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**Identification of a Ca^{2+} Binding
Protein as a New Bermuda Grass Pollen
Allergen Cyn d 7: IgE Cross-Reactivity
with Oilseed Rape Pollen Allergen Bra r 1****Key Words** Ca^{2+} binding allergen
Pollen Allergen
Bermuda grass
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cDNA clones encoding two isoforms of an allergen from pollen of Bermuda grass (*Cynodon dactylon*) have been isolated using IgE from allergic patients. Homologous transcripts are present in pollen of 15 other grasses tested. This allergen, tentatively designated as Cyn d 7, contains two calcium binding domains and shows significant sequence similarity with other Ca^{2+} binding pollen allergens, namely Bet v 4 from birch and Bra r 1 from oilseed rape. Approximately 10% of allergic sera tested showed IgE reactivity to this allergen. IgE cross-reactivity was observed between this allergen and Bra r 1 of oilseed rape. IgE reactivity of this allergen requires protein-bound Ca^{2+} . Using IgE affinity-purified from the recombinant allergen to probe Western blots of pollen extracts Cyn d 7 has been identified as a 12 kDA protein.

Introduction

Grass pollen allergens trigger type I allergic symptoms such as rhinitis, conjunctivitis and bronchial asthma. Allergenic components of grass pollen can be classified into different groups according to their physicochemical and immunological properties [1]. The most important allergens in grass pollen are group 1 and 5 allergens, as judged by both criteria of number of allergic patients responding and relative amount of IgE binding to the allergens [2]. Together, members of these two allergen groups account for most of the IgE reactivity of crude grass pollen extract [3].

Group 1 and 5 allergens from grass genera share cross-reactive IgE epitopes [4–6]. Thus, presence of IgE antibodies reactive to group 1 and 5 allergens from one grass genus is indicative of general hypersensitivity to pollen allergens from a large number of taxonomically related grasses. Group 1 and 5 allergens have been cloned from number of grass genera and their nucleic acid sequences determined [2, 7, 8–11].

Grass pollen hypersensitivity occasionally predisposes individuals for reactivity to pollen from other plants and for food allergy to various vegetables and fruits [5, 12]. Examples of grass pollen-associated allergy have been reported for olive pollen [13], birch pollen [14] and potato tuber antigens [15]. Recently, it has been reported that individuals allergic to oilseed rape are almost always allergic to grass pollen [16]. The common explanation of this observation is the presence of related allergens with cross-reactive IgE epitopes in pollen of both plants. Pollen from the above non-grass genera do not contain antigens cross-reactive with group 1 and 5 allergens of grass pollen. Identification of individual allergens other than those cross-reactive with group 1 and group 5 is of importance for defining the molecular basis of the observed concordant reactivity.

Allergens responsible for reactivity to diverse plant sources are likely to be defined as 'minor' by the criterion of frequency of reactivity in a population. One such minor allergen is profilin which was first shown to be an allergen in birch pollen with 10–15% of allergic subjects showing reac-

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tivity [17]. Profilin is a highly conserved protein present in almost all eukaryotic organisms. It has also been described as an allergen from grass pollen as well as in a wide range of vegetable foods such as potato and celery [5].

In this paper, we describe the identification of Ca^{2+} binding proteins as a new class of pollen allergens from Bermuda grass (*Cynodon dactylon*). We have tentatively named these allergens Cyn d 7. Three cDNA clones, B1, B3 and B4, coding for Cyn d 7 were isolated by screening of a Bermuda grass pollen cDNA expression library with IgE from allergic patients. A high level of Cyn d 7 transcript was detected in mature pollen of Bermuda grass and in a number of grass genera tested. Cyn d 7 shows structural homologies with Ca^{2+} binding allergens, Bet v 4 from birch pollen [18] and Bra r 1 from oilseed rape pollen [19]. IgE cross-reactivity is observed between Cyn d 7 of Bermuda grass and Bra r 1 of oilseed rape pollen allergens. Recently, a high molecular weight allergen from timothy grass (*Phleum pratense*) pollen was found to show significant IgE cross-reactivity with ragweed allergen Amb a 1 [20]. The observed cross-reactivity among pollen allergens from botanically unrelated plant genera may explain in part general hypersensitivity to pollen from any source observed in a certain class of atopic individuals.

Materials and Methods

Serum

Human serum was collected by Dr. Krishnaswamy (Doncaster, Victoria), from patients with a case history of allergy to grass pollen and a positive skin test to ryegrass or Bermuda grass pollen extract.

Grass Pollen

Grass pollen was obtained from Greer Laboratories (N.C., USA) and Hollister Stier (Wash., USA) as dry, nondefatted pollen and stored at -20°C until use. The grasses used in this study were Bermuda grass (*C. dactylon*), quack grass (*Agropyron cristatum*), orchard grass (*Dactylis glomerata*), meadow fescue (*Festuca elatior*), ryegrass (*Lolium perenne*), Kentucky bluegrass (*Poa pratensis*), cultivated oat (*Avena sativa*), timothy grass (*P. pratense*), salt grass (*Distichlis stricta*), smooth brome (*Bromus inermis*), sweet vernal (*Anthxanthum odoratum*), wheat (*Triticum aestivum*), Johnson grass (*Sorghum halepensis*) and maize (*Zea mays*).

Construction and Screening of cDNA Library

Total RNA was isolated from Bermuda grass pollen as described [21]. Poly A⁺ RNA was obtained using a mRNA purification kit (Pharmacia) and a cDNA expression library was constructed in λ gt11 using a Time-Saver cDNA synthesis kit according to the manufacturer's instructions (Pharmacia). Immunological screening of the cDNA library was conducted by probing the recombinant protein lifts with serum of a grass pollen allergic patient. The positive clones were identified using ^{125}I -labelled antihuman IgE antibody (Kallestad, USA).

Nucleotide Sequencing and Sequence Analysis

The cDNA inserts recovered from the positive clones were subcloned into pBluescript SK (+) (Stratagene) and used for sequencing. DNA sequencing was performed on double-stranded DNA by the dideoxy chain-termination method using a T7 DNA sequencing kit (Pharmacia). Sequences were determined on both strands of DNA. Sequence analysis was performed using ANGIS (Australia National Genomic Information Service) which has access to the major databases including GenBank, EMBL and NBRF nucleic acid libraries, NBRF PIR protein and Swiss-Prot libraries.

Northern Blot and Southern Analysis

Total RNA was extracted from pollen, leaves and roots using the method described by Chomczynski and Sacchi [21]. RNA (10 μg) was denatured, size-fractionated on 1.2% denatured agarose gel and transferred onto a Hybond N⁺ Nylon membrane (Amersham) under vacuum using the Vacugene gel blotting system (Pharmacia). Genomic DNA extracted from leaf tissues of Bermuda grass was digested, separated in a 0.7% agarose gel and transferred onto a Hybond N⁺ Nylon membrane under alkaline conditions as described by Xu et al. [22]. Northern and Southern blots were hybridized with ^{32}P -labelled cDNA probes prepared using a GIGaprime DNA labelling kit (Bresatec, Australia). Hybridizations were conducted as described by Xu et al. [22].

IgE Binding Assays

Protein plaque lifts from clone B1 and Bra r 1 were prepared using standard methods as described in Sambrook et al. [23]. The filters containing recombinant allergens were divided into sections and incubated with allergic patients' sera diluted 1:5 in (1) PBS containing 0.5% BSA, and (2) PBS containing 0.5% BSA and 5 mM EGTA. The bound IgE antibodies were detected using ^{125}I -labelled antihuman IgE (Kallestad, USA).

Affinity Purification of IgE Antibodies

Protein plaque lifts from cDNA clones were incubated overnight in sera of allergic patients. The lifts were then washed twice in PBS containing 0.5% Tween 20 and twice in PBS. The bound IgE antibodies were eluted from the plaque lifts by two 5-min incubations (with shaking) in 5 ml of 0.1 M glycine-HCl containing 1% BSA, pH 2.6. The eluted IgE antibodies were pooled and immediately neutralized using 2 M Tris-HCl, pH 9.0. The affinity-purified IgE was used to probe Western blots of pollen proteins.

Gel Electrophoresis and Immunoblot Analysis

Soluble proteins were extracted from pollen by shaking 100 mg of pollen in 1 ml of extraction buffer (15 mM phosphate buffer saline, pH 7.2, 1 mM phenylmethyl-sulfonyl fluoride) for at least 1 h on ice. The proteins (10 μg) were separated under denaturing conditions, on 0.1% SDS-12% PAGE gels using the buffer system described by Laemmli [24]. Protein gels were either stained with Coomassie brilliant blue to visualize proteins or transferred to nitrocellulose as described by Towbin et al. [25]. For immunoblot analysis, Western blots were incubated in sera from allergic individuals or in affinity purified IgE overnight at room temperature. IgE binding was detected by incubation of the blots in ^{125}I -labelled antihuman IgE (Kallestad, USA) overnight at room temperature.

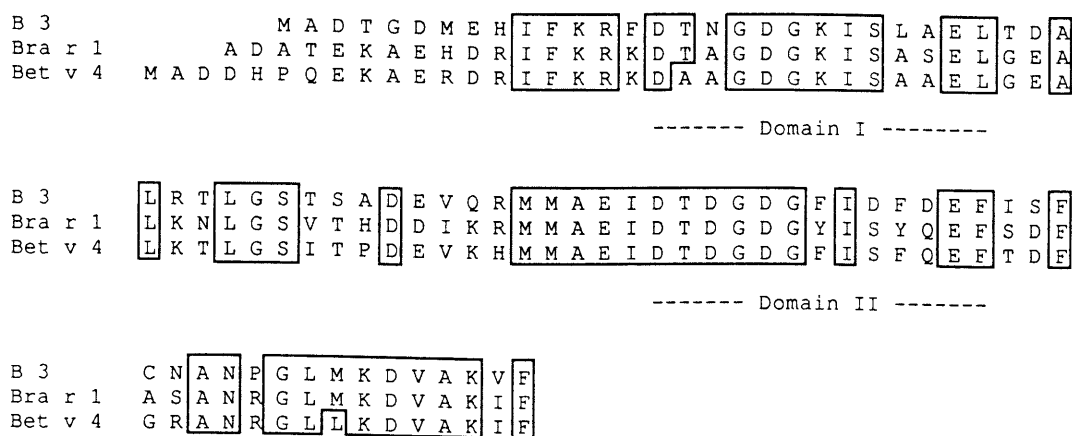


Fig. 1. Comparison of deduced amino acid sequences of B3, Bra r 1 and Bet v 4. Identical amino acids are boxed and the two Ca²⁺ binding domains are indicated.

Results

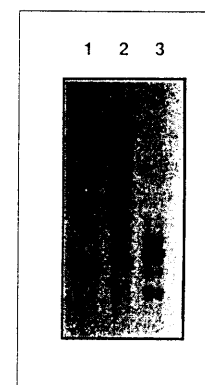
Isolation of cDNA Clones and Sequences Analysis

A cDNA expression library constructed from mature Bermuda grass pollen mRNA was screened with serum IgE from an allergic individual (K.R.). The serum from this individual contains IgE antibodies directed against epitopes present on a large number of proteins in Bermuda grass pollen as shown by Western immunoblotting analysis [26]. Three clones, B1, B3 and B4, showing strong IgE reactivity were isolated from this library.

The complete nucleotide sequences of these clones were determined (GenBank accession number U75585). All three clones encode the same protein. However, clone B4 shows differences in the 3' nontranslated region, indicating that it may be encoded by a separate, closely related gene of the same gene family as B1 and B3. We have tentatively designated these allergens Cyn d 7.

The longest cDNA, B3, encodes an 85-amino acid protein with a calculated molecular mass of 8.7 kDA. Sequence similarities were found with calmodulins from a number of sources including spinach, human and *Arabidopsis*, as well as three Ca²⁺ binding pollen allergens, Bet v 3 and Bet v 4 of birch, and Bra r 1 of oilseed rape. Sequence similarities with calmodulins and Bet v 3 are primarily due to the presence of a conserved Ca²⁺ binding site in these clones and no significant sequence similarities were observed outside Ca²⁺ binding domains. Sequence similarity with Bet v 4 (69% identity) and Bra r 1 (69% identity) are found at the Ca²⁺ binding domains and extend beyond the Ca²⁺

Fig. 2. Southern blot analysis of genomic DNA of Bermuda grass. *Bgl*/II (1)-, *Eco*RI (2)- and *Hind*III (3)-digested genomic DNA was probed with a ³²P-labelled B1 cDNA fragment.



binding regions (fig. 1). Sequence analysis showed that the B1, B3 and B4 clones are similar to Bra r 1 and Bet v 4, containing two Ca²⁺ binding domains, while Bet v 3 has been reported to have three such domains [27].

Southern blot analysis indicated that the clone B1 corresponds to a multigene family in Bermuda grass (fig. 2).

Presence of Homologous Transcripts in Other Grasses

Transcripts corresponding to the cDNA clone B1 were detected by Northern blot analysis of Bermuda grass. The B1 cDNA hybridized to a transcript of approximately 900 nucleotides in pollen RNA but no transcript was detected in RNA from leaves and roots (fig. 3). Homologous transcripts of a similar size were detected in pollen RNAs from the 15 other grasses tested (fig. 3b). Strong hybridiza-

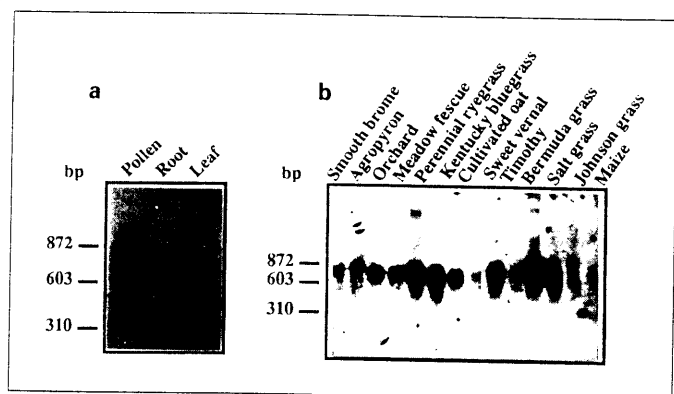


Fig. 3. Northern blot analysis of RNA transcripts corresponding to the clone B1. Total RNA was separated on denaturing agarose gels, transferred to nylon membranes and probed with a ^{32} P-labelled B1 cDNA fragment. A transcript of approximately 900 bp was present in pollen, but not in root and leaf of Bermuda grass (a). Transcripts homologous to B1 were detected in pollen of different grasses (b).

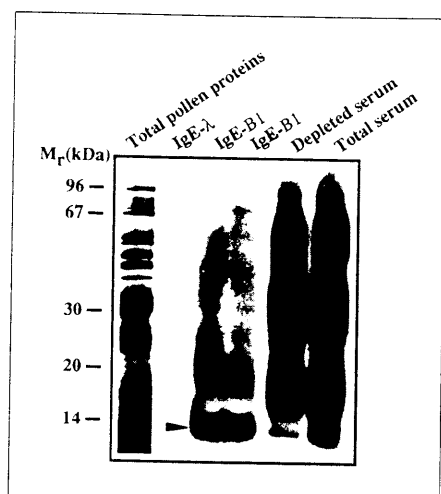


Fig. 4. Identification of Bermuda grass pollen allergens encoded by the clone B1. Soluble pollen proteins were separated by SDS-PAGE and were either stained with Coomassie blue or transferred to nitrocellulose. Western blots were probed with IgE affinity-purified from non-recombinant λ gt11 (IgE- λ), IgE affinity-purified from clone B1 fusion protein (IgE-B1), serum preincubated with B1 protein plaque lifts (depleted serum) and total serum. IgE affinity-purified from clone B1 fusion protein showed specific binding to a protein of 12 kDa (arrowhead). Preincubation of serum with plaque lifts containing B1 protein depleted IgE antibodies to this protein.

tion was observed in some grasses such as quack grass, orchard grass, meadow fescue, ryegrass, Kentucky bluegrass, cultivated oat, timothy and salt grass. In other grasses such as smooth brome, sweet vernal, wheat, Johnson grass and maize, hybridization was detected at a much lower level.

Identification of Bermuda Grass Pollen Allergens Encoded by the cDNA Clones

In order to identify the pollen proteins encoded by the clones B1, B3 and B4, IgE antibodies were affinity purified using the recombinant fusion protein of clone B1. Purified IgE had specificity to a 12 kDa protein of Bermuda grass pollen, as indicated by Western blot analysis (fig. 4). Preincubation of the B1 protein plaque lifts with allergic patient sera resulted in selective depletion of IgE antibodies against this 12 kDa polypeptide (fig. 4).

Concordant Reactivity of Allergic Individuals Sensitive to Cyn d 7 and Oilseed Rape Pollen Allergen, Bra r 1

To determine the IgE reactivity of Cyn d 7 and Bra r 1 proteins, we tested 30 grass pollen allergic sera with recombinant protein expressed by clone B1 and that by Bra r 1, the homologous oilseed rape pollen allergen. Three patients, representing 10% of the tested grass pollen allergic sera, showed IgE antibodies which recognized the recombinant protein expressed by clone B1. The same individuals showed IgE reactivity towards recombinant Bra r 1.

We further investigated whether cross-reactive IgE epitopes are present in Cyn d 7, Bra r 1 and calmodulins of human or spinach. Our results showed that preincubation of the allergic sera with the recombinant Cyn d 7 protein depleted the sera of the IgE reactivity to oilseed rape pollen allergen, Bra r 1 (fig. 5a). Similarly, preincubation of sera with recombinant Bra r 1 depleted IgE reactivity to recombinant Cyn d 7 protein (fig. 5a). In contrast, preincubation of sera with purified spinach or human calmodulin had no effect on IgE reactivity towards B1 and Bra r 1 protein. Preincubation of allergic sera with Birch pollen allergen Bet v 3 had no effect on IgE reactivity towards either Cyn d 7 or Bra r 1 (data not shown).

Effect of Ca^{2+} on IgE Reactivity of Cyn d 7 and Bra r 1

It was reported that IgE binding of Bet v 3, a birch pollen allergen, depended on native protein conformation and required protein-bound Ca^{2+} [27]. Similar experiments using the recombinant B1 and Bra r 1 proteins showed that depletion of Ca^{2+} using EGTA led to a reversible loss of IgE reactivities of both recombinant proteins (fig. 6).

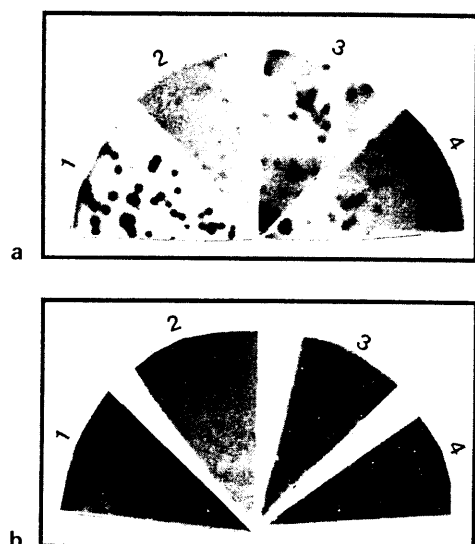


Fig. 5. Analysis of cross-reactive IgE epitopes in allergens encoded by clone B1 of Bermuda grass and Bra r 1 of oilseed rape. Plaque lifts of clone B1 (**a**) and Bra r 1 (**b**) were probed with allergic sera (sections 1), sera preincubated with human calmodulin (sections 3) and sera preincubated with spinach calmodulin (sections 4). Section 2 of **a** was probed with sera preincubated with clone Bra r 1 and section 2 of **b** with sera preincubated with clone B1.

Discussion

In this paper we report the molecular cloning of cDNA clones, B1, B3 and B4, encoding Bermuda grass pollen allergens. All three clones encode the same protein although one amino acid substitution was found in the protein encoded by the clone B4. The 3' nontranslation sequences of the B1 and B3 clone differ from that of clone B4, indicating that these clones may represent different members of a multigene family. We have tentatively designated the allergens encoded by these cDNA clones as Cyn d 7.

These cDNA clones encode a protein of 85 amino acids and the protein contains two EF-hand Ca^{2+} binding domains. It has significant sequence similarity with calmodulins from a number of sources as well as Ca^{2+} binding pollen allergens, Bra r 1 of oilseed rape [19], and Bet v 3 [27] and Bet v 4 of birch. The sequence similarity between Cyn d 7, calmodulins and Bet v 3 occurs in the Ca^{2+} binding domains but not elsewhere in their sequences. Subsequent analysis has revealed that similar Ca^{2+} binding and other regions outside the Ca^{2+} binding domains occur in Bra r 1 and Bet v 4. In addition, Bet v 3 and calmodulins are larger proteins with three or four Ca^{2+} binding domains, respectively. This suggests that the Bermuda grass Ca^{2+} binding allergen Cyn d 7 which we have cloned belongs to a different class of Ca^{2+}

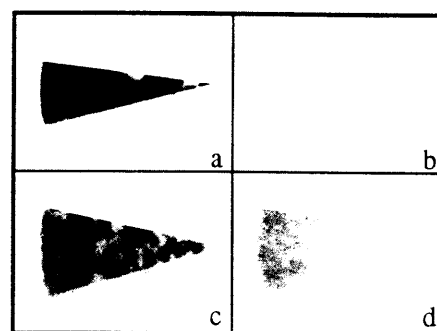


Fig. 6. Effect of calcium on IgE reactivity of the clone B1 (**a**, **b**) and Bra r 1 (**c**, **d**). Plaque lifts of the clone B1 and Bra r 1 were incubated with allergic sera (**a**, **c**) and sera containing 5 mM EGTA (**b**, **d**) showing that depletion of Ca^{2+} using EGTA abolishes IgE reactivity to both B1 and Bra r 1 recombinant proteins.

binding proteins. Further analysis revealed that Cyn d 7 displays a much higher level of sequence similarity with two other pollen allergens, Bra r 1 of oilseed rape and Bet v 4 of birch. Furthermore, all three allergens, Cyn d 7 of Bermuda grass, Bra r 1 of oilseed rape and Bet v 4 of birch, showed a similar protein profile including a similar number of amino acid residues and two Ca^{2+} binding domains. These results strongly suggest that Cyn d 7 may represent a homolog of Bra r 1 and Bet v 4, representing a new class of Ca^{2+} binding allergens of grass pollen.

The IgE binding capacity of Cyn d 7 allergen and its cross-reactivity with Bra r 1 was tested using sera from grass pollen allergic individuals. It was found that approximately 10% of the grass pollen allergic individuals contain IgE to this allergen. The same patients showed IgE binding to the oilseed rape allergen, Bra r 1. Our further studies showed that preincubation of Bra r 1 recombinant protein with allergic serum is able to completely deplete the IgE binding to the recombinant Cyn d 7 protein. Similarly, preincubation of the Cyn d 7 recombinant protein abolishes IgE binding to the Bra r 1 protein. These results suggest that Cyn d 7 and Bra r 1 allergen have shared IgE epitopes which may be partly responsible for the associated hypersensitivity to oilseed rape and grass pollen. It has been reported that approximately 10% of grass pollen allergic patients tested showed IgE reactivity to Bet v 4 [18]. The presence of a Bet v 4 homolog in Bermuda and 15 other grasses, as evident from our studies, may partly explain such cross-reactivity. The presence of this new class of Ca^{2+} binding allergens in a number of plants including a crop plant (oilseed rape), a tree (birch) and grasses (Bermuda and 15 other grasses tested) indicates that this allergen may have the potential to be an important pollen allergen.

It has been reported that IgE binding of Bet v 3, a birch pollen allergen, depended on native protein conformation and required protein bound Ca^{2+} [27]. Removal of Ca^{2+} from Bet v 3 using EGTA led to a reversible loss of the allergens' IgE binding capacity which could be restored by subsequent addition of Ca^{2+} . We have performed similar experiments with Cyn d 7 and Bra r 1 allergens and found that depletion of Ca^{2+} from these proteins results in greatly reduced IgE reactivity. It is unlikely, however, that the amino acid sequences which constitute Ca^{2+} domain are exactly the same as those required for IgE recognition, since preincubation of sera with purified human and spinach calmodulins did not deplete IgE reactivity towards either Cyn d 7 or Bra r 1 allergens. It appears that IgE epitopes of these allergens are localized in amino acid sequences flanking the Ca^{2+} binding domain. It is notable that these flanking regions are almost identical in Cyn d 7, Bra r 1 and Bet v 4 [18, 19]. The absence of cross-reactivity between Cyn d 7 and Bet v 3 is probably due to lack of identical sequences in these flanking regions. The effect on Ca^{2+} removal on IgE reactivity implies that the local conformational changes due to Ca^{2+} binding are important on IgE reactivity. Further work on site-directed mutagenesis studies is needed to clarify structure function relationship of these allergens. The availability of recombinant Cyn d 7 has the potential to im-

prove the precise determination of the allergens to which individuals are sensitized. The presence of Cyn d 7-specific antibodies in atopic serum possibly indicates sensitization to pollen allergens from a wide number of plant genera. 10% of the patients who had anti-Cyn d 7 IgE antibodies complained of a 'nonspecific hyperreactivity' during the pollen season. In case of such patients, desensitization therapy with only major allergens (group I and 5) may not be effective.

As regards the biological significance of Cyn d 7 allergen in pollen, it is known that the Ca^{2+} level in pollen is regulated by a number of different Ca^{2+} binding proteins and Ca^{2+} is essential for pollen germination [28, 29]. The fact that transcripts homologous to Cyn d 7 have been found in all other grasses examined and also in birch and oilseed rape pollen suggests that the protein may have an important role in control of Ca^{2+} binding metabolism in pollen during germination and early pollen tube growth.

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