

A cDNA clone encoding an IgE-binding protein from *Brassica* anther has significant sequence similarity to Ca^{2+} -binding proteins

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Abstract

Thirteen cDNA clones encoding IgE-binding proteins were isolated from expression libraries of anthers of *Brassica rapa* L. and *B. napus* L. using serum IgE from a patient who was specifically allergic to *Brassica* pollen. These clones were divided into two groups, I and II, based on the sequence similarity. All the group I cDNAs predicted the same protein of 79 amino acids, while the group II predicted a protein of 83 amino acids with microheterogeneity. Both of the deduced amino acid sequences contained two regions with sequence similarity to Ca^{2+} -binding sites of Ca^{2+} -binding proteins such as calmodulin. However flanking sequences were distinct from that of calmodulin or other Ca^{2+} -binding proteins. RNA-gel blot analysis showed the genes of group I and II were preferentially expressed in anthers at the later developmental stage and in mature pollen. The recombinant proteins produced in *Escherichia coli* was recognized in immunoblot analysis by the IgE of a *Brassica* pollen allergic patient, but not by the IgE of a non-allergic patient. The cDNA clones reported here, therefore, represent pollen allergens of *Brassica* species.

Introduction

Inhalation of pollen in the atmosphere provokes an IgE-mediated response of hay fever characterized by symptoms such as asthma, sneezing attacks, watery discharge, nasal obstruction and eye itching [2]. Diagnosis and treatment of IgE-mediated allergy disorders require the production

of large amounts of pure and well defined proteins. cDNA cloning and expression of allergens should provide information regarding their structural and functional features and elicit new data about how these allergens promote the IgE response in affected individuals. From the point of view of plant biology, it is of interest to know the functions of such proteins in pollen [22].

The nucleotide sequence data reported will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers D63153 (*Bra r I*), D63151 (*Bra n I*), D63154 (*Bra r II*) and D63152 (*Bra n II*).

Recombinant DNA technology has been used recently for the isolation and characterization of cDNAs encoding pollen allergens in several species [2 for a review], such as rye-grass [9, 23], Kentucky bluegrass [21], timothy grass [4], ragweed [8, 17], birch [3, 20, 26] and olive [27].

Oil seed rape, a member of the *Brassica* family, has been introduced to a number of countries and cultivated widely. The genus includes broccoli, kale, cabbage, mustard, turnip as well as rape. Due to the commercial interest, this genus is an expanding crop throughout the world [24].

Being insect pollinated, the *Brassica* plants produce little airborne pollen. The allergy to *Brassica* pollen is uncommon in the population at large [24]. In a number of epidemiological studies, a very small percentage <1.5% of population showed positive RAST (radioallergosorbent test) to oil seed rape. In contrast, the incidence of rape pollen allergy is very much higher in those subjects occupationally exposed. In an epidemiological study of 29 subjects at Rothamsted Experimental Station (UK), 9 were skin test positive to oil seed rape pollen [7]. In Japan, three cases of occupational pollinosis caused by *Brassica* pollen have been reported, and specific IgE against pollen of *B. rapa* and *B. napus* has been demonstrated by way of an intradermal test and its threshold test, ELISA titration, and inhibition methods [1].

In this study, we isolated cDNA clones encoding IgE-binding proteins from expression libraries of anthers of *Brassica rapa* and *B. napus*. The predicted proteins showed sequence similarity to Ca^{2+} -binding proteins. However, the proteins were distinct from calmodulin or other Ca^{2+} -binding proteins. Therefore, the pollen allergen reported here will belong to a novel class of Ca^{2+} -binding proteins.

Materials and methods

Plant materials

Mature anthers and immature anthers at the microspore stage were taken from *Brassica rapa* L.

homozygous for *S* alleles, *S*⁸ or *S*⁹, which has been maintained in Tohoku University, Sendai, Japan [28]. For construction of cDNA libraries, anthers were also taken from *B. napus* L. cv. Canola grown at the Crops Research Institute (Horsham, Victoria, Australia).

Protein gel-blot

Extracellular proteins were extracted from dehiscent anthers by vigorous shaking in 10 mM Tris-HCl buffer at pH 8.0 for 5 min. Total soluble proteins were also extracted by grinding anthers with liquid nitrogen. The extracts were centrifuged at 15000 rpm for 15 min at 4 °C, and 40 µg of protein was subjected to thin-layer polyacrylamide gel isoelectric focusing (IEF; pI 3–10), as previously described [28].

After electrophoresis the proteins in the acrylamide gel were transferred onto nitrocellulose membranes (BA45; Schleicher & Schuell) at a constant current of 2.5 mA/cm² of gel area for 30 min in a semi-dry electroblot apparatus (Nova blot; Pharmacia) using a solution containing 39 mM glycine, 48 mM Tris, 0.0375% (w/v) sodium dodecyl sulfate and 20% (v/v) methanol.

The blots were blocked with 1% Blocking Reagent (Boehringer Mannheim) in TBS (150 mM NaCl, 10 mM Tris HCl pH 8.0) for 3 h, and reacted with human serum at a dilution of 1: 5 in TBS containing 1% bovine serum albumin, followed by reaction with peroxidase-labelled antibodies to human IgE at a dilution of 1: 5000 (Kirkegaard & Perry Laboratories). The signal was detected by enhanced chemiluminescence according to the ECL Western Blotting Analysis System (Amersham). The pI Calibration Kit (pH 3–10; Pharmacia) was used to estimate pI values of proteins.

cDNA library and immunoscreening

Poly(A) RNA was extracted from anthers at a defined stage of development using the Fast Track mRNA Isolation Kit (Invitrogen) or Quick Prep

mRNA Purification Kit (Pharmacia). cDNA was synthesized, ligated to an *Eco* RI/*Not* I adaptor (Pharmacia), and cloned into the *Eco* RI site of the vector λ gt11 according to the manufacturer's instruction (Pharmacia).

Plaques were lifted onto nitrocellulose filters (BA85; Schleicher & Schuell) by the method of Sambrook *et al.* [19]. After blocking the filters with 10% skim-milk or 1% Blocking Reagent (Boehringer Mannheim), the filters were incubated with 5-fold diluted serum of a *Brassica* pollen-allergic patient in TBS overnight at room temperature. The patient was selected for case history, positive skin-prick test, and positive RAST which indicated class 2 (case 1 described in [1]). IgE-binding was detected by incubation in rabbit immunoglobulins to human IgE (Dakopatts), followed by incubation in peroxidase-labelled anti-rabbit IgE (Promega) as described [16]. Positive signals were visualized using 4-chloro-1-naphthol and H_2O_2 [19], or using enhanced chemiluminescence according to the ECL Western Blotting Analysis System (Amersham).

Nucleotide sequencing

cDNA was isolated from plaque-purified phages, subcloned into *Eco* RI or *Not* I site of pBluescript SK (Stratagene). PCR amplification of cDNA using λ gt11 primers (Promega) was also used to subclone cDNA into a pCRII vector (Invitrogen), as described [29]. DNA sequences were determined and analysed as described [29]. Rescreening of the cDNA library by phage-lift hybridization [29] was carried out to obtain full-length cDNA.

Construction of expression plasmid

An expression plasmid was constructed by inserting each cDNA into *Not* I sites of the PinPoint Xa-3 vector containing the tag sequence encoding the 12.5 kDa subunit of the transcarboxylase complex of *Propionibacterium shermanii* (Promega). The recombinant cDNA was ex-

pressed in *E. coli* XL-1 Blue starin, and crude cell supernatant was prepared by sonication according to the instruction manual of the PinPoint Protein Purification System (Promega). Human serum used for probing blots of the crude cell extract was preincubated overnight with 1/100 volume of sonicated extracts of *E. coli* Y1090 cells to remove antibodies to *E. coli* proteins.

RNA-gel blot

Total RNA was isolated from *B. rapa* using an SDS-proteinase K extraction procedure [14]. RNA was prepared from anthers at the uninucleate microspore stage, the bicellular pollen stage and the tricellular pollen stage, and mature pollen, stigmas and leaves. A 10 μ g portion of total RNA was resolved on 1% agarose gel after denaturation with glyoxal. DNA probes were labelled with digoxigenin-dUTP, and hybridization was detected by treatment with an anti-digoxigenin antibody conjugated with alkaline phosphatase and 3-(2'-spiroadamantane)-4-methoxy-4-(3''-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) according to the manufacturer's instruction (Boehringer Mannheim). Hybridization and washing were performed as described [29]. An RNA ladder (Gibco BRL) was used to estimate the molecular size of mRNAs. The same RNA blot was stripped and reprobed with tubulin cDNA of *Zinnia elegans* L. (kindly provided by H. Fukuda, Tohoku University) to check the integrity of RNA.

Results

Identification of IgE-binding proteins in anther extracts

The serum from the individual who was specifically allergic to *Brassica* pollen detected a unique major band at pI 4.5 on the IEF-gel blots of total soluble proteins extracted from mature anthers of *Brassica rapa* (Fig. 1). A unique band at the same position was also detected on the blots of extra-

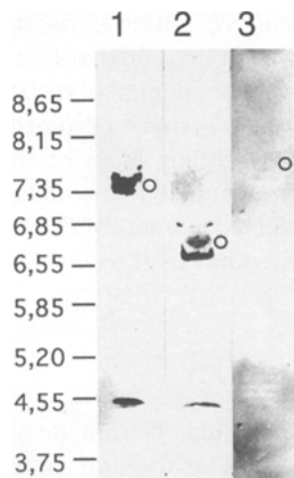


Fig. 1. IgE-immunoblot of anther proteins of *Brassica rapa* separated by IEF. Lane 1, total proteins reacted with IgE of a *Brassica* allergic individual; lane 2, extracellular proteins reacted with IgE of a *Brassica* allergic individual; lane 3, total proteins reacted with IgE of a non-allergic individual. A circle indicates the position of sample application on a piece of filter paper and does not indicate the signals. pI markers are indicated on the left.

cellular proteins from dehiscent anthers. No bands were detected when either blot was probed with serum of an individual who was not allergic to *Brassica* pollen (Fig. 1). The same results were obtained when the anthers of *B. napus* were used as materials (data not shown).

Isolation of cDNA clones

The serum of the allergic individual was used for immunoscreening of the expression cDNA libraries in λ gt11. Screening was initially carried out on the cDNA library made from immature anthers of *Brassica rapa*. Two positive plaques (clones 4 and 5) were identified out of 84000 plaques. Further screening was performed on two cDNA libraries of *B. napus*; one was from immature anthers at the microspore stage, the other was from the mature stage. Eleven positive plaques (clones 41, 42, 43, 44, 51, 52, 53, 61, 62, 63 and 65) were isolated out of 120000 plaques in the mature-anther library. No positive plaques were obtained from

the library of 120000 plaques of immature anthers at the microspore stage.

Cross-hybridization experiments indicated that the cDNA clones were divided into two groups, I and II. Group I was comprised of clone 5 of *B. rapa* and clones 43, 51, 52, 53, 61, 63, 65 of *B. napus*, whereas group II contained clone 4 of *B. rapa* and clones 41, 42, 44, 62 of *B. napus* (Fig. 2). All the cDNAs in group I contained an *Eco* RI cleaved site within the cDNA, together with the *Eco* RI/*Not* I adaptors. Three (clones 43, 53 and 63) were restricted with *Not* I, ligated into pBlue-script and sequenced. The cDNAs in group II did not contain an internal *Eco* RI site. Two of the clones (4 and 41) were restricted with *Eco* RI, ligated into pBluescript and sequenced.

As for the other clones, cDNA inserts were subcloned into a pCRII vector after amplification by PCR, and partial sequences, which covered entire coding region, were determined.

Nucleotide sequences

At first, we determined the sequences of clone 43 in group I and clone 41 in group II. Clone 43 is 437 bp long and contains an open reading frame (ORF) starting from the 5' end of the cDNA which follows the β -galactosidase of λ gt11. Clone 41 is 458 bp long with an ORF starting from the 5' end of the cDNA, as in clone 43. When clone 43 in group I was compared with clone 41 in group II, the identity of the sequences of the entire cDNA was 64%, whereas those of the coding and noncoding regions were 74% and 55% respectively.

In order to obtain longer cDNA and define the first ATG codon, rescreeing by phage-lift hybridization, using clone 43 and clone 41 as probes, was carried out on another cDNA library made from mature anthers of *B. rapa*. We chose *B. rapa* for this purpose, as *B. napus* is known to be an amphidiploid between *B. rapa* and *B. oleracea* and would yield more complicated kinds of cDNAs. Out of 25000 plaques, three were identified to hybridize to clone 43, and three to clone 41. The longest clones, clones 973 and 921, were

<i>Bet v III</i>	QEEADSFSGF	KVFDGDG	ISARELQ	MVL	GKLG	SEGEIDR	
Calmodulin	DSEELKEAF	RVFDKONGE	ISAAELRHVM		TNLGEKLSDEE		
	:	:	:	:	:	:	:
Group I							
<i>B. rapa</i> 5, 973	MADA	EAHERIF	KKFDT	DGDGK	ISAAE	LEEAL	KKLGSVTPDD
<i>B. napus</i> 43,51,52,53,61,63,65	*****	*****	*****	*****	*****	*****	*****
	:	:	:	:	:	:	:
Group II							
<i>B. rapa</i> 921	MADA	TEKAEH	DRIF	KKFD	ANGDGK	ISASEL	GDAL
<i>B. napus</i> 41,62	***	*****	*****	*****	*****	*****	*****
<i>B. rapa</i> 4	E	**R*****	*****	*****	*****	*****	*****
<i>B. napus</i> 42	****	**T*****	*****	T	**ST*****	*****	*****
<i>B. napus</i> 44	****	*****	F	*****	T	**ST*****	*****

<i>Bet v III</i>	VEKMIVSVDS	NRDGRVDFFE	FKDDDMR	SVLVRSS	203
Calmodulin	VDEMIREADV	DGDGQINYE	E	FKVVMMAK	149
	:	:	:	:	:
Group I					
<i>B. rapa</i> 5, 973	VTRMMAKIDT	DGDGNISFQE	FTEFASANPG	LMKDVAKVF	79
<i>B. napus</i> 43,51,52,53,61,63,65	*****	*****	*****	*****	79
	:	:	:	:	:
Group II					
<i>B. rapa</i> 921	IKRMAEIDT	DGDGYISYQE	FSDFASANRG	LMKDVAKIF	
<i>B. napus</i> 41,62	*****	*****	*****	*****	83
<i>B. rapa</i> 4	*****	*****	*****	*****	
<i>B. napus</i> 42	*****	*****	F*****	*****K*****	83
<i>B. napus</i> 44	*****	*****	F*****	*****K*****	83

Fig. 2. Comparison of amino acid sequences of cDNA clones of group I and group II allergens in *Brassica rapa* and *B. napus*. Stars indicate identical residues within each group. Birch pollen allergen, *Bet v III* (accession number X79267 in the EMBL databases) and calmodulin of *Arabidopsis thaliana* (accession number S25131 in the EMBL databases) are also compared. Colons and dots indicate identical and similar residues, respectively, between calmodulin and *Bra r I* and between *Bra r I* and *Bra r II*. The Ca^{2+} -binding domains of calmodulin are underlined.

chosen and sequenced. Clone 973 was 456 bp long, showed 93% identity to clone 43, and contained an in-frame stop codon, TAG, 15 nucleotides upstream from the first ATG initiation codon. Clone 921 was 469 bp long, showed 99% identity to clone 41, and contained an in-frame stop codon, TAA, 6 nucleotides upstream from the ATG initiation codon. We therefore concluded the cDNA clones encoded the full-length protein starting from the first methionine.

Deduced amino acid sequences

The ORF of clone 973 of *B. rapa* in group I predicts a protein of 79 amino acids. All the other clones in group I, clone 5 of *B. rapa* and clones 43, 51, 52, 53, 63, 65 of *B. napus* predicted a protein identical to that of clone 973 (Fig. 2). It

is noteworthy that the clones of *B. rapa* and *B. napus* encode the same protein. We tentatively designated the protein as *Bra r I* for *B. rapa* and *Bra n I* for *B. napus*, in accordance with the International Union of Immunological Societies approved nomenclature system [13]. Because *Bra r I* and *Bra n I* represent an identical protein, we designate it as *Bra r I* in this report.

The ORF of the clones in group II predicted a protein of 83 amino acids, although some clones lacked the first methionine. There was microheterogeneity at several positions of its primary structure (Fig. 2). Clone 921 of *B. rapa* and clones 41 and 62 of *B. napus* predicted an identical protein. This was considered the representative of the protein in group II. We designated the protein encoded by clone 921 of *B. rapa* as *Bra r II*, and that of clones 41 and 62 of *B. napus* as *Bra n II*. As *Bra r II* and *Bra n II* are identical proteins, we

therefore refer to them as *Bra r II* in this report.

The amino acid sequences of *Bra r I* and *Bra r II* showed 78% identity. *Bra r II* had four amino-acid extension near the N terminus of *Bra r I* (Fig. 2). The predicted molecular mass for *Bra r I* was 8.6 kDa with pI value of 4.34, whereas that for *Bra r II* was 9.1 kDa with pI value of 4.54. The pI values were in good agreement with those of the antigenic protein of pI 4.5 observed in the IgE immunoblot of the anther extract (Fig. 1).

Hydropathy profiles calculated using the values and algorithm of Kyte and Doolittle [12] indicated that *Bra r I* and *Bra r II* were remarkably hydrophilic except at the C terminus.

Similarity of *Bra r I* and *Bra r II* to calmodulin

A search of databases identified similarity among *Bra r I*, *Bra r II* and Ca^{2+} -binding proteins such as calmodulin and troponin c from a number of different sources. Similarity was particularly strong in the Ca^{2+} -binding domains of calmodulin. Figure 2 shows similarity between *Bra r I* and calmodulin of *Arabidopsis thaliana*. The calmodulin has 149 amino acids and contains four Ca^{2+} -binding domains. In contrast, *Bra r I* and *Bra r II* had almost half the number of amino acid (79 amino acids for *Bra r I* and 83 amino acids for *Bra r II*) and contained two regions with sequence similarity to Ca^{2+} -binding domains. There was little sequence conservation outside the Ca^{2+} -binding domains, with a one amino acid deletion in the sequence between Ca^{2+} -binding domains and an 11 amino-acid extension to its C terminus.

A pollen allergen, *Bet v III*, with significant homology to calmodulin, has been recently identified in birch [20]. Similarity between *Bra r I*, *Bra r II* and *Bet v III* was evident only in the Ca^{2+} -binding domains (Fig. 2).

Computer-aided predictions of secondary structure indicated that *Bra r I* was particularly well-fitted to an EF-hand structure. The Ca^{2+} -binding domain was flanked on both side by α -helical domains.

RNA-gel blot analysis

The temporal and spatial expression of the genes was examined by northern hybridization analysis (Fig. 3). When the RNA blot was probed with clone 43 to detect *Bra r I* transcripts, the hybridization signal was observed in anthers at all the three stages and in mature pollen, but not in stigmas or leaves (Fig. 3A). The strongest expression was observed in anthers at the bicellular stage and mature stage. A weak hybridization signal was detected in anthers at the uninucleate microspore stage. The same expression pattern was obtained when the RNA blot was probed with clone 41 to detect *Bra r II* transcripts (Fig. 3B). The intensity of the band was stronger in *Bra r I* transcripts than in *Bra r II* transcripts. The amount of the transcripts was consistent with the fact that the number of cDNA clones in group I was more abundant than that in group II.

The size of the transcripts was estimated to be 540 bases for both *Bra r I* and *Bra r II* transcripts. This result indicated that cDNA clone 973 for *Bra r I* and clone 921 for *Bra r II*, which were 456 bp and 469 bp respectively, were nearly full-length. When the same RNA blot was stripped and reprobed with tubulin cDNA, the transcripts

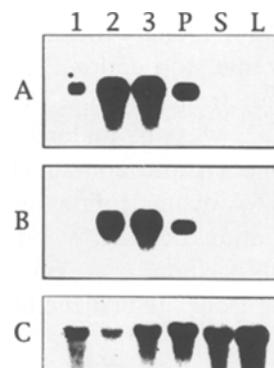


Fig. 3. Northern blot of total RNA isolated from different tissues of *Brassica rapa*. The blot was probed with cDNA clone 43 for detecting *Bra r I* transcripts (A), clone 41 for *Bra r II* (B) and tubulin cDNA (C). Lane 1, anthers at the uninucleate microspore stage; lane 2, anthers at the bicellular pollen stage; lane 3, anthers at the tricellular pollen stage (= mature stage); lane P, mature pollen; lane S, stigmas; lane L, leaves.

were observed in all tissues tested, indicating the integrity of RNA (Fig. 3C).

IgE-binding capacity of recombinant Bra r I and Bra r II

An expression plasmid was constructed by inserting cDNA clones 43 and 41 into a PinPoint Xa-3 vector, and the recombinant *Bra r I* (= *Bra n I*) and *Bra r II* (= *Bra n II*) were produced in *E. coli* as fusion proteins with the tag sequence encoding the transcarboxylase complex of *P. shermanii*. The serum IgE used for the immunoscreening identified a single protein on the IEF-gel blot at pI 4.6 for the *Bra r I* fusion protein and at pI 4.7 for the *Bra r II* fusion protein (Fig. 4). These pI values were consistent with the calculated pI values of the fusion proteins, which were pI 4.62 for the *Bra r I* fusion protein and pI 4.67 for the *Bra r II* fusion protein. The *Bra r I* fusion

protein yielded stronger signals than the *Bra r II* fusion protein. The IgE did not react with the fusion proteins when the cDNA was inserted into the expression vector in an antisense orientation (Fig. 4), indicating that the IgE did not react with the protein encoded by the tag sequence. The IgE of a non-allergic patient did not react with either the *Bra r I* or *Bra r II* fusion proteins (Fig. 4).

Discussion

Patients have been reported to be allergic to pollen of *Brassica* species, including *B. napus*, *B. rapa* and *B. oleracea* [1]. In this study we used the anthers of *B. rapa* and *B. napus*, and 13 cDNA clones encoding IgE-binding proteins were obtained by IgE immunoscreening. These cDNA clones predicted two kinds of proteins *Bra r I* (= *Bra n I*) and *Bra r II* (= *Bra n II*). *B. rapa* and *B. napus* were shown to have the same allergens (Fig. 2). The nomenclature used here is tentative, because we have no information about the purified native allergens. No other cDNA were obtained, probably because the specific reactivity of the IgE used in this study. The IgE reacted to a unique protein in the IEF-gel blot of the anther extract (Fig. 1). It remains unclear whether this IgE-bound anther protein is indeed corresponds to either *Bra r I* or *Bra r II* protein. We carried out IgE immunoblot inhibition experiments by the methods described for the study of pollen allergen of timothy grass [4]. However, the patient's serum depleted of antibodies specific for the recombinant *Bra r I* and *Bra r II* still reacted with the anther protein at pI 4.5 on the IEF-gel blot of the anther extract (data not shown). This result alone can not rule out the possibility that the anther protein is not either *Bra r I* or *Bra r II* protein. Further study is necessary to identify *Bra r I* and *Bra r II* proteins in anthers.

We have successfully expressed the recombinant *Bra r I* and *Bra r II* as fusion proteins. Because immunoblot of fusion proteins showed the recombinant *Bra r I* reacted with the human IgE more strongly than recombinant *Bra r II* (Fig. 4), and because in anthers *Bra r I* was shown to be

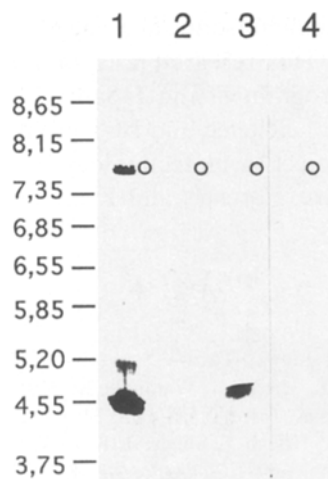


Fig. 4. IgE immunoblot of recombinant *Bra r I* and *Bra r II* separated by IEF. Lane 1, recombinant *Bra r I* reacted with IgE of a *Brassica* allergic individual; lane 2, antisense-recombinant *Bra r I*, in which cDNA was expressed in antisense orientation, reacted with IgE of a *Brassica* allergic individual, showing no signals; lane 3, recombinant *Bra r II* reacted with IgE of a *Brassica* allergic individual; lane 4, recombinant *Bra r I* reacted with IgE of a non-allergic individual, showing no signals. A circle indicates the position of sample application on a piece of filter paper and does not indicate the signals. pI markers are indicated on the left.

expressed in greater amounts than *Bra r II* (Fig. 3), *Bra r I* seems to be the major allergenic protein in *Brassica* pollen. It is clear that the *Bra r I* and *Bra r II* proteins are pollen allergens to the single patient described in this report. However, the IgE from two other patients who were sensitive to *Brassica* pollen (case 2 and case 3 described in Ashida *et al.* [1]) hardly bound to the fusion proteins (data not shown). It is necessary to investigate more patients to know whether *Bra r I* and *Bra r II* proteins are general allergens or not. The production of large amounts of recombinant allergens will be useful for diagnosis and treatment of *Brassica* allergic patients.

Bra r I and *Bra r II* have similarity to calmodulin and other Ca^{2+} -binding proteins. A pollen allergen, *Bet v III*, having homology with Ca^{2+} -binding proteins has been reported in birch [20]. However, *Bra r I* and *Bra r II* did not share any similarity to *Bet v III* outside the Ca^{2+} -binding domains. *Bra r I* and *Bra r II* are therefore likely to be novel allergens not reported previously.

The Ca^{2+} metabolism in pollen is controlled by a number of different Ca^{2+} -binding proteins, and pollen germination is known to depend critically on Ca^{2+} [10, 18]. Although we have not yet demonstrated that *Bra r I* and *Bra r II* do bind to Ca^{2+} , we speculate from their similarity to Ca^{2+} -binding proteins that the function of *Bra r I* and *Bra r II* is to control Ca^{2+} metabolism in mature pollen. This is supported by the observation that the *Bra r I* and *Bra r II* transcripts were present in mature pollen (Fig. 3). *Bra r I* and *Bra r II* are likely to be preferentially expressed in anthers after mitosis of microspores (Fig. 3). The amount of the transcripts during anther development was consistent with the fact that larger numbers of cDNA clones were obtained in the library of mature anthers than from the library of immature anthers.

The function of other pollen allergens has been estimated only in some allergens of birch. The major birch pollen allergen *Bet v I* was shown to be highly homologous to pathogenesis-related plant proteins and was considered to be involved in pathogen resistance of pollen [3]. *Bet v II* was shown to have homology to profilins and was

considered to play a role in actin organization [26]. *Bet v III*, which had homology to Ca^{2+} -binding proteins, was speculated to control the Ca^{2+} metabolism [20].

The role of pollen allergens, however, has not been directly demonstrated in any plant. *Brassica* provides a good material for studying the role of pollen allergens in plant sexual reproduction, because extensive cytological observations of anthers and pollen have been reported in *Brassica* species [5, 6], pollen-stigma interactions have been well characterized in the study of self-incompatibility [11, 15], and transformation is easily achievable [25]. Studies of antisense inhibition of the expression of *Bra r I* and *Bra r II* in transgenic plants are in progress to estimate the role of these allergens in plants.

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