

Genetic Control of Heartwood Formation in Black Walnut

Z. Huang¹, K.E. Woeste¹, C.-J. Tsai², S.A. Harding², and R. Meilan¹

We are investigating the spatial and temporal regulation of heartwood formation in black walnut (*Juglans nigra* L.), one of the most valuable fine hardwood species. We extracted total RNA from the transition zone (TZ) and other tissues of black walnut from replicate trees in two seasons (summer and fall) in each of two years. Hybridizing black walnut TZ cDNA to a poplar microarray led to the identification of candidate genes associated with transcriptional regulation of heartwood formation. After preliminary screening using semi-quantitative RT-PCR, three genes from black walnut were characterized more fully: *JnRAP2-like*, an AP2 domain-containing transcription factor similar to ethylene response element-binding proteins; *JnKNAT3-like*, a *KNOTTED-LIKE3* transcription factor containing two KNOX domains and a homeodomain; and *JnCML-like*, a gene predicted to encode a protein with two EF-hand motifs. Transcripts from all three were found to be more abundant in the TZ when compared to other xylem tissues; however, none of the three genes were expressed at high levels in senescing leaves, indicating that plant cell death (PCD) processes in xylem may differ from leaf senescence. In addition, transcript abundance was higher for all three genes in the fall relative to the summer, lending support to the previous observation that black walnut heartwood is formed in the fall. *JnKNAT3-like* and *JnCML-like* were also expressed in the pith meristem, a tissue spatially related to heartwood and where PCD also occurs; again, this was not true for *JnRAP2-like*. The further characterization of the temporal and spatial regulation of these genes and their expression in heterologous systems is underway.

METHODS AND MATERIALS

Black walnut trees (~20 cm in diameter) growing at the Martell Research Forest, near West Lafayette, IN, were felled on July 1 and Oct. 14, 2004 and 2006. Immediately after the trees were felled, stem cross-sections (“cookies”), about 2.5 cm thick, were cut. The cookies were immediately frozen in liquid nitrogen, and after transport to the lab, stored at -80 °C. Transition zones were identified under UV light and carefully excised from the cookies with a chisel. Other tissues, including interior sapwood, exterior sapwood, and cambium were also harvested. Roots were collected from young, greenhouse-grown walnut trees. Pith meristem, male and female flowers, green leaves, and partially and fully senescent leaves were collected from a 15-year-old black walnut tree growing on the Purdue campus.

Total RNA was isolated using the protocol of Kolosova et al. (2004). Xylem tissue was ground to a fine powder in a freezer mill. The extraction buffer has been described by Huang (2009). Total RNA was used to synthesize cDNA, which was labeled with fluorescent dyes via random hexamer priming. Cy3 and Cy5 were used to label cDNA from different trees. Array hybridization was performed in an HS 400 (Tecan Instruments), following the procedure of Harding et al. (2005) and Huang et al. (2009a).

Semi-quantitative (reverse transcription) RT-PCR was done with 10-15 µg total RNA. Details for RNA purification, primer design, and PCR reaction conditions were described by Huang (2009). Amplified products were separated on 1% agarose gels.

¹Hardwood Tree Improvement and Regeneration Center, Department of Forestry and Natural Resources, Purdue University, West Lafayette, IN 47907-2061

²Warnell School of Forestry and Natural Resources and Department of Genetics, University of Georgia, Athens, GA 30602-2152

Quantitative real-time PCR reactions were carried out using 5 µg of DNase-treated total RNA, and an iQTM SYBR Green Supermix and the iQ5 Multicolor Real-time PCR Detection System (BioRad). Control (18S rRNA) and six samples (TZ, interior sapwood, and exterior sapwood of both summer tree and fall trees) were run in triplicate and repeated twice (technical replicates). Primer design and reaction conditions were previously reported by Huang (2009). BioRad's iQ5 software was used to choose cycle threshold levels and define the log-phase cycle number for comparing gene-expression levels. The fold change of expression relative to an 18S rRNA standard 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).

Full-length cDNA was isolated via 5'- and 3'-RACE, using the Smart RACE cDNA Amplification kit (Clontech). The PCR product was subcloned into the pGEM-T vector (Promega) and recombinant clones were sequenced. Contigs were aligned with SequencerTM 4.1 (Gene Codes).

RESULTS AND DISCUSSION

To analyze the expression of genes found in the TZ of the walnut, we used an aspen 7K microarray, representing ~5,000 unique aspen sequences primarily associated with wood formation. Only 17% (1,253) of the microarray probes hybridized with sequences from black walnut (Huang et al. 2009a). Most of the hits were genes that had not been annotated, but some were deemed potentially useful. Semi-quantitative RT-PCR was used to analyze transcript abundance of genes selected via microarray analysis and from 80 ESTs previously identified in a black walnut TZ cDNA library.

Semi-quantitative RT-PCR was used to identify genes that appeared to be differentially expressed in the TZ in the fall. Three genes were selected for further characterization, including: *JnRAP2-like*, an AP2 domain-containing transcription factor (TF) similar to ethylene response element-binding proteins; *JnKNAT3-like*, a *KNOTTED-LIKE3* TF containing two KNOX domains and a homeodomain; and *JnCML-like*, a gene predicted to encode a protein with two EF-hand motifs associated with calcium signaling. We will focus here on *JnKNAT3-like*.

Knotted-like homeobox (KNOX) genes are a subfamily of the TALE (Three Amino acid Loop Extension) homeodomain family. They were first reported to be genes that regulate cell specification and patterning in *Drosophila melanogaster* (Gehring et al. 1987). The first homeobox gene identified in a plant was *Knotted1* (*KN1*) from *Zea mays* L. (Vollbrecht et al. 1991). Subsequently, many other homeobox plant genes have been cloned and characterized. The KN1-type homeodomain proteins, are subdivided into two classes, I and II (Kerstetter et al. 1994). The *Arabidopsis thaliana* genome contains four class II *KNOTTED-like* genes, *KNAT3*, *-4*, *-5*, and *-7*. *KNAT3*, *-4*, and *-5* genes show distinct expression patterns. *KNAT3* is mainly expressed in early organ development and at junctions between organs (Serikawa et al. 1997).

Sequence analysis revealed that *JnKNAT3-like* contains the requisite KNOX motifs and a homeodomain (Figure 1; from Huang 2009). Quantitative RT-PCR revealed that *JnKNAT3-like* is, indeed, expressed predominantly in the TZ, and more highly in the fall than in the summer (Figure 2; from Huang et al. 2009b). It is widely believed that heartwood formation occurs in the fall, at the beginning of dormancy with the onset of lower temperatures (Hillis 1987).

JnKNAT3-like also was shown to be expressed in the pith meristem, roots, embryogenic callus, vascular cambium, female flowers, male flowers, green leaves, and partially and fully senescent leaves of black walnut (Figure 3; from Huang 2009). In *Arabidopsis*, KNOX class II genes are expressed in all developing tissues except meristems, so we were surprised to observe expression

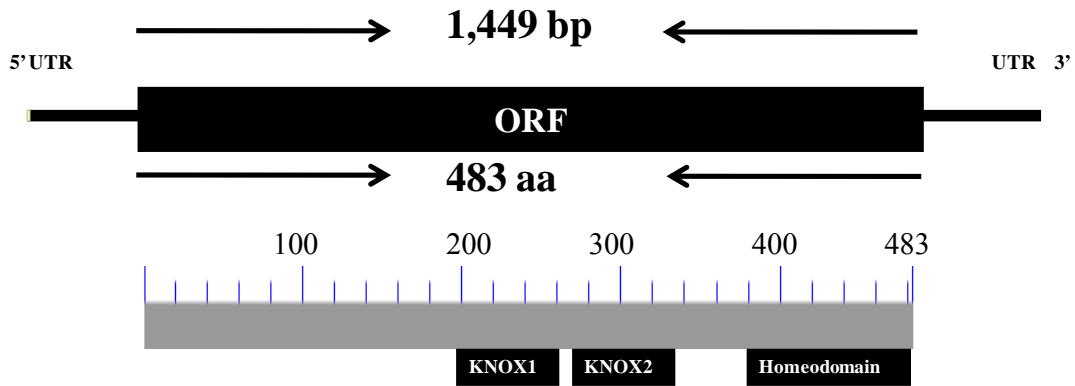


Figure 1. Structure of *JnKNAT3-like* transcription factor. The full-length coding sequence of *JnKNAT3-like* is 1,449 bp, starting with ATG and ending with TAA. The locations of the KNOX1 and KNOX 2 domains and the homeodomain are indicated.

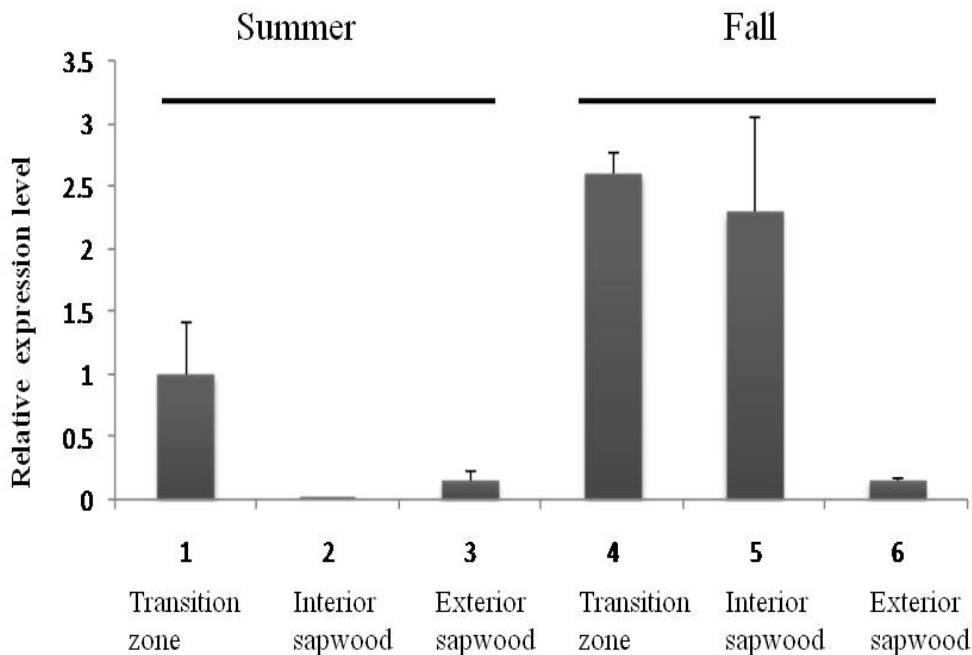


Figure 2. Transcript level of *JnKNAT3-like* in the TZ, interior sapwood, and exterior sapwood in summer and fall. The fold changes were quantified by real-time PCR and were analyzed by the comparative C_T method by comparison with the *JnKNAT3-like* transcript level in TZ of the summer tree. Values are the means \pm SD for three biological replicates. 18S rRNA was used as the standard.

of *JnKNAT3-like* in cambium. Perhaps KNOX class II homeobox gene expression in perennials is unlike that of annuals. A Southern blot revealed one copy of *JnKNAT3-like* in black walnut when using the conserved homeodomain of *JnKNAT3-like* as a probe (data not shown).

Given its known function in heterologous systems and its timing and location of expression in black walnut, *JnKNAT3-like* may be involved in heartwood formation. The role of this and other candidate genes indentified in this study will be investigated more fully.

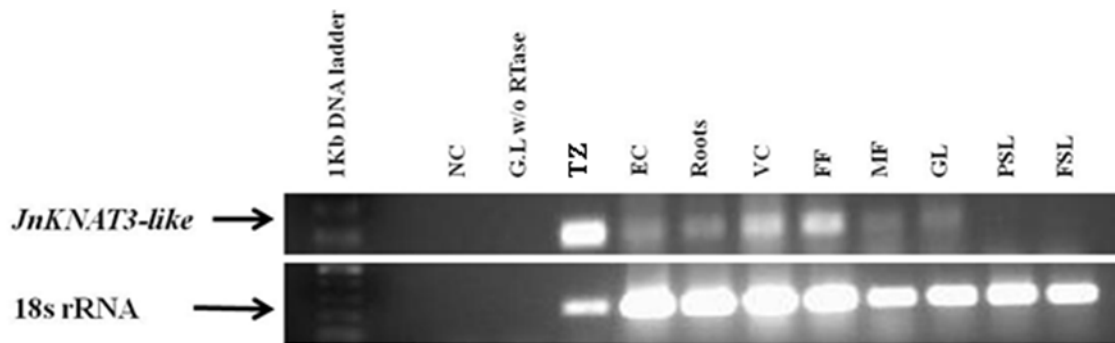


Figure 3. Quantification of *JnKNAT3-like* expression in black walnut tissues. The fold changes were quantified and analyzed by the comparative C_T method. Values are the means \pm SE for two technical replicates. NC = no template negative control; GL w/o RTase = green leaves without reverse transcriptase; TZ = transition zone, as positive control; EC = embryogenic callus; VC = vascular cambium; FF = female flowers; MF = male flowers; GL = green leaves; PSL = partially senescent leaves; FSL = fully senescent leaves. 18S rRNA was used as an internal control.

Acknowledgements: Funds for this research were provided by the U.S. Forest Service (USFS), the Department of Forestry and Natural Resources at Purdue University, and the NSF I/UCRC program. We thank Charles Michler for providing embryogenic callus. The use of trade names is for the information and convenience of the reader and does not imply official endorsement or approval by the USDA or USFS of any product to the exclusion of others that may be suitable.

REFERENCES

- Gehring, W.J. 1987. Homeoboxes in the study of development. *Science* 236: 1245–1252
- Harding, S.A, H.Y. Jiang, M.L. Jeong, F.L. Casado, H.W. Ling, C.-J. Tsai. 2005. Functional genomics analysis of foliar condensed tannin and phenolic glycoside regulation in natural cottonwood hybrids. *Tree Physiol.* 25: 1475–86
- Hillis, W.E. 1987. Heartwood and tree exudates. Springer Series in Wood Science, Springer-Verlag, Heidelberg, New York. 268 pp
- Huang, Z. 2009. Identification and characterization of genes involved in heartwood formation in black walnut (*Juglans nigra* L.). Ph.D. dissertation. Purdue University, 192 pp.
- Huang, Z., C.-J. Tsai, S.A. Harding, R. Meilan, K.E. Woeste. 2009a. A cross-species transcriptional profile analysis of heartwood formation in black walnut. *Plant Mol. Biol. Rep.* DOI: 10.1007/s11105-009-0144-x
- Huang, Z., R. Meilan, K.E. Woeste. 2009b. A *KNAT3-like* homeobox gene from *Juglans nigra*, *JnKNAT3-like*, is highly expressed during heartwood formation. *Plant Cell Rep.* DOI: 10.1007/s00299-009-0771-6
- Kerstetter, R, E. Vollbrecht, B. Lowe, B. Veit, J. Yamaguchi, S. Hake. 1994. Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. *Plant Cell* 6: 1877–87
- Kolossova, N., B. Miller, S. Ralph, B.E. Ellis, C. Douglas, K. Ritland, J. Bohlmann. 2004. Isolation of high-quality RNA from gymnosperm and angiosperm trees. *Biotech.* 36: 821–24
- Serikawa, K.A., A. Martinz-Laborda, H. Kim, P.C. Zambryski. 1997. Localisation of expression of *KNAT3*, a class 2 *knotted1-like* gene. *Plant J.* 11:853–61
- Vollbrecht, E., B. Veit, N. Sinha, S. Hake. 1991. The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature* 350: 241–3