pith meristem, roots, embryogenic callus, vascular cambium, female flowers, male flowers, green leaves, and partially and fully senescent leaves of black walnut, although transcript abundance varied considerably among tissues. These analyses may provide insight into the mechanism regulating heartwood formation in walnut and other hardwood trees.

Keywords Transition zone · Transcription factor · Black walnut · Hardwood

Abbreviations

EST	Expressed sequence tag
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription polymerase chain reaction

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Introduction

The quantity and color of heartwood in a stem strongly influences its value as an industrial raw material. Heartwood also plays important biological roles, including resistance to pathogens (Findlay 1959); mechanical support (Long et al. 1981); nutrient cycling (Andrews and Siccama 1995); response to a hydraulic stimulus (Berthier et al. 2001); and other processes (Taylor et al. 2002). Factors that influence heartwood durability include the types and amounts of extractives, especially tannins, which can inhibit pathogens or insects that invade the heartwood (Taylor et al. 2002).

Heartwood formation is a complex biological process driven by the death of parenchyma cells oriented both axially and radially within the stem. It is also considered to

be the last step in the life cycle of xylem cells (Plomion et al. 2001). During differentiation of vascular cambium into heartwood, xylem cells undergo various fates, beginning with the division of xylem mother cells. These cells elongate and form secondary cell walls by the deposition of cellulose, hemicelluloses, and lignin. Most xylem cells lose their protoplasm, but ray parenchyma cells remain alive and functional for several years. Eventually, the parenchyma cells die and heartwood is formed (Plomion et al. 2001).

Research has shown that the formation of heartwood resembles programmed cell death (PCD) in that both processes involve loss of cell integrity, nuclear fragmentation, mitochondrial degeneration, vacuolar collapse, and release of phenolic extractives (Frey-Wyssling and Bosshard 1959; Bosshard 1965; Hauch and Magel 1998; Magel 2000). Previous studies of heartwood formation have focused on the role of hormones or other physiological changes that occur during heartwood formation, such as changes in parenchyma cell numbers and radial carbohydrate distribution (Frey-Wyssling and Bosshard 1959; Carrodus 1971; Shain and Hillis 1973; Miller et al. 1985; Hillis 1987; Nobuchi et al. 1987; Abeles et al. 1989). Other researchers have analyzed the activity of enzymes that are related to synthesis of sugar, lipids, polypropanoids, and phenolics during heartwood formation, such as sucrose-phosphate synthase (Schrader and Sauter 2002), triacylglycerol acylhydrolase (Hillinger et al. 1996), cinnamate 4-hydroxylase (C4H), 4-coumarate CoA ligase (4CL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), and dihydroflavonol reductase (DFR) (Hauch and Magel 1998; Magel 2000; Yang et al. 2003). The exact mechanism of heartwood formation remains unclear, as do the pathways involved in heartwood development.

Knotted-like homeobox (KNOX) plant genes are a subfamily of the Three Amino acid Loop Extension (TALE) homeodomain family. They were first characterized as regulatory genes that control cell specification and patterning in Drosophila (Gehring 1987). Homeobox genes share a unique structure referred to as the homeodomain, which consists of a highly conserved 60 amino acid stretch, including three α helices, and a helix-turn-helix-type DNA-binding domain (Desplan et al. 1988; Otting et al. 1990). This domain recognizes and binds to specific DNA sequences, regulating their expression. The first homeobox gene identified in plants was Knotted1 (KN1) from maize (Vollbrecht et al. 1991). Since then, many other homeobox genes have been cloned and characterized from other plant species. The most intensively investigated, KN1-type homeodomain proteins, have been subdivided into two groups, classes I and II (Kerstetter et al. 1994). Class I genes are more similar to the maize KN1 gene, sharing major amino acids in the homeodomain, and are strongly expressed in meristematic tissues, mostly the apical meristem, and minimally in differentiated tissues such as leaves, roots, and vascular cambium (Lincoln et al. 1994; Kerstetter et al. 1994; Groover et al. 2006). Class II genes are less similar to the maize KN1 than are class I, and are differentially expressed in all plant organs (Kerstetter et al. 1994; Serikawa et al. 1997). Class II genes have been identified in several plant species, such as Medicago truncatula (Giacomo et al. 2008); maize (Kerstetter et al. 1994); tomato (Sakamoto et al. 1999); and Arabidopsis (Serikawa et al. 1996). The Arabidopsis genome contains four class II KNOTTED-like genes, KNAT3, KNAT4, KNAT5, and KNAT7. KNAT3 and KNAT4 share 84% homology outside of the homeodomain, based on the predicted amino acid sequences (Serikawa et al. 1996). The first systematic survey of KNAT gene expression indicated that KNAT3,-4, and -5 genes show distinct cell type-specific expression patterns in the Arabidopsis root along the longitudinal root axis and in lateral root primordia (Truernit et al. 2006). KNAT4 transcript is most abundant in leaves and young siliques (Serikawa et al. 1996). It is reported that KNAT3, KNAT4, and KNAT5 proteins form heterodimers with BEL proteins, especially BLH1, known as KNOX–BEL interactions, which are thought to regulate plant development (Hackbusch et al. 2005). Localization results using promoter-GUS fusion analysis and in situ hybridization revealed that KNAT3 was mainly expressed in early organ development of leaves, buds, and pedicels, at the junction between two organs, and in tissues such as the petioles of maturing leaves and most of the root (Serikawa et al. 1997). In root development, KNAT3 expression is repressed by cytokinin indicating that plant hormones may regulate the function of some KNOX gene family members (Truernit et al. 2006). In this study, we present the isolation and basal characterization of a KNOX gene from black walnut (Juglans nigra L.), JnKNAT3-like. These experiments provide insight into the genetic pathways regulating heartwood formation in walnut, and potentially in other hardwood species.

Materials and methods

Plant materials and growth conditions

Thirty-nine-year-old black walnut trees grown at the Martell Research Forest, Tippecanoe County, IN, were cut down on July 1, 2004, and October 14, 2004. These trees were labeled "summer tree" and "fall tree", respectively. Another four black walnut trees were felled on the same dates in 2006. Immediately after the trees were cut, stem cross-sections ("cookies"), approximately 2.5-cm-thick, 20 cm in diameter, were cut with a chainsaw. The cookies were immediately submerged in liquid nitrogen. After

returning to the laboratory, the cookies were transferred to an ultra-low freezer (−80°C) for storage. Transition zones (TZ) were identified under UV light and carefully chiseled out of the cookies from each tree. Other tissues, including interior sapwood, exterior sapwood, and cambium were also removed (Fig. 1a). Roots were collected from young walnut trees growing in a greenhouse. Embryogenic calli were from in vitro cultures. Pith meristem, female flowers, male flowers, green leaves, and partially and fully senescent leaves were obtained from a 15-year-old black walnut tree growing on the Purdue University campus. Pith meristems were identified as the region subtending the apical meristem superior to the pith and inside the vascular cylinder (Fig. 1b).

RNA isolation

RNA was isolated as described previously (Kolosova et al. 2004). Xylem tissue was ground to a fine powder in a 6750 SPEX CertiPrep freezer mill (SPEX CertiPrep, INC; Metuchen, NJ). Extraction buffer consisted of the following: 200 mM Tris, pH 8.5, 1.5% lithium dodecyl sulfate, 300 mM lithium chloride, 10 mM disodium salt EDTA, 1% sodium deoxycholate, 1% tergito powder P-40, 5 mM thiourea, 1 mM aurintricarboxylic acid, 10 mM dithiothreitol, 2% polyvinylpolypyrrolidone, and 2% β-mercaptoethanol. RNA concentration was measured at 260 nM with an NanoDrop 1000 spectrophotometer (Wilmington, DE).
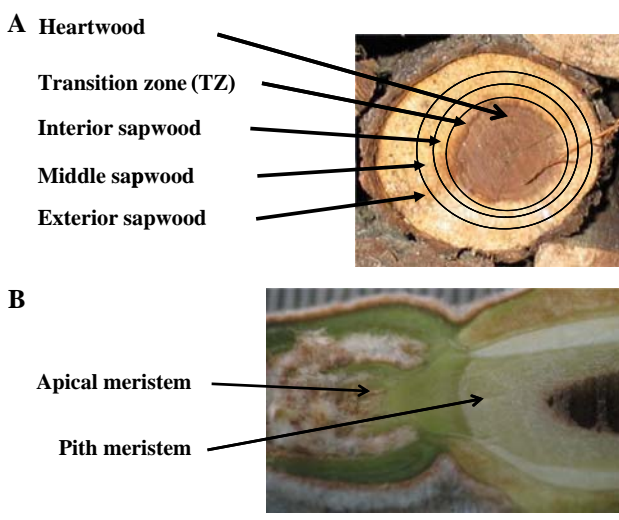
Semi-quantitative RT-PCR

To analyze transcript abundance, 10 μg total RNA was incubated with DNase (Ambion, Foster City, CA) at 37°C for 30 min, followed by the addition of 5 μl DNase inactivation solution (Ambion, Foster City, CA) at room temperature. After 2 min, the mixture was spun for 2 min full-speed in a table-top centrifuge at room temperature, and the supernatant was recovered for reverse transcription (RT). 18S rRNA was used as the internal standard for normalization. RT-PCR primers were designed with the aid of the web-based program "Primer3" (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), and potential inhibitory secondary structures of primers and the predicted amplicon were checked using the web-based program "mFold" (Zuker 2003) (http://www.bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html). Primers were designed to produce amplicons of 150–500 bp in size. The primers included: *JnKNAT3*-like forward, 5′-TTCAGACTGGAGCCGTTTCT-3′, and reverse, 5′-CAAGACCTGCACAGGTACGA-3′; sequences of the 18S rRNA forward and reverse primers were 5′-AGAGGCCTACAATGGTGGTG-3′ and 5′-CTCCAATGGATCCTCGTTA-3′, respectively. First-strand cDNA was synthesized as described by the manufacturer (Invitrogen, Carlsbad, CA). One microliter of cDNA derived from 10 μg of total RNA was added to a PCR consisting of 1× reaction buffer, 100 μM MgCl$_2$ 100 μM dNTPs, 10 pmol each primer, 0.25 U of TaqGo polymerase (Promega, Madison, WI) in a total volume of 25 μl. The PCR program consisted of a 2-min denaturation step at 94°C, followed by 24 cycles of 94°C (30 s), annealing at 54°C (30 s), 68°C (45 s), followed by a 10-min extension at 68°C. Amplified products were electrophoresed through 1% agarose gels, detected by ethidium bromide staining, and photographed under UV light (UVP, BioDoc-It, Upland, CA).

Real-time PCR

We used iQ^{TM} SYBR Green Supermix (Bio-Rad; Hercules, CA) and the iQ5 multicolor real-time PCR Detection System (BioRad, Hercules, CA). Control (18S rRNA) and six samples (TZ, interior sapwood, and exterior sapwood, for both summer tree and fall trees) were run in triplicate and repeated twice (technical replicates). Three biological replicates were from different "cookies" harvested in summer and fall of 2004. Each 25-μl reaction consisted of 12.5 μl of SYBR Green PCR Master Mix (Bio-Rad), 1 μl of each primer (10 μM, *JnKNAT3*-like forward and reverse; these primers were also used for semi-quantitative RT-PCR) and 1 μl of cDNA derived from 10 μg of total RNA. The reaction was run at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s, and an extension phase of 81 cycles of melt-curve analysis as described by the manufacturer. BioRad's iQ5 software was allowed to choose cycle threshold levels and define the log-phase cycle number used for comparing gene-expression levels. The fold change of

A Heartwood
Transition zone (TZ)
Interior sapwood
Middle sapwood
Exterior sapwood

B
Apical meristem
Pith meristem

Fig. 1 **a** Cross-section of a black walnut stem under white light. The transition zone fluoresces blue under UV light. **b** The location of the pith meristem in a branch of black walnut. *Scale bar* = 1 mm

gene expression relative to the standard (18S rRNA) was defined by the formula $2^{-\Delta\Delta C_T}$ (comparative C_T method) (User's Manual, ABI PRISM 7700 Sequence Detection System, Perkin-Elmer Applied Biosystems), where $\Delta C_T = C_T$ (sample) $- C_T$ (18S rRNA), $\Delta\Delta C_T = \Delta C_T$ of samples $-\Delta C_T$ of the TZ harvested from fall. C_T values are the number of PCR cycles at which signal significantly rises above the background; a consistent C_T was applied across all replicates.

DNA isolation

Fresh plant tissues (1 g) was ground in 10 ml of DNA extraction buffer (50 mM Tris–Cl, pH 8.0, 20 mM EDTA, 1.4 M sodium chloride, 0.4 M lithium chloride, 2% CTAB, 2% PVP, 2% SDS) using a mortar and pestle. Two percent (v/v) β-mercaptoethanol and 0.1 g PVP were added to each sample and before incubation at 65°C for at least 1 h with periodic shaking. Two chloroform extractions were performed followed by one extraction with phenol/chloroform/isoamyl alcohol (25:24:1), pH 8.0, and two additional chloroform extractions. The supernatant was transferred to a clean tube and 0.9 volume of cold isopropanol and 0.1 volume 3 M sodium acetate were added. After gentle swirling, the DNA was precipitated by centrifugation for 15-min full-speed in a table-top centrifuge at 4°C. The pellet was washed with 1 ml of 70% ethanol followed by 10 additional minute of centrifugation. Pellets were air-dried and then dissolved in 100 μl TE buffer, pH 8.0, or sterile water. RNase A (10 μg/ml) was added to each sample followed by incubation at 37°C for 30 min. DNA purity was checked by means of absorbance ratios. DNA quality was evaluated electrophoretically.

Isolation of full-length cDNA by 5′- and 3′-RACE

The SMART RACE cDNA Amplification kit (Clontech; Mountain View, CA) was used to perform 5′- and 3′-RACE following the manufacturer's instructions. Samples of 1 μg of total RNA were used for reverse transcription. The gene-specific primers GSP1 (5′-AAGCGCAAAACCAAGGCTTG CCTGAC-3′), designed from the antisense strand was used for 5′-RACE, and the GSP2 (5′-CTTGCGTACGGAGGAGA GCGCTGTTG-3′), from the sense strand, was used for 3′-RACE. All RACE reactions were performed using the following PCR program: 94°C for 30 s, 68°C for 30 s and 72°C for 3 min for 30 cycles. The PCR product was subcloned into pGEM-T vector (Promega; Madison, WI) and recombinant clones sequenced. The contigs were aligned with Sequencer™ 4.1 (Gene Codes; Ann Arbor, MI).

Multiple alignment and phylogenetic tree construction

Orthologs of JnKNAT3-like in different plant species, including *Prunus persica*, *Medicago truncatula*, *Kalanchoe × houghtonii*, *A. thaliana*, *P. trichocarpa*, *Vitis vinifera*, and *Nicotiana tabacum*, were selected based on the results from basic local alignment search tool (BLAST) and their sequences were aligned using Clustal W (Thompson et al. 1997) with the purpose of discerning the Knox I, Knox II, ELK, and Homeobox domains. For phylogenetic analysis, the neighbor-joining trees were constructed with software Mega4 (Molecular Evolutionary Genetics Analysis) (Kumar et al. 2004).

Results and discussion

Isolation and sequence analysis of the putative *JnKNAT3*-like transcription factor cDNA

To understand the genetic regulation of heartwood formation, we partially sequenced a cDNA library using RNA isolated from the TZ of black walnut (data not shown). Candidate genes potentially associated with heartwood formation were screened using semi-quantitative RT-PCR. Preliminary results showed that a cDNA clone containing homeobox domain was differentially expressed in the TZ of the trees entering dormancy (data not shown). Based on the partial cDNA sequence, the full-length coding sequence of a *KNAT3*-like gene was isolated (*JnKNAT3*-like; Accession no.: FJ388876) by 5′- and 3′-RACE. The full-length coding sequence of this putative transcription factor is 1,449 bp in length. It encodes a predicted protein of 483 amino acids with a molecular weight of 53 kD (Fig. 2a). Analysis via the conserved domain database and search service, v2.13 NCBI (Jackson et al. 1994) showed that *JnKNAT3*-like contains a Knox1 ($N_{206} - A_{250}$) domain, Knox2 ($L_{262} - Q_{317}$) domain, ELK ($E_{379} - D_{394}$) domain, and homeodomain ($R_{396} - R_{458}$) (Fig. 2a). Sequence alignment showed that the JnKNAT3-like protein is 67% and 66% similar to *Arabidopsis* KNAT3 and KNAT4, respectively. Comparison of the deduced amino acid sequences with orthologs in other species showed that

Fig. 2 Deduced amino acid sequence for *JnKNAT3-like*. **a** Alignment of the Knox domain and homeodomain of JnKNAT3-like with proteins of other species. The consensus sequence derived from the alignment is *underlined* and invariant residues are indicated as *asterisks* in the consensus. The *bold*, underscored amino acids represent a Knox 1 domain from amino acids 206 to 250, Knox 2 domain from amino acids 262 to 317, ELK domain from amino acids 379 to 394, and homeodomain from amino acids 396 to 458. **b** UPGMA dendrogram of JnKNAT3-like proteins produced using the software Mega4. The distance along the horizontal axis is proportional to the difference between sequences, whereas the distance along the vertical axis has no significance. Pairwise similarity scores were expressed as percentage of identical residues in comparison of amino acid sequences deduced from *JnKNAT3-like* in black walnut with KNOX genes from other species. The comparisons were based on full-length proteins
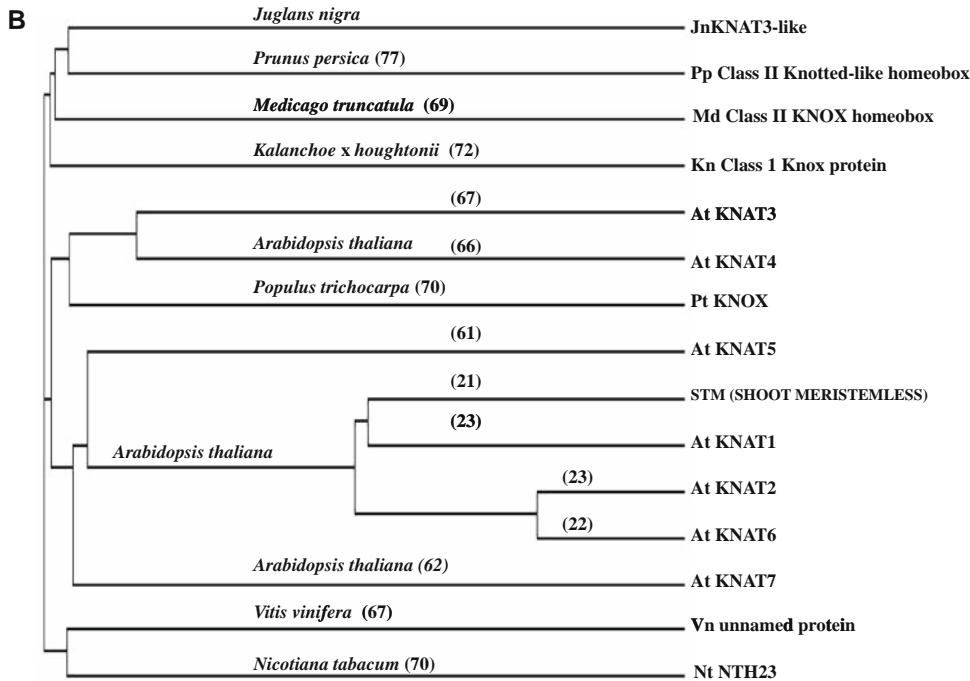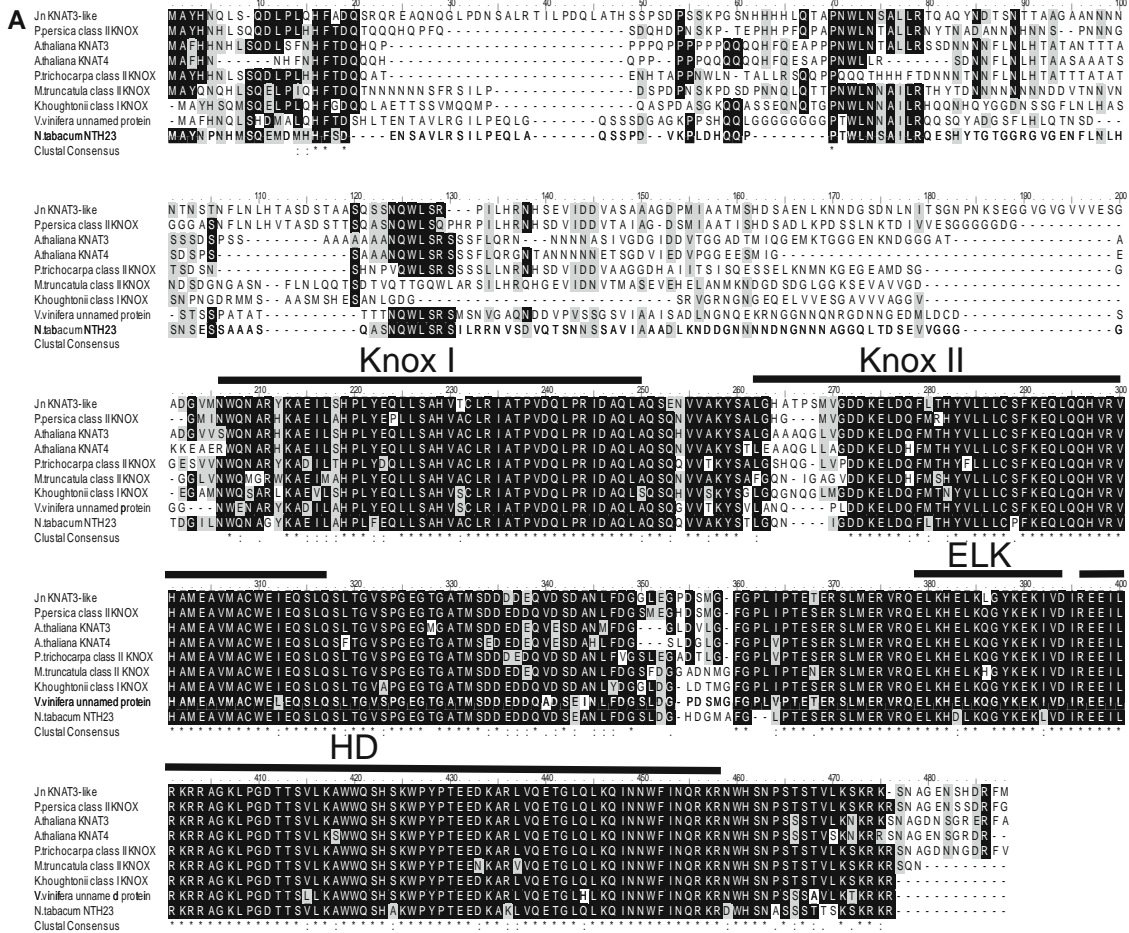
JnKNAT3-like shared 77% similarity with a class II knotted-like homeobox protein from *Prunus persica* (Fig. 2b), and 70% similarity with the ortholog estExt_fgenesh4_pg.C_LG_XVIII0454 [Poptr1_1:825765], in *Populus*. We conclude that *JnKNAT3*-like is most likely a class II KN1-type homeodomain proteins.

Expression of the putative *JnKNAT3-like* transcription factor in black walnut tissues

To better understand the role of the *JnKNAT3*-like gene in tree growth and development, we examined its expression in a series of tissues, including pith meristem, vascular cambium, embryogenic callus, roots, female and male flowers, green leaves, and partially and fully senescent leaves. Total RNA was extracted from these tissues and cDNA was synthesized using random primers. RT-PCR with 35 cycles was performed to test the level of expression in each tested tissue, and the results showed that *JnKNAT3*-like was expressed in nearly all tissues (Fig. 3a). The widespread expression of *JnKNAT3*-like is consistent with the previous research showing that class II genes are expressed in differentiated organs or tissues (Serikawa et al. 1997) indicating that these homeodomain proteins may also be important for controlling developmental transitions in black walnut. RT-PCR with 35 cycles usually has reached the saturated stage.

To quantify the transcript levels of *JnKNAT3-like* in these tissues, semi-quantitative RT-PCR (24 cycles) (data not shown) and real-time PCR (Fig. 3b) were performed. The results showed that *JnKNAT3*-like was weakly detectable in male flowers, partially and fully senescent leaves, but was most abundant in female flowers (11-fold greater), followed by roots (1.9-fold greater) and green leaves (1.3-fold greater), weakly expressed in embryogenic callus (0.26 time as much), when compared with the expression in vascular cambium (Fig. 3b), demonstrating that *JnKNAT3*-like is expressed at different levels in different tissues. The conflicting results between semi-quantitative RT-PCR and real-time PCR for transcript levels in green leaves may be attributed to the greater sensitivity of real-time PCR when compared with semi-quantitative RT-PCR.

Interestingly, the expression of *JnKNAT3*-like was readily detected in female flowers, indicating that it may play an important role in female gametophyte development. Previous research has shown that the class II KNOX protein KNAT3 interacts with BEL1-like homeodomain 1 (BLH1) proteins as heterodimers whose activity is, in turn, regulated by the OVATE protein family (Pagnussat et al. 2007). The *Arabidopsis* OVATE family protein AtOFP5 affects the subcellular re-localization of BLH1 from the nucleus to the cytoplasmic space and appears to be essential for embryo sac development in *Arabidopsis*
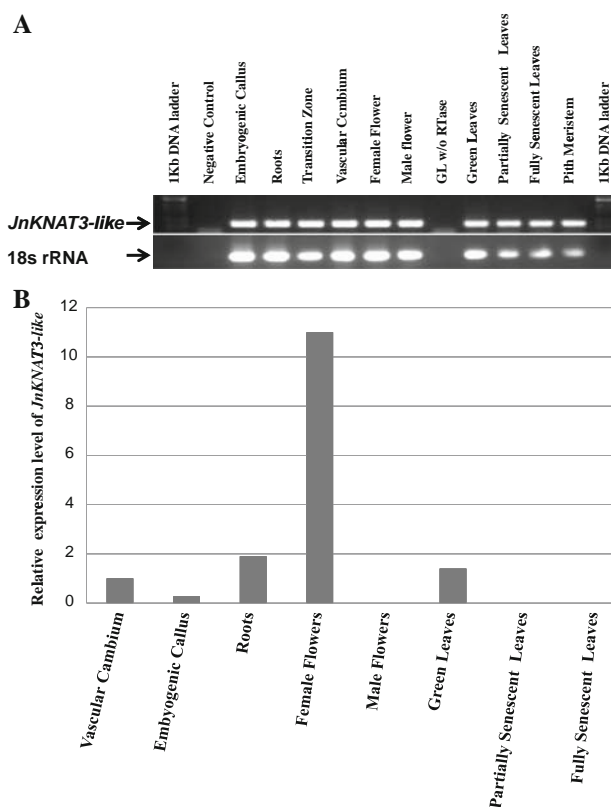
Fig. 3 Transcript abundance of *JnKNAT3*-like transcription factor in tissues of black walnut. **a** Test for the presence of transcript of *JnKNAT3*-like transcription factor in tissues of black walnut by RT-PCR (35 cycle). Equal loading of total RNA samples was confirmed by 18S rRNA. **b** Quantification of *JnKANT3*-like expression in black walnut tissues using real-time PCR. 18S rRNA was used as a standard. The fold changes were quantified and analyzed by the comparative C_T method. Values are the mean ± SE for two biological replicates

(Hackbusch et al. 2005; Pagnussat et al. 2007). Thus, *JnKNAT3*-like might play a similar role in the embryo sac development of black walnut.

Class I KNOX genes are also known to play an important role in meristem maintenance in plants (Miller et al. 1985). In *Arabidopsis*, three class I KNOX family genes, *KNAT1*, *KNAT2*, and *SHOOTMERISTEMLESS* (*STM*), are expressed in shoot apical meristems (SAM), but not in leaves or other differentiated organs (Jackson et al. 1994). Class II genes are typically expressed in all differentiated plant organs (Kerstetter et al. 1994; Serikawa et al. 1997). *JnKNAT3*-like is thought to be a class II gene, so it was not surprising that *JnKNAT3*-like was expressed at different levels in tested tissues based on quantitative RT-PCR (Fig. 3b). Vascular cambium is a secondary meristematic tissue that divides bi-directionally, producing xylem and phloem. Pith meristem is found in the zone of rapidly dividing cells in the central zone of dicot shoot tips that gives rise to the pith of a stem or branch (Fig. 1b).

Interestingly, *JnKNAT3*-like was expressed in both vascular cambium and pith meristem (Fig. 3a), in contrast to the previous research showing that only class I genes are expressed in meristematic tissues (Kerstetter et al. 1994). These results indicate that class II genes might also be expressed in meristematic tissues in some species.

Expression of the putative *JnKNAT3*-like transcription factor in the transition zone of black walnut

To investigate the expression of *JnKNAT3*-like in the TZ versus sapwood in more detail, the sapwood portion of the black walnut logs harvested in summer and fall was divided into three zones: an interior sapwood zone, which was next to the transition zone; a middle sapwood zone; and an exterior sapwood zone, close to the cambium (Fig. 1a). Semi-quantitative RT-PCR was first performed (data not shown), and it revealed that the expression of *JnKNAT3*-like was upregulated in the transition zone of the tree harvested in the fall when compared with other parts of stems harvested in the fall and summer. Real-time PCR (Fig. 4) revealed that *JnKNAT3*-like was expressed most abundantly in TZ and interior sapwood and weakly expressed in exterior sapwood harvested in fall. The expression of *JnKNAT3*-like was similar in TZ and inner sapwood of the tree harvested in fall, possibly indicating that the developmental processes taking place in the interior sapwood in the fall are more like those in the TZ than other portions of the sapwood, or that there was contamination of the inner sapwood with TZ cells. The abundance of *JnKNAT3*-like in xylem tissues harvested in fall displayed a radial gradient. This pattern corresponds to the radial gradient of other components, such as the radial
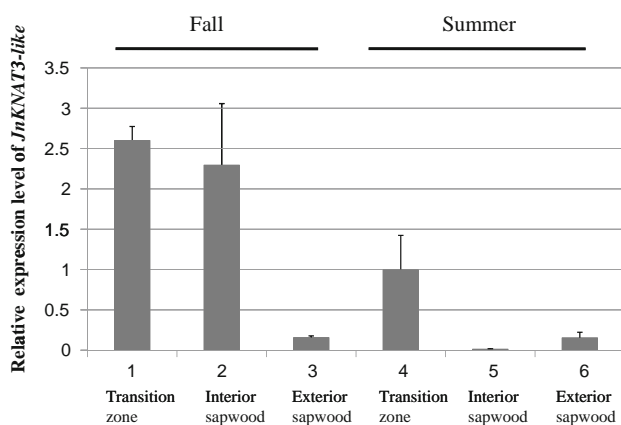
pattern of phospholipase C, sucrose synthase (SuSy), and sucrose-phosphate synthase (SPS) in the trunkwood of *R. pseudoacacia* L. (Hillinger et al. 1996; Hauch and Magel 1998), and the radial distribution of starch in trunk tissue of four tropical woods, *Cecropia*, *Urera*, *Anacardium*, and *Luehea* (Newell et al. 2002).

In the tissues harvested in summer, *JnKNAT3*-like was mostly expressed in TZ and very weakly expressed in interior and exterior sapwood. Furthermore, *JnKNAT3*-like expression was 2.5-fold higher in the TZ in the fall when compared with the TZ harvested in summer (Fig. 4), and was more than 2.5 times more abundant in the TZ in October versus other tissues including interior sapwood harvested in summer, and exterior sapwood of trees harvested in both summer and fall (Fig. 4). Because heartwood formation is thought to occur in the autumn, the expression of *JnKNAT3*-like in xylem tissues harvested in fall suggests that it might be associated with this process, though its role remains unknown. Future study will focus on how this transcription factor affects downstream genes or interacts with other proteins during heartwood formation.

Conclusions

Both sequence alignment and phylogenetic analysis support the view that *JnKNAT3*-like isolated from black walnut belongs to class II of the KNOX gene family. Real-time PCR and semi-quantitative RT-PCR revealed that *JnKNAT3*-like was expressed at different levels in various tissues. Interestingly, *JnKNAT3*-like was expressed in meristematic tissues (i.e., vascular cambium and the pith meristem) indicating that class II KNOX genes can also be expressed in meristems, not only in differentiated organs or tissues. Our data also indicated that *JnKNAT3*-like was highly expressed in female flowers, consistent with a role demonstrated in embryo sac development through interaction with BLH1 protein (Pagnussat et al. 2007). Furthermore, our results show that *JnKNAT3*-like transcript is more abundant in the fall than the summer in both the TZ and inner sapwood. *JnKNAT3*-like was also more highly expressed in TZ than all other xylem tissues, indicating a possible role for this gene in the formation of heartwood.

Fig. 4 Transcript level of *JnKNAT3*-like in the TZ, interior sapwood, and exterior sapwood in summer and fall. The fold changes were quantified by real-time PCR and were analyzed by the comparative C_T method by comparison with the *JnKNAT3*-like transcript level in TZ of the summer tree. Values are the mean $\pm$ SD for three biological replicates. 18S rRNA was used as a standard

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