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Molecular cloning and characterization of anther-preferential cDNA encoding a putative actin-depolymerizing factor

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Abstract

A cDNA clone, LMP131A, which is preferentially expressed in mature anther was isolated from a lily cDNA library. Northern blot analysis and plaque hybridization experiments showed that the LMP131A mRNA is present at ca. 0.3% of the mRNA in mature pollen and is not detectable in carpel, petal, floral bud, leaf, or root. The clone contains an open reading frame of 139 amino acid residues which shows greater than 40% sequence identity in a 91 amino acid overlap to animal actin-depolymerizing factors (ADF), cofilin and destrin. The sequences at and near the actin-binding site are most conserved. Using the lily clone as a probe, a cDNA clone, BMP1, was isolated from a mature anther library of *Brassica napus*. The expression pattern of the BMP1 clone was the same as that of the lily clone. The *Brassica* anther-preferential clone contains an open reading frame which is 79% identical to the lily LMP131A protein. Southern blot analysis showed that there are one or a few copies of the putative ADF genes in *B. napus* and *Arabidopsis thaliana*.

Introduction

Male reproductive processes in flowering plants occur in the anther which is composed of several tissue and cell types. In early developmental stages, the sporogenous tissue within the anther produces pollen mother cells which generate a tetrad of microspores by meiosis. The microspore gradually develops into the male gametophyte which is composed of a vegetative cell and two sperm cells. The role of the male gametophyte is to produce and transport the sperm cells for double fertilization. One sperm fertilizes the egg and the second sperm fuses with two previously fused nuclei of the central cell. This results in the formation of the zygote and primary endosperm cell, respectively.

It was estimated that mature pollen of maize expresses about 24 000 different mRNAs [37]. Approximately 35% of these pollen-expressed mRNAs are very abundant and comprise about 240 sequences. Recently, pollen- or anther-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z14109 (LMP131A) and Z14110 (BMP1).

expressed genes have been isolated from a variety of plant species. Several anther-expressed genes have been studied in tomato. An LAT52 clone from tomato is expressed in pollen, anthers and at 20- to 50-fold lower levels in petals [35]. The LAT52 clone codes for a putative protein of 17.8 kDa which shows 32% identity to the protein sequence of the maize clone, Zmc13 [14]. Both gene products have some sequence homology to trypsin inhibitors [20]. LAT56 and LAT59, which are preferentially expressed in tomato anther, have a significant homology to pectate lyases from the plant pathogen *Erwinia* [38]. A pollen-specific cDNA from maize, Zmc58, and the major ragweed pollen allergen, *AmbaI*, also have striking sequence homology to the LAT59 and LAT56 proteins [18, 27]. Three pollen-preferential gene families from *B. napus* have been isolated and characterized [3, 4, 5]. One of the clones, Bp4, is expressed during the early stages of microspore development. Two other pollen-expressed genes in *B. napus*, Bp10 and Bp19, start to express in uninucleate microspores and the mRNA level is increased during development, reaching a peak in the late stage but declining considerably in mature pollen. Bp19 codes for proteins putatively identified as pectin esterases which may be involved in pollen wall reorganization. The Bp10 gene family codes for proteins of 62 kDa showing approximately 30% sequence identity to cucumber and pumpkin ascorbate oxidase. Putative ascorbate oxidase genes which are preferentially expressed in pollen have also been identified from tomato and tobacco [36]. It was shown that one of the *Arabidopsis* α-tubulin genes is expressed preferentially in mature pollen [11, 16]. A family of pollen-specific genes which produces polygalacturonase was isolated from *Oenothera organensis* [9] and maize [24]. Many allergenic proteins of pollen are responsible for IgE binding in pollen-allergic patients [17]. A cDNA clone, *BetvI*, from the pollen cDNA library of white birch has been isolated by immunoscreening using the serum of a birch pollen allergic patient [8]. The deduced amino acid sequence of *BetvI* has shown 55% sequence identity with a pea disease resistance response gene. A similar approach has been used to clone several pollen-specific allergen cDNAs from Kentucky bluegrass [32] and rye-grass [13, 33].

In this study, we report the isolation of cDNA clones from lily (*Lilium longiflorum* Thunb. cv. Nellie White) and rapeseed (*B. napus*) which are preferentially expressed in mature anther. The protein encoded for by the clones is significantly homologous to animal actin-depolymerizing factor (ADF). It has been suggested that destrin, a member of the animal ADF family, is capable of directly interacting with F-actin, thereby cutting and depolymerizing the F-actin [25]. The action of destrin is insensitive to pH whereas cofilin binds to F-actin at near neutral pH. Cofilin is capable of reversibly controlling actin depolymerization and polymerization in response to various stimuli. Cofilin molecules are almost completely translocated into nuclei in mammalian cells exposed to heat shock or 10% dimethyl sulfoxide [26]. Both destrin and cofilin cDNA clones have been isolated from mammals [1, 2, 19, 22]. However, there is little information on plant ADF.

Materials and methods

Plant materials

Pollen from Easter lily harvested from commercial bulb fields and kept at −20°C was kindly provided by Dr Frank A. Loewus. For Northern analysis, lily plants were grown to the flowering stage under greenhouse conditions. Field-grown rapeseed (*B. napus*) at the flowering stage was used for library construction and northern analysis.

cDNA library construction and molecular characterization

cDNA libraries were constructed using the λ ZapII vector (Stratagene) and poly(A)$^+$ mRNA isolated from various organs. An adapter containing *Eco* RI and *Not* I sites (Pharmacia LKB) was used to ligate the λ vector and cDNA. The libraries were divided into 5 to 20 sublibraries and

amplified in an *E. coli* host strain, XL-1 Blue [F′::Tn*10* *proA+B+*, *lacIq*, *(lacZ)M15/recA1*, *endA1*, *gyrA96*(Nal^r), *thi*, *hsdR17*(r$_k^-$ m$_k^+$), *supE44*, *relA1*, *lac*] (Stratagene). Plaque hybridization was performed with 10^5 plaques which were lifted onto nitrocellulose membranes. The plasmid pBluescript containing cDNA clones was *in vivo* rescued from the bacteriophage λ using f1 helper phage, R408 (Stratagene). Both strands of the cDNA inserts were sequenced by the dideoxynucleotide chain termination method using double-stranded DNA as a template [29]. The GCG programs (Genetics Computer Group) were used for sequence analysis. Southern and northern blot analyses were performed as described by Sambrook *et al.* [28].

Results

Isolation of an anther-preferential clone from lily cDNA library

A clone LMP131A, which is expressed in mature pollen but not in leaf, was isolated from a lily pollen cDNA library by differential hybridization.

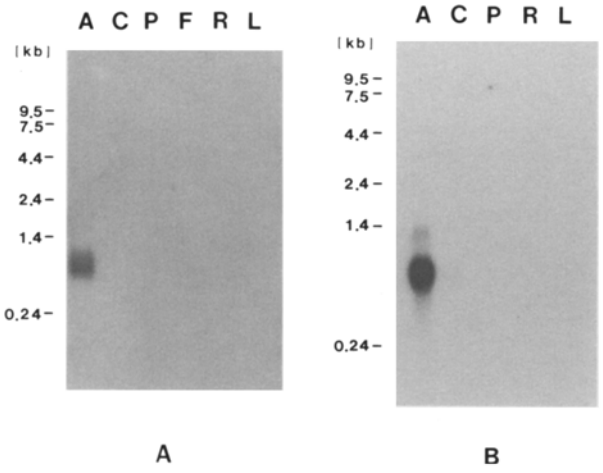

Fig. 1. Expression level of LMP131A and BMP1. A. Northern blot analysis of LMP131A. Ten μg of total RNA prepared from mature anther (A), carpel (C), petal (P), floral bud (B), root (R), or leaf (L) was hybridized with the radioactively labeled LMP131A probe. B. Northern blot analysis of BMP1. Two μg of mRNA prepared from anther (A), carpel (C), petal (P), leaf (L), or root (R) was hybridized with the BMP1 probe. The numbers are the RNA size marker (BRL) in kb.

Northern blot analysis showed that a mRNA of ca. 0.7 kb, present in mature anther, hybridized with the LMP131A probe (Fig. 1A). No detectable level of the LMP131A mRNA was present in carpel, petal, root, or leaf of lily at the flowering stage. Young flower buds also did not show any expression of the mRNA. Therefore, it appears that the LMP131A is preferentially expressed in mature anther. The expression level of the LMP131A clone was estimated indirectly by measuring the frequency of this cDNA clone in 10^5 plaques of the pollen library as well as other cDNA libraries made from carpel, leaf, or root. This experiment showed that the LMP131A clone is present at about a frequency of 0.3% of the lily pollen library and that the clone is not present in other libraries.

Characterization of LMP131A clone

Since the LMP131A clone is preferentially expressed in mature anther and the expression level is high, the protein encoded by the LMP131A clone may play a major role in anther. In order to facilitate characterization of the cDNA clone, the pBluescript vector containing the LMP131A insert was *in vivo* excised from the λ ZapII vector. DNA sequencing of the clone revealed that it contains a 544 nucleotide sequence with one open reading frame coding for 139 amino acid residues (Fig. 2). Homology search to the Swissprotein database with the LMP131A sequence revealed that a family of ADF proteins is the most homologous to the LMP131A (Fig. 3). The human cofilin is 42.9% identical to the lily LMP131A at amino acid residues 34–123. The most conserved area between the animal ADFs and LMP131A is the putative actin binding site [12] at amino acid residues 108–118 and the surrounding region (Fig. 2).

Isolation and characterization of a B. napus *cDNA clone homologous to LMP131A*

To study whether the LMP131A protein is also present in dicot species, we screened a cDNA

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  1  GGAACAAACAACATGGCGAACTCATCGTCCGGAATGGCGGTGGACGATGAATGCAAGCTC
                M  A  N  S  S  S  G  M  A  V  D  D  E  C  K  L    16

 61  AAGTTCATGGAGCTGAAGGCGAAGCGGAACTTCCGGTTCATCGTTTTCAAGATTGAGGAG
     K  F  M  E  L  K  A  K  R  N  F  R  F  I  V  F  K  I  E  E    36

121  AAGGTGCAGCAGGTGACAGTGGAGCGGCTCGGGCAGCCGAATGAGAGCTACGATGATTTC
     K  V  Q  Q  V  T  V  E  R  L  G  Q  P  N  E  S  Y  D  D  F    56

181  ACTGAGTGCCTCCCTCCTAATGAGTGCCGCTATGCTGTTTTCGATTTCGACTTTGTCACT
     T  E  C  L  P  P  N  E  C  R  Y  A  V  F  D  F  D  F  V  T    76

241  GATGAGAACTGTCAGAAGAGCAAGATCTTCTTCATCTCCTGGTCTCCTGACACATCAAGG
     D  E  N  C  Q  K  S  K  I  F  F  I  S  W  S  P  D  T  S  R    96

301  GTGAGGAGTAAGATGCTATATGCTAGTACCAAGGATAGATTCAAGAGAGAGCTTGATGGG
     V  R  S  K  M  L  Y  A  S  T  K  D  R  F  K  R  E  L  D  G   116

361  ATTCAAGTTGAATTGCAAGCAACAGATCCAAGCGAGATGAGCATGGATATTATAAAAGCA
     I  Q  V  E  L  Q  A  T  D  P  S  E  M  S  M  D  I  I  K  A   136

421  AGAGCTTTCTGAAGCATTGGTTGTTCACCCTTCACTTCGAGCTTTCTTTGATTAAATTGT
     R  A  F                                                       139

481  TTCTGTTGACCTTTGTCCTTCATTCATCTTGGTGTGATTAGTTCTAAACTATGTAAAAAT

541  GATT
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Fig. 2. DNA sequence and deduced amino acid sequence of the LMP131A. The positions of nucleotides and amino acids are shown on the left and right, respectively. The putative actin-binding domain is underlined.

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MANSSSGMAV DDECKLKFM- ---------- ELKANGNFRF IVFK------  1
          EDNCKLKFL- ---------- ELKKRI-FRF IIFR------  2
   MASGVQV ADEVCRIFYD MKVRKCSTPE EVKKRKKAVI FCLSPDKKCI  3
   MASGVQV ADEVCRIFYD MKVRKCSTPE EIKKRKKAVI FCLSADKKCI  4
   MASGVAV SDGVIKVFND MKVRKSSTPE EVKKRKKAVL FCLSEDKKNI  5
   MASGVAV SDGVIKVFND MKVRKSSTPE EVKKRKKAVL FCLSEDKKNI  6
   MASGVTV NDEVIKVFND MKVRKSSTPE EIKKRKKAVL FCLSDDKKQI  7

IEEKVQQVTV ERLGQP-NES YDDFTECLPP NECRYAVFDF DFVTDENCQK  1
IDG--QQVVV EKLGNP-QET YDDFTASLPA DECRYAVFDF DFTTNENCQK  2
IVEEGKEILV GDVGVTVTDP FKHFVEMLPE KDCRYALYDA SFETKE-SKK  3
IVEEGKEILV GDVGVTITDP FKHFVGMLPE KDCRYALYDA SFETKE-SRK  4
ILEEGKEILV GDVGQTVDDP YATFVKMLPD KDCRYALYDA TYETKE-SKK  5
ILEEGKEILV GDVGQTVDDP YATFVKMLPD KDCRYALYDA TYETKE-SKK  6
IVEEATRILV GDIGDTVEDP YTAFVKLLPL NDCRYALYDA TYETKE-SKK  7

SKIFFISWSP DTSRVRSKML YASTKDRFKR ELDGIQVELQ ATDPSEMSMD  1
SKIFFIAWSP DSSRVRMKMV YASSKDRFKR ELDGIQVELQ ATDPSEMSFD  2
EELMFFLWAP EQAPLKSKMI YASSKDAIKK KFQGIKHECQ ANGPEDL-NR  3
EELMFFLWAP ELAPLKSKMI YASSKDAIKK KFQGIKHECQ ANGPEDL-NR  4
EDLVFIFWAP ESAPLKSKMI YASSKDAIKK KLTGIKHELQ ANCYEEVKDR  5
EDLVFIFWAP ECAPLKSKMI YASSKDAIKK KLTGIKHELQ ANCYEEVKDR  6
EDLVFIFWAP ESAPLKSKMI YASSKDAIKK KFTGIKHEWQ VNGLDDIKDR  7

IIKARAF                    1. lily LMP131A
IIKSRAL                    2  rapeseed BMP1
ACIAEKLGGS LVVAFEGSPV      3. chicken destrin
ACIAEKLGGS LIVAFEGCPV      4. pig destrin
CTLAEKLGGS AVISLEGKPL      5. human cofilin
CTLAEKLGGS AVISLEGKPL      6. pig cofilin
STLGEKLGGN VVVSLEGKPL      7. chicken cofilin
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Fig. 3. Comparison of ADF sequences. Amino acid sequences were aligned for maximum homology by introducing gaps (–). In bold type are the plant sequences identical to at least one mammalian ADF amino acid.

library of *B. napus* mature anther using the LMP131A clone as a probe. One of the positively hybridizing clones, BMP1, was sequenced in order to deduce the amino acid sequence encoded for by the clone (Fig. 4). The *Brassica* clone contains an open reading frame which is 78.6% identical to the lily LMP131A protein, suggesting that both proteins are functionally similar to each other. However, the first 10 amino acids of the lily protein are not present in the BMP1 clone, indi-

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  1  GGAAGACAACTGCAAGCTGAAGTTTTTGGAGCTAAAGAAAAGAATCTTTCGGTTCATAAT
       E  D  N  C  K  L  K  F  L  E  L  K  K  R  I  F  R  F  I  I   30

 61  ATTCAGGATTGACGGGCAGCAAGTGGTGGTGGAAAAGTTAGGAAACCCCCAAGAGACTTA
       F  R  I  D  G  Q  Q  V  V  V  E  K  L  G  N  P  Q  E  T  Y   50

121  CGATGATTTCACCGCTTCCCTCCCTGCCGACGAGTGCCGCTATGCGGTTTTCGATTTCGA
       D  D  F  T  A  S  L  P  A  D  E  C  R  Y  A  V  F  D  F  D   70

181  TTTTACCACCAATGAAAATTGCCAGAAGAGCAAAATCTTCTTCATAGCCTGGTCACCGGA
       F  T  T  N  E  N  C  Q  K  S  K  I  F  F  I  A  W  S  P  D   90

241  TTCATCTAGAGTGAGGATGAAGATGGTGTACGCAAGCTCTAAGGATAGGTTCAAGAGAGA
       S  S  R  V  R  M  K  M  V  Y  A  S  S  K  D  R  F  K  R  E  110

301  ATTGGATGGCATTCAGGTGGAGTTGCAAGCCACTGATCCTAGCGAGATGAGTTTCGACAT
       L  D  G  I  Q  V  E  L  Q  A  T  D  P  S  E  M  S  F  D  I  130

361  TATCAAAAGCCGAGCTCTCTAGATCTTTTCGTTGTTTTTCATCAAAAAAGCATCTAAGAT
       I  K  S  R  A  L                                           136

421  ATCATCTTTCGTATTTCTGATATTCCAAAAAAGAAAAAACTGAAACATTTTTCAGTATTA

481  TGTAATTTCCTTGGTTTAAGAACTGAATTGTTCATCGTCGTTTTCATTCATATATTCACT

541  TGTCTTTGTAAATAATGTAATACTCCCGTTTGGGAGAAGTTGAACCCATGTTGAGCATGT

601  TTGTGTAACATCTCAAAGAGTAAATTATTCTCTGTAT
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Fig. 4. DNA sequence and deduced amino acid sequence of the BMP1. The positions of nucleotides and amino acids are shown on the left and right, respectively. The positions of amino acid begin with number 11 in order to align it with the lily LMP131A sequence. The putative actin-binding domain is underlined.

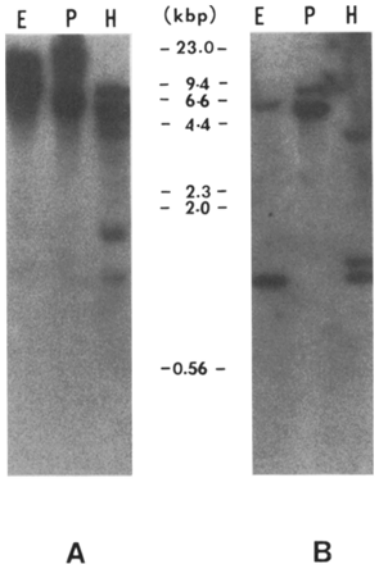

Fig. 5. Southern blot analysis of the BMP1. Genomic DNAs of *B. napus* (A) and *A. thaliana* (B) were digested with *Eco* RI (E), *Pst* I (P), or *Hin*d III (H) and hybridized with the BMP1 cDNA clone. The *A. thaliana* blot was washed at a low stringency condition. The bacteriophage λ DNA digested with *Hin*d III was used as a size marker (center).

cating that this clone is partial. The amino acid sequence at the putative actin binding site is identical between these two proteins, supporting the hypothesis that they are actin binding proteins. Northern blot analysis showed that the BMP1 mRNA is present only in mature anther but not in carpel, leaf, or root (Fig. 1B). Southern blot analysis showed that there is one or a few copies of the putative ADF genes in *B. napus* and *A. thaliana* (Fig. 5).

Discussion

In this article, we report isolation of a lily cDNA clone, LMP131A, which is highly expressed in mature anther. The plaque hybridization experiment showed that the LMP131A clone is present at a frequency of 0.3% in the mature pollen library. Although this is an indirect way of measuring the level of gene expression, the result is probably similar to the actual level of gene expression since the northern blot analysis showed a strongly hybridizing band. The initial size of the lily cDNA libraries was 10^7 to 10^8 pfu which should be large enough to cover the entire mRNA

population present in pollen. To avoid any artifact due to uneven amplification of the libraries, we have divided the primarily packaged phage into 20 sublibraries and maintained them independently. Plaque hybridization to different sublibraries showed a similar frequency, indicating that there is no major difference in the frequency of the LMP131A clone. Northern blot analysis indicated that the LMP131A clone and BMP1 clone are expressed in mature anther but not in other organs examined. Since we have not tested every organ and tissue at different growth stages, it cannot be definitely stated that these clones are anther- or pollen-specific. *In situ* localization experiments are currently in progress to further study the expression pattern of these genes.

The LMP131A clone encodes a 139 amino acid protein which shows a significant sequence homology to animal ADFs. The most conserved region between animal ADFs and the LMP131A protein is the sequence at and near the putative actin-binding site [12]. Actin is the most abundant protein in many eukaryotic cells, often constituting 5% or more of the total cell protein. Actin filaments form a dense network which gives mechanical strength to the surface of the cells and enables the cells to change shape. The dynamic equilibrium existing between unpolymerized actin molecules and actin filaments drives many cell surface movements [10]. Actin gene families have been characterized from various plant species, including soybean [30, 31], maize [31], *A. thaliana* [23], petunia [6], tomato [7], carrot [34], and rice [21]. It was suggested that various members of the actin genes from soybean and rice are differentially expressed [15, 21]. However, it is unknown whether any of these actin genes are preferentially expressed in anther. The finding that the anther-preferential clone, LMP131A, codes for a putative ADF suggests that the equilibrium between actin filament formation and depolymerization plays an important role in anther. However, it will be necessary to prove that the LMP131A protein is indeed ADF.

Southern blot analysis showed that the gene is a member of a small gene family in *B. napus* and *A. thaliana*. Whether other members are also expressed primarily in anther or whether they are present in different tissues is unknown. Isolation of an anther-preferential cDNA clone from *B. napus* which has a significant homology to the lily clone shows that the putative ADF gene is present in both monocots and dicots and suggests that the gene product plays an important function in anther.

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