Journal of Plant Biology, March 1999, 42(1) : 75-80 **SHORT COMMUNICATION**

A cDNA Clone from Broccoli (*Brassica oleracea*) for a New Type of Oleosin

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From a differential screening of a cDNA library for broccoli anther, a cDNA clone for oleosin was isolated and named *BASC1*. *BASC1* did not contain the amino-terminus but probably coded for most of the protein. The open reading frame extending for 212 amino acids had a very long hydrophobic region flanked by amphiphatic regions, the typical pattern for oleosins. In the long hydrophobic region, *BASC1* showed strong homologies to the previously reported oleosins. To the carboxy-terminus from the center of ORF, *BASC1* is unique in having a region of hydrophilicity in the middle of the amphiphatic stretch. Transcripts of *BASC1* were detected from the anther of uninucleate and binucleate pollens, and from the tapetum and pollen.

Keywords: oleosin, cDNA, broccoli, carboxy-terminus extension

Lipid stored in subcellular particles is one of the major food reserves for plants. These subcellular lipid particles are commonly found in the seeds in large amounts, but they are also found in pollens, flowers, roots, and stems of flowering plants (Huang, 1996). These lipid particles, triacylglycerol matrix surrounded by a layer of phospholipid and commonly called oil bodies, are structurally stable and do not coaggregate with each other. The usual size of the oil bodies is about 1 μm in diameter, and this small size provides a large surface area which would facilitate lipase binding for the utilization of stored lipids. The stability of these lipid particles is thought to be provided by a unique protein, oleosin (Huang, 1996; Napier et al., 1996).

Oleosin is a protein with a molecular weight ranging from 14 to 60 kDa. Although the size of the molecule varies to a large extent, all the proteins classified as oleosins and oleosin-like proteins have typical characteristics in common. Each oleosin molecule has a highly conserved central hydrophobic stretch of 70-77 amino acid residues which is considered to function as an anchor into the matrix lipids. In the amino-terminus region and to the carboxylic sides from the highly conserved, long hydrophobic region, amphipathic stretches are usually found. In these regions, similarity is low between oleosins in amino acid sequences, but amphipathicity is commonly found that is considered to provide interactions with the phospholipids on the surface of the oil body. In addition to these regions, an extension at the carboxy-terminus is present in some oleosins (Huang, 1996; Chen et al., 1997; Wu et al., 1998).

We report here a cDNA clone for oleosin from broccoli. The expression pattern is typical for an anther-specific oleosin gene, but the clone is unique in detecting transcripts from both tapetum and microspores and in coding for an oleosin with a distinct carboxy-terminus extension.

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MATERIALS AND METHODS

Plant Material

Broccoli (*Brassica oleracea*) was grown in the field of Hungnong Breeding and Research Station, and flowers at the several stages of development were collected. Collected flowers were immediately frozen and stored in liquid nitrogen.

cDNA Bank Preparation and Differential Screening

Total RNAs were extracted from various parts of broccoli frozen in liquid nitrogen in a mortar with extraction buffer containing guaidium thiocyanate and then purified by CsCl density gradient centrifugation (Hong and Jeon, 1987). Total RNAs from anther were then passed through an oligo(dT)-cellulose column to extract poly(A)$^+$ RNA. cDNA was synthesized using reverse transcriptase and RNaseH (cDNA syn-

thesis kit, Amersham, USA) and blunt-end ligated to the *Eco*RV site of pBR322. A cDNA bank was prepared in *Escherichia coli* strain HB101. Clones in the cDNA bank were subgrouped with 10 clones in each subgroup, and each subgroup was cultured overnight in L-ampicillin liquid medium. Plasmid DNAs extracted from the culture were restriction digested with *Eco*RI and *Hin*dIII and run on an agarose gel. DNA blot hybridization for the restriction digests was performed with ^{32}P-labeled first strand cDNA synthesized from mRNAs of broccoli leaf (Sambrook et al., 1989). Any subgroup which had a clone showing a negative signal was selected and rescreened with the same process for individual clones.

RNA and Genomic DNA Blot Hybridizations

Total RNAs extracted were run on a 0.8% agarose gel with formaldehyde and blotted onto nylon membrane. RNA loaded on each lane was normalized to 10 μg which was confirmed by the measurement of A_{260} and staining of the gel with methylene blue (Sambrook et al., 1989). An RNA ladder (BRL, USA) was used for size markers. For the genomic DNA blot analysis, 2 μg of genomic DNA extracted as described by Junghans and Metzlaff (1990) was digested with *Bam*HI, *Eco*RI, and *Pst*I, electrophoresed on a 0.8% agarose gel and blotted onto nylon membrane. A 1-kb DNA ladder (BRL) was used for size markers. cDNA clone was labeled with [alpha-^{32}P]dCTP by the random priming method using a Prime-a-Gene kit (Promega, USA) and then used for RNA and genomic DNA blot hybridizations. Hybridizations were carried out in hybridization solution (5X SSPE, 5X Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA) at 65°C overnight. After hybridization, the membrane was washed with 0.2X SSPE and 0.1% SDS at 65°C, and the blot was exposed to an X-ray film for 2 days or for 7 days at −70°C with two intensifying screens (DuPont, USA) (Sambrook et al., 1989).

DNA Sequencing

Insert of the cDNA clone was subcloned into pBluescript II SK(+), and a set of unidirectional deletion derivatives was obtained by exonuclease III using the deletion kit for kilo-sequencing (Takara, Japan). Double-stranded plasmid templates were sequenced in both directions by the dideoxy chain termination method (Sanger et al., 1977) using a USB Sequenase 2.0 kit (United States Biochemicals, USA). The nucleotide and the deduced amino acid sequences were analysed using the current GenBank and Swiss-Prot databases.

In Situ Hybridization

Nonradioactive in situ hybridization of the sectioned samples of broccoli anther was performed using digoxigenin-labeled antisense and sense probes of the cDNA. Anthers of broccoli were fixed with FAA (35% formaldehyde: glacial acetic acid:distilled water = 5:5:3.5:1.5, v/v/v) for 8 h, dehydrated and embedded in paraffin. Sections 5 μm thick were produced by an 820 spacer microtome (American Optical, USA) and mounted on the slides coated with 3-aminopropyltriethoxy silane (Sigma, USA), pretreated with 0.1 mg/ml proteinase K at 37°C for 12 min, and then hybridized with the digoxigenin-labeled RNA probe at 42°C overnight. The digoxigenin-labeled RNA probe was prepared as described by the manufacturer's instructions (Boeringer Mannheim, Germany). Slides were washed in several changes of 2X SSC in 0.1% SDS at room temperature, followed by one rinse with 2X SSC in 0.1% SDS at 52°C and two rinses with 0.1X SSC in 0.1% SDS at 52°C. Immunological detection of the hybridized probe was carried out by a digoxigenin-nucleic acid detection kit (Boeringer Mannheim). For color development, slides were incubated overnight in 0.34 mg/mL nitroblue tetrazolium salt in a buffer (100 mM Tris-HCl, 50 mM $MgCl_2$, pH 9.5, 100 mM NaCl). The reaction was stopped with TE solution (100 mM Tris-HCl, 1 mM EDTA pH 8.0). Photography was carried out using a BX50 microscope (Olympus, USA).

RESULTS AND DISCUSSION

Differential screening of the cDNA library prepared from broccoli anther allowed us to isolate clones showing anther-specific expression. Among the putative anther specific cDNA clones analyzed, nucleotide sequencing of *BASC1* identified a putative open reading frame (ORF) extending for 636 nucleotides. The amino acid sequence deduced from the ORF coded for 212 amino acids which started with arginine at the very end of the ORF. Thus, *BASC1* is likely a partial cDNA clone missing the amino-terminus. The ORF showed a long hydrophobic stretch which started from the 5th amino acid, methionine, and extended to the 85th amino acid, valine. The carboxy-terminus half of the ORF did amphiphatic region exist which showed alternating short hydrophobic and

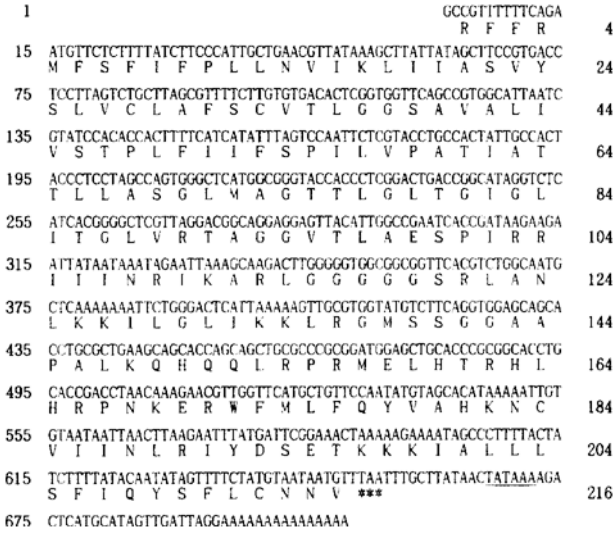

Figure 1. Nucleotide sequence and the deduced amino acid sequence for *BASC1*. Amino acids are denoted by one-letter symbols. The numerals on the left are for the nucleotides. The numerals on the right are for the amino acids. Translation termination codon is marked with ***. Putative polyadenylation signal is underlined.

hydrophilic regions. In the middle of this amphiphatic region, there was a stretch of hydrophilic amino acids. Another unique feature of this protein was that basic amino acids comprised most of the charged amino acids. Only 5 acidic amino acids could be located while 32 basic amino acids were spread throughout the hydrophilic region, resulting in a very basic pI of the ORF, 11.28 (Fig. 1).

Amino acid sequence comparison of BASC1 with other reported proteins in the database identified a strong homology in the long hydrophobic region with the proteins classified as oleosins. The highest homology was marked with the oleosin of *Brassica napus*, C98 (Roberts et al., 1993), i.e. in the long hydrophobic region, six substitutions were shown with four synonymous substitutions between BASC1 and C98. In the region close to the carboxy-terminus, variation in the amino acid sequence was significant. The amino acid sequence of BASC1 in this region did not show meaningful homology with other oleosins (Fig. 2). Comparison of the hydropathicity of BASC1 with other oleosin proteins again showed the characteristic long hydrophobic region of oleosin. Next to the long hydrophobic region, the amphipathic region could be generally observed in oleosin proteins including BASC1. In the region close to the carboxy-terminus, hydropathicity did not show a pattern which can be generalized for oleosin proteins, i.e. in some oleosins hydrophilicity dominated and in other oleosins

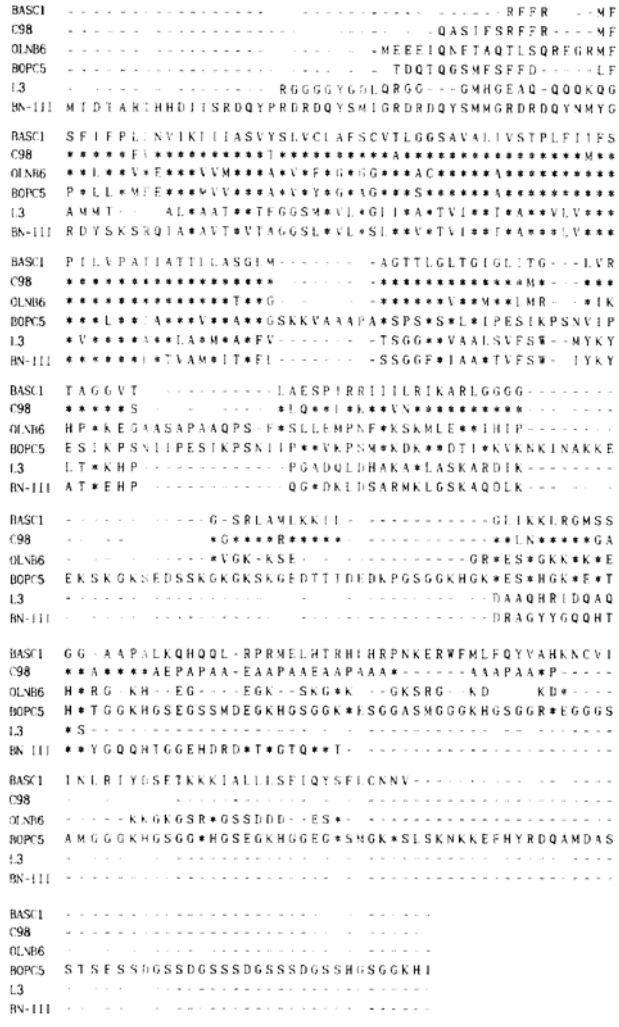

Figure 2. Comparison of the amino acid sequence of BASC1 with other reported oleosin proteins. A gap is introduced to maximize the alignment. * represents identical amino acids. C98, oleosin from *B. napus* (Roberts et al., 1993); OLNB6, oleosin-like protein from *B. napus* (Ross and Murphy, 1996); BOPC5, pollen coat oleosin from *B. oleracea* (EMBL accession X96408); L3, lipid body-associated major protein from *Zea mays* (Vance and Huang, 1987); BN-III, oleosin from *B. napus* (Keddie et al., 1992).

amphipathicity extended to the carboxy-terminus. BASC1 is unique in having a region of hydrophilicity in the middle of the amphiphatic stretch (Fig. 3).

Genomic DNA blot for *BASC1* showed one major band and a couple of minor bands from three digests, *Bam*HI, *Eco*RI and *Pst*I. The pattern indicated that in the genome of broccoli there is one locus for *BASC1* gene and a few homologous sequences are also present (Fig. 4).

In situ hybridization of cross-sections of anther at the stage of immature pollen for *BASC1* transcripts

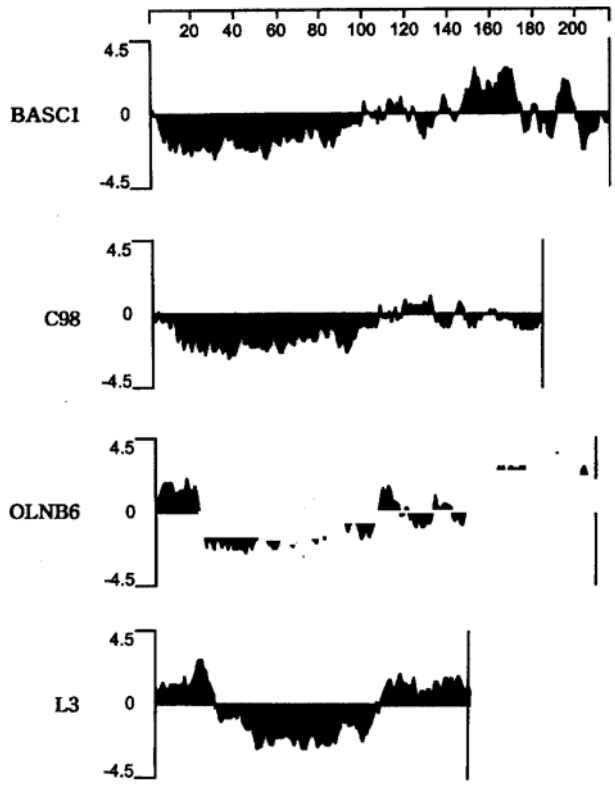

Figure 3. Hydropathy plots for the oleosin proteins. Negative side is for hydrophobicity. C98, OLNB6, and L3 are as in figure 2.

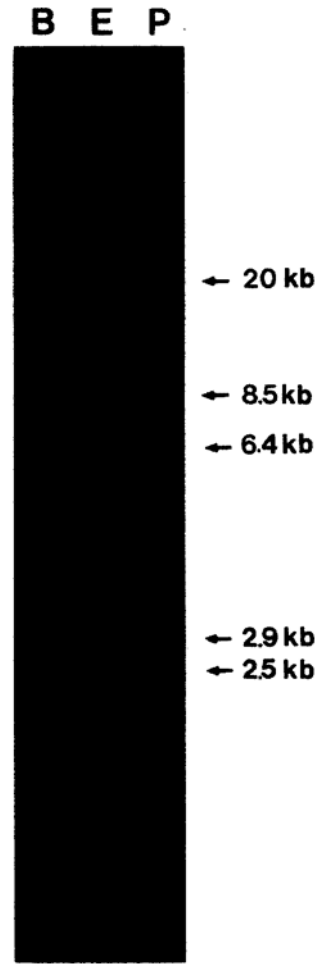

Figure 4. Genomic DNA blot analysis for *BASC1*. Broccoli genomic DNA was digested with *Bam*HI (B), *Eco*RI (E), or *Pst*I (P) and subjected to DNA blot hybridization for the *BASC1* cDNA clone.

showed positive signals from the tapetum and the pollen (Fig. 5A). The antisense probe of *BASC1* showed a thick blue signal from the tapetum and the pollen, but the sense probe did not show a meaningful signal from the cross section of the anther overall (Fig. 5B). RNA blot analysis for *BASC1* transcripts identified a signal from the anther at the developmental stage of pollen after the tetrad stage and before the maturity. From either earlier or later anther developmental stages, the signal from RNA blot was not detected. From all other organs assayed, including the pistil and leaves, the RNA signal could not be obtained. The size of the RNA blot hybridization band was about 1 kb (Fig. 5C).

The hydrophilic and/or amphipathic extensions of oleosins in the regions close to the amino-terminus and carboxy-terminus prevent the phospholipid layers of adjacent oil bodies from coalescing. The negative charge exhibited by the extension to the carboxy-terminus provides the electrical repulsion force between oil bodies and thus helps the oil bodies keep their small, average diameter ranges from 0.6 to 2.0 μm (Napier et al., 1996). The hydrophobic core is very well conserved in amino acid sequence and size among the oleosin proteins, and the main difference is found in the extension to the carboxy-terminus. The extensions so far reported range from 50 to 400 amino acid residues in length, but are much longer in the oleosins of anther (Robert et al., 1994; van Rooijen and Moloney, 1995). Regarding the anther oleosins, most of the earlier reports concern oleosins in the tapetum (Ross and Murphy, 1996; Wang et al., 1997; Tzen et al., 1998). In *B. napus*, oleosin transcripts were localized in the tapetum of anther having microspores at the uninucleate and dinucleate stages (Ruiter et al., 1997). Although lipid bodies and oleosin proteins have been often found in the pollen, detection of oleosin transcripts from the pollen has not been reported so far.

BASC1 reported here has several unique characteristics. The amphiphatic stretch is interrupted by a

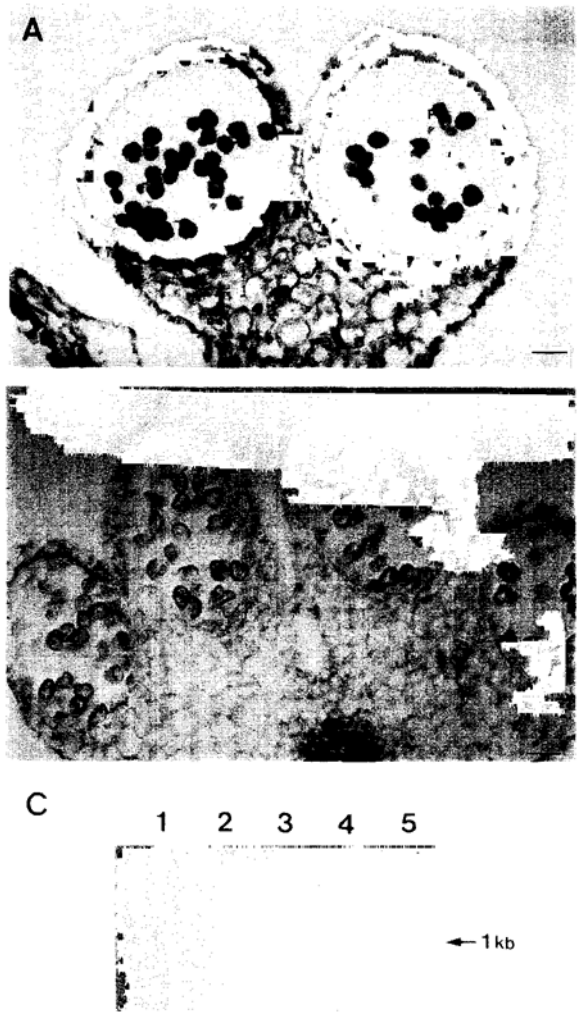

Figure 5. In situ hybridization result of the anther cross-section with the *BASC1* antisense and sense probes, and RNA blot analysis for *BASC1*. Broccoli anthers at the late development stage, i.e. after the tetrad and before pollen maturity, were cross-sectioned and hybridized in situ with either antisense probe (**A**) or sense probe (**B**). Total RNA extracted from several organs of broccoli was subjected to RNA blot hybridization for the *BASC1* transcript (**C**). 1, anther of less than 2 mm in length; 2, anther of 2-4 mm in length; 3, anther of 4-6 mm in length; 4, pistil; E, leaf; P, pollen; T, tapetum. Bar is for 100 μm.

hydrophilic region extending for almost 20 amino acid residues. The carboxy-extension has patches of amino acid repeats, for examples glycine residues and lysine residues. In situ hybridization for *BASC1* transcript to the cross-section of anther showed that the transcript recognized by the *BASC1* antisense probe appeared not only in the tapetal layer but also in the pollen. Oleosin is certainly one of the most interesting proteins related to the storage. Its characteristic structure, namely the very long hydrophobic core and the amphiphatic extensions, can explain the chemical nature necessary to keep oil bodies for efficient storage and utilization of oils. But there are unique natures needed to accommodate the diverse requirements of oil storage, and we think *BASC1* is a cDNA clone coding for a new oleosin probably functioning in the tapetum and pollen of broccoli.

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