

Isolation of candidate genes for apomixis in *Poa pratensis* L.

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Abstract

The essential feature of apomixis is that an embryo is formed autonomously by parthenogenesis from an unreduced egg of an embryo sac generated through apomeiosis. The genetic constitution of the offspring is, therefore, usually identical to the maternal parent, a trait of great interest to plant breeders. If apomixis were well understood and harnessed, it could be exploited to indefinitely propagate superior hybrids or specific genotypes bearing complex gene sets. A fundamental contribution to the understanding of the genetic control of the apomictic pathway could be provided by a deep knowledge of molecular mechanisms that regulate the reproductive events. In *Poa pratensis* the cDNA-AFLP method of mRNA profiling allowed us to visualize a total of 2248 transcript-derived fragments and to isolate 179 sequences that differed qualitatively or quantitatively between apomictic and sexual genotypes at the time of flowering when the primary stages of apomixis occur. Three ESTs were chosen for further molecular characterization because of their cDNA-AFLP expression pattern and BLAST information retrieval. The full-lengths of the newly isolated genes were recovered by RACE and their temporal expression patterns were assessed by RT-PCR. Their putative role in cell signaling transduction cascades and trafficking events required during sporogenesis, gametogenesis and embryogenesis in plants is reported and discussed.

Introduction

Seed production generally requires the mating of opposite sex gametes. Apomixis, an asexual mode of reproduction, avoids both meiotic reduction and egg fertilization, because the embryo is formed autonomously by parthenogenesis from either an unreduced egg generated from a megaspore mother cell (diplospory) or a nucellar cell (apospory) (Koltunow, 1993). The main advantages of apomixis are that it reduces the cost and breeding time, avoids the complications of sexual reproduction, such as incompatibility barriers, and viral transfer in plants typically propagated vegetatively (Bicknell and Koltunow, 2004). A deeper knowledge of the mechanisms that regulate

reproductive development would contribute fundamentally to understanding the genetic control of the apomictic process. Although many years of descriptive studies have provided solid documentation of the types of apomictic pathways that occur in a wide variety of plant species, an understanding of the molecular and genetic basis of apomixis lags far behind. Zygotic embryogenesis and apomeiotic parthenogenesis are thought to follow similar pathways during embryo and seed production. Specific genes are activated, modulated or silenced in the primary steps of plant reproduction to ensure that functioning embryo sacs develop from meiotic spores and/or apomeiotic cells. Additional genes may be specifically or differentially expressed between sexually

and apomictically reproducing plants and operate during embryo development. Our molecular understanding of apomixis would be greatly boosted, if genes that are specifically or differentially expressed during embryo sac and embryo formation could be detected.

The generation of transcriptional profiles has numerous applications in plant biology, including identification of tissue specific or developmental stage-specific, and stress-induced transcripts. Molecular differential screening of plants with contrasting modes of reproduction is one of the most powerful tools that can be applied to identifying, mapping and isolating the gene(s) putatively involved in apomixis. Many new techniques have been designed in recent years (Green *et al.*, 2001). All assess new genes, but while some focus on obtaining expression data and high-throughput data, others aim at identifying new and rare differentially expressed transcripts. Some require large amounts of material to be analyzed and pre-existing genomic knowledge. One of the new techniques is based on microarrays (Brown and Botstein, 1999), which allows a genome-wide expression profile of thousands of genes to be performed in one experiment. Though powerful, this approach is expensive and can be readily applied only to model species for which significant genomic information is available (Baldwin *et al.*, 1999). Unfortunately, genetic annotation in higher eukaryotes is limited to a few models and information on less well-characterized species is poor, and likely to remain so for some time. Moreover, because rarely expressed transcripts are usually missing from cDNA libraries due to over-representation of abundant messengers, microarrays could fail to detect genes that are rare but fundamental for traits like apomixis. Differential display (DD), PCR-derived techniques which share gel separation and visualization procedures, but differ in the methods adopted for generating amplified cDNA fragments, would be more suitable for identifying low-expressed genes (Reijans *et al.*, 2003). mRNA fingerprinting strategies permit a large number of transcript-derived fragments (TDFs) to be analyzed and increase the reliability of differentially expressed transcript detection that starts from very small amounts of messengers (Bachem *et al.*, 1996). This feature is essential when DD is applied to tissues where it is hard to isolate stage-specific mRNAs, such as small florets.

cDNA-AFLP (Bachem *et al.*, 1996) has proved the most popular procedure because of its ability to detect differentially expressed genes. It has good reliability and sensitivity, and correlates well with Northern analysis (Durrant *et al.*, 2000; Jones *et al.*, 2000; Barcaccia *et al.*, 2001; Donson *et al.*, 2002; Cnudde *et al.*, 2003). Compared to microarray and GeneChip technologies (Reijans *et al.*, 2003) the reproducibility is very high. A possible drawback of the technique is that more than one band is expected to be visualized for each transcript (Matz and Lukyanov, 1998). However, redundancy can be very informative in cases of alternative splicing. Gene expression-based techniques combined with differential display should extend our knowledge of apomixis and some progress has been made in this direction by studying the gene expression of model system mutants in *Arabidopsis* and rice, where some genes that resemble features of apomixis have been cloned and characterized: *SPL* (Schieffthaler *et al.*, 1999), *NZZ* (Yang *et al.*, 1999), *SERK* (Schmidt *et al.*, 1997; Hecht *et al.*, 2001), *LEC1*, *LEC2* and *PKL* (Ogas *et al.*, 1999), *FIS1* (or *MEA*), *FIS2* and *FIS3* (or *FIE*) (Ohad *et al.*, 1996; Grossniklaus and Schneitz, 1998; Kiyosue *et al.*, 1999; Luo *et al.*, 1999; Vielle-Calzada *et al.*, 1999). Comparative gene expression studies have been carried out during the early stages of apomictic and sexual embryo sac development in *Panicum maximum* (Chen *et al.*, 1999), *Brachiaria* species (Leblanc *et al.*, 1997; Dusi, 2001; Rodrigues *et al.*, 2003), *Pennisetum* (Vielle-Calzada *et al.*, 1996; Jessup *et al.*, 2003), *Paspalum* (Pessino *et al.*, 2001), *Poa pratensis* (Albertini *et al.*, 2003) and apomeiotic mutants of *Medicago falcata* (Barcaccia *et al.*, 2001). However, most were based on subtractive hybridization techniques and isolated only a few genes to which, disappointingly, no clear function could be assigned. Hybridization-based studies, even if negative in context, add support to the proposal that sexual and apomictic developmental pathways differ primarily in their ability to regulate common elements (Bicknell and Koltunow, 2004).

Kentucky bluegrass (*Poa pratensis* L.) is a hardy, persistent, attractive forage and turf grass adapted to a wide range of soils and climate (van Wijk, 1997). Its mode of reproduction is extremely versatile and ranges from naturally obligate apomixis to complete sexuality. Although this species

can hybridize with and absorb entire genomes, it reproduces mainly through facultative apomixis (Barcaccia *et al.*, 1997), which is functionally composed of two processes: apospory and parthenogenesis. In *P. pratensis*, apospory involves the development of embryo sacs from somatic cells that differentiate into the nucellus. If unreduced polar nuclei positioned centrally within the embryo sac fuse with a sperm cell released from the pollen tube (pseudogamy), the unreduced egg can develop autonomously through parthenogenesis to form viable apomictic seeds. Because different plants may have contrasting modes of reproduction, *P. pratensis* could serve as a model species for investigating apomixis and its inheritance (Barcaccia *et al.*, 1998; Albertini *et al.*, 2001a), and differential display techniques should prove effective for cloning candidate apomixis genes in a species as complex as Kentucky bluegrass.

We isolated 179 ESTs whose expression differed quantitatively or qualitatively in apomictic and sexual *P. pratensis* genotypes. The temporal expression of three selected genes was monitored and their putative involvement in apomixis investigated.

Materials and methods

Plant material

A segregating F₁ population of 68 plants was produced by crossing a completely sexual clone (S1/1-7, non-aposporic and non-parthenogenetic) with a highly apomictic genotype (RS7-3, aposporic and parthenogenetic) of *P. pratensis*. Cytological investigations showed that the chromosome number of parental genotypes was $2n = 36$ for S1/1-7 and $2n = 64$ for RS7-3. All progeny plants investigated were sired by agamosperous pollen and had a complement of 50 chromosomes (Porceddu *et al.*, 2002). Plants were investigated for the genetic capacity for apospory and parthenogenesis for 2 years. On the basis of this information, F₁ genotypes were classified into three classes (Albertini *et al.*, 2001b): apomictic (aposporic and parthenogenetic), sexual (non-aposporic and non-parthenogenetic) and recombinant (aposporic but non-parthenogenetic).

mRNA profiling by cDNA-AFLP

Florets of an apomictic, a sexual and a recombinant genotype of *P. pratensis* were harvested and classified into four developmental stages (pre-meiosis, meiosis, post-meiosis and anthesis) according to cyto-histological investigations. Nucleic acids were isolated from about 0.5 g of fresh tissues (spikelets of inflorescences at different developmental stages, leaves and roots) using the GenElute Mammalian Total RNA miniprep kit (Sigma) according to the manufacturer's instructions. Total RNA was purified from residual genomic DNA by using the DNA-free (Ambion) and the mRNA poly(A)⁺ purified by using the GenElute mRNA miniprep kit (Sigma). Reverse transcription and second-strand synthesis was carried out with 1 µg of mRNA polyA⁺ and the standard procedure followed (Sambrook and Russell, 2001).

The mRNA fingerprinting was based on the detection of *EcoRI/MseI* restriction fragments by PCR amplification (using 33 different primer combinations) of cDNA from developmentally staged inflorescences of one apomictic and one sexual genotype. Leaf samples from each genotype were used to eliminate transcript-derived fragments that could be plant-specific rather than reproductive system-related. The restriction of double-stranded cDNA samples and ligation of adaptors were performed simultaneously at 37 °C for 4 h. 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. The selective amplification step was performed with a fluorescein-labeled *EcoRI*+2 or +3 and an unlabelled *MseI*+2 or +3 primers. Interesting fragments were eluted from the gel with 200 µl of sterile distilled water and re-amplified with the same *EcoRI/MseI* primer combination that yielded the specific marker. An aliquot of the re-amplified DNA was cloned into a pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen). Plasmid DNA was purified from 5 ml of an overnight culture of *Escherichia coli* in LB medium using the GenElute Plasmid miniprep kit (Sigma). The sequence of both strands of the plasmid was determined by

using the Big Dye Terminator v. 1.1 Cycle Sequencing kit (Applied Biosystem).

Sequence data analysis

Similarities for all cDNA amplified fragments were searched in the National Center for Biotechnology Information, NCBI (<http://www.ncbi.nlm.nih.gov/>) database using the 2.2.9 release with BLASTN (est_others database), BLASTX and BLASTP (nr_database) applications (Altschul *et al.*, 1990; Altschul and Lipman, 1990; Gish and States, 1993) to compare nucleotide, translated sequences and deduced proteins, respectively. The PAM30 substitution matrix was used for scoring sequence alignments. The most significant amino acid sequence homologies were used for multiple sequence alignments with the VECTOR NTI Suite 8 AlignX software (InforMax) to highlight conserved and variable regions with respect to the cDNA sequence adopted as query. For differentially expressed mRNAs, clone-specific primers were designed using the Primer3 program.

Rapid amplification of cDNA ends analysis

Clone-specific primers were used for performing both 5'- and 3'-rapid amplification of cDNA ends (RACE) to obtain the full-length of genes. The SMART RACE cDNA amplification kit (BD Biosciences) was applied according to the manufacturer's instructions using the mRNA poly(A)⁺ isolated from spikelets at the stage where each differentially expressed TDF was visualized by cDNA-AFLP profiling. Single amplification products from RACE experiments were sticky-end cloned into a pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen). For each transformation eight colonies were used for the sequencing of cDNA inserts to eventually discriminate multiple alleles. The full-length cDNA clones were reconstructed from 5' to 3' RACE fragments using the Vector NTI Suite 8 Contig Express (InforMax).

RT-PCR analysis

First strand cDNA was synthesized from 1 μ g of *P. pratensis* total RNA in a 50 μ l volume (Sambrook and Russell, 2004). RT-PCR reactions were performed in a total volume of 50 μ l containing 1 μ l of

the first-strand cDNA, 1 μ M of each primer, 1 \times PCR buffer, 1.5 mM MgCl₂, 0.2 μ M dNTPs and 2 U of Taq DNA polymerase recombinant (Invitrogen). The reaction samples were denatured at 94 °C for 1 min, and then subjected to 15–30 cycles of denaturation at 94 °C for 1 min, annealing at a temperature specific of each primer pair for 1 min and polymerization at 72 °C for 1 min, plus a final extension at 72 °C for 10 min. PCR products were collected after 18, 20, 22, 24, 26, 28 and 30 cycles to determine the linearity of the PCR. The *P. pratensis* β -*tubulin* gene was cloned using degenerate primers designed on the most conserved region of this gene in grass species, sequenced, and then specific primers (5'-GTGGAGTGGATCCCCAACAA-3' and 5'-AAAGCCTTCCTCCTGAACATGG-3') were designed. The *Pp* β -*tubulin* gene was used as control in all experiments. PCR products of *Pp* β -*tubulin* were collected after 14, 16, 18 and 20 cycles. Within each experiment and for each gene analyzed, the complete set of samples was processed in parallel in a single PCR using aliquots of the same master mix. RT-PCR experiments were carried out on three apomictic, three sexual and two recombinant genotypes. Each set of determinations was performed in triplicate. Amplification products were directly sequenced to verify the allele-specificity of the primer pair used for RT-PCR and then separated on 2% agarose gel, blotted by capillary transfer and linked at 80 °C for 2 h to a Nitran N nylon membrane (Schleicher & Schuell). The ³²P-labelled probes were synthesized by PCR from purified PCR-derived fragments. Filters were hybridized as in Vicient *et al.* (1999). All blots of the same gene were hybridized together, so the probe concentration was identical for all filters. Hybridized filters were washed successively with 2 \times SSC, 0.1% SDS and 0.1 \times SSC, 0.1% SDS at 55 °C. The hybridized blots were first exposed with Kodak AR films (Kodak) then used for the analysis of bound radioactivity with the InstantImager (Packard). The background corrected gross counts (absolute gross counts subtracted by the background levels from corresponding lanes) for each PCR product were quantified using the InstantImager electronic autoradiography software (Packard). To evaluate the temporal changes in relative levels of mRNAs, the corrected gross counts for the target gene were normalized against those of the housekeeping β -*tubulin* mRNA. RT-PCR patterns were reported by using the following terminology: 'upregulated' or

'downregulated' when the expression of a gene increased or decreased, respectively, from pre-meiosis to post-meiosis. The term 'modulated' was used when the expression increased from pre-meiosis to meiosis then decreased from meiosis to post-meiosis or vice versa.

Results

Cloning of differentially expressed genes in apomictic and sexual P. pratensis

The transcript profiling technique used in this study is based on the original cDNA-AFLP

technique (Bachem *et al.*, 1996). When applied on florets of an apomictic and a sexual genotype of *P. pratensis* harvested at three developmental stages (pre-meiosis, meiosis and post-meiosis), it retrieved useful information on gene transcript expression levels and their modulation during flowering. Floral-specific transcripts with qualitative (presence vs. absence) and quantitative differences in apomictic and sexual genotypes could be detected (Figure 1). A total of 2248 TDFs ranging from 80 to 450 bp were visualized by the 33 *EcoRI/MseI* primer combinations, with an average of about 68 bands per assay. As spikelets and leaves shared the vast majority of

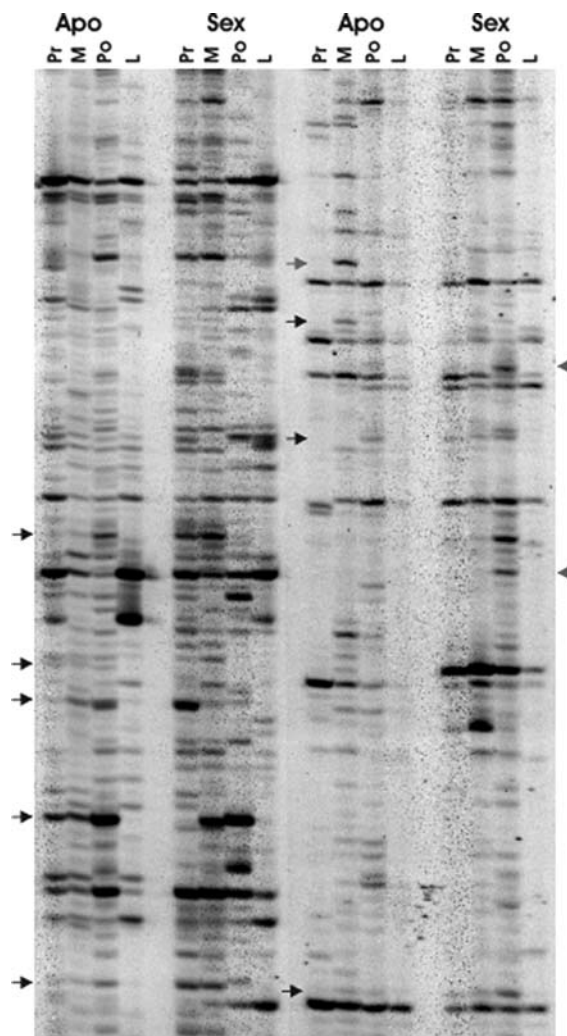


Figure 1. A typical cDNA-AFLP gel picture using cDNA from Poly(A) mRNA extracted from inflorescences at pre-meiotic, meiotic and post-meiotic stages and from leaves of an apomictic and a sexual genotype. Examples of qualitative (grey arrows) and quantitative (black arrows) differentially expressed bands are highlighted.

TDFs, they could be attributed to constitutive clones or clones that were unmodulated during flower development. However, amplified fragments with upregulated and downregulated expression were also scored. Comparison of cDNA-AFLP patterns revealed 179 TDFs (7.9% of total) whose expression was differentially modulated in apomictic and sexual genotypes during flowering (i.e. pre-meiotic, meiotic and post-meiotic stages). These bands were excised from gels, cloned and sequenced and the sequences obtained compared to those available in genomic databases using the BLAST program (Altschul *et al.*, 1990; Gish and States, 1993). As our aim was to detect changes in gene expression in the developing reproductive organs of apomictic and sexual genotypes of *P. pratensis*, the most interesting TDFs are listed in Table 1 together with their expression profiles during female sporogenesis and gametogenesis.

Most importantly about 60% of the 179 qualitatively and quantitatively differentially expressed TDFs manifested highly significant similarity either with genes involved in megasporogenesis, embryo and seed development, or with ESTs isolated from developing spike, flower, embryo sac or pollen-specific libraries. Another 29% of fragments were classified as non-specific, as they shared similarities with genes or ESTs related to flower and vegetative tissue of other plants. BLAST analysis detected no similarities in the remaining 11% of fragments.

A cDNA fragment differentially expressed in apomictic and sexual tissues, recorded as EST #50, shared 63% identity with a GTP-binding protein from *Zea mays* (Table 1). GTP-binding proteins play an important role in the regulation of basic processes in all living organisms. They are, for example, involved in signal transduction, translocation and cell-cycle regulation (Sikora-Borgula *et al.*, 2002). This cDNA fragment was named *PpRABI*.

EST #58 showed 96% identity with a putative nuclear protein from *Oryza sativa* (Table 1), as well as with an EST isolated from an embryo library of *Hordeum vulgare* (accession number A143619). In particular, our cDNA fragment shared 77% identity (e-value of $2e - 04$) with the *CTNNB1* (catenin caderin-associated protein β -like 1, Jabbour *et al.*, 2003) gene of *Homo sapiens* (accession number AAH36739), which encodes for

a protein having predicted structural homology with β -catenin and other armadillo (arm) family proteins. This gene was designated *PpArmadillo-like (PpARM)*.

BLAST analysis of another differentially expressed cDNA fragment, EST #60, present only in the pre-meiotic stage of sexual genotypes, indicated that it shared 71% identity (Table 1) with a probable Ankyrin protein kinase (RING finger homology) of *Arabidopsis thaliana*, 81% (e-value of $3e - 09$) with an EST isolated from an apomictic pistil library of *Pennisetum ciliare* (Jessup *et al.*, 2003) and 88% (e-value of $7e - 69$) with an EST derived from a day after pollination embryo cDNA library of *Hordeum vulgare*. This cDNA fragment was nominated *PpAnkyrin-like Protein Kinase (PpAPK)*.

Because of their cDNA-AFLP expression pattern, BLAST information retrieval and linkage with reproduction-specific genes as shown by comparative genome browsing, these three ESTs were chosen for further molecular characterization.

Cloning of full-length cDNAs of PpRABI, PpARM and PpAPK

The forward and reverse primers designed for 5' and 3' RACE identified two *PpRABI* alleles. *PpRABI-1* is 1021 nt in length. It contains a 612 nt ORF (accession number AJ810707), which encodes a putative 203 amino acid protein, a 304 nt 3'-UTR and a 105 nt 5'-UTR region. The full-length cDNA *PpRABI-2* sequence is 938 nt and contains a 612 nt region that putatively codes for a 203 amino acid protein (accession number AJ810708). The 5'-UTR and 3'-UTR are 110 and 216 nt, respectively. *PpRABI-1* and *PpRABI-2* sequence comparison showed that they share 87% identity. The coding regions were up to 98.4% identical (6 mismatches), whereas the shared identity of UTRs was only 70.2%. While the 5'-UTRs differed for a few residues, mostly due to insertion/deletion, the 3'-UTRs were strikingly diverse and displayed not only several mismatches, but also two long rearranged traits.

The final *PpARM* protein deduced from RACE-derived full-length is 197 amino acids long (accession number AJ810710). Intriguingly, comparison with database proteins reveals that similar proteins in plants are at least 454 amino acids long.

EST	Size (bp)	BLAST result	e-value	Identity	Positivity	Expression ^a							
						APO			SEX				
						S _{pre}	S _M	S _{post}	L	S _{pre}	S _M	S _{post}	L
75	297	Putative DnaI domain containing protein from <i>Oryza sativa</i> (NP_922251)	1e-56	80 (78/97)	90 (88/97)	■	■						
82	144	Putative phosphatidic acid phosphatase beta from <i>Oryza sativa</i> (BAB90492)	4e-08	88 (16/18)	94 (17/18)	■	■						
83	129	Expressed protein from <i>Arabidopsis thaliana</i> (NP_564471)	2e-15	64 (27/42)	83 (35/42)			■	■				
84	95	Phosphatidylinositolglycan class N (PIG-N) family protein from <i>Arabidopsis thaliana</i> (NP_186787)	8e-15	81 (22/27)	96 (26/27)								
90	86	Photosystem II reaction center J protein from <i>Saccharum cereale</i> (P19053)	3e-09	95 (19/20)	95 (19/20)								
91	97	Beta-adaptin, putative from <i>Arabidopsis thaliana</i> (NP_194077)	2e-05	83 (15/18)	88 (16/18)								
92	317	Putative 6b-interacting protein 1 from <i>Oryza sativa</i> (NP_916877)	8e-13	44 (32/72)	65 (47/72)								
94	263	Methyl binding domain protein MBD109 from <i>Zea mays</i> (AAM93219)	1e-13	40 (44/108)	50 (55/108)								
95	149	Putative Ras-GTPase activating protein SH3 domain-binding protein 2 from <i>Oryza sativa</i> (BAD07751)	4e-34	91 (45/49)	97 (48/49)								
97	130	Putative ATP-dependent RNA-helicase from <i>Oryza sativa</i> (BAC98579)	1e-11	95 (21/22)	95 (21/22)								
100	103	Putative SEC6 from <i>Oryza sativa</i> (BAD15629)	2e-22	96 (32/33)	96 (32/33)								
107	82	Aspartate aminotransferase, cytoplasmic isozyme 1/transaminase A (ASP2) from <i>A. thaliana</i> (NP_197456)	9e-08	94 (16/17)	94 (16/17)								
113	102	Poly(ADP)-ribose polymerase from <i>Zea mays</i> (AAC79704)	3e-11	100 (19/19)	100 (19/19)								
114	94	Proline transport protein [imported] from <i>Oryza sativa</i> (T50690)	4e-12	100 (21/21)	100 (21/21)								
115	1100	Putative trehalose-6-phosphate phosphatase from <i>Oryza sativa</i> (NP_922665)	2e-06	84 (16/19)	94 (18/19)								
116	59	Lactoylglutathione lyase family protein/glyoxalase I family protein from <i>Arabidopsis thaliana</i> (NP_200514)	9e-08	89 (17/19)	89 (17/19)								
117	122	Putative CLB1 protein from <i>Oryza sativa</i> (BAD46564)	5e-25	97 (34/35)	100 (35/35)								
118	267	Putative casein kinase 1, delta isoform 1 from <i>Oryza sativa</i> (BAC83610)	1e-59	82 (62/78)	93 (70/75)								
120	146	Putative vacuolar-type H ⁺ -translocating inorganic pyrophosphatase from <i>Oryza sativa</i> (BAD27918)	2e-26	94 (36/38)	97 (37/38)								
122	93	Beta-glucosidase BQG60 precursor, barley, from <i>Hordeum vulgare</i> (A57512)	2e-06	93 (15/16)	93 (15/16)								
133	124	Cytochrome b from <i>Oryza sativa</i> (P14833)	3e-07	100 (16/16)	100 (16/16)								
139	185	OJ000114_01.2 from <i>Oryza sativa</i> (CAE03121)	2e-10	77 (21/27)	88 (24/27)								
143	158	P0490D09.24 from <i>Oryza sativa</i> (similar to A5g43560) (NP_916347)	2e-11	58 (24/41)	70 (29/41)								
144	148	60S ribosomal protein L34 (RPL34B) from <i>Arabidopsis thaliana</i> (NP_177120)	2e-27	88 (39/44)	90 (40/44)								
146	115	P0592G05.9 from <i>Oryza sativa</i> (hypothetical protein similar to A5g63490) (BAB90823)	6e-23	94 (35/37)	97 (36/37)								
149	90	Probable protein phosphatase 2A/B regulatory chain 55K from <i>Oryza sativa</i> (T02952)	2e-11	76 (23/30)	86 (26/30)								
153	81	Putative oxysterol binding protein from <i>Oryza sativa</i> (BAC99388)	2e-11	82 (19/23)	91 (21/23)								
165	140	Cytochrome c oxidase subunit III from <i>Artemia franciscana</i> (NP_007113)	3e-16	79 (27/34)	94 (32/34)								
166	117	NADH dehydrogenase subunit 4 from <i>Oryza sativa</i> (NP_039444)	1e-25	92 (36/39)	94 (37/39)								
171	271	Hypothetical protein Cwai025369 from <i>Crocophlaera watsonii</i> WH 8501 (ZP_00201346)	3e-24	61 (46/75)	66 (50/75)								
172	114	Kinesin motor protein-related from <i>Arabidopsis thaliana</i> (NP_190534)	8e-16	89 (25/28)	96 (27/28)								
173	310	Eukaryotic initiation factor 4A-7 from <i>Nicotiana tabacum</i> (Q0470)	1e-27	75 (39/52)	88 (46/52)								



^aSpikels at pre-meiosis (S_{pre}), meiosis (S_M), and post-meiosis (S_{post}); leaves (L).

Even though derived from conceptual translation, the nearest sequence from human (CTNNBL1 protein, accession number AAH36739) contains only 311 amino acids.

The full-length *PpAPK* is 2501 nt and includes a 300 nt 5'-UTR, an ORF of 1845 nt for 614 amino acids and a 356 nt 3'-UTR (accession number AJ810709).

Sequence analysis

Bioinformatic characterization of isolated cDNAs was carried out using the predicted proteins obtained from the full-length sequences with VECTOR NTI Suite Software (Invitrogen). PpRAB1-1 protein alignments showed it was very similar (e-value $e - 104$, 91% identities and 96% positivities) to the RAB1C protein from *Lotus corniculatus* (accession number CAA98160). It also showed 77% identities and 86% positivities (e-value of $3e - 88$) with the RAB1A protein (member of the RAS oncogene family) of *Homo sapiens* (accession number NP004152). Alignment revealed the RAB domain (e-value $1e - 72$, score 266 bits, 100% aligned), which belongs to the rab subfamily of small GTPases operative in vesicle trafficking.

BLAST analysis revealed high similarity (e-value $e - 103$, 88% identities and 94% positivities) between PpRAB1-2 and the Ras-related protein ARA-5 from *A. thaliana* (accession number P28188). The PpRAB1-2 protein contained a conserved domain very similar to the RAB domain and not unlike that of the Rho domain (e-values $3e - 71$ and $9e - 30$, scores 261 and 124 bits, 100% and 93.8% aligned, respectively). Multiple sequence alignments of a subset of RAB-like, RAS-related and GTP-binding proteins were used to compare available plant sequences. Characterized RAB proteins from mammals were adopted as reference standards. The homology phylogram of RAB-like proteins derived from various genome databases is reported in Figure 2. Ordination analysis of accessions according to protein similarity estimates distinguished subgroups for plant proteins. PpRAB-like proteins were tightly grouped with GTP-binding proteins from other monocotyledons (OsGTP and ZmGTP) and clearly separated from the rest of selected proteins, which mostly belonged to dicotyledons and mammals. *Arabidopsis*, rice and birdfoot trefoil appear to contain at least two distinct types of RAB-like genes. Some of the selected plant

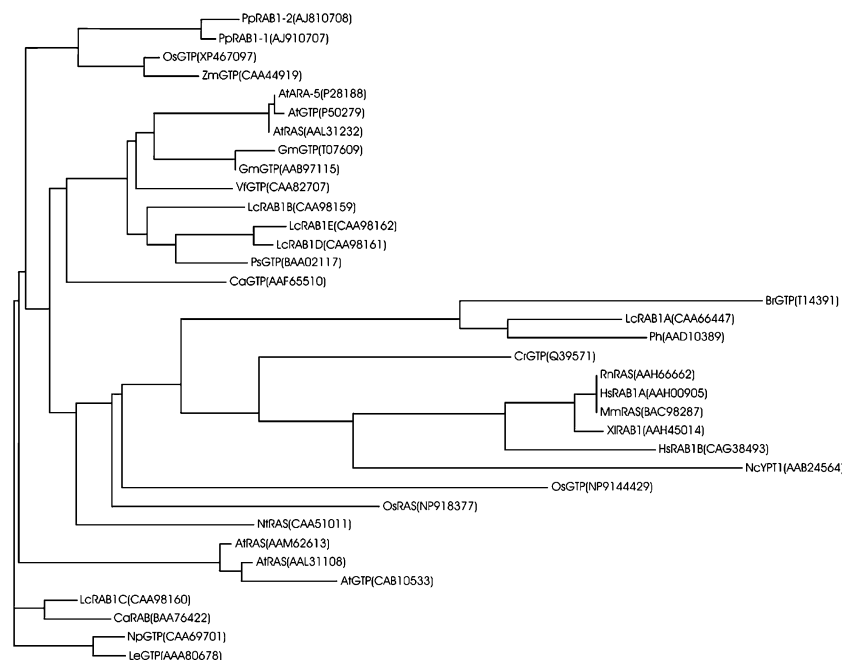


Figure 2. Homology phylogram of RAB-like proteins derived from genome databases. Multiple sequence alignments of a subset of RAB-like, RAS-related and GTP-binding proteins were used to compare available plant sequences. Characterized RAB proteins from mammals were adopted as reference standards.

GTP-binding proteins (e.g. *Brassica rapa*, *O. sativa*, *L. corniculatus*, *Petunia hybrida* and *Nicotiana tabacum*) were grouped with RAB proteins from non-plant organisms apart from other plant accessions (Figure 2), even when they belonged to the same species (i.e. *O. sativa* and *L. corniculatus*).

There was a strong similarity (e-value e^{-136} , 91% identities) between PpARM and a putative nuclear protein from *O. sativa* (accession number XP468544). No known conserved domains were recovered either for this protein or for the most similar proteins of other plant species, despite their being longer.

Database searches revealed marked similarity (e-value 0.0, 92% identities and 97% positivities) between PpAPK and the dehydration-responsive protein-like from *O. sativa* (accession number BAD28913). The PpAPK protein contained a conserved domain very like the uncharacterized DUF248 domain (e-value 0.0, score 804 bits, 100% aligned). Several aligned sequences of DUF248-containing plant proteins either match a methyltransferase profile or contain a sterile alpha motif (SAM)-binding motif, a domain that is widespread in signaling and nuclear proteins. Some family members are described as ankyrin-like.

Owing to the lack of information in genomic databases, neither a multiple sequence alignment nor phylogram construction were performed for the PpARM and PpAPK proteins. Only one accession each for these two genes is currently available for plant genomes (*Arabidopsis* and rice).

Expression of the PpRABI, PpARM and PpAPK in apomictic and sexual genotypes

To confirm the putative relationship of *PpRABI*, *PpARM* and *PpAPK* with apomixis, the expression profiles of each allele of these genes were assessed by RT-PCR analysis. cDNAs of four developmentally staged inflorescences, leaves and roots were probed with primers designed according to allele specific sequences of the corresponding full-length cDNAs. Reactions were performed in triplicate on independently isolated and retrotranscribed mRNAs from three apomictic, three sexual and two recombinant genotypes. Results for each class of genotypes were similar and the mean square ratios from one-way ANOVA never reached the critical value for a

significance at $P = 0.05$. During the whole process, the expression level of β -tubulin cDNA was used as standard for calculating the relative abundance of mRNA for target genes. There was no amplification of mRNAs with specific primers when the RT step was omitted. Figure 3 shows the results for one apomictic, one sexual and one recombinant genotype. The standard deviation for three replicated experiments is given for each sample.

PpRABI-1 was upregulated in sexual and recombinant genotypes from pre-meiosis to anthesis, whereas the expression decreased in apomictic plants from pre-meiosis to meiosis then increased from meiosis to anthesis (Figure 3 A–C).

PpRABI-2 transcript dropped to a very low level, comparable to that of roots, in apomictic genotypes during meiosis. In contrast, the level remained high in sexual and recombinant genotypes, but was higher in the first. Average expression over all stages was modulated in apomictic and recombinant genotypes, whereas it was upregulated from pre-meiosis to post-meiosis in sexual genotypes (Figure 3 D–F). Moreover, *PpRABI-1* and *PpRABI-2* differed in overall expression. In fact, gross counts reported in Figure 3 (A–F) refer to 24 cycles of RT-PCR for *PpRABI-1* and 28 cycles of RT-PCR for *PpRABI-2*.

The expression pattern of *PpARM* was striking. While in sexual and recombinant genotypes it was upregulated, in apomictic genotypes it was downregulated. The expression level in anthesis, roots and leaves was comparable in all genotypes (Figure 3 G–I).

PpAPK expression was modulated in apomictic genotypes, while it was downregulated from pre-meiosis to post-meiosis in sexual and recombinant genotypes. Moreover, expression was higher during pre-meiosis of apomictic genotypes than in those of sexual and recombinant genotypes (Figure 3 J–L).

Discussion

By applying the cDNA-AFLP transcriptional profiling technique to *P. pratensis* in the present research, we isolated messengers from developmental staged inflorescences; 2248 TDFs were detected, as well as 179 mRNAs that differed quantitatively or qualitatively in apomictic and

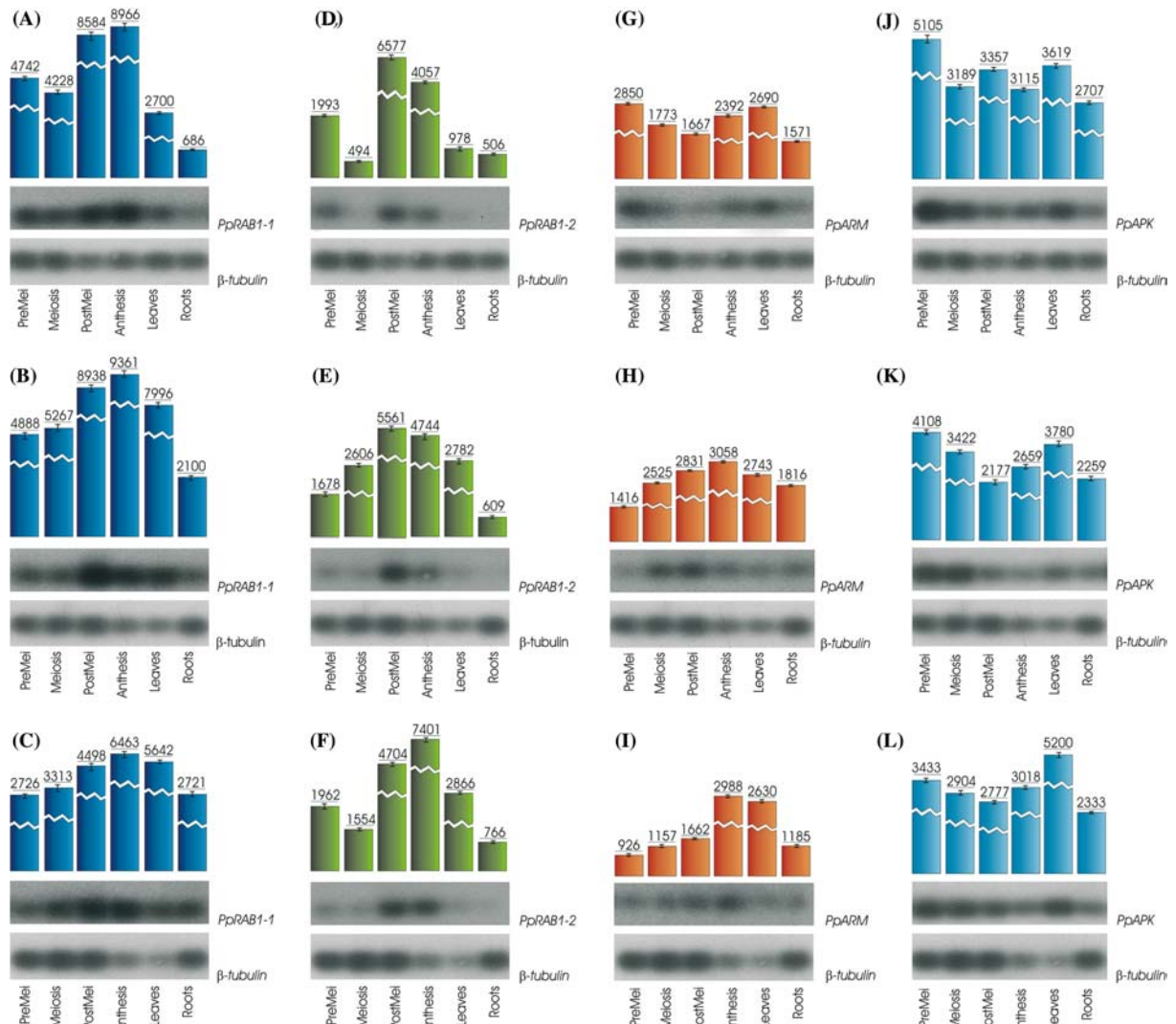


Figure 3. Expression patterns and level of mRNA encoded by *PpRAB1-1* (A–C), *PpRAB1-2* (D–F), *PpARM* (G–I), *PpAPK* (L–N) in apomictic (A, D, G and J), sexual (B, E, H and K) and recombinant (C, F, I and L) genotypes of *P. pratensis*. The PCR products were blotted to a nylon membrane and hybridized. Hybridized blots were exposed to X-ray films (figures) and also used for the analysis of bound radioactivity. The background corrected gross counts (absolute gross counts subtracted by the background levels from corresponding lanes) for each PCR product were used for relative mRNA quantification with the InstantImager electronic autoradiography software (Packard). Gross counts were performed after 24 cycles of RT-PCR for *PpRAB1-1*, *PpARM* and *PpAPK* and 28 cycles for *PpRAB1-2*. To evaluate the temporal changes in relative levels of mRNAs, the corrected gross counts for the target gene were normalized against those of the housekeeping β -tubulin mRNA. Values are expressed in gross counts. Each experiment was performed in triplicate. The standard deviation is shown for each sample. Figures show the results for one apomictic, one sexual and one recombinant genotype since quantitative differences between genotypes with same mode of reproduction were statistically not significant.

sexual genotypes. When used as queries for gene bank searches, 60% of the TDFs retrieved sequence similarity with ESTs or proteins found in developing spikes, reproductive organs or tissues (ovary, ovule and embryo sac) and seed components (embryo and endosperm).

Since the vast majority of genes expressed in florets differed in their expression in timing of activation/inactivation rather than for presence vs. absence of specific transcripts, zygotic embryogenesis and apomeiotic parthenogenesis processes probably share a pathway. Only 1.6% of total

TDFs were sexual- or apomictic-specific. Even this could be an overestimation seeing that some bands present only in a single stage of a single genotype could have been due to a typical AFLP polymorphism rather than a gene specifically expressed at this stage.

Apomixis could reflect a deregulation of the sexual reproductive program in time and space that leads to changes in the cell fate and the omission of steps critical to sexual progression (Bicknell and Koltunow, 2004). Signalling changes resulting from ovule malformation could stimulate a parthenogenetic embryo-development program in a set of somatic cells in a manner not unlike that met in stress-induced somatic embryogenesis (Mordhorst *et al.*, 1997; Bicknell and Koltunow, 2004). A similar mechanism could also explain why, in ovules, an apomictic program leads to unreduced embryo sacs. The cDNA-AFLP investigation allowed us to isolate a group of genes putatively involved in signaling and trafficking events required during sporogenesis, gametogenesis and embryogenesis in plants. Several kinases (ESTs #32, #51, #60, #72, #118) were differentially expressed in apomictic and sexual genotypes. Some of them were of major interest because they are known to be involved in the GTPase activating program (EST #32) and the osmotic stress response (EST #62). Two other amplified fragments were of interest. One (EST #72) was a putative kinase interacting protein that contains the chromosome segregation ATPase domain required for cell division and chromosome partitioning, the other (EST #118) a casein kinase CK1. Although most of these proteins has not been well characterized in plants, their homologues have been studied in mammals. For example, *CKI* is believed to be an integral part of a molecular machine whose purpose is to mark β -catenin (similar to EST #58) for degradation. Based on its role in the destruction of β -catenin, the loss of *CKI* function would be expected to stabilize β -catenin and activate expression of target genes. Mammalian β -catenin is required for both cell-cell adhesion and regulating gene expression during development (Conacci-Sorrell *et al.*, 2002). Stabilized β -catenin is targeted to the nucleus where it can exert an effect on gene expression. The transcriptional targets of β -catenin include regulators of cell proliferation required during development (Coates, 2003). Mammalian β -catenin is the homologue of *Drosophila* Arm (Armadillo), so

named because of the appearance of embryos that are mutant for the *Drosophila* segment polarity gene *armadillo*, the founding member of the family (Nusslein-Volhard and Wieschaus, 1980; Riggleman *et al.*, 1989). Although originally characterized in animals, proteins containing Arm repeats also exist outside the animal kingdom (Coates, 2003). Moreover, the presence of Arm repeat proteins in unicellular eukaryotes, animals and plants suggests that this protein family has ancient evolutionary origins. One hypothesis is that Arm family proteins have function in plants similar to those in other organisms, and several recent studies have shown that this suggestion is partly correct. One of the most important Arm-containing group proteins in *Arabidopsis* are kinesins (similar to EST #172) that likely interact with microtubules because they possess an N-terminal kinesin domain. It is possible that *Arabidopsis* kinesin Arm proteins function analogously to a complex of Arm repeat proteins that interact with microtubules in mammalian cells. Mammalian kinesin family proteins interact with an Arm family protein called SMAP (Smg-GDS-associated protein), which in turn interacts with Smg-GDS, an Arm repeat exchange factor for small GTPases (GTP-binding protein). GTP-binding proteins (EST #50) are involved in signal transduction pathways in animal and plant systems (Lee and Assmann, 1999). cDNAs that encode G proteins α and β subunits have been isolated in *Nicotiana plumbaginifolia* (Kaydamov *et al.*, 2000) and their involvement in early somatic embryo development investigated. It was hypothesized that at least one of these genes regulates cell division and differentiation and/or nutrient accumulation/transport, and also triggers several transduction events during plant growth and development (Kaydamov *et al.*, 2000). A GTP-binding protein recently cloned in starfish (*Asterias amurensis*) (Lamash, 2001) is known to be implicated in the 1-methyladenine-induced maturation of starfish oocytes (Hoshi *et al.*, 1992; Jaffe *et al.*, 1993).

We selected three amplified fragments involved in signal transduction for deeper investigation. All three were chosen because of their expression pattern and database information retrieval. The first two are a *RABI*-like gene and a β -catenin-like/*Armadillo*-like gene (ESTs #50 and #58, respectively); the third an *Ankyrin protein kinase*-like gene (EST #60).

The full-lengths of the selected ESTs were obtained by RACE and the protein deduced. Two members were found for the PpRAB1 protein. They were similar to a RAB1 protein and contained the RAB domain. The RAB1 gene is involved in nodular membrane proliferation in *Glycine max* (Cheon *et al.*, 1993). Moreover, the RAB1 3'-UTR region sequence is reported to have been conserved over infinite time in amniote vertebrates with some exceptions. In fact, there were no significant homologies between the 3'-UTR of *LcRAB1A* and that of other genes, including other members of the *RAB* family (Wedemeyer *et al.*, 2000). Even if all RAB proteins were involved in intracellular vesicle transport, the cellular compartment in which they were active would be clearly distinguished. It has been suggested that the function of *RAB1* 3'-UTRs is to localize the messenger to specific regions of the cytoplasm, so that translation occurs close to the localization of the respective RAB protein (Wedemeyer *et al.*, 2000). The two *PpRAB1* genes we found differed profoundly in their 3'-UTR regions, leading us to believe that they are involved in different localization of *PpRAB1-1/2* transcripts and their respective proteins. For example, the almost complete absence of the messenger in meiosis of the apomictic genotypes could mean that timing of expression is different in *PpRAB1-1* and *PpRAB1-2* transcripts and their localization diverse. It is thought that there is a point at which ovule nuclear cells act as a sink for all sources of nutrients and shunt the regular nutrient traffic from the dividing megaspore mother cell to themselves. We speculate that there is temporary inactivation of the gene during meiosis, but that although the function is restored during post-meiosis, the gene products are translocated to compartments other than their typical ones.

The third EST we chose for further characterization was an Ankyrin protein kinase. ANK repeats are presumed to mediate protein-protein interaction among diverse groups of proteins (Albert *et al.*, 1999), including those that take part in signal transduction processes (Artavanis-Tsakonas *et al.*, 1991), cytoskeleton interactions (Bennett, 1992) and regulation of the cell cycle (Breedon and Nasmyth, 1987). Very few proteins containing ANK repeats have been characterized in plants and their precise molecular functions

are poorly understood (Albert *et al.*, 1999). An Ankyrin protein kinase was recently isolated in *Medicago sativa* and it was postulated that *APKs* are a novel type of protein kinase genes and that they are involved in osmotic stress responses (Chinchilla *et al.*, 2003). Moreover, *MsAPK1* was induced in spontaneous nodules despite the fact that basal expression levels were detected in all *Medicago* tissue tested (Chinchilla *et al.*, 2003). It, therefore, seems that *APKs* may be involved in the cell shape response to osmotic stress in alfalfa.

A feasible interpretation of our data is that the changes provoked by malformed ovules trigger a parthenogenetic program in a set of cells, not unlike the stress-induced by somatic embryogenesis, and that the differential expression of the genes we isolated from apomictic and sexual genotypes of *P. pratensis* is involved in the apomictic pathway of seed formation.

Conclusions

The cDNA-AFLP differential display method retrieved useful information on gene expression levels and their changes during reproduction in *P. pratensis* florets. Informative mRNA fingerprints were also obtained and more than two thousand TDFs visualized. A major finding was that most genes expressed in florets displayed similar patterns in sexual and apomictic pathways. Of the about 8% of mRNAs differentially expressed in apomictic and sexual genotypes, the vast majority proved to be attributable to genes whose temporal expression was differentiated during flowering. The fact that very few sexual- or apomictic-specific mRNAs were detected is evidence that a highly conserved developmental program exists in embryos during zygotic embryogenesis and apomeiotic parthenogenesis. Signaling mechanisms in ovules may promote an apomictic and/or parthenogenetic pathway that leads to embryo sac or embryo development and so replaces the regular pathway of sporogenesis and embryogenesis typical of sexual species. The studies we are presently carrying out are aimed at ascertaining whether the three isolated genes function in the apomictic pathway, and whether, in ovules, their expression correlates with embryo sac and embryo development.

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