# Genetic Control of Heartwood Formation in Black Walnut

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## Abstract

Black walnut (*Juglans nigra* L.) is a valuable hardwood tree species, but log value is affected by the amount of heartwood present. In this project, we are exploring ways to minimize sapwood production. Our goal is to identify genes that are associated with the transition from sapwood to heartwood. We are using a microarray on which ~5,000 unique aspen expressed sequence tags (ESTs) have been spotted. To identify gene expression profiles, these probes have been hybridized with target cDNA isolated from different black walnut tissues (i.e., transition zone, interior sapwood, and exterior sapwood) harvested at different times of the year (trees 1 and 3 were harvested on 1 July and 14 October 2004, respectively). Analysis of the array data revealed clusters of genes that were significantly up- or down-regulated in the transition zone of tree 3. Real-time PCR was performed to verify the expression changes detected via microarrays. These analyses suggest that heartwood formation in black walnut might be associated with vacuolar collapse, ethylene- and auxin-signal pathways, and stress and defense responses. Functionality of selected candidate genes will be investigated using transgenesis. Ultimately, these analyses should provide insight into the mechanism regulating heartwood formation in black walnut and other hardwoods.

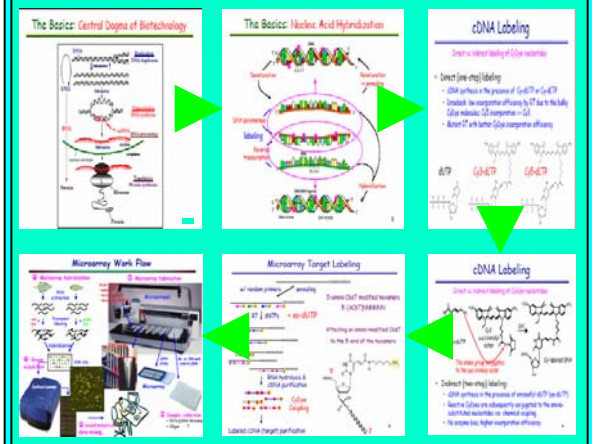
## Introduction

The wood of many hardwood tree species has two distinct regions: sapwood, characterized by a pale color, and heartwood, which is dark in color. Percent 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. 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 real-time PCR. These approaches allow us to evaluate differential expression of thousands of genes simultaneously. This research should ultimately provide insights into the molecular regulation of heartwood formation.

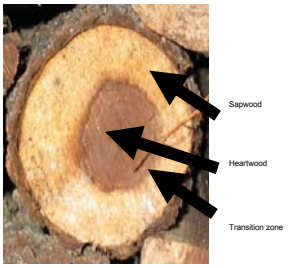
## Materials and Methods

Four black walnut trees, grown at the Martell Research Forest, were cut down on a series of four dates: July 1, 2004, September 1, 2004, October 14, 2004, and November 22, 2004. These trees were labeled 1-4, respectively. 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 ℃) for storage. Transition zones were identified under UV light and chiseled out of cookies from each of the four sample dates. Total RNA was isolated from these transition zones as described by Kolosova (2004), followed by treatment with DNase. Integrity of the RNA was confirmed via agarose gel. cDNA was synthesized from total RNA (via random hexamer priming) and labeled using a protocol provided by the 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 $\geqslant 1.5$ or $\leqslant 0.67$, respectively. Degenerate primers of up- or down-regulated genes of interest selected from microarray analysis were designed via using BLASTN and Clustal W. Primer pairs of genes of interest chosen from cDNA library of transition zone in black walnut were designed using primer 3. Quantitative real-time reverse transcriptase (RT)-PCR assays of those genes were performed using two-step RT-PCR with SYBR Green I (Bio-Rad) on the iQ5 Real Time PCR Detection system (Bio-Rad). Relative quantification was performed using comparative threshold methods, and all relative gene expression levels were normalized to the quantity of 18s rRNA from black walnut. Normalized gene quantities were averaged for the two biological replicates in this experiment.

## Microarray Analysis

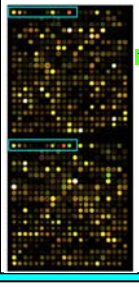



## Cross-section of a black walnut stem




## The design of microarray experiment

The aspen 7K array, representing ~5,000 unique aspen sequences



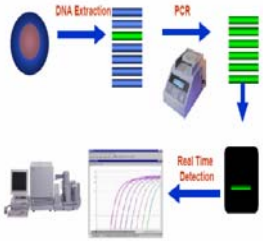
Tree 1: Transition zone, Sap_interior, Sap_exterior
Tree 3: Transition zone, Sap_Interio r, Sap_exterior
Reference Design

**Spike controls:**

Known-quantity DNA spots for monitoring labeling and hybridization efficiency

Need to add spike RNA during target preparation

## Real-time PCR work flow




## The cluster of up-regulated genes in transition zone

## The cluster of up-regulated genes of T3 transition zone


**Table 1. The list of up-regulated genes of interest in Microarray analysis in Transition Zone of Tree3**

| Systematic | Tree1_TZ | Tree1_Sap_inner | Tree1_Sap_out | Tree3_TZ | Tree3_Sap_inner | Tree3_Sap_out | |
|---|---|---|---|---|---|---|---|
| MTU6TR.P6.B01 | 0.737 | | 1.0902 | 1.3969 | 2.7483 | 0.6803 | At4g38510 vacuolar-type H+ATPase subunit B2 (VHA-B2) |
| MTU2TA.P6.G09 | 1.5821 | 1.416667 | 0.743869 | 1.5596 | 3.594527 | 1.27282 | At1g75830 vacuolar sorting protein 35, putative |
| MTU2TA.P7.C03 | 2.8863 | | 2.635302 | 2.4 | 5.55 | 2.587513 | At3g16660 unknown protein |
| MTU4CA.P23.E04 | 0.9036 | 0.8148 | 0.7188 | 1.7003 | 1.2107 | 1.049 | At3g12580 putative protein |
| MTU5CS.P7.D01 | 1.284389 | 0.290323 | 0.903106 | 1.764442 | 0.503931 | 0.852731 | At3g06030 HEAT SHOCK PROTEIN 81-2 (HSP81-2) (sp\|P55737) |
| MTU6CR.P7.F06 | 0.723 | 1.2902 | 0.548 | 2.1476 | 1.0213 | 0.7096 | At3g12580 putative protein |
| MTU7TL.P5.H09 | 0.8326 | 1.2545 | 0.6323 | 2.7024 | 0.7957 | 0.6142 | At1g56410 hypothetical protein |
| MTU6CR.P16.H01 | 2.2271 | 0.5483 | 1.7195 | 21.493 | 1.9235 | 1.5809 | At2g46140 putative desiccation related protein |
| MTU2TA.P1.D05 | 1.6278 | 0.964286 | 1.223656 | 1.84 | 1.220478 | 1.441141 | At1g55660 ubiquitin-protein ligase 1, putative |
| MTU7CL.P17.H06 | 12.252 | 1.3012 | 1.3787 | 30.704 | 1.039 | 0.8538 | At1g12520 Transport inhibitor response 1, putative |
| MTU5CS.P16.E09 | 1.900759 | 5.900001 | 10.75668 | 1.388924 | 20.15 | 13.16543 | At1g15820 unknown protein |
| MTU7TL.P5.H09 | 0.8326 | 1.2545 | 0.6323 | 2.7024 | 0.7957 | 0.6142 | At1g56410 hypothetical protein |
| MTU7TL.P15.D11 | 3.237985 | 6.388888 | 8.560555 | 12.13471 | 14.8 | 0.707247 | At3g04710 ankyrin-like protein |
| MTU4TA.P22.A09 | 4.1037 | | 0.5506 | 9.4 | 0.5901 | 0.4808 | At3g02780 unknown protein |
| MTU5CS.P1.G10 | 2.6641 | | 0.9479 | 3.983 | 1.0141 | 1.0198 | At2g38670 putative xyloglucan endo-transglycosylase |
| MTU7TL.P11.C07 | 0.9481 | 1.2785 | 0.7237 | 2.4889 | 0.9913 | 0.8094 | At1g23310 putative alanine aminotransferase |

## The differential expression fold changes of genes of interest via real-time PCR

| Tissue | Ct (ethylene responsive element binding factor (EREBP-3 ) ) | Ct (18s rRNA) | ΔCt ( EREBP-3 -18s rRNA) | ΔΔCt (ΔCt - ΔCt, 18s rRNA) | Relative to Tree1_Transition Zone |
|---|---|---|---|---|---|
| Tree1_Transition Zone | 27.91±0.51 | 12.4±0.24 | 15.51±0.57 | 0±0.57 | 1 ( 0.68~1.48) |
| Tree1_sapwood_exterior | 29.44±1.27 | 12.74±0.11 | 16.7±1.27 | 1.19±1.27 | 0.44 ( 0.18~1.06) |
| Tree3_Transition Zone | 26.62±0.03 | 10.69±0.02 | 15.93±0.04 | 0.42±0.04 | 0.75 ( 0.72~0.77) |
| Tree3_sapwood_exterior | 27.73±0.03 | 9.65±0.02 | 18.08±0.04 | 2.57±0.04 | 0.17 ( 0.16~0.17) |
| Tissue | Ct (ARF1-binding protein) | 18s rRNA | ΔCt ( ARF1-binding protein - 18s rRNA) | ΔΔCt (ΔCt - ΔCt, 18s rRNA) | Relative to Tree1_Transition Zone |
| Tree1_Transition Zone | 26.92±0.06 | 12.4±0.24 | 14.52±0.25 | 0±0.25 | 1 ( 0.84~1.19) |
| Tree1_sapwood_exterior | 29.22±0.02 | 12.74±0.11 | 16.48±0.11 | 1.96±0.11 | 0.26 ( 0.24~0.28) |
| Tree3_Transition Zone | 23.95±0.23 | 10.69±0.02 | 13.26±0.23 | (-)1.26±0.23 | 2.39 ( 2.04~ 2.8) |
| Tree3_sapwood_exterior | 25.49±0.19 | 9.65±0.02 | 15.84±0.19 | 1.32±0.19 | 0.4 ( 0.35~0.46) |
| Tissue | Ct (early auxin-inducible protein 11 (IAA11)) | 18s rRNA | ΔCt ( IAA11 -18s rRNA ) | ΔΔCt (ΔCt - ΔCt, 18s rRNA) | Relative to Tree1_Transition Zone |
| Tree1_Transiton Zone | 28.5±2.56 | 13.46±0.38 | 15.04±2.59 | 0±2.59 | 1( 0.17~ 6.02 ) |
| Tree1_sapwood_exterior | 23.4±2.33 | 10.15±0.43 | 13.25±2.37 | (-)1.79±2.37 | 3.46 ( 0.67~ 17.88) |
| Tree3_Transition Zone | 27.05±1.05 | 10.69±0.02 | 16.36±1.05 | 1.32±1.05 | 0.4 ( 0.19~0.83) |
| Tree3_sapwood_exterior | 29.47±8.02 | 9.65±0.02 | 19.82±8.01 | 4.78±8.01 | 0.036 ( 0.0001~9.38) |
| Tissue | Ct (senescence-associated protein) | 18s rRNA | ΔCt ( senescence-associated protein - 18s rRNA) | ΔΔCt (ΔCt - ΔCt, 18s rRNA) | Relative to Tree1_Transition Zone |
| Tree1_Transition Zone | 29.01±0.14 | 13.46±0.38 | 15.55±0.41 | 0±0.41 | 1( 0.75~1.33) |
| Tree1_sapwood_exterior | 25.69±0.22 | 10.15±0.43 | 15.54±0.48 | (-)0.01±0.48 | 1( 0.72~1.4) |
| Tree3_Transition Zone | 22.4±0.44 | 10.69±0.02 | 11.71±0.44 | (-)3.84±0.44 | 14.32 (10.56~ 19.43) |
| Tree3_sapwood_exterior | 23.45±0.22 | 9.65±0.02 | 13.8±0.22 | (-)1.75±0.22 | 3.36 ( 2.89~3.92) |
| Tissue | Ct (hypothetical protein-Vascular associated death) | 18s rRNA | ΔCt ( hypothetical protein-Vascular associated death - 18s rRNA) | ΔΔCt (ΔCt - ΔCt, 18s rRNA) | Relative to Tree1_Transition Zone |
| Tree1_Transition Zone | 28.63±0.28 | 13.46±0.38 | 15.17±0.48 | 0±0.48 | 1( 0.72~ 1.39) |
| Tree1_sapwood_exterior | 27.21±0.14 | 10.15±0.43 | 17.06±0.45 | 1.89±0.45 | 0.27( 0.2~0.37) |
| Tree3_Transition Zone | 25.35±0.22 | 10.69±0.02 | 14.66±0.23 | (-)0.51±0.23 | 1.42 ( 1.21~ 1.67) |
| Tree3_sapwood_exterior | 25.67±0.14 | 9.65±0.02 | 16.02±0.15 | 0.85±0.15 | 0.56 ( 0.5~ 0.63) |

**Table2.** The ΔCt value is determined by subtracting the average 18s rRNA Ct value from the average of each gene Ct value. The standard deviation of the difference is calculated from the standard deviations of the genes and 18s rRNA values. The calculation of ΔΔCt involves subtraction by the ΔCt calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of ΔΔCt is the same as the standard deviation of the ΔCt value. The range given for genes of interest relative to 18s rRNA is determined by evaluating the expression: $2^{-\Delta\Delta Ct}$. With ΔΔCt +s and –s, where s= the standard deviation of the ΔΔCt value. Here we use 18s rRNA as the reference gene. The relative value of 18s rRNA is equal to 1, we assumed that the gene of interest is up-regulated if the value is above 1, down-regulated if the value is below 1.

## Ongoing and Future Work

- RT-PCR to verify the microarray
- Select genes of interest based on RT-PCR
- Obtain full-length cDNAs via 5' or 3' RACE or inverted PCR
- Construct binary vectors for genes of interest
- Transform candidate genes into model plant to test for functionality

## Conclusions

- Expression of several genes was up or down regulated in tree 3 transition zone of black walnut based on microarray analysis and were re-confirmed by real-time PCR assays.
- Microarray analysis and real-time PCR assays suggested that auxin, senescence-associated gene and vascular associated death may be involved in heartwood formation.
- Differences in the physiological processes occurring in the transition zone during the summer and the onset of dormancy might be keys to heartwood formation.
- The functionality of genes of interest must be confirmed via transgenesis in a model system.

## Literature Cited

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