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Molecular cloning of a cDNA encoding a pollen extracellular protein as a potential source of a pollen allergen in *Brassica rapa*

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Abstract A polyclonal antiserum was raised against the extracellular pollen proteins of *Brassica rapa* and used for screening the expression cDNA libraries made from immature anthers. We obtained five groups of cDNA clones, including cDNAs similar to PCP1, thioredoxin, and lipid transfer protein (LTP). Recombinant protein of the cDNA clone showing sequence similarity to LTP was demonstrated to bind IgE of a patient allergic to *Brassica* pollen. The cDNA clone reported here, therefore, represents a novel pollen allergen of *Brassica rapa*.

Key words: Anther; *Brassica rapa*; cDNA cloning; Immunoglobulin E; Lipid transfer protein; Pollen allergen

1. Introduction

Pollen allergens bind to human IgE and lead to various allergic manifestations in susceptible individuals. Pollen allergens are readily released from pollen cytoplasm or the pollen coat into human mucus [1]. EM localization of pollen allergen has revealed that *Lol p*Ia and *Lol p*Ib, major pollen allergens of ryegrass (*Lolium perenne*), are located within the cytosol and the starch granules, respectively [2]. In contrast, *Cry j* I, a major pollen allergen of Japanese cedar (*Cryptomeria japonica*), is reportedly located in exine layers and orbicles derived from tapetal tissue as well as Golgi bodies in the protoplasm [3].

Most of the pollen coat proteins on the exine are assumed to be derived from the tapetum, which plays an essential role in pollen development. Tapetum is known to supply nutrients to the developing microspores and precursors for the synthesis of pollen exine. Tapetum degrades at a later stage of pollen development and the remnants of the tapetum are also deposited on the pollen exine [4,5]. Pollen coat proteins have been extensively studied in *Brassica* species [6–8], because they are considered to play an important role in all aspects of pollination, dispersal, adhesion, and pollen-stigma interactions. Several cDNAs encoding pollen coat proteins have been isolated and characterized, including cDNAs encoding oleosin-like proteins [9], PCP1 [10], and transmembrane channel proteins [11].

Pollen coat proteins are a potential source of pollen allergens for humans. Allergy to *Brassica* pollen has been reported in some countries [12–14]. There has been only one report of the isolation of cDNA for a *Brassica* pollen allergen. We have previously isolated cDNA clones, *Bra r* I and *Bra r* II from *B. rapa* and *Bra n* I and *Bra n* II from *B. napus*, as cDNAs for pollen allergens [15].

In this study, a mouse polyclonal antiserum was raised against the extracellular pollen proteins of *B. rapa* and used for screening expression cDNA libraries made from immature anthers at the stage before the tapetum was degraded. Protein plaque lifts from the cDNA clones thus obtained were tested for IgE binding. RNA gel blot analysis of anthers at different developmental stages was also conducted.

2. Materials and methods

2.1. Plant materials

Mature anthers were collected from *B. rapa*, homozygous for the *S9* haplotype, and stored dry with silica gel at −30°C [16]. Pollen grains were removed from the anthers (1 g DW) with a paint brush and a sieve (100 μm mesh). Lipid was removed from the pollen with cold, anhydrous acetone, and then extracellular proteins were extracted by vigorous shaking for 5 min in 3 ml of a solution containing 0.1 M Tris (pH 8.0), 15 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl metabisulfate according to Evans et al. [17].

2.2. Production of antiserum

The extracted proteins (100 μg) were injected intraperitoneally into a female mouse (BALB/c). Freund's complete adjuvant (Difco) was included in the first and the second injections, and the serum was collected 3 days after the third injection, as described for other proteins [16].

2.3. Protein gel blot

Protein (40 μg) was subjected to 15% SDS-polyacrylamide gel electrophoresis or thin-layer polyacrylamide gel isoelectric focusing (IEF). The ampholine PAGplate (p*I* 3.5–9.5) and Multiphor II system (Pharmacia) were used for IEF. After electrophoresis, the proteins were transferred to nylon membrane (Immobilon), as previously described [15].

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 the mouse serum at a dilution of 1:1000 in TBS, followed by reaction with alkaline phosphatase-labelled antibodies to mouse IgG at a dilution of 1:5000 (Promega). The signal was visualized using 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salts and nitroblue tetrazolium chloride.

2.4. cDNA libraries and immunoscreening

cDNA libraries were constructed using vector λgt11 from anthers containing uninucleate microspores and bicellular pollen of *B. rapa*, and from anthers containing uninucleate microspores of *B. napus* cv. Westar. These cDNA libraries were successfully used for screening of cDNAs for *Brassica* pollen allergens, *Bra r* I, *Bra r* II, *Bra n* I, and *Bra n* II, as previously described [15].

Plaques were lifted onto nitrocellulose filters (BA85; Schleicher and Schuell) by the method of Sambrook et al. [18]. After blocking the filters with 1% blocking reagent (Boehringer Mannheim), the filters were incubated for 16 h at room temperature with 1000-fold diluted serum, which had been pre-absorbed with *Escherichia coli* extracts, as described by Toriyama et al. [15]. The filters were then reacted with peroxidase-labelled anti-mouse IgG antibody at a dilution of 1:5000

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(Kirkegaard and Perry Laboratories). Positive signals were visualized using 4-chloro-1-naphthol and H_2O_2 [18].

2.5. Nucleotide sequencing

cDNA was isolated from plaque-purified phages by PCR amplification using λgt11 primers (Promega) and subcloned into a pCRII vector (Invitrogen). DNA sequences were determined and analyzed as previously described [19]. Hydropathy profiles were calculated using the values and algorithm of Kyte and Doolittle [20] and a signal peptide was predicted using the PSORT program (http://psort.nibb.ac.jp/)

2.6. RNA gel blot

Total RNA was isolated using ISOGEN (Nippon Gene) from anthers at the uninucleate microspore stage, the bicellular and tricellular pollen stages, mature pollen, stigmas, and leaves. A 20 μg portion of total RNA was resolved on a 1.5% agarose gel after denaturation with glyoxal. The gel was stained with ethidium bromide before blotting. RNA gel blot analysis was performed as described [15].

2.7. IgE binding assays

Protein plaque lifts from cDNA clones were prepared using standard methods [18]. Briefly, λgt11 phages containing a cDNA clone were plated on LB plates with *E. coli* Y1090 and protein plaque lifts were taken on nitrocellulose filters (BA85, Schleicher and Schuell) presoaked in 10 mM IPTG. Filters containing a recombinant protein were divided into sections and incubated with allergic patients' sera diluted 1:5 in TBS containing 0.5% BSA. The bound IgE antibodies were detected using peroxidase-labelled anti-human IgE as described above.

3. Results

3.1. Characterization of antiserum

Extracellular protein of *Brassica* pollen was extracted in 0.1 M Tris buffer from the lipid-free mature pollen using the methods of Evans et al. [17]. Microscopic observation revealed that the pollen grains hydrated but did not burst using this treatment. Therefore, we assume the extracellular protein

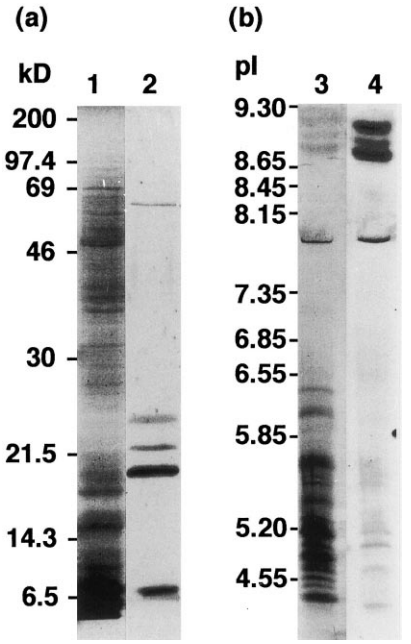

Fig. 1. Coomassie brilliant blue staining (lanes 1 and 3) and immunoblots (lanes 2 and 4) of extracellular pollen proteins of *Brassica rapa* separated by SDS-PAGE (a) or IEF (b). The immunoblots were reacted with a mouse antiserum raised against total extracellular pollen proteins.

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(a)

PEC-1          MKIMVL TLMVFVILLT LFPAPNEAAD TNVEAACDPK QLQPCLAAIT
               : . .        .... .  . ...:: ....::#.:  ::. # .::
HVLTPMR   MAMAMGMAMR KEAAVAVMMV MVVTLAAGAD AGAGAACEPA QLAVCASAIL


PEC-1     GGGQPSGDCC AKLKEQQPCL CGFSKNPAFA QYISSPNSRKV LTACGIPYP
          ::..:::.## ..:..:: #: # . :.:... .:.:::..:.. :. #::: :
HVLTPMR   GGTKPSGECC GNLRAQQGCL CQYVKDPNYG HYVSSPHARDT LNLCGIPVP


PEC-1     SC   98
           #
HVLTPMR   HC  102


(b)

PEC-2     MAATAELIPA GEVIACHTVE DWNNKLKAAK ESNKLIVIDF TAVWCPPCRF
          ::::::.::: :::::::::: :::::::::: :::::::::: ::::::::::
THL-1     MAATAEVIPA GEVIACHTVE DWNNKLKAAK ESNKLIVIDF TAVWCPPCRF


PEC-2     IAPIFVELAK KHLDVVFFKV DVDELATVAK EFDVQAMPTF VYMKGEEKLD
          :::::::::: :::::::::: :::::::::  :::::::::: ::::::::::
THL-1     IAPIFVELAK KHLDVVFFKV DVDELATVAQ EFDVQAMPTF VYMKGEEKLD


PEC-2     KVVGAAKEEI EAKLLKHSQV AAA   123
          :::::::::: :::::::::: :::
THl-1     KVVGAAKEEI EAKLLKHSQV AAA   123


(C)

PEC-3     MKIYYRSSLI GFVMLTILLL GVVANAQKGG PVRKQCVEQY PDPNGKCVID
          ::. ... :: :: ::.: .: : .: :: ..  . .:#  .   ...:.#. .
gPCP1     MKVAFKPWLI GFFMLAI-FL GETAIAQ-EE KTDNQCNLPL INQSGTCAAN


PEC-3     QCKAQCAKNR KGGLARCIDT GKGHMQCRCD YHC    83
          :#.:.#.::. :.:...#...  . .:.# #  : #
gPCP1     QCQAACVKRH KDGVGKCTTN PDKKMRCICF YLCPR  83
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Fig. 2. Comparison of amino acid sequences of PEC-1 (a), PEC-2 (b) and PEC-3 (c) with similar proteins in the EMBL database. HVLTPMR: phospholipid transfer protein from immature barley aleurone cells (Kalla et al., unpublished; accession number X57270); THL-1: thioredoxin that interacts with the kinase domain of a *Brassica* S-locus receptor kinase (accession number U59379 [24]); PCP1, a member of a family of pollen coat protein (PCP) genes from *B. oleracea* encoding novel cysteine-rich proteins (accession number X97056 [10]). Colons and dots indicate identical and similar residues, respectively. Conserved cysteine residues in (a) and (c) are indicated by #. The nucleotide sequences of clone 24 for PEC-1, clone 20 for PEC-2 and clone S8-10 for PEC-3 have been submitted to the EMBL, Genbank and DDBJ databases under the accession numbers AB010433, AB010434 and AB010435, respectively.

mainly consists of pollen coat protein, although we cannot exclude the possibility that the extract included some proteins released from inside the pollen protoplasm.

A polyclonal antiserum was raised against total extracellular proteins. The resulting antiserum detected major bands at 7.5 kDa and 20 kDa on SDS gel blots of the total extracellular pollen proteins (Fig. 1a). The antiserum did not react evenly with all the protein identified by Coomassie brilliant blue (CBB) staining. On the IEF gel blot, highly antigenic proteins were detected at p*I* 8.7 and p*I* 9.0, although a large amount of protein was detected by CBB in the acidic region (Fig. 1b). Thus, the antiserum obtained in this study reacted only with a subset of the protein mixture used for immunization. It probably represents proteins with strong antigenic structures.

3.2. Isolation of cDNA clones

The antiserum against the extracellular proteins of mature pollen was used for immunoscreening of the expression cDNA

libraries in λgt11. Screening was initially carried out on the cDNA library made from anthers containing uninucleate microspores and bicellular pollen of *B. rapa*. Fourteen positive plaques (clones 1, 2, 4, 10, 12, 13, 20–22, 24–28) were identified out of 25 000 plaques. Further screening was carried out using the cDNA library made from anthers at the uninucleate microspore stage of *B. napus*. Four positive plaques (clones 6, 15–17) were isolated out of 40 000 plaques.

Cross-hybridization experiments and partial sequencing indicated that the cDNA clones could be classified five groups (pollen extracellular; PEC-1–5). Group PEC-1, exemplified by clone 24, comprised clones 1, 4, 10, 13, 21, 22, 24–28 of *B. rapa*. Group PEC-2 contained clones 12 and 20 of *B. rapa*. Group PEC-3, exemplified by clone 17, comprised clones 15–17 of *B. napus*. Clone 2 of *B. rapa* and clone 6 of *B. napus* comprised PEC-4 and PEC-5, respectively. PEC-1, PEC-2 and PEC-3 were further characterized.

3.3. Nucleotide sequences and deduced amino acid sequences

The longest cDNA clone of group PEC-1 was clone 24, which was 450 bp long with a 55 bp poly(A) tail. This clone does not contain the first ATG; however, the 5′ region of the cDNA was complemented with an EST clone of *B. pekinensis* (accession number L38539 in the EMBL database [21]), whose sequence was found to be completely identical to that of clone 24 within a 208 bp overlap region. Supplementing clone 24 with the first 12 amino acids from the EST clone resulted in a predicted ORF of 98 amino acids for PEC-1 (Fig. 2a). Hydropathy profiles of PEC-1 show that the predicted protein contains 24 amino acids of the hydrophobic N-terminus, characteristics of a signal peptide.

Computer-aided homology searches indicated that the predicted amino acid sequences of PEC-1 showed similarities with various kinds of lipid transfer proteins (LTPs). PEC-1 contains eight cysteines which are well conserved in other LTPs [22]. The highest similarity (44%) was found in a phospholipid transfer protein from immature barley aleurone cells (Kolla et al., unpublished; accession number X57270 in the EMBL database), as shown in Fig. 2a. However, PEC-1 was rather different from clone E2, which has been reported to encode a phospholipid transfer protein and be expressed in the tapetum and microspores of *B. napus* [23].

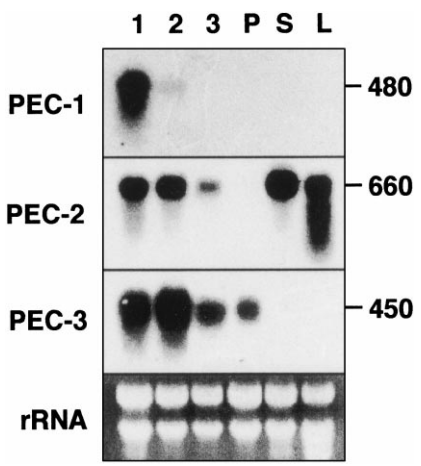

Fig. 3. RNA gel blot of total RNA isolated from different tissues of *B. rapa* probed with clone 26 of PEC-1, clone 20 of PEC-2 and clone 17 of PEC-3. Lane 1, anthers at the uninucleate microspore stage; lane 2, anthers at the bicellular pollen stage; lane 3, anthers at the tricellular pollen stage; lane P, mature pollen; lane S, stigmas; lane L, leaves. A gel stained with ethidium bromide before blotting is also shown in the lower panel.

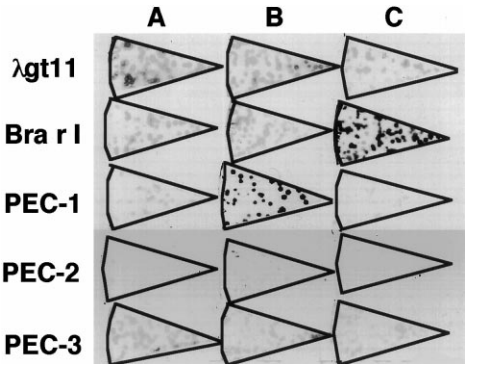

Fig. 4. Protein plaque lifts of cDNA clone 26 of PEC-1, clone 20 of PEC-2, and clone 17 of PEC-3 reacted with serum IgE from individuals (A–C) allergic to *Brassica* pollen. Non-recombinant λgt11 control and *Bra r* I, a cDNA for a *Brassica* pollen allergen, were also tested.

Clone 20 in group PEC-2 was 535 bp long with a predicted ORF of 123 amino acids, starting from the first ATG (Fig. 2b). Hydropathy profiles indicated that the predicted protein contains 15 amino acids of a signal peptide.

A search of databases showed that the predicted amino acid sequence of PEC-2 is the same as that of the thioredoxin-h gene of *B. napus* (THL-1) [24] except for two amino acids, as shown in Fig. 2b.

The longest clone of group PEC-3 was clone 17, consisting of 246 bp nucleotides, but lacking the first ATG. The cDNA library of *B. rapa* was therefore rescreened. Twelve plaques were identified out of 15 000 plaques. The longest clone, S8-10, was 358 bp long with a 8 bp poly(A) tail and a predicted ORF of 83 amino acids, which is the same as that of clone 17 within an overlapping region. Hydropathy profiles indicate that the predicted protein contains 24 amino acids of a signal peptide.

When compared with sequences in the major databases, the predicted amino acid sequence of PEC-3 showed 37% identity with those of PCP1 (Fig. 2c) and 35% with PCP2. PCP1 and PCP2 genes were reported to encode cysteine-rich pollen coat proteins involved in pollen-stigma interactions in *B. oleracea* [10]. Cysteine residues were strongly conserved among these proteins (Fig. 2c).

3.4. RNA gel blot analysis

RNA gel blot analysis showed that transcripts of PEC-1 were present at a high level exclusively in anthers at the uninucleate microspore stage. A low level of expression was detected in the anthers at the bicellular pollen stage. No signal was detected in anthers at the tricellular pollen stage, mature pollen, leaves or stigmas. The size of transcripts was 480 bases (Fig. 3).

When the blot was probed with clone 20 to detected PEC-2, a hybridization signal was observed in the anthers at the uninucleate microspore and bicellular pollen stages and in stigmas and leaves. A weak signal was also detected in the anthers at the tricellular pollen stage. No signal was detected in mature pollen. The size of the transcripts was 660 bases (Fig. 3).

A hybridization signal for PEC-3 was detected in the

anthers at all the stages. The highest level of expression was observed in anthers at the bicellular pollen stage. A weak signal was also observed in mature pollen. No signal was detected in stigmas or leaves. The size of transcripts was 450 bases (Fig. 3).

Ethidium bromide stained rRNA indicates the integrity of RNA and that equal amounts of RNA were loaded in each lane (Fig. 3).

3.5. IgE binding assays

Protein plaque lifts from cDNA clones were reacted with IgE of three individuals who were allergic to *Brassica* pollen [12]. A cDNA clone of *Bra r* I, which has been shown to be a *Brassica* pollen allergen [15], was also tested as a positive control. *Bra r* I bound to IgE of individual C (Fig. 4). In contrast, PEC-1 was shown to bind IgE of individual B. PEC-2 and PEC-3 did not bind to the IgE of any of the three individuals, nor did λgt11 (Fig. 4).

4. Discussion

Mouse antiserum to extracellular pollen protein was used to characterize proteins of *B. rapa* in a manner similar to that recently used with *B. oleracea* [8]. Screening of an anther cDNA expression library with antibodies raised against pollen coat proteins has been used to isolate cDNAs that encode sequences showing homology to transmembrane channel proteins [11].

We carried out screening of cDNA libraries made from immature anthers when the tapetum was very active because we are interested in genes expressed during pollen exine formation. Three groups of cDNA clones were characterized in the current study.

PEC-3 is considered to be a member of PCP1, which has been reported to represent a gene family encoding cysteine-rich proteins involved in pollen-stigma interactions [10]. PCP1 is reportedly expressed in the cytoplasm of trinucleate pollen grains and is believed to be released from the pollen protoplasts into the pollen coating. A similar export of protein is likely carried out for PEP-3.

PEC-2 is considered to be an allele of thioredoxin-h-like clones, THL-1 and THL-2, of *B. napus*. Recombinant proteins of these clones have been reported to interact with the protein kinase domain of the S-locus receptor kinase based on a yeast two-hybrid assay [24]. THL-1 is reportedly expressed in a variety of tissues including anthers, pistils, petals, leaves, stems and roots. An identical sequence to THL-1 was also found using DNA databases; this cDNA encodes a thioredoxin-h-like protein in the pollen coat of *B. oleracea* (Ruiter et al., unpublished; accession number X89759 in the EMBL database). It is unclear how these thioredoxins are incorporated in the pollen coat.

PEC-1 showed some similarity to lipid transfer protein. LTP genes identified in the anthers of *B. napus* (clone E2) [23] and *Lilium henryi* (clone LHM7) [25] have been shown to be expressed in the tapetum. Tapetum-specific expression has been also reported for two LTP-like genes, TA32 and TA36, of tobacco [26]. Expression of PEC-1 exclusively in the anthers of the uninucleate microspore stage (Fig. 3) strongly suggests that the gene for PEC-1 is also expressed in the tapetum. The PEC-1 protein is probably deposited in the pollen coat from the tapetum.

During microgametogenesis, lipids participate in sporopollenin and membrane biogenesis, but also accumulate to significant levels within the pollen wall and in oil bodies within the pollen cytoplasm [27]. Thus, significant movement of lipids or lipid derivatives must occur within both the tapetum and the microspores. The protein encoded by PEC-1 may take part in the transfer of lipids from the tapetum to the pollen wall. It is also possible that PEC-1 acts as a plant defense protein against several pathogens, as recently reported for other LTPs [22].

The function of pollen allergens has been established in only a few allergens. For example, 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 [28]. *Bet v* II has strong homology to profilins and is considered to play a role in actin organization [29]. *Bet v* III, which has homology with Ca^{2+}-binding proteins, is speculated to control Ca^{2+} metabolism [30]. A Bermuda grass pollen allergen, *Cyn d* 7, has also been shown to be a Ca^{2+}-binding protein [31]. In contrast, the major pollen allergen of Japanese cedar, *Cry j* I, has been shown to have pectate lyase activity, and thus may play a role in facilitating pollen tube growth [32]. Recently we demonstrated that *Bra r* I, one of the *Brassica* pollen allergens, was a Ca^{2+}-binding protein and is localized in pollen tubes upon pollination (Okada et al., in preparation). *Bra r* I is strongly implicated in the control of the Ca^{2+} gradient of the pollen tube during elongation.

This study demonstrates that PEC-1 binds strongly to the IgE of a patient allergic to *Brassica* pollen and indicates that the cDNA clone reported here represents a novel pollen allergen of *B. rapa*. The function of this pollen allergen in plant reproduction is suggested to involve lipid transfer or antipathogen defense as discussed above. We employed only three patients to test the IgE binding of the recombinant proteins. More patients should be investigated to determine whether PEC-1 protein is a general pollen allergen or not. IgE from the other patients may bind to proteins encoded by PEC-2 or PEC-3. The pollen coat protein is likely a potential source of pollen allergens.

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References

[1] Knox, R. and Suphioglu, C. (1996) Trends Plant Sci. 1, 156–164.
[2] Singh, M.B., Hough, T., Theerakulpisut, P., Avjioglu, A., Davies, S., Smith, P.M., Taylor, P., Simpson, R.J., Ward, L.D., McCluskey, J., Puy, R. and Knox, R.B. (1991) Proc. Natl. Acad. Sci. USA 88, 1384–1388.
[3] Miki-Hiroshige, H., Nakamura, S., Yasueda, H., Shida, T. and Takahashi, Y. (1994) Sex. Plant Reprod. 7, 95–100.
[4] Echlin, P. (1971) in: Pollen: Development and Physiology (Heslop-Harrison, J., Ed.), pp. 41–61, Butterworth, London.
[5] Heslop-Harrison, J. (1975) Annu. Rev. Plant Physiol. 26, 403–425.
[6] Doughty, J., Hedderson, F., McCubbin, A. and Dickinson, H. (1993) Proc. Natl. Acad. Sci. USA 90, 467–471.
[7] Hiscock, S.J., Doughty, J., Willis, A.C. and Dickinson, H.G. (1995) Planta 196, 367–374.

[8] Ruiter, R.K., Mettenmeyer, T., van Aarhoven, D.M.C., van Eldik, G.J., Doughty, J., van Herpen, M.M.A., Schrauwen, J.A.M., Dickinson, H.G. and Wullems, G.J. (1997) J. Plant Physiol. 150, 85–91.
[9] Ross, J.H.E. and Murphy, D.J. (1996) Plant J. 9, 625–637.
[10] Stanchev, B.S., Doughty, J., Scutt, C.P., Dickinson, H. and Croy, R.R.D. (1996) Plant J. 10, 303–313.
[11] Ruiter, R.K., van Eldik, G.J., van Herpen, M.M.A., Schrauwen, J.A.M. and Wullems, G.J. (1997) Plant Mol. Biol. 34, 163–168.
[12] Ashida, T., Ide, T., Tabata, S., Etoh, Y., Yoshikawa, T., Toriyama, K., Hinata, K., Watanabe, M. and Kishitani, S. (1992) Jpn. J. Palynol. 38, 31–36.
[13] Fell, P.J., Soulsby, S., Blight, M.M. and Brostoff, J. (1992) Clin. Exp. Allergy 22, 501–505.
[14] Singh, B.P., Verma, J., Rai, D., Sridhara, S., Gaur, S.N. and Gangal, S.V. (1995) Int. Arch. Allergy Immunol. 108, 43–48.
[15] Toriyama, K., Okada, T., Watanabe, M., Ide, T., Ashida, T., Xu, H. and Singh, M.B. (1995) Plant Mol. Biol. 29, 1157–1165.
[16] Watanabe, M., Shiozawa, H., Isogai, A., Suzuki, A., Takeuch, T. and Hinata, K. (1991) Plant Cell Physiol. 32, 1039–1047.
[17] Evans, D.E., Taylor, P.E., Singh, M.B. and Knox, R.B. (1991) Plant Sci. 73, 117–126.
[18] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989): Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[19] Watanabe, M., Takasaki, T., Toriyama, K., Yamakawa, S., Isogai, A., Suzuki, A. and Hinata, K. (1994) Plant Cell Physiol. 35, 1221–1229.
[20] Kyte, J. and Doolittle, R.F. (1982) J. Mol. Biol. 157, 105–132.
[21] Lim, C.O., Kim, H.Y., Kim, M.G., Lee, S.I., Chung, W.S., Park, S.H., Hwang, I. and Cho, M.J. (1996) Plant Physiol. 111, 577–588.
[22] Kader, J.C. (1997) Trends Plant Sci. 2, 66–70.
[23] Foster, G.D., Robinson, S.W., Blundell, R.P., Roberts, M.R., Hodge, J., Draper, J. and Scott, R.J. (1992) Plant Sci. 84, 187–192.
[24] Bower, M.S., Matias, D.D., Fernandes-Carvalho, E., Mazzurco, M., Gu, T., Rothstein, S.J. and Goring, D.R. (1996) Plant Cell 8, 1641–1650.
[25] Crossley, S.J., Greenland, A.J. and Dickinson, H.G. (1995) Planta 196, 523–529.
[26] Koltunow, A.M., Truetter, J., Cox, K.H., Wallroth, M. and Goldberg, R.B. (1990) Plant Cell 2, 1201–1224.
[27] Evans, D.E., Taylor, P.E., Singh, M.B. and Knox, R.B. (1992) Planta 186, 343–354.
[28] Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. and Breitenbach, M. (1989) EMBO J. 8, 1935–1938.
[29] Valenta, R., Duchene, M., Pettenburger, K., Sillaber, C., Valent, P., Bettelheim, P., Breitenbach, M., Rumpold, H., Kraft, D. and Scheiner, O. (1991) Science 253, 557–560.
[30] Seiberler, S., Scheiner, O., Kraft, D., Lonsdale, D. and Valenta, R. (1994) EMBO J. 13, 3481–3486.
[31] Suphioglu, C., Ferreira, F. and Knox, R.B. (1997) FEBS Lett. 402, 167–172.
[32] Taniguchi, Y., Ono, A., Sawatani, M., Namba, M., Kohno, K., Usui, M., Kurimoto, M. and Matuhashi, T. (1995) Allergy 50, 90–93.