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ORIGINAL ARTICLE

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Analysis of gene expression during flowering in apomeiotic mutants of *Medicago* spp.: cloning of ESTs and candidate genes for 2*n* eggs

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Abstract Mutants showing features of apomixis have been documented in alfalfa (*Medicago sativa* L.), a natural outcrossing sexual species. A differential display of mRNAs that combines cDNA-AFLP markers and bulked segregant analysis was carried out with the aim of selecting expressed sequence tags (ESTs) and cloning candidate genes for apomeiosis in mutants of alfalfa characterized by 2*n* egg formation at high frequencies. The approach enabled us to select either mutant- or wild type-specific transcript derived-fragments and to detect transcriptional changes potentially related to 2*n* eggs. Sequence alignments of a subset of 40 polymorphic clones showed significant homologies to genes of known function. An EST with identity to a β-tubulin gene, highly expressed in the wild type and poorly expressed in the apomeiotic mutants, and an EST with identity to a Mob1-like gene, qualitatively polymorphic between pre- and post-meiotic stages, were selected as candidate genes for apomeiosis because of their putative roles in the cell cycle. A number of clone-specific primers were designed for performing both 5′ and 3′ rapid amplification of cDNA ends to obtain the full-length clones. Southern blot hybridization revealed that both clones belong to a multi-gene family with a minimum of three genomic DNA members each. Northern blot hybridization of total RNA samples and in situ hybridization of whole buds enabled the definition of their temporal and spatial expression patterns in reproductive organs. Experimental achievements towards the elucidation of apomeiotic megasporogenesis in alfalfa are presented and discussed.

Keywords Differential display cDNA-AFLP · Bulked segregant analysis · Diplospory · Tubulins · Mob (Mps-One-Binder)

Introduction

The cultivated form of alfalfa (*Medicago sativa* subsp. *sativa* L.) is an outcrossing sexual species characterized by tetrasomic inheritance and pronounced inbreeding depression. It is likely that, as alfalfa breeding programs become more sophisticated, future varieties may be genetically uniform because of the constitution of hybrids or the utilization of apomixis. Apomixis would give a unique opportunity for selecting new cultivars, based on one, or only a few, superior genotypes, with permanently fixed heterosis. Although apomictic reproduction has never been detected in the *M. sativa-coerulea-falcata* complex, mutants showing features of apomixis (e.g. apomeiosis and parthenogenesis) have been documented (reviewed by Barcaccia and Veronesi 1999).

In alfalfa mutants in which megasporogenesis is affected, the production of 2*n* eggs was mostly associated with the absence of the final cytokinesis, genetically equivalent to abnormal meiosis of the second division restitution (SDR) type. The formation of 2*n* eggs through diplosporic apomeiosis of the first division restitution (FDR) type has only been observed in a mutant of alfalfa, named PG-F9. Overall, cytohistological and molecular data independently provided evidence that SDR (~43%) and FDR (~21%) mechanisms are active in different ovules of a given ovary of this mutant (Tavoletti et al. 1996; Barcaccia et al. 1997). The fact that normal tetrads

The nucleotide and amino acid sequence data of the cDNA clones isolated and characterized here have been recorded in the GenBank/EMBL sequence databases under the accession numbers AJ319667 [beta-tubulin (β-Tub) mRNA] and AJ319713 [Mob1-like protein (Mob1) mRNA].

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are usually present together with SDR dyads and FDR monads most likely implies that the specific timing of gene expression during megasporogenesis in a common genetic system may direct events towards distinct 2*n* egg formation mechanisms (Barcaccia et al. 2000a).

Molecular differential screening of plants with contrasting modes of reproduction is considered one of the most powerful tools for identifying, mapping and isolating the gene(s) underlying the expression of apomixis. Even in a remarkably complex species like alfalfa, a differential display based on the cDNA-AFLP technique (Bachem et al. 1996) in combination with bulked segregant analysis (BSA; Michelmore et al. 1991) may be effective for detecting expressed sequence tags (ESTs) and cloning candidate genes for a given trait (Barcaccia et al. 2000b). In the case of apomictic mutants, such a method relies on pooling cDNA subsets from reproductive organs at specific developmental stages of individuals sharing the same genetic background but showing extreme classes for the mode of reproduction, and then screening for molecular polymorphisms between mutant and wild-type using AFLP markers. This approach makes possible the analysis of a large number of transcript-derived fragments (TDFs) and increases the reliability of amplification-based polymorphisms.

Our research deals with the analysis of gene expression during megasporogenesis in reproductive mutants of alfalfa characterized by 2*n* egg formation at high frequencies. An mRNA differential display technique combining cDNA-AFLP markers and BSA enabled cloning of ESTs and candidate genes for apomeiosis in alfalfa. The characterization of some of these clones and possible implications for their role in apomeiosis are presented and discussed.

Materials and methods

Plant material

The apomeiotic mutant PG-F9 of *M. sativa* ssp. *falcata* (L.) Arc. (2*n*=2*x*=16) was hand-pollinated with a wild-type plant of *M. sativa* ssp. *coerulea* (Less.) Schm. (2*n*=2*x*=16). F_1 progeny plants were hand-crossed with pollen from a tetraploid plant of *M. sativa* ssp. *sativa* (2*n*=4*x*=32). Since there is a strong triploid embryo block in interploid crosses, which prevents the formation of 3*x* progeny due to endosperm imbalance, the number of seeds produced per pollinated floret (i.e. seed set) was used as a measure of the genetic capacity for 2*n* egg production (Barcaccia et al. 2000a). On the basis of seed set values in 2*x*–4*x* crosses, F_1 plants that exhibited null or very low seed sets were classified as normal egg producers and plants with high seed set as 2*n* egg producers.

Bulked segregant analysis

A total of 24 progeny plants with a contrasting megasporogenesis pattern were selected and used for BSA to identify differentially expressed TDFs putatively related to apomeiosis. cDNA bulks of six plants each from two 2*n*-egg mutant and two wild-type progeny sets were prepared by combining total RNA from flower buds of plants that scored an average seed set of 0.73 and 0.03, respectively. Flower buds were preliminarily classified into four developmental stages: pre-meiosis, initial meiosis, final meiosis, and post-meiosis according to their size (Barcaccia et al.2000b). All bulked cDNA samples were investigated by AFLP markers using parental cDNA pools from somatic tissues such as leaves and stems as constitutive controls.

Total RNA isolation and cDNA synthesis

Nucleic acids were isolated from about 0.5 g of fresh tissue using the Nucleon Phytopure extraction kit (Pharmacia) following the manufacturer's instructions. Total RNA was purified from residual genomic DNA according to Barcaccia et al. (2000b). Reverse transcription was performed using 25 µg of total RNA and 300 ng of oligo-dT with AMV reverse transcriptase (Promega) according to the manufacturer's instructions. For second cDNA strand synthesis, DNA Polymerase I and Ribonuclease H (Pharmacia) were used following a standard procedure (Sambrook et al. 1989). The double-stranded DNA synthesized was extracted with phenol-chloroform, precipitated with ethanol at –20°C and the pellet resuspended in distilled water.

Differential display cDNA-AFLP

The mRNA fingerprinting was based on the detection of *Eco*RI/*Mse*I restriction fragments by PCR amplification with 50 different primer combinations. The restriction of double-stranded cDNA samples and ligation of adaptors were performed simultaneously following the protocol of Barcaccia et al. (2000b). Pre-amplification of prepared templates was carried out using primers with no selective bases and a temperature profile including an initial step of 2 min at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C and a final step of 10 min at 72°C was adopted. To maximize the number of amplification products, hot-PCRs were performed according to Barcaccia et al. (1999) with ^{33}P-ATP-labeled *Eco*RI+2 or +3 and unlabeled *Mse*I+2 primers.

Sequencing of subcloned TDF products

Polymorphic TDFs were excised from the dried acrylamide gel, eluted with 100 µl of sterile distilled water and re-amplified with the same *Eco*RI/*Mse*I primer combination that yielded the specific marker. A 0.7-µl aliquot of the re-amplified DNA was sticky-end ligated into a pCRII-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmid DNA was purified from 25 ml of an overnight culture of *Escherichia coli* in LB medium using procedure "B" of the Flexi-prep kit (Pharmacia). The sequence of both strands of the plasmid was determined by the dideoxynucleotide chain reaction termination method using M13 forward and reverse primers and a Thermosequenase cycle sequencing kit (Amersham).

Rapid amplification of cDNA ends analysis

A number of polymorphic clone-specific 24-mer primers were designed using the Primer3 program (http://genome.wi.mit.edu/cgi-bin/primer) for performing both 5′ and 3′ rapid amplification of cDNA ends (RACE) to obtain the full-length clones. The SMART RACE cDNA amplification kit (Clontech) was applied to total RNA according to the manufacturer's instructions.

Southern and Northern blot hybridization

For Southern analysis, alfalfa genomic DNA was isolated using a CTAB protocol, according to Dellaporta et al. (1983). Three 20 µg samples of genomic DNA were digested to completion with 40U of *Eco*RI, *Hind*III and *Xho*I, respectively (New England Biolabs). Restriction fragments were resolved by electrophoresis in a 0.8% agarose gel, blotted by capillary transfer and linked by UV light to a Hybond-N nylon membrane (Amersham). For Northern analysis,

Table 1 List of alfalfa expressed sequence tags (*ESTs*) related to flower buds at different stages of mega-sporogenesis and -gametogenesis[a]

EST	Size (bp)	Gene product and genebank identification	Species	Homology (%)
001	383	18S ribosomal gene (AF093506)	Medicago truncatula	99
002	70	Late embryonic abundant – LEA protein EMB8 (T08400)	Arabidopsis thaliana	80
003	172	Ubiquitin-like protein (AJ270957)	Lycopersicon esculentum	99
004	161	Chlorophyll a/b-binding protein LCHPII (S73603)	Pinus thunbergii	100
005	129	Early light inducible protein – ELIP (U82810)	Glycine max	89
006	217	Non-functional folate binding protein (AAD00154)	Homo sapiens	51
007	283	60 S ribosomal protein L12 (U93168)	Prunus armeniaca	87
008	178	Putative hydrolase T7H20.280 (AL162508)	Arabidopsis thaliana	67
009	177	Alpha-1 tubulin (BF598276)	Glycine max	67
010	238	Unknown protein (AC007651)	Arabidopsis thaliana	94
011	114	Phosphate-starved leaf cDNA clone (BF638159)	Medicago truncatula	95
012	274	Ethylene-regulated protein ER6 (AF096262)	Lycopersicon esculentum	66
013	103	Alpha-1 tubulin (AA063914)	Homo sapiens	75
014	133	Cytoskeletal keratin-like (AW767031)	Xenopus laevis	74
015	84	Seed lipoxygenase LOX-2 (AW395627)	Glycine max	80
016	157	Beta-1 tubulin (M12296)	Glycine max	98
017	156	Alpha-*N*-acetylglucosaminidase (Y18209)	Nicotiana tabacum	86
018	214	Early light inducible protein-precursor (CAA29399)	Pisum sativum	92
019	340	Allene oxide synthase (AJ271093)	Lycopersicon esculentum	79
020	207	Putative protein F22K18.140 (T05568)	Arabidopsis thaliana	84
021	159	Chlorophyll a/b-binding protein type II/1B precursor (X14506)	Pynus sylvestris	100
022	124	Adenosyl-homocysteinase (L36119)	Medicago sativa	100
023	295	S-beta-1 tubulin (AA34009)	Glycine max	97
024	183	Mitosis and maintenance of ploidy (Mob-like) protein (T40465)	*Schizosaccharomyces pombe*	80
025	175	Sugar transporter – integral membrane protein (U64902)	Beta vulgaris	94
026	122	Nod factor binding lectin-nucleotide phosphohydrolase LNP (AF156782)	Medicago sativa	96
027	188	Alcohol dehydrogenase class III L-chain (P81601)	Pisum sativum	71
028	107	Indole-3-acetate beta-glucosyltransferase (BAA93039)	Citrus unshiu	88
029	145	Alanine:glyoxylate aminotransferase AGT2 (AF166351)	Arabidopsis thaliana	99
030	168	Flower bud cDNA clone cTOD6P5 (BE353911)	Lycopersicon esculentum	72
031	140	Ovary cDNA clone cLED32K8 (AI898230)	Lycopersicon esculentum	56
032	201	Pistil-specific protein sts15 (T07677)	Solanum tuberosum	87

[a] There are nine more ESTs without significant homologies

20 µg of total RNA isolated from flower buds, pods, leaves, petioles and stems were resolved by electrophoresis in a 1% agarose-formaldehyde denaturing gel. About 100 ng of each selected cDNA probe was labeled in the presence of ^{32}P-dCTP using a random priming kit (Promega). Pre-hybridization, hybridization and post-hybridization washing of DNA and RNA membranes were performed according to Ruperti et al. (2001).

In situ hybridization

Alfalfa flower buds collected at three different developmental stages (pre-meiosis, meiosis and post-meiosis) were fixed with 4% paraformaldeyde in PBS buffer pH 7.0 and embedded in Paraplast (Sigma). Sections of 10–12 µm were cut using a microtome (Leica RM 2135) and deparaffinized, pre-treated and hybridized as reported in Liu et al. (1999). Sense and antisense probes were obtained by in vitro transcription using cloned TDFs as templates. Digoxigenin (DIG)-labeled RNA was detected using an anti-DIG alkaline phosphatase conjugate, followed by enzymatic NBT (4-nitro blue tetrazolium chloride; Roche) and BCIP (5-bromo-4-chloro-3-indolyl-phosphatase 4-toluine salt; Roche) staining.

Sequence data analysis

Homologies for all cDNA clones were searched for in public databases with the BLASTN and BLASTX applications (Altschul et al. 1990) to compare nucleotide and translated sequences, respectively. cDNA end sequences upstream and downstream of the clones subjected to RACE analysis were assembled to obtain the full sequence using the PCGENE program (IntelliGenetics, Campbell, Calif.). The most significant amino acid sequence homologies were used for multiple sequence alignments with the CLUSTALW program (Higgins et al. 1992) to highlight conserved and variable regions with respect to the cDNA sequence adopted as query.

Results

The cDNA-AFLP differential display method retrieved useful information on gene expression levels and on changes thereof during flowering in alfalfa. The BSA approach enabled us to select either mutant- or wild type-specific TDFs and to detect transcriptional changes potentially related to the apomeiotic trait. Various expression patterns were detected across all reproductive and vegetative alfalfa samples. The detection of both qualitative (presence vs. absence) and quantitative differentially expressed mRNAs between reproductive and vegetative tissues enabled buds and pods to be clearly distinguished from leaves and stems (data not shown). The vast majority of TDFs were shared and thus attributable to constitutive clones, but clones with increasing or decreasing expression as well as those whose expression was modulated during flowering (i.e. pre-meiotic, meiotic and post-meiotic stages) were scored. An average of four differential TDFs per assay was recorded over all primer combinations used, for a total of about 200 clones. A

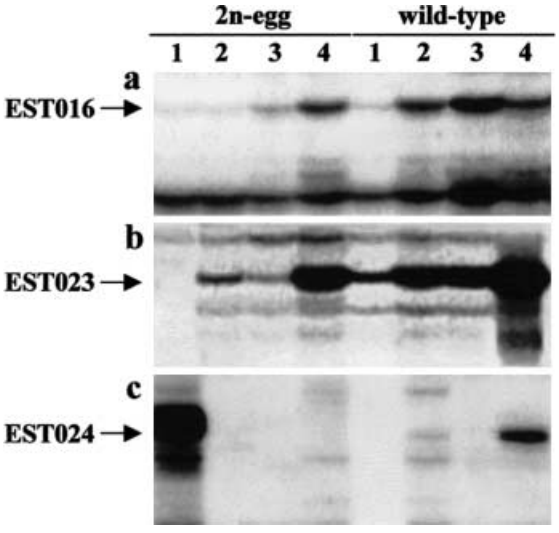

Fig. 1 cDNA-AFLP expression patterns of **a** *EST016* and **b** *EST023*, with homology to β-tubulin, and **c** *EST024* with homology to the Mob1-like clone, in flower buds of alfalfa at different developmental stages: *1* pre-meiosis, *2* and *3* meiosis, *4* post-meiosis

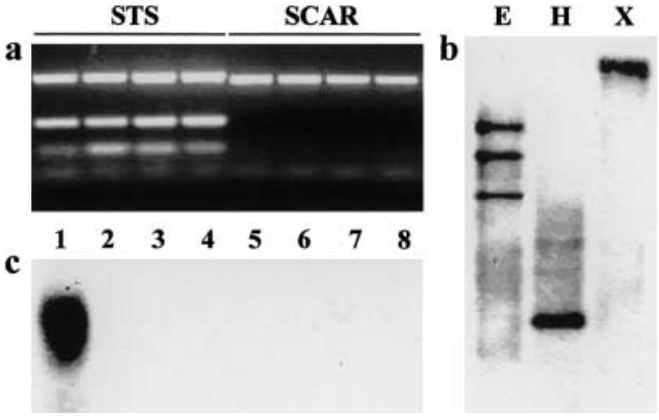

Fig. 2 **a** PCR amplification of four genomic DNA samples of mutants with primers designed in the most conserved regions (*STS* primers) and terminal ends (*SCAR* primers) of β-tubulin. **b** Southern analysis of the Mob1-like clone with *Eco*RI (*E*), *Hind*III (*H*) and *Xho*I (*X*) revealed three, two and one genomic members, respectively. **c** Northern blot hybridization showing the Mob1-like putative transcript in mutant PG-F9. Lanes: *1* total RNA extracted from flower buds at pre-meiosis, *2, 3* total RNA extracted from buds at meiosis, *5–8* total RNA from pods, leaves, petioles, and stems, respectively

subset of 40 TDFs with differential expression between apomeiotic mutants and wild-type was sequenced and this information used for gene data bank queries. Table 1 reports a list of the selected EST clones with their putative gene product identification and corresponding gene bank accession number. In particular, two sequences of 295 and 157 bp similar to β-tubulins that shared a long stretch with full nucleotide homology except for the restriction sites of the longer one, and two clones of 103 and 177 bp similar to α-tubulins were isolated. An additional cDNA clone of 183 bp, with identity to a Mob1 (Mps-One-Binder)-like gene, was found. Pistil-specific transcripts highly homologous to ESTs of unknown function were also detected (data not shown).

EST016, EST023 and EST024 were chosen for further molecular characterization because of their expression patterns and their homology to β-tubulin and Mob-like protein, which have roles in cytoskeleton structure and the cell cycle. EST016 and EST023 were shown to be highly expressed in wild-type and poorly expressed in apomeiotic mutants, whereas EST024 was qualitatively polymorphic, being expressed in the pre-meiotic stage in apomeiotic mutants and in the post-meiotic stage in wild-type (Fig. 1).

A number of β-tubulin and Mob-like specific-primers were designed for performing both 5′ and 3′ RACE. After assembling, the putative β-tubulin clone was shown to be 1544 nt in length and to contain an ORF of 1281 nt. On the basis of multiple alignments, this alfalfa cDNA clone, named β1-Tub, shares between 93% and 95%, and up to 98%, similarity with the vast majority of β-tubulins of higher plants, both monocots and dicots. Although the most similar β-tubulin was that of soybean (404/426 aa of the ORF), the alfalfa clone was also closely related to sequences of corn, wheat, barley, oat and rice.

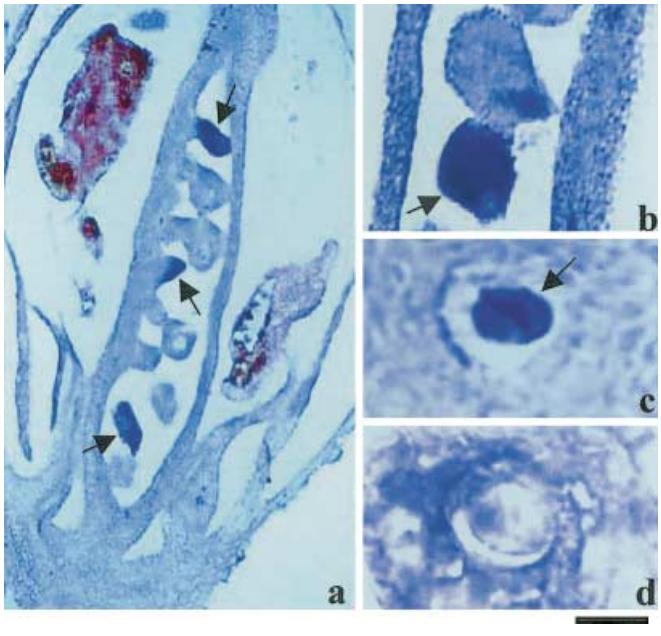

Fig. 3 **a** Longitudinal section of the PG-F9 mutant ovary hybridized with a digoxigenin (DIG)-labeled β-tubulin mRNA antisense probe. **b** Some ovules show the hybridization signal in the outer integument. **c** Hybridization signal localized in a functional megaspore of a mutant ovule. **d** Ovule containing a functional megaspore hybridized with a mRNA sense probe. *Arrows* indicate hybridization signals (*bar*=120 μm in **a**, 30 μm in **b,** 10 μm in **c** and **d**)

Sequence-tagged site amplification of genomic DNA with β-tubulin specific primers designed in the most conserved regions revealed a multiple gene family in alfalfa with at least three separate members (Fig. 2a). Southern blot hybridization with genomic DNA samples and Mob-specific probes revealed a minimum of three genomic

DNA copies (Fig. 2b). Northern blot hybridization indicated that the Mob1-like transcript is expressed in mutant flower buds harvested at the beginning of meiosis when the vast majority of ovules contain primary sporogeneous cells or megaspore mother cells (MMCs) (Fig. 2c) confirming the stage-specificity revealed by cDNA-AFLP (Fig. 1). Transcripts were detected neither in flower buds during gametogenesis nor in pods, leaves, petioles or stems (Fig. 2c).

The spatial distribution of the β-tubulin transcript within reproductive organs was determined by in situ hybridization with DIG-labeled probes. A strong hybridization signal was found in the outer cell layer of some ovules of both the apomeiotic mutants and wild-type as well as in some of the functional megaspores of apomeiotic mutants (Fig. 3a–c).

The final putative Mob1-like clone was 836 nt long. Comparisons with other Mob1-like sequences (e.g. from *Arabidopsis*) suggest that it lacks 23 aa at the 3′ end. This cDNA clone of alfalfa, named Mob1-like, showed significant amino acid sequence similarity with sequences derived from genes from a variety of organisms, including plants, insects, animals, humans, yeast and fungi (data not shown). In particular, it shares 97% similarity and 91% identity with predicted ORFs of unknown function of *Arabidopsis thaliana* and *Cicer arietinum*. The similarity was as high as 82% with Mob1 proteins of *Homo sapiens* and *Mus musculus* and 74% and 68% with Mob1 proteins of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, respectively. Even with the probable Mob1-homologues of *Drosophila melanogaster*, *Neurospora crassa*, and *Caenorhabditis elegans*, the similarity at the amino acid level is high (81%, 70%, and 69%, respectively).

Discussion

Of the differentially expressed cDNAs isolated, the putative β-tubulins and the putative Mob1-like clone seem of particular interest due to their patterns of expression during megasporogenesis in mutants and wild-type, and their roles in chromosome movement and cell cycle. Tubulins are a component of the cytoskeleton, both in microtubules (MTs) and microfilaments (MFs), and play an important role in spindle formation and chromosome separation during cell division (Baskin 2000), whereas Mob1 is an essential yeast gene required for spindle pole body duplication, completion of mitosis and maintenance of ploidy (Luca and Winey 1998).

The physical movements of chromosomes as well as cytokinesis are both accomplished by means of cytoskeletal structures: the spindle and the phragmoplast, respectively. The formation of 2*n* gametes may depend upon alterations of the cytoskeleton, especially for defects in protein that affect the function and integrity of spindle and phragmoplast MTs, structures directing chromosome separation and cytoplasm division, respectively. Mechanisms of 2*n* egg formation in alfalfa could involve a failure of the cytokinetic machinery. Changes in configurations of both MTs and MFs in meiosis II and in cytokinesis were related to 2*n* gamete formation in *ps* (parallel spindles) and *pc* (premature cytokinesis) meiotic mutants of potato (Genualdo et al. 1998). Moreover, examination of several meiotic mutants of corn, e.g. *dv* (divergent spindle), *Mei1* (meiosis) and *ms* (male sterile), suggested that an abnormal sporogenesis can be correlated with disruption of the cytoskeleton. For instance, abnormalities that affect the spindle pole structure during prometaphase were related to disorganized MT and MF arrays leading to defects in chromosome movement and to a lack of, or abnormal, chromosome segregation. In addition, cytoskeletal arrays seem to be crucial in ensuring the correct completion of meiosis since a partial or total failure of cytokinesis was associated with altered MT and MF dynamics (Staiger and Cande 1993).

The β1-Tub clone isolated from alfalfa proved to be highly expressed in wild type and poorly expressed in the apomeiotic mutants and was shown to be highly homologous to the β1-tubulin gene of soybean (Guiltinan et al. 1987), which is expressed in a very peculiar way given that its transcript is mainly located in etiolated cells. Similarly, light could be an important factor regulating β-tubulin expression also in alfalfa. However, the observation that most plant organs synthesize various mixtures of tubulin isotypes while the male and female gametophytes predominantly express a single, or only a few, tubulin isotypes, points toward a more developmentally defined specificity of the isotypes expressed in reproductive organs (Baskin 2000). Additional investigations are therefore needed to ascertain whether β1-Tub expression is regulated by an environmental factor, such as the absence of light, or by a developmental factor acting in the reproductive organs.

Proper cell division requires precise coordination and execution of several events in the cell cycle, including DNA replication, centromere duplication, mitotic spindle assembly, chromosome segregation, and cytokinesis. A failure in the occurrence or proper timing of any of these events could lead to chromosome segregation defects resulting in aneuploidy or polyploidy. The Mob1 gene product binds to Mps1, a protein kinase that has two important functions with regard to genomic stability. It is required for proper spindle pole body duplication and mitotic checkpoint regulation (Winey et al. 1991; Luca and Winey 1998). Yeast strains harboring Mob1 mutations arrest in late nuclear division at the restrictive temperature. Other alleles also cause a complete increase in ploidy at the permissive temperature – haploid strains appear to become diploid – the gene being required for a cell cycle reset function necessary for the initiation of spindle pole body duplication (Luca and Winey 1998). In particular, amino acid changes caused by point mutations in the conserved domains of Mob1 proved to arrest the mitotic process in late anaphase with elongated bipolar spindles and to yield cells with separated chromosomes and elevated cyclin-dependent kinase activity (Cerutti and Simanis 2000).

The Mob1-like alfalfa clone scored a high similarity, ranging from 68% to 82%, of the deduced amino acid sequence with several Mob1 genes from a variety of organisms, as evolutionarily distant as human, *Drosophila*, *C. elegans*, *Neurospora*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. The presence of this gene in a plant suggests that it may play a key role in normal cell cycle progression in eukaryotes in general. Southern blot hybridization showed that there are multiple copies of the Mob1 gene in the wild alfalfa genome while the analysis of the expression pattern in different plant organs, as detected by Northern blot hybridization, indicated that the Mob1 transcript is present in flower buds at the beginning of meiosis and absent in all the other vegetative organs in mutant plants. As it is difficult to envisage that this gene, essential for mitosis in other eukaryotes, is involved only in plant meiosis, further molecular analyses will be necessary to elucidate its expression as well as the expression of Mob-homologues.

Because apomictic development may be initiated by the inhibition of meiosis in the MMC, the elucidation of megasporogenesis alterations responsible for unreduced egg formation may be crucial for the understanding of apomixis.

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