The project set out to use comparative (genotype and treatment) and transgenic approaches to investigate the determinants of condensed tannin (CT) accrual and chemical variability in Populus. CT type and amount are thought to effect the decomposition of plant detritus in the soil, and thereby the sequestering of carbon in the soil. The stated objectives were:

1. Genome-wide transcriptome profiling (microarrays) to analyze structural gene, transcription factor and metabolite control of CT partitioning;
2. Transcriptomic (microarray) and chemical analysis of ontogenetic effects on CT and PG partitioning; and
3. Transgenic manipulation of flavonoid biosynthetic pathway genes to modify the control of CT composition.

Objective 1: A number of approaches for perturbing CT content and chemistry were tested in **Objective 1**, and those included nitrogen deficit, leaf wounding, drought, and salicylic acid spraying. Drought had little effect on CTs in the genotypes we used. Plants exhibited unpredictability in their response to salicylic acid spraying, leading us to abandon its use. Reduced plant nitrogen status and leaf wounding caused reproducible and magnitudinally striking increases in leaf CT content.

Microarray submissions to NCBI from those experiments are the following:

GSE ID 14515: Comparative transcriptomics analysis of Populus leaves under nitrogen limitation: clone 1979. Public on Jan 04, 2010; Contributor(s) Harding SA, Tsai C

GSE ID 14893: Comparative transcriptomics analysis of Populus leaves under nitrogen limitation: clone 3200. Public on Feb 19, 2009; Contributor(s) Harding SA, Tsai C

GSE ID 16783 Wound-induced gene expression changes in Populus: 1 week; clone RM5. Status Public on Dec 01, 2009; Contributor(s) Harding SA, Tsai C

GSE ID 16785 Wound-induced gene expression changes in Populus: 90 hours; clone RM5 Status Public on Dec 01, 2009; Contributor(s) Harding SA, Tsai C

Although CT amount changed in response to treatments, CT composition was essentially conserved. Overall phenylpropanoid composition exhibited changes due to large effects on phenolic glycosides containing a salicin moiety. There were no effects on lignin content. Efforts to publish this work continue, and depend on additional data which we are still collecting. This ongoing work is expected to strengthen our most provocative metabolic profiling data which suggests as yet unreported links controlling the balance between the two major leaf phenylpropanoid sinks, the CTs and the salicin-PGs.

Objective 2: Ontogenic effects on leaf CT accrual and phenylpropanoid complexity (**Objective 2**) have been reported in the past and we contributed two manuscripts on how phenylpropanoid sinks in roots and stems could have an increasing effect on leaf CT as plants grow larger and plant proportions of stem, root and leaf change.

Tsai C.-J., El Kayal W., Harding S.A. (2006) **Populus, the new model system for investigating phenylpropanoid complexity. International Journal of Applied Science and Engineering 4: 221-233.** We presented evidence that flavonoid precursors of CT rapidly decline in roots under conditions that favor CT accrual in leaves.

Harding SA, Jarvie MM, Lindroth RL, Tsai C-J (2009) **A comparative analysis of phenylpropanoid metabolism, N utilization and carbon partitioning in fast- and slow-growing *Populus* hybrid clones. *Journal of Experimental Botany*. 60:3443-3452**. We presented evidence that nitrogen delivery to leaves as a fraction of nitrogen taken up by the roots is lower in high leaf CT genotypes. We presented a hypothesis from our data that N was sequestered in proportion to lignin content in stem tissues. Low leaf N content and high leaf CT in genotypes with high stem lignin was posited to be a systemic outcome of N demand in lignifiying stem tissues. Thereby, stem lignin and leaf CT accrual might be systemically linked, placing control of leaf phenylpropanoids under systemic rather than solely organ specific determinants. Analyses of total structural and non-structural carbohydrates contributed to the model presented.

Harding SA, Xue L, Du L, Nyamdari B, Sykes R, Davis M, Lindroth RL, Tsai CJ (submitted March 2013) **Condensed tannin biosynthesis in leaves conditions carbon use, defense and growth in Populus**. (Invited submission to ***Tree Physiology***) MS abstract: Condensed tannins (CT) are flavonoid end products that can comprise a large fraction of leaf, bark and root biomass in Populus species. CT accrual was investigated in relation to metabolic carbon and nitrogen use in young leaves and shoot tips (ST) where CT biosynthesis was most active. A slow-growing genotype (SG) and a fast-growing genotype (FG) were compared. Both genotypes exhibited the capacity to accrue similarly large reserves of salicortin a phenolic glycoside (PG), but the slow-growing line also produced CT. PG accrual was developmentally delayed in the slow-growing line, SG. Irrigation with low-N nutrients promoted PG accrual in FG plants, but PG accrual was suspended in CT-producing SG plants. In addition, the low C:N amide asparagine accumulated and glucose was depleted in ST and expanding leaves of SG plants. The monoamine phenylethylamine (PEA) was abundant in SG leaves and absent in FG leaves. Leaf metabolite and gene expression differences were observed between SG and FG that would be expected to impinge upon glycolysis, acetyl-CoA production and flavonoid production. A model that integrates PEA with those activities and CT accrual was developed. Briefly, the data support a model in which flavonoid biosynthesis depleted the acetyl-CoA pool, thereby promoting glycolytic and shikimic pathway fluxes in SG plants. PEA results from decarboxylation of the shikimic pathway end-product phenylalanine, and is proposed to have facilitated CT polymerization, thereby promoting the continued biosynthesis of flavonoid CT precursors in SG leaves. The leaf differentials described here were absent in young roots, as was PEA. The potential contribution of PEA to CT polymerization constituted a metabolic carbon drain in developing leaves that was not observed in the roots. We propose that PEA, in addition to other

factors, including flavonoid pathway Myb transcription factors, is an important contributor to carbon management and plant defense in *Populus*.

Objective 3: From work related to the first two objectives, it appeared that CT chemistry, at least in terms of the proportions of mono, di and tri hydroxylation at the phenylpropanoid-derived B-ring, changed little if at all when CT accrual per unit time was increased. A large number of transgenic Populus plants with alterations in the expression of flavonoid pathway genes and the potential to produce B-ring, chemically altered CT were generated during the project. Transgenic lines of *Populus tremula* Michx. × *Populus alba* L. clone 717-1B4, a low CT producer, were produced that over- or under-express several mid and late flavonoid pathway genes including dihydroxyflavonol reductase (DFR-2 isoforms), leucoanthocyanidin reductase (LAR-3 isoforms), anthocyanidin reductase (ANR-2 isoforms), flavonol synthase (FLS-2 isoforms). A large number of additional transformation constructs (chalcone synthases, flavone synthases, and flavanol hydroxylases) were developed that failed to result in transgenic plants. We have purified CT from several of the successful lines and have obtained evidence from pyrolysis GC-MS that CT chemical composition was altered in transgenic lines harboring overexpression constructs for one of the two DFR isoforms. We have also observed increased CT levels in leaves of those lines, but the increases vary substantially in magnitude from experiment to experiment which has led to ongoing efforts to understand the variation before attempting to publish the findings.

Preliminary results from some of the transgenic work were presented:

An C*, Luo K, El Kayal W, Harding SA, Tsai C-J (2009) Transgenic manipulation of condensed tannins in Populus. IUFRO Tree Biotechnology Conference, Whistler, BC, Canada

Work on the design of some of the constructs for the CT transgenics work has been published:

Luo K, Harding SA, Tsai C-J (2008) A modified T-vector for simplified assembly of hairpin RNAi constructs. *Biotechnology Letters* 30: 1271-1274.

DOE support from this project was also acknowledged in a book chapter:

Douglas CJ, Ehlting J, Harding SA (2009) **Phenylpropanoid and Phenolic Metabolism in *Populus*: Gene Family Structure and Comparative and Functional Genomics** *In* Joshi, C.P., and S.P. DiFazio (eds). Genetics, Genomics and Breeding of Crop Plants: Poplar. Science Publishers, Enfield, New Hampshire. Pp. 304-326

Other work directly related to and supported in part by this project include:

Qin H, Feng T, Harding SA, Tsai C-J, Zhang S (2008) **An efficient method to identify differentially expressed genes in microarray experiments.** Bioinformatics 24: 1583-1589.

Tsai C-J, Ranjan P, DiFazio SP, Tuskan GA, Johnson V (2011) **Poplar genome microarrays.** *In*: Joshi CP, DiFazio SP and Kole C (*eds*), Genetics, Genomics and Breeding of Poplars. Science Publishers, Enfield, NH. pp. 112-127.

Street N, Tsai C-J (2010) *Populus* resources and bioinformatics. *In*: Jansson S, Bhalerao R, and Groover AT (*eds*), **Genetics and Genomics of *Populus*.** Plant Genetics and Genomics: Crops and Models book series. Springer, New York, pp. 135-152.

Manuscript submitted March 2013:
Harding SA, Xue L, et al. **Developmental trajectories of condensed tannin biosynthesis condition carbon use and growth in Populus.** Invited manuscript submitted to Tree Physiology.

Abstract:

The project set out to use comparative (genotype and treatment) and transgenic approaches to investigate the determinants of condensed tannin (CT) accrual and chemical variability in Populus. CT type and amount are thought to effect the decomposition of plant detritus in the soil, and thereby carbon sequestration in the soil. A number of approaches for perturbing CT content and chemistry were tested, and those included nitrogen deficit, leaf wounding, drought, and salicylic acid spraying. Drought had little effect on CTs in the genotypes we used. Plants exhibited unpredictability in their response to salicylic acid spraying, leading us to abandon its use. Reduced plant nitrogen status and leaf wounding caused reproducible and magnitudinally striking increases in leaf CT content. Although CT amount changed in response to treatments, CT composition was essentially conserved. Overall phenylpropanoid composition exhibited changes due to large effects on phenolic glycosides containing a salicin moiety. There were no effects on lignin content. Efforts to publish this work continue, and depend on additional data which we are still collecting from stored materials. This ongoing work is expected to strengthen our most provocative metabolic profiling data which suggests as yet unreported links controlling the balance between the two major leaf phenylpropanoid sinks, the CTs and the salicin-PGs. We published evidence that flavonoid precursors of CT rapidly decline in roots under conditions that favor CT accrual in leaves. We published evidence that nitrogen delivery to leaves as a fraction of nitrogen taken up by the roots is lower in high leaf CT genotypes. We presented a hypothesis in that published report that N was sequestered in proportion to lignin content in stem tissues. Low leaf N content and high leaf CT in genotypes with high stem lignin was posited to be a systemic outcome of N demand in lignifiying stem tissues. Thereby, stem lignin and leaf CT accrual might be systemically linked, placing control of leaf phenylpropanoids under systemic rather than solely organ specific, metabolic determinants. Analyses of total structural and non-

structural carbohydrates contributed to the model presented. In carrying out this work, it appeared that CT chemistry, at least in terms of the proportions of mono, di and tri hydroxylation at the phenylpropanoid-derived B-ring, changed little if at all when CT accrual per unit time was increased. A follow-up study using metabolite and gene profiling on an updated agilent populus microarray platform has presently been submitted to Tree Physiology. The report highlights our discovery that the monoamine phenylethylamine (PEA) accumulates to high levels in leaves, but not in roots of high CT-producing lines. With gene and metabolite data, a systems-level model was developed to integrate glycolysis, shikimate pathway, flavonoid Myb transcription factors and PEA production with carbon use and CT accrual in Populus--submitted to Tree Phys March 2013. A large number of transgenic Populus plants with alterations in the expression of flavonoid pathway genes and the potential to produce B-ring, chemically altered CT were generated during the project. Transgenic lines of Populus tremula Michx. × Populus alba L. clone 717-1B4, a low CT producer, were produced that over- or under-express several mid and late flavonoid pathway genes including dihydroxyflavonol reductase (DFR-2 isoforms), leucoanthocyanidin reductase (LAR-3 isoforms), anthocyanidin reductase (ANR-2 isoforms), flavonol synthase (FLS-2 isoforms). Effects on CT accrual were minimal at best in most lines. A large number of additional transformation constructs (chalcone synthases, flavone synthases, and flavanol hydroxylases) were developed that ultimately were determined to have failed due to construct errors. We have purified CT from several of the successful lines and have obtained evidence from pyrolysis GC-MS that CT chemical composition was altered in transgenic lines harboring overexpression constructs for one of the two DFR isoforms. We have also observed increased CT levels in leaves of those lines, but the increases vary substantially in magnitude from experiment to experiment which has led to ongoing efforts to understand the variation before attempting to publish the findings.