**Note**

Isolation and Characterization of cDNAs That Encode Homologs of a Pathogenesis-related Protein Allergen from *Cryptomeria japonica*

Norihiro Futamura,1 Yuzuru Mukai,2 Masahiro Sakaguchi,3 Hiroshi Yasueda,4 Sakae Inouye,3,* Terumi Midoro-Horiuti,5 Randall M. Goldblum,5 and Kenji Shinohara1,†

1Department of Molecular and Cell Biology, Forestry and Forest Products Research Institute, Ibaraki 305–8687, Japan
2Faculty of Agriculture, Shizuoka University, Shizuoka 422–8529, Japan
3Department of Immunology and Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo 162–8640, Japan
4Clinical Research Center for Allergy and Rheumatology, National Sagamihara Hospital, Kanagawa 228–8522, Japan
5Department of Child Health Research Center, University of Texas Medical Branch, Galveston, TX 77555, U.S.A.

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Many plant pathogenesis-related (PR) proteins are allergenic. We isolated three cDNAs, *Cry j 3.1*, *Cry j 3.2*, and *Cry j 3.3*, that encoded homologs of Jun a 3, a PR protein allergen in *Juniperus ashei*, from a cDNA library derived from the pollen of *Cryptomeria japonica*. The predicted amino acid sequences encoded by the three cDNAs were more than 85% identical to each other and about 57% identical to the sequence of Jun a 3. The *Cry j 3* genes seemed to form a small multigene family in the genome of *C. japonica*. Expression of *Cry j 3* was strong in roots and in female and male strobili; expression was weaker in cotyledons, leaves, stems, and pollen grains.

Key words: cDNA cloning; *Cryptomeria japonica*; pathogenesis-related protein; pollen allergen

Sugi, *Cryptomeria japonica* D. Don (Taxodiaceae), is one of the most commercially important conifers in Japan. Sugi pollinosis is a serious allergic disease in this country. Two major allergens, the Cry j 1 and Cry j 2 proteins, in the pollen of *C. japonica* have been identified.1,2 cDNAs for these two proteins have been cloned,3–5 and Cry j 1 and Cry j 2 have been found to have pectate lyase6 and polymethylgalacturonase7 activity, respectively. Allergens homologous to Cry j 1 and Cry j 2 have been isolated from *Chamaecyparis obtusa* and *Juniperus ashei*, and their cDNAs have been cloned and sequenced.8–13

Several members of the pathogenesis-related (PR) protein families 2–5, 8, 10, and 14 are allergenic.14 Recently, a novel PR protein allergen, Jun a 3, in the pollen of *J. ashei* was identified.15 Jun a 3 is homologous to members of the PR-5 protein family. To find whether a homolog of Jun a 3 might be found in *C. japonica*, we attempted to isolate corresponding cDNAs. We found three cDNAs that encoded homologs of Jun a 3 and designated the encoded proteins *Cry j 3*.1, the genomic organization of the *Cry j 3* genes and their patterns of expression in *C. japonica*.

For plant materials, cuttings of ‘Boka-sugi’,16 a local cultivar of *C. japonica*, were grown to 2-year-old saplings in a Phytotron at 25°C for 12 h and 20°C for 12 h with natural light and 75% relative humidity. Flowering was brought about by treatment with gibberellin (290 μM GA₃) once at the end of July and once at the beginning of August.17 The saplings were harvested at the beginning of November and divided into leaves, stems, and roots. The developing and mature strobili and the mature pollen was collected in October, January, and March, respectively, from specimens of *C. japonica* that had been planted in the experimental field of the Forestry and Forest Products Research Institute. Cotyledons were harvested from 1-month-old seedlings. The various organs were stored at –80°C until being used.

Total RNA was isolated from mature pollen5 and
Fig. 1. Alignment of the Deduced Amino Acid Sequences of Cry j 3.1, Cry j 3.2, and Cry j 3.3 with That of Jun a 3.

Amino acids in black and gray boxes are identical and similar, respectively, in at least three of the sequences. A putative processing site is indicated by the vertical arrow. A potential site for N-glycosylation of Cry j 3.1, Cry j 3.2, and Jun a 3 is indicated by the arrowhead. The amino acid sequence corresponding to the antisense primer used for cDNA cloning is underlined by the horizontal arrow. Asterisks indicate the 16 Cys residues that are conserved in the PR-5 proteins.

from various other organs. Polyadenylated RNA was isolated by affinity chromatography on oligo(dT)-cellulose. Genomic DNA was extracted from young needle buds of a local cultivar of sugi, ‘Kumoto-oshii’. DNA and RNA gel blotting was done as described previously. A cDNA library in λZAP II (Stratagene, La Jolla, CA), consisting of 8.5 × 10⁵ recombinants, was constructed from 5 μg of polyadenylated mRNA isolated from the pollen of C. japonica. For identification of cDNA clones that encoded Cry j 3, we first used 32P-labeled Jun a 3 cDNA as the probe. However, we were unable to isolate any Cry j 3 cDNA clones. Therefore, we used the PCR to isolate cDNAs using an excised pollen cDNA library in plasmid form (pBluescript SK(−); Stratagene) as the template. The PCR cDNA library in λZAPII was excised in vivo in plasmid form by coinfection with ExAssist helper phage, as instructed by the manufacturer. The antisense primer for PCR (5'-ATGTTGAG-CCATCGACAGAGGA-3') was based on the sequence from position 361 to position 383 of the nucleotide sequence of Jun a 3 cDNA. The sense primer for PCR (5'-TCTAGAATCTGGAT-CGCCGG-3') was based on the sequence of the multiple cloning site in pBluescript SK(−). After PCR, the amplified fragment of cDNA was cloned into pBluescript II SK(−) and sequenced, to check that we had isolated a homolog of Jun a 3, by the dideoxy-nucleotide chain-termination method with an automated DNA sequencer (ABI Prism 377; Applied Biosystems, Foster City, CA) and cycle sequencing kits (ABI Prism DNA sequencing kits; Applied Biosystems). To obtain cDNA clones that contained entire open reading frames, we screened a cDNA library in λZAPII using the newly isolated cDNA fragment as the probe. Hybridization was done as described previously. Thirteen phage plaques were isolated from the library and plaques were purified by two additional rounds of screening. Both strands of the 13 excised cDNA in pBluescript SK(−) were sequenced as described above. Six, five and two of the 13 cDNAs we sequenced were classified as Cry j 3.1 (accession number AB081303), Cry j 3.2 (AB081304) and Cry j 3.3 (AB081305), respectively (Fig. 1). The Cry j 3.1 and Cry j 3.2 cDNAs contained full-length coding regions. However, the Cry j 3.3 cDNAs lacked part of a putative signal peptide. The predicted amino acid sequences of the three Cry j 3 proteins were more than 85% identical to one another. They were about 57% identical to Jun a 3 and all contained the 16 Cys residues that are conserved in PR-5 proteins.

Figure 2 shows the nucleotide sequences of the six Cry j 3.1 cDNAs (Cry j 3.1.1 through Cry j 3.1.6) and the deduced amino acid sequences. The predicted polypeptide Cry j 3.1 had a calculated molecular mass of 24.4 kDa. Application of the rules proposed by von Heijne, together with comparison to Jun a 3, led to the prediction that 26 amino acids at the
**Fig. 2.** Nucleotide Sequences of *Cry j 3.1* cDNAs and the Deduced Amino Acid Sequences. The main nucleotide sequence is that of *Cry j 3.1.1*. The deletions detected in the two other cDNAs are boxed. The sites of two single-base substitutions in the 5' noncoding region and two synonymous substitutions are indicated by the four filled arrowheads. The sites of two nonsynonymous substitutions are indicated by open arrowheads. An asterisk shows the position of the termination codon. The names of the cDNA clones (*Cry j 3.1.2* through *Cry j 3.1.6*) and their alterations are shown above the boxes and arrowheads.

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Amino terminus constituted a signal peptide. A single potential site for N-glycosylation was found at N<sup>168</sup>YS. A similar putative site for N-glycosylation was found in *Cry j 3.2* and *Jun a 3* (Fig. 1). Six single-base substitutions and two deletions were detected in *Cry j 3.1* cDNAs (Fig. 2). The two single-base substitutions in the coding region changed a tryptophan residue at position 19 and a glutamine residue at position 72 to leucine and arginine, respectively. The lengths of amino acid sequences deduced from cDNAs that included a deletion were reduced because of a frame-shift or a termination codon. No substitutions and deletions were detected in *Cry j 3.2* and *Cry j 3.3* cDNAs.

Genomic DNA gel blotting was done to examine the distribution of *Cry j 3* genes in the genome of *C. japonica* (Fig. 3). We used *Cry j 3.1* cDNA as the probe, and hybridization and washing were done under high-stringency conditions. We detected several discrete restriction fragments that hybridized to...
genomic DNA digested with BamHI, HindIII, or EcoRI. The patterns of bands were identical when Cry j 3.2 or Cry j 3.3 cDNA was used as the probe, although the intensity of the signal was different from that of Cry j 3.1 (data not shown). We also detected the three Cry j 3 genes in the genome of C. japonica by PCR with three pairs of specific primers (data not shown). The results indicated that the Cry j 3 genes form a small multigene family in the genome of C. japonica. Cry j 3.1, Cry j 3.2, and Cry j 3.3 seem to belong to the same family.

Figure 4 shows the results of RNA gel blotting of total RNA extracted from various organs of C. japonica. A transcript of about 1.2 kb was detected in all organs examined. Cry j 3 mRNA was most abundant in developing and mature female strobili, and also in mature male strobili; expression was strong in roots and developing male strobili. The levels of Cry j 3 mRNA in cotyledons, leaves, stems, and pollen were much lower. In contrast, expression of Cry j 1 and Cry j 2 was strong only in mature male strobili and mature pollen. The pattern of expression of Cry j 3 was different from that of Cry j 1 and Cry j 2.

We described here the structural characteristics of three Cry j 3 proteins, that are homologs of Jun a 3, and the patterns of constitutive expression of the corresponding genes. The Cry j 3 proteins resembled PR-5 proteins and the Cry j 3 proteins were about 57% identical to Jun a 3. Genomic DNA gel blotting showed that the Cry j 3 genes form a small multigene family of which we have identified several members. We found several variants in the nucleotide sequences of Cry j 3.1. The cDNA library in this study was derived from pollens of several specimens of C. japonica, so these variants probably reflected the polymorphism of Cry j 3.1. In addition, one deletion was detected in the nucleotide sequences of Cry j 3.1 and Cry j 3.1.5. C. japonica, like other conifers, has a high degree of heterozygosity because of its allogamous mating system. Therefore, these nonsense mutations might be retained in heterozygotes.
The analysis of IgE-reactive tryptic peptides of Jun a 3 indicated that peptides 120–131, 132–145, and 152–165 are IgE epitopes. These peptides corresponded to peptides 126–137, 138–151, and 158–171 of the Cry j 3 proteins (Fig. 1). That one third of sugi pollinosis patients react with Jun a 3 suggests the presence of common IgE epitopes in Cry j 3 and Jun a 3. However, the overall homology of the three peptides to Jun a 3 was about 57%. The allergenicity of the Cry j 3 proteins is not known.

Various cDNAs encoding PR-5 proteins have been isolated from a variety of tissues of many plant species. Several of the corresponding genes are expressed in floral tissues. Stigma- and style-specific genes for PR-5 proteins have been isolated from tobacco and Japanese pear, and a gene the expression of which is specific to male cones has been cloned from Pinus radiata. We detected strong expression of Cry j 3 genes in female and male strobili. The PR-5 proteins might be important in the growth of pollen tubes and the development of microspores during gametogenesis.

Most PR-5 proteins have antifungal effects, but thaumatin-like proteins from cherry and banana fruits have no such effects. The expression of some PR-5 proteins is induced by osmotic stress, wounding, and UV light. PR-5 proteins have many different functions. Further studies are needed to determine the role of Cry j 3 proteins in C. japonica.

Midoro-Horiuti et al. showed that levels of Jun a 3 vary up as much as fivefold among samples of pollen collected from J. ashei in different years or at different locations. We also found that Cry j 3 mRNA in mature pollen varied as much as two- or threefold among different clones (data not shown). It is likely that clonal variations in levels of PR-5 proteins in pollen occur at the transcriptional level. It will be of interest to examine the relationships among environmental conditions, expression of PR-5 proteins and the allergenic potencies of pollen in future studies.

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