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Yanghuan Bao for the degree of Master of Science in Genetics presented on March 28, 2008.

Title: Genome Scale Transcriptome Analysis and Development of Reporter Systems for Studying Shoot Organogenesis in Poplar.

Abstract approved:

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Steven H. Strauss

Vegetative propagation allows the amplification of selected genotypes for research, breeding, and commercial planting. However, efficient *in vitro* regeneration and genetic transformation remains a major obstacle to research and commercial application in many plant species. Our aims are to improve knowledge of gene regulatory circuits important to meristem organization, and to identify genes that might be useful for improving the efficiency of *in vitro* regeneration. In this thesis, we have approached these goals in two ways. First, we analyzed gene expression during poplar (*Populus*) regeneration using an Affymetrix GeneChip® array representing over 56,000 poplar transcripts. We have produced a catalog of regulated genes that can be used to inform studies of gene function and biotechnology. Second, we developed a GUS reporter system for monitoring meristem initiation using promoters of poplar homologs to the meristem-active regulatory genes *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*). This provides plant materials whose developmental state can be assayed with improved speed and sensitivity.

For the microarray study, we hybridized cDNAs derived from tissues of a female hybrid poplar clone (INRA 717-1 B4, *Populus tremula* x *P. alba*) at five sequential time points during organogenesis. Samples were taken from stems prior to

callus induction, at 3 days and 5 days after callus induction, and at 3 and 8 days after the start of shoot induction. Approximately 15% of the monitored genes were significantly up-or down-regulated based on both Extraction and Analysis of Differentially Expressed Gene Expression (EDGE) and Linear Models for Microarray Data (LIMMA, FDR<0.01). Of these, over 3,000 genes had a 5-fold or greater change in expression. We found a very strong and rapid change in gene expression at the first time point after callus induction, prior to detectable morphological changes. Subsequent changes in gene expression at later regeneration stages were more than an order of magnitude smaller. A total of 588 transcription factors that were distributed in 45 gene families were differentially regulated. Genes that showed strong differential expression encoded proteins active in auxin and cytokinin signaling, cell division, and plastid development. When compared with data on *in vitro* callogenesis from root explants in *Arabidopsis*, 25% (1,260) of up-regulated and 22% (748) of down-regulated genes were in common with the genes that we found regulated in poplar during callus induction.

When ~3kb of the 5' flanking regions of close homologs were used to drive expression of the GUSPlus gene, 50 to 60% of the transgenic events showed expression in apical and axillary meristems. However, expression was also common in other organs, including in leaf veins (40% and 46% of *WUS* and STM transgenic events, respectively) and hydathodes (56% of *WUS* transgenic events). Histochemical GUS staining of explants during callogenesis and shoot regeneration using *in vitro* stems as explants showed that expression was detectable prior to visible shoot development, starting 3 to 15 days after explants were placed onto callus inducing

medium. Based on microarray gene expression data, a paralog of poplar *WUS* was detectably up-regulated during shoot initiation, but the other paralog was not. Surprisingly, both paralogs of poplar STM were down-regulated 3- to 6-fold during early callus initiation, a possible consequence of its stronger expression in the secondary meristem (cambium) than in shoot tissues. We identified 15 to 35 copies of cytokinin response regulator binding motifs (ARR1AT) and one copy of the auxin response element (AuxRE) in both promoters. Several of the *WUS* and *STM* transgenic events produced should be useful for monitoring the timing and location of meristem development during natural and *in vitro* shoot regeneration.

Genome Scale Transcriptome Analysis and Development of Reporter Systems for Studying Shoot Organogenesis in Poplar

by
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Yanghuan Bao, Author

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CONTRIBUTIONS OF AUTHORS

Dr. Palitha Dharmawardhana and Dr. Todd Mockler gave extensive advice on microarray data analysis. Dr. Renee Arias and Ms. Megan Allen played a major role in construction of the *ProWUS::GUSPlus* and *ProSTM::GUSPlus* vectors, and recorded the expression patterns of *WUS* and *STM* transgenic events. Ms. Cathleen Ma and her student assistants performed most of the in vitro propagation and genetic transformation. Dr. Strauss provided funding, directed the overall project, and played a significant part in writing and interpretation.

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CHAPTER 1

GENERAL INTRODUCTION

Value of Tree Breeding and Biotechnology

Trees have great economic and ecological value to humankind. However, these two major values have increasingly come into conflict. On the one hand, societies are under great pressure to produce more resources from forests, such as timber for buildings; renewable energy and chemicals; and fiber for paper production. On the other hand, we rely on forests to maintain our ecosystems and the biological diversity they support. They supply oxygen, clean water, mitigate climate, and provide habitat--thus supporting large amounts of biological diversity.

As a result of these needs and conflicts, great efforts have been made to increase forest productivity and reduce the ecological impacts of resource extraction. Tree breeding and biotechnology is internationally recognized as powerful and cost-effective means for improving forest quality and productivity (White et al., 2007). Unfortunately, the biology of forest trees provides substantial impediments to traditional breeding and biotechnology. This includes long production cycles and generation times;, the limited resolution of their genomic maps and sequences; inability to inbreed due to low self-fertility and high genetic load; high cost of large,

long term breeding trials; difficult or costly vegetative propagation, especially from mature, proven trees; and inefficient and costly gene transfer methods for most species, even poplars (Jansson and Douglas, 2007).

Biotechnology, when informed by genomics data and methods, offers a possible means for accelerating forest tree improvement. In addition to the ability to gather large amounts of data on the genes underlying important traits by DNA sequencing and mapping, comparative genomics approaches enable the vast resources on gene function and interaction based in *Arabidopsis* and other model organisms to be transferred to trees. However, because of their distinct phylogeny and development, translation of this information requires elaboration and confirmation in trees. In addition, there are some fundamental differences between *Arabidopsis* and forest trees that require direct study in trees. These includes traits related to their perennial life cycle such as seasonal dormancy; delayed onset of flowering; slow maturation of crown form and other vegetative traits; and the activity of secondary meristems to enable wood formation. For these needs, a model tree species provides a means for rapid progress.

Poplar as a Model Tree

The *Populus* genus (including poplars, cottonwoods, aspens, and many hybrids) is considered the model tree species for genomics and biotechnology (Bradshaw et al., 2000; Taylor, 2002; Brunner et al., 2004; Strauss and Martin, 2004): (1) it has a

relatively small genome, only 480 Mbp (similar to rice, ~4X larger than *Arabidopsis*, and 40X smaller than pine); (2) it grows fast, speeding experimental evaluations; (3) it has abundant genetic variation to aid in genetic analysis, (4) it can be effectively transformed by *Agrobacterium*-mediated techniques (5) it can be easily propagated vegetatively to increase precision of phenotypic evaluation; and (6) there are large collections of genetic markers, maps, and ESTs available. Of most importance, an annotated draft of the *Populus* genome sequence (6.8X) is available at the web site of the Joint Genome Institute (Tuskan et al., 2006) (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). This provides basic information that informs all types of genomics and gene-associated biotechnologies. The main disadvantages of poplar as a model trees are its lesser economic value, reducing research investments in its study; a long generation interval that cannot be effectively reduced by horticultural treatments as it can in conifers and eucalyptus; and its predominant dioecy, making most genotypes impossible to self-pollinate (Nehra et al., 2005b; Jansson and Douglas, 2007).

Diversity of Tree Biotechnologies

The United Nations Convention on Biological Diversity defines biotechnology as: "Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" (http://www.cbd.int/convention/convention.shtml). The activities of forest tree

biotechnology fall under four categories: 1) micropropagation; 2) diversity studies; 3) mapping, marker-assisted selection (MAS), and genomics; and 4) genetic modification (FAO, 2004).

Propagation

Propagation allows the amplification of selected genotypes for research, breeding, or commercial planting. Two major pathways have been used for propagation: somatic embryogenesis and organogenesis. Somatic embryogenesis is the regeneration of a whole plant by embryo formation *in vitro*. The advantage of embryogenesis over organogenesis is that it has higher volume and less costly production in large-scale operations. The embryogenic cultures can be cryopreserved and stored with less concerns for aging and associated epigenetic instability (Nehra et al., 2005b). Most publications on embryogenesis of forest tress are on conifers. Most current research focuses on initiation of embryogenesis in conifers, production of high-quality somatic embryos, and improving quality and reducing cost of derived planting stock (Nehra et al., 2005b).

Organogenesis is the regeneration of a whole plant by sequential organ formation. The starting explants can be leaf disks, stem, hypocotyls, or cotyledons. Organogenesis is predominately used for regeneration of poplars and other dicotyledonous trees such as eucalyptus where embryogeneis is relatively difficult. With both systems, major challenges to commercial use include high cost; aging effects that prevent proven genotypes from being propagated, or impart epigenetic

alterations to important traits; and the induction of genetic or epigenetic change during the regeneration process.

Genetic Modification

Plant genetic transformation requires several steps: introduction of DNA into the genome of a cell, selection and growth of the transformed cell, and regeneration of a whole plant. *Agrobacterium*-mediated method and biolistic bombardment are most commonly used ways to introduce foreign DNA into a plant cell. Other than *Populus* species, many of which are amenable to transformation and regeneration, there are very few reliable transformation systems for forest trees (Nehra et al., 2005b). Conifers were difficult to transform with *Agrobacterium tumefaciens* for many years, thus transformation was originally limited to biolistic bombardment. Recently, *Agrobacterium*-mediated methods became a major option for conifers (Nehra et al., 2005b). The most widely used selectable maker is the *nptII* gene which confers resistance to the antibiotic kanamycin. Other selection markers have also been developed, such as several types of herbicide tolerance genes (Meilan et al., 2002).

Genetic Markers and Maps

Molecular markers and genetic linkage maps can be used to identify quantitative trait loci (QTLs). QTLs are statistical associations between markers and genes that control quantitative traits. The traditional QTL studies use pedigreed populations for within-family selection. However, association studies have grown in prominence in recent

years; they rely on populations of unrelated individuals to map genes with minimal linkage disquilibrium.

The first developed markers (RAPDs, RFLPs, AFLPs, and SSR) provided a way of estimating genetic diversity and gave rise to low density maps. Co-segregation of RAPD and AFLP markers were often used in construction of genetic maps. Development of new markers (AFLP, EST banks, SNP, and cDNA), transcriptomics, and proteomics makes the application of candidate genes in MAS more feasible because far more genes can be mapped. In association genetics, if a SNP in a candidate gene is frequently associated with a phenotype in a population of unrelated trees, the SNP is likely to contribute directly to the genotype—enabling direct functional gene identification (Brown et al., 2004; Neale and Savolainen, 2004). Such inferences were not possible with traditional linkage maps because of the large sections of chromosomes they usually indexed. Most of the genetic linkage maps for forest trees have developed in conifers and poplars (FAO, 2004).

Genome Sequencing and Functional Genomics

In 2006, the first full genome sequence for a forest tree was made available to public (Tuskan et al., 2006). The Joint Genome Institute (JGI) and the Oak Ridge National Laboratory (ORNL), funded by the US Department of Energy (DOE), initiated the project of poplar genome sequencing in 2002. The sequenced tree was female Nisqually-1 (*Populus trichocarpa*, black cottonwood), the largest native angiosperm tree in western North America. The basal annotation was based on the microsatellite

maps developed at ORNL and the EST collections mainly contributed by the Swedish *Populus* Genome Project and Genome British Columbia (Wullschleger et al., 2002; Brunner et al., 2004). Now the poplar nuclear, mitochondrial, and chloroplast genomes–consisting of 45,555 gene models–is available at the websites of the Joint Genome Institute (JGI) (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html).

Expressed sequence tags (ESTs) are an efficient way to study gene expression. The study of ESTs for forest trees began in the Swedish *Populus* Genome Project, which was the earliest and largest poplar genomics project in the world. They first analyzed 5,692 ESTs from the wood-forming tissues of two *Populus* cDNA libraries, and 10,000 ESTs from the leaves of two aspen cDNA libraries (Sterky et al., 1998; Bhalerao et al., 2003). Other poplar EST studies included analysis of 7,000 ESTs from two root cDNA libraries in France (Kohler et al., 2003), a developing xylem library (Dejardin et al., 2004), and a stress-induced leaves library (Nanjo et al., 2004) with >12,000 ESTs from different tissues of quaking aspen (*P. tremuloides*).

As discussed in more detail below, microarray technology can monitor global gene expression changes in different tissues, at different developmental stages, under different environmental conditions, and at different time points after a biochemical or physiological stimulus. As a result, many of the genes and regulatory factors related to specific traits and physiological states can be identified. Wood formation has been intensively studied in poplar and other species using microarrays. For example, transcription changes in the developing xylem treated by GA was determined using a cDNA-based microarry (Israelsson et al., 2003). By comparing gene expression

among stem micro-sections, the roles of many genes in xylem, phloem, and cambium development were characterized (Schrader et al., 2004). Like these studies, most published studies on forest tree used the cDNA array method. Subsequent to the completion of the poplar genome, two commercial oligonucleotide full-genome microarrays were designed, one by by Affymetrix, Inc. and one by NimbleGen, Inc. In *STM*-homolog over-expressed poplars, 102 and 173 genes were up- or down-regulated by two-fold or greater, respectively, using a NimbleGen microarray platform (Groover et al., 2006).

Evolutionary and Comparative Genomics

Tree species represent a diverse group of genera and families of terrestrial plants that includes both angiosperms and gymnosperms. They do not form a monophylogenetic group. Instead of presence of some unique regulatory genes, modifying the expression of genes already present in herbaceous plants is thought to enable secondary growth (Groover, 2005). Poplar is in the Eurosid clade in which *Arabidopsis* is also located. As a result, poplar is closer to *Arabidopsis* than trees in most other dicot taxa (Jansson and Douglas, 2007). Compared with the genome of *Arabidopsis*, 97.9% of the >1,000bp *Populus* EST sequences have a BLASTX score >100 in the analysis of 102,019 *Populus* ESTs. This supports the hypothesis that the main difference in the life histories and development of the two species is in differential gene regulation, rather than gene content (Sterky et al., 2004).

Due to the large salicoid duplication present in all poplars and willows (*Salicaceae*), many single-copy genes in *Arabidopsis* have two close homologs in poplar, and many of these duplicated genes have undergone subfunctionalization to take on modified patterns of gene expression (Jansson and Douglas, 2007). One example is the meristem-active regulatory gene *SHOOTMERISTEMLESS* (*STM*). In *Arabidopsis,* it functions by preventing the incorporation of cells in the meristem center into differentiating organ primordia (Long et al., 1996). In poplar, however, it is expressed in both apical meristem and secondary meristems (Groover, 2005; Groover et al., 2006). Comparative studies of *Arabidopsis* and poplar facilitate discovery of mechanisms that are conserved among eudicots, but also teach how different phylogenetic lineages can evolve distinct adaptive mechanisms using essentially the same set of genes (Jansson and Douglas, 2007).

Plant Meristems and Regeneration

Plant Meristems

Plant meristems consist of stem cells and rapidly dividing daughter cells derived from stem cells. Pluripotent stem cells are incompletely differentiated cells which continue dividing to generate new cells for differentiation of specific tissues and initiation of new organs. Three major types of meristems are present in plants: apical, secondary, and primary meristems (Laux, 2003; Scofield and Murray, 2006).

Apical meristems are comprised of completely undifferentiated stem cells and are located at shoot and root tips. A small population of slowly dividing stem cells located at the Central Zone (CZ) in both shoot apical meristems (SAM) and root apical meristems (RAM) maintain the identity of stem cells. Apical meristems give rise to three types of primary meristems which contribute to the primary growth of a plant: protoderms, procambiums, and ground meristems. The three types of primary meristems develop into epidermis, primary xylem and phloem, and pith, respectively. Primary meristems in turn differentiate into two types of secondary meristems: vascular and cork cambium. Vascular cambium is derived from procambium. During secondary growth, the vascular cambiums divide to produce secondary xylem (wood) towards the inside and secondary phloem towards the outside (bark), resulting in production of wood. Cork cambium is produced by the ground meristem and produces outer bark.

In vitro *Regeneration*

The *in vitro* regeneration of plants can be induced in two different tissue culture systems: somatic embryogenesis and organogenesis. A whole plant is regenerated through embryo formation from explants or from cell masses in somatic embryogenesis, or though organ formation from various tissues other than gametes (e.g., leaf disks, stem, hypocotyls, and cotyledons) in organogenesis. During organogenesis explants usually go though four sequential stages: direct or indirect callus induction, adventitious shoot formation, adventitious root formation from

shoots, and whole plant propagation via repetitive shoot or root production from existing meristematic tissues (e.g., axillary nodes). The developmental fates of explants are largely controlled by the balance of cytokinin and auxin in the growth medium. High, medium, and low cytokinin/auxin ratios induce the formation of shoots, callus, and roots, respectively.

Auxin Signaling

Auxin effects are mediated by both F-box protein-dependent transcriptional pathways and non-transcriptional pathways (Figure 1.1 A; reviewed in (Quint and Gray, 2006; Teale et al., 2006). TIR1 and other three auxin signaling F-box proteins (termed AFB1, 2 and 3 thereafter) are auxin receptors involved in the transcriptional regulation of auxin-responsive genes (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). Ubiquitin-dependent protein degradation is also an important step in auxin signaling (Gray et al., 2001). Members of the auxin response factor (ARF) family bind to auxin-responsive elements (AREs, TGTCTC) in the promoter of primary auxin-responsive genes (e.g. the AUX/IAA, SAUR and GH3 families), which mediates their effects. Auxin binds to TIR1 contained in SCF-like complex (SCFTIR1), which promotes the interaction between TIR1 and AUX/IAAs. The ubiquitin (Ub)-modified AUX/IAAs by SCFTIR1 are then targeted to the 26S proteasome and subsequently degraded. Aux/IAAs present early in the auxin-response pathway and their specific binding to ARFs blocks the ARE-mediated gene transcription. However, TIR1-mediated transcriptional signaling pathway does not

explain rapid cellular responses to auxin. This suggests the existence of another pathway mediating non-transcriptional effects. Auxin-binding protein 1 (ABP1) specifically binds to auxin with high affinity, but does not seem involved in auxin-regulated transcription (Dharmasiri et al., 2005b). Extracellularly localized ABP1 appears to be connected to some rapid auxin-dependent cellular responses, especially cell expansion. This suggests that ABP1 may mediate a non-transcriptional auxin signaling pathway.

Cytokinin Signaling

Cytokinin signaling is similar to prokaryotic two-component systems involving mutil-step phosphorelays (Ferreira and Kieber, 2005; Muller and Sheen, 2007). There are four major steps: cytokinin perception by histidine kinases (HKs), phosphor transfer by histidine phosphotransfer proteins (HPs), transcription activation by response regulators (RRs), and negative feedback by other RRs. The *Arabidopsis* cytokinin kinase (AHK2, AHK3, and AHK4) function as cytokinin receptors (Inoue et al., 2001; Schmulling, 2001; Ueguchi et al., 2001). They contain a conserved extracellular CHASE (cyclase/HK-associated sensing extracellular) domain which binds to cytokinin, a histidine kinase domain, and a receiver domain. HPs interact with various HKs and RRs and mediate cytokinin phosphotransfer.

All three receptors and five *Arabidopsis* HP genes are expressed ubiquitously in different tissue types in *Arabidopsis* and function in a distinct but overlapping manner (Hutchison and Kieber, 2002). The expression of HPs is not affected by

exogenous cytokinin treatment. There are two major types, termed A and B, for the 23 RR genes in *Arabidopsis* (Ferreira and Kieber, 2005). The expression of the type-B RRs remains unchanged in response to cytokinin treatment, while that of the type-A RRs is rapidly elevated. Both types have a conserved receiver domain at the N-terminus. The type-A RRs have been proposed to act as a negative feedback loop in the pathway. The type-B RRs also have a DNA-binding GARP domain and a transcriptional activation domain at their C-terminus. They act as transcription factors that localize in the nucleus and activate or depress the components downstream of the primary cytokinin signaling cascade. A consensus DNA-binding sequence (G/A)GGAT(T/C) has been identified in the type-B RRs and the promoters of many cytokinin response genes.

Meristem Function

Among all the meristem niches, the SAM is best characterized, and *Arabidopsis* meristem function has been extensively reviewed (Vernoux and Benfey, 2005; Williams and Fletcher, 2005; Bhalla and Singh, 2006). Stem cells are located in the central zone (CZ) and are maintained by the signals from the underlying organizing center (OC). The stem cells rapidly divide and displace some of their descendants to the peripheral zone (PZ), where cells give rise to the primordia of aerial tissues.

Among the dozens of identified regulatory factors, *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) have been extensively studied. As of early 2008, approximately 240 research articles on these genes were listed at The *Arabidopsis*

Information Resource (TAIR). The maintenance of the SAM in *Arabidopsis* is regulated by a feedback loop between *WUS* and *CLAVATA* (*CLV*) (Laux et al., 1996; Mayer et al., 1998). *WUS*, located in the OC, is sufficient to induce expression of *CLV3*, which is assumed to be a ligand for the *CLV1* receptor kinase. When CLV1 interacts with CLV3, it triggers a signaling pathway that results in the repression of the expression of *WUS*.

STM is a Class I *knotted*-like homeodomain protein required for SAM formation during embryogenesis (Long et al., 1996). It functions by preventing incorporation of cells in the meristem center into differentiating organ primordia. Organ development takes place when *STM* is down-regulated in primordium cells. The regulation of *Arabidopsis* root apical meristem (RAM) have common themes with that of the SAM (Byrne et al., 2003).

In contrast to the SAM, there has been far less research on the secondary meristem. However, secondary growth is evolutionarily ancient, evolving prior to the separation of gymnosperms and angiosperms (Jansson and Douglas, 2007). Thus, even plants which are phylogenetically unrelated, as well as annuals and perennials, may share common mechanisms and regulatory factors. As discussed above, secondary growth is considered to be a result of modified expression of meristem-regulatory genes, rather than a trait which is unique within in a single lineage (Groover et al., 2006). Even *Arabidopsis* can be induced to undergo secondary growth in certain conditions (Chaffey et al., 2002).

Microarray Methods for Functional Genomics

The basis of DNA microarrays is hybridization of probes and targets (transcripts of interest) (Murphy, 2002). High-quality RNAs isolated from the cells or tissues are usually reverse transcribed to cDNA, then amplified and fluorescently labeled. The labeled RNAs are used as targets for hybridization. There are two major types of probes that are placed on microarrays: cDNAs and oligonucleotides. In cDNA microarrays, the competitive hybridization of two samples indicates the relative abundance of each sample in the original RNA. In oligonuleotide microarrays, only one sample is hybridized on each chip and a comparison between two chips hybridized with cDNA derived from different sources indicates their relative abundances.

For the Affymetrix Genechip, 11 to 20, 25mer oligos, each placed in a region of the target gene that has the least similarity to other genes, are chosen as perfect matches (PM) to identify the tested transcripts (http://www.affymetrix.com/technology/index.affx). Mismatch (MM) control probes are sometimes used to adjust for errant hybridization; they are identical to their perfect match partner except for the central position of the oligomer. The probes are synthesized through mask-directed photolithography. Other technologies, such as ink-jet and digital micromirrors, are also used in target synthesis for other platforms (e.g., Agilent).

Quality Assessment of Microarray Data

Due to the complexity and high cost of microarray platforms and hybridization, after RNA extraction most steps in analysis are usually carried out by trained professionals in genomics facilities (labeling, hybridization, scanning, image gridding, segmentation, intensity extraction, and background corrections). Researchers usually start with the probe set data (signal intensity) and perform specific quantitative analysis relevant to the biological hypotheses being tested.

To ensure that the data generated is of high quality, several kinds of quality control are usually provided on commercial platforms. For example, Affymetrix arrays have a large number of 'housekeeping genes' on its GeneChips, and provide several kinds of information on array quality. This includes image inspection, B2 oligo performance, average background reports, noise values, poly-A controls (*lys*, *phe*, *thr*, *dap*), hybridization controls (*bioB*, *bioC*, *bioD*, and *cre*), and internal control genes (3' to 5' ratios of *β-actin* and *GAPDH*). The reliability and repeatability of the microarray data is summarized by the correlations between biological replicates (association between normalized array intensities on different arrays from the same biological treatment, but with independently extracted RNA and probe hybridization). Residual images can be used to detect chip-wide hybridization problems (Reimers and Weinstein, 2005).

Statistical Analysis

Systematic (technical) variation often exists across experimental conditions, which is unrelated to the biological differences of interest. To compensate for systematic technical differences, and to reveal systematic biological differences across samples, data normalization is carried out before statistical analysis. More than a dozen methods are available for normalizing probe level data, including a variety of graphic methods and summary statistics (http://affycomp.biostat.jhsph.edu/). (GC)RMA (Robust Multichip Average) outperforms the other common methods (Wu et al., 2004), and thus is recommended for Affymetrix data (Allison et al., 2006). It also takes the CG percentage of the probes into consideration during analysis. The algorithm computes gene expression summary values for Affymetrix GeneChip® data in three steps: a background adjustment using sequence information, quantile normalization, and finally summarization. The summary values are based on a log2 scale.

Statistical Significance Determinations

Increasing the number of biological replicates is the most powerful means for improving the ability to declare changes in gene expression as statistically significant. The higher the number of biological replicates, the lower the False Discovery Rate (FDR) (Wolfinger et al., 2001). At least two replicates are necessary for standard t-tests and ANOVA.

Due to the high number of genes on a microarray, even if the p-value assigned to a gene is low, the gene still could be a false positive caused by random rather by a true treatment effect. This is a result of the very large number of unplanned comparisons made during array analysis. The False Discovery Rate (FDR, Q-value) has been proposed as a method to control the number of false declarations of statistical significance that would otherwise occur. Some statistical methods that use FDRs have been tailored to microarray analyses, including SAM (Significance Analysis of Microarrays, http://www-stat.stanford.edu/~tibs/SAM/) and EDGE (Extraction and Analysis of Differential Gene Expression, http://www.biostat.washington.edu/software/jstorey/edge/).

Biological Interpretation Tools

To find changes in expression pattern associated with specific types of biological processes, differentially expressed genes can be categorized by their functional classes. Gene Ontology (GO, http://www.geneontology.org/) provides a standard categorization and vocabulary with respect to various types and scales of biological function (Lewis, 2005). It enables the biological processes underlying expression data to be compared among different species, even when specific homologs cannot.

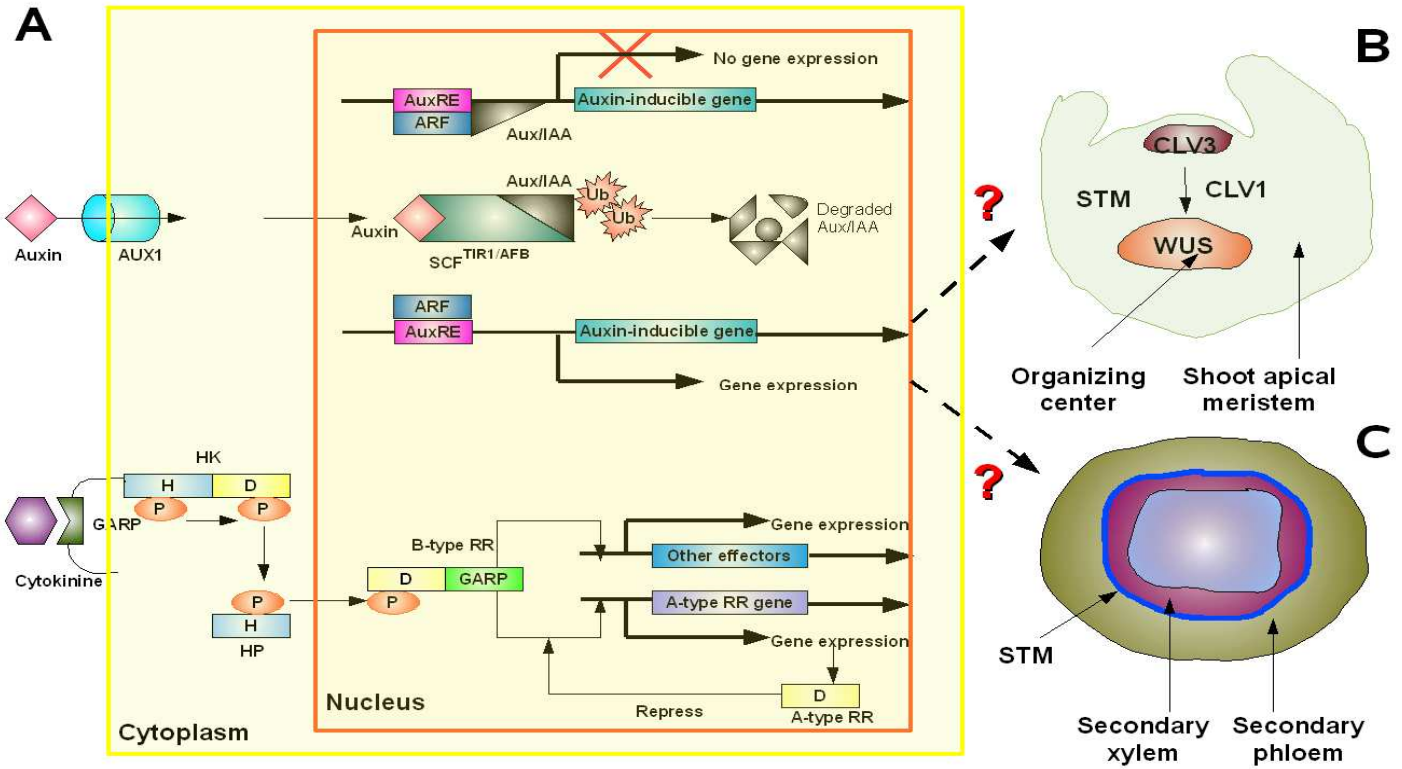
Genes in the same regulatory circuit or with similar function tend to be correlated in their expression patterns. In order to find groups of functionally related genes, hierarchical clustering, K-means clustering, and other methods can be used to detect similarity in expression pattern. The regulatory regions and introns of similarly

regulated genes can be scanned for conserved motifs that may link their expression with the binding or processing by common regulatory factors (Choe et al., 2005; Allison et al., 2006).

Data Format and Deposit

Community standards have been put in place to ensure that published microarray data is of high quality, and can be interpreted and reanalyzed by others. The most prevalent standard is that called “Minimum Information About a Microarray Experiment” (MIAME, http://www.mged.org/Workgroups/MIAME/miame.html). It helps to standardize file formats and descriptions of array data and experiments, both in publishing and in online databases. Its checklist has been adopted by many journals as a requirement for the submission of papers incorporating microarray results. There are also microarray databases that collect data from microarray experiments to facilitate use by others. They are the Stanford Microarray database (http://genome-www5.stanford.edu/), Gene Expression Omnibus - NCBI (http://www.ncbi.nlm.nih.gov/geo/), and ArrayExpress at EBI (http://www.ebi.ac.uk/microarray-as/aer/?#ae-main[0]).

Figure 1.1. Overivew of hormone signaling and meristem regulation. See text for a detailed description. (A) Auxin and cytokinin signaling. (B) Regulation of the shoot apical meristem (SAM). (C) Secondary meristem regulation.



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CHAPTER 2

GENOME SCALE TRANSCRIPTOME ANALYSIS OF SHOOT ORGANOGENESIS IN POPLAR

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Abstract

We analyzed gene expression during poplar regeneration using an Affymetrix GeneChip® array representing over 56,000 poplar transcripts. Our aims are to improve knowledge of gene regulatory circuits important to meristem organization, and to identify regulatory genes that might be useful for improving the efficiency of regeneration during transformation. Regeneration of transgenic cells remains a major obstacle to research and commercial deployment of transgenic plants for most species. We focused on callus induction and shoot formation, thus sample RNAs were collected from tissues: prior to callus induction, 3 days and 15 days after callus induction, and 3 days and 8 days after the start of shoot induction. We used a female hybrid white poplar clone (INRA 717-1 B4, *Populus tremula* x *P. alba*) that is used widely as a model transgenic genotype. Approximately fifteen percent of the monitored genes were significantly up-or down-regulated based on both Extraction and Analysis of Differentially Expressed Gene Expression (EDGE) and Linear Models for Microarray Data (LIMMA, FDR<0.01); over 3,000 genes had a 5-fold or greater change in expression. We found a large initial change in expression after initial hormone treatment (at the earliest stage of callus induction), and then a much smaller number of additional differentially expressed genes at subsequent regeneration stages. A total of 588 transcription factors that were distributed in 45 gene families were differentially regulated. Genes that showed strong differential expression included components of auxin and cytokinin signaling, selected cell division genes, and genes related to plastid development and photosynthesis. When compared with data on *in*

vitro callogenesis in *Arabidopsis*, 25% (1,260) of up-regulated and 22% (748) of down-regulated genes were in common with the genes regulated in poplar during callus induction.

Keywords: *Populus, in vitro* shoot organogenesis, transcriptome, auxin, cytokinin, transformation, regeneration, dedifferentiation.

Introduction

In vitro regeneration is a common research tool and important method for plant propagation. It is also essential for most forms of genetic transformation, which require the regeneration of single transgenic cells into non-chimeric organisms (Nehra et al., 2005; Poupin and Arce-Johnson, 2005). Both embryogenic and organogenic regeneration pathways are widely employed, with the system of choice varying among species and research or propagation goal.

Organogenesis systems are more widely applied than embryogenic systems, particularly in dicotyledenous plants, because the explants and *in vitro* conditions are less complex. During organogenesis, explants are generally subjected to four sequential stages: direct or indirect callus induction, adventitious shoot (or root) formation, adventitious root (or shoot) formation, and micropropagation using axillary or apical meristem containing tissues based on either shoot or root cuttings.

About a half century ago, researchers found that the developmental fates of explants *in vitro* are controlled by the balance of cytokinin and auxin (Skoog and Miller, 1957). When cytokinin is high relative to auxin, shoots are induced; when the reverse is true, roots are induced. When both hormones are present, but usually with dominance of auxin, undifferentiated growth of callus often occurs. Although there has been a great deal of progress in identification of key genes that regulate embryogenesis and organogenesis (Zhang and Lemaux, 2004; Castellano and Sablowski, 2005; Cairney and Pullman, 2007), as well as genome scale studies of *in vitro* regeneration (Che et al., 2002; Che et al., 2006; Su et al., 2007), the studies have

focused on few species and specific regeneration systems. For example, the studies in *Arabidopsis* have all focused on indirect regeneration via root explants rather than shoot explants (Che et al., 2006), and used the Affymetrix ATH1 GeneChip which represents only 22,810 genes. Root explants were pre-incubated on callus induction medium (CIM) for 4 days and then transferred to a cytokinin-rich shoot induction medium (SIM). Near half (10,700 out of 22,810) of probe sets exhibited regulated expression profiles. During early shoot development, 478 and 397 genes were specifically up-regulated and down-regulated, respectively. In rice, a monocot, somatic embryos regenerated from cell culture were used to induce shoots. By comparing gene expression at 7 days on SIM with somatic embryos using a 70-mer cDNA microarray containing 37,000 probe sets, 433 and 397 genes were up-or down-regulated, respectively (Su et al., 2007).

The genus *Populus* has grown rapidly as a model system for plant and tree biology (Jansson and Douglas, 2007). Its utility is likely to grow further as a result of the publication of a complete genome of *Populus trichocarpa* (Torr. & Gray) produced by the USA Department of Energy Joint Genome Institute (Tuskan et al., 2006). The value of poplar as a model tree results from its modest sized genome, facile transformation and clonal propagation, rapid growth, extensive natural diversity, many natural and bred interspecific hybrids, and diverse environmental and economic values (Bradshaw et al., 2000; Taylor, 2002; Brunner et al., 2004). Its natural ability for vegetative regeneration, even from mature tissues, and its amenability to organogenic regeneration and transformation *in vitro*, has motivated a large number of studies of

the biology and management of regeneration systems (Nehra et al., 2005; Poupin and Arce-Johnson, 2005).

Microarrays have successfully identified many of the genes and regulatory factors related to specific physiological states in poplar. Wood formation has been intensively studied using microarrays. For example, transcription changes in the developing xylem treated by GA was studied using a cDNA-based microarry analysis (Israelsson et al., 2003). By comparing gene expression among stem micro-sections, the roles of many genes in xylem, phloem, and cambium development were characterized (Schrader et al., 2004). Subsequent to the completion of the poplar genome, two commercial oligonucleotide genome-scale microarrays were designed. One was produced by Affymetrix and another by NimbleGen. In *STM*-homolog over-expressed poplars, 102 and 173 genes were up- or down-regulated by two-fold or greater, respectively, using a NimbleGen platform (Groover et al., 2006)

The goal of this study was to characterize the changes in gene expression that accompany dedifferentiation and organogenic regeneration in *Populus*, and compare them to results from *Arabidopsis* and other species. Characterization of the regulatory networks from poplar—with its distinct *in vitro* system and phylogeny compared to the other species studied to date—should give new insights into the conserved mechanisms for maintenance and regulation of plant stem cells. In this chapter, we describe a genome-scale transcriptome analysis of dedifferentiation to callus, and subsequent regeneration of shoots, using the Affymetrix Poplar Genome GeneChip. It monitors more then 56,000 transcripts based on the poplar genome and EST

sequences. We describe the identities and biological roles of more then 9,000 unique regulated genes observed over five stages of regeneration.

Materials and Methods

Plant Material and Culture Conditions

Hybrid poplar clone INRA 717-1 B4 (female, *Populus tremula* x *P. alba*) was used for all experiments. Plants were *in vitro* propagated according to published protocols (Filichkin et al., 2006; Meilan and Ma, 2006). In brief, inter-nodal stem segments (3-4 mm in length) from *in vitro* micropropagated plants were cut and incubated on callus induction medium (CIM, MS containing10 μMnaphthaleneacetic acid (NAA) (Sigma, St. Louis, MO) and 5 μM N^6-(2-isopentenyl) adenine (Sigma) at 22°C in darkness for 15 days. Shoots were induced by culturing explants on shoot induction medium (SIM, MS containing 0.2 μM TDZ (NOR-AM Chemical Co., Wilmington, DE).

RNAs were extracted separately from two batches (biological replications) that had been grown under the same growth conditions but three weeks apart in February 2007. For both, samples were collected at five time points: prior to callus induction, 3 days on CIM, 15 days CIM (then transferred to SIM), and 3 days and 8 days on SIM. Approximately 10-15 stem explants from the same plate (~3 to 4 mm in length, with nodes removed) were pooled for RNA extraction for each biological replication

Microarray Platform

The GeneChip® Poplar Genome Array was designed by Affymetrix; it contains more than 61,000 probe sets representing over 56,000 transcripts and gene predictions. The probes are based on content from UniGene Build #6 (March 16, 2005), GenBank® mRNAs, and ESTs for all *Populus* species (up to April 26, 2005) from the predicted gene set v1.1 from the *Populus* genome project (U.S. Department of Energy, Joint Genome Institute, downloaded on May 4, 2005; http://www.affymetrix. com/ products/arrays/ specific/ poplar.affx). The genome sequence is based on reads from a single tree of black cottonwood of the pacific northwestern USA (*P. trichocarpa*; Tuskan et al. 2006)

RNA Extraction and Quality Examination

Total RNA was isolated and purified according to the RNeasy Mini Protocol for Isolation of Total RNA from Plant Cells and Tissues and Filamentous Fungi (QIAGEN Inc., Valencia, CA). A260/A280 ratios of RNA samples dissolved in 10 mm Tris pH 7.6 ranged from 1.9 to 2.1. The integrities of RNA samples were examined by the Agilent 2100 Bioanalyzer; their RINs (RNA Integrity Number) ranged from 8.6 to 10.0, and they showed no evidence of degradation.

Array Hybridization and Quality Assessment

The arrays were labeled and hybridized at the Center for Genomics and Biocomputing at Oregon State University (http://www.cgrb.oregonstate.edu/) according to

Affymetrix protocols. The quality of data was assessed by a series of parameters associated with assay and hybridization performance developed by Affymetrix. These include probe array image inspection, B2 oligo performance, average background, and noise values, poly-A controls (*lys*, *phe*, *thr*, *dap*), hybridization controls (*bioB*, *bioC*, *bioD*, and *cre*), internal control genes (3' to 5' ratios of *β-actin* and *GAPDH*), percent presence, scaling, and normalization factors. The reliability and repeatability of this microarray platform was also evaluated by the correlations between the two biological replicates.

Quantitative Analysis

The probe-level data were normalized by the GC Robust Multichip Average (GCRMA) (Wu et al., 2004) algorithm using affylmGUI (http://bioinf.wehi.edu.au/affylmGUI/). The algorithm computes gene expression summary values for Affymetrix GeneChip® data in three steps: a background adjustment using sequence information, quantile normalization, and finally summarization. The summary values are based on a log2 scale.

Differentially expressed genes were identified by two methods: Extraction and Analysis of Differential Gene Expression (EDGE, http://www.biostat.washington.edu/software/jstorey/edge/) (Leek et al., 2006) and Linear Models for Microarray Data (LIMMA, http://bioinf.wehi.edu.au/limma) (Smyth, 2005). EDGE is designed for time course microarray experiments, and tests differential expression patterns globally. It fits two models of expression for each gene

during the time course, assuming that there is differential expression and there is no differential expression, then tests the null hypothesis via an F-test. A false discovery rate (FDR) of 0.01 was used as cutoff to identify differentially expressed genes.

LIMMA identifies differential expression via a modified t-test of gene expressions between two points, using B statistics to rank differentially expressed genes. A P-value adjustment of HM (FDR) was applied and an adjusted P-value of 0.01 was used as a cutoff. The genes identified in both EDGE and LIMMA were compared to identify those in common and those that were unique.

To reveal both global expression changes compared to the starting explant developmental state, and the specific expression changes taking place at each stage, two sets of contrasts between time points were used. First, the expression during the each of the stages was compared with the baseline explant (CIM0). Second, the expression at each stage was compared with that of the previous time point.

Biological Interpretation

All annotation information for the Affymetrix Poplar Genome Array was retrieved from PopARRAY (http://popgenome.ag.utk.edu/mdb/N_Affy_annot.php). The annotation for each Affymetrix probe set ID consists of corresponding public ID, JGI poplar gene models, predicted *Arabidopsis* homolog, and functional annotation.

The JGI gene model IDs of all transcription factors were download from the Database of Poplar Transcription Factors (DPTF) (http://dptf.cbi.pku.edu.cn/). It collects known and predicted transcription factors from *Populus trichocarpa*. DPTF

currently contains 2,576 putative transcription factors gene models, distributed in 64 families.

Hierarchical clustering was performed using MeV 4.0 (MultiExperiment Viewer, http://www.tm4.org/mev.html) with the Pearson correlation and average linkage model. The ratios of expression of a gene at each time point with its highest expression value among the five time points were used for scaled clustering.

GO annotation and categorization were done at the Bio-Array Resource for *Arabidopsis* Functional Genomics (BAR, http://www.bar.utoronto.ca/) with predicted *Arabidopsis* matches. The normalized frequencies were calculated as frequency of the class in the input data set divided by the frequency of the class in the whole genome. The class frequency was calculated as the ratio of the number of regulated genes in that class divided by the total number of genes in the class in the input data set, and the frequency of the class in the genome was calculated as the ratio of the total number of genes for that class in the genome divided by the total number of genes in the genome. Because of a lack of available detailed genome annotation statistics for poplar, the percentage of each functional class in the poplar genome was assumed to be approximately equal to that in *Arabidopsis*.

Comparative studies were carried out by comparing the regulated *Arabidopsis* homologs to a group of regulated poplar or rice genes detected under similar conditions. Data on *Arabidopsis* and rice was downloaded form the online supporting materials of the relevant publications (Che et al., 2006; Su et al., 2007). For *Arabidopsis*, root explants had been preincubated on CIM for 4 days and then

transferred to cytokinin-rich SIM. Among the monitored 22,810 transcripts on the Affymetrix ATH1 GeneChip, 5,038 (up-regulated) and 3,429 (down-regulated) genes exhibited regulated expression profiles with a false discovery rate of 0.01. During early shoot development, 478 and 397 genes were specifically up-regulated and down-regulated, respectively. For rice, somatic embryos generated from cell culture were used to induce shoots. By comparing gene expression 7 days on SIM with somatic embryos with a 70-mer cDNA microarray containing 37,000 probe sets, 433 and 397 gene were found up-or down-regulated, respectively (p-value < 0.05, $\geq$ two-fold change, Su et al., 2007). For comparison of regulated genes identified between species, the *Arabidopsis* homolog ID (identification) numbers of the rice genes that were given in the online supporting tables (Su et al., 2007), and the preferred *Arabidopsis* homolog IDs of the poplar genes from the PopArray database (http://popgenome.ag.utk.edu/mdb/N_Affy_annot.php), were compared with the *Arabidopsis* IDs in Che et al. (2006). A gene is considered to be in common with *Arabidopsis* if their *Arabidopsis* homolog ID matches the *Arabidopsis* ID.

Results

Callus and Shoot Development during Regeneration

To determine the time points for taking tissue samples during *in vitro* shoot organogenesis, we carried out a preliminary regeneration experiment where 3 to 4 mm internodal stem segments were placed on auxin-rich CIM in dark for 15 days, then transferred them to cytokinin-rich SIM following our optimized transformation

protocol (described under methods). No observable morphological change occurred during the first three days on CIM (Figure 2.1 B). The explants began to form callus at the two ends starting at 7 days on CIM, and the size of callus continued to grow (Fig 2.1 C). Individual or multiple shoot buds emerged from callus beginning from 8 days on SIM. Shoots were observed in approximately 10% of explants by 10 days on SIM (Fig 2.1 D), and the percentage grew to around 20% at 20 days on SIM. Based on the above observations, explants were collected at 3 days both on CIM and SIM to detect early genetic regulation of callus induction and shoot induction, respectively. Eight days on SIM was chosen to study regulatory events just prior to shoot emergence.

Quality Assessment of Array Data

We inspected graphical images of the raw hybridization intensity for each of the 10 arrays, and found no severe spatial artifacts (Fig S1 A) that appear likely to prevent accurate estimation of transcript expression levels over the 11 randomly located probes per transcript (Reimers and Weinstein, 2005). The quality report files (GeneChip Expression Analysis Data Analysis Fundamentals) —which consist of average backgrounds, scaling factors, percentages of presence, internal controls, poly-A controls, and hybridization controls—indicated that no significant flaws were detected (Fig S1, B-G). Approximately 48,000 transcripts out of over 56,000 had detectable expression for at least one time point. The squared correlations between the two biological replicates ranged from 0.94 to 0.99 (Fig S1 F).

Identification of Differentially Expressed Genes

The numbers of differentially expressed genes identified by EDGE and LIMMA were 8,848 and 12,513, respectively. A total of 8,045 (14% of all monitored transcripts on the genechip) were in common among the two methods, accounting for 91% of the total number of genes identified by EDGE, and 64% of the genes identified by LIMMA (Fig 2.2A). The larger set of genes identified by LIMMA were considered in further analyses.

When expression at each stage was compared to that prior to regeneration (Fig 2.2 B), we found up to 4,312 genes were up-regulated, and up to 4,772 genes were down-regulated. The largest number of regulated genes were identified at the earliest stage of callogenesis, though morphological changes were not yet visible at this time point. When comparing the expression at each time point with that of the previous time point, the difference among the numbers of differentially expressed genes declined nearly an order of magnitude with sequential time points (Fig 2.2 C). In contrast to the thousands of regulated genes during early callogenesis, there were only 132 and 90 genes up- and down-regulated, respectively, during the early stages of shoot induction.

Gene Ontology Categorization of Differentially Expressed Genes

To identify the over-represented molecular functions and biological processes at each stage, we categorized the groups of the up-or down-regulated genes at each stage by their Gene Ontology (GO) class. Due to incompleteness of poplar GO annotations and

the conservation of gene families between poplar and *Arabidopsis,* we used the *Arabidopsis* matches of the identified differentially expressed poplar gene for GO categorization. We used normalized frequencies to test if a functional class is over-represented; when the normalized frequency of a functional class is larger than 1, this functional class is likely to be over-represented in a group of genes.

Most of the GO biological process categories classes had similar numbers of genes that were up- and down-regulated (Table 2.1). However, at the onset of callogenesis—where the large majority of regulated genes were detected—there was a preponderance of up-regulated genes for the GO cellular components related to ribosome, cytosol, mitochondria, cell, wall, and endoplasmic reticulum functions. In contrast, there was strong down-regulation for chloroplast and plastid functions. For GO molecular function categories, a preponderance of up-regulation during the start of callogenesis was observed for structural molecule activity, nucleotide binding, and nucleic acid binding.

Clustering of Differentially Expressed Genes

To identify genes with similar expression patterns during regeneration, we clustered the 9,033 genes identified by LIMMA that had expression levels above those flagged as absent or marginal in Affymetrix data quality reports at the stages when they are regulated. At least five major clusters are visible (Fig. 2.3). Prior to callus induction, about half of the regulated genes are strongly expressed, but most of these are shut down or repressed immediately and permanently upon callogenesis (Cluster 1, 5,434

genes). Small numbers of genes form the next three clades. One group has genes that are very weakly expressed prior to callogenesis, activated during late callogenesis, then sequentially shut down as shoot induction proceeds (Cluster 2, 587 genes). Another group's genes are strongly expressed then largely shut down throughout the rest of regeneration (Cluster 3, 1,028genes); others are mostly turned off, further reduced in expression during initial callogenesis, then activate late in callogenesis and are subsequently turned off during shoot induction (Cluster 4, 734 genes). Finally, a very large group of genes have very weak expression prior to regeneration, activated rapidly and strongly during early callogenesis, then was largely down-regulated for the remainder of regeneration (cluster 5, 3,525 genes). There does not appear to be a cluster of genes that are specifically up-regulated during shoot induction.

Clustering of Differentially Expressed Transcriptional Factors

We found that 588 transcriptional factors (23% of total) distributed in 42 families were differentially expressed (Table 2.2). Transcription factors involved in auxin signaling are among the most abundant regulated transcription factor families. Approximately 70% of Aux/IAA and 40% of ARF genes were up- or down-regulated during at least one stage. Other abundant families—involving at least 40% of its members—included SRS, TLP, CCAAT-HAP2, GRF, and C2C2-Dof.

When only transcription factors were considered in cluster analysis, several distinct clusters emerged, but were somewhat different in their patterns from the full gene list (Figure 2.4, Supplemental Table 4). Similar to the complete gene list, prior to

callus induction more than half of the regulated genes were strongly expressed (A), but mostly shut down or repressed immediately and permanently upon callogenesis (Cluster 1, 316 genes). A small group had genes that were also expressed prior to callogenesis, then shut down but many reactivated during later stages of shoot induction (E) (Cluster 2, 35 genes). Another small group's genes were largely unexpressed prior to callus induction, but then strongly up-regulated during early callogenesis (B) and then largely deactivated again thereafter (Cluster 3, 52 genes). A large heterogeneous group had genes that were variably, but generally weakly, expressed prior to callus induction, but then reactivated at various times in callus and shoot induction (Cluster 4, 132 genes). Finally, a small group of genes were conspicuously strongly expressed during late callogenesis (C), but weakly and variably expressed at other stages (cluster 5, 45 genes). As with the full gene set, there does not appear to be a cluster of genes that are specifically up-regulated during shoot induction.

Auxin, Cytokinin, and Cell-Cycle Associated Genes

Two F-box proteins were differentially regulated upon callus induction that are closely related to *Arabidopsis TIR1* (*Transport Inhibitor Response 1*) genes (Fig 2.7 A). After early callus induction stage, their expression stabilized for the remainder of the regeneration period. A number of F-box proteins are thought to take part in auxin signaling (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). Twenty-three Aux/IAAs and fifteen ARFs were differentially expressed during

at least one stage (Figure 2.5 B and C). The majority of both classes of genes were down-regulated at the onset of callus induction and throughout subsequent regeneration, but specific groups of Aux/IAA genes were then up-regulated late in callus and during shoot development, or up-regulated during early callus induction and then down-regulated thereafter (Figure 2.5C)

A number of genes that take part in cytokinin signaling were regulated during regeneration (Figure 2.6). Key components of the cytokinin signaling and reception pathways include receptor kinases, phosphotransfer proteins, and various response regulators (Ferreira and Kieber, 2005; Muller and Sheen, 2007). A putative cytokinin receptor histidine kinase was down-regulated upon callus induction. Three differentially expressed histidine phosphotransfer proteins were down-regulated during callus induction, the up-regulated during subsequent growth and shoot regeneration. All three A-type response regulators were up- then down-regulated during callus development, then strongly up-regulated during shoot induction. Only one of two B-type response regulators was substantially down-regulated upon callus induction. A regulated pseudo response regulator was strongly down-regulated at callus induction, then strongly up-regulated during shoot induction.

Cell cycle genes are of obvious importance for regeneration, as slow growing explant tissues must be reactivated to grow rapidly during callus and shoot development. The cell cycle genes showed complex patterns of regulation, some being up- and others down-regulated at various points in regeneration (Figure. 2.7). A group (A) was rapidly up-regulated, then mostly down-regulated after callus induction.

Another group (B) was not up-regulated until late in callus induction, but then was also mostly reduced in expression during shoot induction; some of these genes, however, did reactivate later in shoot induction. A third major group (C) was strongly expressed prior to callus induction, then showed diverse patterns of reduced expression in subsequent stages.

Comparison of Regulated Genes to Arabidopsis *and Rice*

To identify genes whose function in regeneration is conserved among plant families, we compared our results to that of a similar microarray experiment in *Arabidopsis* (Che et al., 2006). They reported changes in expression after four days on CIM to pre-induction root tissues, and found 5,038 up-regulated and 3,429 down-regulated genes at an FDR of 0.02. Our comparison revealed that 16% to 22% of down-regulated genes were in common, and 25 to 27% of up-regulated genes were in common, depending on the direction of comparison (poplar to *Arabidopsis*, or the reverse; Figure 2.9). Thus, approximately 2,000 genes were conserved in their basic roles among the two species. Of these genes approximately 8% were transcription factors. The largest GO classes of genes that were common and up-regulated include those related to cell growth, such as ribosome expression and DNA/RNA metabolism (Figure 2.8 A). By far the largest common down-regulated class was genes related to plastid development (Figure 2.8 B).

By using data on shoot regeneration from rice (Su et al., 2007) *and Arabidopsis* (Che et al., 2006), were able to compare up-regulated genes among all

three species. Of approximately 500 genes from each species, only 6 were common among all three. There were more than 10-fold fewer genes in common between poplar and rice than there were between poplar and *Arabidopsis*. Among the 6 common genes, three are putative oxidoreductases with a NAD-binding domain.

Discussion

Although some spatial variation in variability in hybridization intensity was visible on our arrays, we found that they gave a high degree of precision for estimates of gene expression. For example, 31,939 genes (out of a total 61,413 genes on the array) were flagged "Present" for the both biological replicates prior to callus induction (i.e., above background, as determined by the Affymetrix software). Based on variance between biological replications after normalization, the mean, standard deviation, and coefficient of variation of signal intensity over biological replicates was 7.70, 0.20, and 3.18%, respectively. The mean standard error over biological replicates was 0.14 (1.84% relative to the mean).

From the sequential comparisons of regulated genes, we found that there was a massive reorganization of gene expression shortly after the start of callus induction, but before visible changes in explant morphology were obvious. Changes in gene regulation after this point were far smaller, and decreased over time. Surprisingly, there were no substantial changes in gene expression observed after transfer to shoot induction medium. This may reflect the limited percentage of explants that actually responded and produced shoots in this system (~20%), as well as the considerable

variation within explants in regeneration activity. Most explants that produced shoots formed only one to two shoots that were visible in this time frame. It may also reflect the observation that even after callus induction there was some meristematic activity observed in a number of explants, including the production of root initials. This may have coincided with a large and complex set of alterations in gene expression that are not substantially reset with the increase in cytokinin provided by the SIM medium.

The changes in GO categories reflect the large reorganization that tissues are undergoing during regeneration. Genes involved mitochondria, cell wall, ER, cell organization, and biogenesis were highly up-regulated during callus induction. This is a likely consequence of increased proteins synthesis to support cell division and wall formation during callus induction. In contrast, chloroplast/plastid genes are strongly down-regulated gene during callus induction, which likely corresponds to the transition from autotrophy to heterotrophy at this developmental transition. It also likely reflects the suppressive effect of callus development in the dark in our regeneration systems on light regulated, photosynthesis associated genes.

Two F-box proteins were regulated during regeneration. TIR1 and other three auxin F-box proteins have been suggested as auxin receptors involved in the regulation of auxin-responsive genes (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). Auxin binds to TIR1 that is contained in SCF-like complex (SCFTIR1), which promotes the interaction between TIR1 and AUX/IAAs (reviewed by (Quint and Gray, 2006; Teale et al., 2006). By comparison to auxin

associated genes, only a small number of genes related to cytokinin signaling appear to be regulated in our dataset. However, the A-type response regulators and the pseudo-response regulator appear to be specifically induced during shoot induction, suggesting a direct role in cytokinin signaling. The type-A ARRs, are considered negative regulators of cytokinin signaling that are rapidly up-regulated in response to cytokinin. (To et al., 2007).

There was strong and complex regulation of cell-cycle genes. In JGI, 110 genes have been assigned to GO:0007049, the cell cycle category (http://genome.jgi-psf.org/cgi-bin/ToGo?species=Poptr1_1). Of these, 21 were differentially expressed during our regeneration treatments. Approximately half of these are hypothetical proteins, and 6 are cyclin genes. As expected given the rapid tissue growth that occurs during callogenesis, the majority (17 out of 21) were up-regulated around the time of callus induction. Among the four genes that were down-regulated during callus induction, estExt_fgenesh4_pg.C_LG_V0508 was identified as a cyclin dependent kinase inhibitor (Ralph et al., 2006).

MYB proteins are a large group of transcription factors that have a wide variety of roles in development. For example, the expression of many are correlated with secondary wall formation, both in *Arabidopsis* and poplar (Rogers and Campbell, 2004; Jansson and Douglas, 2007). During regeneration, we found that 41 (19% of the 216 poplar *MYBs*) showed regulated expression, and the number of down-regulated *MYBs* were roughly double the number of up-regulated *MYBs* at any stage. Not surprisingly, it therefore appears that many play important roles in organogenesis.

The catalogs of regulated genes we have identified provide candidates for analysis of *in vitro* development, and for modifying development for better control of regeneration. For example, the many new gene family members and unknown genes could be characterized biochemically or via reverse genetic screens such as with RNAi or overexpression to identify their roles in control of regeneration. Induced expression of genes that appear to regulate cell cycle such as the cyclins, or of transcription factors that are associated with dedifferentiation such as some of the *MYB*s, might be useful for promoting regeneration of transgenic plants (Arias et al. 2006). Microarray analysis of transgenic plants with these misexpressed genes would also provide insight into the regulatory networks in which they play a part. The low level of conservation of the regulated gene sets between poplar, Arabidopsis, and rice demonstrates that transcriptome studies of a number of species and regeneration systems are needed in order to understand—and thus more rationally modify—*in vitro* regeneration pathways.

Figures

Figure 2.1 Tissue samples during *in vitro* shoot organogenesis.

Internode explants from *in vitro* micropropagation were sampled for RNA extraction at five sequential time points. They were first placed on callus induction medium (CIM) and then on shoot induction medium (SIM). The sample times were: (A) directly after removal from parent plants and prior to placement on CIM; (B) 3 days after placement on CIM; (C) 15 days on CIM; (D) 3 days on SIM after CIM treatment; and (E) 8 days on SIM after CIM treatment.

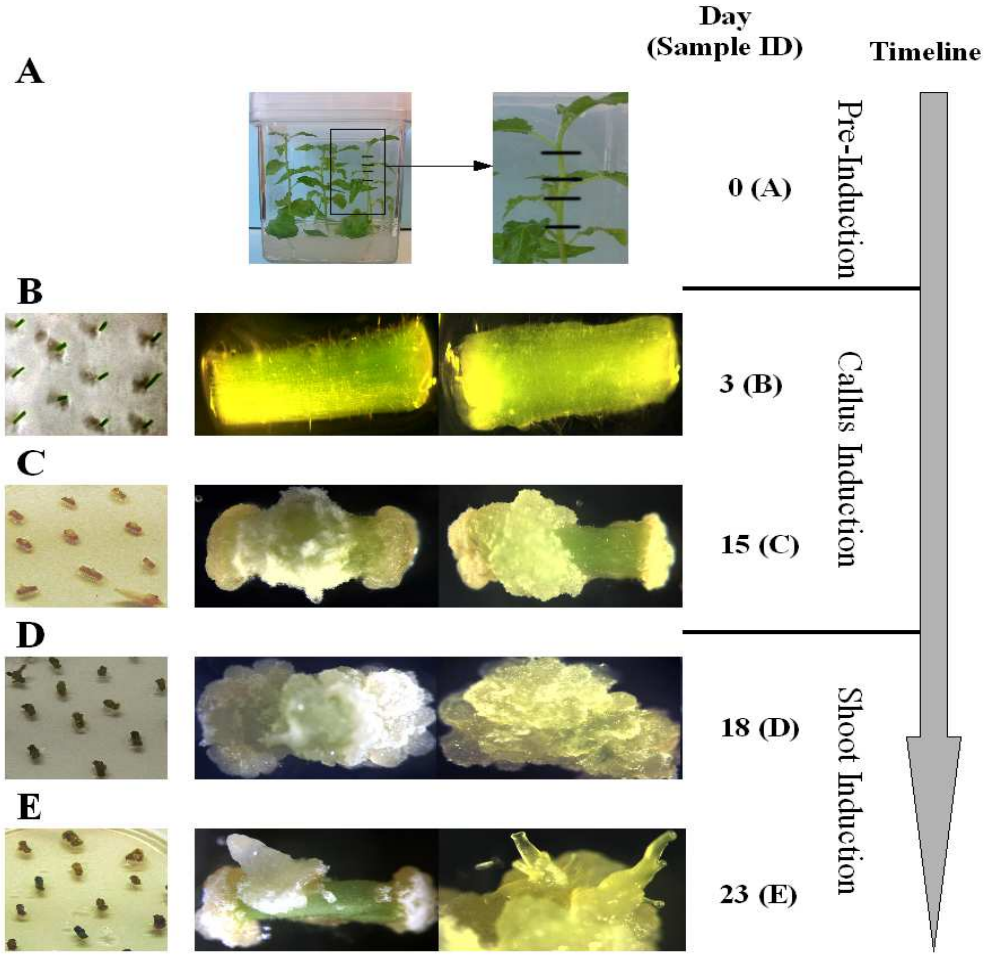
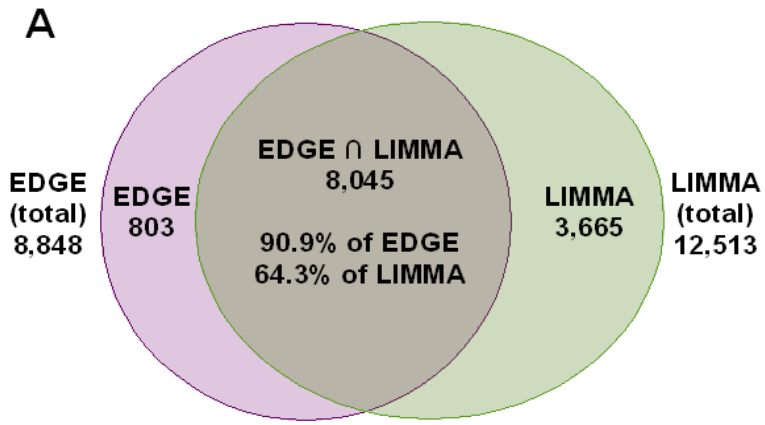


Figure 2.2 Numbers of differentially expressed genes during regeneration.

(A) Venn diagram showing the numbers of differentially expressed genes identified using Extraction and Analysis of Differential Gene Expression (EDGE) and Linear Models for Microarray Data (LIMMA). The number of genes identified by both methods and the percentages are given. Empty bars above line are up-regulated genes; gray bars below are down-regulated genes.
(B) Differential expression calculated by comparison with the pre-induction stage (baseline).
(C) Differential expression calculated by comparison with the prior sample point (sequential).



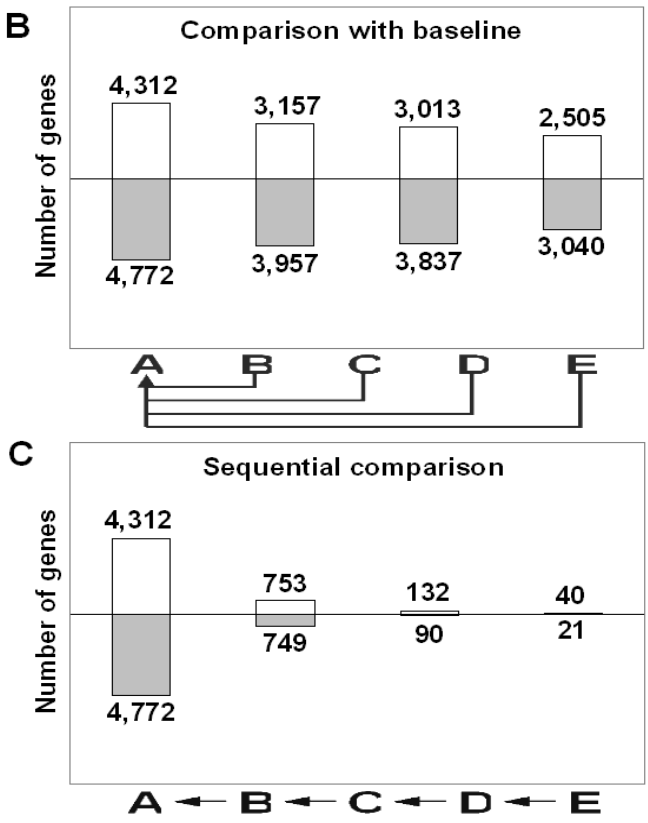

B
Comparison with baseline
Number of genes
4,312
3,157
3,013
2,505
4,772
3,957
3,837
3,040
A
B
C
D
E
C
Sequential comparison
Number of genes
4,312
753
132
40
4,772
749
90
21
A
B
C
D
E

Figure 2.3 Clustering of differentially expressed genes identified by LIMMA.

The ratios of the gene expression at each time point and the highest level of expression of that gene among the five time points (i.e., a within-gene scale) were used for scaled clustering. Five distinctive expression patterns are labeled and discussed in the text (1-5).

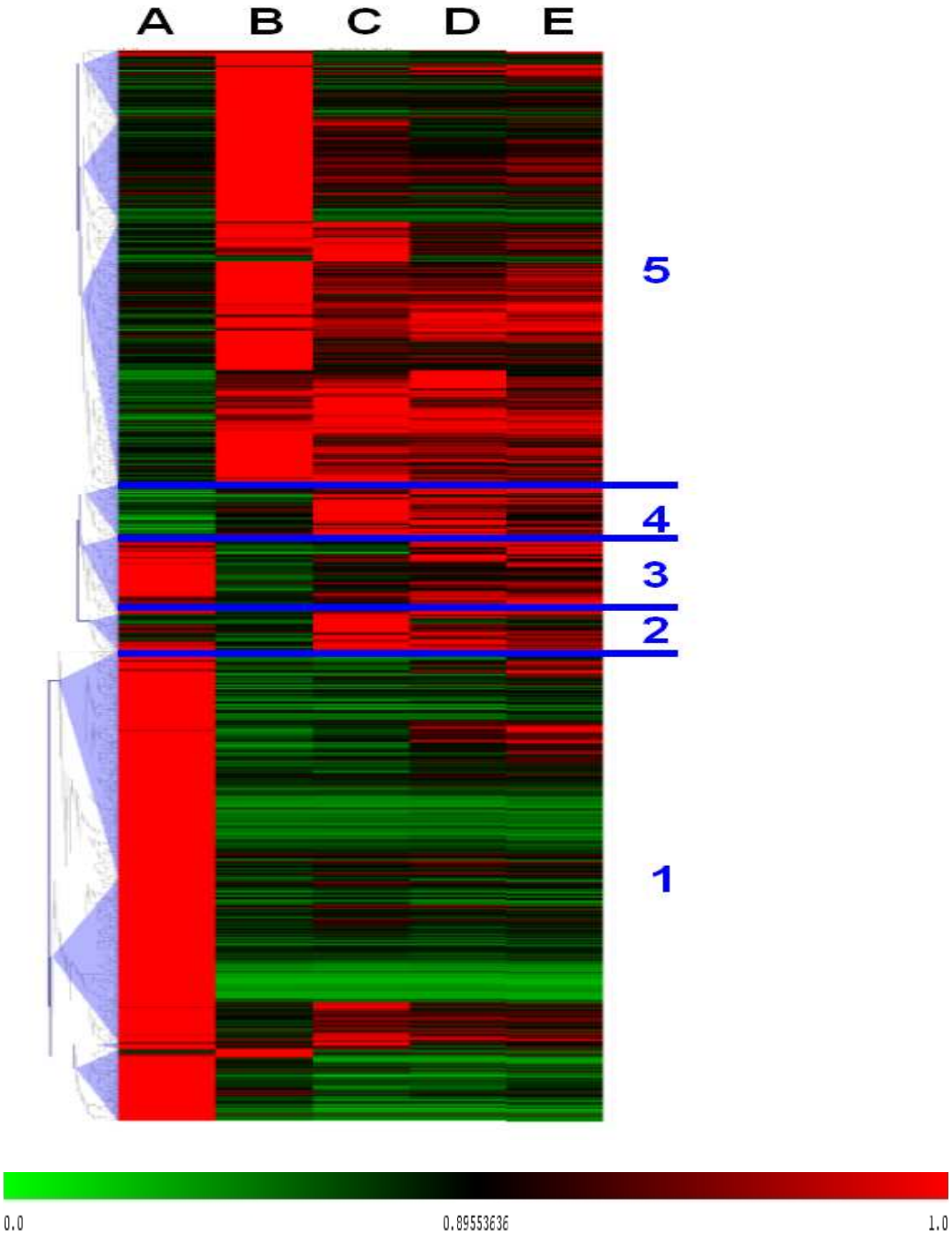


Figure 2.4 Clustering of significantly regulated transcription factors.

The ratios of the gene expression at each time point and the highest level of expression of that gene among the five time points (i.e., a within-gene scale) were used for scaled clustering. Five distinctive expression patterns are labeled and discussed in the text (1-5).

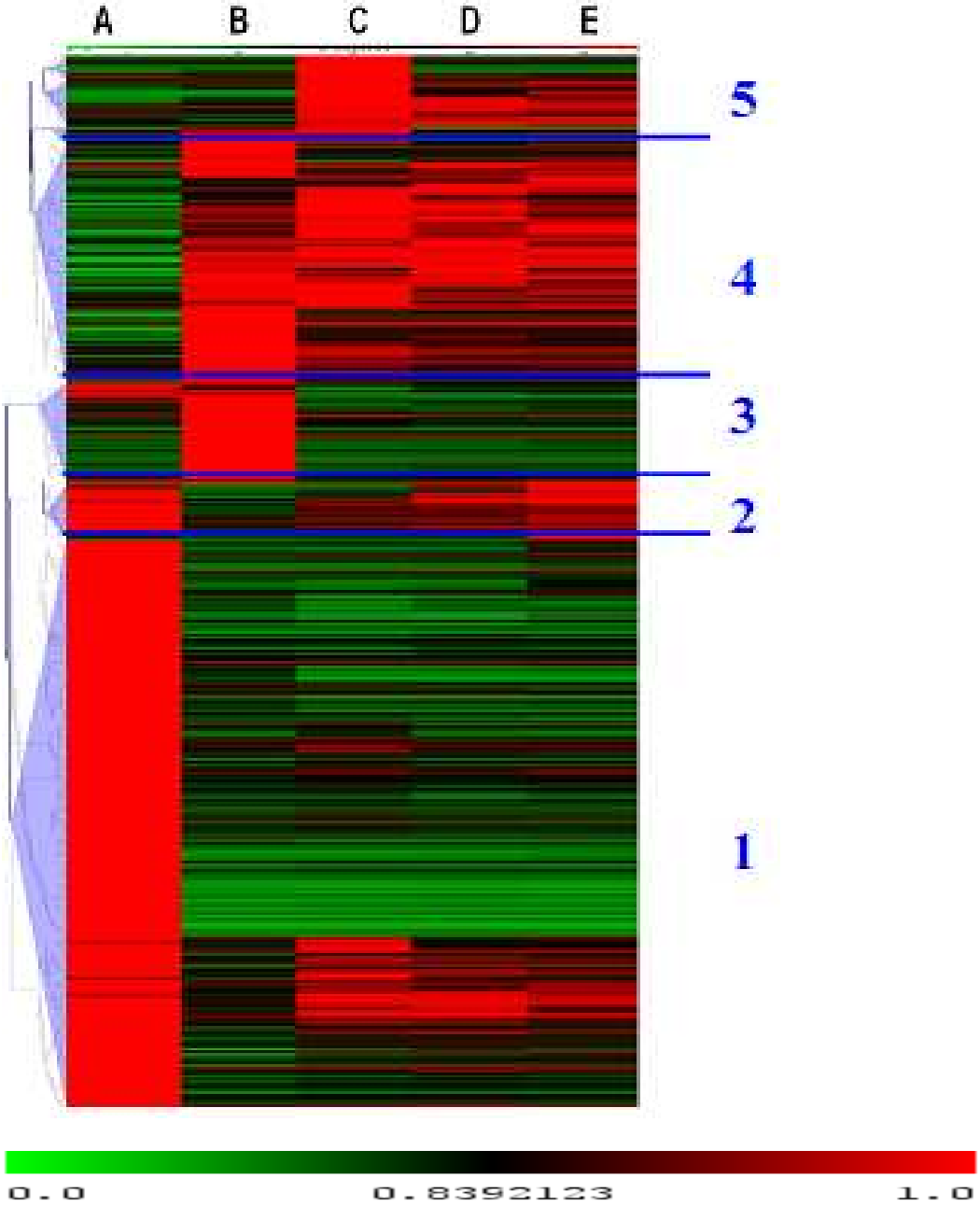
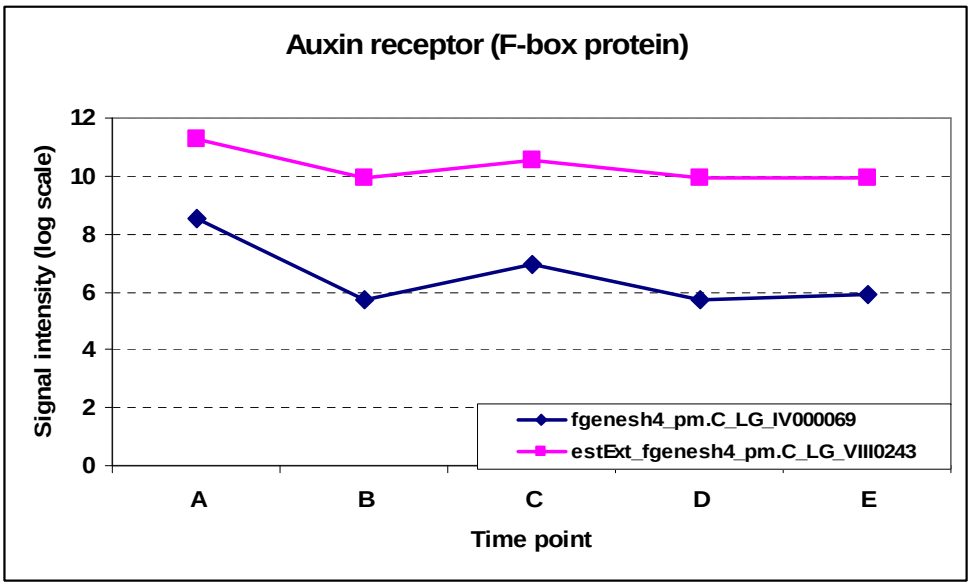


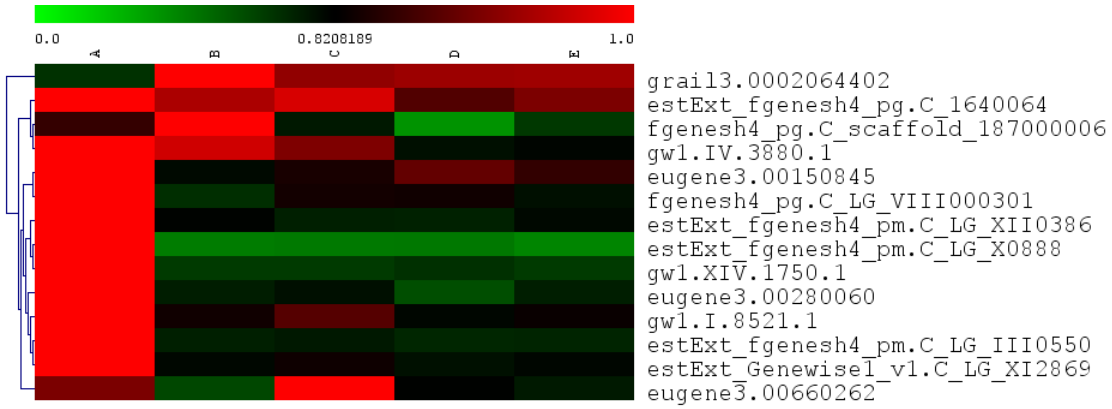
Figure 2.5 Expressions of regulated components in auxin signaling.

(A) Two auxin-receptor F-box genes.
(B) Differentially expressed members of the ARF family.
(C) Differentially expressed members of the Aux/IAA family.

A



B


0.0
0.8208189
1.0
A
B
C
D
E
grail3.0002064402
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fgenesh4_pg.C_scaffold_187000006
gw1.IV.3880.1
eugene3.00150845
fgenesh4_pg.C_LG_VIII000301
estExt_fgenesh4_pm.C_LG_XII0386
estExt_fgenesh4_pm.C_LG_X0888
gw1.XIV.1750.1
eugene3.00280060
gw1.I.8521.1
estExt_fgenesh4_pm.C_LG_III0550
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eugene3.00660262

C

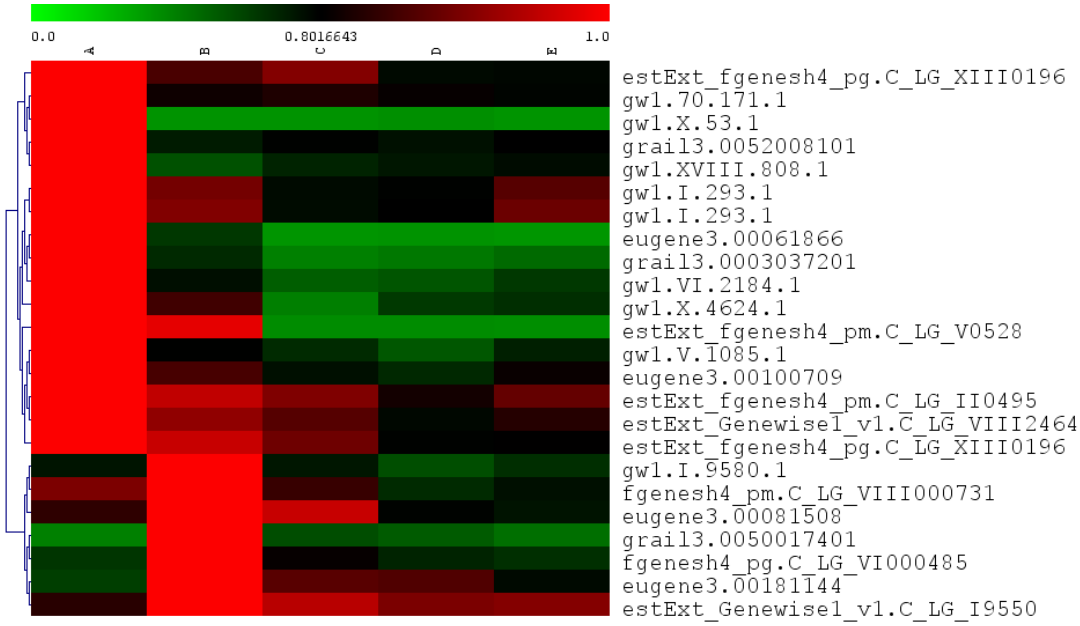
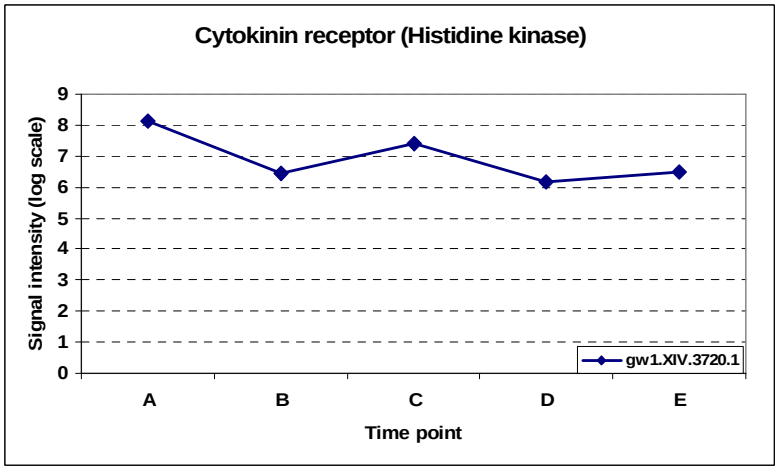

0.0
0.8016643
1.0
A
B
C
D
E
estExt_fgenesh4_pg.C_LG_XIII0196
gw1.70.171.1
gw1.X.53.1
grail3.0052008101
gw1.XVIII.808.1
gw1.I.293.1
gw1.I.293.1
eugene3.00061866
grail3.0003037201
gw1.VI.2184.1
gw1.X.4624.1
estExt_fgenesh4_pm.C_LG_V0528
gw1.V.1085.1
eugene3.00100709
estExt_fgenesh4_pm.C_LG_II0495
estExt_Genewise1_v1.C_LG_VIII2464
estExt_fgenesh4_pg.C_LG_XIII0196
gw1.I.9580.1
fgenesh4_pm.C_LG_VIII000731
eugene3.00081508
grail3.0050017401
fgenesh4_pg.C_LG_VI000485
eugene3.00181144
estExt_Genewise1_v1.C_LG_I9550

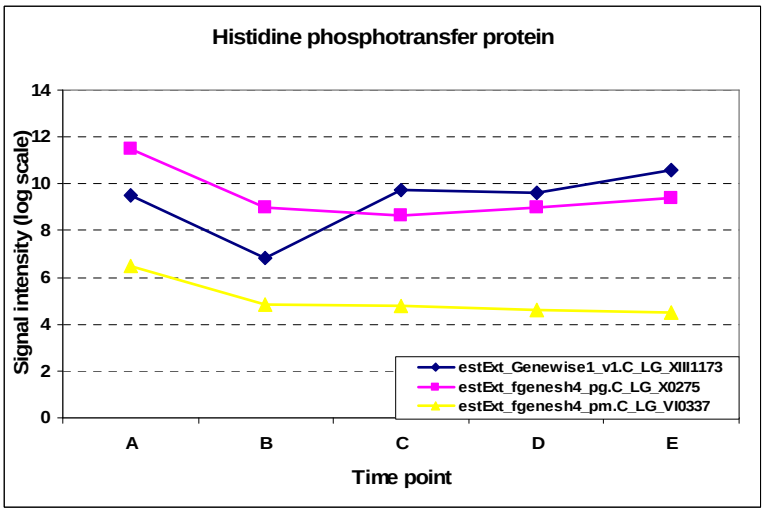
Figure 2.6 Expressions of regulated components in cytokinin signaling.

(A) Differentially expressed cytokinin receptor (histidine kinase).
(B) Differentially expressed histidine phosphortransfer proteins.
(C) – (E) Differentially expressed A-type (C), B-type (D), and pseudo cytokinin response regulators (E).

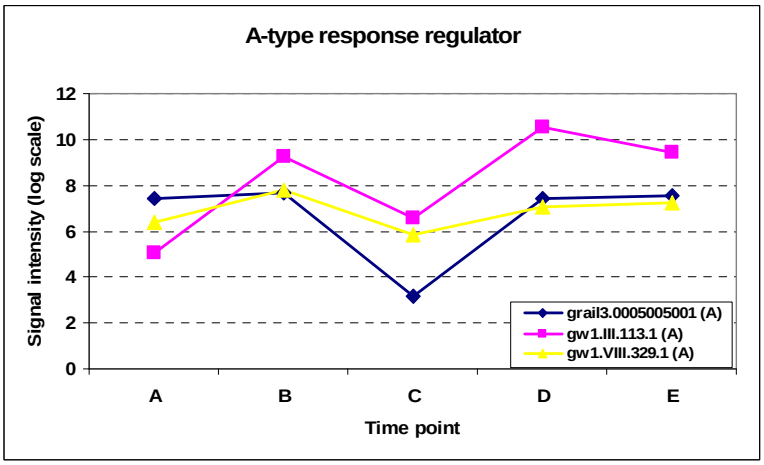
A



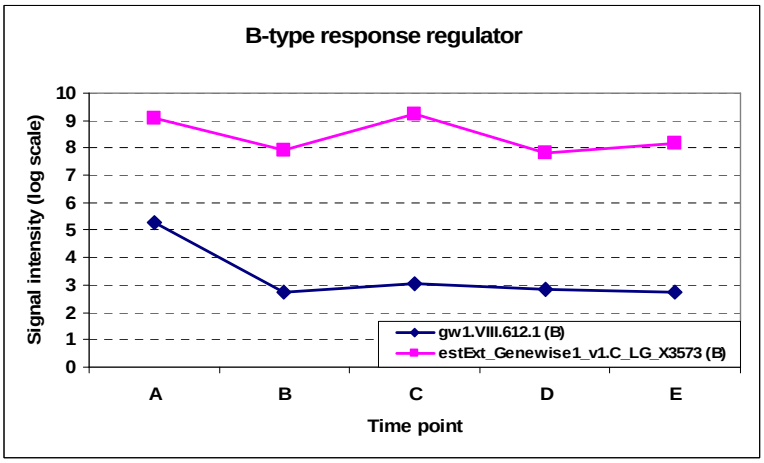
B



C


A-type response regulator
Signal intensity (log scale)
12
10
8
6
4
2
0
A
B
C
D
E
Time point
grail3.0005005001 (A)
gw1.III.113.1 (A)
gw1.VIII.329.1 (A)

D


B-type response regulator
Signal intensity (log scale)
10
9
8
7
6
5
4
3
2
1
0
A
B
C
D
E
Time point
gw1.VIII.612.1 (B)
estExt_Genewise1_v1.C_LG_X3573 (B)

E

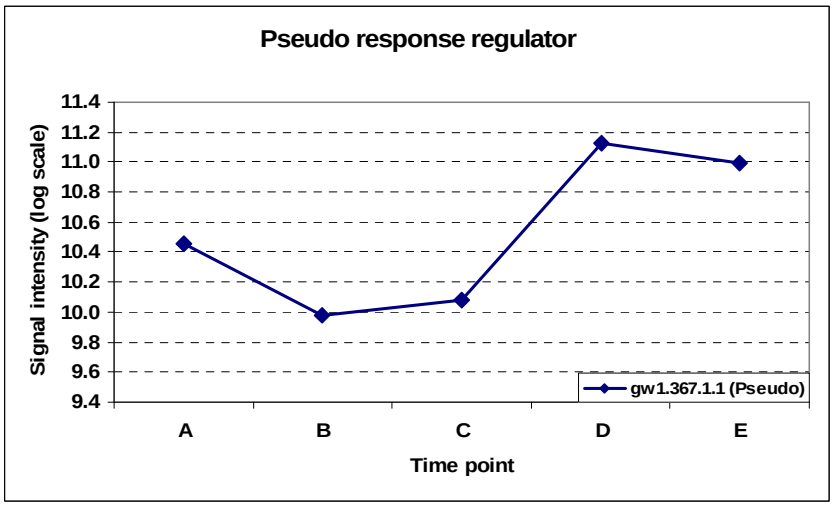

Pseudo response regulator
Signal intensity (log scale)
11.4
11.2
11.0
10.8
10.6
10.4
10.2
10.0
9.8
9.6
9.4
A
B
C
D
E
Time point
gw1.367.1.1 (Pseudo)

Figure 2.7 Clustering of regulated cell cycle genes in poplar.

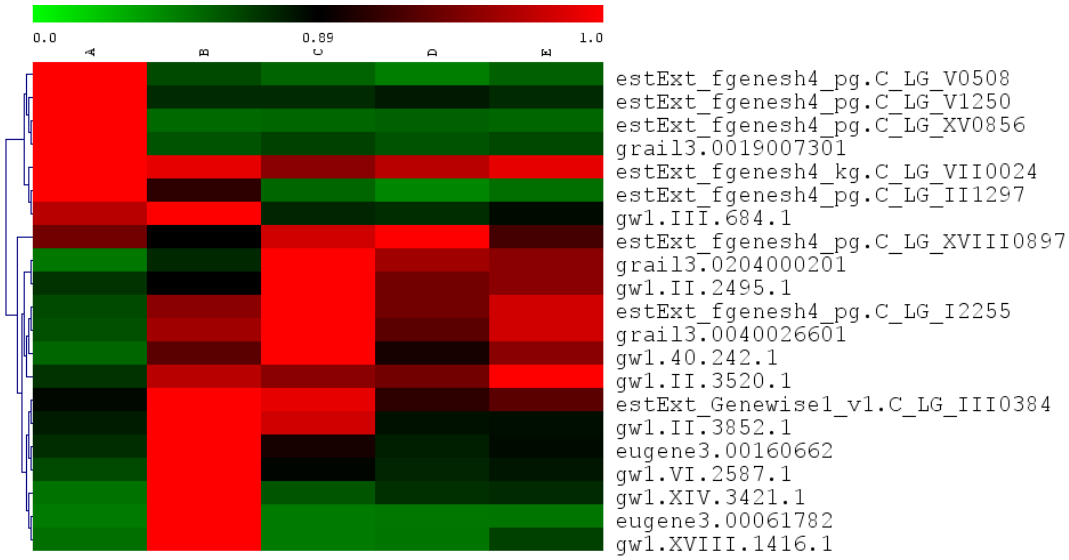
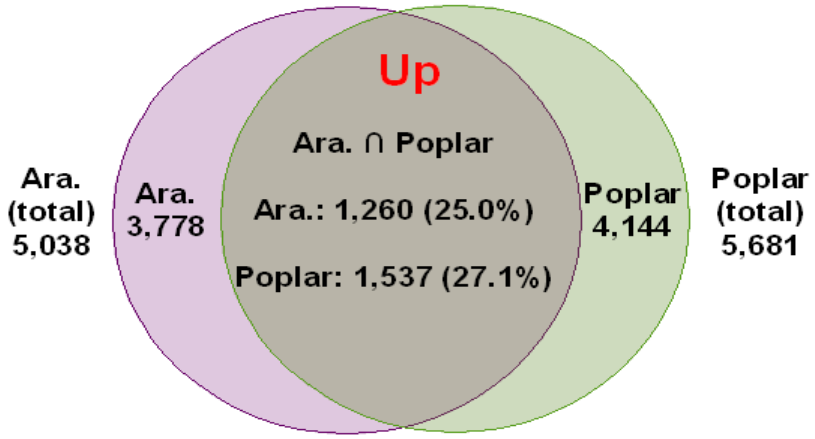


Figure 2.8 Genes involved in callus induction common to *Arabidopsis* and poplar.

A



B

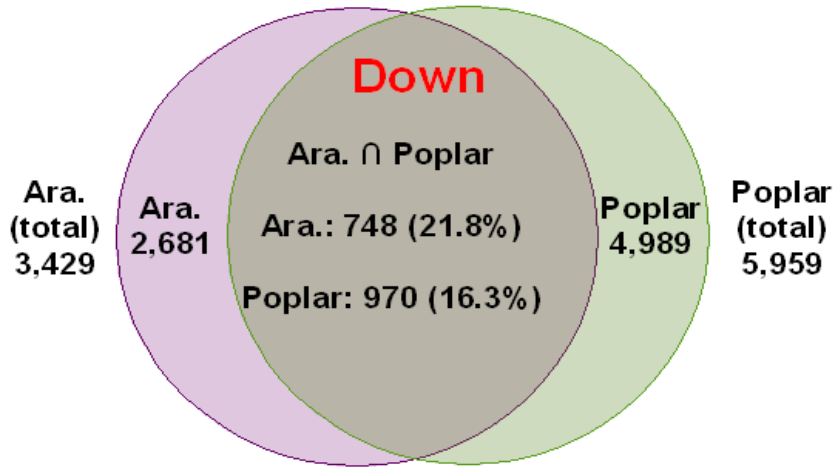
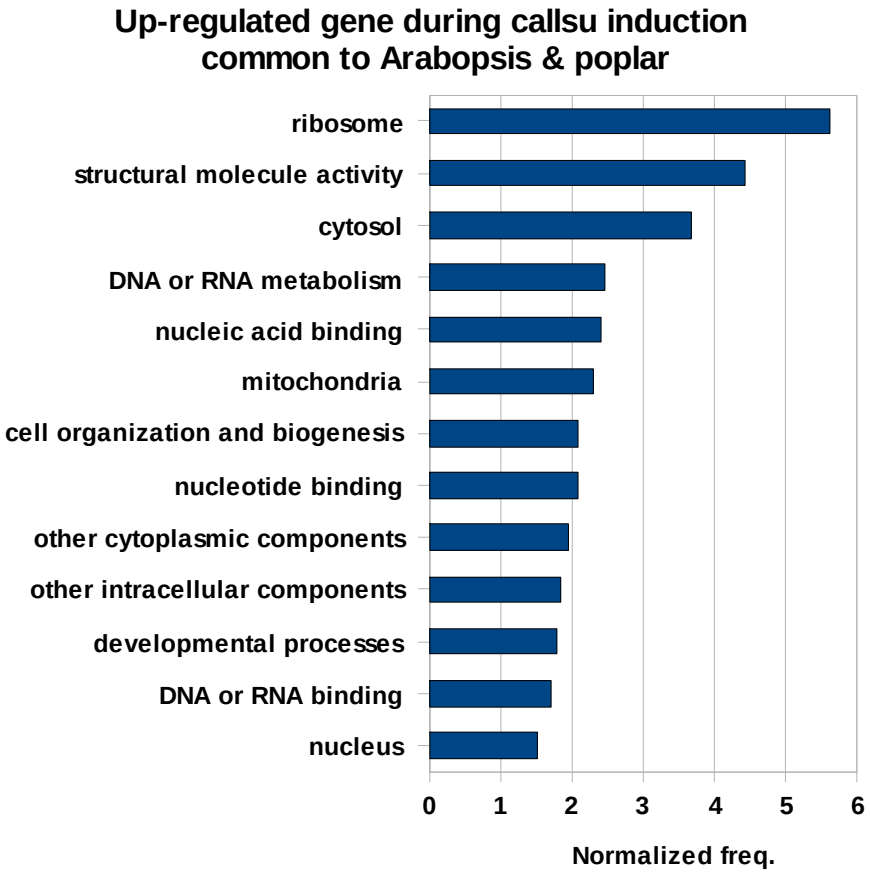


Figure 2.9 Over-represented GO classes during callus induction common to *Arabidopsis* and poplar.

A



B

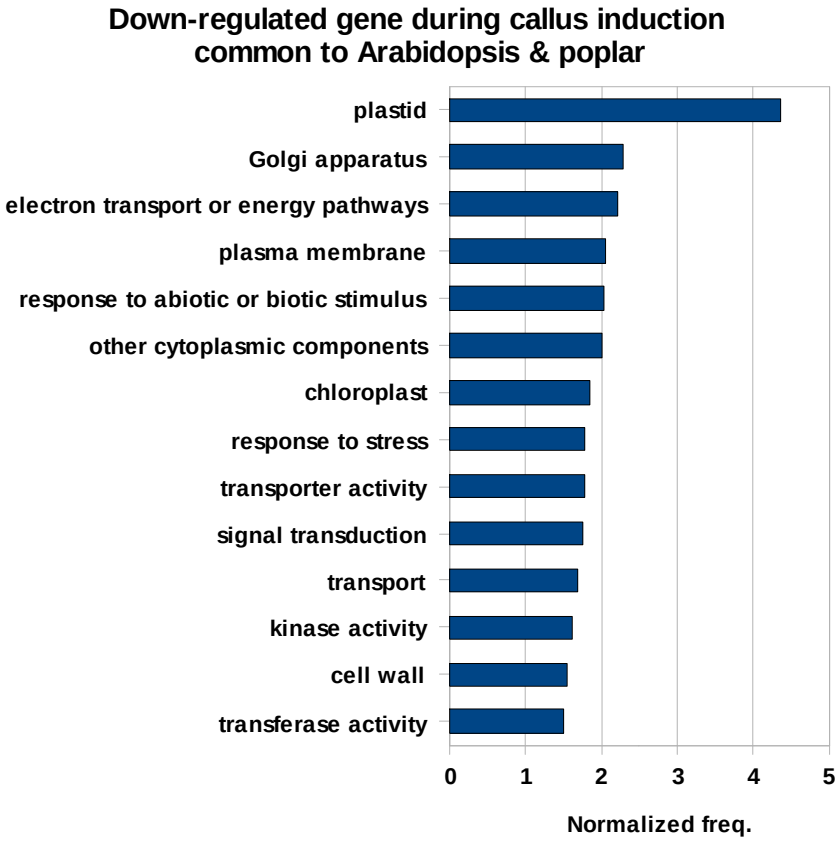
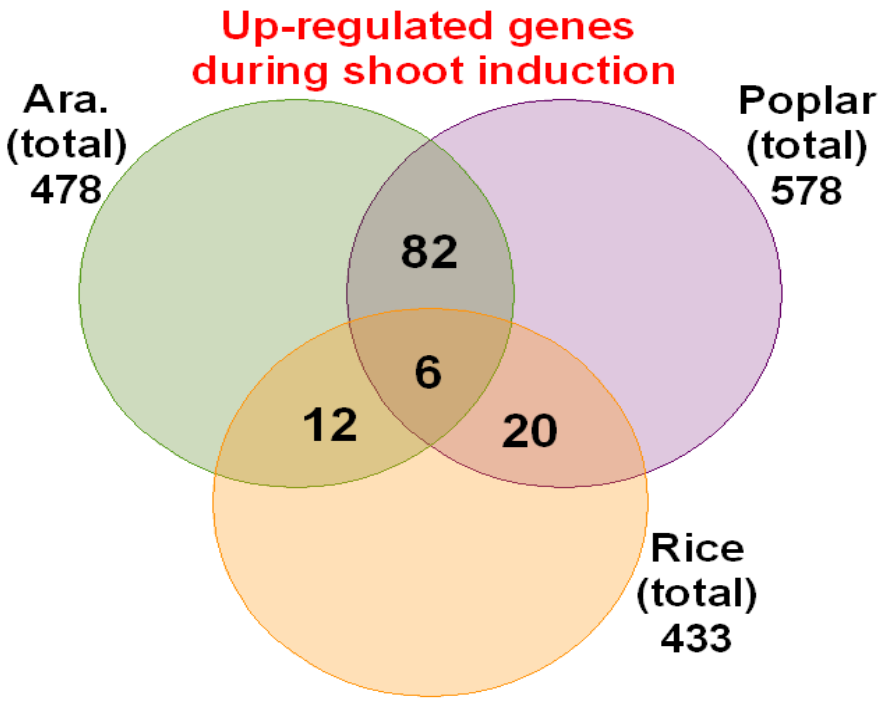

Down-regulated gene during callus induction
common to Arabidopsis & poplar
plastid
Golgi apparatus
electron transport or energy pathways
plasma membrane
response to abiotic or biotic stimulus
other cytoplasmic components
chloroplast
response to stress
transporter activity
signal transduction
transport
kinase activity
cell wall
transferase activity
0
1
2
3
4
5
Normalized freq.

Figure 2.10 Up-regulated genes during shoot induction common to *Arabidopsis*, poplar and rice.



Tables

Table 2.1 GO categorization of differentially expressed poplar genes during in vitro organogenesis.

The *Arabidopsis* homologs of the identified differentially expressed poplar gene were used for GO categorization. The percentage of each functional class in the poplar genome is assumed to equal to that in *Arabidopsis*.

		Up-regulated >2	Up-regulated 1.5-2	Up-regulated 1.2-1.5	Down-regulated >2	Down-regulated 1.5-2	Down-regulated 1.2-1.5

GO category	Function category	B vs. A Up	B vs. A Down	C vs. A Up	C vs. A Down	D vs. A Up	D vs. A Down	E vs. A Up	E vs. A Down
Biological Process	response to stress	1.8	1.8	2.1	1.7	2.3	1.6	2.3	1.8
	cell organization and biogenesis	1.6	1.2	1.1	1.2	1.0	1.3	1.0	1.3
	response to abiotic or biotic stimulus	1.5	2.0	1.8	2.0	1.9	1.8	1.8	1.9
	developmental processes	1.5	1.3	1.4	1.4	1.3	1.6	1.5	1.6
	other metabolic processes	1.3	1.2	1.3	1.2	1.4	1.1	1.4	1.1
	other cellular processes	1.3	1.2	1.3	1.2	1.3	1.2	1.3	1.1
	protein metabolism	1.3	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	electron transport or energy pathways	1.2	1.8	1.5	1.7	1.6	1.0	1.6	1.1
	transport	1.1	1.2	1.1	1.2	1.1	1.4	1.1	1.3
	DNA or RNA metabolism	1.1	0.4	0.6	0.5	0.6	0.6	0.6	0.6
	other biological processes	0.9	0.9	0.9	0.9	0.9	1.0	0.9	1.0
	signal transduction	0.9	1.4	1.0	1.4	1.1	1.6	0.9	1.8
	transcription	0.8	1.1	1.0	1.1	1.0	1.3	1.0	1.4
	ribosome	2.8	0.9	0.9	1.0	1.1	0.2	1.5	0.2

Cellular Component	cytosol	2.8	1.1	2.1	1.1	2.3	0.9	2.8	1.0
	mitochondria	2.6	0.9	1.9	0.8	1.9	0.8	1.8	0.7
	cell wall	2.2	0.9	2.0	1.2	2.1	1.3	2.5	1.3
	other cytoplasmic components	2.0	2.1	1.4	1.9	1.5	1.2	1.6	1.0
	ER	2.0	0.2	2.1	0.5	2.1	0.8	2.4	0.4
	other intracellular components	1.6	1.6	1.0	1.4	1.0	1.1	1.1	1.0
	Golgi apparatus	1.3	1.2	0.7	1.5	0.7	2.0	0.4	1.8
	nucleus	1.1	1.1	1.2	1.1	1.0	1.3	1.0	1.3
	plasma membrane	1.1	1.9	1.3	2.3	1.4	2.5	1.1	2.6
	other cellular components	1.0	1.0	0.9	0.9	0.9	0.9	0.9	0.9
	chloroplast	1.0	2.5	0.9	2.1	1.0	1.2	1.1	1.2
	other membranes	0.9	1.2	1.1	1.3	1.2	1.2	1.1	1.1
	plastid	0.9	4.9	0.9	3.9	1.0	1.4	1.1	1.2
	extracellular	0.8	0.6	1.1	0.8	1.2	0.7	1.4	0.5
Molecular Function	structural molecule activity	2.2	1.4	0.7	1.3	0.9	0.8	1.2	0.8
	other enzyme activity	1.8	1.3	2.0	1.3	2.2	1.0	2.2	1.0
	nucleotide binding	1.7	0.9	1.4	1.1	1.3	1.2	1.2	1.2
	nucleic acid binding	1.6	0.9	1.2	0.9	0.9	0.9	1.0	0.9
	transferase activity	1.5	1.2	1.9	1.2	1.8	1.3	1.9	1.3
	transporter activity	1.4	1.3	1.4	1.4	1.4	1.6	1.4	1.6
	hydrolase activity	1.4	1.1	1.3	1.2	1.2	1.1	1.4	1.1
	kinase activity	1.1	1.3	1.4	1.4	1.4	1.7	1.2	1.8
	DNA or RNA binding	1.0	1.0	0.9	0.9	0.9	1.0	0.8	1.1
	protein binding	1.0	1.3	1.0	1.3	0.9	1.5	0.8	1.6
	transcription factor activity	0.8	1.4	1.0	1.5	1.1	1.7	1.1	1.8
	other binding	0.8	1.0	0.8	0.9	0.8	0.9	0.7	0.9
	other molecular functions	0.7	0.8	0.7	0.8	0.7	0.8	0.6	0.8
	receptor binding or activity	0.5	0.7	0.7	0.6	0.6	0.6	0.6	0.7

Table 2.2 Up- or down-regulated transcription factors during CIM and SIM.

The JGI gene model IDs were downloaded from the Database of Poplar Transcription Factors (DPTF) and were searched against the list of the differentially expressed genes identified by LIMMA. The list is ranked by the percentage of the total number of each transcription factor class in poplar. The total number of regulated transcription factors were corrected for redundancy among the array probes; the number of up-or down-regulated transcription factors at each stage were not corrected for redundancy (i.e., multiple probe sets targeting the same transcript may be present).

		NO.	NO.	B vs. A		C vs. A		E vs. A		F vs. A	
Gene family	Percentage	(regulated)	(total)	Up	Down	Up	Down	Up	Down	Up	Down
SRS	80.0%	8	10	3		5		2		4	
TLP	72.7%	8	11		6	4	4	2	6	2	4
AUX-IAA	69.7%	23	33	9	11	2	16		27		15
CCAAT-	45.5%	5	11		5		2		4		4
GRF	44.4%	4	9							4	
C2C2-Dof	42.9%	18	42	5	11		13	3	13		14
WRKY	38.5%	40	104	22	12	27	14	29	14	25	10
ARF	37.8%	14	37		13		10		13		10
HB	37.7%	40	106	15	23	7	22	7	24	6	24
AS2	36.8%	21	57	8	4	15	2	8	4	6	3
FHA	36.8%	7	19		7		8		4		4
ZIM	36.4%	8	22	8	3	7	2	5	3	4	2
GARP-G2-	35.8%	24	67	2	19	4	14	6	19	3	15
TCP	35.3%	12	34		11	2	9		9	3	8
HMG	33.3%	4	12	2	2	2	2	2	3	2	3
LIM	33.3%	7	21		7		7		7		7
ULT	33.3%	1	3			1		1		1	
ZF-HD	32.0%	8	25		6		4		3	2	
E2F-DP	30.0%	3	10	2	2						
SBP	27.6%	8	29		8	4	5		7		6

CCAAT-	26.3%	5	19		5		3		3		3
Trihelix	25.5%	12	47	4	6	4	4	5	4	4	3
bHLH	25.0%	37	148	9	25	5	27	5	24	4	19
PLATZ	25.0%	5	20	5	4	4	2	3	2	3	2
bZIP	22.4%	19	85	4	22	2	18	4	17	3	17
Alfin	22.2%	2	9		2						
GRAS	20.8%	20	96	7	12	5	7	8	12	5	7
MYB-	20.2%	17	84	5	14	3	6	2	7	2	6
HSF	19.4%	6	31	7		11		7		5	
MYB	19.0%	41	216	13	27	11	27	12	29	11	27
AP2-EREBP	18.9%	40	212	28	10	23	8	25	8	25	
NAC	18.6%	32	172	16	15	10	13	6	15	7	13
C2C2-CO-	17.9%	7	39		10		6		7		5
C3H	17.9%	14	78	10	3	9	2	7	5	6	2
PcG	17.8%	8	45	2	6	2	3		3		2
C2H2	16.0%	13	81	5	11	3	9	3	9	2	9
C2C2-GATA	15.6%	5	32		2		2	3	3		
C2C2-	15.4%	2	13							3	
JUMONJI	15.0%	3	20					2			
TAZ	14.3%	1	7				1		1		
GARP-ARR-	13.3%	2	15		4			2	3		3
ABI3-VP1	11.1%	12	108	9	4	9	2	10	2	9	6
MADS	10.8%	12	111		8	2	7	6	5	2	6
CCAAT-	10.5%	2	19					2		2	
PHD	9.3%	8	86	7	3	5		3	2	4	2
Total	22.8%	588	2576								

Online Supporting Materials

Available at ScholarsArchive@OSU
(http://ir.library.oregonstate.edu/dspace/, search for "Yanghuan Bao")

S1. Detail on quality assessment of microarray hybridization.
Background on quality parameters are provided in Affymetrix GeneChip Expression Analysis: Data Analysis Fundamentals (Page 36-40). (A) Average background, typical ranging from 20 to 100. (B) Scaling factor, usually around 3, less than 5 is considered acceptable; (C) Percent of probes detected; 50% is common. (D) Internal controls genes *β-actin* and *GAPDH* used to assess RNA sample and assay quality. Specifically, the ratio of the 3' probe set to the 5' probe set is generally no more than 3. However, a high 3' to 5' ratio of only one group of the internal control genes does not necessarily indicate RNA degradation. (E) Poly-A controls used to monitor the entire target labeling process. All controls should be called "Present" with increasing signal value in the order of *lys, phe, thr,* and *dap*. (F) Hybridization controls independent of RNA sample preparation, and used to evaluate sample hybridization; their signal values should reflect their relative concentrations (*bioB:bioC:bioD:cre* = 1.5:5:25:100). For (A) – (G), A-E at X-axis indicates five time point for collecting samples. R1 and R2 indicate biological replicate group 1 and 2, respectively. (G) Correlation efficiency between biological replicates at five time points for collecting samples.

S2. Regulated genes at each stage identified by LIMMA.

S3. Counts and percentages of regulated genes by GO category.

S4. Up-regulated genes during shoot induction.

S5. Regulated transcription factors at each stage.

S6. Regulation of auxin signaling.

S7. Regulation of cytokinin signaling.

S8. Up-regulated genes at early callus induction common to *Arabidopsis* and poplar.

S9. Down-regulated genes at early callus induction common to *Arabidopsis* and poplar.

S10. Differentially expressed cell cycle genes.

S11. Up-regulated genes during shoot induction common to *Arabidopsis*, poplar, and rice.

S12. Down-regulated genes during shoot induction common to poplar and rice.

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CHAPTER 3

DEVELOPMENT OF *WUS* AND *STM*-BASED REPORTER GENES FOR STUDYING MERISTEM DEVELOPMENT IN POPLAR

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Abstract

We describe the development of a reporter system for monitoring meristem initiation in poplar using promoters of poplar homologs to the meristem-active regulatory genes *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*). When ~3kb of the 5' flanking regions of close homologs were used to drive expression of the GUSPlus gene, 50 to 60% of the transgenic events showed expression in apical and axillary meristems. However, expression was also common in other organs, including in leaf veins (40% and 46% of *WUS* and *STM* transgenic events, respectively) and hydathodes (56% of *WUS* transgenic events). Histochemical GUS staining of explants during callogenesis and shoot regeneration using *in vitro* stems as explants showed that expression was detectable prior to visible shoot development, starting 3 to 15 days after explants were placed onto callus inducing medium. Based on microarray gene expression data, a paralog of poplar *WUS* was detectably up-regulated during shoot initiation, but the other paralog was not. Surprisingly, both paralogs of poplar *STM* were down-regulated 3- to 6-fold during early callus initiation, a possible consequence of its stronger expression in the stem secondary meristem (cambium). We identified 15 to 35 copies of cytokinin response regulator binding motifs (ARR1AT) and one copy of the auxin response element (AuxRE) in both promoters. Several of the events recovered may be useful for studying the process of primary and secondary meristem

development, including treatments intended to stimulate meristem development to promote clonal propagation and genetic transformation.

Keywords: *WUS, STM, Populus,* meristem, organogenesis, secondary meristem, cambium, promoter, stem cells.

Introduction

Plant meristems consist of stem cells and rapidly dividing daughter cells with restricted development potential, often called pluripotent stem cells. These cells continue dividing to generate new cells for differentiation into different tissues and organs. The three major types of meristems that are generally recognized in plants are the shoot apical meristem (SAM), the secondary meristem (SM), and the primary meristem (PM) (Laux, 2003; Scofield and Murray, 2006). Apical meristems include completely undifferentiated stem cells, and are located at shoot tips and root tips. A small population of slowly dividing stem cells is located in the Central Zone (CZ) in both shoot and root apical meristems that maintain the identity of stem cells. The analysis of meristem differentiation and structure, especially in *Arabidopsis,* has been the subject of numerous studies and recent reviews (e.g., (Vernoux and Benfey, 2005; Williams and Fletcher, 2005; Bhalla and Singh, 2006).

Among dozens of identified meristem regulatory factors, *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) are two meristem-predominant genes whose function has been well studied. Approximately 240 research articles on these genes

were cataloged in The Arabidopsis Information Resource (TAIR) in early 2008. The maintenance of the SAM in *Arabidopsis* is regulated by a feedback loop between *WUS* and *CLAVATA* (*CLV*) (Laux et al., 1996; Mayer et al., 1998). *WUS* is expressed in the organizing center, and induces expression of *CLV3*—the hypothesized ligand for the CLV1 receptor kinase. When CLV1 interacts with CLV3, it triggers a signaling pathway which results in the repression of the expression of *WUS*. *STM* is a Class I *knotted*-like homeodomain protein required for SAM formation during embryogenesis and other points in plant development (Long et al., 1996). It functions by preventing the incorporation of cells in the meristem center into differentiating organ primordia. Organ development takes place when *STM* is down-regulated in primordial cells. The regulation of *Arabidopsis* root apical meristems (RAMs) have common themes with those of SAMs (Byrne et al., 2003).

Poplar shares the majority of its meristem regulatory framework with *Arabidopsis*, however, additional regulatory signaling networks appear to be present. For example, the *Populus* homologs of *STM* are expressed in both the SAM and the vascular cambium (Groover, 2005; Groover et al., 2006). *Populus WUS* appears to be expressed only in the SAM whereas *WUS*-like genes are expressed in the VC zone (Schrader et al., 2004). Examination of the expression patterns of *WUS*, *STM* and their related genes in poplar can help to reveal the specialized roles of the vascular cambium regulatory genes in woody plants, as well as help identify the conserved themes of SAM and VC development.

To better understand the roles of the multiple paralogs of meristem regulatory genes in woody plants, we cloned the promoters of close *Populus* homologs of *WUS* and *STM*, and transformed promoter::reporter constructs into poplar. We describe the expression patterns of transgenic *WUS* and *STM* events, and the relation of these expression patterns to that observed in our ongoing microarray studies of poplar development. The transgenic plants we have produced should also provide useful tools for further dissection of the factors that control meristem development in poplar. The production and development of adventitious meristematic organs are considered to be important limiting factors to vegetative propagation and transformation of many plant species (Arias et al., 2006).

Materials and Methods

Plant Material, Transformation and Regeneration

Hybrid poplar clone INRA 717-1 B4 (female, *Populus tremula* x *P. alba*) was used for all transformation and microarray studies. *Populus trichocarpa* Nisqually-1 (Tuskan et al., 2006) was used as a source of DNA for the *WUS* and *STM* promoters. Plants were *in vitro* propagated and transformed according to the protocol described by (Filichkin et al., 2006). Forty- to fifty-day-old plantlets served as explant sources.

Bioinformatic Analysis of Populus *Homologs to* WUS *and* STM

The amino acid sequences of the *Arabidopsis WUS* (AT2G17950.1) and *STM* (AT1G62360.1) were retrieved from The Arabidopsis Information Resource (TAIR,

http://www.arabidopsis.org/index.jsp). The sequences were BLASTed against the database of poplar protein sequences at the Joint Genome Institute (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html). Phylogenetic analysis was conducted using the Neighbor-Joining method in MEGA version 4 (Tamura et al., 2007). Tests of inferred phylogenetic groups were conducted by bootstrapping with 500 replications. Sequence alignments were done with MUSCLE at the Center for Genome Research and Biocomputing (CGRB) of Oregon State University (http://www.cgrb.oregonstate.edu/). EST information from The DFCI poplar Gene Index (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=poplar) was used to obtain evidence for expression in different tissue types for the putative *Populus WUS* and *STM* homologs. Cis-acting regulatory elements were identified by scanning the 3kb of 5' untranslated sequences against the Database of Plant Cis-acting Regulatory DNA Elements (PLACE, http://www.dna.affrc.go.jp/PLACE/index.html) (Higo et al., 1999).

Construction of ProWUS::GUS *and* ProSTM:GUS Constructs

The 35S promoter driving GusPlus in pCAMBIA1305.1 was removed using HindIII and NcoI, blunt ended, and self ligated. The *HPTII* gene was also removed from it as an XhoI fragment and replaced by *NPTII* using an XhoI fragment from pCAMBIA 2300. The resulting plasmid requires kanamycin as a selection agent both for plant and bacterial transformation, and has no promoter driving GusPlus. The resulting construct was called pPROTEST, and was used to test the promoters of *WUS* and *STM*.

Based on phylogenetic analysis grail3.0019031001 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) was used as a target to amplify a poplar *WUS* promoter from *P.trichocarpa* genomic DNA using WUS2proForward, and WUS2proReverse primers (Table S3). The 3,492 nt fragment upstream from the *WUS* gene start codon was cloned into the pCR4-TOPO vector (Invitrogen) and sequenced using T7 and T3 promoters primers from outside of the promoter sequence, and also internal primers WUSpro1, WUSpro2, WUSpro3, WUSpro4, and WUSpro5.

estExt_Genewise1_v1.C_LG_II1820 was used as a target to amplify a poplar *STM* promoter using STM3pro-F03, and STM3pro-R01. The 3,320 nt fragment upstream from poplar STM Start codon was cloned into pCR4-TOPO vector (Invitrogen) and sequenced using T7 and T3 promoters primers from outside of the promoter sequence and also using the internal primers STMinternal#1, STMinternal#2, STMinternal#3, STMinternal#4, and STMinternal#5.

For both genes, the forward and reverse primers contained an inserted SacI site at the 5’ end and a KpnI site at the 3’ end to allow directional cloning into the pPROTEST vector. DNA sequencing was used to verify the integrity of junction sequences. The constructs (Fig 3.3) were transferred to *Agrobacterium* strain AGL1, PCR confirmed, and transformed into hybrid poplar clone 717-1B4 (*P. tremula* x *P. alba*).

WUS transgenic plants were confirmed by PCR amplification of a 1,580 bp product with primers WUSpro4 and GPLUS287R. *STM* transgenic plants were confirmed via PCR amplification of a 732 bp product with primers KNX91F and

GPLUS287R. PCR confirmed plants were also confirmed via GUS staining, as described below.

Histochemical GUS Staining

For histochemical GUS staining, tissues of entire regenerated plants from Magenta boxes (roots and shoots) were incubated overnight in 2 mM of 5-bromo-4-chloro-3-indolyl-β-d-glucuronic acid (X-Gluc) solution at 37°C essentially as described (Weigel and Glazebrook, 2002). After staining, whole plants were treated for 30 min in 10% aqueous solution of commercial bleach (5.25% sodium hypochlorite), transferred to 70% ethanol, and photographed using an Olympus C5050 digital camera. Individual plant organs were examined and photographed using Zeiss Stemi SV 11 dissection microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). For study of expression during *in vitro* organogenesis for two transgenic events, to help link the two studies we used the same time points for collecting explants as had been used in our microarray studies (Chapter 2). These were 3 days (d) and 15d on Callus Induction Medium (CIM), and 3d and 8d on Shoot Induction Medium (SIM).

Microarray Analysis

An Affymetrix GeneChip® Poplar Genome Array was used to analyze the expression of poplar *WUS*, *STM* and closely related genes during *in vitro* shoot organogenesis. Sample collection, RNA extraction, probe labeling and hybridization, and quantitative analysis were described in Materials and Methods in Chapter 2.

For expression of poplar *WUS*, *STM* and related genes from the previous phylogenetic analysis, their coding sequences were submitted to the Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) to search for the probe sets targeting these genes (Table S4). The expression for a probe set on a single array is estimated by the signal intensities detected from 11 distinct probes randomly located on the array. Not all genes have corresponding probe set with 11 perfectly matching probes on the array. Only probe sets with at least half of the 11 probes targeting a gene were used to estimate the expression of the gene in our study.

For *WUS* and its related genes, the single intensity from PtpAffx.207414.1.S1_at was used to estimate the expression of *PopWUS1*. It was targeting the less conserved region of *PopWUS1* (alignment not shown). PtpAffx.54684.1.A1_at, targeting the less conserved region of *PopWUS2*, was used for study of *PopWUS2*. PtpAffx.218777.1.S1_s_at targeted the conserved region of *PopWUS1* and *PopWUS2*, and gw1.21516.1.1. As a result, the signal intensity from PtpAffx.218777.1.S1_s_at reflected the expression of all the three genes. For STM, only *PopSTM1* and grail3.0036024801 are detectable on the array (among *STM* and its related genes only these two have more than 7 matching probes in their own corresponding probe sets).

Microarray analysis of tissue-specific expression patterns: A NimbleGen custom oligonucleotide microarray was used to study patterns of tissue-specific expression of the *WUS* and *STM* gene families in poplar (Brunner et al., 2007). A description of this platform can also be found in (Groover et al., 2006).

Results

Identification of Populus *Homologs of* WUS *and* STM

To identify *Populus* homologs of *WUS* and *STM* that are expressed in meristems, we searched the JGI *Populus* (*Populus trichocarpa* v1.1) genome database using the *Arabidopsis WUS* and *STM* amino acid sequences as query sequences, respectively. The 291bp amino acids encoded by the *WUS* gene contains consist two conserved domains, a 66 bp homeodomain and a 8 bp *WUS* Box (TLPLFPMH) located downstream of the homeodomain (Mayer et al., 1998; Haecker et al., 2004). The top five BLAST genes that had complete open reading frames were subject to further phylogenetic analysis. Three of these gene models, gw1.21516.1.1, gw1.XII.25.1, and gw1.XV.1017.1, contained the conserved homeodomain regions; they appeared to truncations upstream of the conserved regions and thus were not considered as promoter candidates (data not shown). Two poplar genes, grail3_0019031001 and estExt_fgenesh4_pg_C_570090 (hereafter called *PopWUS1* and *PopWUS2*, respectively), were most closely related to *Arabidopsis WUS* (Fig 3.1A). These two genes are highly similar, sharing 79% identity and 87% similarity in amino acid sequences. Bootstrapping showed 100% replication of their close association, and a strong association with *Arabidopsis WUS* (above 90%). The identity of these two genes and *Arabidopsis WUS* is approximately 40%, and their similarity 63%. Alignment of their amino acid sequences showed that both poplar paralogs of *WUS* have the highly conserved homeodomain and *WUS* Box (Fig 3.1B), but also are

differentiated by a considerable number of amino acid indels in otherwise conserved areas.

Arabidopsis WUS gene has three exons and two introns. The lengths of the exons are 620, 88, and 516 bp, and the introns are 601 and 90 bp, respectively. *PopWUS1* and *PopWUS2* also has three extrons and two introns, but two of three of the exons are shorter. The lengths of the exons of *PopWUS1* are 364, 104, and 310 bp, respectively. *PopWUS2* has very similar size exons to *PopWUS1*. When searching the EST database, both *PopWUS1* and *PopWUS2* had high identity with ESTs TC60159, TC50140, TC49749, and TC76257, which were from cDNA libraries made from male catkins, flowers, bark, cambium, and apical shoots. Because of variation among the different *Populus* species in EST sequences, we could not determine which ESTs corresponded to *PopWUS1* vs. *PopWUS2*. As cited above, only grail3_0019031001 (*PopWUS1*) was selected for promoter analysis.

The *Arabidopsis STM* encodes a class I *KNOTTED*-like protein that is 382 amino acid long (Long et al., 1996). The conserved domains of *STM* consist of KNOX, ELK and homeodomains. Five poplar genes with the highest BLAST scores were subject to phylogenetic analysis. As shown in Fig 3.2A, gw1_XI_1499_1 and fgenesh4_pm_C_scaffold_166000014 are in the same clade with *Arabidopsis STM*, with a bootstrapping value of 100%. estExt_Genewise1_v1_C_LG_II1820 is also closely related to *Arabidopsis STM*, with a 100% bootstrapping value compared to the two other poplar *STM* genes. All of these poplar genes share the highly conserved KNOX1, KNOX2, ELK, and homeodomains with *Arabidopsis STM*, however,

gw1_XI_1499_1 is truncated at the 5' end and was thus not considered for promoter selection. We hereafter refer to estExt_Genewise1_v1_C_LG_II1820 and fgenesh4_pm_C_scaffold_166000014 as *PopSTM1* and *PopSTM2*, respectively.

Arabidopsis STM has three large introns, ranging in size from 460 bp to 690 bp. The sizes of its four exons range from 254 to 429 bp. *PopSTM1* and *PopSTM2* also have four exons, ranging from 205 to 419 bp. *PopSTM1* has 99.9% identity with a 1,107-long EST (TC29178) derived from cDNA libraries of cambium, apical shoots, tension wood, and secondary xylem. *PopSTM2* had 86% identity with a 1,124-long EST (NP1274762) which was found in secondary xylem. Based on its broader and less xylem-dominant EST pattern, and similar phylogenetic profile to fgenesh4_pm_C_scaffold_166000014 (*PopSTM2*), we chose estExt_Genewise1_v1_C_LG_II1820 (*PopSTM1*) for our promoter studies.

Expression Patterns Conferred by Populus WUS *and* STM *Promoters*

A total of 45 *PopWUS1* and 54 *PopSTM1* independent kanamycin-resistant events were PCR-positive (Table 3.1). Based on the number of independent transgenic regenerants produced compared to explants cocultivated, the transformation efficiency for both constructs was approximately 2.3%.

To study tissue-specific expression patterns, we performed histochemical GUS staining of all PCR-positive events. Transformants for both genes showed a great diversity of expression patterns. Of 45 *PopWUS1* transgenic events, GUS expression was detected in nearly all major types of tissues (Table 3.2, Fig 3.4). Approximately

half (47%) of the events have GUS expression in apical and axillary meristems (Fig 3.4 A). A large number also had expression in putative hydathodes (56%, Fig 3.4B) and in leaf lamina and veins (40%, Fig 3.4 C). We found only three event with exclusive expression in apical and axillary meristems, but their expression was very weak (Table S1). Over 80% of the events with expression in meristems also had expression in putative hydathodes.

Compared with *PopWUS1* transgenic events, *PopSTM1* transgenic events had a higher percentage of events with expression in meristems (65% of 54 events, Fig 3.5 A) and leaf lamina & veins (46%) (Table 3.3, Fig 3.5 C). We detected GUS expression in putative hydathodes in only 9% (Fig 3.5 B) of transgenic events. Similar to *PopWUS1*, the events showed a great diversity of expression patterns. Eleven events had exclusive expression in meristems (20.4%), but the expression level of all these events was low (Table S2).

Reporter Expression during in vitro *Shoot Organogenesis*

We chose a single transgenic event from both *PopWUS1* (event 47) and *PopSTM1* (event 130) that showed strong and predominant meristem expression and performed GUS staining at several stages during *vitro* shoot organogenesis. No GUS expression was detected in the non-transgenic 717 controls (Fig 3.6). In *PopWUS1* and *PopSTM*, expression was detectable prior to visible shoot development, starting 3 to 15 days after explants were placed onto callus inducing medium (Fig 3.6 C). GUS expression

grew increasingly strong in calli over time, especially at the edges where shoots tend to emerge (Fig 3.6 D, E).

In regenerated plants, expression in apical and axillary meristems was still dominant (Fig 3.7 A, B, D), but GUS expression was also clearly detected in putative hydathodes in *PopSTM1* transformants (Fig 3.7 E). We also observed minor expression in *PopWUS1* transformant stems, and in older leaves of *PopSTM1* transformants.

Microarray Analysis of Gene Family Expression

We retrieved gene expression estimates of all close homologs of poplar *WUS* and *STM* genes from two related microarray studies. The first microarray study was described in Chapter 2. The other microarray study is an analysis of tissue-specific expression of poplar genes using a NimbleGen custom microarray that was described under methods. For the latter study, most sample tissues were collected from two-year-old poplar tress in the field.

The expression of *PopWUS1*, *PopWUS2*, and two other related genes could be detected by unique probe sets on the Affymetrix microarray. Due to low expression, *PopWUS1* and fgenesh4_pg.C_LG_X001013 did not show detectable changes during regeneration (Fig 3.8 A). *PopWUS2* and possibly gw1.21516.1.1 were up-regulated 7-fold during the first 8 days of shoot induction. estExt_fgenesh4_pm.C_400124 was down-regulated by more than 10-fold change during late callus induction, and then up-regulated to the level prior to hormone treatment during the first 3 days of shoot

induction. In the tissue-specific expression array study, *PopWUS1* and *PopWUS2* were still below the detection level in nearly all types of tissues. However, estExt_fgenesh4_pm.C_400124 and estExt_Genewise1_v1.C_LG_II2767 had very high expression in phloem/cambium compared with both that in other tissues, and that of other *WUS* genes (Fig 3.9 A).

Only *PopSTM1* and grail3.0036024801 were detectable on the Affymetrix microarray. Both of them were down-regulated upon callus induction 3- and 6- fold, respectively (Fig 3.8 B). The tissue-specific expression results showed that *PopSTM1* was distinct from all other paralogs in having its highest expression in both apical and axillary buds, and in phloem/cambium (Fig 3.9 B).

Promoter Motifs Related to Meristem Regulation of WUS *and* STM

To test if *WUS* and *STM* in both *Arabidopsis* and poplar have similar cis-acting regulatory elements, we analyzed their promoter sequences. The 3kb upstream sequences of *WUS* and *STM* in *Arabidopsis*, *PopWUS1*, *PopWUS2*, *PopSTM1*, and *PopSTM2* were submitted to the Database of Plant Cis-acting Regulatory DNA Elements (PLACE). Special attention was paid to auxin and cytokinin related motifs, since the ratio of these two hormones directs the developmental fates of cells during shoot organogenesis.

Approximately 800 motifs were identified for each gene; two motifs were related to cytokinin response, and three motifs to auxin response (Table 3.4). The distribution of hormone-associated motifs is listed in Table 3.5. Our analysis shows

that ARR1ATs are present in both strands of the promoters in abundance; their numbers ranged from 17 to 45 (Table 3.5). One to two copies of AuxRE were identified.

Discussion

The gene expression results from our microarray datasets, and the diversity of expression from *WUS* and *STM* promoters, suggests that expression of *WUS* and *STM* may be more complex in poplar than it is in *Arabidopsis*. A great diversity of expression has also been reported in rice transformed with a *WUS*-like gene driving GUS (Kamiya et al., 2003). Due to the large salicoid duplication in poplar, many single-copy genes in *Arabidopsis* have two close homologs in poplar, and many of these duplicated genes have undergone subfunctionalization (Jansson and Douglas, 2007). For example, *STM* is expressed in both apical meristem and secondary meristems in poplar (Groover, 2005). Our regeneration microarray study gave the surprising results that *STM* and its related genes were more highly expressed in stems than in the tissues undergoing shoot organogenesis. In addition, our primary *STM* paralog (*PopSTM1*) had its highest expression in phloem/cambium, xylem, and roots in 2 year-old trees from the field. For *WUS*, at least one paralog was up-regulated during shoot induction, despite very low expression during callus induction. Similar results were also reported in *Arabidopsis* (Che et al., 2006). However, the cloned paralog of *WUS* was undetectable in most tissues of adult trees, while three other

WUS-like genes were highly expressed either in phloem/cambium, roots, catkins, xylem, and other tissues.

We identified a single copy of an auxin response element (AuxRE) and multiple copies of response regulator binding motifs (ARR1AT) (Ross et al., 2004) in both the *WUS* and *STM* promoters, and both in *Arabidopsis* and poplar. ARR1AT binds to *Arabidopsis Response Regulator 1* (*ARR1*), which has an activation domain and serves as a type-B response regulator involved in cytokinin signaling (Ross et al., 2004). *CPBCSPOR* is found in the promoter of the cucumber (CS) POR (NADPH-protochlorophyllide reductase) gene, and is critical for cytokinin-dependent protein binding *in vitro* (Fusada et al., 2005). ARFAT, also called AuxRE, is an ARF (Auxin Response Factor) binding site found in the promoters of many primary/early auxin response genes of Arabidopsis such as *SAUR* (Small Auxin-Up RNA) (Goda et al., 2004). NTBBF1ARROLB is the NtBBF1 (Dof protein from tobacco) binding site in regulatory domain B in *Agrobacterium,* which is required for tissue-specific expression and auxin response (Baumann et al., 1999). SURECOREATSULTR11 is the core of sulfur-responsive element (SURE) which contains the complementary sequence (TGTCTC) to the auxin response factor (ARF) binding sequence (GAGACA) (Maruyama-Nakashita et al., 2005).

The abundance of ARR1AT motifs suggests their possible role as link between cytokinin signaling and meristem regulation. Very few direct connections between cytokinin signaling and downstream meristem development, for which *WUS* is a central regulator, have been made. *WUS* has been found to repress ARR 5, 6, 7, and 15

by direct interaction with ARR7 (Leibfried et al., 2005). The abundance of ARR1ATs in the promoters of both *WUS* and *STM* therefore lead us to propose that ARR1 and its homologs in *poplar* are possible binding site for cytokinin—implying tight regulation of *WUS* and *STM* activity by cytokinin.

WUS and *STM* have been cloned and characterized in several species in addition to *Arabidopsis* (Kamiya et al., 2003; Groover et al., 2006; Nardmann and Werr, 2006). They function mainly in the SAM, and their closely related genes have similar roles of regulating meristems in the RAM and the secondary meristems. The dominant expression in shoot apical and axillary meristems seen in the transgenic plants demonstrates that the 3.5kb-promoters used cover the majority of the elements needed for SAM regulatory functions, for which similar results were found in *Arabidopsis* (Baurle and Laux, 2005; Uchida et al., 2007). A 57-bp regulatory region was sufficient there to provide all the information required for *WUS* transcription in the SAM. The activity could be further assigned to two adjacent short sequence motifs within the region (Baurle and Laux, 2005).

In addition to the dominant expression in meristems, two other significant patterns we observed (>50% of transgenic events) in both *WUS* and *STM* transgenics was strong expression in leaf veins, and for *WUS* strong expression in hydathodes. Leaf veins are vascular tissues made up of xylem and phloem, and hydathodes are connected to the vascular tissues by a vascular bundle (Mauseth, 1988). WUS and STM may take part in the precise spatial and temporal expression needed for differentiation of these organs. Veinal areas often show strong regeneration capacity

in vitro (e.g., Lee-Stadelmann et al., 1989). Micro-cross sections (MCS) of mid-veins from hybrid *Populus* leaves were approximately 25-fold more efficient in shoot regeneration than were entire explants. Perhaps the common veinal patterns of GUS expression we observed with the WUS reporter genes was a result of cryptic meristematic tissues associated with leaf veins. *Arabidopsis* transformed with the promoter of an auxin-induced IAA gene from zinnia that was driving GUS showed a very similar expression pattern in leaf veins to what we observed. This is not surprising given that *WUS* is certain to be responsive to auxin signaling cascades during organogenesis (Groover et al., 2003). A hydathode is a type of secretory tissue that enables guttation, where water in the form of drops is released from the terminal tracheids of the veins and may help in transport of nutrients from roots to leaves (Fahn, 1990). The pluripotentency of hydothode cells is well known, most prominently in the Bryophyllum section of the succulent *Kalanchoe*,where miniature plantlets form on the margins of leaves and enable vegetative propagation (http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?21076). Finally, the 35S enhancers present in the promoter used to drive the selectable marker gene in our vector may have broadened the variation in expression (Nilsson et al., 1992; Yoo et al. 2005, Wei et al., 2007) beyond what would normally be produced due to position effects alone (van der Hoeven et al., 1994; Gallie, 1998). However, the lack of preferential expression in hydathodes from the *STM* promoter—which was transformed using the same vector—makes it unlikely that the 35S enhancers had a substantive influence.

Although *STM* appears to have strong cambial expression based on array results, this was not observed in the transgenic plants that we studied. This may be a result of their small size and limited secondary meristem development of the regenerated plants when GUS stained. In addition, for the event where *in vitro* regeneration was studied, our selection of an event with strong apical meristem expression may have biased our sample away from one with strong cambial expression.

The efficient *in vitro* regeneration, propagation and transformation of woody plants remains a major obstacle to research and commercial application (Nehra et al., 2005). There are a number of developmental obstacles that may exist—ranging from dedifferentiation to organ initiation—for which there are often not morphological indicators. The transgenic reporter plants we developed would appear to be useful for identifying very early stages of meristem initiation, which based on strong GUS activity for both the *WUS* and *STM* promoters that we observed in callus, appear to being early in callus development in poplar. GUS expression grew increasingly strong in calli over time, especially at the edges where shoots tend to emerge. The reporters therefore appear to be active up to two weeks before shoot induction conditions are even imposed via SIM medium, and several weeks before organ primordia become visible. Given the diverse expression patterns of *WUS* and *STM* gene family members in poplar revealed by our microarray analyses, it would also appear feasible to also create reporter systems for other meristematic populations, particularly the vascular cambium.

Figures

Figure 3.1 Analysis of the amino acid sequences of the putative *Populus WUS* encoding genes and their relationship to *Arabidopsis WUS*.

(A) Phylogenetic analysis of *Arabidopsis* WUS and its homologs in poplar. Bootstrapping values (%) are based on 500 replications. grail3.0019031001 was used for promoter studies.

(B) Alignment of the amino acid sequences of *Arabidopsis* WUS and its homologs in poplar. The signature domains of the WUS homeodomain and WUS box are indicated.

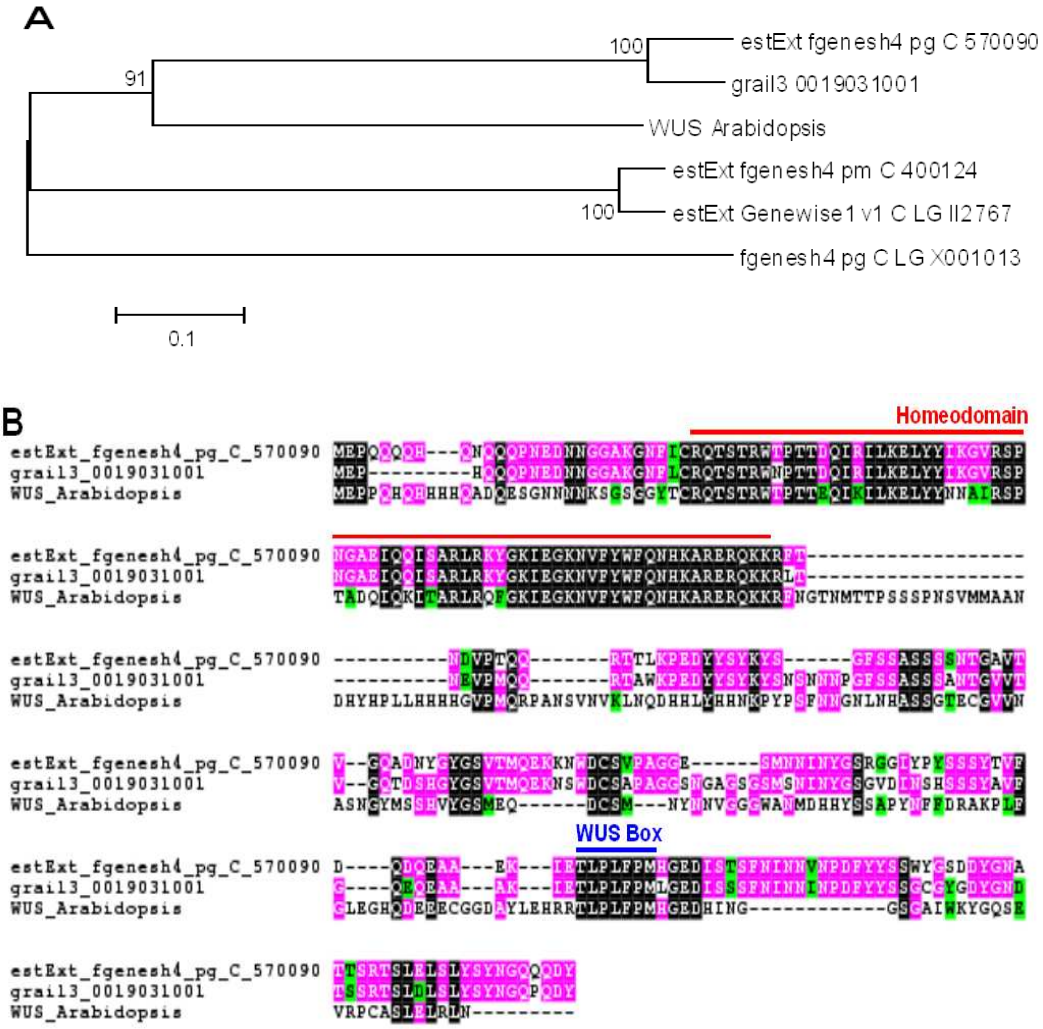


Figure 3.2 Analysis of the amino acid sequences of the putative *Populus STM* encoding genes and their relationship to *Arabidopsis STM*.

(A) Phylogenetic analysis of *Arabidopsis STM* and its *Populus* homologs. Bootstrapping values (%) are based on 500 replications.
(B) Alignment of the **amino acid** sequences of *Arabidopsis STM* and *Populus* homologs. estExt_Genewise1_v1.C_LG_II1820 was used for promoter analysis. The signature motifs of STM, KNOX1, KNOX2, ELK and homeodomain are indicated.

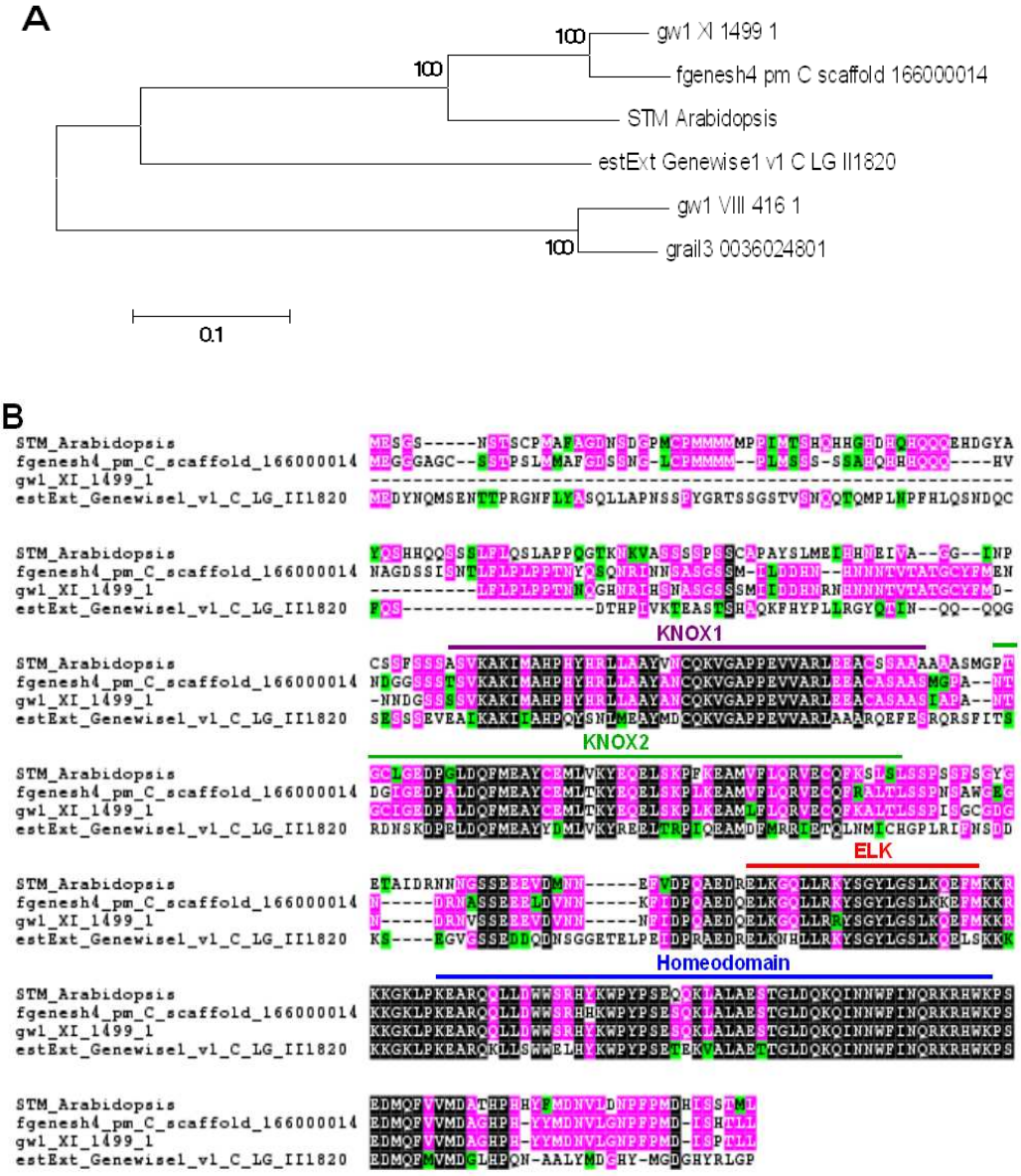


Figure 3.3 Schematic representations *of ProWUS::GUSPlus* and *ProSTM::GUSPlus* vectors.

LB and RB – left and right T-DNA borders, respectively; *tNOS – nopaline synthase* terminator; *nptII – neomycin phosphotransferase II*; *uidA – gusPlus* reporter gene containing an intron. Diagram is not drawn to scale.

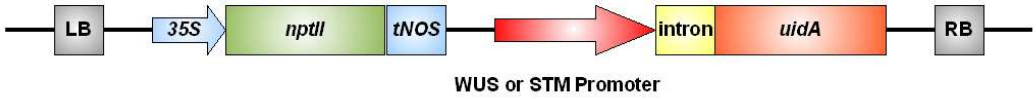


Figure 3.4 Expression patterns of *ProWUS::GUSPlus* transgenic events.

Examples of GUS expression in (A) apical and axillary meristems; (B) hydathodes; (C) major leaf veins and edges; (D) leaf veins & petiole; (E) stipules; (F) root tips; (G) root tissues. Arrows indicate the locations of prominent expression. Scale bars: 4mm.

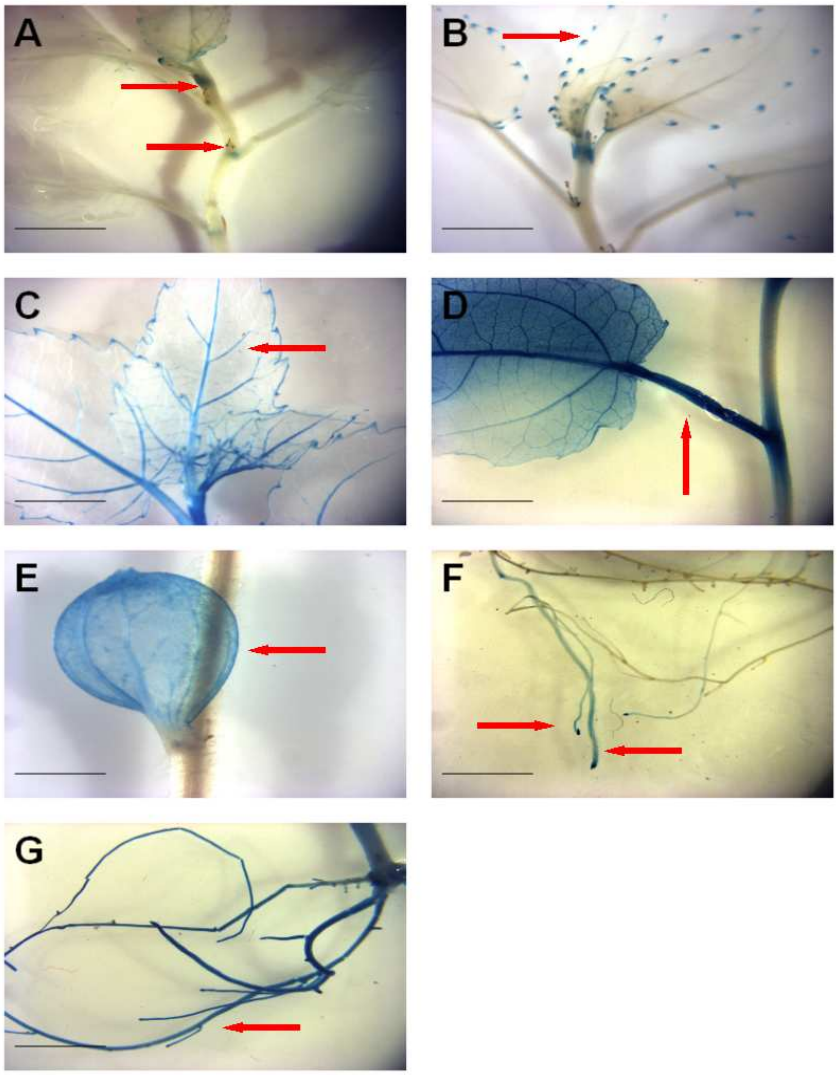


Figure 3.5 Expression patterns of *Populus ProSTM::GUSPlus* events.

GUS expression in (A) apical and axillary meristems; (B) hydathodes; (C) leaf lamina, veins, and petioles; (D) stipules; (E) root tips; (F) other root tissues. Red arrows indicate the expression locations. Scale bars: 4mm.

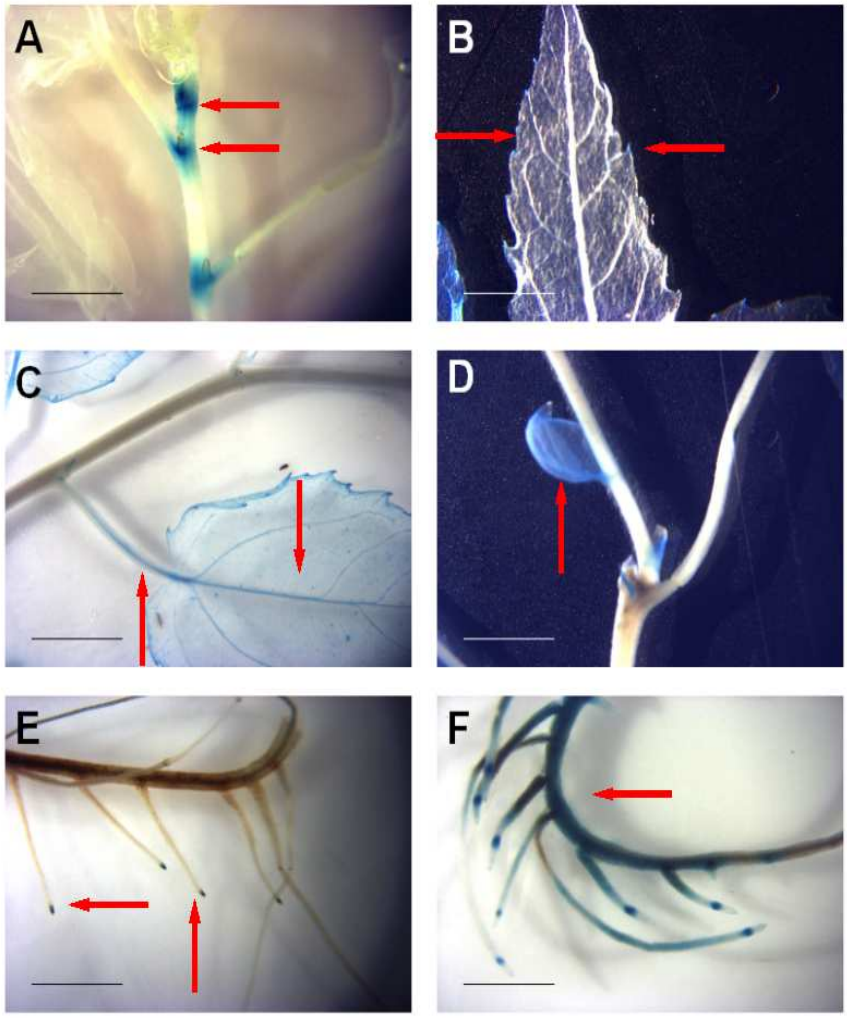


Figure 3.6 GUS staining of recovered transgenic explants.

A single *PopWUS1:: GUSPlus* event (left panel), *PopSTM1::GUSPlus* event (middle panel), and control 717 (right panel) during *in vitro* regeneration. (A) – (E): The five sequential time points for collecting and staining explants: before transfer to callus induction medium (CIM), 3 days on CIM, 15 days on CIM, 3 days on shoot induction medium (SIM), and 8 days on SIM. Scale bars: 2mm.

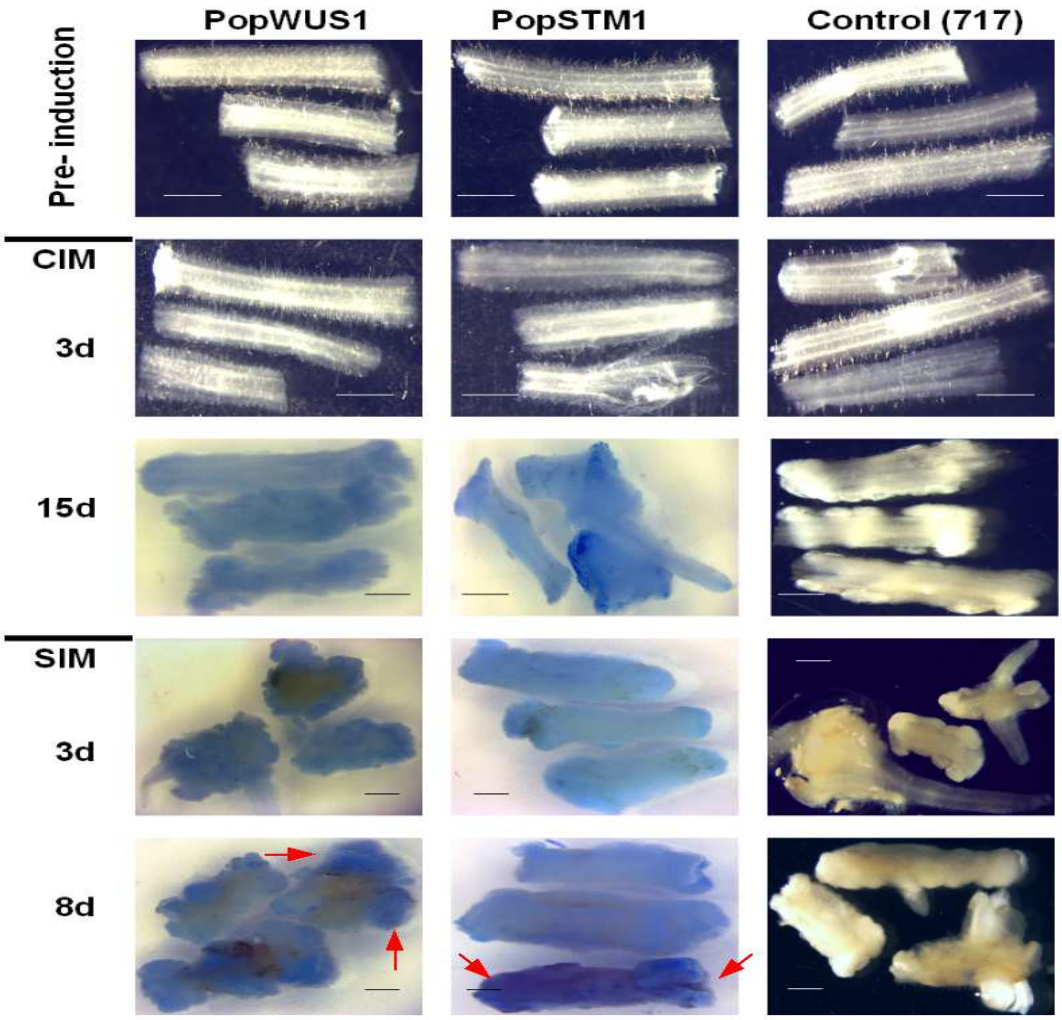


Figure 3.7 GUS expression patterns of *WUS* and *STM* transgenic events.

A single *PopWUS1:: GUSPlus* event (left panel, A-C) and *PopSTM1::GUSPlus* event (D-E, right panel) during *in vitro* regeneration.
(A) – (C) *PopWUS1:: GUSPlus* event GUS expression in (A) the whole plant; (B) apical and axillary meristem; (C) stem. (D) – (F) *PopSTM1::GUSPlus* event GUS expression in (D) the whole plant; (E) apical and axillary meristem and hydathodes; and (F) in leaves. Scale bars: 4mm.

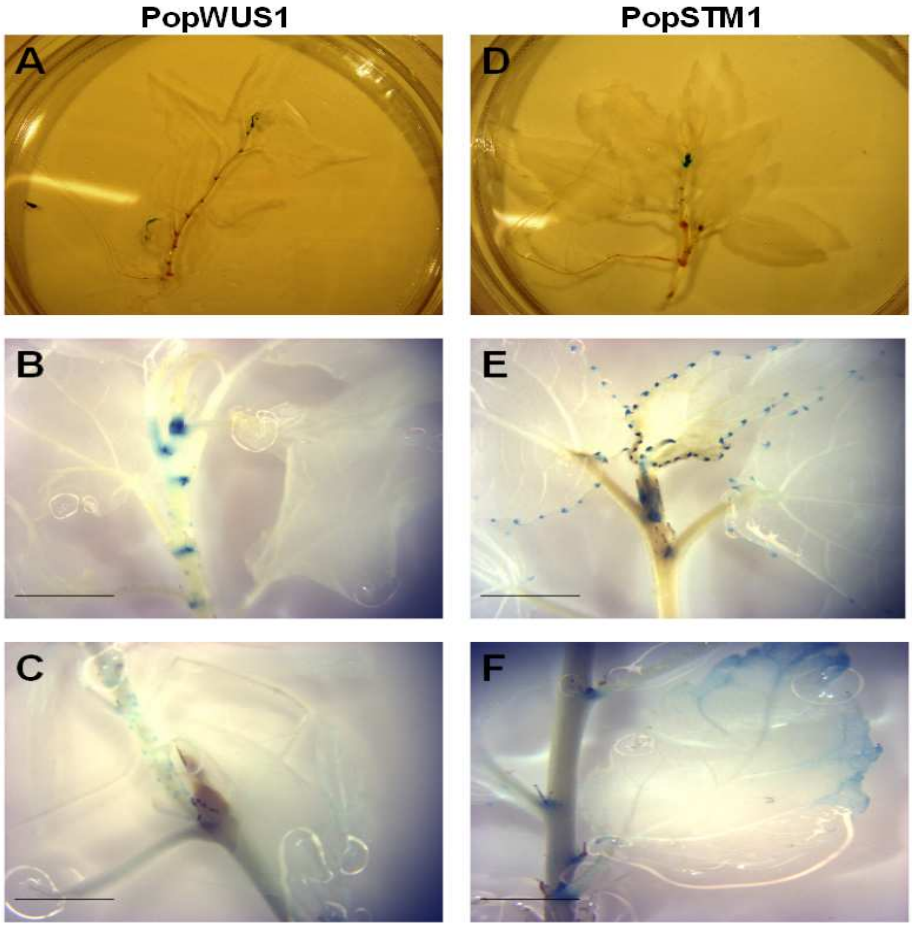
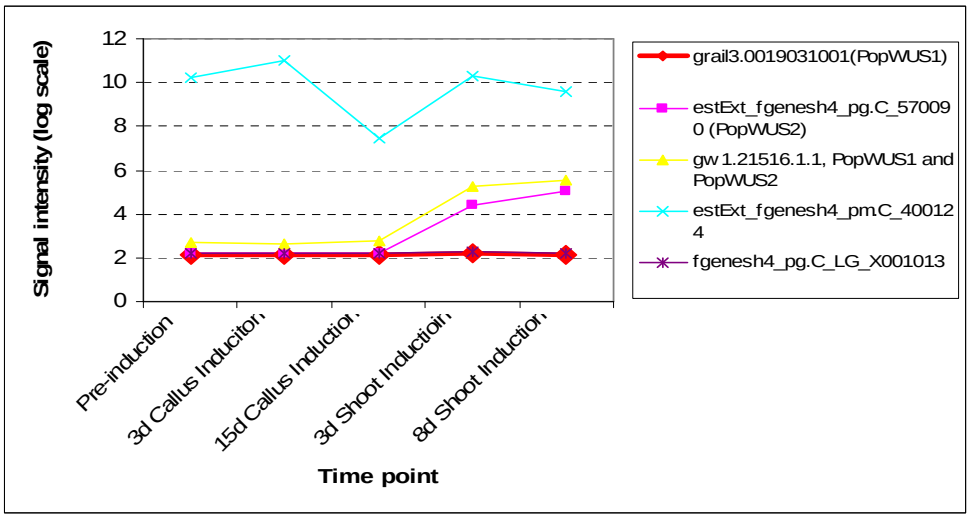


Figure 3.8 Expression of poplar *WUS*, *STM* and closely related genes during *in vitro* shoot organogenesis.

RNAs for micorarray were extracted from the explants at five time points (Fig 3.6 and methods). The logarithms of intensities detected from hybridizations, after normalization, are plotted. Red arrows indicate stronger GUS expression where presumably shoot emerge.

A



B

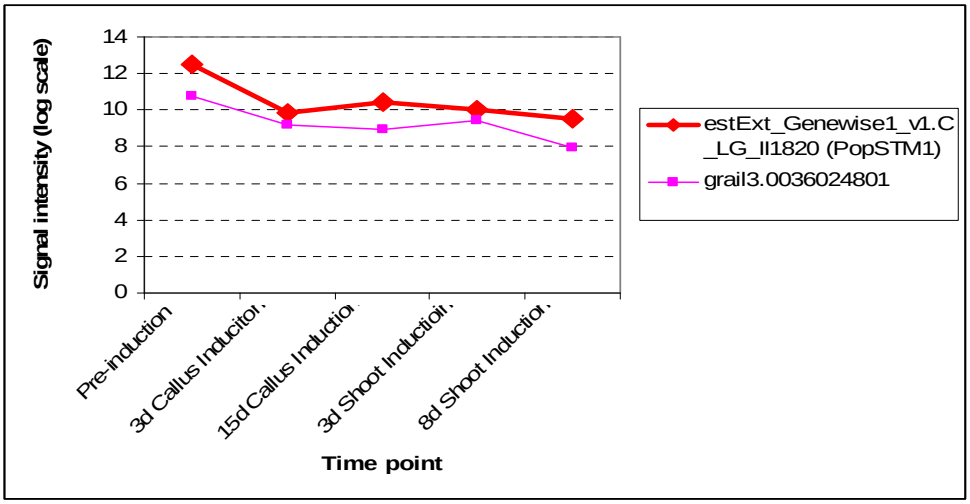
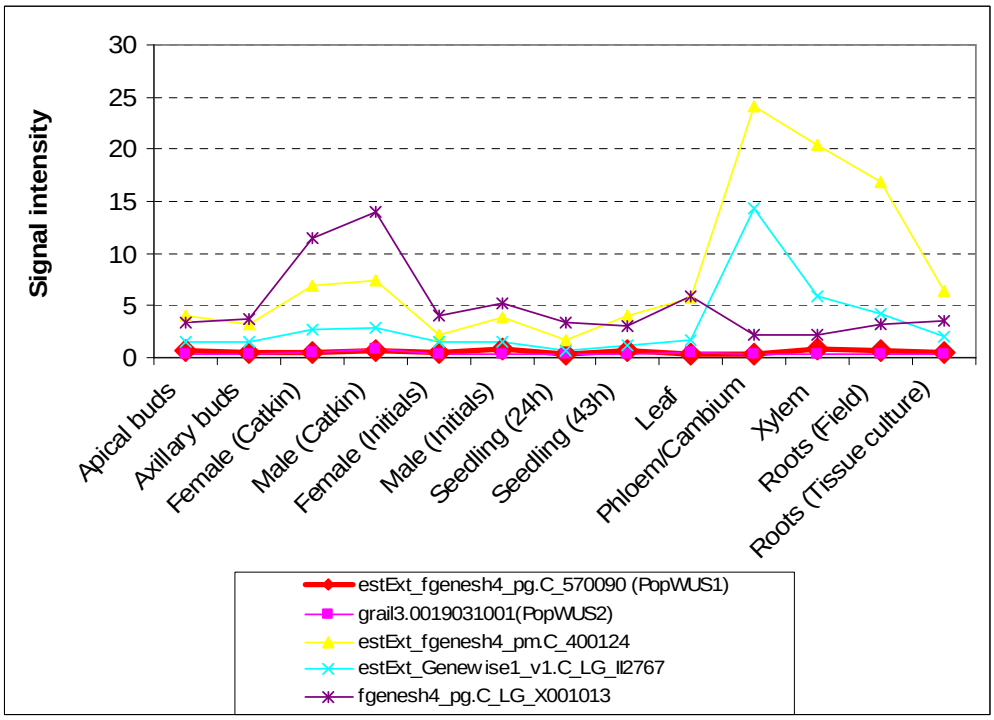


Figure 3.9 Microarry analysis of tissue-specific expression of poplar *WUS*, *STM*, and closely related genes.

Different types of tissues collected from 2-year-old trees from the field were analyzed by a NimbleGen genome scale microarray. Genes with a normalized intensity <0.8 are not significantly different from background.

A



B

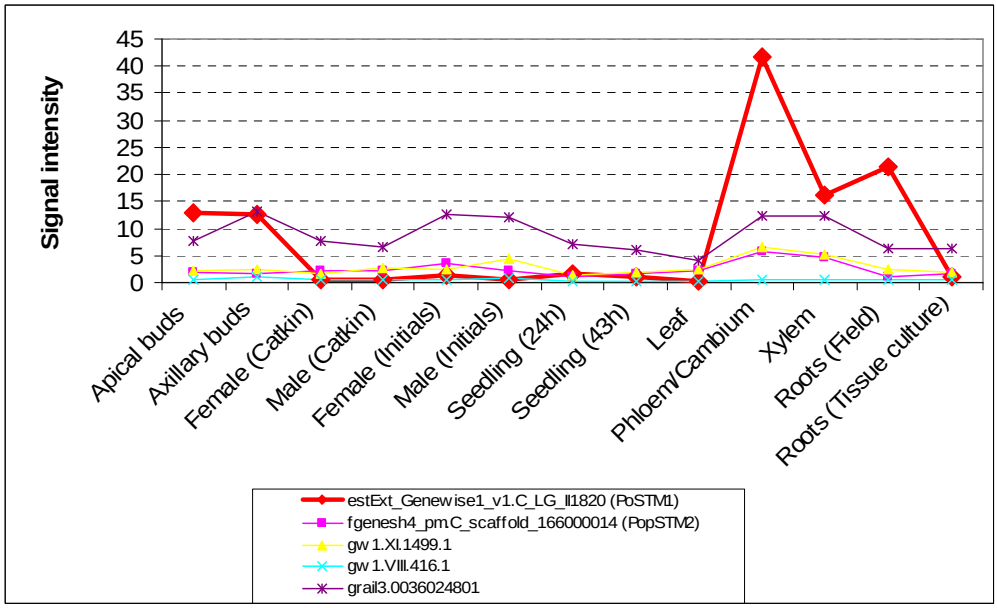
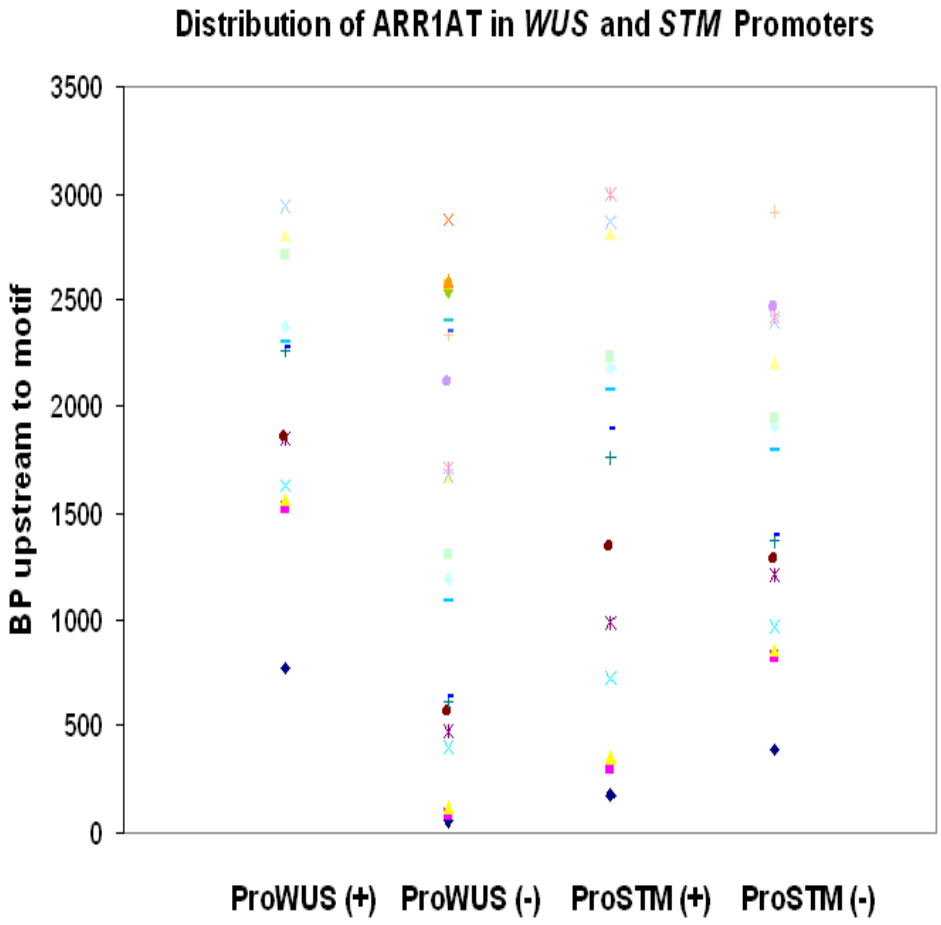

Signal intensity
45
40
35
30
25
20
15
10
5
0
Apical buds
Axillary buds
Female (Catkin)
Male (Catkin)
Female (Initials)
Male (Initials)
Seedling (24h)
Seedling (43h)
Leaf
Phloem/Cambium
Xylem
Roots (Field)
Roots (Tissue culture)
estExt_Genewise1_v1.C_LG_II1820 (PoSTM1)
fgenesh4_pm.C_scaffold_166000014 (PopSTM2)
gw1.XI.1499.1
gw1.VIII.416.1
grail3.0036024801

Figure 3.10 Distribution of ARR1AT motifs in *Arabidopsis WUS* and *STM* promoters.

The dots indicate the number of base pairs upstream of the translation initiation codons (ATGs) of *WUS* and *STM*; numbers are given for both (+) and (-) strands.



Tables

Table 3.1 Transformation efficiency of *PopWUS1:: GUSPlus* and *PopSTM1:: GUSPlus* transgenic events.

Overall transformation efficiency is the percentage of explants cocultivated that gave rise to an independent transgenic plant.

Construct	*ProWUS1::GUSPlus*	*ProSTM1::GUSPlus*
No. of explants co-cultivated	2,066	1,976
No. of explants with shoots	259	281
No. of shoots selected	145	176
No. of shoots rooted	68	80
No. of plants PCR positive	47	59
Overall transformation efficiency	2.27%	2.99%

Table 3.2 Summary of expression patterns of *PopWUS1:: GUSPlus* transgenic events.

All PCR positive *PopWUS1:: GUSPlus* transgenic events were GUS stained. The numbers of events and percentages of the type of tissues in which GUS was expressed are presented; photographic examples are given in Fig. 3.4.

Populus WUS	**Apical and**	**Other aerial**				**Subaerial**	
	axillary		**Leaf lamina**	**Leaf lamina**		**Root**	
45 events	**meristems**	**Hydathodes**	**& veins**	**& petioles**	**Stipules**	**tips**	**Other**
Number of events	21	25	18	5	9	4	7
Percentage	46.7%	55.6%	40.0%	11.1%	20.0%	8.9%	15.6%
Photograph (Fig. 3.4)	A	B	C	D	E	F	G

Table 3.3 Summary of expression patterns of *PopSTM1:: GUSPlus* events.

All PCR positive *PopSTM1:: GUSPlus* transgenic events were GUS stained. The numbers of events and percentages of the type of tissues in which GUS was expressed are presented; photographic examples are given in Fig. 3.5.

Populus STM	**Apical and**	**Other aerial**				**Subaerial**	
54 events	**axillary meristems**	**Hydathodes**	**Leaf lamina & veins**	**Leaf lamina & petioles**	**Stipules**	**Root tips**	**Other**
Number of events	35	5	25	9	6	12	14
Percentage	64.8%	9.3%	46.3%	16.7%	11.1%	22.2%	25.9%
Photograph (Fig. 3.5)	A	B	C	C	D	E	F

Table 3.4 Selected motifs related to cytokinin and auxin response from the Plant Cis-acting Regulatory DNA Elements (PLACE) database.

Two cytokinin-related and three auxin-related motifs were found in the promoter regions of *WUS* and *STM* genes from both *Arabidopsis* and poplar.

	PLACE ID	PLACE Accession Number	Sequence	Description	Selected Reference
Cytokinin related	ARR1AT	S000454	NGATT	"ARR1-binding element" found in Arabidopsis and rice; ARR1 is a response regulator; N=G/A/C/T.	(Ross et al., 2004)
	CPBCSPOR	S000491	TATTAG	Critical for cytokinin-enhanced Protein Binding *in vitro*.	(Fusada et al., 2005)
	ARFAT(AuxRE)	S000270	TGTCTC	ARF binding site found in the promoters of primary/early auxin response genes.	(Goda et al., 2004)
	NTBBF1ARROLB	S000273	ACTTTA	Required for tissue-specific expression and auxin induction.	(Baumann et al., 1999)
Auxin related	SURECOREATSULTR11	S000499	GAGAC	Core of sulfur-responsive element (SURE); containing ARF binding sequence GAGACA (complementary AuxRE TGTCTC).	(Maruyama-Nakashita et al., 2005)

Table 3.5 Distribution of identified cytokinin-related and auxin-related PLACE motifs in *WUS* and *STM* genes from *Arabidopsis* and poplar.

The numbers of the motifs are listed for the both strands of the promoters of both *Arabidopsis* and *Populus WUS* and *STM*. *PopWUS1* = grail3_0019031001; *PopWUS2* = estExt_fgenesh4_pg_C_570090. *PopSTM1* = estExt_Genewise1_v1_C_LG_II1820; *PopSTM2* = fgenesh4_pm_C_scaffold_166000014.

	Motif ID	Strand	WUS	PopWUS1	PopWUS2		STM	PopSTM1	PopSTM2
Cytokinin related	ARR1AT	(+)	13	9	7		14	17	7
		(-)	22	14	14		16	5	10
	CPBCSPOR	(+)	1	0	2		2	0	0
		(-)	4	1	2		0	0	0
Auxin related	ARFAT(AuxRE)	(+)	1	0	0		1	0	1
		(-)	1	0	1		0	1	0
	NTBBF1ARROLB	(+)	0	3	6		3	0	4
		(-)	5	1	3		2	0	4
	SURECOREATSULTR11	(+)	1	3	3		0	1	1
		(-)	6	2	2		1	1	5

Supplemental Materials

Available at ScholarsArchive@OSU
(http://ir.library.oregonstate.edu/dspace/, search for "Yanghuan Bao")

S1. **Expression patterns of all *PopWUS1:: GUSPlus* transgenic events.** Entire regenerated plants from all 45 PCR positive *PopWUS1:: GUSPlus* transgenic events were GUS stained, and their expression patterns summarized. A darkened box indicates observed expression in that tissue/organ.

S2. **Expression patterns of *PopSTM1:: GUSPlus* transgenic events.** Entire regenerated plants of all 54 *PopSTM1:: GUSPlus* transgenic events were GUS stained and their expression patterns summarized. A darkened box indicates observed expression in that tissue/organ.

S3. Sequences of primers used in cloning of the promoters of *WUS* and *STM*.

S4. **Affymetrix probe sets targeting *WUS, STM* and closely related poplar genes.** The number of probe sets (of the 11 per gene on the array with a perfect match to the gene models), are listed. Expression from each probe set at the five sample points during regeneration is given in the rightmost columns.

S5. **Expression of genes with similar expression patterns to the up-regulated paralog of *Populus WUS* during shoot organogenesis**. A total of 42 genes share a similar expression pattern during *in vitro* shoot organogenesis (Pearson's correlation coefficient higher than 0.8, one-tailed $P<0.05$). Each gene expression value is normalized to the largest expression value observed for that gene over all the tissue samples studied. The analysis was conducted using HAYSTACK (http://haystack.cgrb.oregonstate.edu/); the up-regulated paralog of *Populus WUS* is boxed at the right.

S6. **Highest-count motifs in the 3kb 5' region of the 42 *Populus* genes with similar patterns to the up-regulated paralog of *Populus WUS* during shoot organogenesis.** The over-represented motifs were identified by Z-scores with corrected P-values$\leq$0.05 using Element (http://element.cgrb.oregonstate.edu/).

S7. **Map of *PopWUS1:: GUSPlus* binary vector.**

S8. **Map of *PopSTM1::GUSPlus* binary vector.**

Online Supporting Materials

S9. **Genes showing a similar expression pattern to *Populus WUS* during shoot organogenesis based on microarray analysis.** A total of 42 genes that showed significant regulation in the data set ($P<0.05$) had a similar expression pattern with *Populus* WUS during *in vitro* shoot organogenesis (Pearson's correlation coefficient above 0.8, and a one-tailed P-value below 0.05; conducted using HAYSTACK.

S10. **Over-represented motifs in the 3kb regions (putative promoters) of the genes with similar expression patterns to *Populus WUS* during *in vitro* shoot organogenesis.** The over-represented motifs were identified by Z-scores with corrected P-values above 0.05 using Element software. The links to annotations for the motifs are in the PLACE database.

S1

Populus WUS	Expression location							Number of events	Percentage
	Apical and axillary meristems	Other aerial				Subaerial			
		Hydathodes	Leaf lamina & veins	Leaf lamina & petioles	Stipules	Root tips	Other		
								9	20.0%
			■					3	6.7%
	■							3	6.7%
		■						3	6.7%
	■	■			■			1	2.2%
	■	■			■		■	1	2.2%
	■	■	■		■			1	2.2%
	■	■				■	■	1	2.2%
	■	■	■	■			■	1	2.2%
	■	■	■			■		1	2.2%
	■	■				■		1	2.2%
	■	■	■	■				1	2.2%
	■	■	■					1	2.2%
	■	■						1	2.2%
	■	■			■			1	2.2%
	■	■						1	2.2%
		■	■		■			1	2.2%
		■	■					1	2.2%
		■	■					1	2.2%
	■	■	■	■	■		■	1	2.2%
	■	■						1	2.2%
		■			■			1	2.2%
	■	■	■				■	1	2.2%

								1	**2.2%**
								1	**2.2%**
								1	**2.2%**
								1	**2.2%**
								1	**2.2%**
								1	**2.2%**
								1	**2.2%**
								1	**2.2%**
Number of events	**21**	**25**	**18**	**5**	**9**	**4**	**7**	**Total events: 45**	
Percentage	**46.7%**	**55.6%**	**40.0%**	**11.1%**	**20.0%**	**8.9%**	**15.6%**		

S2

Populus STM	Expression location							Number of events	Percentage
	Apical - axillary meristems	Other aerial				Subaerial			
		Hydathodes	Leaf lamina & veins	Leaf lamina & petiole	Stipules	Root tips	Other		
								13	24.1%
	■							11	20.4%
	■		■					5	9.3%
	■		■		■			2	3.7%
	■		■	■	■			2	3.7%
	■	■	■	■		■	■	1	1.9%
	■	■	■			■		1	1.9%
	■	■	■			■	■	1	1.9%
	■	■	■		■	■	■	1	1.9%
	■	■	■	■				1	1.9%
	■		■	■		■	■	1	1.9%
	■			■		■	■	1	1.9%
	■		■			■	■	1	1.9%
	■		■	■			■	1	1.9%
	■		■				■	1	1.9%
	■						■	1	1.9%
	■			■				1	1.9%
	■		■			■		1	1.9%
	■		■					1	1.9%
	■		■				■	1	1.9%
	■		■	■		■	■	1	1.9%
	■					■	■	1	1.9%
			■	■	■	■		1	1.9%

								1	**1.9%**
								1	**1.9%**
								1	**1.9%**
Number of events	**35**	**5**	**25**	**9**	**6**	**12**	**14**		
Percentage	**64.8%**	**9.3%**	**46.3%**	**16.7%**	**11.1%**	**22.2%**	**25.9%**	**Total: 54**	

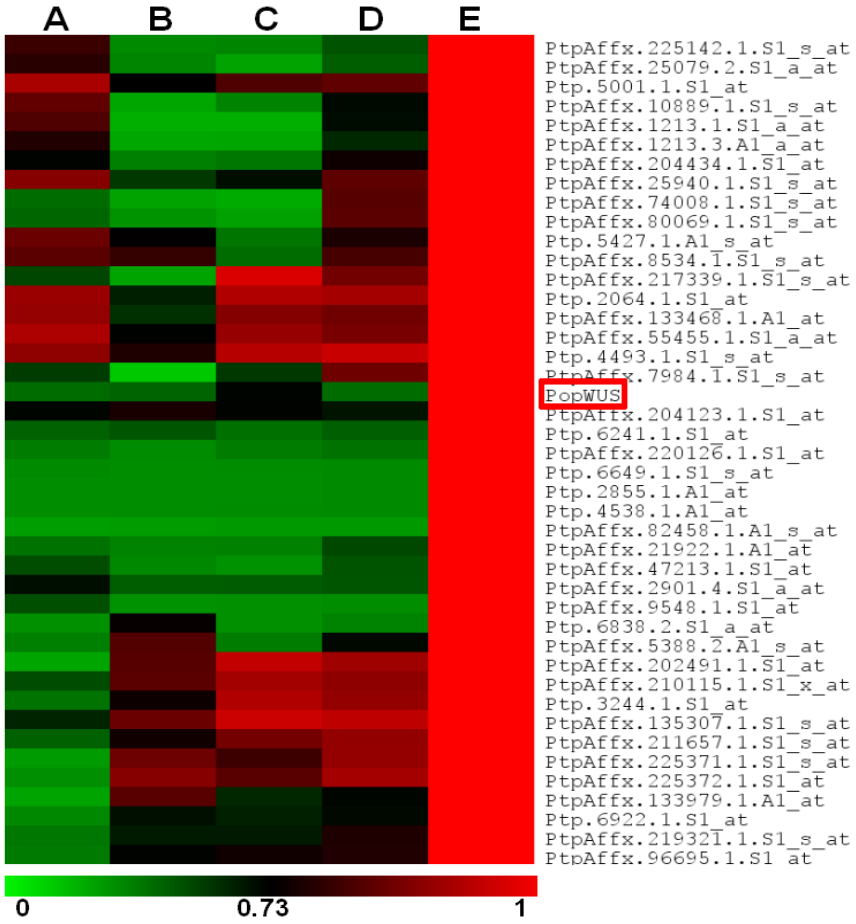
S3

Primer ID	Sequence
WUS2proForward	5' CGGCTGGGCAATATCACTAATAG 3'
WUS2proReverse	5' GATGGATTGAGAAGCCAGAAC 3'
WUSpro1	5' ATGATGAAGTGTCAAACTCAA 3'
WUSpro2	5' AACCTGGTAGTAAATCATGCAC 3'
WUSpro3	5' TGAACCCAATTGCCGACATTAC 3'
WUSpro4	5' TATGATCAGGGAGCAAGAGATG 3'
WUSpro5	5' AGACATGAACACACTACATCG 3'
STM3pro-F03	5' ACGAGCTCTCATGCTACTGGTAACCCTT 3'
STM3pro-R01	5' TAGGTACCCTCTCTCGACAAACCCAGTT 3'
STMinternal#1	5' GTCAAGGATTTATTGCAAGAGT 3'
STMinternal#2	5' GTTAGGATAGAAAAAAGATCAT 3'
STMinternal#3	5' ATTTTTATTATAGATTAGTTTT 3'
STMinternal#4	5' ATCCAGTTTGTGCGCACCTCGA 3'
STMinternal#5	5'AGTAGAGAATGTTTTATATCCA 3'
GPLUS287R	5' AGTCCTTTCCCGTAGTCC 3'
KNX91F	5' CACCAAGAAACGCAGCCCTTAG 3'

S4

	Affymetrix probe set ID	Poplar gene model	Expression Values					
			Number	Pre-	3d	15d	3d	8d
WUS	PtpAffx.218777.1.S1_s_at	grail3.0019031001(PopWUS1)	11	2.72	2.66	2.77	5.23	5.55
	PtpAffx.207414.1.S1_at	grail3.0019031001(PopWUS1)	11	2.15	2.14	2.16	2.20	2.15
	PtpAffx.218777.1.S1_s_at	estExt_fgenesh4_pg.C_570090 (PopWUS2)	9	2.72	2.66	2.77	5.23	5.55
	PtpAffx.54684.1.A1_at	estExt_fgenesh4_pg.C_570090 (PopWUS2)	7	2.23	2.19	2.21	4.38	5.01
	PtpAffx.5866.1.A1_a_at	estExt_fgenesh4_pm.C_400124	11	10.24	10.99	7.46	10.27	9.58
	PtpAffx.208881.1.S1_at	fgenesh4_pg.C_LG_X001013	11	2.23	2.22	2.23	2.29	2.23
	PtpAffx.218777.1.S1_s_at	gw1.21516.1.1 (truncated)	11	2.72	2.66	2.77	5.23	5.55
	no match	estExt_Genewise1_v1.C_LG_II2767	0					
STM	Ptp.5813.1.S1_at	estExt_Genewise1_v1.C_LG_II1820 (PopSTM1)	11	12.49	9.89	10.42	10.02	9.51
	Ptp.5742.1.S1_at	grail3.0036024801	11	10.78	9.18	8.96	9.44	7.96
	PtpAffx.3110.1.S1_at	gw1.XI.1499.1(truncated)	5	11.37	8.00	7.63	7.68	7.81
	PtpAffx.3110.2.S1_at	fgenesh4_pm.C_scaffold_166000014 (PopSTM2)	3	9.34	6.35	2.87	2.95	2.84
	PtpAffx.20353.1.A1_s_at	gw1.VIII.416.1	2	10.30	9.17	8.59	8.93	7.95

S5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PopWUS
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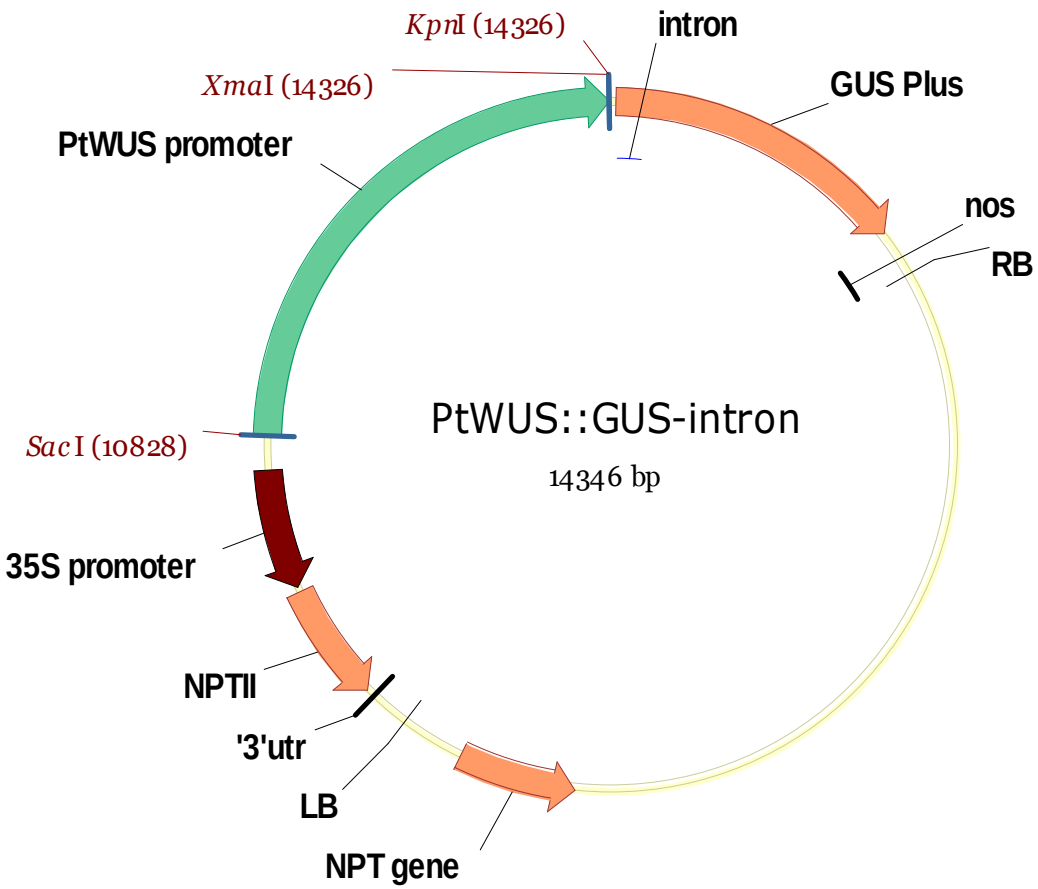
0.73
1

S6

Motif	Count	Z-score	Corrected P-Value	PLACE ID	PLACE Definition
AATAAAT	334	5.217	0.000	POLASIG1	"PolyA signal," l found in *legA* gene of pea and rice alpha-amylase; -10 to -30 bp in the case of animal genes. Near upstream elements (NUE) in Arabidopsis.
AAATTAT	295	3.914	0.007		
AATGAA	288	3.250	0.050		
TATTAAA	248	3.279	0.047		
GAAAAAAA	206	3.643	0.017	GT1CONSENSUS	Consensus GT-1 binding site in many light-regulated genes; GT-1 can stabilize the TFIIA-TBP-DNA (TATA box) complex; The activation mechanism of GT-1 may be achieved through direct interaction between TFIIA and GT-1; binding of GT-1-like factors to the PR-1a promoter influences the level of SA-inducible gene expression.
AATTTTG	133	3.995	0.005	CANBNNAPA	Core of "(CA)n element" in storage protein genes in *Brassica napus*(B.n.); embryo- and endosperm-specific transcription of napin (storage protein) gene, *napA*; seed specificity; activator and repressor.
TGAAAAAA	118	3.449	0.030		See "GT1CONSENSUS"
AATTTTAT	114	3.627	0.018		
TAATTTAA	109	3.319	0.043		

S7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intron
XmaI (14326)
GUS Plus
PtWUS promoter
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RB
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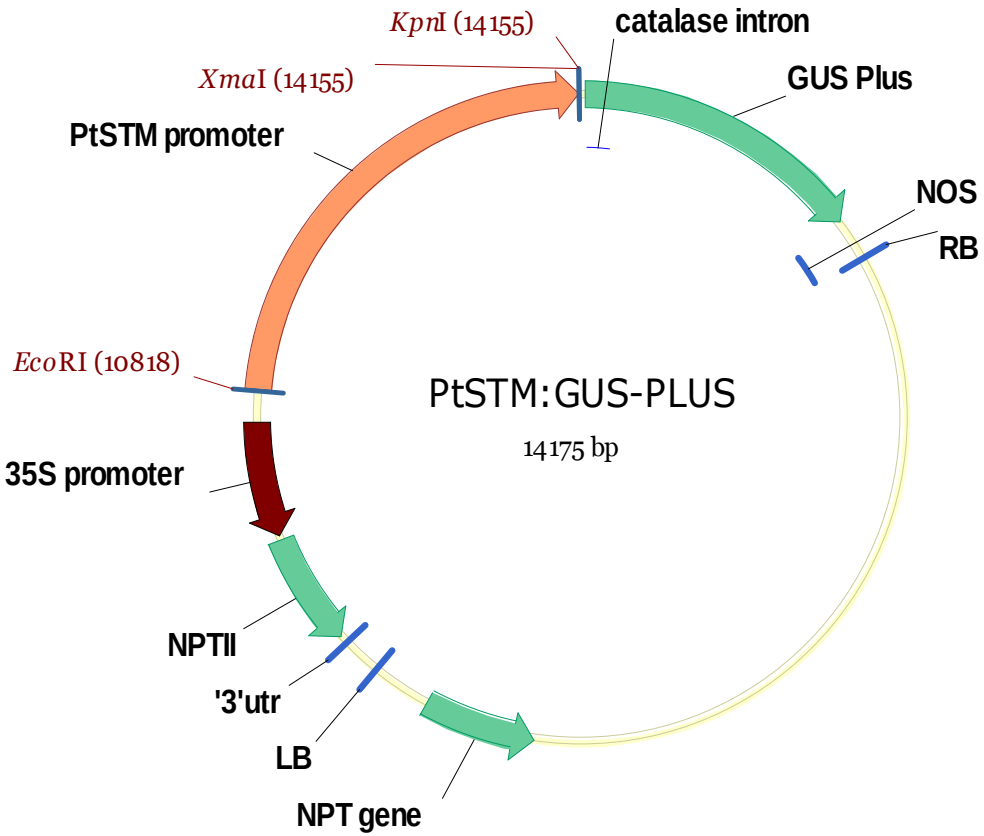
35S promoter
NPTII
'3'utr
LB
NPT gene

S8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XmaI (14155)
GUS Plus
PtSTM promoter
NOS
RB
EcoRI (10818)
PtSTM:GUS-PLUS
14175 bp
35S promoter
NPTII
'3'utr
LB
NPT gene

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CHAPTER 4

GENERAL CONCLUSIONS

1. The major genetic events in regulation of *in vitro* organogenesis in poplar occurred during the early stages of dedifferentiation. Nearly 10,000 genes were differentially expression during the onset of callus induction. A much smaller number of differentially expressed genes were detected at subsequent regeneration stages.
2. A total of 588 transcription factors that were distributed in 45 gene families were differentially regulated. Genes involved in auxin signaling, cytokinin signaling, and secondary meristem regulation (eg. *MYB*s) were among the most abundantly regulated classes of transcription factors.
3. Genes related to auxin signaling were highly regulated during regeneration. Two auxin F-box receptors, and more than a dozen Aux/IAAs and ARFs, showed differential expression. Clustering of Aux/IAAs and ARFs showed evidence of redundant genes within each class.
4. Differentially expression of genes associated with cytokinin signaling included regulation of cytokinin histidine kinase receptors, two phosphotransfer proteins, and A-, B-type, and pseudo response regulators.
5. Most of the identified cell cycle genes were up-regulated during callus induction.
6. Many aspects of the regulatory circuits were conserved between *Arabidopsis* and poplar during callus induction, though different explants (stems vs. roots)

were employed. Approximately one-fourth of the regulated genes in *Arabidopsis* were shared with poplar.

7. We cloned and characterized poplar homologs to *Arabidopsis WUS* (grail3.0019031001) and *STM* (estExt_Genewise1_v1.C_LG_II1820) using phylogenetic analysis of amino acid sequences, and microarray expression data from diverse poplar tissues.
8. *WUS*::*GUS* and *STM*::*GUS* promoter::reporter fusions were predominantly expressed in apical and secondary meristems in transgenic poplars. However, we also observed a wide diversity of expression patterns, including prominent expression in leaf veins and hydathodes. This may reveal the existence of cryptic meristematic cells.
9. At least one copy of *WUS* responds to cytokinin treatment and is up-regulated during shoot organogenesis.
10. Two *STM* paralogs are down-regulated during early callus induction, a possible consequence of its strong expression in the secondary meristem (cambium).
11. We identified 15 to 35 copies of cytokinin response regulator binding motifs (ARR1AT) and one copy of the auxin response element (AuxRE) in promoters of both *WUS* and *STM*.
12. Differential expression of the paralogs of *WUS* and *STM* in different stem cell niches provide an example of subfunctionalization in the highly redundant and duplication rich poplar genome.

13. Several of the *WUS* and *STM* transgenic events could be useful for studying the process of meristem development, including treatments intended to stimulate organogenesis and genetic transformation.
14. The large catalog of regulated genes that we produced provides numerous candidates for studies of the function of unknown genes and gene family members. This will advance knowledge of meristem development, and provide new tools for manipulation of regeneration.

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