

GENE 07453

Cloning of a cDNA encoding a group-V (group-IX) allergen isoform from rye-grass pollen that demonstrates specific antigenic immunoreactivity

(cDNA cloning; allergy; *Lol p* IX; *Lol p* V; IgE binding; polymorphism)

Eng Kok Ong^a, Irwin J. Griffith^b, R. Bruce Knox^a, and Mohan B. Singh^a

^aSchool of Botany, University of Melbourne, Parkville, Vic. 3052, Australia. Tel. (61-3) 344 5056; and ^bImmuLogic Pharmaceutical Corporation, Waltham, MA 02154, USA. Tel. (61-7) 466 6000.

Received by P.A. Manning: 13 April 1993; Revised: Accepted: 6 July 8 July 1993; Received at publishers: 23 July 1993

SUMMARY

We have isolated and characterized the cDNA clone, 19R, that encodes an isoform of a major rye-grass pollen allergen, *Lol p* V [previously referred to as *Lol p* 1b; Singh et al., Proc. Natl. Acad. Sci. USA 88 (1991) 1384–1388; and *Lol p* IX; Suphioglu et al., Lancet 339 (1992) 569–572]. Clone 19R was isolated from a rye-grass pollen cDNA expression library using grass pollen-specific immunoglobulin E (IgE) antibodies (Ab) from an allergic serum pool. The nucleotide (nt) sequence of clone 19R potentially encodes a 33.8-kDa protein of 339 amino acids (aa). It possesses a leader peptide essentially identical to the previously characterized isoform of *Lol p* V (*Lol p* VA). This indicates a mature processed 31.3-kDa protein of 314 aa, correlating well with the size of the polypeptides revealed by Western analysis of pollen proteins using IgE Ab affinity purified from recombinant fusion protein (reFP) encoded by clone 19R as solid matrix. There is no *N*-glycosylation motif. The protein encoded by clone 19R, designated *Lol p* VB, has 66.4% identity and 80.4% similarity with *Lol p* VA. However, a *Lol p* VA-specific monoclonal Ab, FMC A7, does not recognize reFP encoded by clone 19R, indicating that *Lol p* VB does not share this epitope. Cross-reactivity studies using affinity purified IgE Ab showed that both isoforms share similar allergenic epitopes. Immunoblot analysis using sera from a population of 30 patients showed that 80% possess IgE Ab that recognize both *Lol p* V isoforms. Variation occurred in the signal intensities of IgE binding. Like *Lol p* VA, *Lol p* VB is pollen specific and is part of the same multigene family.

INTRODUCTION

Grass pollen allergy afflicts up to 25% of the population in cool temperate climates. Of the grasses, rye-grass is the major cause of these diseases due to its copious production of pollen during flowering (Smart et al., 1979). Pollen of rye-grass contains 17 allergens ranging in size from 12 to 89 kDa (Ford and Baldo, 1986). Among these

allergens, *Lol p* I and *Lol p* IX (previously referred to as *Lol p* 1b, Singh et al., 1991) have been shown to be the most important allergens in rye-grass pollen as judged by both criteria of number of allergic patients responding and relative amount of IgE binding to the allergens (Singh et al., 1991). Together, both of these allergens could almost completely inhibit IgE binding to rye-grass soluble pollen extract (Bond et al., 1993).

Correspondence to: Dr. R.B. Knox, School of Botany, University of Melbourne, Parkville, Victoria 3052, Australia. Tel. (61-3) 344 5055; Fax (61-3) 344 5063.

Abbreviations: aa, amino acid(s); Ab, antibody(ies); Blotto, fat-free produced milk; BSA, bovine serum albumin; cDNA, DNA complementary to RNA; Exo, *E. coli* exonuclease III; Ig, immunoglobulin; IUIS, International Union of Immunological Societies; *Lol p*, allergen(s)

(see INTRODUCTION) and their gene(s) or cDNA: mAb, monoclonal Ab; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate-buffered saline (3.3 mM NaH₂PO₄ 2H₂O/6.7 mM Na₂HPO₄ 0.15 M NaCl pH 7.2); reFP, recombinant fusion protein(s); SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/15 mM Na₂citrate pH 7.6; SSPE, 0.18 M NaCl/10 mM Na₂HPO₄/1 mM EDTA pH 7.4; ' (prime), denotes a truncated gene at the indicated site.

mAb FMC A7 has previously been shown to bind reFP-*Lol p* VA (Singh et al., 1991) but does not show any reactivity with reFP-*Lol p* VB. However, IgE Ab affinity purified using reFP-*Lol p* VA as a solid matrix, recognized reFP-*Lol p* VB. Similarly, IgE Ab affinity purified using reFP-*Lol p* VB as a solid matrix, recog-

nized reFP-*Lol p VA* (data not shown). These two isoforms apparently share epitope(s) that are recognized by IgE Ab.

The affinity purified IgE Ab from reFP-*Lol p VB* were also used for Western analysis of rye-grass pollen proteins. The purified IgE bound to protein components forming a triplet of 28, 30 and 32 kDa (Fig. 3). These values are in good correlation with the polypeptides predicted by clones 12R (*Lol p VA*) and 19R.

MAb PpV1, PpV4 and PpV8 raised against Timothy grass group V allergen, *Phl p V* (Matthiesen et al., 1990) bound to reFP-*Lol p VB*. However, only mAb PpV4 and PpV8 recognized reFP-*Lol p VA* (data not shown). Similarly, Olsen et al. (1991) showed that both mAb PpV1 and PpV4 recognized *Poa p V* allergens. Thus there is antigenic cross-reactivity between group V allergens from different grasses.

(e) Relative clinical significance

Lol p VA and *Lol p VB* showed reactivity with 80% (24/30) of IgE Ab from sera of a sampled population of grass pollen-allergic patients (data not shown). Thus we conclude that reFP-*Lol p V* in its native state is a major allergen in pollen of rye-grass. The IgE immunoblots revealed variation in the signal of IgE binding intensities. Some patients' sera showed more intense IgE binding to *Lol p VA* than to *Lol p VB* and a few of them have more intense IgE binding to *Lol p VB* than to *Lol p VA*. These differences have also been observed in short ragweed, *Amb a 1* isoforms; a minority of patients' IgE reacts with only the recombinant *Amb a 1.1* whereas most patients' IgE reacts with the recombinant *Amb a 1.1* as well as with the *Amb a 1.2* and *Amb a 1.3* proteins (Bond et al., 1991).

(f) Northern and Southern analyses

A Southern analysis of rye-grass genomic DNA revealed hybridization to several fragments when full length cDNA of clone 19R was employed as probe (data not shown). These results suggest that *Lol p V* allergens are encoded by a multigene family. Like *Lol p VA*, *Lol p VB* is pollen specific. In Northern blot analysis, a cDNA probe specific to *Lol p VB* hybridized to a single transcript (approximately 1353 nt) in pollen but not other tissues (Fig. 4). The size of the transcript identified is similar to that of *Lol p VA* (Singh et al., 1991). However, the specificity of the probe showed the two transcripts to be different. The level of expression of these transcripts in pollen is not yet known.

(g) Conclusions

(1) An isoform of *Lol p V* allergen has been cloned and sequenced from rye-grass pollen. The cDNA of

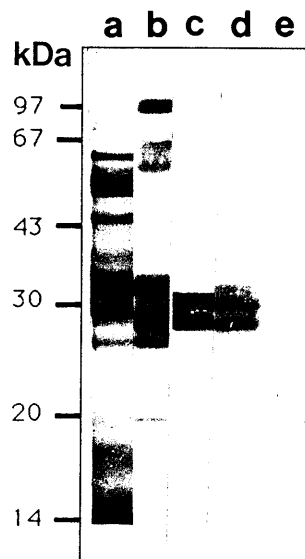


Fig. 3. One-dimensional Western analysis of rye-grass pollen proteins. The positions of size markers are shown at left. In all cases, 10 µg of rye-grass pollen proteins was loaded per lane. (a) SDS-PAGE of total proteins stained in Coomassie blue R250. Western blots probed with (b) total IgE Ab from pooled sera of grass pollen allergic patients, (c) mAb FMC A7, (d) affinity-purified IgE Ab from reFP-19R and (e) affinity-purified Ab from non-recombinant λgt11 as control. **Methods:** Soluble proteins were extracted from rye-grass pollen by vigorous shaking in PBS and 1 mM phenylmethylsulfonylfluoride on ice for 3 h. Conditions for 0.1% SDS-10% PAGE were essentially as described (Ong et al., 1990). Immediately after electrophoresis, the separated proteins were either Coomassie stained or transferred at 4 °C to nitrocellulose (Towbin et al., 1979). For IgE Ab binding, blots were incubated in pooled allergic sera or affinity purified IgE in PBS containing 0.5% BSA. The bound IgE was detected (Ong et al., 1990) using ¹²⁵I-labeled anti-human IgE (Kallestad Lab., Chaska, MN). For mAb binding, the bound IgG was detected using horseradish peroxidase-labeled sheep anti-mouse Ig (Silenus, Australia). The blot was developed using enhanced chemiluminescence system (Amersham Int., UK) as described (Ong et al., 1990). To affinity purify IgE Ab, cDNA clones in λgt11 phages encoding allergens were expressed as reFP in *E. coli*. Plaque lifts containing the reFP were incubated in pooled sera overnight, washed once with PBS containing 0.1% Tween 20 and twice with PBS only. The bound IgE Ab were eluted with 0.2 M glycine HCl, pH 2.6/0.5% BSA/0.1% sodium azide. The preparation and characterization of mAb FMC A7 have been described elsewhere (Smart et al., 1983). Sera were obtained from grass allergic subjects selected on the basis of skin prick test and radioallergen sorbent test (RAST).

Lol p VB encodes a putative 339-aa protein and possesses an essentially similar 25 aa leader peptide as *Lol p VA*.

(2) *Lol p VB* is immunologically distinct from *Lol p VA* in terms of binding to mAb FMC A7 and mAb PpV1. Both allergens appear to share similar IgE binding epitopes.

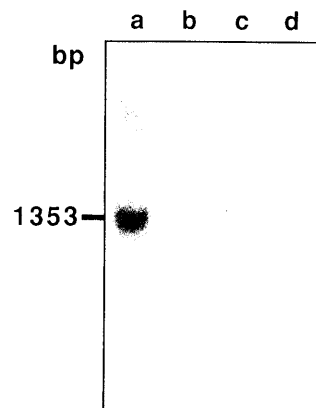


Fig. 4. Northern blot analysis of clone 19R. A cDNA fragment containing sequence unique to clone 19R was hybridized to total RNA of rye-grass pollen (a), leaf (b), root (c), and seed (d). **Methods:** Total RNA was denatured in 20 mM 3-(N-morpholino)-propanesulfonic acid 50% deionized formamide 2.2 M formaldehyde at 65 °C for 5 min, electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, and electroblotted onto nitrocellulose. The filter was prehybridized for 2 h at 70 °C in a solution containing 2 × SSPE 0.5% Blotto 1% poly(ethylene glycol) 20000 7% SDS and 0.5 mg ml⁻¹ (final vol.) carrier DNA. Hybridization was carried out for 24 h at 70 °C in an identical solution containing 0.5–1.0 × 10⁶ cpm ml⁻¹ ³²P-labeled cDNA probe prepared by random oligonucleotide priming using an oligolabeling kit (Bresatec, Adelaide, Australia). The filter was washed as follows: (a) 2 × SSC 0.5% SDS at room temperature for 5 min, (b) 2 × SSC 0.1% SDS at room temperature for 15 min, (c) 0.1 × SSC 0.5% SDS at 37 °C for 30 min, (d) 0.1 × SSC 0.5% SDS at 65 °C for 30 min, (e) rinse briefly with 0.1 × SSC at room temperature. At these washing conditions, the probe bound specifically to cDNA of 19R but not to cDNA of 12R. The cDNA probe containing sequence unique to clone 19R was prepared by cleaving full length 19R cDNA with *Dde*I restriction enzyme, resulting in a 465-bp fragment.

(3) Comparison between the aa sequences of *Lol p VA* and *Lol p VB* reveals 80.4% similarity. Knowledge of all the aa sequences of *Lol p V* isoforms and the extent of polymorphism will be useful to determine the number of allergenic and antigenic epitopes in *Lol p V* allergen.

(4) *Lol p VB* is pollen specific, like *Lol p VA*, and is part of the same multigene family

ACKNOWLEDGEMENTS

The authors gratefully thank Dr. F. Matthiesen for the gifts of mAbs PpV1, PpV4 and PpV8 and the Australian National Health and Medical Research Council, Asthma Foundation of Victoria, Baker Shaw Trust for financial support.

REFERENCES

- Birnsteil, M.L., Busslinger, M. and Strub, K.: Transcription termination and 3' processing: The end is in site! *Cell* 41 (1985) 349–359.
- Bond, J.F., Garman, R.D., Keating, K.M., Briner, T.J., Rafnar, T., Klapper, D.G. and Rogers, B.L.: Multiple *Amb a 1* allergens demonstrate specific reactivity with IgE and T cells from ragweed-allergic patients. *J. Immunol.* 146 (1991) 3380–3385.
- Bond, J.F., Segal, B.S., Yu, X.-B., Theriault, K.A., Pollock, M.S. and Yeung, H.: Human IgE reactivity to purified recombinant and native grass allergens. *J. Allergy Clin. Immunol.* 91 (1993) 339.
- Cassab, G.I. and Varner, J.E.: Cell wall proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39 (1988) 321–353.
- Cavener, D.R. and Ray, S.C.: Eukaryotic start and stop translation sites. *Nucleic Acids Res.* 19 (1991) 3185–3192.
- Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J.: Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18 (1979) 5294–5299.
- Ford, D. and Baldo, B.A.: A re-examination of rye-grass (*Lolium perenne*) pollen allergens. *Int. Arch. Allergy Appl. Immunol.* 81 (1986) 193–203.
- Griffith, J.L., Pollock, J., Klapper, D.G., Rogers, B.L. and Nault, A.K.: Sequence polymorphism of *Amb a 1* and *Amb a 1.1*, the major allergens in *Artemisia artemisiifolia* (short ragweed). *Int. Arch. Allergy Appl. Immunol.* 96 (1991a) 296–304.
- Griffith, J.L., Smith, P.M., Pollock, J., Theerakulpit, P., Avcioglu, A., Davies, S., Hough, T., Singh, M.B., Simpson, R.J., Ward, I.D. and Knox, R.B.: Cloning and sequencing of *Lol p 1*, the major allergenic protein of rye-grass pollen. *FEBS Lett.* 279 (1991b) 210–215.
- Gubler, U. and Hoffman, B.J.: A simple and very efficient method for generating cDNA libraries. *Gene* 25 (1983) 263–269.
- Herrin, D. and Michaels, A.: Isolation and fractionation of intact, translatable RNA from walled algal cells. *Plant Mol. Biol. Rep.* 2 (1984) 24–28.
- Klysner, S., Welinder, K.G., Lowenstein, H. and Matthiesen, F.: Group V allergens in grass pollens. IV. Similarities in amino acid compositions and NH₂-terminal sequences of the group V allergens from *Lolium perenne*, *Poa pratensis* and *Dactylis glomerata*. *Clin. Exp. Allergy* 22 (1992) 491–497.
- Matthiesen, F. and Lowenstein, H.: Group V allergens in grass pollens. Purification and characterization of the group V allergen from *Phleum pratense* pollen. *Phl p V*. *Clin. Exp. Allergy* 21 (1991a) 297–307.
- Matthiesen, F. and Lowenstein, H.: Group V allergens in grass pollens. Investigation of group V allergens in pollens from 10 grasses. *Clin. Exp. Allergy* 21 (1991b) 309–320.
- Matthiesen, F., Klysner, S. and Lowenstein, H.: Monoclonal antibodies against group I and group V allergens of grass pollens. *Clin. Exp. Allergy* 20 (Suppl. 1) (1990) 47.
- Meese, E., Olson, S., Leis, L. and Trent, J.: Quick method for high yields of lambda bacteriophage DNA. *Nucleic Acids Res.* 18 (1990) 1923.
- Olsen, E., Zhang, L., Hill, R.D., Kisil, F.T., Schon, A.H. and Mohapatra, S.S.: Identification and characterization of the *Poa p 1X* group of basic allergens of Kentucky bluegrass pollen. *J. Immunol.* 147 (1991) 205–211.
- Ong, E.K., Suphioglu, C., Singh, M.B. and Knox, R.B.: Immunodetection methods for grass pollen allergens on Western blots. *Int. Arch. Allergy Appl. Immunol.* 93 (1990) 338–343.
- Perez, M., Ishioka, G.Y., Walker, L.E. and Chestnut, R.W.: cDNA cloning and immunological characterization of the rye-grass allergen *Lol p 1*. *J. Biol. Chem.* 265 (1990) 16210–16215.
- Roberts, A.M., van Ree, R., Emly, J., Cardy, S.M., Rottier, M.M.A. and Walker, M.R.: N-terminal amino acid sequence homologies of group

- V grass pollen allergens. *Int. Arch. Allergy Appl. Immunol.* 98 (1992) 178–180.
- Sambrook, J., Fritsch, E.F. and Maniatis, T.: *Molecular Cloning. A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
- Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74 (1977) 5463–5468.
- Silvanovich, A., Astwood, J., Zhang, L., Olsen, E., Kisil, F., Sehon, A., Mohapatra, S. and Hill, R.: Nucleotide sequence analysis of three cDNAs coding for *Poa p 1X* isoallergens of Kentucky bluegrass pollen. *J. Biol. Chem.* 266 (1991) 1204–1210.
- 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.: Isolation of cDNA encoding a newly identified major allergenic protein of rye-grass pollen: intracellular targeting to the amyloplast. *Proc. Natl. Acad. Sci. USA* 88 (1991) 1384–1388.
- Smart, I.J., Tuddenham, W.G. and Knox, R.B.: Aerobiology of grass

pollen in the city atmosphere of Melbourne: effects of weather parameters and pollen sources. *Aust. J. Bot.* 27 (1979) 333–342.

- Smart, I.J., Hedde, R.J., Zola, M. and Bradley, L.: Development of monoclonal mouse antibodies specific for allergenic components in rye-grass (*Lolium perenne*) pollen. *Int. Arch. Allergy Appl. Immunol.* 72 (1983) 243–248.
- Smith, P.M., Ong, E.K., Avjioglu, A., Singh, M.B. and Knox, R.B.: Analysis of rye-grass pollen allergens using two dimensional electrophoresis and immunoblotting. In: Kraft, D. (Ed.), *Molecular Biology and Immunology of Allergens*. CRC Press, Boca Raton, FL, 1993.
- Suphioglu, C., Singh, M.B., Taylor, P., Bellomo, R., Holmes, P., Puy, R. and Knox, R.B.: Mechanism of grass-pollen-induced asthma. *Lancet* 339 (1992) 569–572.
- Towbin, H., Staehelin, T. and Gordon, J.: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76 (1979) 4350–4354.
- Young, R.A. and Davis, R.W.: Efficient isolation of genes by using antibody probes. *Proc. Natl. Acad. Sci. USA* 80 (1983) 1194–1198.

Gene, 134 (1993) 241–243

© 1993 Elsevier Science Publishers B.V. All rights reserved. 0378-1119/93/\$06.00

GENE 07348

Sequences of two cDNA clones from the medfly *Ceratitis capitata* encoding antibacterial peptides of the cecropin family*

(Insect immunity; ceratotoxin; sarcotoxin; magainin; recombinant DNA; nucleotide sequence; amino acid sequence homology; medfly cDNA library)

Marco Rosetto^a, Andrea G.O. Manetti^a, Daniela Marchini^a, Romano Dallai^a, John L. Telford^b and Cosima T. Baldari^a

^aDepartment of Evolutionary Biology, 53100 Siena, Italy; and ^bI.R.I.S., Via Fiorentina 1, 53100 Siena, Italy. Tel. (39-577) 293470

Received by G. Bernardi: 18 January 1993; Revised/Accepted: 3 May/10 May 1993; Received at publishers: 17 June 1993

SUMMARY

Using a backtranslated oligodeoxyribonucleotide probe, encoding a conserved motif in insect antibacterial peptides, we have isolated two cDNA clones from the medfly, *Ceratitis capitata*. Sequence determination shows that the cDNAs encode two closely related peptides which are members of the cecropin family.

INTRODUCTION

Antimicrobial peptides have been isolated from both invertebrates and vertebrates. They include, among others, the cecropins (Cec) and the dipterocins from insects (reviewed in Boman and Hultmark, 1987; Hoffmann and Hoffmann, 1990), the magainins from amphibians (Zaslouff, 1987) and the defensins from mammalian polymorphonuclear lymphocytes (Selsted et al., 1985). Although different in primary structure, many of these peptides can form amphipathic α -helices and kill bacteria by attacking the cell membrane, probably through the formation of ion channels (Okada and Natori, 1985a; Wade et al., 1990).

Most antibacterial peptides are produced in insects in response to infection. Among the most potent of the in-

ducible antimicrobial peptides are the Cec (reviewed in Boman and Hultmark, 1987). Interestingly, the Cec locus in *Drosophila melanogaster* has been shown to include the gene encoding andropin, a male-specific antimicrobial peptide which is constitutively produced in the ejaculatory duct (Samakovlis et al., 1991). Recently two closely related antibacterial peptides have also been purified from the female reproductive accessory glands secretion in *Ceratitis capitata* (Marchini et al., 1993).

Here we present the sequence of two cDNA clones from the medfly *Ceratitis capitata* encoding peptides of the Cec family.

EXPERIMENTAL AND DISCUSSION

(a) Cloning and sequence determination of two medfly cDNAs

We have screened a λ gt11 cDNA library from adult females of *Ceratitis capitata* for the genes coding for the female antibacterial peptides ceratotoxins (Ctx) (Marchini et al., 1993). The oligo which was used in the screening was designed by backtranslation of the middle portion of medfly Ctx A and Ctx B (Marchini et al., 1993) using *Drosophila* codon bias (see Fig. 1). This region con-

Correspondence to: Dr. C.T. Baldari, Department of Evolutionary Biology, Via Mattioli 4, 53100 Siena, Italy. Tel. (39-577) 293470; Fax (39-577) 298898.

*On request, the authors will supply detailed experimental evidence for the conclusions reached in this Short Communication.

Abbreviations: aa, amino acids(s); bp, base pair(s); cDNA, DNA complementary to RNA; Cec, cecropin(s); Ctx, ceratotoxin; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; SP, signal peptide(s).