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Mapping the Gene Encoding Cry j 1: a Major *Cryptomeria japonica* Pollen Allergen

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Abstract

Cryptomeria japonica D. Don (Sugi, Japanese cedar) causes a serious allergic disease in Japan. In this report, we describe cDNA polymorphisms of Cry j 1, one of the major allergens involved in *C. japonica* pollinosis. We detected two cDNA sequences encoding Cry j 1, one of which was different from all sequences previously reported. A cleaved amplified polymorphic sequence (CAPS) marker that distinguishes a nucleotide difference between the detected cDNA sequences was developed and designated CRYJ1-352 because it has a polymorphic site at position 352 of mature Cry j 1. In an inheritance analysis based on 65 progeny trees from a controlled cross between Iwao-sugi x Boka-sugi, this CAPS marker showed a Mendelian segregation pattern. In addition, the location of CRYJ1-352 was determined on the linkage map of Iwao-sugi.

Key words: Cry j 1, *Cryptomeria japonica,* pollinosis, CAPS, linkage map.

Introduction

Cryptomeria japonica D. Don (Sugi, Japanese cedar) is a coniferous tree species that covers 4.53 million ha and comprises about 45% of the man-made forests in Japan. An allergic disease caused by pollen released from *C. japonica* has become a serious problem. More than 10% of Japanese people currently suffer from *C. japonica*-associated pollinosis, and the number of affected individuals is increasing. Reports have been published on two major allergens responsible for *C. japonica* pollinosis, Cry j 1 and Cry j 2, that include details of their physiological functions (OHTSUKI *et al.,* 1995; TANIGUCHI *et al.,* 1995), partial amino acid sequences (YASUEDA *et al.,* 1983; TANIAI *et al.,* 1988; SAKAGUCHI *et al.,* 1990), and cDNA sequences (GRIFFITH *et al.,* 1993; KOMIYAMA *et al.,* 1994; NAMBA *et al.,* 1994; SONE *et al.,* 1994; WANG *et al.,* 1998). The Cry j 1 and Cry j 2 contents in pollen (w/w) are known to vary widely among individual trees (SASAKI *et al.,* 1996; GOTO *et al.,* 1999; SAITO and TERANISHI, 2002). The Cry j 1 content, however, shows minor variations among pollen samples from the same clones, indicating that the Cry j 1 content in pollen is under strong genetic control. This suggests that planting trees that produce relatively small amounts of the two allergens could be a useful tool for reducing pollinosis. However, the selection of such trees requires a marker that would indicate trees of reduced pollen allergenicity. We report now on a molecular marker for the Cry j 1 gene, which would enable the mapping of the corresponding locus within the genome of *C. japonica.*

Materials and Methods

Plant materials

Pollen samples for RNA isolation were collected from a single ramet of Chichibusyo 3, a plus tree clone, in March 1999. In addition, young leaf samples of eight plus trees (Chichibusyo 3,

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Higashishirakawa 12, Ishikawasyo 3, Kinada 7, Kitashitara 8, Nishitama 5, Nishitama 8 and Syouchiku 3) were collected and DNA isolated from them according to the CTAB method (MURRAY and THOMPSON, 1980). An inheritance analysis of the newly developed DNA marker was performed using 65 F_1 offspring trees, which were obtained from a controlled cross between Boka-sugi x Iwao-sugi (KURAMOTO *et al.,* 2000).

Preparation of Cry j 1 cDNA

Total RNA was extracted from pollen as previously described by SONE *et al.* (1994). Poly A^+ RNA was isolated from total RNA using Oligotex-dT30 (Super) latex beads (JSR Corp., Japan) as recommended by the manufacturer. An RT-PCR was then performed for the amplification of Cry j 1-encoding cDNA using a TaKaRa RNA LA PCR Kit (AMV) (TaKaRa Shuzo Co., Ltd., Japan) and both a forward (5' TCATAATCATAGCATAGCCG 3') and a reverse primer (5' CAACATGCTAGAATATATGC 3'. A reverse transcription was performed at 55°C for 30 min. For the hot-start PCR, the reaction tube was preheated to 94°C for 2 min, followed by 40 cycles, each of 94°C for 30 s, 55°C for 1 min and 72°C for 3 min. The final extension step was carried out at 72°C for 2 min. Cry j 1-encoding cDNA fragments were purified using a GENECLEAN II kit (Qbio gene, Inc., Montreal, Canada), following electrophoresis on a 1.5% agarose gel.

Cloning and sequencing

Cry j 1-encoding cDNA was cloned using the pCR 2.1 vector system and a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA USA) according to the manufacturer's instructions. DNA sequencing was performed using the dideoxynucleotide chain termination method and a 373 DNA sequencing instrument (Applied Biosystems, Foster City, CA USA). Nucleotide alignments and amino acid translations were undertaken using the DNASIS v 3.7 program (Hitachi Software Engineering Co., Ltd., Japan).

Marker development

A cleaved amplified polymorphic sequence (CAPS) marker was developed based on differences between the detected Cry j 1-encoding cDNA sequences. To detect possible DNA polymorphism, cDNA sequence data were thoroughly inspected for the presence of recognition sites of different endonucleases using Infobiogen software (http://www.infobiogen.fr/services/analyseq). PCR primers that targeted the Cry j 1 gene were derived from clone pCCI-2-2, which represented a cDNA sequence reported by SONE *et al.* (1994). The OLIGO 5.1 program (National Biosciences Inc., Plymouth, MN USA) was applied for primer design. PCR conditions were the same as those described for the preparation of Cry j 1 cDNA. The PCR products, obtained from eight plus trees and subsequently digested with a selected restriction endonuclease, were separated by electrophoresis on 1.5% agarose gels.

Inheritance analysis and mapping of the Cry j 1 gene

An inheritance analysis was performed by comparing the polymorphic DNA pattern amongst both the parents and their offspring using the controlled cross mentioned above. By analyzing the previously reported mapping population (KURAMOTO *et al.,* 2000), the newly developed DNA marker was introduced to the existing linkage map.

Results and Discussion

Cry j 1 cDNA sequences

Two different sequences were derived from 12 Cry j 1-encoding cDNA clones. The Cry j 1 isoforms encoded from the two derived cDNA sequences were designated as Cry j 1a and Cry j 1b. The Cry j 1a-encoding cDNA sequence was found to be identical to a previously reported sequence (NAMBA *et al.,* 1994; WANG *et al.,* 1998), which was recorded under Genbank accession number D34639 by NAMBA *et al.* (1994). The Cry j 1b was found to be a new sequence. A total of 15 nucleotide transitions were detected between Cry j 1a and Cry j 1b, including one amino acid replacement mutation occurring at position 14 of the signal peptide (Phe/Ser) and three replacement mutations occurring at positions 45, 121 and 352 of the mature protein (Ala/Val, Tyr/His and Arg/His, respectively). The Cry j 1b-encoding cDNA sequence showed 98.3 and 99.2% identities to cDNA sequences of two clones reported by SONE *et al.* (1994), namely pCCI-2-2 and pCCI-15, respectively. Comparing the amino acid sequence deduced from clone pCCI-2-2, Cry j 1b had two amino acid replacement mutations in the signal peptide, positions 12 (Leu/Phe) and 14 (Phe/Ser), and three in the mature protein, positions 45 (Ala/Val), 181 (Ser/Thr) and 352 (Arg/His). Between Cry j 1b and the deduced amino acid sequence encoded from clone pCCI-15, one amino acid replacement mutation was detected at position 14 (Phe/Ser) of the signal peptide and six in the mature protein at positions 45 (Ala/Val), 121 (Tyr/His), 200 (Ser/Leu), 337 (His/Gln), 340 (Gln/Lys) and 352 (Arg/His).

Development of the CAPS marker

Following inspection of the sequence data, a restriction endonuclease, *Psp*1406I (*Acl*I), was selected to distinguish between Cry j 1a and Cry j 1b. This enzyme recognizes a polymorphic site causing an amino acid difference at position 352, which is Arg in Cry j 1a and His in Cry j 1b. PCR primers which amplified fragments including the recognition site by *Psp* 1406I were designed (forward, 5'-ACGATATGGACTTGTAC-3'; 5'-ACAACAATATACATCATGAT-3'). After PCR amplification, the expected single band of 440 bp was obtained from DNA samples of all eight trees tested. Following digestion, the PCR

Table 1. – CRYJ1-352 genotypes of eight *C. japonica* plus trees.

clone	genotype
Chichibusyo 3	AB
Higashishirakawa 12	AA
Ishikawasyo 3	AA
Kinada 7	AA
Kitashitara 8	AA
Nishitama 5	BB
Nishitama 8	AA
Syouchiku 3	AB

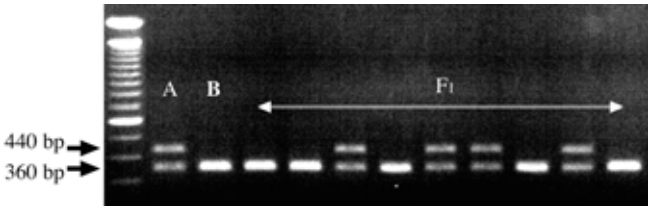

Figure 1. – Co-dominant inheritance of marker CRYJ1-352, indicated by comparing the patterns of the father tree (A; Iwao-sugi), mother tree (B; Boka-sugi) and nine offspring. The fragments of 80 bp are not indicated.

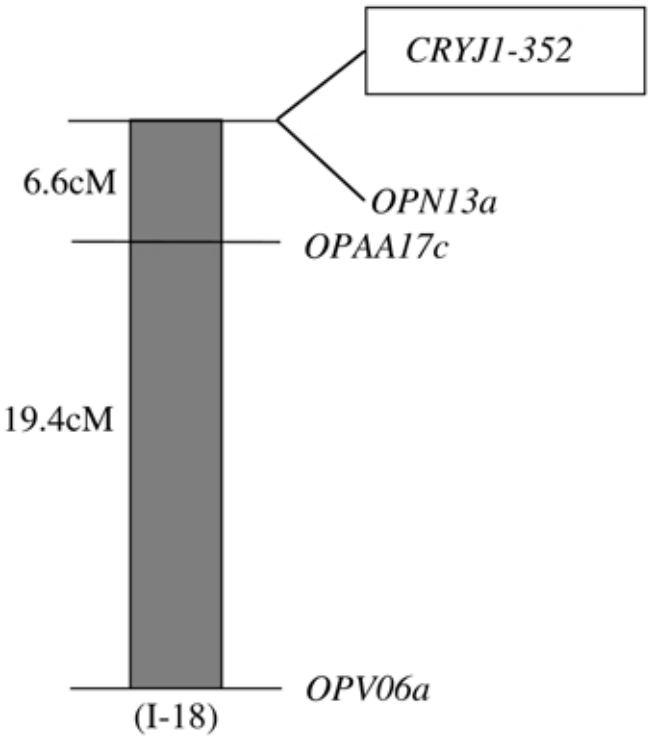

Figure 2. – Location of the marker CRYJ1-352 on the genomic map of Iwao-sugi. CRYJ1-352 co-segregates with a RAPD marker, OPN13a. Neighboring markers and their distances (cM) are indicated (LOD score of 4.0).

product of Chichibusyo 3 yielded two additional fragments of 360 and 80 bp, together with the uncut fragment of 440 bp, as anticipated from the polymorphism between cDNA sequences encoding Cry j 1a and Cry j 1b. These two alleles, corresponding to Cry j 1a and Cry j 1b, were designated A and B, respectively. Thus, the genotype of Chichibusyo 3 was denoted AB. Furthermore, the PCR products from the other seven trees were also digested and run on a 1.5% agarose gel. As a result, all tested trees showed band patterns of AA, AB or BB. The genotypes of each tree are listed in *Table 1*. Among them, five had AA genotypes and there was one each of AB and BB. This result indicates that the developed marker was specific to the *Cry j 1* gene. Thus, this marker was designated CRYJ1-352 as it had a polymorphic site at position 352 in the amino acid sequence of mature Cry j 1.

Inheritance analysis and mapping of the Cry j 1 gene

PCR amplification and restriction analyses were carried out on Iwao-sugi and Boka-sugi, the parents of a controlled cross. The results suggest that the genotype of Boka-sugi is AA and Iwao-sugi is the heterozygous AB (*Fig. 1*). An inheritance analysis of the Cry j 1 locus was performed by testing 65 offspring individuals of Boka-sugi × Iwao-sugi and the band patterns of 33 of the 65 trees were AA, and 32 were AB. Thus, CRYJ1-352 segregated in a co-dominant manner with an AA:AB ratio of 1:1 in the progeny family ($\chi^2 = 0.0154$, $p > 0.05$). These results strongly indicate that CRYJ1-352 provides a marker for different alleles of a single locus inherited in a Mendelian fashion. Furthermore, CRYJ1-352 co-segregated with the RAPD marker, OPN13a, belonging to the I-18 linkage group, which was previously constructed by KURAMOTO *et al.* (2000) (*Fig. 2*). Because there is a large variation in Cry j 1 content in pollen among trees, it is assumed that several genes probably regulate the expression of Cry j 1. A quantitative trait loci (QTL) analysis of Cry j 1 content in pollen using the mapping population would clarify the location of the other genes that regulate the expression of Cry j 1, as well as the relationships between them and the *Cry j* 1 gene.

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