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Isolation and Characterization of a Pollen-specific cDNA Clone from Easter Lily

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A pollen-specific cDNA clone, LMP50, was isolated from the mature pollen cDNA library of the Easter lily. The *LMP50* transcript was highly abundant in mature pollen grains but not detectable in other organs. The LMP50 cDNA clone contains 1383 nucleotides and two open reading frames. The first codes for a peptide of 15 amino acid residues. The role of this peptide is unclear. The second encodes a protein containing 329 amino acid residues. This protein exhibited a significant homology to human tartrate-resistant acid phosphatase and porcine uteroferrin. Both of these enzymes have been suggested to play a role in iron transport. Therefore, LMP50 may act as an iron carrier protein in mature pollen grains.

Keywords : pollen, Easter lily, acid phosphatase, uteroferrin, iron transport

Reproduction plays a major role in the plant life cycle. Male reproductive processes in flowering plants occur within the anther, which is composed of several types of tissues and cells (Esau, 1977). Pollen mother cells are produced in the sporogenous tissue within the anther. After meiotic division, a tetrad of microspores is generated from a pollen mother cell. The role of the male gametophyte is the production of sperm cells and their transport within the pollen tube through the tissues of the style and into the embryo sac in the ovule. In the double fertilization that follows, fusion of one sperm with the egg and of the second sperm with two previously fused nuclei results in the formation of the zygote and primary endosperm cell, respectively.

It is well established that pollen is transcriptionally and translationally active during development. For example, the maize alcohol dehydrogenase gene specifies a dimeric enzyme that is responsible for the ADH activity of pollen grains (Felder *et al.*, 1973; Freeling and Schwartz, 1973). Evidence for the presence of mRNAs in the pollen grains of several plants has been obtained by their isolation and translation in cell-free systems into polypeptides, many of which show similarity to proteins made during germination (Frankis and Mascarenhas, 1980; Mascarenhas *et al.*, 1984, Schrauwen *et al.*, 1990).

Several anther-specific or anther-preferential genes have been isolated from both monocot and dicot plant species (Mascarenhas, 1990; McCormick, 1991). The tissue specificity and developmental regulation of these genes have been analyzed by RNA blot analysis and *in situ* localization of a variety of plant species including tobacco (Seurinck *et al.*, 1990), tomato (Twell *et al.*, 1989; Smith *et al.*, 1990), *Arabidopsis thaliana* (Ludwig *et al.*, 1988), *Brassica napus* (Albani *et al.*, 1990; 1992; Theerakulpisut *et al.*, 1991), *Oenothera* (Brown and Crouch, 1990), sunflower (Domon *et al.*, 1990; Evrard *et al.*, 1991), maize (Hamilton *et al.*, 1992), ryegrass (Singh *et al.*, 199), ragweed (Rafnar *et al.*, 1991) . Sequence similarity searches and antisense approaches were used in an attempt to gain insight into the possible roles of these gene products during pollen development.

We have previously isolated a cDNA clone encoding a putative actin depolymerizing factor which was expressed specifically in the mature pollen grains of lily, *B. napus*, and *A. thaliana* (Kim *et al.*, 1993). In addition, we have reported the isolation and characterization of three pollen-specific cDNA clones from the Easter lily (Kim et al. 1994). Two of these clones encode proteins with a significant

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homology to a pectate lyase of the tomato and a variety of thioredoxins. The third clone did not show any significant homology to known sequences. In this present study, we report the isolation and characterization of an additional cDNA of the Easter lily that exhibits a pollen-specific expression pattern.

MATERIALS AND METHODS

Plant samples and bacterial strains

Easter lily (*Lilium longiflorum* Thunb., cv Nellie White) plants were grown as described previously (Kim *et al.*, 1993). Pollen was kindly supplied by Dr. Frank Loewus of the Institute of Biological Chemistry at Washington State University. Pollen was stored in plastic containers at −20°C until used.

Escherichia coli strains, MC1000 [F-, *araD139,* △*(araABC-leu)7679, galU, galK,* △*(lac)X74, thi, rpsL (Str^r)*], JM83 [F-, *ara,* △*(lac-proAB), rpsL(Str^r),* ϕ*80d(lacZ)*△*M15*], and XL-1 Blue [F'::Tn*10 proA*+ *B*+, *lacIq, (lacZ)M15/ recA1, endA1, gyrA96(Nalr), thi, hsdR17*(rk-mk+), *supE44, relA1, lac*] were used as hosts for molecular cloning. The f1 helper phage, R408, was used for in vivo excision of the pBluscript plasmid vector from the λZapII phage (Stratagene).

Plaque hybridization experiments

The pBluscript plasmid containing an insert was rescued from a lambda phage using a helper phage, R408. Plaque hybridization was performed as described (Kim *et al.*, 1994). The *in vivo* excised plasmid was cut with *Eco*RI and the cDNA inserts were separated on 0.7% agarose gel. The 1.3 kb LMP50 cDNA fragment was recovered by electroelution and used as a hybridization probe after labelling the fragment with [α-^{32}P]dCTP.

RNA blot analysis

Total RNAs were prepared from various organs using the method developed by Davis *et al.* (1986). Ten μg of total RNA were run on a 1.2% formaldehyde agarose gel and blotted onto a nylon membrane (BioRad). The blot was hybridized with a radioactively labelled probe (Davis *et al.* 1986) for 24 h at 42°C in a solution containing 5X SSPE, 50% formamide, 2X Denhardt's, 0.1% SDS, and 50 μg/mL denatured salmon sperm DNA (Sambrook *et al.*, 1989). The membrane was washed in a solution containing 2x SSC and 0.1% SDS at room temperature. When the background radioactivity was high, the membrane was further washed in a solution carrying 0.1x SSC and 0.1% SDS at 65°C.

cDNA blot analysis

Total bacteriophage DNA was isolated from cDNA libraries according to Chisholm (1989). The phage DNA was digested with *Eco*RI, separated on a 0.8% agarose gel, and transferred to a nylon membrane. The blot was hybridized with the cDNA probe at 65°C for 24 h in a solution containing 6x SSC, 0.05x BLOTTO, and 100 μg/mL salmon sperm DNA. The membrane was washed as described for the RNA blot analysis.

Preparation of labeled probes

The probe DNA was radioactively labelled using [α-^{32}P]dCTP (3000 Ci/mmol) by the random priming method (Feinberg and Vogelstein 1983). The radioactively labelled probe was generated by T7 DNA polymerase (Pharmacia). The specific activity of the probe was approximately 5×10^7 dpm/μg. Unincorporated nucleotides were removed by G-50 Sephadex column chromatography. The labelled probe was denatured at 95°C for 3 min before use for hybridization.

DNA sequencing and analysis

Both strands of the cDNA insert were sequenced according to the dideoxynucleotide chain termination method using double stranded DNA as a template (Sanger *et al.*, 1977). Computer software GCG programs (Genetics Computer Group, Inc.) was used for sequence analysis. Genbank, EMBL and Swissprot databases were searched for sequence homology. Protein sequence similarity comparisons were based on Lipman and Pearson (1985). The hydropathy profile of the deduced protein of cDNA clone was according to Kyte and Doolittle (1982). Asn-linked glycosylation sites were searched based on the consensus sequence, asparagine-X-threonine or asparagine-X-serine (Wagh and Bahl 1981) when a putative signal peptide sequence was present at the NH_2-terminal region.

RESULTS

Isolation of a pollen-specific cDNA clone, LMP50

We have previously reported the isolation of several pollen-specific cDNAs from the Easter lily (Kim *et al.*, 1994). This was achieved by analyzing the expression patterns of the randomly selected clones. The rationale behind this method was based on the observation that a significant portion of pollen-expressed mRNAs are pollen-specific. It was reported that a large portion of the mRNA present in mature pollen are the transcripts of highly-expressed genes. Among 25 randomly selected cDNA clones, eight belonged to a high abundance class in which six were expressed preferentially in pollen grains. Characterization of three of these clones were previously reported (Kim *et al.*, 1994). In this study, we have further characterized one of the abundant clones, designated LMP50.

Expression pattern of the LMP50 clone

The expression pattern of the LMP50 clone was studied by RNA blot analysis. The result revealed that the *LMP50* mRNA is present in anthers and absent in carpels, petals, flower buds, roots, and leaves (Fig. 1A). The size of the *LMP50* transcript was approximately 1,500 nucleotides. The expression level of the clone was indirectly measured by cDNA blot analysis which was shown to be more sensitive than the conventional RNA blot analysis (Fig. 1B). This analysis showed that this clone is present only in the pollen cDNA library but not in the leaf, root, or carpel cDNA library. A colony hybridization experiment with the LMP50 clone revealed that 0.89% of the clones in the mature pollen cDNA library were the LMP50 cDNA, indicating that LMP50 is highly abundant in mature pollen grains.

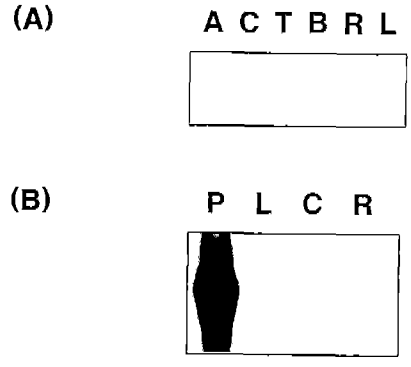

Fig. 1. RNA and cDNA blot analysis of LMP50 clone. A. RNA analysis. Ten μg of total RNA were prepared from mature anthers (A), carpels (C), tepals (T), flower buds (B), roots (R), or leaves (L) of the Easter lily. The probe was the full length LMP50 cDNA clone. The numbers indicate the size of RNA markers (BRL) in kb. B. cDNA blot analysis. Two μg of total DNA isolated from cDNA libraries of pollen grains (P), leaves (L), carpels (C), or roots (R) were digested with *Eco*RI and hybridized with the LMP50 probe. The numbers indicate the size of DNA markers in kb.

DNA sequence analysis

The DNA sequence of the LMP50 clone was determined (Fig. 2). It contains two open reading frames (ORF). The first ORF is very short; it consists of 15 codons including the start codon. The second ORF is 987 bp, encoding a protein of 329 amino acid residues. It is followed by a 217 bp 3' untranslated region. A putative polyadenylation signal is present 15 bp upstream of the poly(A)+tail.

The deduced amino acid sequence of the longer

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1    CAATTTAAAACATAAAAGCAGTGCATACATCACATAAAGGTTCTACAGAAGCATTAAGAA
61   CTAACAAACAAGCAGCTTCAGTTTTACTCATCAATAATAGCAGTTTGCAAAAACTAAATA
                                                        M
121  TGGACCAAAAAGCCAACCAGAGCTCCTCTTTCCCAGTCCCTCAATAAAGCAGAGCCATGG
       D  Q  K  A  N  Q  S  S  S  F  P  V  P  Q           M  A    2
181  CAGCAAGCTCCTCTTGCGAATCCATGGCTCTGTCTCTCATCCTTGTGATGGGACTTCTTT
       A  S  S  S  C  E  S  M  A  L  S  L  I  L  V  M  G  L  L  S   22
241  CCCTTGTGGCCGCCGAGCTGCCGAGGCTCGAGCAGCCGATCAAGGCCGACGGCTCACTGA
       L  V  A  A/E  L  P  R  L  E  Q  P  I  K  A  D  G  S  L  S   42
301  GTCTCCTTGTAATTGGAGATTGGGGAAGGAATGGGGGCTACAATCAATCCCAAGTAGCCA
       L  L  V  I  G  D  W  G  R  N  G  G  Y  N  Q  S  Q  V  A  T   62
361  CTCAGATGGGTAAGATTGGAGAGGAGCTAGACATTGATTTTGTAGTATCAACCGGCGATA
       Q  M  G  K  I  G  E  E  L  D  I  D  F  V  V  S  T  G  D  N   82
421  ACTTCTACGACAGTGGATTGACCAGCACCGATGACAAGGCCTTCGAGGCATCCTTCACAA
       F  Y  D  S  G  L  T  S  T  D  D  K  A  F  E  A  S  F  T  N  102
481  ATATATACACTGCAAAGAGCTTGCAGAAGCAATGGTACAGTGTCTTGGGGAACCATGACT
       I  Y  T  A  K  S  L  Q  K  Q  W  Y  S  V  L  G  N  H  D  Y  122
541  ACCGCGGTGACGCTGTGGCACAGCTGAGCCCCGTGCTGCAGAAGATCGATAGCCGATGGC
       R  G  D  A  V  A  Q  L  S  P  V  L  Q  K  I  D  S  R  W  L  142
601  TCTGCATGAGATCATTCATCCTCGATGCAGAAATCGTTCAATTCTTCTTCGTAGACACAA
       C  M  R  S  F  I  L  D  A  E  I  V  Q  F  F  F  V  D  T  T  162
661  CTCCTTTCGTGATGAACTACTGGGACGACCCACAAGATCATCACTATGACTGGAGGGACG
       P  F  V  M  N  Y  W  D  D  P  Q  D  H  H  Y  D  W  R  D  V  182
721  TCGCTCCACGCGATAACTACATCAAGAATCTCAGGAACGATTTGGACTCCGCACTGAAGG
       A  P  R  D  N  Y  I  K  N  L  R  N  D  L  D  S  A  L  K  E  202
781  AATCAACAGCGACATGGAAGATTGTTGTTGGTCATCATACCATGAGGAGCGTAAGTGAAC
       S  T  A  T  W  K  I  V  V  G  H  H  T  M  R  S  V  S  E  H  222
841  ATGGAGATACCAAAGAGCTGTTGGATTTGCTTCTTCCCATTCTTAAGGCTAATGAAGTTG
       G  D  T  K  E  L  L  D  L  L  L  P  I  L  K  A  N  E  V  D  242
901  ATCTTTATATCAATGGACATGACCATTGCCTTGAGCATGTTAGTAGCAATGACAGCCCAA
       L  Y  I  N  G  H  D  H  C  L  E  H  V  S  S  N  D  S  P  I  262
961  TTCAGTACCTCACAAGTGGAGCAGGTTCGAAGGCGTGGAGGGGTACCTACATTCCGAACT
       Q  Y  L  T  S  G  A  G  S  K  A  W  R  G  T  Y  I  P  N  S  282
1021 CGGACAAGTTACAGTTCTTCTACGACGGGCAAGGGTTCATGTCGCTGCAATTGACCGGAT
       D  K  L  Q  F  F  Y  D  G  Q  G  F  M  S  L  Q  L  T  G  S  302
1081 CCGACGCGAAGGTGGCGTTCTACGATGCTTTCGGGAAAGTTCTTCACACATGGAGTGTTA
       D  A  K  V  A  F  Y  D  A  F  G  K  V  L  H  T  W  S  V  T  322
1141 CCAAGGATCTTCATACATCTATCTAAGAGGAGCTTAGCTAGAAGGGATTTTTGTAGCATT
       K  D  L  H  T  S  I                                        329
1201 ACTCTTTACATATATCGCCATTAAACTTATGTGGTGTATTGTGAAGTACTGCATGCTGGT
1261 TTTGCAGTTTGTTTGGAGTCTATTTGTAGTGGAAGTTTGGTTAGGGGAGGAGAGTGACGA
1321 TATGAGTCACACCATTTGTATTGGTCTTTCTTGTAGTGATGTTAATATACAGATTGGTCT
1381 GTT(A)n
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Fig. 2. Nucleotide and deduced amino acid sequences of LMP50. The positions of the nucleotides and amino acids are indicated on the left and right margins, respectively. The putative signal peptidase cleavage site is indicated with a slash. The two potential attachment sites for N-linked oligosaccharides are underlined.

ORF shows that there is a hydrophobic region at the N-terminus, suggesting the presence of a signal peptide. The first amino acid of the mature protein is assigned as glutamic acid. This assignment was determined by application of the (-3,-1) rule of von Heinje (1987), but remains tentative. The molecular mass of the putative polypeptide encoded by the ORF is approximately 37 kD. LMP50 protein contains two potential attachment sites for N-linked oligosaccharides. The sequences Asn-Gln-Ser and Asn-Asp-Ser occur at amino acids 56-58 and 258-260, respectively.

Homology search with the databases showed that the LMP50 protein was most homologous to human tartrate-resistant acid phosphatase (Ketcham *et al.*, 1989) and porcine uteroferrin (Simmen *et al.*, 1989). The LMP50 protein showed 28.1% identity and 72.1% similarity in a 313 amino acid overlap to the human tartrate-resistant acid phosphatase (Fig. 3).

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1  MAASSSCESM ALSLILVMGL LSLVAAELPR LEQPIKADGS LSLLVIGDWG
2  -------MDM WTALLILQA- LLLPSLAD-- -----GATPA LRFVAVGDWG
3  -------MDT WTVLLILQAS LVLPGAVGTR TNTRTAPTPI LRFVAVGDWG

1  --RNGGYNQS QV---ATQMG KIGEELDIDF VVSTGDNFYD SGLTSTDDKA
2  GVPNAPFHTA REMANAKEIA RTVQILGADF ILSLGDNFYF TGVQDINDKR
3  GVPNAPFHTA REMANAKAIA TTVKTLGADF ILSLGDNFYF TGVHDAKDKR

1  FEASFTNIYT AKSLQK-QWY SVLGNHDYRG DAVAQL--SP VLQK--IDSR
2  FQETFEDVFS DRSLRKVPWY VLAGNHDHLG NVSAQIAYSK ISKRWNFPSP
3  FQETFEDVFS DPSLRNVPWH VLAGNHDHLG NVSAQIAYSK ISKRWNFPSP

1  WLCMRSFILD AEI-VQFFFV DTTPFVMNYW DDPQDHHYDW RDVAPRDNYI
2  FYRLHFKIPQ TNVSVAIFML DTVTLCGN-S DDFLSQQPER PRLTARTQ-L
3  YYRLRFKIPR SNVSVAIFML DTVTLCGN-S DDFVSQQPER NLALARTQ-L

1  KNLRNDLDSA LKESTATWKI VVGHHTMRSV SEHGDTKELL DLLLPILKAN
2  SWLKKQLAAA RED----YVL VAGHYPVWSI AEHGPTHCLV KQLRPLLATY
3  AWIKKQLAAA KED----YVL VAGHYPVWSI AEHGPTHCLV KQLLPLLTTH

1  EVDLYINGHD HCLEHVSSND SPIQYLTSGA G----SKAWR GTYIPNSDKL
2  GVTAYLCGHD HNLQYL-QDE NGVGYVLSGA GNFMDPSKRH QRKVPNGY-L
3  KVTAYLCGHD HNLQYL-QDE NGLGFVLSGA GNFMDPSKKH LRKVPNGY-L

1  QFFYDGQ--- -GFMSLQLTG SDAKVAFYDA FGKVLHTWSV TKDLHTSI
2  RFHYGTEDSL GGFAYVEISS KEMTVTYIEA SGKSLFKTRL PRRARP
3  RFHFGAENSL GGFAYVEITP KEMSVTYIEA SGKSLFKTKL PRRARSENQHRRA
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Fig. 3. Comparison of the LMP50 protein (1) with human tartrate-resistant acid phosphatase (2), and pig uteroferin (3). Amino acid sequences are aligned for maximum homology by introducing gaps (-). Bold typed are the identical sequences with at least one mammalian enzyme.

DISCUSSION

In this study, we report isolation and characterization of a cDNA clone LMP50. RNA blot analysis and cDNA blot analysis showed that the *LMP50* mRNA is present only in anthers. The clone was highly abundant in the mature pollen library, indicating that the gene product plays a major role in mature pollen grain. In the previous studies, we have shown that pollen grains contain several mRNA species that are highly abundant in the tissue but not in other parts of the plant (Kim *et al.*, 1993; 1994).

The cDNA clone contains two ORFs. Both of the ORFs contain the consensus sequence at the eukaryotic start sites (Kozak, 1984), indicating that both ORFs may be translated. The functional role of the short ORF is unclear. Although it is not common, the 5' untranslated region of plant mRNAs occasionally contains a short ORF. It may be involved in the regulation of mRNA stability or translation efficiency.

The LMP50 protein is most homologous to the porcine uteroferrin and the human tartrate-resistant acid phosphatase. Both the uteroferrin and the acid phosphatase are iron-containing glycoproteins and resemble each other closely in electrophoretic mobility, in substrate specificity, and in their responses to a variety of activators and inhibitors (Hunt *et al.*, 1987). Both of the enzymes have been suggested to play a role in iron transport in the uterus. The uteroferrin can bind two atoms of iron per molecule and is postulated to act as a major iron carrier from the maternal uterus to the developing fetus where the iron molecule is largely used for the fetal hemoglobin synthesis (Roberts *et al.*, 1986). The tartrate-resistant acid phosphatase is an orthophosphoric monoester phosphohydrolase active at acid pH. It will be interesting to study whether the lily LMP50 protein functions as iron carrier and/or phosphohydrolase in mature pollen.

The LMP50 clone will be useful in obtaining pollen-specific genes from other plant species. The pollen-specific genes are useful in understanding the regulatory mechanisms of gene expression in pollen and for genetic improvement of crop plants.

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