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Cloning, expression and immunological characterization of *Ory s 1*, the major allergen of rice pollen

(cDNA cloning; cross reactivity; IgE binding; epitope; allergy; *Oryza sativa*)

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SUMMARY

We have isolated and characterized a cDNA clone, *Ory s 1*, encoding a group-1 allergen of rice pollen. The *Ory s 1* protein shows significant sequence identity to the major allergen of rye-grass pollen, *Lol p 1*. RNA gel blot analysis shows that the *Ory s 1* gene is expressed in mature anthers, but not in vegetative or other floral tissues tested. Southern blot analysis indicates that this clone represents a member of a small gene family in rice. Western blot analyses of total rice pollen proteins with the group-1 allergen-specific monoclonal 3A2 and IgE antibodies from grass pollen-allergic patients, revealed the presence of cross-reactive antigenic and allergenic epitopes in *Ory s 1*.

INTRODUCTION

A substantial proportion (20–25%) of the human population living in temperate climates suffers from IgE-mediated atopic diseases such as allergic rhinitis and seasonal asthma (Fleming and Crombie, 1987; Wutrich, 1989). These symptoms are caused by airborne allergens from pollen, mites, mould, and cat and

dog dander, all of which cross-link mast-cell-bound-specific IgE (Segal et al., 1977). The major outdoor source of pollen allergens is grasses. Rye-grass (*Lolium perenne*) is considered as a dominate source of pollen allergens and, consequently, has been well studied (Smart et al., 1983; Singh and Knox, 1985; Ford and Baldo, 1986; Esch and Klapper, 1989a; Smith et al., 1994a). The major allergens of rye-grass pollen are *Lol p 1* and *Lol p 5*, as indicated by the number of reactive allergic patients and the relative amount of IgE binding (Singh et al., 1991; Griffith et al., 1991). Together, both of these allergens can almost completely inhibit IgE binding to rye-grass soluble pollen extract (Bond et al., 1993). We have cloned and characterized genes encoding both *Lol p 1* and *Lol p 5* proteins (Singh et al., 1991; Griffith et al., 1991).

In this paper, we report the cDNA sequence (*Ory s 1*) and immunological characterization of a rice pollen protein, which shows significant sequence homology to *Lol p 1* from rye-grass pollen. Rice (*Oryza sativa*) is widely cultivated as the major cereal crop in Japan, China, Thailand and other Asian countries. Our report of a rice pollen protein homologous to group-1 allergens is sig-

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); Blotto, fat-free milk; bp, base pair(s); BSA, bovine serum albumin; cDNA, DNA complementary to RNA; DTT, dithiothreitol; Ig, immunoglobulin; IUIS, International Union of Immunological Societies; kb, kilobase(s) or 1000 bp; *Lol p 1*, major allergen of rye-grass pollen; *Lol p 1*, gene encoding *Lol p 1*; mAb, monoclonal Ab; nt, nucleotide(s); ORF, open reading frame; *Ory s 1*, major allergen(s) of rice pollen; *Ory s 1*, gene (DNA) encoding *Ory s 1*; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (3.3 mM NaH₂PO₄·2H₂O 6.7 mM Na₂HPO₄·0.15 M NaCl pH 7.2); PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl 15 mM Na₃ citrate pH 7.6; SSPE, 0.18 M NaCl/10 mM Na₂HPO₄ 1 mM EDTA pH 7.4; X, any aa.

nificant because airborne rice pollen originating from extensive rice fields can be a seasonal trigger of hayfever and a major source of outdoor allergy for field workers and people living in the areas. Matsumura et al. (1969) have shown a significant correlation between atmospheric rice pollen counts and asthma episodes in Japan. In 34 of 41 asthmatic children studied, the asthma attacks occurred at a time (autumn) closely corresponding to the flowering season of rice plants. Bronchial provocation tests on 16 of these asthmatic children showed positive response in 13 cases. In contrast, provocation tests failed to cause bronchial constriction in non-asthmatic children. Moreover, the same asthmatic children gave a positive response to rice pollen extract on skin prick test. Further studies show that the soluble extract of rice pollen possess cross allergenicity with extracts from timothy and orchard grasses (Kimura et al., 1969). Our present studies show that this cross allergenicity may be due to shared IgE epitopes present in both *Ory s 1* and group-1 homologues of other grasses. This information will allow the rational design of new diagnostic reagents as well as immunotherapeutic agents with improved efficacy for treatment of rice pollen induced hayfever and seasonal asthma.

EXPERIMENTAL AND DISCUSSION

(a) Isolation and characterization of *Ory s 1*

A mature rice anther cDNA expression library was constructed and screened with group-1 allergen-specific mAb 3A2. This Ab was raised against Cyn d 1, a major allergen of Bermuda grass pollen, and is cross-reactive with group-1 allergens of other grasses (Smith et al., 1994a). Clones that bound strongly to this mAb were identified and named *Ory s 1* in accordance with IUIS nomenclature (Marsh et al., 1988). Purified clones, when incubated with pooled sera from rye-grass pollen allergic patients, showed IgE binding activity. No binding was detected when tested with non-allergic sera.

The complete nt sequence of *Ory s 1* was determined and the aa sequence is deduced (Fig. 1). The sequence contains an ORF of 792 bp encoding a protein of 264 aa (28 kDa). An N-glycosylation motif N-X(S/T) motif is present near the N terminus (Fig. 1). The deduced aa sequence contains a putative leader peptide of 23 aa on the basis of hydrophobicity profiles of the N-terminal sequence. This suggests that the mature processed protein has 241 aa (26 kDa). Comparison of the deduced *Ory s 1* aa sequence with sequences in GenBank revealed a significant sequence homology with major grass pollen allergens of rye-grass Lol p 1 (65.5%) and Bermuda grass Cyn d 1 (62.9%) (Fig. 1).

Or	M...A.SSSLULACVVVAAMVSPSPAGHPKVP	55
Lo	...S...V...VVALF-VFLG-AH-IA-----AE-----D-KSTWYG-K-TGA	56
Cy	AIG.....DK-----AT-S-----RATFYGSN--GA	33
Or	APKDNNGGAGCYKDVDPKAPFLGMNSCGNDPIFKDGKGCSCFEIKCSKPEACSDKPALIHV	115
Lo	G-----N--TG--T-----R-----T--S--GEAVTVTI	116
Cy	--D-H-----RN--P--D--TA--E-----L--A-Y--KE-VE--GE-V-VKI	93
Or	TDMDEPTAAYHFDLSGLA...MAKDGKDEELRKAGIIDTPRRVKCKYPADTKITFHIE	172
Lo	--D-E-----P-----H-FGS--K-EEQK--S-ELEL-----DG--P--V-	175
Cy	--K-Y-H-----K-FGA--K-QEDK-----ELTL-----SG-----	153
Or	KASNPNYLALLVKYVAGDGVVEVEIKESSEWKALKESWGAATWRIIDTPKPLKGPFSVR	232
Lo	-----I-----D-----A-D-----KDK-IE-----V-----DK-T--T--	235
Cy	-G--DH-----A-----NI-A-D-RP-D-D-FIPM-S-----PK-----I-	213
Or	VTTEGARRSSAEDAIPDPGRQRQVNVQAK..	263
Lo	---GTK-EV--V--EGWKADTSY.S....	262
Cy	L-S--GAHLVQD-V--ANWKPD-TYTSKLQFGA	246
	**	

Fig. 1. Comparison of deduced aa sequences of the *Ory s 1* (Or) cDNA clone with group-1 allergens of rye-grass Lol p 1 (Lo) and Bermuda grass Cyn d 1 (Cy). Numbers to the right of the sequences refer to the numbering of each protein. Residues identical to *Ory s 1* are indicated by a dash. The aa defined to be similar are: A, S, T; D, E; R, K; I, L, V, M; N, Q; F, Y, W. The putative glycosylation site of *Ory s 1* is underlined (position 32). 3A2 mAb binding site is marked by asterisks. Motifs which are highly conserved between group-1 allergens and two other proteins are indicated by bold letters. Sequences of Lol p 1 and Cyn d 1 are from Griffith et al. (1991) and Smith (1994), respectively. **Methods:** Total RNA was extracted from mature anthers of rice (*Oryza sativa* L. indica var. IR36) using the method described by Chomczynski and Sacchi (1987). Polyadenylated RNA was isolated using Oligo d(T) affinity column (Pharmacia) and cDNA library constructed in lgt11 expression vector using Time-Saver cDNA synthesis kit (Pharmacia). Immunological screening was done by probing duplicate filters with mAb 3A2. The positive clones were identified using peroxidase-labeled antimouse immunoglobulin as secondary antibody. The cDNA inserts recovered from the positive clones were ligated into plasmid vector, pBluescript KS(+) (Stratagene). DNA sequencing was performed on double stranded DNA by the dideoxy chain-termination method using T7 DNA sequencing kit (Pharmacia). Specific oligodeoxy primers were used to obtain the complete sequence along both strands. Sequence analysis was performed using Melbot/Angis database which incorporates databases from the following major sources: GenBank, EMBL and NBRF nucleic acid libraries; NBRF PIR protein and Swiss-Prot libraries. The nt sequence of the *Ory s 1* cDNA was submitted to GenBank (accession No. U31771).

Genes encoding the group-1 allergens *Lol p 1* from rye-grass show pollen-specific expression (Griffith et al., 1991). Using RNA gel blot analysis, we examined *Ory s 1* expression in leaf, root and anthers of three different developmental stages (unicellular microspore, bicellular pollen and mature pollen). An mRNA transcript of approx. 1.2 kb was detected in mature anther stage (Fig. 2A).

(b) Southern blot analysis

Sequence analyses of the cDNA clones encoding group-1 allergens from rye-grass and Bermuda grass pollen have identified multiple clones which encode different but highly related proteins (Perez et al., 1990; Griffith et al., 1991). In addition, two-dimensional electrophoresis and Western blot analysis of pollen extracts from these genera show the presence of 4 to 6 isoforms which are isoallergenic in nature (Smith et al., 1993;

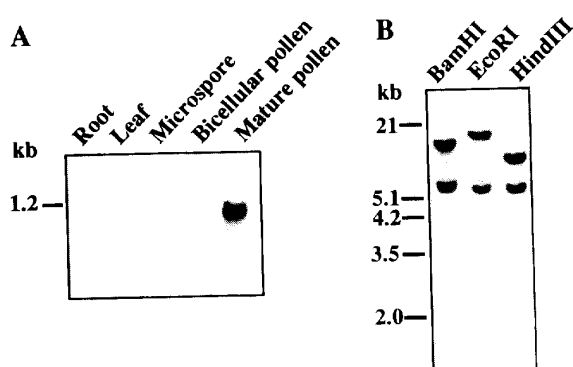


Fig. 2. RNA and DNA gel blot analysis of *Ory s 1* gene. (A) RNA gel blot analysis showing the expression of *Ory s 1* gene in mature pollen, but not in vegetative tissues (leaf and root) or at early pollen developmental stages (unicellular microspore and bicellular pollen). (B) DNA gel blot analysis of rice genomic DNA. **Methods:** Total RNA was extracted from various tissues as in Fig. 1. RNA (20 µg) was denatured in 20 mM 3-(*N*-morpholino)-propanesulfonic acid/50% deionized formamide/2.2 M formaldehyde at 65 °C for 5 min, electrophoresed in 1.2% denatured agarose gel and transferred onto Hybond N⁺ nylon membrane (Amersham) under vacuum using Vacugene gel blotting system (Pharmacia) following manufacturer's instruction. ³²P-labelled *Ory s 1* cDNA probe was prepared by random oligodeoxy priming using GIGAprime DNA labeling kit (Bresatec, Adelaide, Australia). Hybridization was conducted in 2 × SSPE/0.5% Blotto/1% PEG 20000/7% SDS/50% formamide/0.5 mg ml carrier DNA for 12–14 h at 42 °C. The blot was washed 2 × SSC containing 0.1% SDS at room temperature for 15 min, 0.1 × SSC containing 0.1% SDS at 65 °C for 15 min and rinsed briefly in 0.1 × SSC at room temperature. Genomic DNA was extracted from leaf tissues as described by Dellaporta et al. (1983). DNA (20 µg) was digested with *Bam*HI, *Eco*RI and *Hind*III, separated on 0.7% agarose gel and transferred onto Hybond N⁺ nylon membrane (Amersham) under alkaline conditions according to the manufacturer's specifications. Hybridization was conducted as described in Xu et al. (1995).

1994a,b). Isoallergens have been defined as a group of allergens which possess experimentally indistinguishable immunological properties and closely related physicochemical structures (Marsh et al., 1970). Such related proteins are encoded by multigene families. In order to determine the copy number of the *Ory s 1* gene in the rice genome, DNA gel blot analysis was performed. The result (Fig. 2B) shows that digestion of rice genomic DNA with restriction enzymes separates two fragments that hybridize to the *Ory s 1* cDNA probe in each case, thus indicating that this clone represents a member of a small multigene family of two.

(c) Immunoreactivity of the *Ory s 1* protein

To characterize the protein product of the *Ory s 1* gene, protein extracts from root, leaf and anther at three developmental stages were analysed on a Western blot using mAb 3A2. mAb 3A2 recognised a 35-kDa polypeptide that presented only in mature anther extracts (Fig. 3A). This polypeptide was identified as *Ory s 1*, a

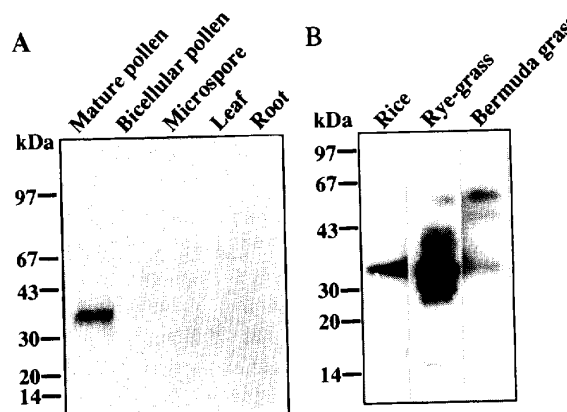


Fig. 3. Immunological analyses of *Ory s 1* protein. Molecular mass markers (Bio-Rad) in kDa are shown on the left. (A) Western blot analysis of proteins from different tissues of rice using 3A2 mAb. The *Ory s 1* protein was detected in mature pollen, but not in any other vegetative tissues (leaf and root) or at early pollen developmental stages (unicellular microspore and bicellular pollen). (B) Cross-reactivity of rice pollen allergens with rye-grass and Bermuda grass as detected using IgE Ab from pooled sera of patients with high sensitivity for rye-grass pollen. **Methods:** Total proteins were extracted from various tissues by grinding the tissues in the extraction buffer containing 50 mM Tris-HCl pH 7.2/1 mM Ca₂Cl₂/10 mM NaCl/1 mM DTT/1 mM phenylmethylsulfonyl fluoride. Protein samples (10 µg per lane) were denatured under reducing conditions by boiling for 5 min in the presence of 25 mM Tris-HCl pH 6.8/2% DTT/10% glycerol and separated by 0.1% SDS-10% PAGE using buffer system described by Laemmli (1970). The separated proteins were then transferred to nitrocellulose as described by Towbin et al. (1979). After protein transfer, unreacted binding sites were blocked by incubation of the nitrocellulose blots in 10% powdered milk in PBS for 1 h. For mAb, the blot was then incubated with 3A2 mAb for 3 h and the bound IgG was detected using horseradish peroxidase conjugated sheep anti-mouse Ig (Silenus, Melbourne, Australia). For IgE, the blot was incubated overnight in pooled allergic sera (diluted 1:5 in PBS containing 0.5% BSA) and the bound IgE was detected using ¹²⁵I-labelled anti-human IgE (Kallestad, Chaska, MN, USA).

rice homologue of Cyn d 1. The difference in the observed molecular mass of *Ory s 1* after SDS-PAGE and the calculated molecular mass based on aa sequences is likely to be due to glycosylation.

mAb 3A2 was originally prepared by immunizing mice with the Bermuda grass allergen Cyn d 1 and has cross-reactive epitopes on group-1 homologues of 8 other grasses from three sub-families (Smith et al., 1994a). The binding site of mAb 3A2 on Cyn d 1 is mapped to be within a linear sequence DPKKPLKGPFISIRLT, as tested by using synthetic peptides (Smith et al., 1994a). The deduced aa sequence of *Ory s 1* contains a region (DTPKPLKGPFISVRVT) which displays a very high level of sequence identity with the 3A2 mAb binding epitope on Cyn d 1 (Fig. 1). This may account for the observed immunological cross-reactivity of the two proteins.

Allergenic cross-reactivity has been shown to exist

between allergens derived from different species of grass pollens (Standing et al., 1987; Van Ree et al., 1992). To test the allergenic cross-reactivity of pollen allergens between rice and other grasses, a Western blot of pollen-protein extracts from rice, rye-grass and Bermuda grass was probed with human IgE Ab from rye-grass pollen allergic individuals. As shown in Fig. 3B, a strong IgE-binding protein band was detected in rice. Pre-incubation of sera with the recombinant Ory s 1 proteins completely abolished the IgE-binding to this protein (data not shown). This indicates that the IgE-binding protein detected in rice is Ory s 1 protein encoded by the cDNA isolated. In comparison to rice, strong IgE binding to the two major allergens Lol p 1 and Lol p 5, as well as minor allergens in rye-grass was observed (Fig. 3B). Similarly, IgE binds to three protein bands including Cyn d 1 in Bermuda grass (Fig. 3B). Since the IgE Ab was obtained from grass allergic patients in Melbourne, Australia, where rice does not exist as an aeroallergen, the Ory s 1 protein is unlikely to be the primary sensitizing antigen. The IgE cross-reactivity of Ory s 1, as observed by Western blot analysis, is likely to be due to the presence of shared epitopes with allergens from temperate grasses, such as Lol p 1 from rye-grass.

By using tryptic digestion of Lol p 1, Esch and Klapper (1989a,b) identified a major IgE epitope which was found at the C-terminal end of this allergen. Similar results have been obtained by random fragmentation of *Lol p 1* cDNA (Smith et al., 1994a). Further studies have shown that a 28-aa synthetic peptide based on the C-terminal sequence of Lol p 1 displays IgE binding ability (Van Ree et al., 1994). In the present study, we prepared a corresponding 28-aa peptide based on the deduced aa sequence of *Ory s 1* cDNA (Fig. 1). This peptide also shows recognition by IgE Ab from grass pollen allergic individuals (data not shown). The result indicates that this region may partly account for the observed cross allergenicity between rice and temperate grasses such as rye-grass, timothy and orchard grasses (Kimura et al., 1969).

(d) Clinical and biological significance of the Ory s 1 protein

Previous studies have shown that group-1 allergens are present in three sub-families of the Poaceae: Pooideae (rye-grass, Kentucky blue grass, timothy grass and orchard grass), Chloridideae (Bermuda grass, grama grass) and Panicoideae (maize, Johnson grass, bahia grass) (Singh and Knox, 1985; Smith et al., 1994a,b). Our present results have revealed that group-1 allergens are also present in the fourth sub-family, Bambusoideae (rice).

It has been reported that the group-1 allergen of rye-grass, Lol p 1, accounts for about 40–50% of total IgE

reactivity of crude pollen extract, and that the rest of the IgE reactivity of rye-grass pollen is largely due to Lol p 5 (Bond et al., 1993). We have not been able to detect the Lol p 5 homologue in rice pollen using either a panel of mAb to group-5 or DNA probes. Moreover, pre-incubation of allergic sera with purified group-1 allergens (Lol p 1 or Ory s 1) deplete almost all of the IgE reactivity to crude rice pollen extract. These results suggest that Ory s 1 is the potential major allergen of rice pollen. This situation is similar to that in Bermuda grass (*Cynodon dactylon*), where group-5 allergens have not been reported (Smith et al., 1994b). Studies using mAb and nucleic acid probes have shown that group-5 allergens seem to be restricted to grasses belonging to the sub-family Pooideae (Smith et al., 1994b).

There is no clue as to the precise biological role of group-1 allergens in pollen. The conservation of group-1 allergens in grasses from many families suggests that they may have important role in pollen function or development. Recently, a cytokinin induced polypeptide, Cim1, from soybean callus tissue (Crowell, 1994) and a protein, PPAL, from pistils of *Nicotiana* (Mariani et al., personal communication) have been shown to have noticeable aa sequence homology with group-1 allergens, including Ory s 1. Sequence analyses have revealed strict conservation of certain aa motifs, especially GGACGY, YHFDLSG and SWGAVW, between these two proteins and group-1 allergens (Fig. 1). These sequence motifs thus must play an important role in a conserved biological function of these proteins in plant tissues, including pollen. These motifs, however, do not have any role in the allergenic reactivity of Ory s 1 and other homologues. Indeed, the *Nicotiana* pistil protein PPAL, which shows homology with group-1 allergens, does not bind IgE from grass pollen allergic patient sera.

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