

Short Communication

Pollen-Specific Expression of the Gene for an Allergen, Cry j 1, in *Cryptomeria japonica*Yunqiu Wang,¹ Yuzuru Mukai,² Mitsue Fukui, Norihiro Futamura, Akinori Nagao, and Kenji Shinohara³

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A cDNA fragment for an allergen, Cry j 1 in sugi (*Cryptomeria japonica* D. Don) was generated by the reverse transcriptase-polymerase chain reaction. Using this cDNA fragment, we determined the number of copies of the *Cry j 1* gene in the sugi genome and the organ-specific expression of the *Cry j 1* gene in sugi trees. The *Cry j 1* genes formed a small multigene family within the sugi genome, with a stoichiometry of approximately five to eight copies of the *Cry j 1* gene per diploid genome. The *Cry j 1* gene was expressed exclusively in male reproductive tissues, particularly in mature pollen.

Key words: a small multigene family, allergen, *Cry j 1* gene, pollen-specific expression, sugi (*Cryptomeria japonica* D. Don)

Sugi, *Cryptomeria japonica* D. Don (*Taxodiaceae*) is one of the commercially most important conifers in Japan. However, sugi pollinosis is the most serious allergic disease in Japan. Two major allergens, the Cry j 1 and Cry j 2 proteins, have been identified in sugi pollen (Yasueda *et al.*, 1983; Sakaguchi *et al.*, 1990). Cloning of cDNAs for the two proteins has been reported (Sone *et al.*, 1994; Namba *et al.*, 1994), and it has been demonstrated that Cry j 1 and Cry j 2 proteins have pectate lyase activity and polymethylgalacturonase activity, respectively (Taniguchi *et al.*, 1995; Ohtsuki *et al.*, 1995). Previous studies have been limited to analyses of the structure and function of the Cry j 1 and Cry j 2 proteins. The patterns of expression of the *Cry j 1* and *Cry j 2* genes, and the structure and genomic organization of these genes in sugi have not been characterized. In this study, we determined the number of copies of the *Cry j 1* gene in the sugi genome and the organ-specific expression of the *Cry j 1* gene in sugi trees.

Materials and Methods

1 Plant materials

'Boka-sugi' (Nagao *et al.*, 1989) and 'Kumotooshi' (Mukai *et al.*, 1995), two local cultivars of *C. japonica* that had been propagated by cutting, were used for the experiments. One-year-old saplings of Boka-sugi were harvested in March and divided into leaves, stems and roots. Flowering of Boka-sugi (2-year-old) was induced by gibberellin treatment (50 ppm GA₃, 3 times; Nagao *et al.*, 1989), and male and female strobili were harvested. Mature pollen was collected in March. The various organs were stored at –80°C prior to analysis.

2 Isolation of RNA and DNA

Total RNA was isolated from pollen as described by Sone

et al. (1994), and separately from other organs by extraction with hexadecyltrimethylammonium bromide and chloroform (Shinohara and Murakami, 1996). Genomic DNA was extracted from young needle buds of individual specimens as described elsewhere (Mukai *et al.*, 1995).

3 Preparation of a fragment of *Cry j 1* cDNA by the reverse transcriptase-polymerase chain reaction (RT-PCR)

The oligonucleotide primers used for RT-PCR were 5'-ACTCAAAGTGGGCACAAAAC-3' (forward) and 5'-AGTTGCATTCCCATTCTCAAC-3' (reverse). These sequences correspond to portions of the nucleotide sequence of pCCI-2-2 (Sone *et al.*, 1994). Pollen RNA was reverse transcribed in 50 µl of a reaction mixture that contained 20 µg of the pollen RNA, 1 µM reverse primer, and 300 units of superscript II RT (Gibco BRL, Gaithersburg, MD) at 37°C for 1 h. PCR was performed in a 50-µl reaction mixture that contained 10 µl of reaction mixture after reverse transcription, 1 µM each primer, and 2 units of Ampli Taq DNA polymerase (Perkin-Elmer, Foster City, CA). Conditions for amplification were incubation at 95°C for 5 min followed by 30 cycles of 96°C for 30 s, 55°C for 30 s, and 72°C for 120 s. The amplified fragment of cDNA was cloned directly into the BSII TSK vector (Ichihara and Kurosawa, 1993). The sequences of both strands of the cDNA fragment were determined by the dideoxynucleotide chain-termination method with an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer) and cycle sequencing kits (ABI PRISM DNA Sequencing kits; Perkin-Elmer) according to the protocol provided by the manufacturer.

4 Southern and Northern blot analyses

Southern and Northern blot analyses were performed as described previously (Mukai *et al.*, 1995; Shinohara and Murakami, 1996). The ³²P-labeled cDNA fragment was used as the probe.

Results and Discussion

We determined the nucleotide sequence of the cDNA fragment (974 bp) that had been generated by RT-PCR. Comparison of the sequence with that encoded by pCCI-2-2 (Sone

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1' ACTCAAAGTGGGCACAAACAGAATGAAGCTCGCAGATTGTGCAGTGGGCTTCGGAAGCTCCACCATGGGAGGCAAGGGAGGAGATCTTT
*****
152" ACTCAAAGTGGGCACAAACAGAATGAAGCTCGCAGATTGTGCAGTGGGCTTCGGAAGCTCCACCATGGGAGGCAAGGGAGGAGATCTTT

91' ATACGGTCACGAACTCAGATGACGACCTGTGAATCCTGCACCAGGAAGCTCTGCGCTATGGAGCAACCCGAGATAGGCCCTGTGGATAA
*** *****
242" ATACAGTCACAACTCAGATGACGATCCTGTGAATCCTGCACCAGGAAGCTCTGCGCTATGGAGCAACCCGAGATAGGCCCTGTGGATAA

181' TTTTCAGTGGGAATATGAATATAAAGCTCAAATGCCTATGTACATTGCTGGGTATAAGACTTTTGATGGCAGGGGAGCACAAGTTTATA
*****
332" TTTTCAGTGGGAATATGAATATAAAGCTCAAATGCCTATGTACATTGCTGGGTATAAGACTTTTGATGGCAGGGGAGCACAAGTTTATA

271' TTGGCAATGGCGGTCCTGTGTGTTTATCAAGAGAGTTAGCAATGTTATCATAACGGTTTGTATCTGTACGGCTGTAGTACTAGTGTTT
*****
422" TTGGCAATGGCGGTCCTGTGTGTTTATCAAGAGAGTGAGCAATGTTATCATAACGGTTTGCATCTGTACGGCTGTAGTACTAGTGTTT

361' TGGGGAATGTTTTGATAAACGAGAGTTTTGGGGTGGAGCCTGTTTCCTCAGGATGGCGATGCTCTTACTCTGCGCACTGCTACAAATA
*****
512" TGGGGAATGTTTTGATAAACGAGAGTTTTGGGGTGGAGCCTGTTTCCTCAGGATGGCGATGCTCTTACTCTGCGCACTGCTACAAATA

451' TTTGGATTGATCATAATTCTTTCTCAATTCTTCTGATGGTCTGGTCGATGTCACCTTACTTCGACTGGAGTTACTATTTCAAACAATC
*****
602" TTTGGATTGATCATAATTCTTTCTCAATTCTTCTGATGGTCTGGTCGATGTCACCTTCTTCGACTGGAGTTACTATTTCAAACAATC

541' TTTTTTCAACCATCATAAAGTGATGTTGTTAGGGCATGATGATGCATATAGTGATGACAAATCCATGAAGGTGACAGTGGCGTTCAATC
*****
692" TTTTTTCAACCATCATAAAGTGATGTTGTTAGGGCATGATGATGCATATAGTGATGACAAATCCATGAAGGTGACAGTGGCGTTCAATC

631' AATTTGGACCTAACTGTGGACAAAGAATGCCAGGGCAGCATATGGACTTGTACATGTTGCAACAATAATTATGACCCATGGACTATAT
*****
782" AATTTGGACCTAACTGTGGACAAAGAATGCCAGGGCAGCATATGGACTTGTACATGTTGCAACAATAATTATGACCCATGGACTATAT

721' ATGCAATTGGTGGGAGTTCAAATCCAACCATCTAAGTGAAGGGAATAGTTTCACTGCACCAATGAGAGCTACAAGAAGCAAGTAACCA
*****
872" ATGCAATTGGTGGGAGTTCAAATCCAACCATCTAAGTGAAGGGAATAGTTTCACTGCACCAATGAGAGCTACAAGAAGCAAGTAACCA

811' TACGTATTGGATGCAAAACATCATCATCTTGTCAAATTGGGTGTGGCAATCTACACAAGATGTTTTTATAATGGAGCTTATTTTGTAT
*****
962" TACGTATTGGATGCAAAACATCATCATCTTGTCAAATTGGGTGTGGCAATCTACACAAGATGTTTTTATAATGGAGCTTATTTTGTAT

901' CATCAGGGAAATATGAAGGGGTAATATATACACAAAGAAAGCTTTCAATGTTGAGAATGGGAATGCAACT
*****
1052" CATCAGGGAAATATGAAGGGGTAATATATACACAAAGAAAGCTTTCAATGTTGAGAATGGGAATGCAACT

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Fig. 1 Nucleotide sequence of the amplified fragment of cDNA for Cry j 1 protein. The top line indicates the nucleotide sequence of the amplified cDNA fragment. The bottom line is that of the original insert in pCCI-2-2 (Sone *et al.*, 1994). Asterisks indicate identical nucleotides. Nucleotide differences at seven positions result in two amino acid exchanges. For details, see the text.

et al., 1994) revealed differences at seven positions (Fig. 1). These differences resulted in changes in amino acids at positions 122 (His to Tyr) and 181 (Ser to Thr) of the protein encoded by pCCI-2-2. The result indicated that the cDNA fragment had been derived from the transcript of the *Cry j 1* gene. Using this cDNA fragment, we performed Southern blot analysis to estimate the number of copies of the *Cry j 1* gene in the sugi genome (Fig. 2). Boka-sugi and Kumotooshi

gave very similar banding patterns with minor differences. A stoichiometry of approximately five to eight copies of the *Cry j 1* gene per diploid genome was estimated by taking the 4-kb plasmid DNA that included the cDNA fragment as the standard and assuming a haploid genome size of 10 pg (Price *et al.*, 1974). Our results confirmed that the Cry j 1 protein is encoded by a small multigene family within the sugi genome, and seem to be consistent with the previous report by Yasueda

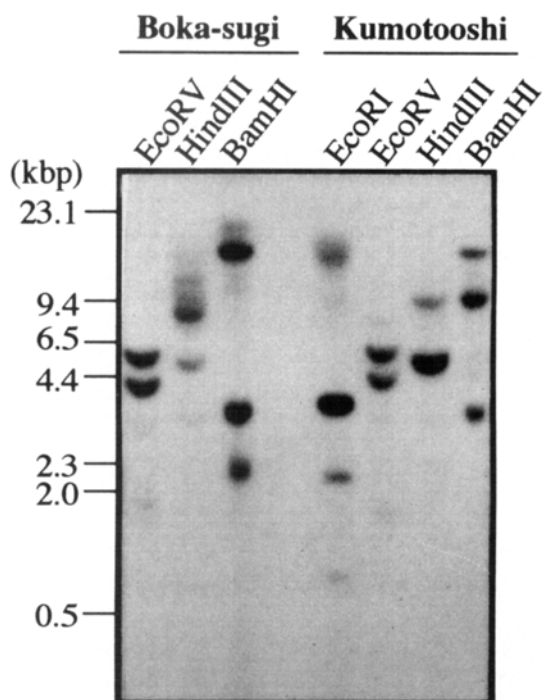


Fig. 2 Genomic Southern blot analysis of *Cry j 1* genes in the genome of *C. japonica*. Genomic DNA (5 µg), prepared from Boka-sugi and Kumotooshi was digested with *EcoRI*, *EcoRV*, *HindIII* or *BamHI*, as indicated. Digested DNA was fractionated by agarose gel electrophoresis. The positions of size markers are shown on the left in kilobase pairs.

et al. (1983), who found four groups of *Cry j 1* proteins, which differ in both isoelectric point and molecular weight from one another.

Cry j 1 protein has pectate lyase activity (Taniguchi *et al.*, 1995), and it is well known that pectic substances, namely, substrates for pectate lyase, are found in the cell walls of all plant tissues. Therefore, we performed Northern blot analysis to determine whether the *Cry j 1* gene is expressed in all plant tissues (Fig. 3). An intense signal was obtained only with the preparation of RNA from mature pollen. A faint signal was also detected in case of male strobili after prolonged autoradiography (data not shown). In fact, the *Cry j 1* transcript was detected only at the late stage of the development of male strobili after induction by GA₃ (Fukui *et al.*, unpublished results). These results suggest that the *Cry j 1* gene is expressed exclusively in male reproductive tissues, in particular, in mature pollen. Using immunocytochemical techniques, Miki-Hirosige *et al.* (1994) demonstrated the ultrastructural localization of *Cry j 1* protein at multiple sites in pollen grains and the accumulation of *Cry j 1* protein during the maturation of pollen grains. We can speculate that the *Cry j 1* gene is expressed only at the late stages of pollen development and that its late expression contributes to the growth of each pollen tube in the nucellar tissue via the depolymerization of pectin in the mature pollen and in female strobili during pollination (cf. Mascarenhas, 1990). The degraded products of pectin in mature pollens can be probably used as

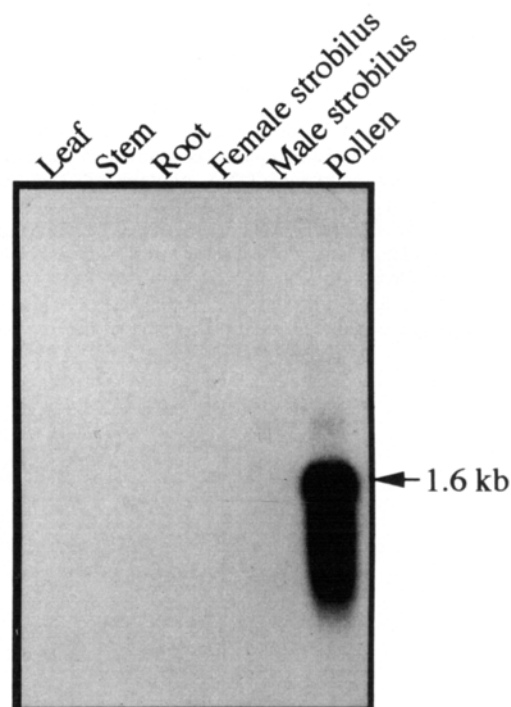


Fig. 3 Northern blot analysis of *Cry j 1* transcripts from different organs of *C. japonica*. Ten micrograms of total RNA were used for the analysis in each case. The approximate size of the mRNA, as estimated from the mobilities of the 28S and 18S rRNAs of *Saccharomyces cerevisiae* and the 23S and 16S rRNAs of *Escherichia coli*, is indicated on the right.

energy source and osmotic pressure regulator during the elongation of the pollen tube.

The information obtained in this study should allow isolation of a pollen-specific promoter that functions in gymnosperms, as well as the genetic engineering of transgenic sugi with allergen-free and infertile pollen grains.

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