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Molecular Characterization of *Brassica* Pollen Allergen

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Abstract

Allergy to *Brassica* pollen has been reported in some countries. We have cloned a cDNA encoding a *Brassica* pollen allergen, Bra r 1. Bra r 1 belongs to a new family of Ca^{2+}-binding proteins, characterized by the presence of two EF-hand calcium-binding domains. Bra r 1 was detected in the tapetum, microspores, pollen coat and pollen tubes, indicating Bra r 1 is involved in pollen pistil interaction and pollen tube growth. We have engineered the hypoallergenic mutants of Bra r 1 for immunotherapy. Here we describe the review of molecular characterization of Bra r 1.

Introduction

Pollen allergens bind to human IgE and lead to various allergic manifestations in susceptible individuals. Many cDNA clones encoding pollen allergen have been isolated from trees, weeds, grasses and other plants, and recombinant proteins were used for the tools for diagnosis, determination of epitopes and immunotherapy (Scheiner, 1992; Valenta and Kraft, 1995; Valenta et al., 1996).

Being insect pollinated, the *Brassica* plants produced some airborne pollen. Recent studies have revealed that *B. napus* and *B. rapa* have been recognized as a potential cause of allergic sensitization (Singh et al., 1995; Focke et al., 1998).

Here we describe the review of molecular characterization of *Brassica* pollen allergen Bra r 1 and engineering of hypoallergenic mutants of Bra r 1 for immunotherapy.

Isolation of cDNA for Brassica pollen allergens and sequence similarity to other pollen allergens

We have isolated cDNA clones encoding *Brassica* pollen allergens, Bra r 1 and Bra r 2, from expression libraries of anthers of *B. rapa* and *B. napus* using serum IgE from a patient who was specifically allergic to *Brassica* pollen (Toriyama et al., 1995). The deduced amino acid sequences of Bra r 1 and Bra r 2 contains two regions with sequence similarity to EF-hand motifs, Ca^{2+}-binding sites of Ca^{2+}-binding proteins such as calmodulin (CaM). However, Bra r 1 and Bra r 2 were found to be distinct from the other Ca^{2+}-binding proteins and were about half the molecular weight of CaM. Bra r 1 and Bra r 2 were characterized as a novel class of Ca^{2+}-binding protein.

Recently, cDNAs encoding such a two EF-hand Ca^{2+}-binding protein were isolated from pollen cDNA libraries of birch (Bet v 4), alder (Aln g 4), olive (Ole e 3), Bermuda grass (Cyn d 7), and timothy grass (Phl p 7). Figure 1 shows alignment of amino acid sequences of these cDNA. Aln g 4 (Hayek et al., 1998), Bet v 4 (Engel et al., 1997) and Ole e 3 (Batanero et al., 1996) were isolated as tree pollen allergens and Cyn d 7 (Suphioglu et al., 1997) and Phl p 7 (Niederberger et al., 1999) as grass pollen allergens. They are highly homologous to Bra r 1, showing 68-77% similarity to Bra r 1. Both EF-hand motifs, especially the Ca^{2+}-binding loop, are well conserved in all sequences. The C-terminal region is also

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well conserved and seven of ten residues are identical in all sequences.

Expression and localization of Bra r 1 in anthers and pollen tubes

Bra r 1 was specifically expressed in anthers and pollen, and protein was mostly accumulated in mature pollen grains during pollen development (Toriyama et al., 1995; Okada et al., 1999). RNA in situ hybridization showed that Bra r 1 was expressed in the diploid tapetum and haploid microspores. Immunogold labeling of thin sections further confirmed the presence of Bra r 1 in the pollen coat (tryphine) and pollen cytoplasm, but not in exine (Okada, et al., 1999). When pollen grains were suspended in an aqueous solution, Bra r 1 was mainly detected in the pollen extracellular fraction, indicating that Bra r 1 is released from the pollen upon hydration (Okada et al., 1999). These results indicate the potential allergenisity of Bra r 1 in *Brassica* pollen.

Calcium plays an essential role during pollen development and pollen tube growth. The Ca^{2+}-mediated processes such as pollen maturation and pollen tube growth appear to be correlated with Ca^{2+}-binding proteins. In orde to study the relationship of Bra r 1 with pollen tube growth, the localization of Bra r 1 in pollen tubes was determined. Immunolocalization of Bra r 1 in the *in vivo* germinated pollen tubes was examined in the longitudinal sections of self- or cross-pollinated pistils of *B. rapa* homozygous for the self-incompatibility locus. When pollen tube growth was inhibited in self-pollination, Bra r 1 was detected only in pollen grains on the stigma. Two hours after cross-pollination, Bra r 1 was detected in pollen tubes elongating in transmitting-tissues (Okada et al., 1999). Hence, we suggest that Bra r 1 may be involved in pollen germination and pollen tube growth.

Engineering of hypoallergenic mutants of Bra r 1 for immunotherapy

Bra r 1 has been shown to be recognized by at least 50% of patients who were allergic to *Brassica* pollen (Focke et al., 1998). Thus Bra r 1 represents one of the major allergen of *Brassica* species. Toward developing specific immunotherapy, engineering and characterization of mutant protein with reduced IgE binding activity is a prerequisite.

Bra r 1 contains two EF-hand calcium-binding domains. A Ca^{2+}-dependent conformational change has been reported for other members of Bra r 1-like Ca^{2+}-binding proteins (Engel et al., 1997; Hayek et al., 1998; Batanero et al., 1996). Human IgE recognizes conformation of pollen allergens. In order to study the effect of Ca^{2+}-dependent conformational change on IgE-reactivity, we introduced point mutations in each EF-hand motif of Bra r 1. The disruption of the EF-hand motif by amino acid substitutions demonstrated that both domains of Bra r 1 constitute functional Ca^{2+}-binding sites. Calcium-binding deficient mutants displayed significantly reduced IgE binding activity, indicating human IgE recognized Ca^{2+}-binding form of Bra r 1 (Okada et al., 1998).

To evaluate the application of Bra r 1 mutants for allergen-specific immunotherapy, we obtained mouse antisera raised against mutant proteins. Injection of these mutated Bra r 1 variants into a murine model system showed that mouse IgG raised against the mutants recognized native Bra r 1 in *Brassica* pollen extracts (Okada et al., 1998). These results indicate that the IgG against the mutant Bra r 1 can be used as a competitor to IgE for binding to native Bra r 1, suggesting the potential use of the engineered Bra r 1 variants for allergen specific immunotherapy.

IgE cross-reactivity have been reported among Bra r 1 type Ca^{2+}-binding pollen allergens such as Bet v 4 , Aln g 4, Cyn d 7 and Ole e 3 (Engel et al., 1997; Smith et al., 1997; Twardosz et al., 1997). Bra r 1 is likely to represent these pollen allergens. Therefore, hypoallergenic variants of Bra r 1 have the po-

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                <----    EF-hand 1    ---->        <----    EF-hand 2    ---->

Bra r 1  MAD------AEHERIFKKFDTDGDGKISAAELEEALKKLGSVTPDDVTRMMAKIDTDGDGNISFQEFTEFASANPGLMKDVAKVF 79
Bra r 2  ...--ATEK...D.......AN.......S..GD...N.....H..IK....E.......Y..Y...SD.....R........I. 83
Aln g 2  ...DHPQDQ........C..AN.......S..GD...T.......E.KH...E.......F..F....N..R..R..V.....I. 85
Bet v 4  ...DHPQDK..R.....R..AN..........G....T...I...E.KH...E.......F.......D.GR..R..L.....I. 85
Ole e 3  ...-DPQEV........R..AN......SS..G.T..T......EEIQ....E.......F...E...V..R..R..V.....I. 84
Cyn d 7  ..------TGDM.H...R...N......L...TD..RT...TSA.E.Q....E.......F.D.D..IS.CN.............. 80
Phl p 7  ..--------DM.....R...N......LS..TD..RT...TSA.E.Q....E.......F.D.N..IS.CN......E...... 78
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Figure 1. Comparison of amino acid sequences of Bra r 1 with putative homologues in other species. The sequences are obtained from DDBJ, EMBL and GenBank databases (accession numbers given in parentheses). Bra r 1, Brassica pollen allergen (D 63153); Bra r 2, Brassica pollen allergen (D63154); Aln g 4, alder pollen allergen (Y17713); Bet v 4, birch pollen allergen (S 54819); Ole e 3, Olive pollen allergen (AF015810); Cyn d 7, Bermuda grass pollen allergen (U35683); Phl p 7, timothy grass pollen allergen (Y17835). The two EF-hand motifs are shown above the sequences. Dots and hyphens indicate identical residues of Bra r 1 and gaps in sequences respectively. Numbers of residues are shown in right.

tential to be used as cross-protective therapeutic agents for effective immunotherapy against allergies to Bra r 1 type Ca^{2+}-binding pollen allergens.

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