

**GENOME-ENABLED DISCOVERY OF CARBON SEQUESTRATION GENES IN  
POPLAR**

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## EXECUTIVE SUMMARY

The goals of the S.H. Strauss laboratory portion of “Genome-enabled discovery of carbon sequestration genes in poplar” are (1) to explore the functions of candidate genes using *Populus* transformation by inserting genes provided by Oakridge National Laboratory (ORNL) and the University of Florida (UF) into poplar; (2) to expand the poplar transformation toolkit by developing transformation methods for important genotypes; and (3) to allow induced expression, and efficient gene suppression, in roots and other tissues.

As part of the transformation improvement effort, OSU developed transformation protocols for *Populus trichocarpa* ‘Nisqually-1’ clone and an early flowering *P. alba* clone, 6K10. Complete descriptions of the transformation systems were published (Ma et. al. 2004, Meilan et. al 2004). Twenty-one ‘Nisqually-1’ and 622 6K10 transgenic plants were generated.

To identify root predominant promoters, a set of three promoters were tested for their tissue-specific expression patterns in poplar and in *Arabidopsis* as a model system. A novel gene, *ET304*, was identified by analyzing a collection of poplar enhancer trap lines generated at OSU (Filichkin et. al 2006a, 2006b). Other promoters include the *pGgMT1* root-predominant promoter from *Casuarina glauca* and the *pAtPIN2* promoter from *Arabidopsis* root specific PIN2 gene.

OSU tested two induction systems, alcohol- and estrogen-inducible, in multiple poplar transgenics. Ethanol proved to be the more efficient when tested in tissue culture and greenhouse conditions. Two estrogen-inducible systems were evaluated in transgenic *Populus*, neither of which functioned reliably in tissue culture conditions.

GATEWAY-compatible plant binary vectors were designed to compare the silencing efficiency of homologous (direct) RNAi vs. heterologous (transitive) RNAi inverted repeats. A set of genes was targeted for post transcriptional silencing in the model *Arabidopsis* system; these include the floral meristem identity gene (APETALA1 or AP1), auxin response factor gene (ETTIN), the gene encoding transcriptional factor of WD40 family (TRANSPARENTTESTAGLABRA1 or TTG1), and the auxin efflux carrier (PIN-FORMED2 or PIN2) gene. More than 220 transgenic lines of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> generations were analyzed for RNAi suppression phenotypes (Filichkin et. al., manuscript submitted).

A total of 108 constructs were supplied by ORNL, UF and OSU and used to generate over 1,881 PCR verified transgenic *Populus* and over 300 PCR verified transgenic *Arabidopsis* events. The *Populus* transgenics alone required *Agrobacterium* co-cultivations of 124,406 explants.

**KEYWORDS:** *Populus*, *Arabidopsis*, root promoter, alcohol-inducible gene expression, RNAi, trichome, AT-hook, *P. trichocarpa* ‘Nisqually-1’

## REPORT DETAIL

The goals of the S.H. Strauss laboratory portion of “Genome-enabled discovery of carbon sequestration genes in poplar” are (1) to explore the functions of candidate genes using *Populus* transformation by inserting genes provided by Oakridge National Laboratory (ORNL) and the University of Florida (UF) into poplar; (2) to expand the poplar transformation toolkit by developing transformation methods for important genotypes; and (3) to allow induced expression, and efficient gene suppression, in roots and other tissues.

### The specific Oregon State University objectives and tasks included:

- 2.1. Develop the *Populus* transformation toolkit
  - 2.1.1. Develop efficient transformation protocols for model *Populus* clones
  - 2.1.2. Identify root-predominant promoters
  - 2.1.3. Create inducible promoter system for *Populus*
  - 2.1.4. Construct RNAi vectors for efficient ubiquitous, root-predominant and/or inducible suppression of target genes
- 2.2. Create a population of transgenics of each candidate gene designed to yield maximum information about gene function
  - 2.2.1. Create invertase transgenics
  - 2.2.2. Create cytokinin response factor and auxin response factor transgenics
  - 2.2.3. Create AUX/IAA transgenics
  - 2.2.4. Create sink strength transgenics

### 2.1. Development of the *Populus* transformation toolkit

2.1.1. As a part of the transformation improvement effort, OSU developed transformation protocols for *Populus trichocarpa* ‘Nisqually-1’, with known genome sequence, and early flowering *P. alba* (6K10) clones.

- A total of 21 ‘Nisqually-1’ transgenic plants were generated. A complete description of transformation system for ‘Nisqually-1’ was published (C. Ma, S. H. Strauss, and R. Meilan. 2004. *Agrobacterium*-mediated transformation of the genome-sequenced poplar clone, ‘Nisqually-1’ (*Populus trichocarpa*). *Plant Molec Biol Reporter.*, 22, p.1-9).

- A total of 622 transgenic plants were generated using the early flowering 6K10 clone. The average frequency of the developed 6K10 clone transformation system ranged from 2 to 4%. Five major classes of constructs were used for 6K10 transformation:
  1. RNAi constructs (a total of 552 transgenic plants);
  2. overexpression constructs (70 transgenics);
  3. ablation constructs (10 transgenics);
  4. promoter – reporter gene constructs (74 transgenics); and
  5. dominant negative mutants (79 transgenics).

**2.1.2. To identify root predominant promoters** a set of three promoters were tested for their tissue-specific expression patterns in poplar and in *Arabidopsis* as a model system.

By analyzing a collection of poplar enhancer trap lines generated at OSU, we identified a novel gene *ET304*. This gene is highly active during formation of adventitious and lateral roots and encodes a putative protein with a single predicted AT-hook motif, DNA binding domain, and nuclear localization signal. An isolated *ET304* promoter fused to the *GUS* reporter gene directed strong auxin-inducible expression in meristematic cells of root tips and lateral root primordia, both in poplar and *Arabidopsis* (Filichkin et al., 2006a). The later raised a possibility that *ET304*, and other genes from this gene family, may play an important role in initiation, development, and branching of lateral roots.

The lateral root-specific expression pattern of pET304 was confirmed by the analysis of T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub> generations of transgenic *Arabidopsis*. We searched the poplar genome for genes that share sequence similarity with *ET304*. In preliminary studies, using poplar whole genome microarrays and relative RT PCR, we found that similar to *ET304*, the majority of the family members display root-predominant expression patterns. Further comparative analysis of poplar, *Arabidopsis*, and rice genomes confirmed that ET304-like families of putative AT-hook transcription factors are phylogenetically conserved among all three genomes. The *pET304* promoter, and potentially the promoters of other root-predominant genes of *ET304*-like families, is excellent candidates for the utilization in tree carbon sequestration research and biotechnology.

- The other tested promoters included: 1) *pCgMT1* - root-predominant promoter from a tropical tree (*Casuarina glauca*) and 2) *pAtPIN2* - promoter from *Arabidopsis* root specific *PIN2* gene. The *pCgMT1* promoter showed high levels of activity in roots of multiple poplar transgenic lines; however, its expression was not limited to root tissues as *pCgMT1*-driven GUS expression was frequently observed in leaves and other aerial parts. The high activity of the *pCgMT1* promoter makes it a good candidate for general use in tree biotechnology although a background expression of *pCgMT* in aerial parts has limited the utility of this promoter in carbon sequestration by roots.
- The *Arabidopsis* promoter from the *PIN2* gene (encoding an auxin carrier protein) was active in *Populus* root tissues and cell files involved in basipetal auxin transport. Therefore, *pAtPIN2* was identified as a good candidate for the utilization in tree carbon sequestration research and biotechnology.

**2.1.3. Strong constitutive expression or silencing** of target genes may interfere with the recovery of viable transgenic plants. To overcome these limitations several chemically inducible systems have been recently developed. To our knowledge, none of these systems were successfully tested

in woody plants. To evaluate the efficiency of two chemically regulated promoter systems, alcohol- and estrogen-inducible, OSU tested them in multiple poplar transgenics.

- We investigated the function of an alcohol-responsive gene induction system in transgenic *Populus* using multiple independent transformation events in two different genetic backgrounds. Upon induction, the activity of GUS enzymes in poplar tissues was measured using fluorometry. The optimal inducer concentration and the duration of induction period were determined in dose response and time course experiments, respectively.
- We demonstrated that among the tested chemical inducers, ethanol was the most effective activator when concentrations were at or below 2%. Acetaldehyde at higher concentrations was limited by its toxicity to poplar plants. In contrast, up to 8% ethanol had no apparent toxic effects to plants both in tissue culture and in greenhouse conditions. The *alcA* promoter system was sensitive to the induction by ethanol in concentrations as low as 2%.
- A long-term (up to 30 days) continuous induction showed significant increase of *GUS* levels. Vapor treatment was an effective induction procedure resulting in high levels of *GUS* activity in leaves.
- The induction by ethanol was more efficient in plant growth in tissue culture as compared to greenhouse conditions. No significant GUS activity was detected in roots of greenhouse-induced transgenic trees.
- The alcohol-inducible system was functional in transgenic poplar and allowed temporal control of gene expression (Filichkin et al., 2006b).
- The alcohol-inducible “gene switch” was proposed for utilizations in studies of developmental genes improving transformation efficiency in poplar and other plants (Arias et al., 2006).
- Two estrogen-inducible systems were evaluated in transgenic *Populus*. Neither of the systems functioned reliably in tissue culture conditions.

#### 2.1.4. Construction of RNAi vectors.

To facilitate high throughput gene silencing, we designed GATEWAY-compatible plant binary vectors with similar backbones in which dsRNAs with either homologous (direct) or heterologous (transitive) inverted repeats and driven by identical constitutive promoters. These vectors allowed direct comparison of silencing efficiency for both types of RNAi. To evaluate the suppression effects of direct and transitive RNA interference methods in *Populus*, a set of genes was targeted for post transcriptional silencing in the model *Arabidopsis* system. This set included:

1. floral meristem identity gene *APETALA 1 (API)*;
  2. auxin response factor *ETTIN*;
  3. *TRANSPARENTTESTAGLABRA1 (TTG1)* gene encoding transcriptional factor of WD40 family (*TTG1* regulates trichome differentiation/anthocyanin biosynthesis); and
  4. auxin efflux carrier, *PIN-FORMED2 (PIN2)* required for gravitropic root growth.
- More than 220 transgenic lines of the 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> generations were analyzed for RNAi suppression phenotypes of the targeted genes listed above.
  - Both direct (self-complementary intron-spliced inverted repeat of the target sequence, hIR) and transitive (sequence fragment of the target gene cloned adjacent to heterologous IR) *API* RNAi constructs induced silencing of selected target genes.

- Both direct IR and transitive *API* RNAi constructs produced highly visible phenotypes consistent with observations reported for *ap1* loss of function mutants. Both direct IR and transitive *API* RNAi constructs showed high penetrance and stable inheritance in T2 progeny. Direct IR *API* RNAi constructs have had a higher proportion of strong, and a lower proportion of weak phenotypes when compared to transitive RNAi constructs. Strong *API* RNAi phenotypes resulted in complete or partial plant sterility. The depletion of *API* mRNA levels were confirmed by a quantitative reverse transcription polymerase chain reaction (qRT-PCR). Expression profiling of a number of genes, including a cluster of floral homeotic genes in the *API* pathway, was affected by both transitive and direct IR RNAi. Changes observed in expression of several floral developmental genes were corroborated by qRT-PCR. Thus, *API* RNAi suppression demonstrated to be an effective tool for regulation of plant sterility in a model system with great potential for applications in poplar biotechnology.
- RNAi suppression of the *ETTIN* target gene resulted in abnormal development and reduced or increased number of floral organs. An overall penetrance of *ETTIN* RNAi suppression was relatively low. In contrast to *API*, the RNAi silencing of *ETTIN* had no or little effect on plant sterility.
- *TTGI* RNAi constructs displayed moderate suppression of trichome development with a low frequency of transgenics with trichomless leaves. Further evaluation of *TTGI* RNAi effects on seed and root hair development is in progress.
- No highly visible agravitropic root phenotypes were observed for RNAi suppression of *PIN2* target gene using both direct and transitive constructs. The analysis of *PIN2* mRNA synthesis showed that approximately 50% T1 transformants had depleted levels of mRNA.
- We further evaluated efficiency and potential off-target effects of *API* gene silencing by transitive and direct IR constructs using genome-scale microarrays and quantitative RT PCR. We report broadly similar levels of gene silencing, and no evidence of significant off-targeting, caused by either RNAi method.
- Overall, the direct IR vector had a higher proportion of events with strongly manifested silencing phenotypes while the recombination-based vector for transitive RNAi had advantages of rapid sequence-independent cloning and targeting of multiple genes useful in high throughput poplar functional genomics. A combination of direct IR and transitive vectors provides a set of effective tools for applications in poplar functional genomics and the discovery of novel genes for modification of carbon sequestration.

**2.2.** A total of 108 constructs supplied by ORNL and UF and several built at OSU, were used to generate transgenic *Populus* plants. The major types of vectors utilized for poplar transformation included cDNA over-expression and RNA interference (RNAi) vectors (for both RNAi-direct and RNAi-transitive gene silencing). Detailed backbone description, vector maps and sequences are available via the ORNL Web site at [http://www.esd.ornl.gov/PGG/cap/belowground\\_poplar\\_project\\_vectors.htm](http://www.esd.ornl.gov/PGG/cap/belowground_poplar_project_vectors.htm).

The production of a panel of *Populus* transgenics required *Agrobacterium* co-cultivation of 124,406 explants. A total of 1,881 *Populus* and approximately 320 of *Arabidopsis* independent transgenic events were produced and verified by PCR. OSU transformation efforts for poplar are summarized in **Table 1**. Specific details and timelines in OSU production of transgenic trees are

shared with project collaborators and can be accessed at [http://www.esd.ornl.gov/PGG/cap/project\\_internal.htm](http://www.esd.ornl.gov/PGG/cap/project_internal.htm).

## CONFERENCE PAPERS/PROCEEDINGS AND JOURNAL ARTICLES

### *Presentations at professional meetings.*

S. A. Filichkin. Alcohol-Inducible Gene Expression In Transgenic Poplar. Plant & Animal Genomes XIII Conference; San Diego, January 15-19, 2005. P519 ([http://www.intl-pag.org/13/abstracts/PAG13\\_P519.html](http://www.intl-pag.org/13/abstracts/PAG13_P519.html)).

S. A. Filichkin. Directed functional genomics. Tree Biosafety and Genomics Research Cooperative Annual Meeting. Oregon State University, November 2004.

### *Publications*

Filichkin, S.A., Q. Wu, V.B. Busov, R. Meilan, C. Lanz-Garcia, C. Ma, P. Dharmawardhana, A.M. Brunner, and S.H. Strauss. 2006a. Enhancer trapping in woody plants: Isolation of the *ET304* gene encoding a putative AT-hook motif transcription factor and characterization of the expression patterns conferred by its promoter in transgenic *Populus* and *Arabidopsis*. *Plant Sci.* 171(2): 206-216.

Filichkin, S.A., Meilan, R., Busov, V.B., Ma, C., Brunner, A.M. and Strauss, S.H. 2006b. Alcohol-inducible gene expression in transgenic *Populus*. *Plant Cell Rep.* 25(7): 660–667.

Arias R.S., Filichkin S.A., Strauss S.H. 2006. Divide and conquer: development and cell cycle genes in plant transformation. *Trends Biotechnol.* 24(6): 267-273.

Busov V.B., Brunner A.M., Meilan R., Filichkin S., Ganio L., Gandhi S., Strauss S.H. 2005. Genetic transformation: a powerful tool for dissection of adaptive traits in trees. *New Phytol.* 167: 9-18.

S. H. Strauss, A. Brunner, S. A. Filichkin, S. Gandhi, E. Jaeger, J. Li and C. Ma. 2004. Improvement of transformation tools for directed functional genomics. Tree Biosafety and Genomics Research Cooperative. Annual Report. pp. 24- 26.

C. Ma, S. H. Strauss, and R. Meilan. 2004. *Agrobacterium*-mediated transformation of the genome-sequenced poplar clone, ‘Nisqually-1’ (*Populus trichocarpa*). *Plant Mol. Biol. Rep.*, 22, 1-9.

Meilan, R., M. Sabatti, C. Ma., E. Kuzminsky. 2004. An early-flowering genotype of *Populus*. *J. Plant Biol.*, 47, 52-56.

Meilan, R. and C. Ma. 2005. *Populus*. In: *Agrobacterium* Protocols, Methods in Molecular Biology, vol. 44. Ed. K. Wang, Humana Press.

Filichkin, S.A., DiFazio, S.P., Brunner, A.M., Davis, J.M., Kalluri, U.C., Yang, Z.K., R.S., Etherington, E., Tuskan, G.A., Arias, Strauss, S.H. Efficiency of gene silencing in

*Arabidopsis* : Direct inverted repeats versus transitive RNAi vectors. Manuscript submitted.

**Table 1. Summary of OSU poplar transformation efforts and service work for collaborator institutions (UF and ORNL)**

<b>Group of Constructs (Institution)</b>	<b>Number of Constructs</b>	<b>Explants Co-Cultivated</b>	<b>PCR verified Events</b>	<b>Status Of Product Delivery</b>
Invertases (UF)	11	14,352	262	Transgenic lines delivered to UF
Tester Lines (OSU & UF)	4	6,925	89	Transgenic lines delivered to UF, tested at OSU
Vector Controls (ORNL)	3	2,294	17	Accomplished, transgenic lines delivered to ORNL
Estrogen Inducible (OSU)	2	2,606	71	Testing accomplished
Alcohol Inducible System (OSU)	1	1,828	33	Testing accomplished , manuscript published
Root Predominant Promoters (OSU)	7	4,922	132	Testing accomplished, manuscript published
Yeast Invertase Overexpression (UF)	3	2,837	80	Accomplished, transgenic lines delivered to UF
Response Regulators (UF)	4	7,218	87	Accomplished, transgenic lines delivered to UF
<i>TRANSPARENT TESTA GLABRA</i> -like loci RNAi (UF)	12	15,227	273	Accomplished, transgenic lines delivered to UF
Auxin/IAAs (ORNL)	26	29,577	625	Accomplished, transgenic lines delivered to ORNL
Auxin Response Factors (ORNL)	16	17,217	150	Transgenic lines are currently being delivered to ORNL
Cytokinin Response Regulators (UF)	19	19,403	62	PCR testing in progress