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***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 ~3 kb of the 5' flanking regions of close homologs were used to drive expression of the *GUS* gene, 50–60% 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 3–15 days after explants were placed onto callus inducing medium. A minority of *WUS* and *STM* events also showed expression in the cambium, phloem, or xylem of regenerated, greenhouse grown plants undergoing secondary growth. Based on microarray gene expression data, a paralog of poplar *WUS* was detectably up-regulated during shoot initiation, but the other paralog was not. Both paralogs of poplar *STM* were down-regulated threefold to sixfold during early callus initiation. We identified 15–35 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.

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Abbreviations

ARR	<i>Arabidopsis</i> response regulator
AuxRE	Auxin response element
CIM	Callus induction medium
CLV	<i>CLAVATA</i>
CZ	Central zone
PLACE	Plant <i>Cis</i> -acting regulatory DNA elements
RAM	Root apical meristem
SAM	Shoot apical meristem
SAUR	Small auxin-up RNA
SIM	Shoot induction medium

<i>STM</i>	<i>SHOOTMERISTEMLESS</i>
SURE	Sulfur-responsive element
TAIR	The <i>Arabidopsis</i> information resource
VC	Vascular cambium
<i>WUS</i>	<i>WUSCHEL</i>
X-Gluc	5-Bromo-4-chloro-3-indolyl- β -D-glucuronic acid

Introduction

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

Among dozens of identified meristem regulatory factors, *WUSCHEL* (*WUS*) and *SHOOTMERISTEMLESS* (*STM*) are the 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 from 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 (VC) (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 to help identify the conserved themes of SAM and VC development.

The production and development of adventitious meristematic organs are considered to be the important limiting factors to vegetative propagation and transformation of plant species (Arias et al. 2006). To provide new tools to understand and modify meristem development, we created two new promoter::reporter systems based on the promoters of close *Populus* homologs of *WUS* and *STM*. We characterized the expression patterns of transgenic plants with these transgenes, and then analyzed the relation of their expression patterns to microarray studies of poplar development. The transgenic plants should also provide useful tools for further dissection of the factors that control meristem development in poplar.

Materials and methods

Plant material, transformation and regeneration

Hybrid poplar clone INRA 717-1 B4 (female, *Populus tremula* $\times$ *P. alba*) was used for all transformation and microarray studies. The genome sequence of *Populus trichocarpa* Nisqually-1 (Tuskan et al. 2006) was used as a source to predict and amplify the *WUS* and *STM* promoters. Plants were in vitro propagated and transformed according to the protocol described by Filichkin et al. (2006). Forty- to 50-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 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 3 kb 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 *Hind*III and *Nco*I, blunt ended and self-ligated. The *HPTII* gene was also removed from it as an *Xho*I fragment and replaced by *NPTII* using an *Xho*I 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 help create the *WUS* and *STM* reporter constructs.

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 S1). 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 (Table S1).

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 promoter primers from outside of the promoter sequence, as well as the internal primers STMinternal#1, STMinternal#2, STMinternal#3, STMinternal#4, and STMinternal#5 (Table S1).

For both genes, the forward and reverse primers contained an inserted *Sac*I site at the 5' end and a *Kpn*I 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. 1; for complete maps, see Electronic Supplemental Materials S2, 3) were transferred to *Agrobacterium* strain AGL1, PCR confirmed, and transformed into hybrid poplar clone INRA 717-1B4 (*P. tremula* × *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 reporter gene expression during in vitro organogenesis, we selected a single transgenic event from each construct that showed strong meristematic expression (*PopWUS*1 event 47 and *PopSTM*1 event 130), and then used the same time points for collecting explants as had been used in our microarray studies (Bao 2008). These were at 3 and 15 days on callus induction medium (CIM), and 3 and 8 days on shoot induction medium (SIM). To study expression in the vascular cambium, 12 *WUS* events and 13 *STM* events were transferred into soil and grown for approximately 100 days, until the transplanted trees were healthy and approximately 0.75 m in height. GUS staining was done as above, on two ramets from each event. Stem sections were taken by hand from internodes ~4 cm above the soil.

Microarray analysis

An Affymetrix GeneChip® Poplar Genome Array was used to analyze the expression of poplar *WUS*, *STM* and closely

Fig. 1 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

related genes during in vitro shoot organogenesis. Sample collection, RNA extraction, probe labeling and hybridization, and quantitative analysis were described in Bao (2008). In brief, RNAs were extracted separately from two batches (biologic replications) that had been grown under the same growth conditions but 3 weeks apart in February 2007. For both replications, samples were collected at five time points: prior to callus induction, 3 days on CIM, 15 days on CIM (then transferred to SIM), and 3 and 8 days on SIM. Approximately, 10–15 stem explants from the same plate (~3–4 mm in length, with nodes removed) were pooled for RNA extraction for each biologic replication. The arrays were labeled and hybridized according to Affymetrix protocols, and the summary value extracted for each gene was based on a log2 scale.

Tissue type microarray data were extracted from a dataset (Brunner et al. 2007) generated using a poplar Nimblegen microarray (<http://www.nimblegen.com/>), and targeting 55,794 predicted transcripts from the *Populus trichocarpa* genome sequencing project, 126 mitochondrial and chloroplast gene models, and 9,995 unigenes derived from aspen EST sequences (Sterky et al. 2004). Each gene model was represented by three replicated 60mer isothermal probes on the array. Array hybridization, quality control, and data extraction were carried out by Nimblegen

using their established microarray processing pipeline (Wang et al. 2006). Microarray data were normalized across all the arrays used in the experiment using the Bioconductor—Robust Multiarray Averaging (RMA) protocol.

For analysis of microarray expression of the poplar *WUS*, *STM*, and related genes, 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 those genes (Table S7). 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 a corresponding probe set with 11 perfectly matching probes on the array. Only probe sets with at least half of the 11 probes targeting the gene were used to estimate the expression of the genes in our study.

For *WUS* and its related genes, the signal intensity from PtpAffx.207414.1.S1_at was used to estimate the expression of *PopWUS1*. It targeted 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

Fig. 2 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

Fig. 3 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

Table 1 Summary of expression patterns of *PopWUS1::GUSPlus* transgenic events

<i>Populus WUS</i>	Apical and axillary meristems	Other aerial				Subaerial	
		Hydathodes	Leaf lamina and veins	Leaf lamina and petioles	Stipules	Root tips	Others
45 events							
Number of events	21	25	18	5	9	4	7
%	46.7	55.6	40.0	11.1	20.0	8.9	15.6
Photograph (Fig. 4)	a	b	c	d	e	f	g

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. 4

PtpAffx.218777.1.S1_s_at reflected the expression of all 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 seven matching probes in their corresponding probe sets).

Results

Development of *WUS*- and *STM*-based reporter genes

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 291 amino acids (aa) encoded by the *WUS* gene consists of two conserved domains, a 66 aa homeodomain and an 8 aa *WUS* Box (TLPLFPMH) located downstream of the homeodomain (Haecker et al. 2004; Mayer et al. 1998). 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 be truncated upstream of the conserved regions and thus were not considered as promoter candidates (data not

Fig. 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 and petiole, **e** stipules, **f** root tips, **g** root tissues. Arrows indicate the locations of prominent expression. Scale bars 4 mm

shown). Two poplar genes, *grail3_0019031001* and *estExt_fgensch4_pg_C_570090* (hereafter called *PopWUS1* and *PopWUS2*, respectively), were most closely related to *Arabidopsis WUS* (Fig. 2a). 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. 2b), but also are differentiated by a considerable number of amino acid indels in otherwise conserved areas.

The *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 have three exons and two introns, but two of the three exons are shorter. The lengths of the exons of *PopWUS1* are 364, 104, and 310 bp, respectively. *PopWUS2* and *PopWUS1* have very similar exon sizes. 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* versus *PopWUS2*. As cited above,

Fig. 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. Arrows indicate the expression locations. Scale bars 4 mm

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. 3a, *gw1_XI_1499_1* and *fgenes4_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 thus was not considered for promoter selection. We hereafter refer to *estExt_Genewise1_v1_C_LG_II1820* and *fgenes4_*

pm_C_scaffold_166000014 as *PopSTM1* and *PopSTM2*, respectively.

Arabidopsis STM has three large introns, ranging in size from 460 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 *fgenes4_pm_C_scaffold_166000014* (*PopSTM2*), we chose *estExt_Genewise1_v1_C_LG_II1820* (*PopSTM1*) for our promoter studies.

A total of 45 *PopWUS1* and 54 *PopSTM1* independently regenerated, kanamycin-resistant events were PCR-positive (ESM S4). Based on the number of independent

Table 2 Summary of expression patterns of *PopSTM1::GUSPlus* events

<i>Populus STM</i>	Apical and axillary meristems	Other aerial				Subaerial	
		Hydathodes	Leaf lamina and veins	Leaf lamina and petioles	Stipules	Root tips	Others
54 events							
Number of events	35	5	25	9	6	12	14
%	64.8	9.3	46.3	16.7	11.1	22.2	25.9
Photograph (Fig. 5)	a	b	c	d	e	e	f

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. 5

Fig. 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. The five sequential time points used were: 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. Arrows in 8 days point to areas of intense GUS staining that represent putative areas of shoot meristem initiation. Scale bars 2 mm

transgenic regenerants produced and compared to explants cocultivated, the transformation efficiency for both constructs was approximately 2.5%.

Expression patterns conferred by *WUS* and *STM* promoters

To study tissue-specific expression, 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 1; Fig. 4). Approximately, half (47%) of the events had GUS expression in apical and axillary meristems (Fig. 4a). A large number also had expression in putative hydathodes (56%, Fig. 4b) and in leaf lamina and veins (40%, Fig. 4c). We found only three events with exclusive expression in apical and axillary meristems, but

Fig. 7 GUS expression patterns of *WUS* and *STM* transgenic events. A single *PopWUS1::GUSPlus* event (left panel, **a–c**) and *PopSTM1::GUSPlus* event (**d–f**, right panel) after 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 4 mm

their expression was very weak (ESM S5). 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. 5a) and leaf lamina and veins (46%) (Table 2; Fig. 5c). We detected GUS expression in putative hydathodes in only 9% (Fig. 5b) 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 (ESM S6).

Reporter expression during in vitro shoot organogenesis

We chose a single transgenic event from both *PopWUS1* and *PopSTM1* and performed GUS staining at several stages during in vitro shoot organogenesis. The two events were chosen based on the observation that they showed strong and predominant meristem expression (*PopWUS1*

event 47 as seen in Fig. 7a and *PopSTM1* event 130 as seen in Fig. 7d). No GUS expression was detected in the non-transgenic 717 controls (Fig. 6). In *PopWUS1* and *PopSTM1*, expression was detectable prior to visible shoot development, starting 3–15 days after explants were placed onto callus inducing medium (Fig. 6 CIM 15 days). GUS expression grew increasingly strong in calli over time, especially at the places where shoots tend to emerge (Fig. 6 SIM 3 and 8 days).

In regenerated plants, expression in apical and axillary meristems was still dominant (Fig. 7a, b, d), but GUS expression was also clearly detected in putative hydathodes in *PopSTM1* transformants (Fig. 7e). We also observed low GUS expression in stems of *PopWUS1* transformants, and in older leaves of *PopSTM1* transformants. In the transplanted events growing in the greenhouse, only one *WUS* event showed GUS expression in both ramets, whereas four *STM* events showed GUS expression in stems of both ramets, of which one was in maturing xylem and three were predominantly in the phloem/cambium (Fig. 8).

Fig. 8 Expression in stem sections of transgenic plants growing in the greenhouse that showed evidence of histochemical expression. Of 12 *WUS::GUS* events, only one event showed expression (**a**); of 13 *STM::GUS* events, only four events showed expression (**b–d**). **b** shows the stem of one event, and **c** shows stems of three events; the *inset* in **c** is magnified in **d**. Scale bars 2 mm

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 designed to analyze the genome scale transcriptome of shoot organogenesis which was represented by five time points: prior to induction, 3 and 8 days callus induction, 3 and 8 days shoot induction. The other microarray study was 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 2-year-old poplar trees 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 *fgenes4_pg.C_LG_X001013* did not show detectable changes during regeneration (Fig. 9a). *PopWUS2* and possibly *gw1.21516.1.1* were up-regulated sevenfold during the first 8 days of shoot induction. *estExt_fgenes4_pm.C_400124* was down-regulated by more than tenfold 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 type expression array study, *PopWUS1* and *PopWUS2* were still below the detection level in nearly all types of tissues; *PopWUS1* was only detectable in catkins. However, *estExt_fgenes4_pm.C_400124* and *estExt_Genewise1_v1.C_LG_II2767* had very high expression in phloem/cambium compared with both than in other tissues, and that of other *WUS* genes (Fig. 10a).

Only *PopSTM1* and *grail3.0036024801* were detectable on the Affymetrix microarray. Both of them were down-regulated upon callus induction threefold and sixfold, respectively (Fig. 9b). 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. 10b).

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 3 kb upstream sequences of *WUS* and *STM* in *Arabidopsis*, *PopWUS1*, *PopWUS2*, *PopSTM1*, and *PopSTM2* were submitted to the PLACE.

Fig. 9 Expression of poplar *WUS*, *STM* and closely related genes during in vitro shoot organogenesis. RNAs for microarray were extracted from the explants at five time points (Fig. 6 and “Methods”). The logarithms of intensities detected from hybridizations, after normalization, are plotted

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). The distribution of hormone-associated motifs is shown in Fig. 11 and listed in Table 4. Our analysis shows that ARR1ATs are abundantly present in both strands of the promoters; their numbers ranged from 17 to 45 (Table 4). One to two copies of auxin response element (AuxRE) were also 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 appear to 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 initially surprising result 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 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

Fig. 10 Microarray analysis of tissue-specific expression of poplar *WUS*, *STM*, and closely related genes. Different types of tissues collected from field- or in vitro-grown plants were analyzed by a NimbleGen genome scale microarray. Genes with a normalized intensity <0.8 were not significantly different from background

Table 3 Selected motifs related to cytokinin and auxin response from the Plant *Cis*-acting Regulatory DNA Elements (PLACE) database

	PLACE ID	PLACE accession number	Sequence	Description	Selected references
Cytokinin related	ARR1AT	S000454	NGATT	“ARR1-binding element” found in <i>Arabidopsis</i> and rice; ARR1 is a response regulator; <i>N</i> = 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)
Auxin related	NTBBF1ARROLB	S000273	ACTTTA	Required for tissue-specific expression and auxin induction	Baumann et al. (1999)
	SURECOREATSULTR11	S000499	GAGAC	Core of sulfur-responsive element (SURE); containing ARF binding sequence GAGACA (complementary AuxRE TGTCTC)	Maruyama-Nakashita et al. (2005)

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

critical for cytokinin-dependent protein binding in vitro (Fusada et al. 2005). ARFAT, also called AuxRE, is an auxin response factor (ARF) binding site found in the promoters of many primary/early auxin response genes of *Arabidopsis* such as *small auxin-up RNA (SAUR)* (Goda et al. 2004). NTBBF1ARROLB is the NtBBF1 (Dof protein from tobacco) binding site in regulatory domain B in

Agrobacterium, which is required for its 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).

Fig. 11 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 (–) strand

The abundance of ARR1AT motifs supports their possible role as links between cytokinin signaling and meristem regulation. *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* suggests that ARR1 and its homologs in poplar are possible binding sites for cytokinin—implying the direct regulation of *WUS* and *STM* activity by cytokinin.

WUS and *STM* have been cloned and characterized in several species in addition to *Arabidopsis* (Groover et al. 2006; Kamiya et al. 2003; 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 3.5 kb-promoters used to 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). 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; Wei et al. 2007) beyond what would normally be produced due to position effects alone (Gallie 1998; van der Hoeven et al. 1994). 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. In addition, when the same poplar clones were transformed with enhancer trap vectors containing 35S enhancers, the expression in hydathodes of the leaf was rare compared with other expression patterns (Groover et al. 2004). Finally, in ongoing studies in our laboratory (unpublished data) that used the same vector backbone and had gibberellic acid gene promoters (*GA2-oxidase* and *GA20-oxidase*) driving GUS in 26 independent events, the expression patterns seen were virtually all distinct from those seen in this study. This further shows that simple insertion of the GUS gene did not cause the meristem-, leaf vein-, and hydathode-dominant patterns seen with the *WUS* and/or *STM* promoters.

Table 4 Distribution of identified cytokinin-related and auxin-related PLACE motifs in *WUS* and *STM* genes from *Arabidopsis* and poplar

	Motif ID	Strand	<i>WUS</i>	<i>PopWUS1</i>	<i>PopWUS2</i>	<i>STM</i>	<i>PopSTM1</i>	<i>PopSTM2</i>
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

The numbers of the motifs are listed for the both strands of the promoters of both *Arabidopsis* and *Populus* *WUS* and *STM*

PopWUS1 = grain3_0019031001; *PopWUS2* = estExt_fgenes4_pg_C_570090

PopSTM1 = estExt_Genewise1_v1_C_LG_II1820; *PopSTM2* = fgenes4_pm_C_scaffold_166000014

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. Hydathodes are primary sites of free-auxin production. The developmental pattern of free-auxin production is suggested to control the basipetal maturation sequence of leaf development and vascular differentiation in *Arabidopsis* leaves (Aloni et al. 2003). The pluripotency of hydathode 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>). The above evidence suggests that the expression of GUS in hydathodes conferred by the *WUS* promoter may be a result of *WUS*'s regulatory role in auxin signaling and synthesis.

Although *STM* appears to have strong cambial expression based on array results, this was only modestly 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 in GA-7 vessels (Magenta Corp.) when most events were GUS stained. We did observe expression during secondary growth in a subset of the *STM::GUS* transgenic poplars, but it was still visible in only a minority of transgenic events. For unknown reasons, similar to what we observed with the apical expression patterns from these promoters, the cambial expression patterns also show large variation in expression among transgenic events.

The efficient in vitro regeneration, propagation and transformation of woody plants remains a major obstacle to research and commercial applications (Nehra et al. 2005). There are a number of developmental obstacles that may exist—ranging from dedifferentiation to organ initiation—for which there is a lack of early morphological indicators. The transgenic reporter plants we

developed would be useful for identifying very early stages of meristem initiation. Based on strong GUS activity for both the *WUS* and *STM* promoters that we observed in callus, this appears to be occurring during early callus development in poplar. GUS expression grew increasingly strong in calli over time, especially at the edges where shoots tend to emerge. The reporter, therefore, appeared to be active up to 2 weeks before shoot induction conditions were 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 seems feasible to create reporter systems for other meristematic tissues using these promoters, particularly the vascular cambium and vein-associated pre-meristematic tissues.

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Electronic Supplemental Materials

S1. Sequences of primers used in cloning of the promoters of *WUS* and *STM*.

S2. Map of *PopWUS1::GUSPlus* binary vector.

S3. Map of *PopSTM1::GUSPlus* binary vector.

S4. 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.

S5. 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.

S6. 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.

S7. 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.

S1

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

S2

S3

S4

Construct	<i>ProWUS1::GUSPlus</i>	<i>ProSTM1::GUSPlus</i>
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%

[illegible]

								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%		

S6

[illegible]

	Affymetrix probe set ID	Poplar gene model	Expression values					
			Number matching probes	Pre-induction	3d CIM	15d CIM	3d SIM	8d SIM
WUS	PtpAffx.218777.1.S1_s_at	grail3.0019031001(<i>PopWUS1</i>)	11	2.72	2.66	2.77	5.23	5.55
	PtpAffx.207414.1.S1_at	grail3.0019031001(<i>PopWUS1</i>)	11	2.15	2.14	2.16	2.20	2.15
	PtpAffx.218777.1.S1_s_at	estExt_fgenes4_pg.C_570090 (<i>PopWUS2</i>)	9	2.72	2.66	2.77	5.23	5.55
	PtpAffx.54684.1.A1_at	estExt_fgenes4_pg.C_570090 (<i>PopWUS2</i>)	7	2.23	2.19	2.21	4.38	5.01
	PtpAffx.5866.1.A1_a_at	estExt_fgenes4_pm.C_400124	11	10.24	10.99	7.46	10.27	9.58
	PtpAffx.208881.1.S1_at	fgenes4_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 (<i>PopSTM1</i>)	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	fgenes4_pm.C_scaffold_166000014 (<i>PopSTM2</i>)	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