RESEARCH PAPER

# Molecular dissection of the pea shoot apical meristem[*]

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## Abstract

**The shoot apical meristem (SAM) is responsible for the development of all the above-ground parts of a plant. Our understanding of the SAM at the molecular level is incomplete. This study investigates the gene expression repertoire of SAMs in the garden pea (*Pisum sativum*). To this end, 10 346 EST sequences representing 7610 unique genes were generated from SAM cDNA libraries. These sequences, together with previously reported pea ESTs, were used to construct a 12K oligonucleotide array to identify genes with differential SAM expression, as compared to axillary meristems, root apical meristems, or non-meristematic tissues. A number of genes were identified, predominantly expressed in specific cell layers or domains of the SAM and thus are likely components of the gene networks involved in stem cell maintenance or the initiation of lateral organs. Further *in situ* hybridization analysis confirmed the spatial localization of some of these genes within the SAM. Our data also indicate the diversification of some gene expression patterns and hence functions in legume crop plants. A number of transcripts highly expressed in all three meristems have also been uncovered and these candidates may provide valuable insight into molecular networks that underpin the maintenance of meristematic functionality.**

**Key words:** Garden pea, meristem, *Pisum sativum*, transcript profiling.

## Introduction

The development of the shoot apical meristem (SAM) has attracted widespread attention during recent years as an excellent model for understanding stem cell maintenance during plant development. Meristems are considered to be a plant's equivalent of stem cell niches which are the cellular microenvironments that provide signals to maintain stem cells in a slowly dividing and undifferentiated state (Bhalla and Singh, 2006; Singh and Bhalla, 2006; Scheres, 2007). Stem cells in the central zone of the SAM provide cells for continuous development of the aerial parts of the plant. Dividing cells, that leave the central zone, enter the flanking peripheral zone and can be recruited for

lateral organ primordia, which involves rapid division and differentiation.

The SAM is located at the tip of the growing shoot. Histological analysis of the SAM shows that it is highly-patterned and well-organized; one of the most obvious properties of the SAM is that it is made up of superimposed cell layers designated as L1, L2, and L3 (Satina *et al.*, 1940). In general, L1-derived cell lineages form the epidermis, L2-derived cells develop into sub-epidermis cells like photo-synthesizing and gametal cells, and L3-derived cells make up the internal tissues (Stewart and Burk, 1970; Szymkowiak and Sussex, 1996). Different plant species may organize

their cell layers differently; for example, the SAM only contains two layers—the single-layered tunica (L1) and the corpus (L2) (Steffensen, 1968)—in maize, but four super-imposed cell layers are present in birch (Rinne and van der Schoot, 1998).

Extensive genetic investigations, especially in the model angiosperm *Arabidopsis thaliana*, have led to the identification of several key meristem regulatory genes. The regulatory interactions between these genes have been elucidated through genetic and molecular analyses. These key regulatory genes include *CLAVATA3 (CLV3)*, *WUSCHEL (WUS)*, and *SHOOTMERISTEMLESS (STM)* (Long *et al.*, 1996; Mayer *et al.*, 1998; Fletcher *et al.*, 1999). *WUS* and *CLV3* form a feedback regulatory pathway that sustains the stem cell pools (Schoof *et al.*, 2000). The functions of *CLV3*, *CLV1,* and *STM* orthologues appear to be well conserved in monocot and dicot systems (Vollbrecht *et al.*, 1991; Sato *et al.*, 1996; Suzaki *et al.*, 2004, 2006; Chu *et al.*, 2006). At the same time, the *STM*-leading pathways maintain cells in the meristem indeterminate, which is analogous to the role of the *CLV–WUS* pathway (Clark *et al.*, 1996; Lenhard *et al.*, 2002). However, the *CLV–WUS* pathway appears to be less conserved than the *STM* pathway. The maize and rice *WUS* orthologues show obvious differences with *Arabidopsis* regarding their expression patterns (Nardmann and Werr, 2006). Another gene, *ROSULATA* in *Antirrhinum*, which is considered to be the counterpart of *Arabidopsis WUS*, also demonstrates different functions due to mutations, resulting in abnormalities in leaf arrangement and branching patterns (Kieffer *et al.*, 2006). These results show that observations for *Arabidopsis* cannot always be extended to other species, including other dicot species. We are particularly interested in exploring the molecular basis of meristem functionality in legumes, which diverged from *Arabidopsis* approximately 92 million years ago (Eckardt, 2001). Moreover, legumes are the second most important family of crop plants—they are widely used as a food and feed source (Graham and Vance, 2003). Legumes are particularly important for sustainable agriculture because of their ability to fix atmospheric nitrogen. In this study, the focus was on the garden pea (*Pisum sativum*), a classic model species for crop and plant development studies. In addition, the availability of various developmental pea mutants (Singer *et al.*, 2002; Foucher *et al.*, 2003) makes this system amenable to genomics studies.

In recent work, isolated SAM was used to generate cDNA libraries enriched in genes expressed in the SAM (Wong *et al.*, 2008). In the present study, sequencing performed using these cDNA libraries allowed us to discover apical meristem-expressed genes. These sequences were used to make a 12K microarray to investigate the transcriptional repertoire of the pea SAM. Furthermore, the spatial expression pattern within the SAM was investigated for selected genes by *in situ* hybridization. Our results show that spatially limited gene activation or the repression of genes underpins meristem development and functionality. Our data also provide a catalogue of target genes that can be used for both reverse-genetics approaches and meristem cell-type specific markers.

## Materials and methods

### Plant materials and RNA isolation

Garden pea (*Pisum sativum*) cultivar Torsdag was grown under greenhouse conditions. 10-day-old pea seedlings at the vegetative stage were used. Four different samples were harvested and these included the shoot apical meristem (SAM), dormant axillary meristem (AM), root apical meristem (RAM), and non-meristem tissue (NM; consisting of tendril, leaf, stem, and mature root tissues). Total RNA was extracted from each sample using the Qiagen RNeasy Mini Kit according to manufacturer's instructions. Three independent tissue collections and RNA extractions were performed for each of the microarray hybridization experiment.

### EST sequencing and the construction of a 12K custom array

Normalized library derived from dissected SAMs as described in Wong *et al.* (2008) was used for the generation of a further 10 000 ESTs. These ESTs were sequenced at the Australian Genome Research Facility (AGRF), Australia using T7 primer and the resulting data were cleaned and assembled using TIGR Gene Indices clustering tools (TGICL, Pertea *et al.*, 2003). These sequences have been deposited in GenBank under the accession numbers FG528667 to FG539012.

In addition to in-house ESTs, approximately 2000 publically available ESTs were also downloaded from NCBI and the IPK (the Leibniz Institute of Plant Genetics and Crop Plant Research) Crop EST database (http://pgrc.ipk-gatersleben.de/cr-est/index.php; ESTs with accession numbers that begin with CL). A number of transcripts ($n=1237$) were also randomly selected to be represented twice on the array for the subsequent construction of a 12K Combimatrix pea array. The array was made based on CombiMatrix technology (hyperlink) using *in situ* synthesis as outlined at www.combimatrix.com.

### Microarray data acquisition and analysis

RNA samples were hybridized to two-colour Combimatrix arrays manufactured with a custom library of 11 958 probes. Target preparation and hybridization to Combimatrix 12K arrays were performed at the Australian Genome Research Facility Ltd (AGRF), according to the standard CombiMatrix (www.combimatrix.com). Images were scanned and quantified using GenePix Pro 4.0. The limma software package for R (Smyth, 2005) was used in the statistical analysis of the data generated. The microarray data has been submitted to GEO (www.ncbi.nlm.nih.gov/geo/) under the accession GSE13451.

On examination of the data during quality control assessment, it became evident that the Cy3 channel showed little dynamic range and background correction and normalization could not correct for this. Therefore only the Cy5 channel was used in the subsequent analysis. This resulted in somewhat unbalanced sample sizes with six replicates of SAM and one each of RAM, AM, and NM. Although this reduces the degrees of freedom for error to 5, there are well-established statistical methods that can handle this situation (Smyth, 2004).

The red channel intensities were pre-processed and normalized using standard methods for single-channel microarray data (Bolstad *et al.*, 2003). The intensities were background subtracted using local median background estimates, then quantile normalized and $log_2$ transformed.

Differential expression was assessed using linear modelling and empirical Bayes moderated *t*-statistics (Smyth, 2004). This approach allows variance information to be borrowed across both samples and genes, resulting in reliable statistical inference when the number of replicates is small. The residual variance is estimated from the available replicates, allowing statistical tests to be performed even for the groups with only one sample. The false discovery rate (FDR) was controlled at less than 1% for each comparison using the method of Benjamini and Hochberg (1995).

The normalization procedure improved consistency between the replicate arrays, as evidenced by our ability to detect a large number of statistically significant differentially expressed transcripts at a very stringent FDR. For example, 1350 transcripts were detected as differentially expressed between SAM and non-meristematic tissue at 1% FDR after normalization, whereas only 17 transcripts are detected for this comparison using un-normalized $log_2$ transformed data. This highlights the importance of performing appropriate normalization of microarray data. As a further quality control check, the consistency of estimated $log_2$ fold changes was examined for the 1237 transcripts which had duplicate probes on the arrays. The duplicates showed strong agreement after normalization (Fig. 1).

### RT- and real time-PCR

For reverse transcription (RT)-PCR studies, 1 µg of DNA-free RNA extract was converted into first-strand cDNA using the SuperScript III system for first-strand cDNA synthesis (Invitrogen) and oligo(dT)$_{12-18}$. 2 µl of cDNA samples were amplified in a 25 µl PCR mixture. Quantitative-PCR was performed using SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen) on Mx3000P instrument (Stratagene). ROX at a final concentration of 50 nM was used as a reference dye. All reactions were run in duplicate and a melting curve analysis was performed to check for specific product amplification. Relative expression was calculated using MxPro QPCR software v. 1.00 (Stratagene) according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).
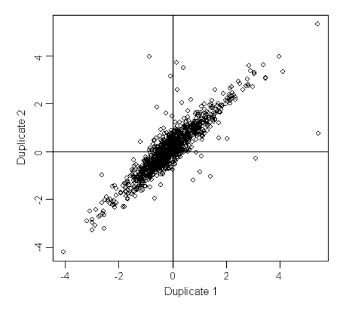


**Fig. 1.** Consistency of $log_2$ fold changes for duplicate spots. The plot compares $log_2$ fold changes for the SAM versus a non-meristematic tissue comparison for the 1237 probes with duplicate spots. The first duplicate of each probe is on the *x*-axis and the second duplicate of the same probe is on the *y*-axis. Duplicates show a very high level of concordance.

### *In situ* hybridization

Non-radioactive *in situ* hybridization was performed as described previously (Haerizadeh *et al.*, 2009; Wong *et al.*, 2009).

## Results and discussion

### Features of generated ESTs

Sequences obtained from 10 346 ESTs were assembled and clustered, resulting in 5754 singlets and 1856 contigs. Close to 97% of these 7610 unique sequences (hereafter referred to as unigenes) could be designated as genes with known or putative functions, or as genes encoding hypothetical proteins based on sequence similarity (see the Materials and methods). Using BLAST2GO, 5124 of the unigenes were classified in terms of their GO biological process (data not shown). The successfully classified unigenes cover a broad range of GO functional categories, ranging from genes involved in cell surface receptor-linked signal transduction to cell differentiation for genes associated with transcription and protein metabolic processes (see Supplementary Table 1 at *JXB* online). Closer inspection of the unigenes grouped in the category 'Cell differentiation' revealed a number of sequences with orthologues known to be essential for regulating developmental activities in the SAM. These orthologues include sequences predicted to encode WUS (FG529966), Homeobox protein KNOTTED-1-like 2 (FG538349), YABBY2-like protein (FG532702), FASCIATA2 (FG529452), ARGONAUTE (FG529161), and Homeobox protein SBH1 (FG536622). The occurrence

of these sequences demonstrates the utility of our EST collection and supports the potential of our experimental approach in providing insight into the processes underlying SAM function and maintenance.

### Functional domains of pea SAM

Base on sequence similarity (at $E \leqslant 10^{-10}$), transcripts have been identified that are putative orthologues to *Arabidopsis* genes known to be essential in the functioning and maintenance of the SAM among our EST collection. The spatial expression of these genes was explored in pea to investigate whether their expression was conserved with

their putative *Arabidopsis* orthologues and in doing so distinguishing the different functional domains of SAM in the garden pea.

Maize *KNOTTED1* (*KN1*) and its *Arabidopsis* counter-part (*STM*) are mainly expressed in the inner layers (L2 and L3) of the SAM (Smith *et al.*, 1992; Jackson *et al.*, 1994; Long *et al.*, 1996; Long and Barton, 2000). A similar gene has been uncovered in pea (Hofer *et al.*, 2001) and our *in situ* hybridization analysis revealed the expression of *PsKN1* in the pea's inner layers (Fig. 2B) consistent with the expression pattern previously reported by Hofer *et al.* (2001); this is probably an indicator of undifferentiated cells in the SAM region, as reported for *KN1* or *STM* in maize
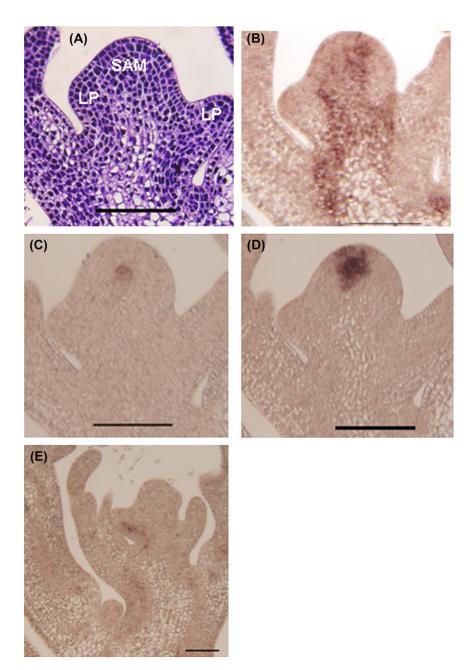


**Fig. 2.** Expression of key meristem genes in the pea SAM. (A) A longitudinal section of a paraffin-embedded SAM from a 10-d-old seedling stained with toluidine blue. (B) *PsKN1* is expressed throughout the meristem. (C) *PsWUS* expression is found in the stem cell niche. (D) *PsCLV3* is expressed in the stem cell region. (E) *PsCLV1* is expressed mainly in the vascular system. Scale bar: 100 μm.

or *Arabidopsis*, respectively. Similar to *Arabidopsis* and maize, the expression of *PsKN1* is down-regulated at leaf primordium initiation sites and this has also been shown by Hofer *et al.* (2001) (Fig. 2B). These results imply the possible conserved functions of *PsKN1* with that of the *Arabidopsis STM*.

In *Arabidopsis*, the domain of *WUS* expression spans the lower part of the central zone (CZ) and the upper part of the rib zone (RZ), defining a functional domain in the CZ called the organizing centre; this centre confers stem cell fates to neighbouring cells (Mayer *et al.*, 1998). Among our EST collection, a likely pea orthologue of *WUS* was identified (*PsWUS*; FG529966; see Supplementary Fig. 1A at *JXB* online). Although *PsWUS* only shows 48% sequence similarity to petunia *WUS* at the amino acid level and 33% to *Arabidopsis* (data not shown), a saturated RT-PCR confirmed the SAM-specific expression of *PsWUS* (Fig. 3). Further *in situ* analysis of this gene revealed the expression of *PsWUS* in a small round area of about two or three cells in diameter in the SAM, just beneath the fourth layer (tunica layer) and in part of the L5 layer (corpus layer; Fig. 2C), which is probably the organizing centre as WUS has been identified to be a marker for the organizing centre (Mayer *et al.*, 1998). This expression pattern strongly suggests that, like the *Arabidopsis WUS*, the *PsWUS* also plays key role in stem cell maintenance in pea.

*WUS* expression in the organizing centre has been shown to activate CLV genes that encode important signalling molecules. While *CLV1* encodes a leucine-rich repeat (LRR) receptor-like protein kinase (Clark *et al.*, 1997), *CLV3* encodes a secreted protein that can be used to indicate stem cell identity (Fletcher *et al.*, 1999). Since no CLV3 orthologue could be found in our EST collection, the *CLV3* gene was cloned using a degenerate PCR method based on the alignment of the reported *CLV3* genes of *Medicago*, *Arabidopsis*, rice, and castor beans. In addition, saturated RT-PCR of the cloned *PsCLV3* showed that it is expressed exclusively in the SAM (Fig. 3). Spatial localization analysis indicated its activity in the four outer layers of the central zone (Fig. 2D), an expression pattern similar to that of *Arabidopsis CLV3* (Fletcher *et al.*, 1999).
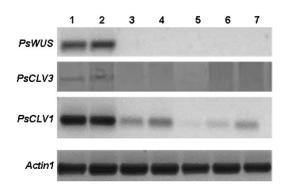


**Fig. 3.** RT-PCR analysis of key stem cell maintenance genes in pea. Lanes 1, 2, 3, 4, 5, 6, and 7 contain samples from SAM, AM, RAM, tendril, leaf, stem, and mature root, respectively.

Although the WUS–CLV3 pathway appears to be conserved in pea, based on their similar spatial expression patterns to that of *Arabidopsis*, *PsCLV1* (FG529170) shows a different expression pattern in pea compared with *Arabidopsis CLV1*. In contrast to the reported expression of *Arabidopsis CLV1* in the SAM (Clark *et al.*, 1997), *PsCLV1* is predominantly expressed in the vascular system (Fig. 2E). This result suggests that the receptor kinase activity of *PsCLV1* has been replaced by other genes or that *PsCLV1* has been recruited to function within different pathways. The latter theory is not unlikely in view of the fact that soybean *GmNark* shares high sequence homology with *CLV1* and is involved in the process of symbiosis (Searle *et al.*, 2003). In addition, the soybean CLV1-like receptor kinase acts predominantly in the vascular tissue (Beveridge *et al.*, 2007). Since CLV3 is expressed in the stem cell region of the pea meristem, the nature of its receptor remains to be resolved.

## Detection of differentially expressed genes using the 12K pea oligonucleotide array

The 7610 unigenes from this study were combined with approximately 3000 publically available pea EST sequences to construct a 12K oligonucleotide array (see Materials and methods) that is representative of the pea genome and is enriched for genes exhibiting SAM-associated expression. This microarray was used to perform an extensive analysis of the transcript profile of the pea SAM in comparison to other types of meristems, namely the axillary meristem (AM) and the root apical meristem (RAM), as well as to non-meristem (NM) tissues.

The microarray results showed that the most significant differential expression occurs between non-meristematic tissue and the shoot apical meristem (see Supplementary Table 2 at *JXB* online), with 1350 transcripts or 10% of the total number of transcripts (11 335) showing differential expression at 1% FDR. As expected, the majority of the SAM up-regulated genes are represented in our collection of SAM-enriched clones, highlighting the significance of constructing an SAM library.

When hierarchical clustering was performed for the 1000 genes exhibiting the most variable expression across the four groups, the resulting heat map illustrated the similarity of the AM's transcript profile to that of SAM (Fig. 4A). Ternary plots of relative expression also showed strong consistency between AM and SAM. Transcripts generally show the same pattern of expression in SAM as in AM relative to the other two tissues (Fig. 4B). Meanwhile, the non-meristematic tissue revealed the most dissimilar profile in comparison to all three meristems (Fig. 4) which is also reflected by the small number of differentially expressed genes in the AM and SAM comparison (Table 1). To verify the results of our microarray analysis, RT-PCR analysis was performed for 25 genes that were selected on the basis of their diverse range of differential expression levels in the SAM and NM comparison. In general, the outcomes are in agreement with the microarray data (Fig. 5).
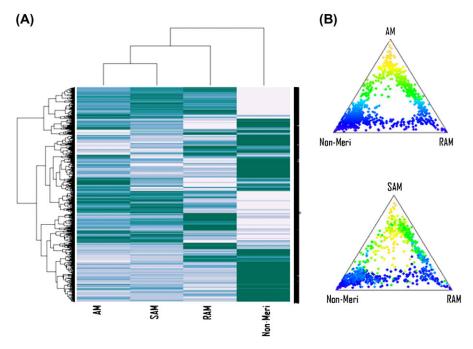
**(A)**

**(B)**



**Fig. 4.** Expression profiles of the 1000 transcripts with the most variable expression across the four tissues used. (A) Hierarchical clustering and heatmap and (B) ternary plots of relative expression. The ternary plots position each transcript according to its relative expression in three different tissues. The distance of each point to each corner is inversely proportional to its expression level in that tissue. Points near each corner of the ternary plot correspond to transcripts expressed exclusively in that tissue whereas transcripts near the centre of the plot are expressed equally in all three tissues. Transcripts are colour-coded according to the first ternary plot, i.e. according to relative expression in AM versus RAM and non-meristem, and the same colour coding is used in the second plot. Both heatmap and ternary plots show strong consistency between AM and SAM.

**Table 1.** Number of transcripts with significant differential expression in the comparison of tissues as indicated (FDR ≤0.01)

| Direction of regulation | SAM versus NM | SAM versus AM | RAM versus NM |
|---|---|---|---|
| Up | 538 | 2 | 218 |
| Down | 812 | 25 | 404 |
| Total | 1350 | 27 | 622 |

A survey of the top 300 genes up-regulated in the pea SAM in comparison to non-meristematic tissues showed that about one-third of the genes can be classified as encoding gene products with DNA or RNA-binding activities. On the other hand, genes in this class represent only 2% of the top 300 genes that are down-regulated in the SAM in comparison to non-meristematic tissues. Based on cellular component classifications, approximately 40% of the genes up-regulated in the SAM resided in the 'nucleus' category (Fig. 6). Among those genes down-regulated in the SAM, 43% belonged to the 'plastid and chloroplast' class consistent with the fact that SAMs are heterotrophic.

*Comparison of differentially expressed AM and SAM transcripts*

AM is the meristem responsible for the secondary axes of plant growth. Many pea orthologues of key meristem identity genes such as *PsWUS*, *PsCLV3*, and *PsKN1* show expression in the SAM as well as in the AM (Fig. 2). This is not surprising as studies have indicated the formation process of AM resembles that of the SAM (reviewed by Bennett and Leyser, 2006). Despite the similarity in transcript profiles of the AM and SAM, a total of 27 transcripts have been successfully identified that potentially distinguish the AM gene expression profile from that of SAM (at FDR ≤0.01; Table 2). Among them is a transcript known as *DRM4* and reported to be associated with AM dormancy (Stafstrom *et al.*, 1998). Further investigation of transcripts in Table 2 should reveal novel dormancy associated genes.

Intriguingly, one transcript that showed lower expression in the AM is one that is predicted to encode the WD-40 repeat MSI4-like protein (BV165610). Members of the MSI-like protein family are known to interact directly or indirectly with histones and hence may target chromatin assembly factors and histone deacetylases and are found to control cell proliferation and differentiation in animals (reviewed by Hennig *et al.*, 2005). The observation that a similar transcript is expressed at a lower level in AMs in comparison to SAMs suggests that it may play roles in regulating activities that are specific to the SAM. Recently, strigolactone has been identified as a hormonal signal that represses axillary bud outgrowth but which does not appear to affect apical growth in pea (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). It will be interesting to determine how it affects the expression of these genes during the

**Fig. 5.** RT-PCR analysis of differentially expressed genes. RT-PCR analysis was carried out for 25 selected transcripts as indicated. The *ACTIN* gene was used as an internal control. FG531364, FG532117, EX570080, FG536909, and FG538772 were identified as those that were significantly down-regulated in the SAM in comparison to the NM. Negative control consisted of samples subjected to PCR analysis with *ACTIN* primers to examine the absence of any genomic DNA. Lanes 1, 2, 3, and 4 contain samples from SAM, AM, RAM, and non-meristematic tissues (a mixture of tendril, leaf, stem, and mature root tissues), respectively.

transition of the AM to a SAM that occurs during axillary bud outgrowth.

*Shared transcriptional signatures of meristematic activity*

Hierarchical clustering of the top 1000 significantly differentially expressed genes revealed a subset of sequences whose up-regulation relative to non-meristematic tissues is shared among all meristem samples, i.e. SAM, AM, and RAM (Fig. 4; see Supplementary Table 3 at *JXB* online). These genes provide a valuable insight into molecular networks that underpin the maintenance of meristematic functionality. As expected, a significant number of these encode the core histones H1, H2, H3, and H4 (see Supplementary

Table 3 at *JXB* online). Other genes whose preferential expression is shared among the three meristems examined include those that encode the WD-40 repeat protein MSI3, nucleolar protein Nop56 (Nucleolar protein 5A), non-specific lipid transfer protein, palmitoyltransferase ZDHHC3, and LIM domain only protein 7. The WD-40 repeat protein MSI3 is a retinoblastoma-binding protein that plays an important role in controlling cell cycle progression (Dennis, 2007). Nucleolar protein Nop56 activity highlights the increasing relevance of the nucleolus in regulating mitosis and cell cycle progression (Boisvert *et al.*, 2007). Shared up-regulation of palmitoyltransferase ZDHHC3 in meristematic tissues is of particular relevance as this enzyme mediates palmitoylation, a post-translational modification that can have multiple effects on protein function and localization. For example, palmitoylation can influence membrane binding and membrane targeting, and palmitoylation of many proteins is required for efficient signal transduction (Resh, 2006). Our findings indicate that the role of post-translational protein palmitoylation in cell signalling is required to maintain meristematic competence in plants.

A survey of the top 1000 significantly differentially expressed genes also showed that gene repression is a more widespread feature of meristematic activity than up-regulation (see Supplementary Table 4 at *JXB* online). The number of genes sharing repression in the SAM, AM, and RAM is nearly five times higher than those undergoing up-regulation. Understandably, a majority of the genes repressed in the meristem are involved in glycolytic and photosynthetic metabolism processes. Significant development-related genes that are repressed in all three meristems include the homeobox protein SBH1, late embryogenesis abundant protein 76 (LEA 76), *Pisum sativum* PsAD2 mRNA, *Pisum sativum* GA 20-oxidase mRNA, and zinc finger protein CONSTANS-LIKE 16. SBH1 belongs to the Knotted-1 family of homeodomain proteins that are strongly expressed in differentiated cell types (Tioni *et al.*, 2003). GA-20 oxidases regulate the final rate-limiting steps in GA biosynthesis (Desgagne-Penix and Sponsel, 2008). CONSTANS-LIKE 16 CO belongs to a family of 17 putative transcription factors defined by two conserved domains (Robson *et al.*, 2001). The first is a zinc finger region near the amino terminus that resembles B-boxes, which regulate protein–protein interactions in several animal transcription factors. Our data thus highlight candidate genes whose spatially limited gene activation or repression probably underpins meristematic competence in plants.

*Apical meristem-specific genes*

As previously mentioned, saturated RT-PCR analysis confirmed the exclusive expression of key SAM regulatory genes such as *PsWUS* and *PsCLV3* in SAMs or AMs (Fig. 2). In order to identify transcripts that demonstrate similar expression profiles, candidates were selected that were up-regulated when the transcript profile of SAM was compared with that of RAM or NM for further saturated
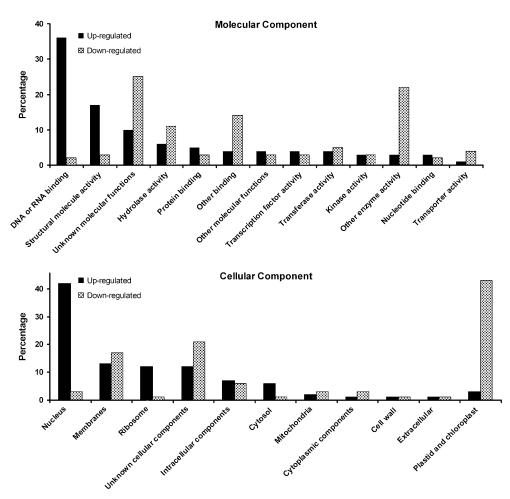
**Fig. 6.** Gene ontology (GO) functional categorization of transcripts differentially regulated in SAM in comparison to non-meristematic tissues. The matching *Arabidopsis thaliana* gene index (AtGI) for the top 300 transcripts was used in the automated GO categorization available at www.arabidopsis.org.

RT-PCR analysis. Among 10 selected candidates, five SAM-specific genes were found (Fig. 7). These genes were expressed only in the SAM or AM and not in any other tissues: tendril, leaf, stem, and root. To validate these observations, further real-time PCR analysis was also carried out for a subset of these candidates (Fig. 8) and the results are in line with that of the RT-PCR analysis.

The SAM-specific genes identified include transcripts predicted to encode PROTODERMAL FACTOR1 (PDF1; CL850CT1007CN1219), a WUS-related product, STIP (FG537183), YABBY2 (FG532702), an unknown protein (FG529949), and AINTEGUMENTA (FG530203) (Fig. 7). As the expression domains of these within the SAM are essential for understanding their functional significance, their spatial expression patterns were investigated.

*PDF1* is a known marker gene for the L1 layer in *Arabidopsis* (Abe *et al.*, 1999). The pea orthologue *PsPDF1* (CL850CT1007CN1219) shows high sequence similarity to *Arabidopsis* PDF1 (Wong *et al.*, 2008) with 60% identity at the amino acid level (data not shown). *In situ* hybridization revealed that the *PsPDF1* is also exclusively expressed in the L1 layer (Fig. 9A) indicating the conserved function of this gene in pea as in *Arabidopsis*. Meanwhile, the expression of

*STIP* (FG537183) was detected in the SAM and leaf primordia (Fig. 9C) and more specifically it is only expressed in the boundary region corresponding to the leaf axil (Fig. 9C), which initiates the axillary meristem. In the SAM region, the expression of *STIP* is restricted to a small domain that probably separates the leaf anlage and the stem cells (Fig. 9C). After a new axillary meristem is initiated, its expression is again restricted to the leaf axil (Fig. 9C). This expression pattern indicates that this gene is recruited to mark the boundary region separating organ domains. Key genes for axillary meristem initiation reported to date share a common expression feature: the boundary expression domain in the SAM (Schmitz *et al.*, 2002; Keller *et al.*, 2006; Muller *et al.*, 2006). Intriguingly, another transcript annotated to encode an unknown protein (EX570896) with enriched expression in the SAM shows similar spatial expression profile to that of *STIP* (Fig. 9E) and thus they may be involved in regulating AM initiation as well as in SAM maintenance. This is not unlikely as there are reports suggesting that boundary genes are required for SAM and AM development (Hibara *et al.*, 2006; Borghi *et al.*, 2007).

When the expression of the transcripts annotated as AINTEGUMENTA-LIKE (FG530203) was examined

**Table 2.** Transcripts that are differentially regulated in the SAM in comparison to the AM (FDR ≤0.01)

| Accession no. | Annotation | Fold change (log$_2$) |
|---|---|---|
| FG531608 | Cytochrome P450-like protein | 3.0 |
| BV165610 | WD-40 repeat protein MSI4 | 2.1 |
| FG536909 | AP2 domain-containing protein | −2.1 |
| EX569019 | No BLASTX match | −2.2 |
| EX569165 | No BLASTX match | −2.2 |
| FG531433 | F-box/LRR-repeat protein 20–like | −2.3 |
| FG528742 | No BLASTX match | −2.5 |
| FG536535 | No BLASTX match | −2.5 |
| FG534876 | No BLASTX match | −2.5 |
| EX569623 | F-box protein family-like protein | −2.5 |
| FG538233 | Albumin-2 (PA2) | −2.5 |
| FG530490 | Hypothetical protein | −2.6 |
| FG537964 | No BLASTX match | −2.6 |
| FG531181 | Ethylene–responsive transcription factor 5 | −2.6 |
| X65154.1 | GA regulated mRNA | −2.7 |
| FG531688 | Histidine kinase-like protein | −2.7 |
| EX569457 | No BLASTX match | −2.8 |
| AF515796.1 | Drm4 mRNA | −2.8 |
| FG535658 | Acetylglucosaminyltransferase SPINDLY | −3.0 |
| FG534193 | 18 kDa seed maturation protein | −3.0 |
| FG530878 | No BLASTX match | −3.0 |
| FG535973 | Peripheral-type benzodiazepine receptor | −3.1 |
| FG530812 | Cold-acclimation specific protein 15 | −3.4 |
| FG529829 | Hypothetical protein | −3.4 |
| FG538772 | Sex determination protein tasselseed-2 | −3.6 |
| FG529247 | Hypothetical protein | −4.0 |
| FG538246 | Hypothetical Cys-3-His zinc finger protein | −4.0 |
| FG530800 | Isoflavone reductase | −4.4 |



**Fig. 8.** Real-time PCR analyses of transcripts with specific (A, B) or enriched (C, D) gene expression in apical meristems.
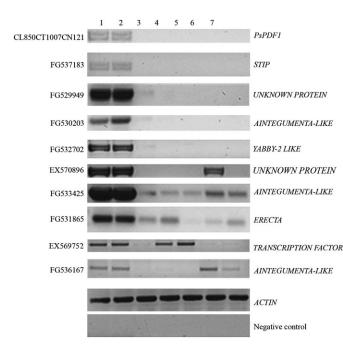


**Fig. 7.** Saturated PCR showing the gene expression of differentially expressed ESTs. Lanes 1, 2, 3, 4, 5, 6, and 7 contain samples from SAM, AM, RAM, tendril, leaf, stem, and mature root, respectively.
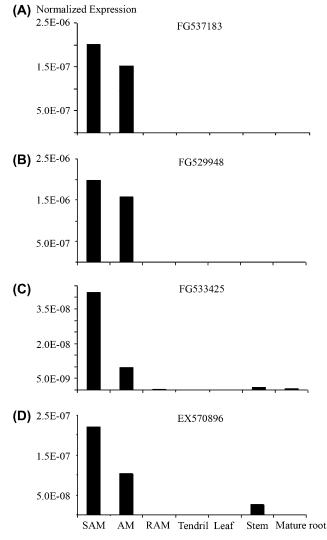
*in situ*, signals were detected related to its expression in the presumptive organ as well as localized to the central and marginal regions of the primordium and developing leaf (Fig. 9G). This is consistent with what has been reported for a similar *Arabidopsis* gene (Elliott *et al.*, 1996), suggesting a likely conserved function of this gene in pea.

### Transcripts with high expression in the SAM

In addition to investigating the spatial expression of SAM-specific genes, the *in situ* expression of five other candidates that have enriched expression in the SAM, in comparison to other tissues, has also been examined.

*Genes involved in the initiation of lateral organs*: Genes involved in leaf primordium initiation are represented in our array data. *YABBY* genes in *Arabidopsis* and maize have been shown to play key roles in leaf primordium initiation and expansion (Bowman, 2000; Eshed *et al.*, 2004; Juarez *et al.*, 2004). FG532839, a putative orthologue of the *Arabidopsis YABBY5* gene based on a BLASTX match, is
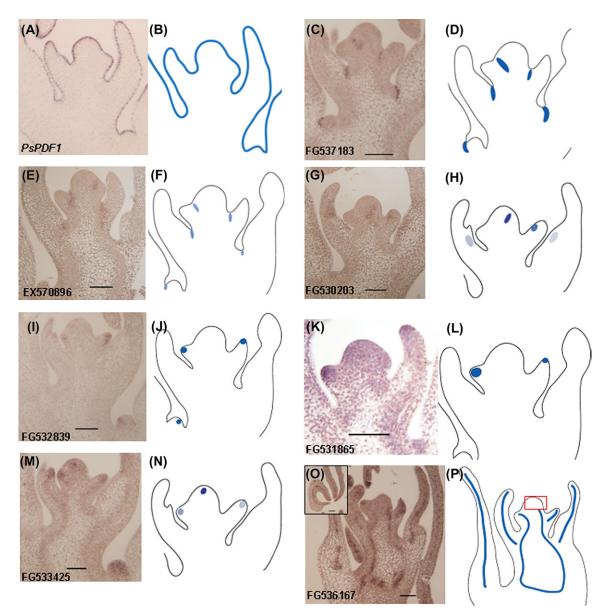
**Fig. 9.** *In situ* localization of transcripts with specific or enriched expression in the apical meristems. Gene expression patterns in the pea shoot apex [(A), (C), (E), (G), (I), (K), (M) and (O)] with the corresponding modelled expression as shown in (B), (D), (F), (H), (J), (L), (N) and (P). (A, B): Pea *PsPDF1*(CL850CT1007CN1219) expression was detected in the outermost layer of the meristem. (C, D) and (E, F): Expressions of *STIP* (FG537183) and an unknown gene (EX570896) were found in the boundary region corresponding to the axillary meristem initiation area. (G, H): ANT-like gene (FG530203) was expressed in a region likely to be the presumptive organ primordium as well as in the central and marginal region of the primordium and developing leaf. (I, J) and (K, L): *YABBY* (FG532839; I, J) and *ERECTA* (FG531865; K, L) were mainly expressed in the organ primordia. (M, N): The expression of FG533425, a putative member of the *ANT*-like genes, is limited to the central zone, an area whereby the expression of *PsWUS* was detected as well as at the tip of leaf primordial (Fig. 2C). (O, P): Another putative *ANT*-like gene (FG536167) is expressed in the provascular region in the stem and leaf primordia suggesting its likely roles in vascular differentiation. All are longitudinal sections of paraffin-embedded shoot apices probed with the antisense strand of digoxigenin-labelled RNA.

highly expressed in the SAM (Fig. 7). *In situ* localization showed that this gene accumulates mainly in the leaf primordium (Fig. 9I) indicating the conserved role of pea *YABBY* genes in lateral organ formation. The discrepancy between the RT-PCR results and the *in situ* data is due to the fact that, while it was attempted to exclude as much leaf primorium from the dissected SAM as possible, the youngest developing leaf primordium often could not be eliminated as it was difficult to identify on the SAM under the dissecting microscope.

Genes important for cell-to-cell signalling in lateral organ development were also identified in this experiment. The *ERECTA* gene was characterized as a receptor-like kinase signalling receptor, a component of cell-to-cell signalling (Torii *et al.*, 1996). A similar pea sequence predicted to encode ERECTA-like kinase (FG531865) is highly expressed

in the lateral primordium (Fig. 9K) and is consistent with the expression pattern described for *Arabidopsis ERECTA* (Shpak *et al.*, 2004; Yokoyama *et al.*, 1998).

*AINTEGUMENTA (ANT)-LIKE* genes: A total of three *ANT-LIKE* genes (see Supplementary Fig. 1B at *JXB* online)—FG533425, FG530203, and FG536167—were highly expressed in the SAM in comparison to the NM (see Supplementary Table 2 at *JXB* online). In addition to the SAM-specific sequence (FG530203), two other sequences are meristem-enriched in their expression (FG533425 and FG536167) (Fig. 7). FG533425 is expressed not only in the leaf primordium, but also in the centre of the SAM (Fig. 9M). The latter expression pattern has not been reported for the *Arabidopsis ANT* that is exclusively expressed in the organ primordia and is used to mark the shoot organ primordia (Long and Barton, 1998, 2000). Another *ANT*-like gene, FG536167, shows a completely different expression pattern compared to the known *ANT* genes. It is expressed in the provascular region throughout the meristem (Fig. 9O) and the expression is confined to the middle vascular system in the developing leaflet or tendril (inset picture in Fig. 9O). This unique expression pattern indicates that ANT-like genes could be involved in the initiation and formation of the provascular system.

Despite the rapid growth in the understanding and identification of key genes controlling meristem development, a comprehensive view of the temporal and spatial gene expression pattern involved in SAM functionality remains elusive. The development of nearly 12 000 ESTs, representing genes transcribed in the pea SAM, and the identification of gene subsets that are up-regulated or specifically-expressed in SAM cell layers/zones represents a significant advance towards identifying the regulatory networks involved in specifying cell types and providing positional information within the meristem. Our data indicate the usefulness of the transcriptome approach to identifying meristem regulatory genes in legumes and offers the possibility of transferring information from pea to other agronomically important legume species. Moreover, analysis of genes in the legume class contributes to the evolutionary context of plant development and should highlight conserved and divergent mechanisms.

## Supplementary data

Supplementary data is available at *JXB* online.

**Supplementary Fig. 1.** Phylogenetic analysis of (A) *PsWUS* and (B) *PsANT-LIKE* genes.

**Supplementary Table 1.** Functional classification of unigenes according to GO biological processes using BLAST2GO (Conesa *et al.*, 2005).

**Supplementary Table 2.** Differentially expressed transcripts in the pea SAM in comparison to NM (at adjusted *P*-value ≤0.05).

**Supplementary Table 3.** Up-regulated transcripts in meristems (SAM, AM, and RAM) in comparison to the NM (at adjusted *P*-value ≤0.01).

**Supplementary Table 4.** Down-regulated transcripts in meristems (SAM, AM and RAM) in comparison to the NM (at adjusted *P*-value ≤0.01).

## Acknowledgements

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