Identification and Characterization of Genes Involved in Heartwood Formation in Black Walnut

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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. To do this, a microarray containing ~5,000 unique *Populus* expressed-sequence tags (ESTs) was hybridized with cDNA isolated from walnut tissues harvested on 1 July and 14 October 2004 and 2006. Analysis of the array data revealed the up-regulation in the autumn of genes associated with vacuolar-sorting and defense responses, indicating that heartwood formation may be related to programmed cell death (PCD). To test our hypothesis that PCD plays an important role in heartwood formation, we analyzed 80 ESTs expressed in the region of heartwood formation in the walnut stem (*i.e.*, the transition zone, TZ) that appeared to be associated with PCD. Semi-quantitative RT-PCR was performed to detect expression changes in the TZ and sapwood of the trees harvested in summer and fall. Results revealed that transcripts P22_T3 with significant similarity to an AP2 transcription factor, F18_T3 with a homeobox protein Knotted-1-like 3 (KNAP3), H12_T3 with a cysteine protease, and N01_T3 with ripening-related protein are differentially expressed in TZ of the tree harvested in the fall compared to the other tissues. Experiments were repeated three times. Real-time PCR was performed to confirm the expression changes detected via semi-quantitative RT-PCR. Full-length sequence analysis revealed that P22_T3 contains AP2 domain, known to be a transcription factor in other plants. Meanwhile, other genes of interest are being explored. Functionality of selected candidate genes will be investigated via transgenesis. Ultimately, these analyses should provide insight into the mechanism regulating heartwood formation in walnut and other hardwoods.

Introduction

The wood of many hardwood tree species has two distinct regions: sapwood, characterized by a pale color, and heartwood, which is dark in color. The percentage of heartwood is a major factor in determining the value and quality of hardwood logs. Heartwood formation, a natural aging process, is characterized by cell death and a change in wood color, and is thought to occur in the fall. Because of many limitations (e.g., the presence of wood extractives and difficulty isolating intact RNA), little is known about the metabolic and physical mechanisms of heartwood formation. In order to increase our understanding of this process, we are seeking to identify genes associated with the transition from sapwood to heartwood using EST-based microarrays and other molecular tools. These approaches allow us to evaluate differential expression of thousands of genes simultaneously. This insights into the regulation of heartwood formation. research should ultimately provide

Materials and Methods

Two black walnut trees grown at the Martell Research Forest were cut down on July 1, 2004, and October 14, 2004, respectively. These trees were labeled summer tree (Tree 1) and fall tree (Tree 3). Another four black walnut trees were cut down on the same dates in 2006. Immediately after the trees were felled, stem cross-sections ("cookies), approximately 2.5 cm thick, were cut with a chainsaw. The cookies were immediately submerged in liquid nitrogen. After returning to the lab, the cookies were transferred to an ultra-low freezer (-80 °C) for storage. Transition zones (TZ) were identified under UV light (Figure 1. A) and chiseled out of cookies from each tree, including sapwood interior and sapwood exterior (Figure 1. A). Total RNA was isolated from the transition zone, sapwood interior, and sapwood exterior of both summer and fall trees as described by Kolosova et al., (2004), followed by treatment with DNase. Integrity of the RNAs was confirmed via agarose gel. cDNA was synthesized from total RNA (via random hexamer priming) and labeled using a protocol provided by Chung-Jui, Tsai lab (Michigan Tech. Univ.). After labeling, array hybridization was performed in an HS400 (Tecan Instruments HS 400 Hybridization station). Cy3 and Cy 5 were used to label cDNA from Tree 1 (control) and Tree 3. Signal intensities were acquired by GenePix Pro software and output was obtained by GeneSpring microarray data analysis. Genes were considered up- or down-regulated when the expression ratios were ≥1.5 or ≤0.67, respectively. 80 primer pairs of genes of interest related to various forms of programmed cell death (PCD) were chosen from a cDNA library of transition zone in black walnut and designed using Primer 3. Transcript levels of genes of interest in transition zone, sapwood interior, and sapwood exterior of summer trees and fall trees were first detected by semi-quantitative reverse transcriptase (RT)-PCR and confirmed by quantitative real-time PCR assays (Bio-Rad). To get the full-length cDNA of genes of interest, 5' and 3' rapid amplification of cDNA ends (RACE) was performed by using SMART™ cDNAs (CLONTECH) and then was gel-purified, cloned into a pGEM-T vector (Promega), sequenced, and BLAST searched.

Results and Discussion

Figure 1. The microarray experiment.

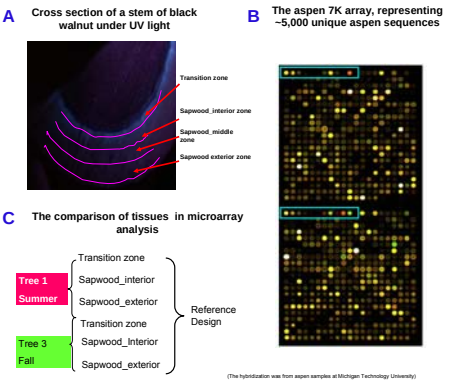
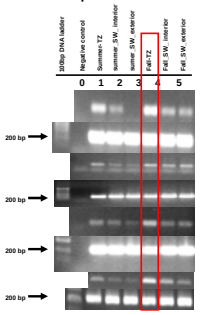


Figure 2. The cluster of up-regulated genes of Tree 3 (fall) transition zone contains many poorly characterized genes.

Figure 2. Results of the hierarchical clustering displayed by TREEVIEW. Each colored box represents the mean signals of two replicates for a particular sample. These data suggest that expression of this cluster of genes is greater in the transition zone of summer trees (Tree 1) or fall trees (Tree 3) since their expression profiles are similar. This is in contrast to other tissues such as sapwood interior and sapwood exterior of Tree 1 and Tree 3 in which expression is dramatically decreased in response to season and location. A column represents a different tissue and a row represents a gene. The mean expression level was determined for each gene across the six tissues, and for each tissue a gene was assigned red if expression was above the mean, green if expression was below the mean, and black if expression was near the mean. The tissues are indicated vertically on the top (T1_TZ = Tree 1(summer tree) transition zone; T1_SW_interior= Tree 1(summer tree) sapwood interior; T1_SW_exterior= Tree 1(summer tree) sapwood exterior; T3_TZ= Tree 3(fall tree) transition zone; T3_SW_interior= Tree 3(fall tree) sapwood interior; T3_SW_exterior= Tree 3 (fall tree) sapwood exterior. Genes are designated by MTU gene ID number of *Populus trichocarpa*.

Figure 3. Differential expression level of genes of interest in Tree 3 (Fall) TZ compared to other tissues via semi-quantitive RT-PCR.



cDNA	Putative protein
P22_T3	AP2 domain containing transcription factor
18S rRNA	Loading control
F18_T3	KNAP3 transcription factor
18S rRNA	Loading control
N01_T3	Ripening-related protein
18S rRNA	Loading control
H12_T3	cysteine protease
18S rRNA	Loading control

Figure 3. Transcript expression levels of P22_T3, F18_T3, N01_T3 and H12_T3 in six tissues of black walnut by semiquantitive RT-PCR. Equal loading of total RNA samples was confirmed by 18S rRNA. Shown is one of three replicates. The symbols of columns are the same as in Figure 2.

Figure 4. Relative abundance of P22_T3, F18_T3 and H12_T3 transcript in black walnut determined by real-time PCR.

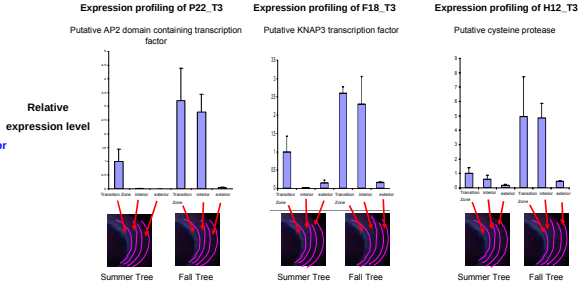
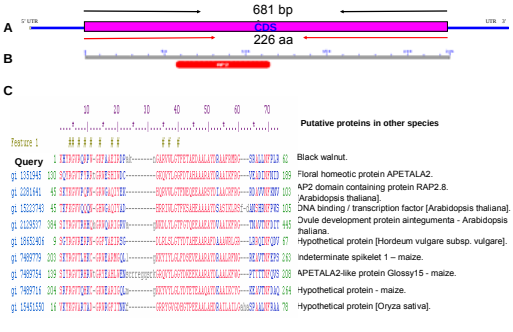


Figure 4. The fold change ratio of P22_T3, F18_T3 and H12_T3 expression to 18S rRNA was defined as the formula $2^{\Delta\Delta-Ct}$, where ΔCt =Ct (sample)−Ct (18S rRNA). ΔΔCt = ΔCt of samples –ΔCt of Tree 1(summer) transition zone. S (standard deviation) = square root of $S_1^2 + S_2^2$. Here is shown one of three replicates. The symbols of y-axis are the same as Figure 2.

Figure 5. Sequence analysis of the P22_T3 cDNA from black walnut encoding a putative AP2 domain protein.



A. The partial cDNA sequence was isolated from a cDNA library of the transition zone in black walnut. The full-length coding sequence of this P22_T3 cDNA was obtained using 5' and 3' RACE. The cDNA was in length with a complete open reading frame (ORF) of 681 bp encoding a polypeptide of 226 amino acids

B. Amino acid sequence analysis of P22_T3 revealed that it contains a conserved AP2 DNA-binding domain.

C. A conserved domain database search demonstrated that P22_T3 shares significant sequence homology with previously characterized AP2 domain-containing proteins from different species.

Ongoing and Future Work

Clone transcription factor F18_T3

↓

Construct binary vectors

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Transform candidate genes into SALK lines in *Arabidopsis thaliana*, *Populus trichocarpa* and *Juglans nigra*. L to test for functionality

Conclusions

- Expression of several genes related to alternative forms of programmed cell death (PCD) was up-or down-regulated in the transition zone of black walnut based on the aspen microarray analysis, indicating that PCD might play an important role in heartwood formation.
- 80 ESTs that are annotated to be associated with PCD were selected from a cDNA library of the transition zone in black walnut and the transcript level of those unique genes was detected via semi-quantitive reverse transcriptase PCR and re-confirmed by real-time PCR. Results showed that the transcripts P22_T3, with significant similarity to an AP2 transcription factor, and F18_T3, with a homeobox protein Knotted-1-like 3 (KNAP3), are highly upregulated in the transition zone of trees in the fall.
- Full-length sequences of the P22_T3 gene was isolated by 3' and 5' RACE using gene specific primers obtained from partial cDNA sequences. Sequence analysis of P22_T3 demonstrated that it contains an AP2 DNA-binding domain that is similar to a DNA-binding domain of previously characterized and putative proteins from *Arabidopsis* and other plants, including *Medicago truncatula*, *Capsicum annuum* , and rice. Previous research showed that the AP2 group of proteins is most similar to ERF proteins involved in transcriptional regulation of biotic and abiotic stress induced gene expression through a GCC box (AGCCGCC). In our experiment, P22_T3 is highly up-regulated in the transition zone of trees in the fall, indicating that P22_T3 may be involved in the regulation of cold-responsive gene expression by binding to GCC box, and ultimately leading to heartwood formation.
- The functionality of genes of interest is being confirmed via transgenesis in a model system.
- This study will shed light on the molecular mechanism of heartwood formation in black walnut and other fine hardwoods.

Literature

Beritognolo I, Magel EA, Abdel-Latif JP, Charpentier CJ, and Breton C (2002) Expression of genes encoding chalcone synthase, flavanone hydroxylase, and dihydroflavonol 4-reductase correlates with flavanol accumulation during heartwood formation in *Juglans nigra* L. Tree Physiology 22: 291–300.

Han KH (2001) Molecular biology of secondary growth. Journal of Plant Biotechnology 3(2): 45-57.

Kolosova N, Miller B, Ralph S, Ellis BE, Douglas C, Ritland K, and Bohlmann J (2004) Isolation of high-quality RNA from gymnosperm and angiosperm trees. Biotechniques 36: 821-824.

Ranjan P, Kao YY, Jang HY, Joshi CP, Harding S, and Tsai CJ (2004) Suppression subtractive hybridization-mediated transcriptome analysis from multiple tissues of aspen (*Populus tmuloides*) altered in phenylpropanoid metabolism. Planta 219: 694-704.

Sahin-cevik M and Moore GA (2006) Two AP2 domain containing genes isolated from the cold-hardy citrus relative *Poncirus trifoliata* are induced in response to cold. Functional Plant Biology 33: 863-975.

Yang J, Kamdem DP, Keathley DE and Han KH (2004) Seasonal changes in gene expression at the sapwood–heartwood transition zone of black locust (*Robinia pseudoacacia*) revealed by cDNA microarray analysis. Tree Physiology 24: 461–474.

Yang J, Park S, Kamdem DP, Keathley D, Retzel E, Paule C, Kapur V, and Han KH (2003) Novel gene expression profiles define the metabolic and physiological processes characteristive of wood and its extractive formation in a hardwood tree species, *Robinia pseudoacacia*. Plant Molecular Biology 52: 935–956.