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Changes in gene expression during male meiosis in *Petunia hybrida*

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Abstract

We analyzed changes in gene expression during male meiosis in *Petunia* by combining the meiotic staging of pollen mother cells from a single anther with cDNA-AFLP transcript profiling of mRNA from the synchronously developing sister anthers. The transcript profiling experiments focused on the identification of genes with a modulated expression profile during meiosis, while premeiotic archesporial cells and postmeiotic microspores served as a reference. About 8000 transcript tags, estimated at 30% of the total transcriptome, were generated, of which around 6% exhibited a modulated gene expression pattern at meiosis. Cluster analysis revealed a transcriptional cascade that coincides with the initiation and progression through all stages of the two meiotic divisions. Fragments that exhibited high expression specifically during meiosis I were characterized further by sequencing; 90 out of the 293 sequenced fragments showed homology with known genes, belonging to a wide range of gene classes, including previously characterized meiotic genes. *In-situ* hybridization experiments were performed to determine the spatial expression pattern for five selected transcript tags. Its concurrence with cDNA-AFLP transcript profiles indicates that this is an excellent approach to study genes involved in specialized processes such as meiosis. Our data set provides the potential to unravel unique meiotic genes that are as yet elusive to reverse genetics approaches.

Introduction

Meiosis is a special type of cell division that produces haploid gametes from diploid parental cells and creates natural variation through crossovers and chromosome recombination. The chromosome number is halved during meiosis because a single round of DNA replication is followed by two consecutive rounds of chromosome segregation. Yeast, *Drosophila melanogaster*, mouse, *Arabidopsis thaliana* and *Caenorhabditis elegans* are well-known attractive models for meiotic studies. Before the 1960s meiosis was mostly studied at the microscopic level using a range of plant and insect

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species (Pearson *et al.* 1997). Despite a long history of research in flowering plants, the molecular and functional analysis of meiotic genes was relatively underdeveloped until recently.

Nowadays investigations into plant meiosis begin to benefit from the immense amount of data generated by large-scale gene expression studies on meiosis in yeast. A genome-wide transcriptome analysis during meiosis in *Sarcharomyces cerevisiae* using DNA microarrays led to the identification of 1600 genes exhibiting a modulated expression at different stages of meiosis (Primig *et al.* 2000). These genes could be grouped into seven clusters, each with a unique expression pattern. Based on these expression data, Rabitsch *et al.* (2001) carried out a targeted mutagenesis program for a further characterization of genes required for meiosis. Comparison with a similar analysis in *S. pombe* revealed a strikingly small overlap of the meiotic transcriptomes (Mata *et al.* 2002). Microarrays have also been used to study gene expression profiles during mouse spermatogenesis, which may lead to the identification of genes with potential relevance to mammalian meiosis (Schultz *et al.* 2003, Yu *et al.* 2003). In a comparative study, Hwang *et al.* (2001) performed a bioinformatics-based search for homologs of meiosis-induced yeast genes in *C. elegans*, *D. melanogaster* and mammals, thus demonstrating that yeast sporulation and meiosis in higher eukaryotes were evolutionary highly conserved. However, intriguing differences do exist, reflecting that distinctive mechanisms govern progression of meiosis in each organism (Cnudde & Gerats 2005).

In plants, a number of genes playing a role in meiosis have been identified based on a gene-by-gene approach, using both reverse and forward genetics. Several mutants, such as *Atrad51* and *Atdmc1* (Doutriaux *et al.* 1998), *Atrad50* (Gallego *et al.* 2001) and *Atmre11* (Bundock & Hooykaas 2002) resulted from a reverse genetics strategy, based on sequence information of known meiotic genes in yeast. In the forward genetics approach, chemical and insertion mutants with sterility phenotypes have been screened followed by a detailed secondary screen to detect meiotic defects. Genes such as the *AtSDS* (Azumi *et al.* 2002), *ZmSGO1* (Hamant *et al.* 2005) and *ZmPHS1* (Pawlowski & Cande 2005) in maize have been identified in this manner. Identification of specific types of meiotic genes remains a challenge due to limited sequence conservation between species, existence of closely related gene families and in some cases functional redundancy between gene family members (Sanchez-Moran *et al.* 2005). For instance, structural proteins involved in the formation of the synaptonemal complex (SC) have been elusive to detection based purely on primary sequence homology. Although functionally analogous, yeast and mammalian SC proteins exhibit no homology. The central element protein AtZIP1 was identified using a combination of primary sequence information and secondary structure prediction (Higgins *et al.* 2005).

Recently, alternative approaches have been developed to identify novel meiotic genes. Peptide mass-finger printing and matrix-assisted laser desorption ionization time of flight mass spectrometry have been successfully employed to analyze proteins expressed in meiocytes during prophase I of meiosis (Sanchez-Moran *et al.* 2005). A cDNA microarray comparing gene expression in rice seedlings, meiotic and mature anthers, as well as suspension cells treated with gibberellic acid (GA3) or jasmonic acid (JA) identified 2155 genes as preferentially expressed in anthers and 47 genes as differentially expressed in meiotic and mature anthers (Wang *et al.* 2005). Microarray analysis of gene expression is exhaustive but depends on the availability of sequence information. Gel-based transcript profiling systems such as mRNA differential display (DDRT-PCR) and cDNA-AFLP are powerful techniques because they allow identification of differentially expressed transcripts without any *a-priori* knowledge of their sequence or function. Once identified, the transcripts of interest can be sequenced for further analysis. The reproducibility of cDNA-AFLP patterns allows great confidence in the acquired data and differences in the intensities of amplified products can be informative and may allow quantitative expression comparision at different meiotic time points (Breyne *et al.* 2003).

In this study, we employ cDNA-AFLP transcript profiling to identify genes in *Petunia hybrida* that are modulated in their expression during male meiosis. The advantage of *Petunia* is that the size of the chromosomes are large enough for a straightforward microscopic staging of meiotic samples. In addition, each anther contain approximately 10 000 relatively synchronously dividing meiocytes, which is much more than seen in *Arabidopsis* anthers with an average of only 30 meiocytes in a locule (Armstrong *et al.* 2001). Sufficient mRNA can be obtained from three anthers derived from a single flower bud, allowing a reproducible gene expression analysis by cDNA-AFLP.

Among the 8000 amplicons obtained we selected 475 candidate cDNA fragments displaying a modulated and/or meiosis-specific gene expression pattern. Frag-

ments that exhibited a modulated expression were further analyzed by direct sequencing. Homologies with diverse classes of known meiosis genes were retrieved, confirming that our screening was effective. We identified five large clusters of co-expressed genes by hierarchical average linkage clustering and adaptive quality-based cluster analysis. Furthermore, we performed *in-situ* hybridizations with five selected fragments as a probe to check the temporal gene expression patterns generated by cDNA-AFLP transcript profiling and to obtain additional spatial expression information.

Materials and methods

Plant material

Petunia hybrida var. Mitchell (W115) (Mitchell *et al.* 1980) plants were grown under greenhouse conditions of 21°C on average and 16 h photoperiod. Immature flower buds of 2–5 mm were dissected under a binocular using a small pair of forceps. The smallest anther of each flower bud was out of phase with the rest and therefore discarded. Of the remaining four anthers, one was fixed in a freshly prepared ethanol–acetic acid (3:1) mixture at room temperature for 12–18 h for microscopic analysis. The remaining three anthers of a single flower bud were frozen in liquid nitrogen and then stored at −80°C until subsequent RNA isolation.

Staining of enzyme-digested spread pollen mother cells

The ethanol–acetic acid-fixed anthers were washed in water, incubated in a mix of pectolytic enzymes for cell wall digestion, spread on a slide and stained with 4′,6-diamidino-2-phenylindole (DAPI) for staining the chromatin as described by Ross *et al.* (1996). The preparations were studied under a fluorescence microscope and meiotic cells were captured with a CoolSNAP CCD camera (Roper Scientific, USA). We used Photoshop for adapting resolution sharpening and cropping of the images.

Total RNA isolation and cDNA synthesis

For a total RNA sample of any meiotic stage we always used the three anthers of one flower bud; whereas for samples of premeiotic stages we used the anthers of two flower buds at the same stage. Using complete anthers implies that, in addition to the meiocytes in the pollen sac, the samples contain other anther tissues such as the epidermis, the endothecium, the connective tissue and the tapetum. Anthers were homogenized in an MM300 bead laboratory mixer mill (Retsch GmbH and Co, Haan, Germany) for 40 s at 20 Hz. Immediately after homogenization, RNeasy-buffer was added and total RNA was isolated using the RNeasy Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Total RNA was treated with DNase (Promega, Madison, WI, USA) for 30 min at 37°C and purified by phenol–chloroform extraction and ethanol precipitation. For the cDNA synthesis we used 3 μg of total RNA. First and second cDNA strands were synthesized according to standard protocols (Sambrook *et al.* 1989) and finally purified by phenol extraction and ethanol precipitation.

cDNA-AFLP analysis

Double-stranded cDNA was used for the cDNA-AFLP-based transcript profiling procedure according to Breyne *et al.* (2003) with the *Bst*YI and *Mse*I restriction enzymes (New England Biolabs, Beverly, MA, USA). For preamplifications an *Mse*I-primer without selective nucleotides was combined with a *Bst*YI-primer containing a T at the 3′-end. We used 5 μl of a 600 times diluted amplification mixture for final selective amplifications following Breyne *et al.* (2002). The *Bst*YI+T- and *Mse*I-primers with two selective nucleotides each were used for the cDNA-AFLP analysis and all 256 possible primer combinations were performed. The ^{33}P-ATP-labeled fragments were separated on a 5% polyacrylamide gel. The dried gels were exposed to Kodak Biomax films and scanned in a Phospho-Imager (Molecular Dynamics, Sunnyvale, CA, USA) to cut out and quantify the bands of interest, respectively.

Reamplification of AFLP fragments and direct sequencing

Fragments corresponding to differentially expressed transcripts were excised from the dried polyacrylamide gel and suspended in 150 μl H_2O for 3 h at 20°C. A 5 μl aliquot of the eluted DNA suspension was used for the reamplification reaction under the same PCR conditions and using the same primer combination as for selective amplification. Reamplified products were checked on a 1% agarose gel. Sequence information was obtained by direct sequencing of one strand using the selective *Bst*YI-primer as a sequencing primer. As

a control we checked whether the sequences corresponded to the excised fragments in size and selective nucleotides of the *Mse*I-primer. The whole procedure was repeated if the quality of the sequenced fragment was insufficiently clear.

Data analysis

Transcript tags were compared to sequences in GenBank using BLASTX (Altschul *et al.* 1997) and FASTX (Pearson *et al.* 1997) algorithms. Transcripts returning scores under the significance cutoff value of E = 0.001 were compared with the database using the Smith–Waterman algorithm (Smith & Waterman 1981).

Quantitative measurements of the expression profiles and data analysis

Gel images were quantitatively analyzed using the AFLP-QuantarPro image analysis software (Keygene, Wageningen, The Netherlands). All AFLP fragments were scored and individual band intensities were measured for quantification of the expression profile of each transcript. The raw data were corrected for differences in total lane intensities, after which individual gene expression profiles were variance-normalized (Breyne *et al.* 2002). The Cluster and TreeView software (Eisen *et al.* 1998) was used for hierarchical, average linkage clustering, followed by a second, adaptive quality-based clustering (De Smet *et al.* 2002). The minimal number of tags in a cluster and the acquired probability of genes belonging to a cluster were set to 2 and 0.95, respectively.

RNA in-situ *hybridization*

RNA *in-situ* hybridization experiments were performed as described by Cox & Goldberg (1988) with minor modifications. The fresh selected flower buds were fixed overnight at 4°C in 0.25% (w/v) glutaraldehyde, 4% (w/v) formaldehyde in 0.01 M phosphate buffer (pH 6.8), and subsequently dehydrated, cleared and embedded in paraffin. We hybridized the [^{33}P]-UTP-labeled RNA probes at a specific activity of 4–5 $\times$ 10^8 dpm/µg onto the 10 µm sections. Sense and antisense RNA probes were synthesized using the cDNA-AFLP tags cloned into a pGEM-T vector. After hybridization and emulsion development the sections were studied under a bright-field, differential interference contrast or dark-field microscope. The slides were also stained with DAPI and studied under the fluorescence microscope to establish the stage of meiosis.

Results

Meiotic staging and synchrony within the anthers

Developmental staging of *Petunia* flowers by Porceddu *et al.* (1999) was based on developmental landmarks linked with morphometric data. However, we found that bud and anther size at male meiosis vary considerably under different environmental conditions, making it difficult to correlate meiotic stages based on size alone. We therefore identified the precise meiotic stage of each sample on the basis of DAPI-stained enzyme-digested pollen mother cells (Figure 1). Analyzing the microscopic slides containing the spread cells from a single anther we observed a high synchrony of meiotic development, especially during the long prophase I stages. During the fast-evolving meiosis II, often three subsequent meiotic stages, e.g. anaphase II, telophase II and tetrad, could be observed in the same preparation.

The progression through meiosis occurred synchronously in four out of the five anthers of a flower bud, while in the smallest anther the meiotic stages had progressed further (data not shown). The special nature of the abaxial anther is not uncommon in the Solanaceae family. While this anther is tiny compared to the other anthers in *Petunia*, it is completely aborted in its close relative *Salpiglossis* (Fries 1911). Hence, the smallest anther of each flower bud was discarded from sampling.

For studying the expression profiles we selected meiotic samples containing pollen mother cells at premeiotic interphase, early prophase I (including leptotene and part of zygotene), synizesis (including part of zygotene and early pachytene), late pachytene up to metaphase I, dyad to telophase II and postmeiotic stages. The morphological criteria are based on meiotic prophase description for tomato (Moens 1964) and *Beta* (De Jong & Oud 1979) and include synizesis and diffuse diplotene. Leptotene and zygotene are not distinguishable in the strict sense as the onset and progress of chromosome pairing cannot be assessed reliably. Diagnostic substaging of early prophase can be made on the basis of heterochromatin patterns. Most plants with pericentromere heterochromatin display a variable number of chromocentres at interphase and

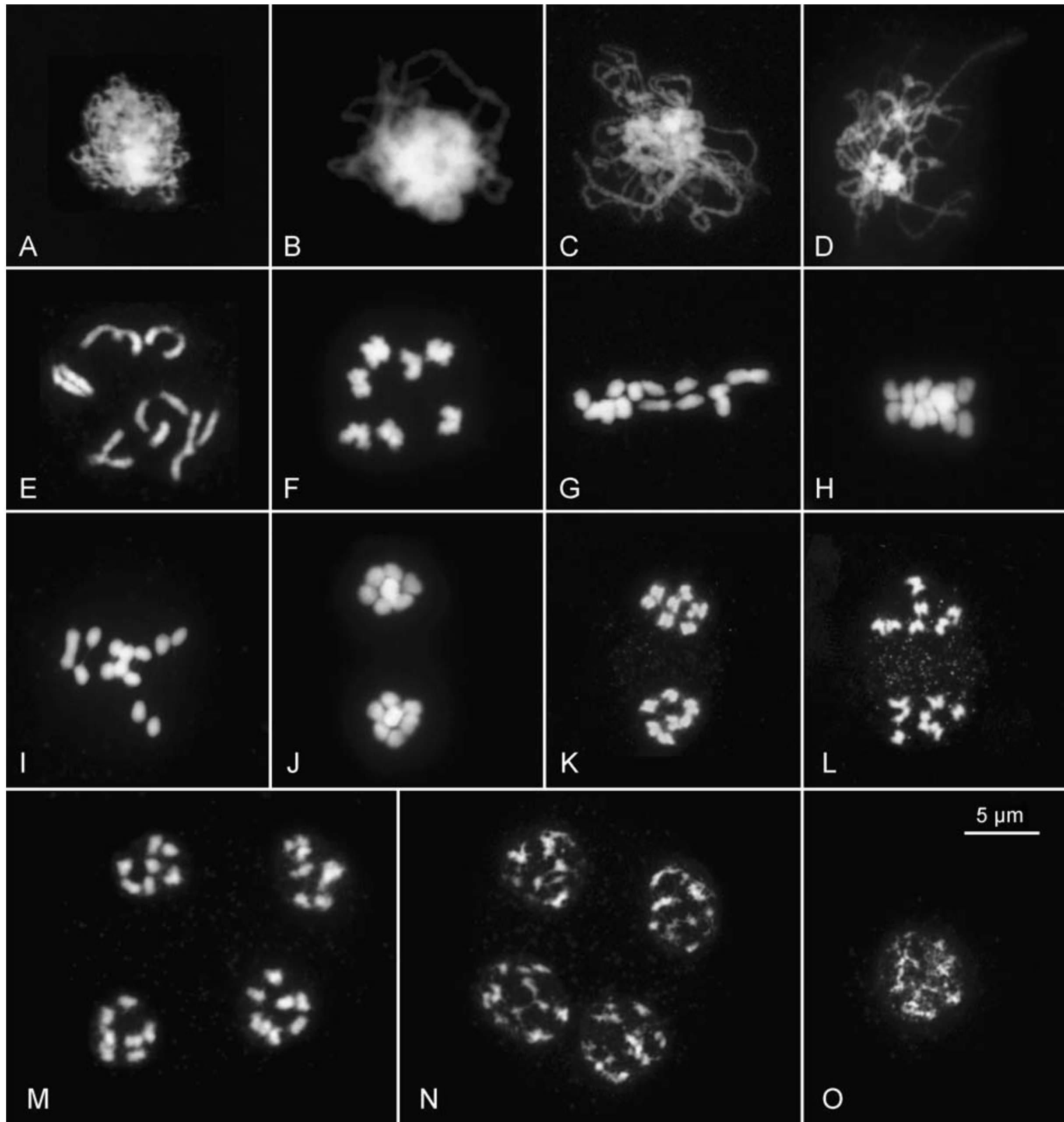

Figure 1. Overview of meiotic stages as determined by DAPI-staining in microsprocytes of *Petunia hybrida*. **A**,**B**: very early meiotic prophase, **C**: synizesis (including pachytene), **D**: early diplotene, **E**: late diplotene, **F**: early metaphase I, **G**: metaphase I, **H**: early anaphase I, **I**: anaphase I, **J**: telophase I, **K**: dyad stage, **L**: metaphase II, **M**: telophase II, **N**: tetrad, **O**: end of tetrad stage.

very early meiotic prophase that aggregate during very early prophase to form ultimately the synizetic knot (De Jong & Oud 1979). The changes in heterochromatin morphology and clustering of the chromocenters, along with pairing in the visible chromosome loops, can be used to substage early prophase I events (Abirached-Darmency *et al.* 1991).

We focused on prophase I stages to investigate the meiosis-specific processes of chromosome pairing and recombination. The high level of synchrony of meiotic

prophase I stages is an important advantage for gene expression analysis. The two premeiotic samples were prepared from anthers derived from two flower buds of 1.8 and 2.2 mm in size, respectively (stage 2 in Porceddu *et al.* 1999). Two postmeiotic samples were taken from anthers derived from single flower buds of 4.75 and 5 mm, respectively (stage 4 in Porceddu *et al.* 1999).

cDNA-AFLP transcript profiling

The cDNA-AFLP transcript profiling with the *Bst*YI+T- and *Mse*I-primers and two additional selective nucleotides generated 30–35 AFLP tags per primer combination, which is sufficient for quantitative analysis. While the majority of the signals were constitutive, cDNA fragments exhibiting a modulated expression pattern were well represented (Figure 2).

We regularly observed an abrupt transition of cDNA profiles between the zygotene and the pachytene sample, which seemed to necessitate a more refined meiotic staging. Therefore, we repeated our transcript profiling experiment on an extended set of 36 samples derived from partially overlapping, meiotic stages, with emphasis on the transition between synizesis and pachytene. The expression profiles were found to be consistent with the first experiment, including the abrupt transition between the zygotene and pachytene stages (data not shown). The experiment with 256 primer combinations resulted in approximately 8000 transcript tags. Based on a comparative restriction site analysis (Breyne *et al.* 2003), we estimated that roughly 30% of the total *Petunia* transcriptome was covered. Six percent (475) of the transcript tags showed a visually modulated gene expression pattern during meiosis.

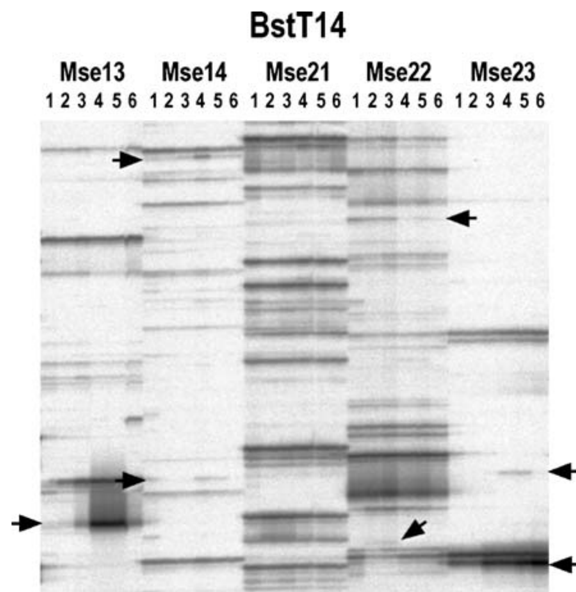

Figure 2. cDNA-AFLP transcript profiling during male meiosis, using BstT/Mse +2/+2 primer combinations. Expression levels are compared during six stages, which are determined by cytological analysis. Arrows indicate fragments with a modulated expression pattern during meiosis: *1*: premeiotic, *2*: very early meiotic prophase, *3*: early synizesis, *4*: pachytene-metaphase I, *5*: dyad stage-telophase II, *6*: postmeiotic.

Cluster analyses of the expression profiles

We performed a cluster analysis on 7408 expression profiles. Approximately 500 expression profiles were not included because of insufficient quality for cluster analysis or because the corresponding PhosphoImager scans were not available. The threshold level chosen for identifying significant differences in signal intensity corresponded to a three-fold transcriptional change, which is a stringent condition to minimize false-positives. As a result, a set of 978 significantly modulated fragments was obtained, which included a significant number of the 475 gene fragments that were selected upon simple visual inspection.

In a first approach, we performed a hierarchical average linkage clustering according to Eisen *et al.* (1998). Hierarchical clustering is a pairwise average-linked algorithm that sorts through all the data to identify pairs of genes that behave most similarly and then progressively adds other genes to the initial pairs to form clusters of genes with a correlated expression profile. An overview of the resulting clusters of genes with similar expression profiles is presented in Figure 3. A transcriptional cascade can be observed that governs initiation and progression through the meiotic cell cycle. These successive waves of transcription reveal a high level of coordination and a precise temporal activation of meiotic genes, indicating a strict regulation at the transcriptional level.

Large clusters of co-regulated genes were expressed exclusively premeiotically or postmeiotically, while other clusters harbored meiosis-specific genes, which displayed high expression levels during early, middle or late meiosis (Figure 3). The highest levels of gene induction were found in the cluster with postmeiotically expressed genes with up to a 60-fold increase in mRNA levels. In contrast, a majority of the premeiotically expressed genes is 20–30-fold down-regulated

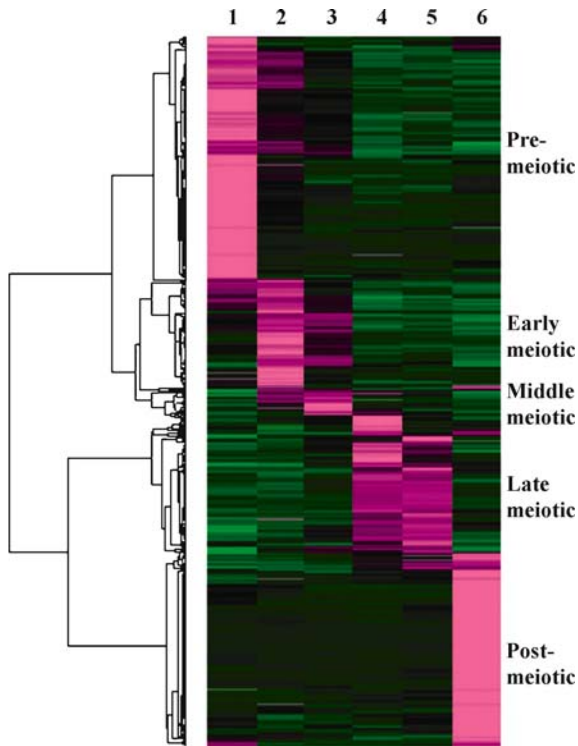

Figure 3. Hierarchical average linkage clustering of the expression patterns of 978 significantly modulated cDNA-AFLP fragments according to Eisen *et al*. (1998). The matching dendrogram shows five different clusters of co-expressed genes, with maximal expression level at different stages during meiosis. The color scale ranges from saturated red (highest expression) to saturated green (lowest expression): 1: premeiotic, 2: very early meiotic prophase, 3: early synizesis, 4: pachytene-metaphase I, 5: dyad stage-telophase II, 6: postmeiotic.

upon meiotic initiation. A second group of premeiotically expressed genes is less repressed and remains transcriptionally active during early prophase. We focused on genes modulated during meiosis. Most of the 475 transcript tags selected for further characterization belonged to the clusters with meiosis-specific gene expression patterns. Two major clusters harbor genes expressed in early prophase (up to synizesis) or later in meiosis (from pachytene to telophase II). A third, smaller cluster contains 'middle' genes reaching their peak expression level at the zygotene stage.

To confirm the results obtained by hierarchical average linkage clustering, the data set was subjected to a second clustering algorithm, called adaptive quality-based clustering (De Smet *et al*. 2002). The program defines separate groups of significantly co-expressed genes without the requirement to specify the number of clusters *a priori*. Expression profiles that do not fit in any cluster are rejected. The substantial discriminatory power of this method allows a further refinement in the classification of co-expressed genes, compared to hierarchical average linkage clustering. As a result, 11 clusters were obtained, representing the expression profiles of 826 genes, which is 85% of the total data set (data not shown). Of these, five major clusters of co-expressed genes were generated (Figure 4) namely, premeiotic (254), down-regulated (50), early meiotic (66), late meiotic (100), and postmeiotic (244), which is 80% of the total data set. Overall, adaptive quality-based clustering confirmed the clustering tree of Figure 3. However, the substantial discriminatory power of the second method allows a further refinement in the classification of co-expressed genes, compared to hierarchical average linkage clustering.

Homology search for known genes

Direct sequencing of 475 selected cDNA-AFLP fragments resulted in useful sequence information for 293 cDNA fragments (62%). The sequences obtained were compared to those present in the GenBank database using BLAST (Altschul *et al*. 1997) and FASTA (Smith & Waterman 1981) algorithms; 90 fragments (30%) showed significant homology (maximum *E*-value 0.001) to genes with known function, while another 25 tags (8%) matched with genes without an allocated function (EST or putative proteins). The remaining 178 fragments (62%) shared no homology with sequences in the database. These figures are comparable to earlier cDNA-AFLP analyses in *Petunia* (Cnudde *et al*. 2003) and tobacco (Breyne *et al*. 2003). The consistent expression pattern seen for the multiple tags corresponding to the same gene served as a good internal control. The successfully sequenced transcript tags were annotated and classified according to their putative functions, based on literature searches. An overview is provided in Table 1, while the analyzed sequence tags (submitted to NCBI) are provided as Supplementary Data*.

In general, the extent of homology between the *Arabidopsis* proteins characterized so far and their meiotic homologs in yeast and humans range from

***Electronic Supplementary Material**
Supplementary material is available for this article at http://dx.doi.org/10.1007/s10577-006-1099-5 and is accessible for authorized users.

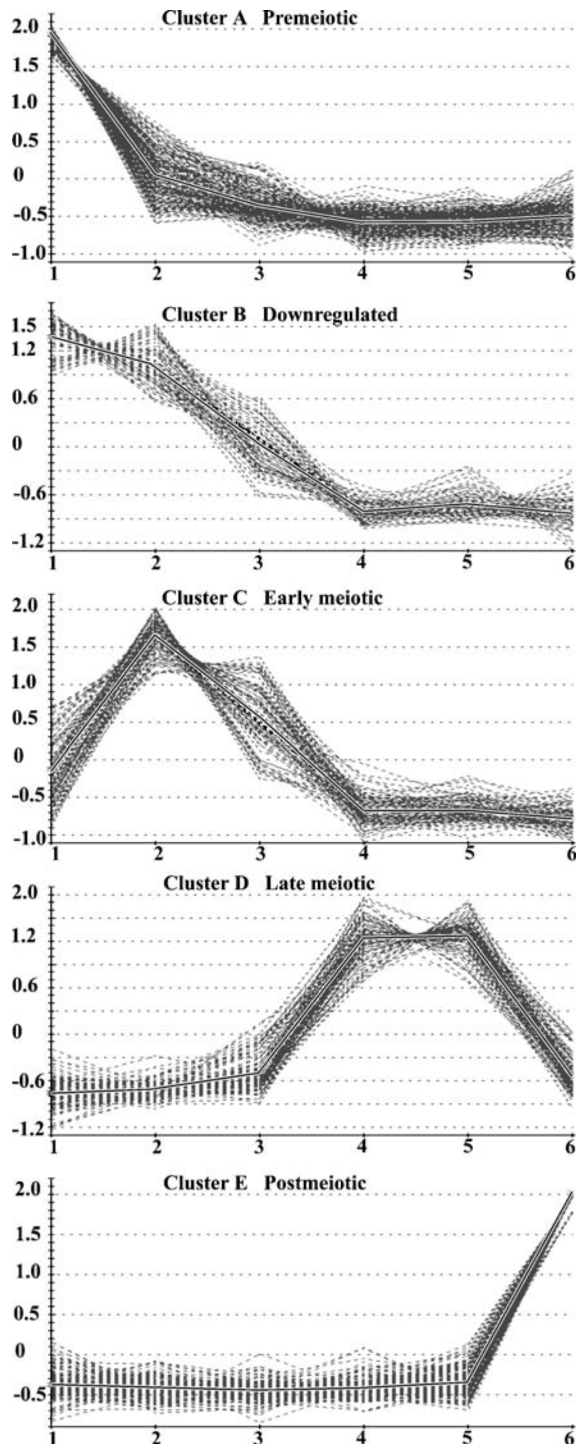

Figure 4. Adaptive quality-based clustering of the expression patterns of 978 significantly modulated cDNA-AFLP fragments according to De Smet *et al.* (2002). **A**–**E**: Five major clusters harboring 87% of all expression patterns; *1–6*: Sampled stages. 1: Premeiotic, 2: very early meiotic prophase, 3: early synizesis, 4: pachytene-metaphase I, 5: dyad stage-telophase II, 6: postmeiotic.

20% to 45% identity and from 40% to 60% similarity (Schwarzacher 2003, Kerzendorfer *et al.* 2006). Homologies with known meiotic genes from plants, yeast and mammals showed that our screening was in principle effective. We were able to retrieve cDNA tags homologous to *Arabidopsis* genes involved in meiosis-specific processes. In the early meiotic cluster, tags 12, 46 and 56 correspond to *AtDMC1*, involved in meiotic recombination (Doutriaux *et al.* 1998). In the down-regulated cluster, tags 265, 285 correspond to *AtASK2*/*AtASK1* with a role in meiosis, flower and embryo development (Yang *et al.* 1999, Yang & Ma 2001) and tags 203, 204 correspond to *AtASY1* involved in synapsis (Ross *et al.* 1997, Caryl *et al.* 2000, Armstrong *et al.* 2002). Several links were found with mammalian testis development, for example protein kinases that are denoted as testis-specific in the database. Another striking homology was found with a RecA-gene of *Streptococcus* (tag 229), adding further proof to the high level of evolutionary conservation of meiotic recombination proteins. Some of the up-regulated expression tags correspond to genes that are also characterized to be essential for male fertility, gametogenesis and embryo development (Table 1). These include homologs of tapetum differentiation gene *AtGPAT1* (tag 358), tomato meiotic

Table 1. Functional classification of transcript tags with a significant level of homology (E-value < 0.001) using BLAST at NCBI.

Function	No. of tags
DNA replication and modification	7
Proteolysis	10
Signal transduction	7
Cytoskeleton	8
DNA repair and recombination	7
Synapsis and sister chromatid cohesion	2
RNA processing	4
Protein synthesis	7
Hormone response	1
Metabolism	33
Transcription factors	1
Transport and secretion	3
Unknown function	25

serine proteinase *LeTMP* (tag 76) essential for microsporogenesis (Riggs *et al.* 2001) and auxin response factor *AtARF4* (tag 393) as auxin is critical for anther/pollen grain development (Feng *et al.* 2006). The cell wall biosynthesis gene *AtTPS1* (tag 40), polyA-specific ribonuclease *AtPARN* (tag 245) are essential for embryogenesis, while the polyamine biosynthetic gene AtADC1 (tag 99) is needed for seed development. However, our finding that their expression is up-regulated in meiotic anthers is new. In addition, the roles of some of the genes known to be essential for meiotic processes in yeast and mammals are yet to be proven in *Arabidopsis*. These include tag 280, corresponding to the plant homolog of Damaged DNA Binding Protein 1 (DDB1), a protein originally identified because of its role in the human disease xeroderma pigmentosa. Tag 106 is a putative ubiquitin-conjugating enzyme. In mammals these enzymes are known to interact with recombination RAD51 protein and with proteins of the synaptonemal complex (Kovalenko *et al.* 1996). *Ubc9* interacts with the meiosis-specific *RecA* homolog, Lim15/Dmc1 in the basidiomycete *Coprinus cinereus* (*CcLim15*), and mediates its sumoylation during meiosis (Koshiyama *et al.* 2006). Other tags of the ubiquitin family correspond to tags 15, 37, 66, 121, 333, 388, 445, 452 and the histone-2B family 223, 237, 246, 414, 019, and are expressed at higher levels during the early meiotic phase.

Transcript tags showing a significant homology in database searches were unequally distributed over the clusters described above. In particular, fragments expressed late during meiosis have a considerably smaller chance of finding a match in the BLAST database than early meiotic fragments. Of the 100 sequenced fragments belonging to cluster D (Figure 4), only eight showed a significant homology (8%), compared to 18 of the 66 sequenced cluster C fragments (27%) and 16 of the 50 sequenced cluster B fragments (32%).

Expression analysis by RNA in-situ *hybridization*

To obtain spatial expression patterns and to confirm the cDNA-AFLP expression profiles, we examined the expression of five tags in reproductive tissues, using RNA *in-situ* hybridization. Two transcript tags are homologs of characterized meiotic genes in *Arabidopsis*, namely, *DMC1* (tag 56) and *ASY1* (tag 203). *DMC1* was expressed during leptotene and zygotene, also showing high levels of meiocyte-specific expression (Figure 5). However, while no premeiotic expression was visible in the cDNA-AFLP pattern, the *in-situ*

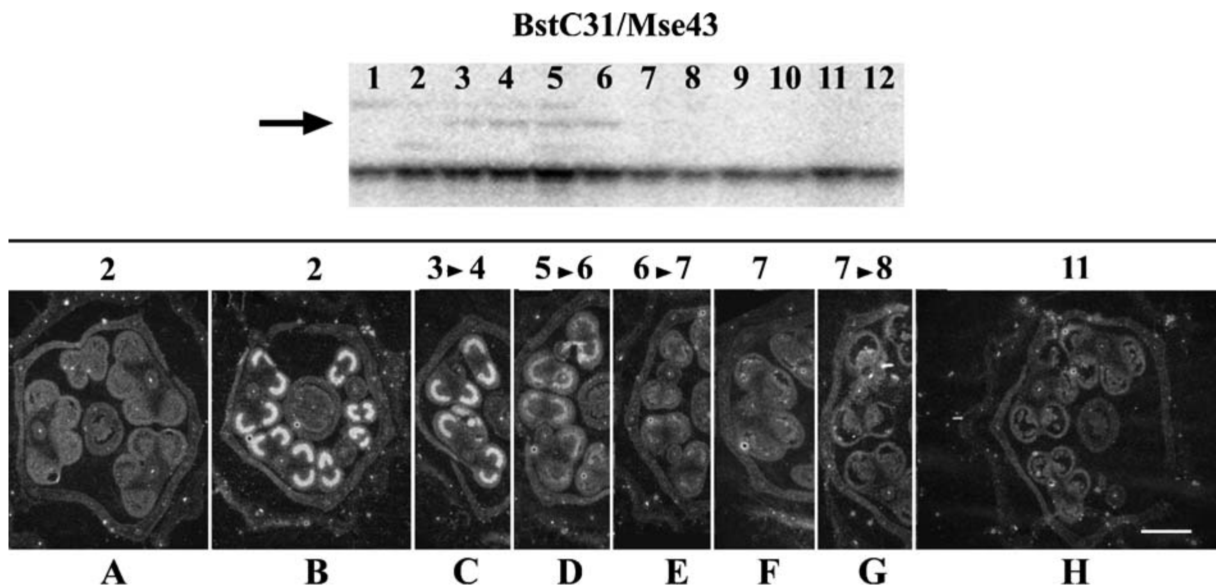

Figure 5. Expression analysis of *DMC1* homolog M56. *Upper panel*: cDNA-AFLP expression pattern. *1*, *2*: premeiotic, *3*: very early meiotic prophase, *4*: early meiotic prophase, *5*: early synizesis, *6*: synizesis, *7*: pachytene, *8*: metaphase I, *9*: dyad stage, *10*: telophase II, *11*, *12*: postmeiotic. *Lower panel*: *In-situ* hybridization using [^{33}P]-UTP-labeled RNA probe on sections of *Petunia* buds, exposed for 35 days and examined using dark-field illumination. **A**: sense; **B–H**: antisense, **A**,**B** premeiotic, **C**: very early meiotic prophase, **D**: synizesis, **E**: synizesis-pachytene, **F**: pachytene, **G**: diplotene, **H**: tetrad. Bar = 0.5 mm.

hybridizations showed a clear signal in the pollen mother cells before meiosis. For *ASY1*, the *in-situ* data confirmed the cDNA-AFLP pattern (Figure 6), showing high levels of *ASY1* expression in the early meiotic stages, localized in premeiocytes and leptotene microsporocytes. The timing of ASY1 expression corresponds to the initiation of synaptonemal complex formation that precedes the start of chromosome synapsis at zygotene.

Furthermore, preliminary *in-situ* data were obtained for fragments homologous to genes encoding a poly(A) ribonuclease (PARN) (tag 245), a checkpoint protein (BUB1) (tag 342) and a C3HC4 zinc finger protein (tag 407) (data not shown). *PARN* expression was detected before meiosis and in the leptotene and zygotene stages. *BUB1* transcripts accumulated in all cell types of the anther during early meiosis, but from pachytene onwards the signals could no longer be detected. The expression of the C3HC4 zinc finger gene appeared to be localized in the tapetum layer, rather than in the meiocytes. Further analysis will show whether this gene does exert its function in the tapetum itself, or whether it has a non-autonomous function in meiocytes. In general, the *in-situ* hybridization experiments confirmed the gene expression patterns for all selected genes, validating our cDNA-AFLP results, while adding spatial information.

Discussion

We employed cDNA-AFLP transcript profiling for the identification of genes that are modulated in their expression during male meiosis in *Petunia hybrida*. Cluster analyses on our data set identified five large clusters of co-expressed genes, representing 83% of all selected gene tags. A transcriptional cascade is thus observed that governs initiation and progression through the meiotic cell cycle, as has been demonstrated in a temporal analysis of the meiotic transcriptome in yeast (Chu *et al.* 1998, Primig *et al.* 2000, Mata *et al.* 2002). These successive waves of transcription reveal a high level of coordination and a precise temporal activation of meiotic genes. As genes with related functions tend to be

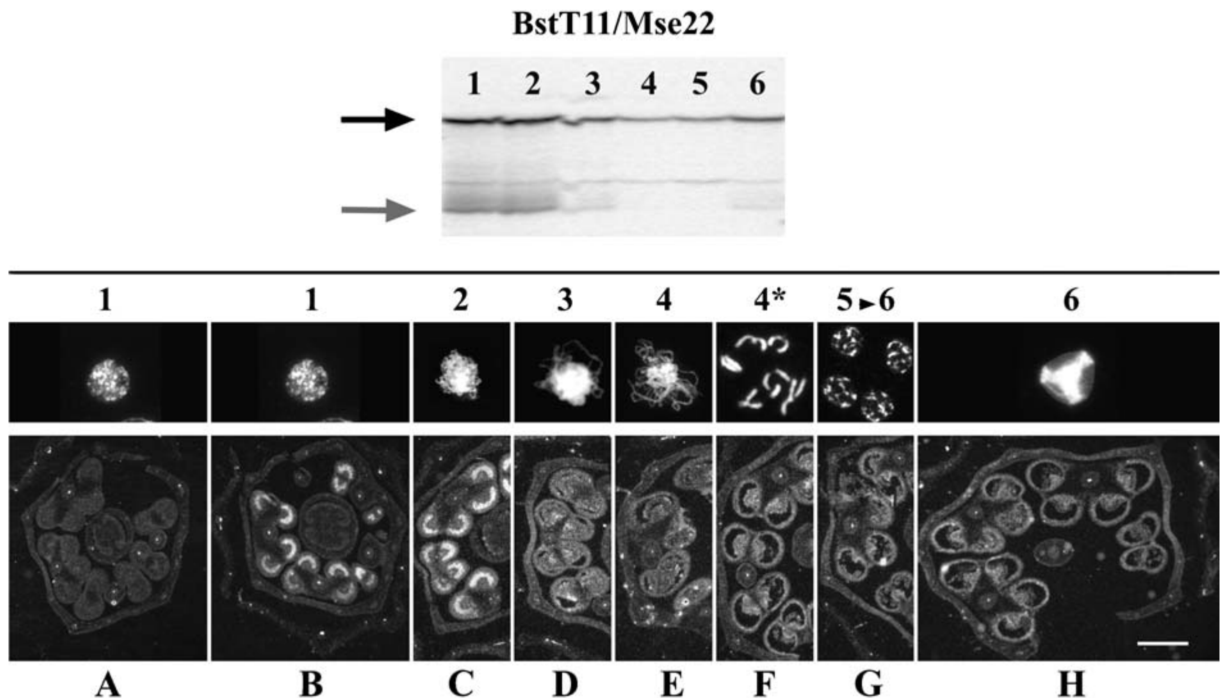

Figure 6. Expression analysis of *ASY1* homologe M203. *Upper panel*: cDNA-AFLP expression pattern. *Middle panel*: *1*: premeiotic, *2*: very early prophase, *3*: synizesis, *4*: pachytene → metaphase I; *4**: diplotene, *5*: dyad stage → telophase II, *6*: postmeiotic. *Lower panel*: *In-situ* hybridization using [^{33}P]-UTP-labeled RNA probe on sections of *Petunia* flower buds, exposed for 35 days and examined using dark-field illumination. **A**: sense, **B**–**H**: antisense, **A**,**B**: premeiotic, **C**: very early prophase, **D**: synizesis, **E**: pachytene, **F**: diplotene, **G**: tetrad, **H**: pollen grain. Bar = 0.5 mm.

expressed in similar patterns, cluster analysis may provide a first clue for the functional characterization of yet-unknown co-expressed genes. A strict co-regulation of genes encoding proteins involved in successive steps of the cell cycle has been demonstrated in plants (Breyne *et al.* 2002) and gene expression profiling data have been successfully used to infer gene functions in yeast meiosis (Toth *et al.* 2000). Accordingly, we may find support for the proposed functions of several isolated fragments with varying levels of homology, based on their temporal association with meiotic genes of known function.

The 254 premeiotically expressed genes in our study are strongly repressed upon entry into meiosis. Fragments belonging to this class have not been characterized further by sequencing. Another 50 *Petunia* cDNA-AFLP fragments were more gradually down-regulated, showing a gradual decrease in transcript levels up to the end of the zygotene stage. Many of the transcript tags with this expression pattern putatively have roles in ubiquitin-mediated proteolysis, RNA processing and protein biosynthesis. Homologies were found to genes involved in DNA replication, synapsis of homologous chromosomes (*ASY1*), SCF complex subunit (*SKP1*/*ASK1*) and spindle assembly *(BUB3)*, indicating the preparation of the meiotic cell for the recruitment of a specialized set of proteins upon commitment to meiosis. Sixty-six fragments showed a pattern of expression characterized by an early induction at leptotene, followed by a decrease in transcript levels at zygotene. Characterized genes with this transient induction pattern encode proteins, involved in meiotic recombination (*DMC1*), DNA modification, checkpoint control and membrane transport. This cluster is comparable to the ‘early gene’ clusters distinguished in budding yeast (Chu *et al.* 1998, Primig *et al.* 2000) and fission yeast (Mata *et al.* 2002).

A fourth cluster of 100 fragments followed a pattern of expression characterized by a ‘late’ induction at pachytene and sustained expression during the meiotic divisions. Compared to the clusters discussed above, fewer homologs were found to genes expressed in the late meiotic phase, which is of a short duration (Armstrong & Jones 2003), indicating that their corresponding sequences are yet underrepresented in databases. Sequenced transcript tags showed homology to genes encoding DNA repair proteins that may be involved in Holliday junction resolution and mismatch repair. Further, homologies are found with proteases, signal transduction proteins (kinases, phosphatases) and those required for proper chromosome segregation such as the structural maintenance of chromosomes (SMC) proteins. Compared to the gene expression patterns of the ‘middle’ genes in yeast (Chu *et al.* 1998, Primig *et al.* 2000, Mata *et al.* 2002), the expression of *Petunia* fragments belonging to this cluster is more repressed in the postmeiotic stage.

Finally, about 244 genes are induced after completion of the meiotic divisions. They putatively represent gametogenesis-related genes, many of which may have unique functions during microspore development. They correspond to the anther-specific transcripts that were identified during a previous cDNA-AFLP transcript profiling experiment on developing *Petunia hybrida* floral organs (Cnudde *et al.* 2003). A similar cluster of postmeiotically induced genes is present in yeast (Chu *et al.* 1998, Primig *et al.* 2000, Mata & Bähler 2003). However, these genes encode proteins involved in spore maturation and ascus formation, which exhibit low levels of homology with plant genes (Cnudde *et al.* 2003).

Strong homologies to known meiotic genes from, e.g., *Arabidopsis* or *Saccharomyces* that resulted from our experiment, include genes such as *DMC1*, *ASY1,* and *ASK1*. Few others, e.g. *BUB1* and *BUB3*, have been identified as essential components of the mitotic and meiotic spindle check point machinery (Amon 1999, Prinz & Amon 1999, Yamaguchi *et al.* 2003). While *BUB3* has a significant hit (tag 21), no obvious *BUB1* homolog (tag 342) is known in the *Arabidopsis* genome. Weak homologies were found with nucleic acid-modifying enzymes, such as ATP-dependent helicases (tags 222, 230) topoisomerases (tags 283, 319), p23 tubulin binding proteins (tag 445), microtubule associated (tag 77). Fragment 287 is induced late in meiosis and shows homology to a meiosis-related, testis-specific protein known as cancer-antigen in humans and to a plant Ruv DNA-helicase-like protein. Proteins related to the bacterial RuvB DNA helicase have been shown to catalyze branch migration of Holliday junctions (Shen *et al.* 2000). In this way a targeted selection can be carried out to identify candidate gene fragments with a putative function in particular aspects of meiosis.

A number of genes that we found in this study are implicated in processes other than meiosis, such as flower development, gametogenesis, embryogenesis, microsporogenesis — namely *AtGPAT1, AtPARN, AtADC1*. Many others are related to genes of major biochemical metabolic and signaling pathways, for example *AtNADH5, AtBAP1, ATIPT2, ATCIMS* and

AtARF4 that are essential for the cells to survive and grow. However, their expression is also up-regulated in meiotic anthers and yet it seems unlikely that they have a meiotic role *per se*. The identification of genes seemingly unrelated to meiosis may be the result of our sampling method. Exclusive sampling of microsporocytes would enrich the pool of meiosis-specific transcripts but is technically challenging and tedious.

Finally we performed RNA *in-situ* hybridization in order to validate the expression pattern of the transcripts. The results are in general agreement in all the cases, with some differences. For example, the strong cDNA-AFLP tag expression of *DMC1* in microsporocytes of *Petunia hybrida* is in agreement with the RNA *in-situ* hybridization data and the results of *AtDMC1* promoter:GUS protein gene fusions in *Arabidopsis thaliana* (Klimyuk & Jones 1997). However, the expression in premeiotic stages seen in the anthers using RNA *in-situ* hybridization was not seen in cDNA-AFLP expression pattern for *DMC1*. This difference could reflect the difficulty in precisely defining the onset of leptotene.

Further studies, including mutational analyses, are required to elucidate the possible meiotic role of several genes identified in this study. While *Petunia hybrida* is amenable to the microscopy-based sampling and cDNA-AFLP transcript profiling, meiotic research in *Arabidopsis* is attractive due to the extensive sequence information and the large mutant collections available for this model species. Because the function of the homologs in *Petunia hybrida* and *Arabidopsis thaliana* is likely to be conserved to a great extent in meiotic processes such as recombination, synapsis and in the synaptonemal complex components, one can make use of the advantages of *Arabidopsis* and look for *Arabidopsis* homologs of candidate *Petunia* gene fragments with a putative function in meiosis.

In conclusion, the combination of accurately staged anthers with cDNA-AFLP transcript profiling in *Petunia hybrida* is a valuable method to identify genes involved in meiosis. Based on their modulated gene expression pattern, candidate meiosis-related AFLP tags can be selected, while a first indication for a possible function can be obtained by sequence homology and their clustering with known meiotic genes. Our data set provides a starting point for unraveling meiosis at a molecular level in plants. This will allow a knowledge-based selection of novel meiotic genes for further characterization.

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