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# Identification of genes specifically expressed in cauliflower reproductive meristems. Molecular characterization of *BoREM1*

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**Abstract**

Using the meristems of the cauliflower curd as a source of tissue and a series of subtractive hybridizations and amplification reactions, we have constructed a cDNA library highly enriched in cDNAs expressed in reproductive meristems. The analysis of a sample of 250 clones from this library identified 22 cDNA clones corresponding to genes specifically expressed in these cauliflower meristems. Apart from two clones that corresponded to *APETALA1*, and two other ones showing similarity to different aminoacyl-tRNA synthetases, the remaining clones showed no similarity to any sequence in the databases and may correspond to novel genes. One of these clones, *BoREM1*, was further characterized and found to correspond to a gene encoding a protein with features of regulatory proteins that follows a expression pattern very similar to the *LEAFY* transcripts.

## Introduction

The transition from vegetative to reproductive growth represents one of the most important and conspicuous developmental switches in plants. The use of experimental systems like *Arabidopsis* and *Antirrhinum*, and a combination of genetic and molecular tools, are allowing the identification of the genetic determinants responsible for the time control of this switch and unravelling the molecular mechanisms involved (see [25] for a recent review). In *Arabidopsis*, more than 30 genes with different roles in the control of flowering time have already been identified [29, 23] and several of them have been cloned and characterized at the molecular level, through positional cloning or insertional mutagenesis approaches (see [25, 23, 37] for recent reviews). The identity of the floral meristems that are formed at the apex is mainly controlled by the activity of genes like *LEAFY* (*LFY*) [47] and *APETALA1* (*AP1*) [28] which are not only required but sufficient for the initiation of flowers [48, 27]. Moreover, other genes like *APETALA2* (*AP2*) [18], *CAULIFLOWER* (*CAL*) [21], *AGAMOUS* (*AG*) [34], *UNUSUAL FLORAL ORGANS* (*UFO*) [16], and *FT* and *FWA* [41], also play a role in the establishment of flower meristem identity.

Despite the success of genetic analysis in the identification of the main genetic determinants responsible for floral initiation, other genes involved in this process could have escaped primary mutant screening. Mutations in those genes could have been missed either because they cause lethality or strong pleiotropic effects, or because they may not provoke any pheno-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF051772.

typic effect due to genetic or functional redundancies [36]. In fact, secondary mutant screenings performed on mutant backgrounds have identified second site mutations that do not produce any phenotypic effect by themselves [21], and many cloned genes have been shown to belong to small gene families [39, 26]. In this context, a molecular approach designed to identify genes with specific expression patterns can be a useful approach for the identification of new genes involved in the vegetative to floral transition at the apex. Several reports have described the isolation of genes expressed in shoot apical meristems during the floral transition by performing differential hybridizations on apical meristems at different stages of the transition [31, 20, 32], or directly on proliferating reproductive meristems from cauliflower or the *anantha* mutant of tomato [30, 38, 45]. However, most of the cDNA clones isolated corresponded to genes not especifically expressed in the shoot apex at the floral transition [31], or which function could not be directly related to the floral transition itself [30, 38, 45]. Only in one case the identified gene has been shown to have an effect on flower initiation when overexpressed in transgenic *Arabidopsis* plants [19]. These molecular approaches have been hampered by the scarcity of meristematic tissue, difficulty in the determination of the developmental stage of meristems and the level of specificity provided by differential hybridization, and generally do not contemplate cDNA samples from all possible tissue sources as negative probes.

In order to identify new genes specifically expressed in reproductive meristems that could be involved in the floral transition, we have tried a novel approach that combines the developmental features of *Brassica oleracea* var. *botrytis* (cauliflower) and other cultivated forms of this species, with the use of subtracted cDNA libraries and probes. In cauliflower, the development of the curd is the result of the proliferation of indeterminate inflorescence meristems that are arrested in a stage previous to floral initiation [42, 21]. For this reason, the curd is considered as an early stage of inflorescence development and represents an interesting source of plant material to identify genes specifically expressed in reproductive meristems. Recently, the orthologues of *LFY* and *AP1* have been cloned in cauliflower and their mRNAs detected in the cauliflower curd, suggesting that the curd contains a combination of inflorescence and floral meristems [2, 3, 4, 5, 9]. Another form of *B. oleracea*, the variety *geminifera* (Brussels sprouts), presenting proliferation of lateral vegetative meristems, was used as a source of vegetative meristem cDNAs for the enrichment. Here, we present the strategy followed for the construction of a highly enriched reproductive meristem cDNA library, the isolation of specific cDNAs from reproductive meristems, and the molecular characterization of *BoREM1*, a gene corresponding to one of these cDNAs.

## Material and methods

### *Plant material*

Organ and tissue samples were obtained from cauliflower (*Brassica oleracea* var. *botrytis* cv. Snow Crown), Brussels sprouts (*Brassica oleracea* var. *gemmifera* cv. Jade) or broccoli (*Brassica oleracea* var. *italica* cv Grande) plants grown in a greenhouse or locally purchased.

### *RNA purification and synthesis of cDNA*

Total RNA from adult plant organs was extracted as described [6]. Poly(A)$^+$ RNA was prepared using the Quick Prep mRNA purification kit (Pharmacia) following the manufacturer's indications. A 5 $\mu$g portion of poly(A)$^+$ RNA from curd meristems, roots, stems, curd stems and flowers from cauliflower, and meristems from Brussels sprouts, were separately employed for synthesis of double-stranded cDNA, according to cDNA Synthesis System Plus (Amersham) instructions, using random priming for first-strand synthesis.

### *Subtractive hybridizations and construction of an enriched cDNA library*

Fractionation of cDNAs, digestion, linker ligation, PCR amplifications, photobiotinylation of driver cDNAs and subtractive hybridizations were performed as previously described [46]. Equalization of cDNAs was performed by subjecting the pool of cDNAs to three succesive cycles of self-subtraction where a 1:1 ratio of biotinylated driver to non-biotinylated tracer was used [22]. Tracer cauliflower meristem cDNA was subtracted twice with a driver biotinylated-equalized pool of cDNAs using a ratio of 1:20. The enrichment procedure was tested by hybridizing samples of the subtracted cDNA populations obtained with a *Bam*HI-*Kpn*I fragment from *LEAFY* plasmid pDW122 [47]. Finally, 100 ng of the cDNA population obtained after 10 rounds of subtraction was *Eco*RI-digested, ligated into $\lambda$ZAP-II arms (Stratagene Cloning Systems) and

packaged into bacteriophage using Gigapack Gold extracts (Stratagene Cloning Systems), following the manufacturer's specifications. A total of 80 000 pfu were obtained in the primary library, with 80% recombinant clones. To identify clones with the required hybridization signal, the library was plated at low density on XL-1 Blue cells and transferred onto Hybond $N^+$ membranes (Amersham). Selected plaques were eluted in SM buffer [43] and the phage DNA amplified by standard PCR, with T3 and T7 primers. Equal amounts of the PCR products derived from cDNA clones were dot blotted under vacuum onto two different Hybond N membranes (Amersham) and hybridized either with the subtracted cDNA population or with a mixture of cDNAs from all the other organs. As a final step in the selection of the cDNA clones, 1 $\mu$g of organ-derived cDNAs obtained through PCR amplifications was dot-blotted as above, and the blots hybridized with the PCR products from cDNA clones as probes.

*DNA and RNA gel blots*

DNA and RNA were size-fractionated by standard techniques [43] and transferred onto Hybond $N^+$ membranes (Amersham). DNA probes were labeled with $\alpha$-$^{32}$P-dCTP using Megaprime DNA Labelling System (Amersham). Hybridization solution was prepared according to Amersham's Hybond $N^+$ protocol. For high-stringency conditions, hybridizations were carried out at 65 °C overnight, and washed in 2× SSPE-0.1% SDS, 1× SSPE-0.1% SDS and 0.1× SSPE-0.1% SDS at 65 °C, 20 min each. Moderate-stringency hybridization was performed at 60 °C and washed in 2× SSPE-0.1% SDS at 60 °C for 30 min. Membranes were exposed to X-ray films (Amersham) at −80 °C until visualization. The integrity and relative quantification of poly(A)$^+$ RNAs were assayed through hybridizations with $\alpha$-$^{32}$P-dCTP-labeled $(dT)_{20}$ with terminal transferase (Promega) [17]. Autoradiographic films were digitalized in a Scanmaster 3+ scanner and analyzed for densitometry with BioImage software (Millipore).

*Rapid amplification of cDNA ends (RACE)*

First-strand cDNA was synthetized from 1.5 $\mu$g of cauliflower meristem poly(A)$^+$ RNA with Superscript reverse transcriptase (Gibco-BRL) and $dT_{30}$ adapter as primer, according to Marathon cDNA Synthesis Kit (Clontech). After second-strand synthesis, cDNA ends were blunt-ended with T4 DNA polymerase and ligated to a partially double-stranded adapter oligonucleotide, using T4 DNA ligase. A 1:50 dilution of the cDNA was employed as template for PCR reactions, using the adapter primer (Clontech) and the CM2 primer (5′-CCCTCTCTCCATCTATTTCTACTC) for the amplification of the 1192 bp fragment, and the CM4 primer (5′-TTTGTCCGAGGCGTCTG) to obtain the 198 bp cDNA fragment. PCR reactions were performed with Long Expand Polymerase (Boehringer Mannheim) at an annealing temperature of 58 °C during 30 cycles. PCR products were excised from the gel, blunt-ended with Klenow polymerase (Promega) and ligated into the *Sma*I site of Bluescript (Stratagene) for sequencing.

*DNA sequencing*

Nucleotide sequence analysis was carried out using Sequenase Ver 2.0 (USB) and synthetic primers. All sequences were analyzed for homology to databases using the website of the NCBI (http://www.ncbi.nlm.nih.gov) running the BLAST programs [1] against 'nr', 'GenBank' and 'dBEST' data banks. Other sequence analyses and manipulations were carried out with the PC-Gene programs (Intelligenetics, Mountain View, CA).

In situ *hybridization*

Antisense and sense *BoREM1* and *BoLFY* probes were labeled with digoxigenin-11-UTP (Boehringer Mannheim) using T3 or T7 polymerase following the protocol of the manufacturer. DNA used as template for the synthesis of the *BoREM1* probe was obtained after linearization of CM6.2 clone (287 bp, 1456–1743 of the cDNA *BoREM1*). The synthesis of the *LFY* probe was accomplished from the *BoLFY* clone isolated from the subtracted cauliflower meristem cDNA library constructed in this work (276 bp, corresponding to 232–324; [2]). Hybridization was carried out on 8 $\mu$m tissue sections that had been fixed in 2% formaldehyde, 5% acetic acid, 60% ethanol, dehydrated in ethanolic series, and embedded in Paraplast Plus (Sigma). Paraffin was removed with xilene and tissue sections were pre-treated, hybridized and washed according to Huijser *et al.* [14]. Immunological detection was performed according to DIG Nucleic Acid Detection Kit (Boehringer Mannheim). The hybridized sections were visualized and photographed under dark-field conditions in a Labophot-2 microscope (Nikon).

## Results

### *Identification of genes specifically expressed in cauliflower reproductive meristems*

With the aim of identifying genes specifically expressed in the reproductive meristems of cauliflower, we constructed a cDNA library enriched in cDNA from the curd meristems. This enrichement was obtained through the three steps that are shown in Figure 1A. The first step consisted in six succesive rounds of subtraction of cDNA from the curd meristem with cDNA from Brussels sprouts vegetative meristems, as previously described [46]. The final goal of this step was to eliminate from the curd cDNA the molecules corresponding to genes related with the meristematic activity itself. The resulting cDNA population was used to construct a library that was screened for cauliflower curd meristem-specific cDNA clones with the same subtracted cDNA as a probe. Most of the clones isolated corresponded to abundant transcripts, and none of them resulted to be specifically expressed in the cauliflower curd meristem (data not shown). The second step consisted in two rounds of subtraction of the reproductive meristem cDNAs with the cDNA clones identified in the first screening. Finally, the third step corresponded to two rounds of subtraction with an equalized cDNA population from different tissues of cauliflower. To obtain this equalized cDNA population, we prepared cDNA samples from roots, stems, curd stems and flowers of cauliflower. Equal amounts of each cDNA were then mixed and subjected to three rounds of self-subtraction, as described [22]. The enrichment process of the succesive inflorescence meristem cDNA populations was followed by cDNA blot hybridizations with a *LFY* probe, whose expression has been shown to be low and restricted to the cauliflower curd meristem [3, 5]. As shown in Figure 1B, as succesive rounds of subtraction were performed, a further enrichment in *LFY*-hybridizing cDNAs was obtained. We estimated that the final cDNA population presented at least a 100-fold enrichment in *LFY* cDNAs with respect to the initial cDNA population from cauliflower meristems (data not shown). Finally, the enriched cDNA population was used to construct a library in λZAPII.

When using a cDNA population as a probe to screen a library, clones giving a strong hybridization signal correspond to the abundant transcripts in the probe. A reconstruction experiment, with *LFY* as a probe, demonstrated that clones carrying *LFY*

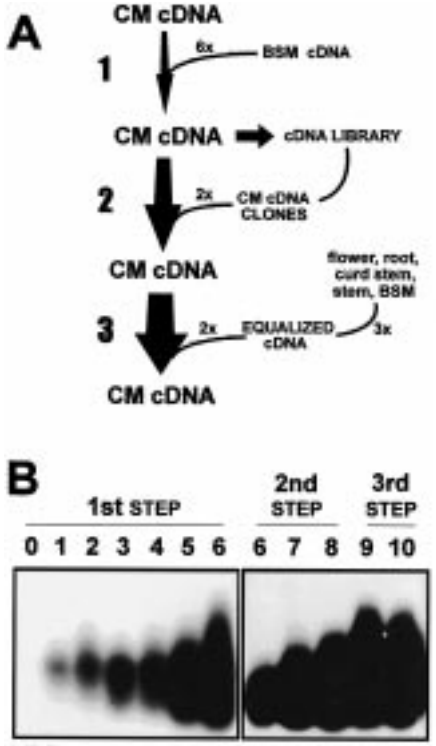


*Figure 1.* Enrichement of the cauliflower curd meristem cDNA population. **A.** Flow diagram representing the three subtraction steps of the cauliflower reproductive meristem cDNA (CM cDNA). In step 1, six subtractive hybridizations were performed with Brussels sprouts shoot apical meristem cDNA (BSM cDNA), according to Wang and Brown [46]. Step 2 consisted in two subtractive hybridizations with cDNA clones corresponding to abundant, non specific, curd meristem transcripts. In step 3, two subtractive hybridizations were carried out with an equalized pool of cDNAs from cauliflower roots, stems, curd stems and flowers, and Brussels sprouts meristems. Equalization of cDNAs was performed according to Ko [22]. **B.** Enrichment in *LEAFY* cDNAs. DNA blot hybridization of cDNA samples (1 $\mu$g) obtained after each round of subtraction with an *Arabidopsis LEAFY* cDNA probe [47]. the last CM cDNA population obtained (10) was used for the construction of an enriched cauliflower curd meristem cDNA library.

sequences gave weak hybridization signals with the enriched cDNA probe, indicating that, even in the enriched cDNA population, these transcripts were not highly abundant. Thus, in order to identify cDNAs specific for reproductive meristems we selected those phage which gave weak or null hybridization signal. These phage were identified as those with similar or lower signal than phage containing a *LFY* insert, identified with the *LFY* probe. DNA from isolated phage was amplified and hybridized with a mixture of cDNAs from roots, stems, curd stems and flowers from cauliflower, and meristems from Brussels sprouts, to eliminate those clones that showed hybridization with

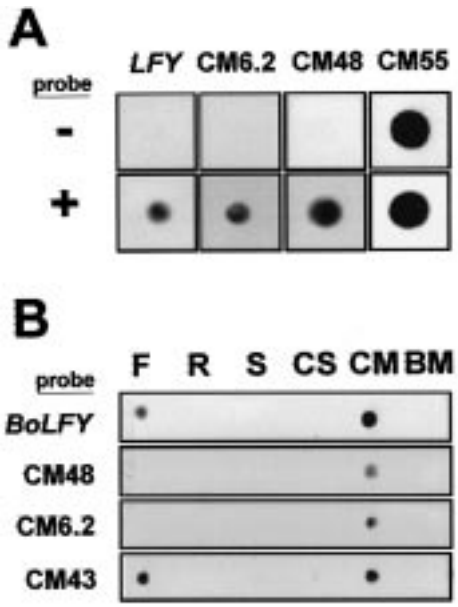


*Figure 2.* Selection of specific cauliflower reproductive meristem cDNA clones. **A.** Differential dot blot hybridizations of different cDNA clones with a curd meristem cDNA probe (+) and a mixed probe containing cDNAs from flower, root, stem and curd stem from cauliflower, and from shoot apical meristems from Brussels sprouts (–). **B.** Dot blot hybridizations of cDNAs prepared from different organs with probes corresponding to several cDNA clones. F, cauliflower flower; R, cauliflower root; S, cauliflower stem; CS, cauliflower curd stem; CM, cauliflower meristem; BM, Brussels sprouts shoot apical meristem. See Table 1 for a brief description of the cDNA clones.

cDNAs from other organs than the curd meristem (see Figure 2A for an example). Finally, a set of diagnostic dot blots containing cDNAs from different organs were hybridized with probes from the selected cDNA clones, allowing to select 22 clones from an initial sample of 250 cDNA clones (an example is shown in Figure 2B). The sequence of these cDNA clones revealed that two clones corresponded to the cauliflower *APETALA1* gene, as could be expected from the subtraction procedure followed for their isolation (Table 1). Two other clones showed high similarities to two different aminoacyl-tRNA synthetases (Table 1). These genes are expected to be expressed at higher levels in proliferating cells. Finally, the other 18 clones did not show any significant similarity to sequences present in the databases, although three of them corresponded to the same gene sequence.

### *Molecular characterization of* BoREM1

One of the resulting 16 cDNA clones, CM6.2, was selected for further characterization. Since sequence analysis of this clone had revealed that it contained a 287 bp insert without any detectable open reading frame (ORF), we screened a cauliflower curd cDNA library to isolate a longer cDNA clone. The only phage identified (out of over 250 000 pfu screened) yielded a cDNA insert which encompassed the entire 3′-untranslated region (3′UTR) and a putative 258 bp long ORF. A longer cDNA clone (1192 bp) and one additional fragment (198 bp), corresponding to the 5′

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1    TACTAATAAGAATACAGAAGTTTCCCTTTCATTATTCTCTCTCTACTTAGCTCTGCTC
61   TGAACAATGGTTCCTCCTCCACAACCCTCTCTGTTTCAACTGACATTTCTTACCGGAGAC
          M  V  P  P  P  Q  P  S  L  F  Q  L  T  F  L  T  G  D    18
121  AAACCAATTCTGACGCTTGATGATGAGTTCATAAGTAGTCACACGAAAGTTTTGCTGATA
       K  P  I  L  T  L  D  D  E  F  I  S  S  H  T  K  V  L  L  I    38
181  TCAGACGCCTCGGACAAAATATGGGAAGTGAAACTGGACGGCAACACAGGCTCGCCGGCGGC
       S  D  A  S  D  K  I  W  E  V  K  L  D  G  N  R  L  A  G  G    58
241  TGGGAAGAGTTCGCCGCCGTTAATAATTTCAGCGAGGGAAACGTCTTGGTTTTAGACAC
       W  E  E  F  A  A  V  N  N  F  S  E  G  N  V  L  V  F  R  H    78
301  AATGGAGAAGAGATCTTTCATGTGGCTGTTTCCAGTGAATCTGATGATGATGAGTCCGAT
       N  G  E  E  I  F  H  V  A  V  S  S  E  S  D  D  D  E  S  D    98
361  GATACTGACGACAGTGAATCTGATGATGAGTCCAATGACACTGACGACAGTGAATCTGAT
       D  T  D  D  S  E  S  D  D  E  S  N  D  T  D  D  S  E  S  D   118
421  GATTCCGAAGATAATGGTGAAGGGGACAGTAGTTTGGTGAACAAGGAAGCAGATTCTTCA
       D  S  E  D  N  G  E  G  D  S  S  L  V  N  K  E  A  D  S  S   138
481  TCAGACTGTTTCCTCAGAGCTCGTGTCACTCCTTATAGCCTCACCAAAGATCGTCTTGAT
       S  D  C  F  L  R  A  R  V  T  P  Y  S  L  T  K  D  R  L  D   158
541  CTTTCTAAAGATTTTAAGTTTATGTTGTTTGATGAGCACAACAAACCGTGTGAGATATAC
       L  S  K  D  F  K  F  M  L  F  D  E  H  N  K  P  C  E  I  Y   178
601  TTAGTTAATGAGAAAGGAAGAAAATGGACACTGAGGCTTTCAAGAAACATATCAAGTGGT
       L  V  N  E  K  G  R  K  W  T  L  R  L  S  R  N  I  S  S  G   198
661  GCGTTTTACATCACAAGAGGCTGGGCTAACTTTTGCTCTGCTAATGGGTTAATCCGAGGT
       A  F  Y  I  T  R  G  W  A  N  F  C  S  A  N  G  L  I  R  G   218
721  GACTTTTGTTACTTCAAACTTTCCGAAAGTGGGGAAAGGCCTGTGCTTTTGTTGTGTTCA
       D  F  C  Y  F  K  L  S  E  S  G  E  R  P  V  L  L  L  C  S   238
781  CACGAGTCTGGCAATGGCCATGAAGACAAAGAAGAAGAAGAAGAATGCCCTGAAGCAGAT
       H  E  S  G  N  G  H  E  D  K  E  E  E  E  E  C  P  E  A  D   258
841  ACATTGAAGATATGTTCTGTAGGAGGCTGCAGCAACGAGAAGAACACTCCTTCTCGCTTT
       T  L  K  I  C  S  V  G  G  C  S  N  E  K  N  T  P  S  R  F   278
901  CTGACACAAAAGTTTACACCAAGCCGTTTCAAGACCGGGCAACTATATATTTCAATGCTT
       L  T  Q  K  F  T  P  S  R  F  K  T  G  Q  L  Y  I  S  M  L   298
961  TCATCGGGTGGCTTGCGTGAGAGCGGCATTAAGAAGACTGGGGAGATAATTCTGCTGGAC
       S  S  G  G  L  R  E  S  G  I  K  K  T  G  E  I  I  L  L  D   318
1021 AATGATGGAAGAAAGTGGCCATCTTATCTAAACAAGACAGGACAACCCGGAGGTGAATGG
       N  D  G  R  K  W  P  S  Y  L  N  K  T  G  Q  P  G  G  E  W   338
1081 TGTTACATAAGAAAAGGTTGGAGAGAGATGTGCGAGGCGAATGGAGTTGAAGTGAATGAT
       C  Y  I  R  K  G  W  R  E  M  C  E  A  N  G  V  E  V  N  D   358
1141 TCATTCGTGTTGGAGTTGATATGCGAAGCTGCAAACCCTATCTTTAAGCTCCATTCTAAG
       S  F  V  L  E  L  I  C  E  A  A  N  P  I  F  K  L  H  S  K   378
1201 ATTAGAAACAAGGGAAAAGGAAACATAGTAACTAGTAAGAAGAGGGCTCTACATGCAAGG
       I  R  N  K  G  K  G  N  I  V  T  S  K  K  R  A  L  H  A  R   398
1261 ACTGTGGAAAGGACTCCAGGAGTAGAAATAGATGGAGAGAGGGGAAGCAAGAGAGGATGC
       T  V  E  R  T  P  G  V  E  I  D  G  E  R  G  S  K  R  G  C   418
1321 ACTAGGGTTTCAAACAGATCCAACACTTACTTGAAGGGCAAGCAACCAGAATCTTGCTCA
       T  R  V  S  N  R  S  N  T  Y  L  K  G  K  Q  P  E  S  C  S   438
1381 GTCTCTGATCAAGTGGCTAACGTGAGACAAAGCGTTCTAGATACTCTGAACACCATCAGA
       V  S  D  Q  (V) A  N  V  R  Q  S  (V) L  D  T  L  N  T  (I) R   458
1441 CATTTCAAAGCGGAGCTCAAGACAAGGGAGAGGAATCTGGAAGCTTCACTACTTGAACTT
       H  F  K  A  E  (L) K  T  R  E  R  N  (L) E  A  S  L  L  E  (L)   478
1501 GACGCGCTAGGTGAGAGGATATTGGGAATCAGCAAAATTCTCAACAATAATCTGGCTTAA
       D  A  L  G  E  R  (I) L  G  I  S  K  I  (L) N  N  N  L  A  *   497
1561 AGTATAATTAAATAGCAGGGGGAAAAGGATCAAAAGACTATCTGCATGCACTTTTTGTAT
1621 CATGGTTTTCACAAGAACGGCCTCTTCCTCAAAACTCCACGTTAAGTTTCCTTTTCAGTA
1681 TTTAAGACTTTTAAGTTCTAGAGACTGCCTATTATCTTGGTGCCTTTGTTCGGTTCCATG
1741 GCTCAACCAAGGTTTGTATATTTGAAGGTCTTGTGCTTACGTTAAAAAAAAAAAAAAAAA
1801 AAAAAAAAAAAA
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*Figure 3.* Nucleotide sequence and predicted amino acid sequence of cDNA *BoREM1*. Sequences corresponding to the translational start consensus and the putative polyadenilation signal are underlined. The acidic/serine-rich domain is double-underlined. Charged residues conforming the basic motifs characteristic of nuclear localization signals are in bold letters. Hydrophobic residues at a spacing of seven amino acids are encircled.

*Table 1.* Summary of the cauliflower meristem-specific cDNA clones isolated. Clones CM67, CM97 and CM222 are different cDNA clones of the same transcript. Only 4 clones showed similarity to known sequences in databases, whereas the rest could represent unknown genes.

| Clone number | Similarity | Reference |
|---|---|---|
| CM46, CM48 | *APETALA1* | [4] |
| CM15 | Gln-tRNA synthetase | [10] |
| CM131.2 | Phe-tRNA synthetase | [8] |
| CM67, CM97, CM222, CM6.1, CM6.2, CM43.1, CM43.2, CM85, CM88, CM89.1, CM89.2, CM108, CM89.3, CM89.4, CM96, CM131.1, CM132, CM168 | Unknown | |

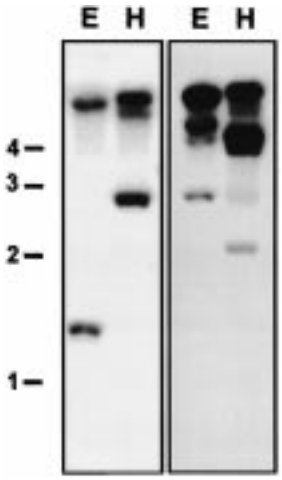


*Figure 4.* Genomic organization of *BoREM1*. The same DNA gel blot containing 5 $\mu$g of cauliflower genomic DNA digested with *Eco*RI (E) and *Hind*III (H) was hybridized with the cDNA clone CM6-2 at high-stringency conditions (left), and the 1.2 kb RACE derived *BoREM1* cDNA at moderate stringency (right). Sizes of the molecular weight marker are in kilobases.

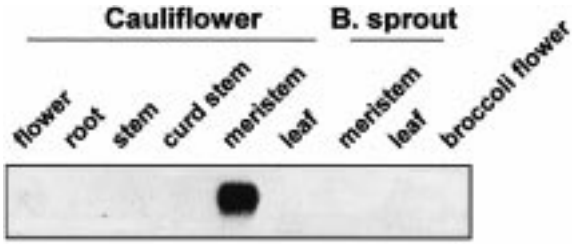


*Figure 5.* RNA blot hybridization analysis of *BoREM1* expression. An RNA gel blot containing 2 $\mu$g of poly(A)$^+$ RNA from organs of different *Brassica oleracea* varieties was hybridized with the CM6.2 cDNA fragment. Equal amounts of poly(A)$^+$, quantified as described in Material and methods, were loaded in each lane.

end of the cDNA, were obtained through two steps of RACE. The nucleotide sequence and the deduced amino acid sequence from this full-length cDNA are presented in Figure 3. The corresponding gene was named *BoREM1* (from *Brassica oleracea* reproductive meristem gene 1). The *BoREM1* cDNA sequence is 1813 bp in length (including the polyadenine tail) and contains a long ORF starting at position 67, with an ATG flanked by sequences that conformed to the consensus for translation initiation in plants [24], and ends at a putative stop codon at position 1557. Downstream of this stop codon, at position 1566, the cDNA shows a polyadenilation signal, 217 bp upstream of the polyadenilation site.

Sequence comparisons with the available databases did not reveal any significant similarity between *BoREM1* and other known nucleotide or peptide sequences. However, significant similarity was detected with and ORF identified in a BAC sequenced through the systematic sequence of the *Arabidopsis* genome (GenBank AF013293). The BoREM1 predicted protein contains 497 amino acids, corresponding to a deduced molecular mass of 55.5 kDa and a pI of 4.84. A special feature of the predicted BoREM1 protein is the presence of a highly acidic domain, near the N-terminus of the protein, at 89–140. In this region, 51% of the residues are negatively charged. We also found in this domain a high proportion of serine residues (29%), which are the most abundant amino acid residues in the whole protein sequence (10.5%). The C-terminal domain contained a large proportion of hydrophobic residues (i.e. leucine, isoleucine and valine), including a potential leucine zipper, with Val-443, Val-450, Ile-457, Leu-464, Leu-471, Leu-478, Ile-485 and Leu-492. Other interesting feature in the BoREM1 amino acid sequence, is the presence of a basic motif at 378–394, resembling the bipartite nu-

clear localization signals found in many proteins that are transported to the nucleus [40].

To determine the presence of other genes related to *BoREM1* in the cauliflower genome, we hybridized genomic DNA blots with two distinct DNA probes, at high and moderate stringency conditions. With the CM6.2 clone as a probe (which mainly contained 3′UTR sequences) under high-stringency conditions, we detected two restriction fragments corresponding to genomic segments of *BoREM1* gene (Figure 4, left). However, when the 1192 bp RACE product was used as a probe under moderate-stringency conditions, several new hybridization bands were detected that could correspond to other sequences similar to *BoREM1* in the cauliflower genome (Figure 4, right). Fragments corresponding to the 3′ end of *BoREM1* were not detected by the RACE probe and did not appeared in this second hybridization (Figure 4).

### *The* BoREM1 *transcript is specifically expressed in the cauliflower reproductive meristem*

To verify that *BoREM1* was specifically expressed in the curd meristem we carried out RNA blot hybridizations with poly(A)$^+$ mRNA obtained from different organs of cauliflower, Brussels sprouts, and broccoli. As shown in Figure 5, the presence of a hybridizing transcript, ca. 2 kb long, could only be detected in the reproductive meristem of cauliflower. To further analyse the tissue-specific expression of *BoREM1*, we performed *in situ* hybridizations on longitudinal sections of shoot apices of cauliflower in different stages of development, with both sense and antisense *BoREM1* digoxigenin-labeled RNA probes (Figure 6). The same experiments were also performed with a *BoLFY* RNA probe, whose tissue expression pattern was used as a control. As shown in Figure 6A, no *BoREM1* expression was detected in vegetative apices of cauliflower, where it was possible to detect some hybridization signal with the *BoLFY* probe, restricted to the leaf primordia (data not shown), an observation that had not previously been shown in cauliflower [5] but that has been reported for the *Arabidopsis LFY* [7]. The *BoREM1* transcripts, as the *BoLFY* transcipts, were already detectable in young inflorescence cauliflower apices (Figure 6B, C) and always detected in the meristematic domes that conform the cauliflower curd. In these meristems both transcripts were always associated to the flanking regions and flanking meristems, being absent from the youngest apical domes and the rest of cell types of the curd (Figure 6D, E). Sense *BoREM1* and *BoLFY* control probes did not yield any detectable hybridization signal, even after 48 h of inmunological detection (data not shown). These results show that the tissue expression pattern of the *BoREM1* is specific to the meristematic region of the curd and very similar to the expression pattern shown by *BoLFY* in cauliflower [5].

## Discussion

Flowering induction and flower development are highly complex proccesses that require the coordinate expression of numerous genes throughout the different stages of development [11]. Mutant analyses performed in *Arabidopsis* and other species like *Antirrhinum* has allowed the identification and cloning of many of the genes involved in these processes [25]. However, the genetic approach is limited to those genes whose mutations yield an altered phenotype. In order to identify genes involved in the flowering transition at the apical meristem that could have escaped mutant analyses, we isolated and characterized cDNAs corresponding to genes specifically expressed in the meristems of the cauliflower curd.

The curd meristem [30] or the highly proliferative meristems of the tomato *anantha* mutant [38, 45] had previously been used to isolate specific meristem cDNA clones using differerential hybridization strategies due to the easy availability of meristematic tissue. However, in both cases, the identified genes corresponded more to house-keeping genes abundantly expressed that were not specific of the reproductive meristematic tissue. Other strategies have tried to identify genes that could be transiently and specifically expressed in the the apex of tobacco [31, 20] or *Sinapis* [32] after floral induction, using subtraction of cDNA from these reproductive meristems with cDNA from leaves or vegetative meristems. Several new genes have been identified in this way but, so far, only one of the *Sinapis* genes, known as *FPF1*, has been shown to be specifically activated after flowering induction in the apex of *Sinapis* [19], where it is later expressed in the floral meristems.

Given the fact that, so far, none of the genes identified as controlling the specification of floral meristems is only transiently expressed in the apex, we took advantage of the availability of reproductive meristematic tissue provided by the cauliflower curd to perform a recurrent subtraction strategy with cDNAs from all the other possible plant sources. In this way, we

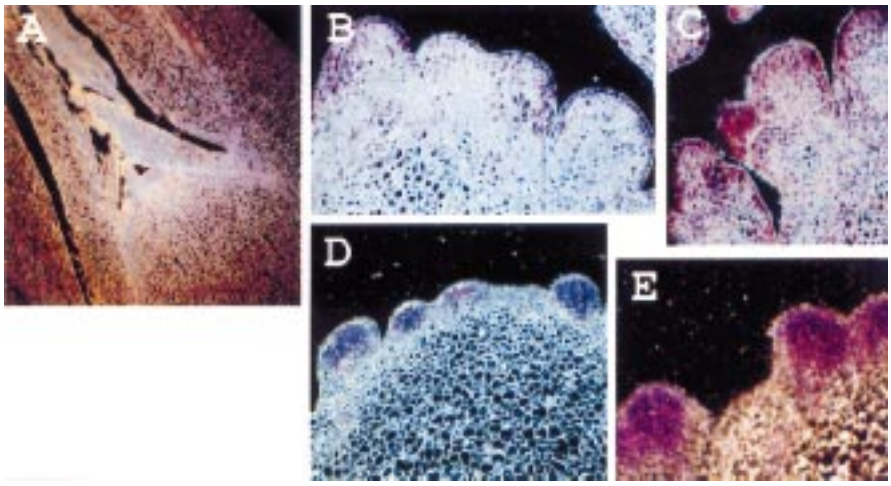


*Figure 6.* Localization of *BoREM1* and *BoLFY* mRNAs in cauliflower tissue sections by *in situ* hybridizations. Longitudinal tissue sections were hybridized with antisense RNA probes corresponding to *BoREM1* (A, B, D) and *BoLFY* (C, E). A. Shoot apical meristem of cauliflower seedlings during vegetative development. B and C. Reproductive meristems of cauliflower plants in early stage of curd development. D and E. Reproductive meristems of cauliflower in a late developmental stage of the curd.

obtained a cDNA population that was ca. 100-fold enriched for cauliflower reproductive meristem cDNAs, with 0.2% of them corresponding to *BoLFY* (data not shown). Furthermore, the use of a cold plaque screening procedure [13], based on the *BoLFY* hybridization signal, allowed to identify clones corresponding to cDNA species that were low abundant in the population of molecules used as a probe. The specificity of their expression patterns in reproductive meristems was assayed by RNA blot hybridizations. Compared to the previously mentioned strategies [20, 32], this approach allowed the identification of a number of specific cDNA clones, that would had not been isolated by classical differential hybridization techniques.

One of the isolated clones named as CM6.2 was further characterized to verify its specificity and to learn more about its molecular structure. The corresponding gene, known as *BoREM1*, belongs to a small gene family in cauliflower and encodes a protein showing several interesting features.

1. The presence of an acidic domain. This kind of domains, which are often Ser-Thr-rich [44], are frequently found in chromatin binding proteins and transcriptional activators, and have been proposed to function as activation domains [33, 44].
2. The presence of a putative nuclear localization signal, suggesting that the BoREM1 protein is transported into the nucleus [40].
3. The presence of eight heptad hydrophobic repeats in its carboxy terminus, that could represent a leucine zipper involved in specific interactions with other proteins [35, 15].

Altogether, these features could suggest a regulatory role for what could represent a new family of regulatory proteins. Additionally, the results of RNA blot and *in situ* hybridizations indicate that the expression of *BoREM1* is specifically detected in the cauliflower reproductive meristems and restricted to flanking regions of young meristematic domes, considered as peripheral zones, and to flanking meristems. *BoREM1* expression was absent from the youngest apical domes and the rest of cell types of the curd, following a pattern very similar to that of the *BoLFY* mRNA [5]. This peripheral zone corresponds to the region of the emerging floral meristems suggesting a putative role for *BoREM1* in the initiation of these meristems. Strong expression of *LFY*, *AGL8* and *AP1* has been shown in these regions of the apex after floral induction in *Arabidopsis* [12], and other genes like *FPF1* from *Sinapis* and *Arabidopsis* follow a similar pattern of expression [19].

In conclusion, by selecting the appropriate tissue, performing a recurrent subtraction and amplification protocol, and a controlled cold-plaque selection of cDNA clones we have shown the feasibility to identify rare messages specifically expressed in reproductive meristems. The close phylogenetic relationship between cauliflower and *Arabidopsis*, which facilitates the identification of orthologous sequences in this species, will allow to test the putative role of *BoREM1* in the initiation of floral meristems In this way, preliminary results in *Arabidopsis* indicate that

the orthologous sequence also belongs to a small gene family and follows a similar expression pattern as *LFY*.

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