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Genome-wide analysis of gene expression in soybean shoot apical meristem

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Abstract The shoot apical meristem (SAM) contains undifferentiated stem cells that are responsible for the initiation of above-ground organs. The nature of genetic programs and the regulatory networks underlying SAM function in a major legume crop, soybean was investigated here. We used soybean GeneChip® (containing 37,744 probe sets) to examine the transcript profiles associated with micro-dissected, actively growing SAMs or growth arrested axillary meristems (AMs) experiencing apical dominance, in comparison to that of non-meristem (NM) tissue. A total of 1,090 and 1,523 transcripts were identified to be significantly up- or down-regulated in the SAM in comparison to the NM. RT-PCR and in situ hybridization analysis were also carried out to verify the experimental approach. The resulting gene expression profiles point to the combinatorial role of diverse regulatory pathways including those associated with cell division and proliferation, epigenetic regulation, auxin-mediated responses and microRNA regulation in meristem function. In situ hybridization analysis on selected transcripts has implicated their roles in SAM maintenance and the establishment of organ polarity. We also identified a gene, *ANGUSITFOLIA3* that could potentially serve as a novel marker for differentiating cells in the meristem. Computational analysis on the promoter regions of *Arabidopsis thaliana* orthologs of genes with high expression in the soybean SAM revealed a conserved over-representation of three *cis*-acting regulatory motifs. Our data show that plant meristems possess a unique transcriptional profile, with shared "molecular signatures" in apical and axillary meristems providing a rich source of novel target genes for further studies into a fundamental process that impacts plant growth and crop productivity.

Keywords Soybean · Legume · Crop growth and development · Crop productivity · Gene expression

Introduction

Plant apical meristems maintain pools of pluripotent stem cells that are required for the continuous formation of new organs such as the stems, leaves, flowers and roots. Similar to animal stem cells, the plant stem cells are undifferentiated cells capable of self-renewal, proliferation and the generation of a large number of differentiated progeny that give rise to diverse tissues and organs. Plant meristems parallel the stem cell niches in animal systems, providing a microenvironment and a molecular milieu in which the neighbouring cells provide signals to maintain the stem cells in an undifferentiated state (reviewed by Singh and Bhalla 2006; Sablowski 2007; Tucker and Laux 2007).

The shoot apical meristem (SAM) that generates the aerial part of a plant persists as a cell dome with both a radial and a longitudinal structure (Steeves and Sussex

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1989). In most dicots, the SAM is divided into three clonally distinct layers. The outermost layer (L1) harbours cells that generate the epidermal tissues, whereas cells of the sub-epidermal layer (L2) and the internal layers (L3) differentiate into vascular and internal tissues. The SAM can also be perceived as a longitudinal structure consisting of three zones with distinct functions. The peripheral zone (PZ) and the rib zone (RZ) contain cells that will be incorporated into lateral organs and the stem core, respectively, while the central zone (CZ) constitutes the self-renewing stem cell reservoir that becomes the source of cells for the PZ and RZ.

To maintain the integrity of the SAM, the dynamic balance between the formation of new tissue and the maintenance of undifferentiated stem cells must be coordinated. This has been a subject of great interest in developmental biology and the past few years have seen notable progress in this area of research. Of particular significance is the identification in *Arabidopsis thaliana* of a feedback loop involving *CLAVATA* (*CLV*) and *WUSCHEL* (*WUS*) genes that are essential in maintaining the stem cell homeostasis, as well as a number of other homeobox genes such as the *SHOOT MERISTEMLESS* (*STM*) that prevent the differentiation of stem cells (Bhalla and Singh 2006). Though the role of WUS in maintaining the SAM appears to have been evolutionarily conserved in angiosperms other than Arabidopsis (Stuurman et al. 2002; Kieffer et al. 2006), the uniqueness of WUS/CLV antagonism in the maintenance of the shoot stem cell niche remains to be demonstrated especially in important crop species. Furthermore, a recent study has suggested the existence of a yet to be unravelled pathway that can redundantly maintain stem cell identity in the absence of WUS (Green et al. 2005). The same group also showed that neither WUS nor CLV is a robust marker for stem cell identity as their expressions can be found in differentiated tissues (Green et al. 2005). It is clear that we have only scratched the surface in understanding the molecular nature and cellular source of signals that direct the formation of a SAM.

There are significant challenges in translating knowledge gained from research from a model plants such as *Arabidopsis* to crop plants (Rothstein 2007), and in developing new technologies that are directly applicable to crop plants. Nevertheless, the use of the model plant *Arabidopsis* in plant development studies has helped the rapid advances in plant biology frontiers. However, the diversity of legume plants due to their unique capacity to fix nitrogen in soil makes the production of health-promoting biochemicals, proteins and oil-rich seeds, and the legume pod (i.e. fruit) impossible in such systems. Moreover, recent studies comparing leaf and flowering mutant genes between *Arabidopsis* and legumes (such as pea and *Medicago truncatula*) have highlighted unique aspects of legume development (Hecht et al. 2005; Domoney et al. 2006). Further, the reproductive phase of legumes involves the development of inflorescence and leaves, in contrast to only the production of flowers in *Arabidopsis* (Hecht et al. 2005; Domoney et al. 2006).

In this study, we addressed fundamental questions regarding transcriptional features distinguishing the SAM from non-meristematic plant tissues (NM), and the extent to which current known SAM gene networks are conserved in soybean, a legume that leads and drives the global oilseed market. Further, we also investigated whether the transcriptional profile differs significantly between the soybean SAM and the axillary meristem (AM), the meristem responsible for the secondary axes of plant growth. We addressed these issues by interrogating the expression of 37,744 soybean unigenes using Affymetrix soybean genome chips and microdissected fresh (unfixed) meristems or non-meristematic tissues unlike previous work that reported the use of dissected shoot apex consisting of leaf and leaf primordia as well as SAM (Schmid et al. 2005), or examined fixed SAM from a monocot, maize (Ohtsu et al. 2007). Our data reveal that plant meristems possess a unique transcriptional profile highlighting gene programs with roles in SAM maintenance and the establishment of organ polarity. The data presented here provides a rich source for identification of novel regulators of meristem identity from functional and evolutionary perspectives.

Materials and methods

Plant materials and RNA extraction

Soybean plants [*Glycine max*. (L) Merr. Cv. Bragg] were grown in a greenhouse located at the University of Melbourne, Victoria, Australia. SAMs were micro-dissected from 10-day-old soybean with a 26G syringe needle (Terumo Medical Corporation, NJ, USA) under the dissecting microscope at 40× magnification (Wong et al. 2008a). Any leaf primordia were excluded in order to create a meristem-enriched tissue collection and the location of tissue sample is indicated in Fig. 1. Dissected samples were quickly frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from dissected SAM or AM experiencing apical dominance (approximately 80 SAMs or AMs per extraction) or other plant parts (NM consisting of equal amount of tissues from primary stem, primary roots and mature leaves) using Qiagen RNeasy Mini Kit with on column DNAse digestion. Two independent tissue collections and RNA extractions were performed for each of the SAM and NM sample. Subsequent cDNA labelling and Affymetrix Soybean GeneChip hybridization was carried out by Australian

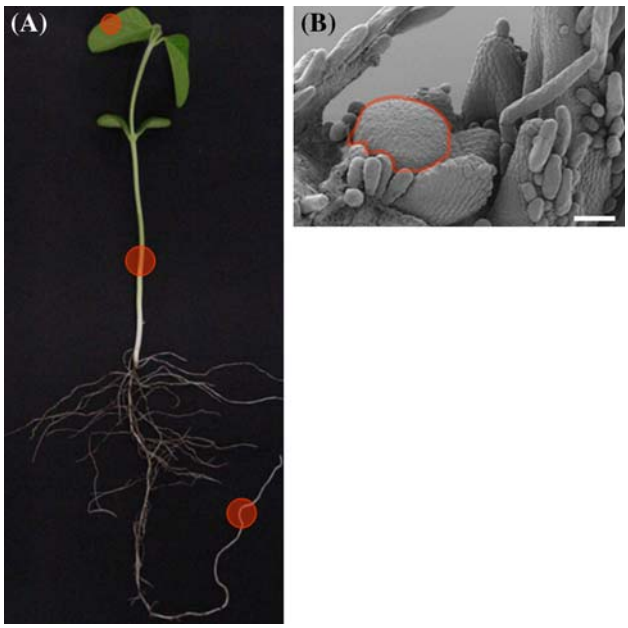

Fig. 1 A 10-day-old soybean seedling used for experiments. **a** The non-meristematic (NM) sampling scheme is indicated by red circle and **b** a representative of the micro-dissected SAM is marked in the scanning electron micrograph of a soybean shoot apex. Scale bar, 50 μm

Genome Research Facility (AGRF, Melbourne, Australia) using 3 μg of total RNA.

Analysis of expression data

Expression levels were estimated from Affymetrix hybridization intensity data using MicroArray Suite 5.0 (Affymetrix 2001). Raw numeric values representing the signal of each feature were imported into Affymetrix linear modeling Graphical User Interface (AffylmGUI) (Wettenhall and Smyth 2004) that uses the Empirical Bayes linear modeling approach of Smyth (2005) for identifying differentially expressed genes in SAMs. The data were normalized using Robust Multiarray Averaging (RMA) method and a linear model was then used to average data between replicate arrays and to look for variability between them (Wettenhall and Smyth 2004). The list of transcripts that were detected to be differentially expressed at adjusted *P*-value of <0.05 was then filtered using a relatively conservative selection method. We applied a cut-off of a 1.5-fold ratio of expression. Hybridization quality was also tested by checking the expression level of the soybean housekeeping control genes that include 18S rRNA, Actin, GSTa, cytochrome P450, SBP, Ubiquitin, and GAPDH. The expression ratios (SAM/NM) of the control genes were consistently in the range of 0.83–1.29. Microarray data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE10607.

RT-PCR analysis

The one-tube, two enzyme Access RT-PCR system (Promega, Annandale, New South Wales, Australia) was used according to manufacturer's instructions in all RT-PCR analysis. Five ng of RNA isolated from the SAM, mature stem, mature leaf, primary root, axillary meristem, and the RAM ($\sim$0.8 mm from the root tip) of 10-day-old soybean seedlings were used as a template in a 10 μl reaction volume. The soybean actin gene (AFFX-GM_ACTIN_M_AT) that showed a similar expression level across the six hybridization experiments was used as an internal control. The number of cycles used for the transcripts investigated was routinely between 25 and 30, ensuring that the amount of amplified product remained in linear proportion to the initial template present in the reaction. Fifty percent of the PCR reaction was separated on 1% agarose gel containing 0.1 μg/μl ethidium bromide and visualized under UV light.

In situ hybridization

The soybean shoot apices were dissected from 10 day-old seedlings and fixed with 4% paraformaldehyde (Sigma) in phosphate buffered saline (PBS) overnight at 4°C after vacuum infiltration. The tissue was then dehydrated and embedded in paraplast (Structure Probe, West Chester, PA) following standard methods. Sections (8 μm) were placed on poly-L-lysine-coated glass slide and the paraplast was removed by immersion in Histoclear (National Diagnostics, Atlanta, GA). Sections were then rehydrated, incubated with 1 μg/ml proteinase K (Sigma) in TE [50 mM Tris–HCl (pH 7.5), 5 mM EDTA] for 15 min at 37°C, 10 min in 4% paraformaldehyde in PBS, and 10 min in 0.5% acetic anhydride in 0.1 M triethanolamine (pH 8). Sections were then dehydrated through an ethanol series and air-dried before application of 200 μl hybridization solution (50% deionized formamide, 12.5% dextran sulphate, 1 μg/ml tRNA, 1× Denhardt's solution, 0.3 M NaCl, 10 mM Tris–HCl pH 6.8, 10 mM $NaHPO_4$ pH 6.8, 5 mM EDTA) that routinely contains about 200 ng of probe, to each slide. Following an overnight incubation at 50°C in a humid box, slides were washed twice in 0.2× SSC for 1 h at 50°C followed by the treatment with 20 μg/ml RNase for 30 min at 37°C. Slides were washed again in 0.2× SSC for 1 h at 50°C. Slides were first incubated in 1% blocking reagent (Roche company) in TBS [100 mM Tris (pH 7.5), 150 mM NaCl] and then in 1% BSA in TBS for 1 h each. Two hundred microliter of anti-digoxigenin antibodies conjugated with alkaline phosphatase (Roche Diagnostics, Castle Hill, New South Wales, Australia) that was diluted 1:1,250 in BXT (1% BSA, 0.3% Triton X-100 in TBS) was applied to each slide, and incubated in moist chambers for 1 h at 25°C. Slides were then washed 4 × 20 min with BXT. Two

hundred microliter of fresh solution [220 μg/ml nitroblue tetrazolium chloride (NBT) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt (BCIP) in 100 mM Tris (pH 9.5), 50 mM $MgCl_2$, 100 mM NaCl] were applied to the slide and incubated in the dark at room temperature overnight. Observations and photography were conducted with a microscope and digital camera (DP70; Olympus, Mount Waverley, Victoria, Australia). Digoxigenin-labeled antisense RNA probes were transcribed from T7 or SP6 promoter of pGEM-Easy vector (Promega) using the DIG RNA Labeling Kit (Roche Diagnostics).

Results and discussion

Differentially expressed transcripts in SAMs

In order to identify regulatory genes that potentially play important roles in meristem development, we compared the transcript profiles of soybean SAMs or AMs that were carefully dissected from 10-day-old seedlings with that of non-meristematic (NM) tissues (Fig. 1). The soybean GeneChip® used contains probe sets for 37,744 transcripts and this represents the largest number of soybean sequences currently available on a commercial microarray. The transcripts have been annotated by HarvEST (www.harvest-web.org) based on the best BLASTX match of the corresponding soybean sequences against TAIR Arabidopsis protein database or Uniprot protein databases. Close to 30,000 of the soybean sequences could be assigned an Arabidopsis locus (expect value $\leq$1e−3). With 8,000 sequences having no significant BLASTX match against Arabidopsis genes and assuming ESTs of the same gene would be assigned to the same Arabidopsis locus, we estimated that the number of unique soybean genes represented by the array is close to 20,000.

Microarray hybridization experiments were performed to obtain transcript profiles of the shoot apical meristems (SAM), axillary meristems (AM) and non-meristematic tissues (NM). The raw intensity data generated were imported into Affymetrix linear modeling Graphical User Interface (AffylmGUI; Wettenhall and Smyth 2004) for linear modeling of the microarray data and for identifying differentially expressed genes in meristems. The data were analysed and filtered as outlined in Materials and methods. The normalized data were visually displayed by scatter-plotting the $\log_2$-transformed signal intensities of the different samples (Fig. 2). The relatively similar transcript profiles of SAM and AM are obvious from the plot while there are distinct differences between that of the NM and SAM/AM samples (Fig. 2). We could not identify any gene to be significantly differentially regulated when the transcript profile of SAM was compared against AM, in spite of the fact that the former was actively growing while the latter experienced apical dominance, and was therefore arrested. This similarity in gene expression profile of the two tissues is nevertheless consistent with studies indicating the formation process of AM resembles that of the

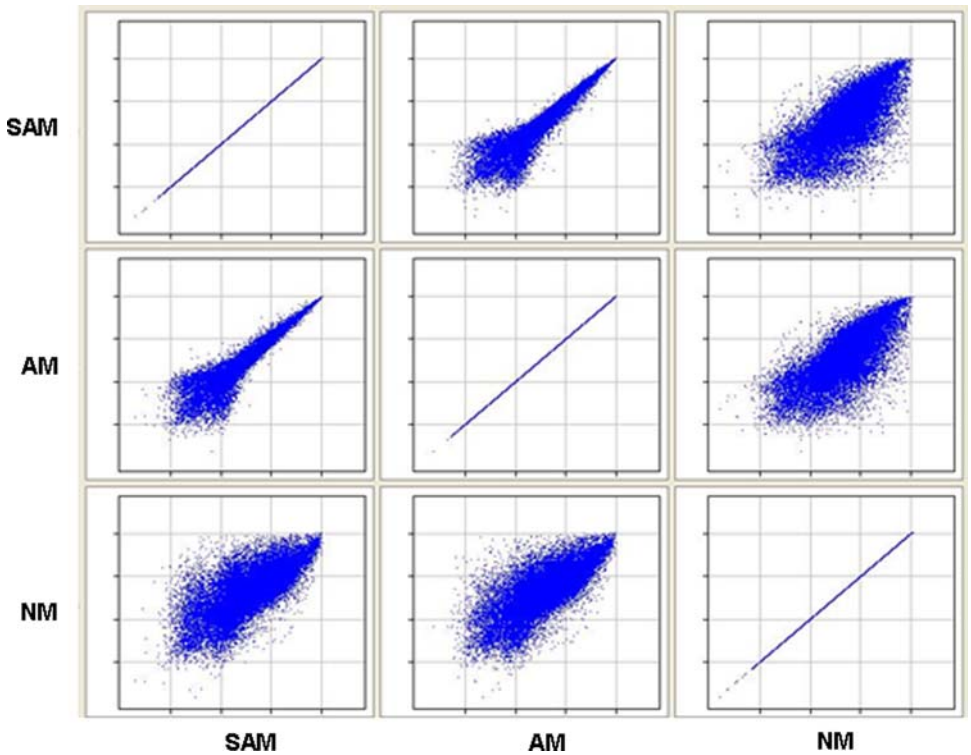

Fig. 2 Scatter plots comparing gene expression in different tissue samples. Log_2 normalized signal intensity for each probe set was used in the plot against different tissue samples as indicated. *SAM* shoot apical meristem, *AM* axillary meristem, *NM* non-meristem tissues

SAM (reviewed by Bennett and Leyser 2006). However, this raises an important question as to the identity of regulatory pathways governing the activities of AM that distinguish AM from the SAM such as its formation only in the axils of leaves. It is likely that regulatory activities specific to AM function do not occur via the change in gene expression rather there are involvements of post-translational or biochemical regulations. It is equally possible that genes involved are rare transcripts that are yet to be represented by the current soybean GeneChip®.

Our analysis on the comparison of SAM and NM dataset indicated that there are a total of 1,090 and 1,523 transcripts identified to be significantly up- or down-regulated in the SAM in comparison to the NM. These range in the fold change relative to the NM from 0.002 ($\log_2$ of −9.1) to over 200 ($\log_2$ of 7.7; Supplemental Tables 1 and 2). The former corresponds to a sequence predicted to encode a chloroplastic carbonic anhydrase while the latter is a *proline-rich extensin-like* gene. The low expression of the putative carbonic anhydrase transcript as well as other photosynthetic-related sequences in SAM (Supplemental Table 2) is not surprising as plant SAMs are heterotrophic, i.e. they do not contain chlorophyll and analysis of transcript patterns for genes such as RUBISCO whose products are required for photosynthesis revealed that these genes are not expressed within the SAM but are highly expressed in regions just outside the meristem (Fleming 2006). On the other hand, upregulation of *extensin-like* gene is consistent with reported abundant expression of similar gene in the meristematic region (Ye and Varner 1991).

The differentially expressed genes identified from the SAM and NM comparison were assigned to different functional categories based on their BLASTX results (Fig. 3). As shown in Fig. 3, categories such as cell growth and division, transcription and post-transcription are more abundantly represented in the induced gene dataset while the percentage of sequences classified under categories linked with general metabolism (primary and secondary metabolism and energy), disease and defence is greater in the repressed gene dataset.

Verification of microarray data

Semi-quantitative RT-PCR was carried out to verify the expression of several randomly selected genes that are detected to be differentially expressed in the SAM. In addition to the RNA samples used in the microarray experiment, RNA from the root apical meristem (RAM) were also included in the RT-PCR experiment to determine whether transcripts with abundant expression in SAM or AM are also highly expressed in RAM on average. As shown in Fig. 4, the gene expression profile detected by RT-PCR is generally consistent with the microarray data and the expression profiles of SAM and RAM are more divergent, unlike that of SAM and AM.

Transcription factors

Examination of differentially expressed genes revealed the presence of many transcription factors with corresponding

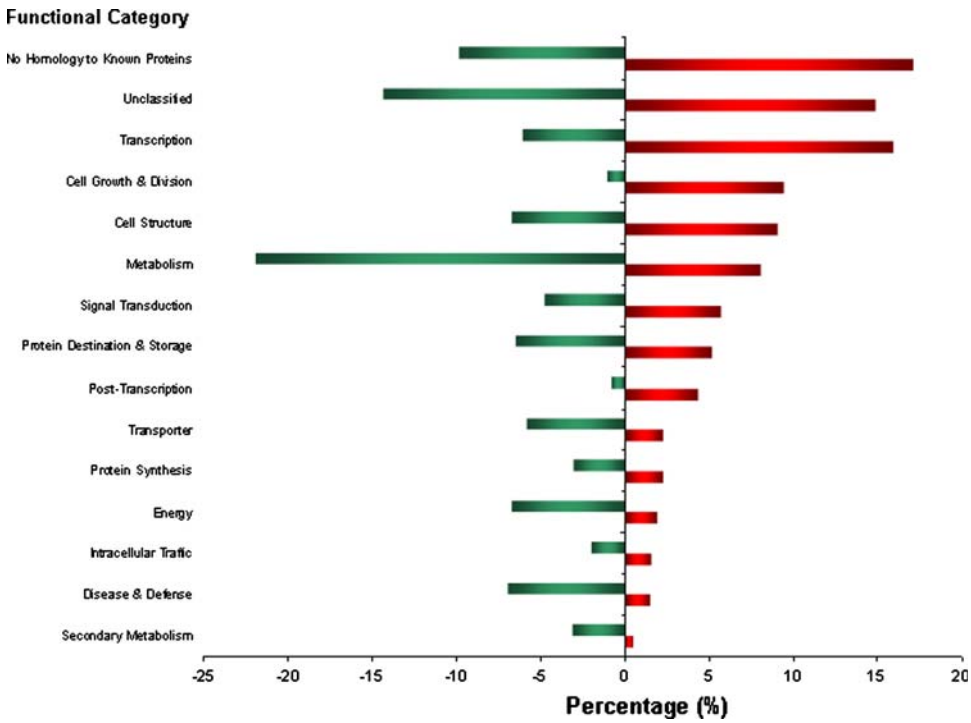

Fig. 3 Functional categorization of up- and down-regulated genes in the soybean SAM, with a probability significance cut-off of $P < 0.05$. *Red* or *green bar* denotes up- or down-regulated categories, respectively

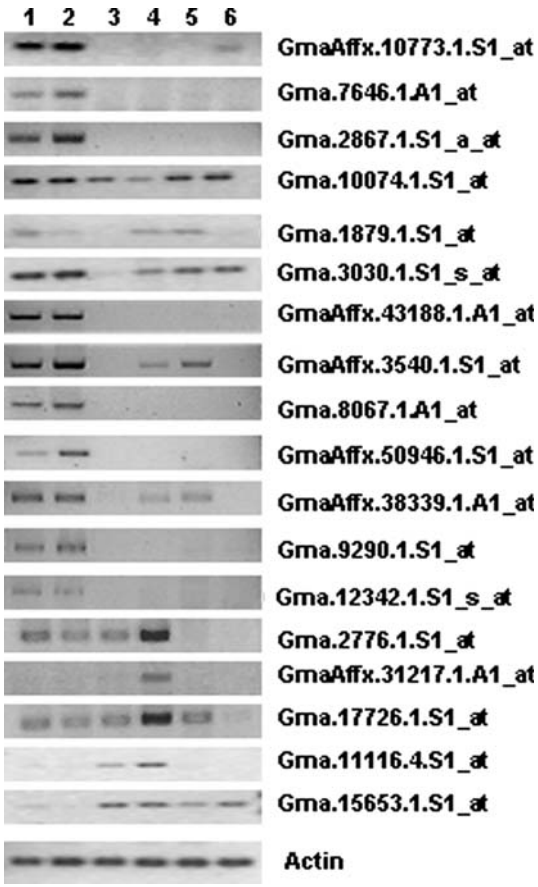

Fig. 4 Verification of microarray data using RT-PCR analysis. Semi-quantitative RT-PCR analysis for 18 randomly selected transcripts as indicated. The *actin* gene was used as an internal control. Transcripts corresponding to Gma.2776.1.S1_at, GmaAffx.31217.1.A1_at, Gma.17726.1.S1_at, Gma.11116.4.S1_at and Gma.15653.1.S1_at were identified as those that were significantly down-regulated in the SAM in comparison to the NM. 1, SAM; 2, Axillary meristem; 3, Stem; 4, Leaf; 5, Root; 6, RAM

orthologues that are known to play a role in the regulation of plant development, in particular the development of the SAM (Table 1A). To reveal the spatial expression patterns and to obtain further insights into the function of some of these transcripts, we performed in situ hybridization analysis for three selected putative transcription factors (Gma.3540.1.S1_at, Gma.3030.1.S1_at, Gma.7714.1.S1_at).

As shown in Fig. 5a, the transcript corresponding to Gma.3540.1.S1_at, annotated to encode an ovule developmental protein AINTEGUMENTA (ANT), is expressed in the outer PZ of the soybean SAM, likely marking cells that will leave the meristem to form lateral organs and hence may function in regulating organ initiation from the SAM. Strong signal related to *ANT* expression is observed in the adaxial side of the emerging leaf primordia suggesting its role in some aspects of adaxial leaf development (Fig. 5a). Recent studies in Arabidopsis have indicated *ANT* playing roles in the maintenance of the meristematic competence of cells during organogenesis as well as in the regulation of genes associated with the establishment of organ polarity (Elliott et al. 1996; Klucher et al. 1996; Mizukami and Fischer 2000; Nole-Wilson and Krizek 2006). As the spatial expression pattern of the soybean *ANT* is similar to that of the Arabidopsis *ANT* (Elliott et al. 1996), the function of the orthologous gene is likely to be conserved.

On the other hand, the transcript of Gma.3030.1.S1_at that is predicted to encode a transcriptional coactivator ANGUSTFOLIA3 (AN3) has gene expression detected mainly in the PZ and parts of the RZ of the SAM, and intriguingly, the signal associated with the expression seems to be absent from the stem cell region (Fig. 5b). This pattern of expression implies that the expression of *AN3* is associated with cells that have exited the stem cell pool in the CZ and are beginning to differentiate. Though there is no in situ hybridization data available for the Arabidopsis *AN3* gene, RNA gel blot analysis has demonstrated its abundant expression in the shoot apex (Kim et al. 2003). Our in situ localisation study provides a higher resolution of the expression of this gene and the results suggest that the gene can serve as a marker for cells showing early signs of differentiation. A recent genetic analysis on the Arabidopsis *an3* mutant has suggested its role in leaf cell proliferation, in particular in the promotion of lateral expansion of the leaf blade, by possibly interacting with a putative transcription factor known as a growth-regulating factor 5 (Horiguchi et al. 2005). Our spatial expression data imply that AN3 likely plays additional role in coordinating activity in the meristem and the molecular mechanism involved awaits further study.

Gma.7714.1.S1_at is a probe set corresponding to a putative *STM* gene. In Arabidopsis, STM is required for SAM initiation and maintenance (Barton and Poethig 1993; Endrizzi et al. 1996). As shown in Fig. 5c, this orthologous gene is expressed throughout the meristem except in the region of what seem to likely be the incipient leaf primordia where its expression is down-regulated similar to the expression in Arabidopsis (Long and Barton 2000). *STM* expression is also detected in inter-primordial regions in line with the proposed role of STM in preventing organ fusion and promoting organ separation in Arabidopsis (Clark et al. 1996; Long and Barton 1998).

There are several differentially expressed transcripts that correspond to putative AP2 (APETALA2) and ethylene-responsive element binding proteins (EREBPs) family of transcription factors. Members of this group play a variety of roles throughout the plant life cycle. Some of the AP2/EREBP transcription factors are key regulators of several developmental processes that include floral organ identity determination or control of leaf epidermal cell identity, while others play important roles in regulating biotic or

Table 1 Representative transcripts up-regulated in the soybean shoot apical meristem in comparison to non-meristem tissues

Affymetrix probe set	Log_2[a]	Putative annotation[b]	AGI No[c]
A			
Homeodomain			
GmaAffx.75004.1.S1_at	2.0	Homeobox-leucine zipper protein	At1g05230
GmaAffx.70395.1.S1_at	2.5	Homeobox transcription factor (KNAT6)	At1g23380
Gma.7714.1.S1_at	4.5	Homeobox protein SHOOT MERISTEMLESS (STM)	At1g62360
Gma.2140.1.S1_at	2.8	Homeobox-leucine zipper protein PHABULOSA	At2g34710
GmaAffx.38738.1.S1_at	4.7	Homeobox protein knotted-1 like 1 (KNAT1)	At4g08150
GmaAffx.88070.1.A1_at	3.1	Homeobox-leucine zipper protein 14	At5g06710
Gma.4824.1.S1_at	2.4	Homeodomain-leucine zipper protein REVOLUTA	At5g60690
AP2/EREBP			
GmaAffx.3540.1.S1_at	7.3	AP2/EREBP AINTEGUMENTA-LIKE 1	At1g72570
GmaAffx.47376.1.S1_at	3.0	AP2 domain-containing	At4g23750
Gma.8249.1.S1_at	4.8	Ovule development protein, putative	At5g10510
Gma.612.1.A1_at	3.7	ANT-like protein (*Nicotiana tabacum*)	UniRef90_Q6SA75
Zinc finger			
GmaAffx.24075.1.S1_at	1.9	C2H2 zinc-finger protein SERRATE (SE)	At2g27100
GmaAffx.30405.1.S1_at	2.7	Zinc finger (C3HC4-type RING finger)	At2g35330
Gma.4704.1.A1_at	2.6	Zinc knuckle (CCHC-type) family protein	At3g02820
Gma.59.1.S1_at	2.4	Zinc finger (C2H2 type) family protein	At5g03740
GmaAffx.78042.1.S1_at	2.1	Zinc finger (C2H2 type) family	At5g14140
Auxin-related			
Gma.7655.2.A1_at	1.5	Auxin-responsive protein (IAA17)	At1g04250
GmaAffx.53937.1.A1_at	6.2	Transcription factor MONOPTEROS (MP)	At1g19850
Gma.12239.1.A1_at	4.5	Axial regulator YABBY1 (YABBY1)	At2g45190
GmaAffx.20323.1.S1_at	5.0	Axial regulator YABBY3 (YABBY3)	AT4G00180
GmaAffx.46821.1.S1_at	2.4	Auxin-responsive factor (ARF9)	At4g23980
Gma.9082.1.S1_at	1.8	Auxin-responsive factor (ARF7)	At5g20730
MADS			
Gma.16078.1.S1_at	3.8	MADS box protein TDR6—tomato	UniRef90_Q533S2
GmaAffx.9006.2.S1_at	1.5	Short vegetative phase protein (SVP)	At2g22540
MYB			
GmaAffx.52983.2.S1_at	3.4	myb family transcription factor (MYB72)	At1g56160
GmaAffx.52983.1.A1_at	6.4	myb family transcription factor (MYB106)	At3g01140
NAC			
Gma.7381.1.S1_at	1.8	No apical meristem (NAM) family	At3g10480
GmaAffx.85080.1.S1_at	2.6	No apical meristem (NAM) family	UniRef90_Q6ZI65
Others			
Gma.3030.1.S1_at	5.0	ANGUSITFOLIA3	UniRef90_Q8L8A5
B			
GmaAffx.26356.S1_at	2	CBL-interacting protein kinase 9	AT1G30270
Gma.9290.S1_at	4.2	Leucine-rich repeat family protein/protein kinase	AT5G62710
GmaAffx.5435.A1_at	2.0	Leucine-rich repeat transmembrane protein kinase	AT5G51350
GmaAffx.40965.A1_at	2.8	Leucine-rich repeat transmembrane protein kinase	AT2G20850
Gma.7646.A1_at	4.2	Leucine-rich repeat transmembrane protein kinase	AT5G51560
GmaAffx.4979.A1_at	3.3	Leucine-rich repeat transmembrane protein kinase	AT5G51560
GmaAffx.26181.A1_at	1.8	Protein kinase family protein	AT5G54590
Gma.4153.S1_at	1.9	Protein kinase family protein	AT3G58690
GmaAffx.30774.S1_at	2.5	Protein kinase family protein	AT1G77720

Table 1 continued

Affymetrix probe set	Log_2[a]	Putative annotation[b]	AGI No[c]
GmaAffx.54819.S1_at	3.9	Protein kinase family protein	AT3G04810
GmaAffx0522.A1_at	3.3	Protein kinase family protein	AT4G16970
Gma7060.S1_at	3.3	Protein kinase family protein,	AT5G46570
GmaAffx.50305.S1_at	2.9	Receptor-like protein kinase ERECTA	AT5G10020
Gma.9652.S1_at	2.8	Ser/Thr kinase	AT2G45490
Gma.9548.S1_at	2.7	SHAGGY-related protein kinase	AT4G00720
GmaAffx.76293.1.S1_at	3.2	Rac-like GTP-binding protein	AT4G28950
C			
Cell division and proliferation			
Gma975.4.S1_at	2.6	Histone H2B, putative	At1g07790
Gma.390.S1_at	2.8	High-mobility-group protein/HMG-I/Y	At1g14900
Gma.77612.S1_at	2.2	Retinoblastoma-related protein	At3g12280
Gma.8420.S1_at	2.4	Cyclin, putative similar to CYCB1-1 protein	At2g26760
GmaAffx.55946.S1_at	2.6	Cell division control protein	At3g25100
GmaAffx.52251.A1_s_at	1.6	DNA-directed RNA polymerase II	At3g52090
GmaAffx.42811.S1_at	2.4	Cyclin family protein	At4g03270
Gma7779.S1_at	3.9	High mobility group (HMG1/2) protein	At4g11080
Gma7555.2.S1_at	2.5	60S ribosomal protein L32 (RPL32A)	At4g18100
GmaAffx.93128.S1_s_at	3.3	High mobility group (HMG1/2) protein	At4g23800
Gma.4863.2.A1_at	2.7	UvrD/REP helicase family protein	At4g25120
Gma.8422.A1_at	3.3	Cyclin, putative (CYC3b)	At5g11300
Gma1176.A1_at	2.4	Histone H3	At5g65360
Epigenetic regulation			
Chromatin remodelling factors			
GmaAffx.45461.S1_at	4.4	SNF2 domain-containing protein/helicase	At5g66750
Gma7365.S1_at	3.0	SWIB complex BAF60b domain-containing	At4g22360
GmaAffx2705.S1_at	2.8	SWIB complex BAF60b domain-containing	At3g01890
GmaAffx.23619.S1_at	2.6	Chromatin assembly factor-1 (FASCIATA1)	At1g65470
Gma0074.S1_at	2.6	Chromatin remodelling complex ATPase chain ISWI	At5g18620
GmaAffx.76576.S1_at	2.5	SWIB complex BAF60b domain-containing	At2g35605
GmaAffx.28735.S1_at	2	SNF7 family protein	At5g44560
GmaAffx.4127.S1_at	1.6	SWIRM domain-containing protein	At2g33610
GmaAffx.34560.S1_at	2.3	WD-40 repeat protein (MSI3)	At4g35050
Gma.6467.2.A1_at	1.92	WD-40 repeat protein (MSI1)	At5g58230
Heterochromatin formation factors			
GmaAffx.27360.S1_at	2.3	SET domain-containing protein (SUVR4)	At3g04380
Histone modification			
GmaAffx.8024.A1_at	2.0	Histone deacetylase family protein	At5g61060
Gma.8550.2.S1_at	1.7	Histone acetyltransferase	At5g64610
GmaAffx.86609.S1_at	2.6	Histone acetyltransferase family	At5g56740
GmaAffx.86609.A1_at	2.7	Histone acetyltransferase family	At5g56740
Gma.8264.S1_at	2.3	Curly leaf protein (CURLY LEAF)	At2g23380
RNA silencing			
Gma.7408.S1_at	2.2	PAZ domain-containing protein/piwi	At5g21150
Gma.6413.S1_at	1.9	PAZ domain-containing protein/piwi	At2g27040
Gma.5747.2.S1_at	1.9	Argonaute protein (AGO1)	At1g48410
Gma.9691.A1_at	2.5	Pinhead protein (PINHEAD)/zwille	At5g43810
GmaAffx.26441.S1_at	2.7	DEAD/DEAH box helicase, DICER family	At2g40700

Table 1 continued

Affymetrix probe set	Log_2[a]	Putative annotation[b]	AGI No[c]
GmaAffx.64309.2.S1_at	2.3	ASF1-like anti-silencing family protein	At5g38110
Protein turnover			
Gma1749.S1_at	2.7	Ubiquitin-specific protease 15 (UBP15)	At1g17110
Gma.5792.S1_at	1.9	26S proteasome regulatory subunit S3	At1g20200
GmaAffx.30405.1.S1_at	2.7	Zinc finger (C3HC4-type RING finger)	At2g35330
Gma.5575.S1_at	1.4	E3 ubiquitin ligase SCF complex subunit	At1g75950
Gma.6337.A1_at	2.2	Proteasome-related similar to 26S proteasome	At3g15180
Gma.7192.A1_at	2	Ubiquitin-specific protease 26 (UBP26)	At3g49600
GmaAffx.63355.S1_at	1.6	Cullin family protein similar to cullin	At4g02570
Gma.1305.S1_at	1.4	26S proteasome AAA-ATPase subunit	At4g29040

Transcripts are annotated based on the best BLASTX match and are grouped under different functional categories: A. Transcription factors; B. Receptor kinases and cell signalling; C. Cell division and proliferation, epigenetic regulation, RNA silencing and protein turnover

[a] Log_2 ratio of the normalized intensity values of SAM over that of NM

[b] Annotation is provided by Harvest website (http://harvest.ucr.edu/) and are based on best BLASTX match against Arabidopsis TAIR database or UniRef90 protein database

[c] Arabidopsis gene index number (AGI No) is given based on the BLASTX match and when there is no match with Arabidopsis protein, the UniRef90 accession is given

abiotic stress response (reviewed by Riechmann and Meyerowitz 1998). This diversity of roles could likely explain the observation that some putative members of this transcription factor family are also represented in the list of transcripts that are down-regulated in the SAM (Supplemental Table 2).

C2H2 zinc-finger proteins are another group of transcription factors that play important roles in plant development including leaf and lateral shoot initiation (for example, see (Prigge and Wagner 2001). There are a number of transcripts with higher expression in SAM and are predicted to encode different members of the C2H2 zinc-finger family, one of which displays a significant similarity to the Arabidopsis *SERRATE* (At2g27100), a gene that is associated with organogenesis and is transcribed in SAMs and in the emerging organ primordia (Prigge and Wagner 2001). SERRATE has been reported to act in a microRNA (miRNA) gene-silencing pathway to regulate expression of *PHABULOSA* while also limiting the competence of shoot tissue to respond to *STM* expression (Grigg et al. 2005).

Auxin has been purported to play a central role in organ initiation and positioning, and floral initiation at the meristem (Reinhardt et al. 2000; Wong et al. 2008b). It is not surprising that among the putative transcription factors, there are members of auxin response factors (ARFs) identified (Table 1A) and one of them is annotated as putative *MONOPTEROS/ARF5*. In Arabidopsis, MONOPTEROS/ARF5 is capable of controlling both axis formation in the embryo and auxin-dependent cell expansion (Hardtke and Berleth 1998; Hardtke et al. 2004).

The presence of various transcription factor families in the microarray dataset reflects the importance of this group of genes in regulating SAM function. In addition to those with orthologues that are known to play a role in regulating stem cell behaviour or primordia formation, other transcription factors listed in Table 1A represent noteworthy candidate genes that may contribute to the functioning of the SAM via mechanism that are currently unknown.

Receptor kinases and cell signalling

It has been suggested that coordinated cell division and differentiation in plant SAMs is controlled by different classes of receptor kinases (Dievart and Clark 2004; Shiu et al. 2004) that include the CLV family of proteins (Stahl and Simon 2005) and ERECTA (Torii et al. 1996; Laufs et al. 1998; Yokoyama et al. 1998; Douglas et al. 2002; Shpak et al. 2003; Woodward et al. 2005). The Clavata1-like protein Gma.7646.1.A1_at was up-regulated in the soybean SAM and we found that the most similar *Arabidopsis* orthologue of this gene was AT5G51560, which is annotated as encoding leucine-rich transmembrane protein kinase. However, the gene annotated as CLV1 in the TAIR database is AT1G75820. There are a number of sequences predicted to encode leucine rich transmembrane protein kinase (Table 1B) that can represent candidates playing similar roles as the CLV or ERECTA. PP2C-type protein phosphatases have been shown to regulate several aspects of plant development. Two PP2C-type phosphatases, KAPP and POL, negatively regulate the CLV1 pathway via dephosphorylation of CLV1. Our microarray data show that the Gma.4656 encoding protein of the phosphatase 2C family is significantly up-regulated in the soybean meristem. Another highly up-regulated gene in the soybean

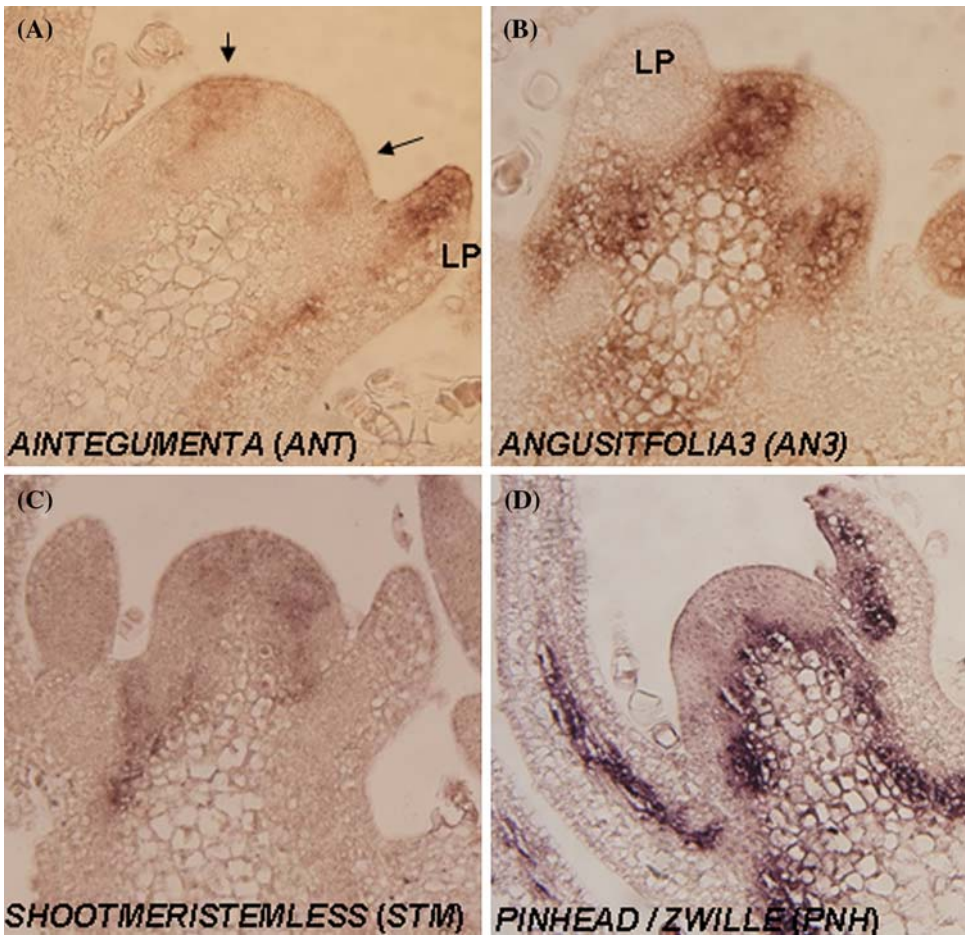

Fig. 5 In situ hybridization of selected transcripts in a 10-day-old soybean SAM. Longitudinal sections of soybean SAMs hybridized with antisense probe corresponding to the transcript of **a** GmaAffx. 3540.1.S1_at, **b** GmaAffx.3030.1.S1_at, **c** Gma.7714.1.S1_at, **d** Gma.9691.1.A1_at. *LP* leaf primordia

SAM is GmaAffx.76293.1.s1_at, which encodes Rac-like GTP-binding protein. The auxin indole-3-acetic acid, a key plant hormone, has been shown to activate Rac-like GTPases (referred to as Rac/Rop GTPases), and they in turn stimulate auxin-responsive gene expression (Tao et al. 2002; Perez-Perez et al. 2004).

Other notable SAM up-regulated transcript is one annotated to encode a SHAGGY-related protein kinase (Gma.9548.1.s1_at; Table 1B). SHAGGY is a highly conserved serine/threonine kinase known to function in eukaryotic signalling pathways. In plants, SHAGGY-like kinases have been implicated in auxin and brassinosteroid signalling (Tao et al. 2002; Perez-Perez et al. 2004). Recent work has also suggested the possible involvement of this group of kinases in cell wall differentiation or homeostasis (Eyuboglu et al. 2007).

Cell division and proliferation at the SAM

A high proportion of the genes that are more abundantly expressed in the SAM are predicted to encode products associated with cell division and proliferation (Table 1C). Among those in this cluster are various chromatin structural proteins such as different histone subunits and HMG proteins, proteins related to DNA, mRNA or protein synthesis machinery, and cell cycle regulatory proteins (Table 1C). This is in agreement with maize microarray data (Ohtsu et al. 2007).

A transcript annotated to encode putative retinoblastoma-related (RBR) proteins (Table 1C) has orthologues reported to control entry into the cell cycle by interacting with cell cycle regulators such as the cyclin/CDK complexes (Huntley et al. 1998; Nakagami et al. 2002; Wildwater et al. 2005; Wyrzykowska et al. 2006). Interestingly, the RBR has recently been reported to play a role in stem cell differentiation (Wildwater et al. 2005; Wyrzykowska et al. 2006). It is likely that some of the transcripts annotated to be encoding products related to fundamental cellular processes may likewise play a direct role in the regulation of the SAM activity.

Epigenetic regulation of the SAM activity

Increases in transcript abundance were also found for genes predicted to encode various products potentially associated with the epigenetic regulation of gene expression and these include putative *chromomethylase*, *histone acetylase* and *deacetylase*, and *SWI2/SNF2* components and *FASCIATA1*.

Similar genes have been known to play roles in DNA methylation, nucleosomal histone modifications and modulation of the chromatin structure that ensure the constant remodelling of the chromatin in order to activate or repress specific sets of genes in response to intracellular or extracellular signals (Wagner and Meyerowitz 2002; Reyes 2006).

Studies done on mammalian stem cells recently have implicated the function of stem cell chromatin in repressing differentiation genes while allowing the activation of stem cell regulators and proliferation factors (reviewed by Scheres 2007). Though the involvement of the plant equivalent of the animal gene in stem cell maintenance is yet to be demonstrated, other factors that contribute to epigenetic regulation have been implicated in plant stem cell maintenance (Kaya et al. 2001; Ono et al. 2006). For example, it has been shown that WUS expression is directly regulated by the SNF2-class ATPase chromatin-remodeling-factor SPLAYED (Wagner and Meyerowitz 2002; Kwon et al. 2005). In addition, as subunits of chromatin assembly factor-1, *FASCIATA1* and *FASCIATA2* genes maintain the cellular and functional organization of the SAM in *Arabidopsis*, and the corresponding mutants show mis-expression of *WUS* in the central zone of the SAM (Kaya et al. 2001). The up-regulation of the expression of sequences associated with epigenetic regulation as observed in this study is therefore consistent with the emerging model of epigenetic regulation of plant stem cells.

Small interfering RNA and meristem function

miRNA, a new class of non-coding ≈20–24 nt RNAs, play crucial regulatory roles in eukaryotes by targeting mRNAs for cleavage or repressing translation. The role of miRNA in plant development has been recently highlighted (Axtell et al. 2007). A number of transcripts encoding putative members of the ARGONAUTE family proteins, and catalytic components of the RNA-induced silencing complex, are among the genes detected to have a higher expression level in the SAM (Table 1C).

Our study on spatial expression of a putative *PINHEAD/ZWILLE* (*PNH*; Gma.9691.1.A1_at) showed a high level of *PNH* expression detection in the border of SAM CZ and RZ (Fig. 5d). *PNH* high-expression is also detected in the provascular tissue and at the adaxial side of primordia and this adaxial expression seemed to become limited to the vascular bundle of the stem at a later stage of primordial development (Fig. 5d).

As members of the ARGONAUTE family proteins have been associated with the regulation and/or the degradation of mRNAs, it is likely that *PNH* exerts its effect by targeting mRNAs related to stem cell maintenance or leaf polarity establishment. A recent study in Arabidopsis has predicted that genes regulating cell cycle and axis determinacy are likely to be among PNH targets (Newman et al. 2002). In addition, *PNH* is also known to act redundantly with *AGO* and are required for the functioning of SAM stem cells and for maintaining organ polarity (Newman et al. 2002; Nishimura et al. 2002; Nagasaki et al. 2007). The *PNH* gene is also specifically required to establish the central-to-peripheral organization of the embryo apex, which is critical for SAM self-perpetuation (Moussian et al. 1998). Further study focusing on the identification of the target genes for PNH/AGO as well as the underlying mechanisms of regulation shall allow us to gain some insight into these very basic aspects of plant development.

Protein turnover and the regulation of SAM activity

A number of up-regulated transcripts in the SAM were predicted to encode proteins involved in the ubiquitin-proteosome pathway (Table 1C). The finding of various ubiquitin-proteosome pathway-related genes indicates the importance of this targeted protein turnover process in the functioning of SAM, consistent with a finding in Arabidopsis that a gene encoding a subunit of the 26S proteasome is essential for the maintenance of meristems (Ueda et al. 2004). In addition to the 26S proteosome, there are three main enzymes involved in the pathway: the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin protein ligase (E3). One class of the E3 ubiquitin ligase, the SCF class, is known to participate extensively in plant development, affecting processes such as hormone response, photomorphogenesis and floral development (reviewed by Moon et al. 2004; Dreher and Callis 2007). The SCF components could be involved in the regulation of stem cell activity through auxin as SCF components have been associated with regulating auxin response or indeed constitute the auxin receptor (Moon et al. 2004; Kepinski and Leyser 2005; Yin et al. 2007). Another likely mode of action of this pathway in regulating the stem cell activity is perhaps through ubiquitination of histones that regulate chromatin dynamics (Wang et al. 2004) and hence possibly affect the transcription of meristem genes.

Comparison with maize and Arabidopsis microarray datasets

A comparison of the functional categories identified to be over- or under-represented in maize (Ohtsu et al. 2007) and this study are generally in good agreement with categories such as photosynthesis-related over-represented among the down-regulated sequences whereas categories such as chromatin remodeling, cell division, transcription and gene silencing were over-represented among the up-regulated

transcripts. However, while high retrotransposon-related transcriptional activity was hypothesized in maize to be contributing to stem cell functions (Ohtsu et al. 2007), this does not appear to be a conserved feature in the soybean SAM as none of the differentially regulated transcripts identified belongs to any of the retrotransposon family. This may reflect the difference in the regulatory mechanism likely deployed by dicot and monocot in controlling stem cell activity. Furthermore, comparative expression analysis have also documented different expression patterns of *WUS* or *CLV* between dicot and monocot plants (reviewed by Nardmann and Werr 2007).

Using the matching AGI, we also investigated the expression of these putative orthologs of soybean transcripts in different organs of Arabidopsis using Genevestigator, a database and Web-browser data mining interface for Affymetrix GeneChip data (Zimmermann et al. 2004). Genevestigator contains microarray expression data for a wide range of Arabidopsis tissues (Schmid et al. 2005) and the closest representation of shoot apical meristem tissue in the collection are those under the category of 'shoot apex'. When the normalized expression levels for putative orthologs of soybean transcripts up-regulated in the SAM was compared with that of cauline leaf as the NM control, 65% of the matching AGI of the up-regulated soybean transcripts has greater than or equal to 2-fold expression level (data not shown). A closer inspection of the transcript level across the different organs illustrated that most of these transcripts have the highest expression in the shoot apex and this includes many unknown genes (Fig. 6a, b). The concordance of expression pattern displayed by these orthologous genes in soybean and Arabidopsis points to a conserved function of these genes in the maintenance of SAM activity for both plants. The finding also highlights a group of sequences with unknown functions that may play novel roles in regulating events taking place in the SAM.

Transcriptional regulation of genes up-regulated in the SAM

The conserved gene expression pattern of many Arabidopsis orthologs (predominantly in the shoot apex) provided an opportunity to identify *cis*-acting elements involved in the transcriptional regulation of these genes. Since promoters of co-regulated genes are likely to share common regulatory motifs, we searched for conserved sequence motifs in the 5′-region 1 kb immediately upstream of 85 *Arabidopsis* orthologs using the MEME motif discovery tool (version 3.5.0) (Bailey et al. 2006). This in silico search revealed three motifs (Fig. 7) conserved within 500 bp upstream of the promoters of all the genes examined. We then used the same tool to examine promoter regions of *Arabidopsis* counterparts of soybean genes that were down-regulated in the SAM and no conserved motif was observed in this case.

Computational prediction of these three motifs for their possible recognition by specific transcription factors showed that these motifs corresponded to consensus binding sites of DNA binding with one finger (Dof) transcription factors, GT-element-binding factors and GAGA-box-binding transcription factors. Dof proteins are plant-specific single-zinc-finger DNA-binding proteins that enhance transcription from the promoters of various genes. Dof proteins possess a highly conserved 52-amino-acid DNA-binding domain that recognizes the (A/T)AAAG sequence as the core motif (Yanagisawa 2001; Umemura et al. 2004). GT elements present in the promoter region of numerous plant genes can exhibit promotion or repression depending on the structural context (Zhou 1999). The corresponding ubiquitously expressed GT-element-binding transcription factors contain one or two trihelix DNA-binding domains. The GT-1 GAGA-box dinucleotide repeat sequence with the pattern (GA)(n)/(TC)(n) is recognized by specific GAGA-box-binding factors (Sangwan and O'Brian 2002). It has been shown in *Drosophila* that GAGA elements may play a role in chromatin remodelling by creating a local chromatin environment via recruiting specific non-histone protein complexes (reviewed by Lehmann 2004). Several *Drosophila* genes, including homeotic and developmental genes contain GAGA motifs that are essential for their transcriptional regulation (van Steensel et al. 2003). Although potential transcription factors present in three conserved sequence motifs in promoters that are up-regulated in the SAM remain to be characterized, co-location of these motifs in promoters of 100% of the genes up-regulated in the SAMs examined in this study points to the critical role of specific transcription factors in SAM function. The specificity of co-location of these motifs in genes up-regulated in the SAM was confirmed by searching for the presence of these motifs in the region 1 kb upstream of *Arabidopsis* genes preferentially expressed in the pollen grains. No such conservation was observed in this set of pollen-expressed genes. The presence of three strictly conserved regulatory motifs in genes up-regulated in the SAM points to a combinatorial interaction of different transcription factors in regulating the specificity and timing of gene expression.

Conclusions

Characterizing active and inactive (repressed) genes is a vital step towards understanding the regulatory circuits that control developmental processes in the SAM. In this study, we have identified a total of 1,090 up-regulated and 1,523 down-regulated genes in the soybean SAM using the soybean GeneChip®. To our knowledge, this is the first report

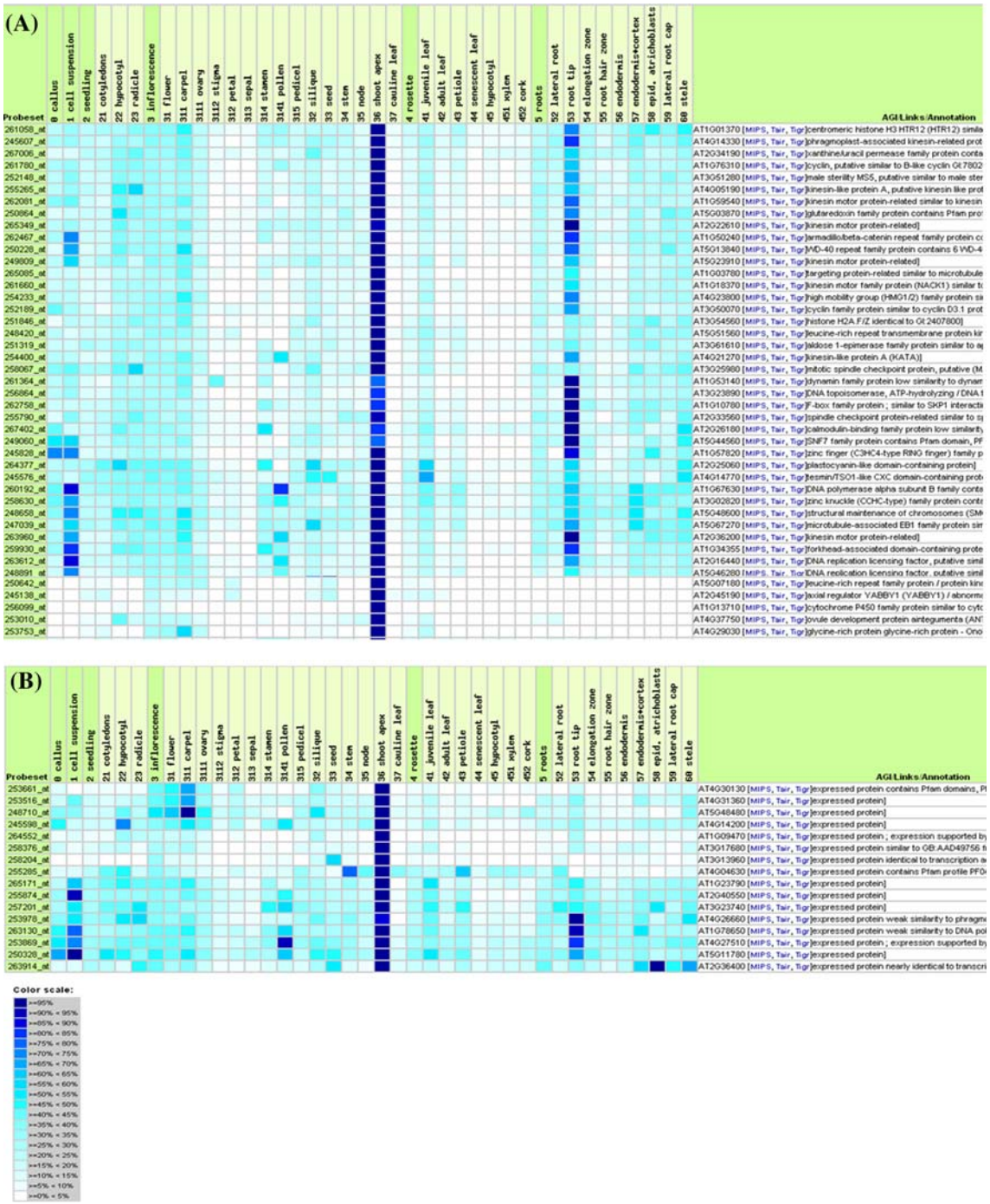

Fig. 6 Meta-Analyzer (Genevestigator) database results, showing differential expression patterns of Arabidopsis orthologues of soybean genes that are highly up-regulated in the SAM. Heat maps of known Arabidopsis meristem genes (**a**), putative annotation (**b**) unknown. *Blue-white* colour scheme: all gene-level profiles as described in Genevestigator website were normalized such that the highest signal intensity for each gene was given a value of 100% (*dark blue*) while the absence of signal is assigned the value of 0% (*white*)

on the transcriptional profiling and identification of genes that are differentially expressed in the SAM of a major dicotyledonous crop plant. The putative annotation of these genes provides an extensive picture of the regulatory mechanisms associated with the SAM. Along with many orthologs of known meristem regulators, we also identified many transcripts with unknown function among the group of up-regulated genes in the soybean SAM. The results from our comparative genomic analysis using Genevestigator database suggest that these genes of unknown function may perform similar functions in Arabidopsis as in soybean. Hence, the approach that we adopted yields a

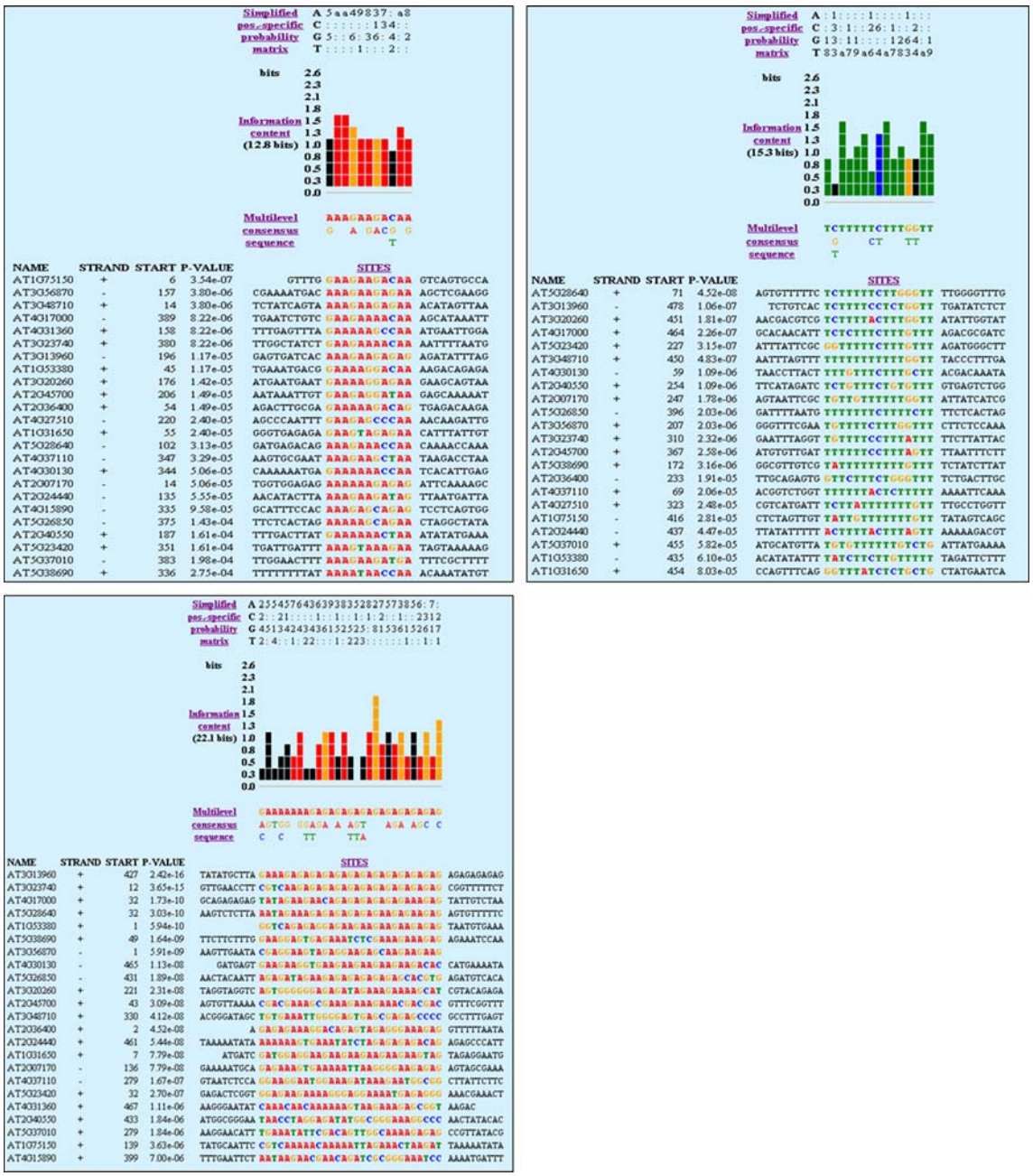

Fig. 7 Conserved motifs in the 500-bp 5′-regions of selected Arabidopsis transcripts. The analysis of possible conserved cis-acting regulatory element was carried out using MEME motif discovery tool on selected Arabidopsis transcripts that are putative orthologs to soybean sequences up-regulated in the SAM and with high transcript expression in the shoot apex as indicated by Genevestigator data

reliable source of novel genes for further functional studies to obtain a comprehensive understanding of active regulatory networks governing the SAM and stem cell fate and differentiation, which has not been possible using classical forward-genetics approaches. Our finding that a portion of Arabidopsis orthologs preferentially expressed in the SAM contains a highly conserved occupancy sites for Dof, GT-1 and GAGA-motif-binding transcription factors in the promoters points to the presence of combinatorial interactions between different transcriptional regulatory complexes in meristem function. Overall, the repertoire of genes reported shall provide a foundation to explore meristem-regulating pathways using mutant and RNA-interference analysis, reporter-gene analysis and other functional genomic approaches.

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