

Characterization of Genes for a Pollen Allergen, Cry j 2, of *Cryptomeria japonica*

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Key Words

Pollen allergen • *Cryptomeria japonica* • Cry j 2 gene • Polymorphism • Gene expression • Clonal variation • Cross-allergy • Coniferous species

Abstract

Background: Cry j 2 is one of the major pollen allergens of *Cryptomeria japonica*. The polymorphism of Cry j 2 isoforms and the conservation of the structure of Cry j 2 in coniferous species remain to be analyzed. **Methods:** A cDNA library derived from the pollen of *C. japonica* was screened using a fragment of Cry j 2 cDNA. Restriction fragment length polymorphism analysis was performed to examine the diversity of Cry j 2 genes. The promoters of Cry j 2 genes were isolated with a commercially available cloning kit. Clonal variations in the expression of Cry j 2 in pollen were examined by RNA gel blot analysis, and the conservation of the structure of the Cry j 2 gene in coniferous species was evaluated by DNA gel blot analysis. **Results:** We isolated three cDNA clones encoding novel isoforms of Cry j 2. We also sequenced a total of 16 promoter regions from 10 specimens. The sequences of promoter regions of Cry j 2 genes were highly divergent. The amount of Cry j 2 mRNA also varied considerably. The Cry j 2 gene was found to be conserved among species belonging to Taxodiaceae and Cupressaceae but to vary between Taxodiaceae and Pinaceae. **Conclusions:** The coding and promoter regions of Cry j 2 genes contain large numbers of polymorphisms. Our analysis revealed large variations in the expression of Cry j 2 at

the transcriptional level, and we suggest that conserved homologs of Cry j 2 confer cross-allergenicity among Taxodiaceae and Cupressaceae.

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Introduction

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 and afflicts more than 10% of the population. To date, four allergens, namely Cry j 1, Cry j 2, CJP-4 and CJP-6, have been identified in the pollen of *C. japonica* [1–4]. Of these, Cry j 1 and Cry j 2 are the major allergens, and more than 90% of patients with sugi pollinosis have immunoglobulin E (IgE) antibodies specific to both of them [5].

Cry j 1 is a basic protein with pectate lyase activity and a molecular mass of 45–50 kDa [6, 7]. The Cry j 1 protein appears to be produced in the tapetum and the Golgi bodies of mature pollen and is localized in the orbicules and pollen wall [8]. Two-dimensional immunolabeling with IgE from sera of patients with pollinosis revealed the presence of at least 12 isoforms of Cry j 1 [9]. Four cDNAs that include entire open reading frames of Cry j 1 isoforms have been reported, and the isoforms have different reactivities to two monoclonal antibodies [10]. Clonal variations in the concentration of Cry j 1 protein have also been reported by several groups [11, 12].

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Cry j 2 is a basic protein with polymethylgalacturonase activity, a molecular mass of 37 kDa, and a pI of 8.6–9.2 [2, 13]. Cry j 2 is localized in the starch grains of pollen [14], and clonal variations in the concentrations of Cry j 2 have been reported [15, 16]. Moreover, three isoforms of Cry j 2 have been detected in two-dimensional IgE-binding analysis [9]. However, the complete sequences of Cry j 2 isoforms have not been determined. In the present study, we examined the diversity of the coding and promoter regions of Cry j 2 genes in an effort to characterize Cry j 2 isoforms. We also examined the patterns of expression of Cry j 2 in various plus trees to monitor variations at the transcriptional level. In addition, we performed DNA gel blot analysis to determine the distribution of Cry j 2 homologs in various coniferous species.

Materials and Methods

Plant Materials and Isolation of RNA and DNA

Mature pollen of *C. japonica* was collected from 17 specimens of plus trees, as well as from local cultivars that had been planted in the botanical garden of the Forestry and Forest Products Research Institute in March 2000. The current needles of 25 specimens of plus tree and of local cultivars of *C. japonica* were collected in April 2000. Current-year needles of *Sequoia sempervirens*, *Cunninghamia lanceolata*, *Chamaecyparis obtusa*, *Chamaecyparis pisifera*, *Thuja dolabrata*, *Thuja standishii*, *Sciadopitys verticillata*, *Abies firma*, *Picea jezoensis* var. *hondoensis*, *Pinus thunbergii*, *Pinus densiflora*, *Tsuga diversifolia*, *Pseudotsuga japonica*, *Taxus cuspidata*, *Torreya nucifera* and *Cephalotaxus harringtonia* were collected in the botanical garden of the Forestry and Forest Products Research Institute in October 1999. Total RNA was isolated from mature pollen as described by Sone et al. [17]. Genomic DNA was extracted from current-year needles of *C. japonica* and all 16 listed species as described by Wagner et al. [18].

Isolation of Full-Size Cry j 2 cDNA Clones

For the preparation of a Cry j 2 cDNA probe, we amplified a fragment of Cry j 2 cDNA by the reverse transcriptase polymerase chain reaction (PCR), with the oligonucleotide primers 5'-GCTC-CAATGGCCTTTGTGGC-3' (forward) and 5'-GCCATGACG-GCTGCACATCC-3' (reverse). These sequences correspond to positions 144–163 and 1633–1652 in the nucleotide sequence of a previously reported Cry j 2 cDNA clone, respectively [19]. One microgram of RNA from *C. japonica* pollen was used for amplification of Cry j 2 with a GeneAMP® RNA PCR kit (Applied Biosystems, Foster City, Calif., USA) according to the protocol provided by the manufacturer. Conditions for amplification were as follows: incubation at 95°C for 5 min, and then 30 cycles of incubation at 95°C for 1 min, 55°C for 1 min and 72°C for 2 min. Amplified fragments were cloned into the pCR® 2.1 vector (Invitrogen, Carlsbad, Calif., USA).

A cDNA library in λZAP® II (Stratagene, La Jolla, Calif., USA) was constructed from 5 µg of polyadenylated mRNA that had been isolated from the pollen of *C. japonica* [20]. To obtain cDNA clones

that contained the entire open reading frames of Cry j 2 genes, we screened a cDNA library in λZAP II using the cloned product of PCR as a probe. The DNA probes were labeled with [α -³²P]dCTP with the Megaprime™ DNA labeling system (GE Healthcare Bio-Science, Piscataway, N.J., USA). Hybridization was performed as described previously [21]. The cDNAs from recombinant phages were excised in vivo in plasmid form (pBluescript® SK-; Stratagene) by coinfection with ExAssist® helper phage (Stratagene), according to the manufacturer's instructions. The sequences of both strands of each cDNA insert were determined.

Restriction Fragment Length Polymorphism Analysis

Samples of genomic DNA (5 µg) from 25 specimens of *C. japonica* were digested with EcoRV, fractionated electrophoretically on a 0.7% agarose gel and transferred to Hybond™-XL membranes (GE Healthcare Bio-Science) by alkaline transfer. Membranes were prehybridized at 42°C for several hours in 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 50% formamide, 1% SDS, 10% dextran sulfate, and 100 µg/ml salmon sperm DNA. The amplified cDNA fragment of Cry j 2 was used as the probe. We used the sense primer CRYJ2-F (5'-GCGGCAGCAGAAGATCAATC-3') and the antisense primer CRYJ2-421R (5'-TGGTACGCAGC-TATTATCCC-3') for PCR, and we labeled the product of PCR with [α -³²P]dCTP using the Megaprime DNA labeling system (GE Healthcare Bio-Science). The labeled probe was denatured and added directly to the prehybridized membranes in the above mentioned solution. After hybridization at 42°C overnight, the membranes were rinsed in 2× standard saline citrate (SSC) plus 0.1% SDS and washed twice for 15 min with 0.5× SSC plus 0.1% SDS at 42°C and twice for 15 min with 0.1× SSC plus 0.1% SDS at 60°C. Then the washed membranes were exposed to Kodak BioMax™ MS film (Eastman-Kodak, Rochester, N.Y., USA).

Identification of the Promoter Regions of Cry j 2 Genes

We used the LA PCR™ in vitro Cloning Kit (Takara Bio, Shiga, Japan) for identification of promoter regions of Cry j 2 genes. Samples of total DNA from the Takasaki 4 and Kuji 13 cultivars were digested to completion with BglII, and resultant fragments were ligated to the Sau3AI cassette. Two rounds of PCR were performed with 30 cycles of incubation at 94°C for 30 s, 57°C for 30 s and 72°C for 4 min. The primers for the first PCR were cassette primer C1, provided by the manufacturer, and the Cry j 2-specific S1 primer 5'-CCTATAGCGACGCAGTACTC-3'. The primers for the second PCR were cassette primer C2 and the Cry j 2-specific S2 primer 5'-CAATCATGCTTTCCATCGCCTACTG-3'. The products of PCR were cloned into the pCR-TOPO® vector using the TOPO TA Cloning Kit (Invitrogen). The sequences of both strands of the products of PCR were determined.

We amplified promoter regions of Cry j 2 genes using samples of genomic DNA that had been extracted from the current-year needles of 8 specimens of plus trees as templates. The sense primer (5'-CCTCTTTTATAGGGGTATGGG-3') and the antisense primer (5'-AAACATGTTTGTGAGTGGGAAAT-3') were based on the common sequence of the Cry j 2 promoter between the Takasaki 4 and Kuji 13 cultivars. Amplification was performed with an initial denaturation at 95°C for 5 min, which was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 60 s, with a final extension at 72°C for 5 min. Products of PCR were cloned and sequences were determined as indicated above.

RNA Gel Blot Analysis

Total RNA (5 µg) was fractionated by electrophoresis on a formaldehyde-agarose gel and blotted onto a Hybond-XL membrane by capillary transfer. Then RNA gel blot hybridization was performed as described previously [22]. The sense primer CRYJ2-F and the antisense primer CRYJ2-R (5'-CCTGCGGCATAAT-ACGATGA-3') were used for PCR to prepare the *Cry j 2* probe. The resultant amplified cDNA fragment of *Cry j 2* was used as the probe. Membranes were exposed to Kodak X-ray film, and the signals on autoradiograms were quantified with a Molecular Imager (Bio-Rad, Hercules, Calif., USA).

DNA Gel Blot Analysis

We digested 3.50 µg of genomic DNA from *C. japonica*, *C. lanceolata*, *C. obtusa*, *C. pisifera*, *T. dolabrata*, *T. standishii* and *S. verticillata*, 5.25 µg of genomic DNA from *T. cuspidata*, *T. nucifera* and *C. harringtonia*, 7.0 µg of genomic DNA from *A. firma*, *P. jezoensis* var. *hondoensis*, *P. thunbergii*, *P. densiflora*, *T. diversifolia* and *P. japonica*, and 10.5 µg of genomic DNA from *S. sempervirens* with *EcoRI*. The procedures for electrophoresis and blotting were the same as those described above for restriction fragment length polymorphism (RFLP) analysis. The blotted membrane was prehybridized at 37°C for several hours in 50 mM Tris-HCl (pH 7.5), 1 M NaCl, 25% formamide, 1% SDS, 10% dextran sulfate, and 100 µg/ml salmon sperm DNA. The same product of PCR as that used for RNA gel blot analysis was used as a probe. After hybridization at 37°C overnight, the membrane was rinsed in 2× SSC plus 0.1% SDS and washed twice for 15 min with 0.5× SSC plus 0.1% SDS at 54°C. The washed membrane was exposed to Kodak X-ray film.

Construction of Reporter Genes and Transformation of *Arabidopsis*

To obtain fragments that contained promoter sequences and the 5'-untranslated regions of *Cry j 2* genes, we performed PCR with a new set of primers, using the cloned *Cry j 2* promoters as templates. The nucleotide sequence of the sense primer was 5'-TAGCCTTATAATTCTCTTGGC-3' and that of the antisense primer was 5'-TTTAATAAAAGAAAATCTTTCTATA. We obtained DNA fragments of 1,248 and 1,396 bp that corresponded to the promoters and the 5'-untranslated regions derived from the *Cry j 2* genes of Kuji 13 and Takasaki 4, respectively. We cloned the products of PCR into the binary vector pSMAH621, which contains genes for β-glucuronidase (GUS) and hygromycin phosphotransferase in its T-DNA region [23]. We also cloned the LAT52 promoter at the same site in pSMAH621 as a positive control for pollen-specific expression [24]. We introduced the resultant constructs into *Agrobacterium tumefaciens* strain GV3101 (pMP90), and we used the transformed bacteria to generate transgenic *Arabidopsis* plants by the floral dip procedure [25]. Transformed plants were selected on the basis of hygromycin resistance. We produced four independent lines for each construct and used homozygous T₃ plants for histochemical analysis of the expression of GUS as described by Jefferson et al. [26]. Tissues were incubated overnight at 37°C in a solution of 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronidase and 50 mM sodium phosphate buffer (pH 7.0). Samples were destained in 70% (v/v) ethanol overnight at room temperature prior to examination.

Results

Sequence Diversity among the Coding Regions of *Cry j 2* Genes

We isolated three cDNA clones (*Cry j 2.1*, *Cry j 2.2* and *Cry j 2.3*; accession numbers AB081403, AB081404 and AB081405) from a cDNA library generated from the pollen of *C. japonica*. The cDNA inserts ranged in length from 1,710 to 1,731 bp, with the longest open reading frame encoding 514 amino acids (fig. 1a). All of the cDNA clones included nucleotide sequences that corresponded to the coding region, as well as the 5'- and 3'-noncoding sequences. The amino acid sequences predicted from the three cDNA clones were not identical to those of *Cry j 2* reported to date (fig. 1a). Comparison of *Cry j 2.1* through *Cry j 2.3* and two *Cry j 2* isoforms reported to date revealed 15 changes in amino acid residues. We found that the restriction endonuclease *EcoRV* should recognize the nucleotide sequence of the *Cry j 2* gene that encoded the amino acids DI at positions 34 and 35, but not that of the gene that encoded VV at the same positions (fig. 1b).

In order to examine the diversity of *Cry j 2* genes among specimens of plus trees, we performed DNA gel blot analysis using, as probe, the *Cry j 2* cDNA fragment of 326 bp that included the recognition site for *EcoRV*. As anticipated, this probe was able to distinguish between genes that encoded VV and those that encoded DI at positions 34 and 35 (fig. 2). Two of 25 plus trees were homozygous for DI, 7 were heterozygous for DI and VV, and the rest were homozygous for VV (fig. 2). Thus, this difference in amino acid sequence was widely detected among our specimens of plus trees.

Sequence Diversity among Promoter Regions of *Cry j 2* Genes

We identified 1,268- and 1,414-bp nucleotide sequences of *Cry j 2* promoters that included the 5'-untranslated region in the genomic DNA of the Kuji 13 and Takasaki 4 cultivars, respectively (accession numbers AB259788 and AB259789) (fig. 3). The most striking difference between the two promoter regions was the presence or absence of a nucleotide sequence of 138 bp. We designated promoters with this insertion as long (L) and those without it as short (S). We amplified the promoter regions of *Cry j 2* genes from 8 specimens to examine the distribution of L and S promoters. We generated 14 kinds of clones and sequenced them (data not shown; accession numbers AB259790 through AB259803). Only one or two *Cry j 2* promoter sequences were identified in each specimen. We detected both the L and the S promoter

Fig. 1. Alignment of the amino acid sequences deduced from the nucleotide sequences of four cDNA clones. **a** Alignment of the entire deduced amino acid sequences of Cry j 2.1 through Cry j 2.3 with those of Cry j 2 reported to date. Accession number D29772 refers to the sequence reported by Komiyama et al. [33] and D37765 to the sequence reported by Namba et al. [19]. The sequence of Cry j 2.1 is shown in the standard one-letter code. Sequences for the other Cry j 2 family members are given relative to Cry j 2.1 with only differences. Dashes indicate identical amino acid residues. The amino acid residue numbering is shown on the right. **b** Alignment of the deduced amino-terminal sequences of Cry j 2.1 through Cry j 2.3 and the corresponding partial nucleotide sequences. The *EcoRV* site in Cry j 2.1 is indicated by a line above the sequence.

Cry j 2.1	MAMKFIAPMAFVAMQLIIMAAEDQSAQIMLSDIEQYLRNRSRLRKVEHSRHDAINIFN:	60
Cry j 2.2	---L-----L-----V-----VV-K-----	60
Cry j 2.3	---L-----L-----VV-K-----	60
D29772	---L-----L-----VV-K-----	60
D37765	-----	60
Cry j 2.1	VEKYGAVGDGKHDCTEAFSTAWQAACKKPSAMLLVPGNKKFVNNLFFNGPCQPHFTFKV:	120
Cry j 2.2	-----N-----S-----	120
Cry j 2.3	-----N-----S-----	120
D29772	-----N-----S-----	120
D37765	-----	120
Cry j 2.1	DGIIAAYQNPASWKNRIWLQFAKLTGFTLMGKGVIDGQKQWWAGQCKWVNGREICNDR:	180
Cry j 2.2	-----	180
Cry j 2.3	-----	180
D29772	-----	180
D37765	-----	180
Cry j 2.1	DRPTAIKFDFSTGLIIQGLKLMNSPEFHLVFGNCEGVKIIGISITAPRDSPTNDGIDIFA:	240
Cry j 2.2	-----R-----	240
Cry j 2.3	-----R-----	240
D29772	-----R-----	240
D37765	-----	240
Cry j 2.1	SKNFHLQKNTIGTGDDCVAIGTGSSNIVIEDLICGPGHGHSIGSLGRENSRAEVSVYVHN:	300
Cry j 2.2	-----	300
Cry j 2.3	-----	300
D29772	-----	300
D37765	-----	300
Cry j 2.1	GAKFIDTQNGLRITKWTQGGSGMASHIIYENVEMINSENPILINQFYCTSASACQNQRS	360
Cry j 2.2	-----	360
Cry j 2.3	-----	360
D29772	-----	360
D37765	-----	360
Cry j 2.1	QIQDVTYKNIRGTSATAAAIQLKCSDSMPCKNINLSDISKLTSGKIASCLNDNANGYFS:	420
Cry j 2.2	-----D-K-----	420
Cry j 2.3	-----D-K-----	420
D29772	-----D-K-----	420
D37765	-----D-K-----	420
Cry j 2.1	GHVIPACKNLSPSAKRKESKSHKPKTMVENMGAYDKGNRTRILLGSRPPNCTNKCHGC:	480
Cry j 2.2	-----R-----	480
Cry j 2.3	-----R-----	480
D29772	-----R-----	480
D37765	-----K-----	480
Cry j 2.1	SPCKAKLVIVHRIMPQEYYPQRWMCSCCHKIYHP	:514
Cry j 2.2	-----I-----	:514
Cry j 2.3	-----I-----	:514
D29772	-----I-----	:514
D37765	-----R-----	:514
a		
Cry j 2.1	MAMKFIAPMAFVAMQLIIMAAEDQSAQIMLSDIEQYLRNRSRLRKVEHSRHDAINIFN:	60
Cry j 2.2	---L-----L-----V-----VV-K-----	60
Cry j 2.3	---L-----L-----VV-K-----	60
Cry j 2.1	GCCCAAATTATGTTGGACAGTGATATCGAACAATATCTTAGATCGAATCGGAGTTAAGA	
Cry j 2.2	-----T-G-----A-----	
Cry j 2.3	-----T-G-----A-----	
b		

sequences in 6 of 8 specimens. One specimen had only an L-type promoter sequence, and another had only an S-type promoter. It appears that the heterozygosity of the promoter region of the *Cry j 2* gene is very high. We also found a large number of nucleotide changes, deletions

and insertions in both L and S promoter sequences (data not shown).

The *Cry j 2* promoter sequences contained the *cis*-elements responsible for expression in pollen, namely LAT52 [27], the core motifs of LAT52/56 and LAT56/59 boxes

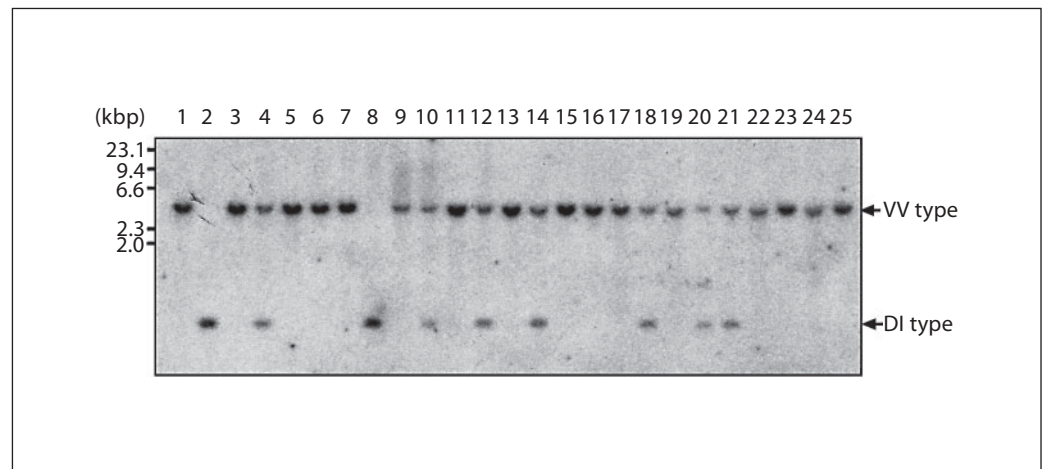


Fig. 2. RFLP analysis for detection of amino acid diversity at the 34th and 35th amino acid residues of *Cry j 2* from 25 specimens of plus trees. Five micrograms of total DNA were prepared from each of 25 specimens of plus trees, digested with *EcoRV*, and subjected to electrophoresis on an agarose gel. After the transfer of bands of DNA to a nylon membrane, the DNA on the membrane

was allowed to hybridize with a ^{32}P -labeled 326-bp cDNA fragment that included the recognition site for *EcoRV*. The *Cry j 2* genes that encoded DI at positions 34 and 35 included the recognition site for *EcoRV*, while the *Cry j 2* genes that encoded VV at the same positions were not recognized by *EcoRV*. The signals derived from the DI and VV types are indicated by arrows.

[27, 28], NTP303 [29], and the enhancing Q (quantitative) element [30] (fig. 3). We fused the *Cry j 2* promoter sequences from Kuji 13 and Takasaki 4, separately, to the gene for GUS and used the resultant constructs for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* plants to examine the activity of the *Cry j 2* promoters. We were unable to detect any GUS activity in flowers, leaves, stems, roots and pollen of transgenic plants. By contrast, the *LAT52* promoter, used as a positive control, was associated with strong GUS activity in pollen grains (data not shown).

Variations among Patterns of Expression of Cry j 2 Gene at the Transcriptional Level

We previously reported that the *Cry j 2* mRNA is expressed only in mature male flowers and pollen grains [20]. We examined levels of *Cry j 2* transcripts in pollen grains from 17 specimens of plus trees. RNA gel blot analysis revealed distinct differences in the levels of *Cry j 2* transcripts, with highest and lowest levels of expression of *Cry j 2* gene differing by as much as 4-fold (fig. 4).

Distribution of Cry j 2 Homologs in Various Coniferous Species

The *Cry j 2* homologs in *C. obtusa* and *Juniperus ashei*, namely Cha o 2 and Jun a 2, are allergenic [31, 32]. We performed DNA gel blot analysis, using total DNA pre-

pared from 17 species of coniferous plants, to examine the conservation of the structure of *Cry j 2* (fig. 5). When we used entire cDNA of *Cry j 2* as probe, we obtained clear signals from species in Taxodiaceae, Cupressaceae, Sciadopityaceae, Taxaceae and Cephalotaxaceae, whereas the signals from species in Pinaceae were smeared. These observations indicate that *Cry j 2* homologs are not conserved between Taxodiaceae and Pinaceae.

Discussion

This study revealed polymorphisms in the coding and promoter regions of *Cry j 2* genes. Two isoforms of *Cry j 2* were reported previously [19, 33], and in this study, we identified three new isoforms of *Cry j 2* (fig. 1). Our RFLP analysis indicated that the *Cry j 2* genes were located at a single locus (fig. 2). The intensity of signals also corresponded to one copy of the gene per haploid genome if we assume that the size of the haploid genome of *C. japonica* is 11 pg according to a previous report [34] (data not shown). Therefore, the diversity of amino acid sequences can be attributed to an allele at a single locus, as suggested by a previous study of the linkage map [35]. The microheterogeneity in the *Cry j 2* gene seems to be relatively high, as judged from the results of RFLP analysis (fig. 2) and the variations among promoter regions (fig. 3).

Kuji 13 -1266 TCTCAATCCCAGGGGGGTAGCCTTATAATCTCTGGCTCTAATACTTGTGGCTAGTCCCAACCTACTA -1197
Takasaki 4 -1414 TCTCAATCCCAGGGGGGTAGCCTTATAATCTCTGGCTCTAATACTTGTGGCTAGTCCCAACCTACTA -1345

F2 52/56 box(+)

Kuji 13 -1196 GGTACTATTTTAGGTAGCCTTCTACCTCTTTTATAGGGGTATGGGGAGGTGAGTTGACCTAATTTTGACAA -1127
Takasaki 4 -1344 GGTACTATTTTAGGTAGCCTTCTACCTCTTTTATAGGGGTATGGGGAGGTGAGTTGACCTAATTTTGACAA -1275

F1 Q(-)

Kuji 13 -1126 GTTTCCTCATCAACAACCTTGTATTCTTTATTTTGCACCCAACAACCTAGTTCAAAGAACTCATAAGG -1057
Takasaki 4 -1274 GTTTCCTCATCAACAACCTTGTATTCTTTATTTTGCACCCAACAACCTAGTTCAAAGAACTCATAAGG -1205

LAT52(-) LAT52(+)

Kuji 13 -1056 AAGGTGTGTTTACGAGTTGTAAAGCCCATGGATGACACTAATGTTGACATTATATCAATTGCTTAATTATA -987
Takasaki 4 -1204 AAGGTGTGTTTACGAGTTGTAAAGCCCATGGATGACACTAATGTTGACATTATATCAATTGCTTAATTATA -1135

Kuji 13 -986 TATTTTTATGTTTATAAATTGAACCTAATCCACAGAAATTCATGAACCTACCCCTTCTACCAGCCTAATT -917
Takasaki 4 -1134 TATTTTTATGTTTATAAATTGAACCTGATCCACAGAAATTCATGAACCTACCCCTTCTACCAGCCTAATT -1065

52/56 box(-)

Kuji 13 -916 TTTTTCAGTAAATATATATTATATTTTTCATTTTAAATACAAAAGAAAGTAAAGATATTTCTTAAAAAT -848
Takasaki 4 -1064 TTTTTCAGTAAATATATATTATATTTTTCATTTTAAATACAAAAGAAAGTAAAGATATTTCTTAAAAAT -995

NPT303(-)

Kuji 13 -847 ATGAACGACTACTTAAATCTTTTAAAAAAATTTAAATTTAAATTTAAATCCACTTGTGGGAATGAGCT -778
Takasaki 4 -994 ATGAACGACTACTTAAATCTTTTAAAAATCTTTTAAATTTAAATTTAAATCCACTTGTGGGAATGAGCT -925

52/56 box(-) 52/56 box(+)

Kuji 13 -777 AAGTAA.....AAATTTATATTATGTTTCTCTAAACATTCCATACAAAACTAAGGCTC -720
Takasaki 4 -924 AAGGGAATGAGCTAAGTAA.....AAATTTATATTATGTTTCTCTAAACATTCCATACAAAACTAAGGCTC -855

LAT52(-)

Kuji 13 -719 AACCCACACGAAGACAAAAAGTCAAAGTTTGCATTGGAACCTGTAAATCCTAATTGACGAAATTATACCA -650
Takasaki 4 -854 AACCCACATGAAGACAAAAAGTCAAAGTTTGCATTGGAACCTGTAAATCCTAATTGACGAAATTATACCA -785

52/56 box(-)

Kuji 13 -649 AAGAGGTAACCGTCGTAAACTAGAACTAAACCAGTTTAAATCGTATAGATATTCTTGGAGCCTTTCAA -580
Takasaki 4 -784 AAGAGGTAACCGT.....ACTAAACCAGTTTAAATCGTATAGATATTCTTGGAGCCTTTCAA -726

Kuji 13 -579 GCAAAAACTAAAAGAAGTATAATCCTCCTCGATACGTGTTATTATCATTCGACCCCTGGGATTCTGCTA -510
Takasaki 4 -725 GCAAAAACTAAAAGAAGTATAATCCTCCTCGATACGTGTTATTATCATTCGACCCCTAGGATTCTGCTA -656

Kuji 13 -509 C..... -509
Takasaki 4 -655 CAGGCTGGAATTTGACGGTCACCCCTACCTGTCTATGAATTTTGTGACGGTTTAGTCATTTCAT -586

Kuji 13 -509 T -508
Takasaki 4 -585 GCCTCTCATTGACATTTTAAAGAAAATATTAACGTCAAAAAACATTAAAGTAGAAGATTGAAGCTACT -516

Kuji 13 -507 TCACAAGTTCTAAACGGTTCACAACAGTTTAAATCGATGAAATATCGTGTCATCGAAAACTAATGCTTT -438
Takasaki 4 -515 TCACAAGTTCTAAACGGTTCACAACAGTTTAAATCGATGAAATATCGTGTCATCGAAAACTAATGCTTT -447

56/59 box(-)

Kuji 13 -437 AAAAGTTCTATGACGGCTGGTCGCCTGGACTGTAGATGACTGCAGAACTTCTCCAAAAAATCTCTAAAA -368
Takasaki 4 -446 AAAAGTTCTATGACGGCTGGTCGCCTGGACTGTAGATGACTGCAGAACTTCTCCAAAAAATCTCTAAAA -377

Kuji 13 -367 TCAAGTGTCTTGATATATCCATCACCTCATACTGAAAAACAATCACGGCATATCAATTATCTTATTC -298
Takasaki 4 -376 TCAAGTGTCTTGATATATCCATCACCTCATACTGAAAAACAATCACGGCATATCAATTATCTTATTC -308

56/59 box(-) 56/59 box(-)

Kuji 13 -297 ATTACTCTATGATATCATGTATCTTATAACCACTAAACGACATTGTAACGTGTAATTCAGAGTTGGAT -228
Takasaki 4 -307 ATTATCTTTGATATCATGTATCTTATAACCACTAAACGACATTGTAACGTGTAATTCAGAGTTCAAT -238

Kuji 13 -227 TTGCAAGAGGAAGTGTACAGAAAT.....CGTCTGATATGGTGCTAAGAAAAAGCATTCTGTTGA -168
Takasaki 4 -237 TTGCAAGAGGAAGTGTACAGAAATATTACTGAATCGTCTGATATGGTGCTAAGAAAAAGCATTCTGTTGA -168

LAT52(+) 52/56 box(+)

Kuji 13 -167 ATTCAAGATCCTGCACGTACCATTTTTTCTGGATTGCCTCTCTATCGAAATGGAATACTCATAGGCGA -98
Takasaki 4 -167 ATTCAAGATCCTGCACGTACCATTTTTTCTGGATTGCCTCTCTATCGAAATGGAATACTCATAGGCGA -98

Kuji 13 -97 CATGTATAAAATGAACAGTGTATTTCCACTCACAACATGTTTCTTCTCAATAGTTGAGTTGAGAGCAA -28
Takasaki 4 -97 CATGTATAAAATGAACAGTGTATTTCCACTCACAACATGTTTCTTCTCTATAGTTGAGTTGAGAGCAA -28

56/59 box(+) 52/56 box(-) 56/59 box(-) LAT52(-)

Kuji 13 -27 GTATAGAAAGAAATTTTCTTTTATTAAT -1
Takasaki 4 -27 CTATAGAAAGAAATTTTCTTTTATTAAT -1

LAT52(+) LAT52(-)
R2

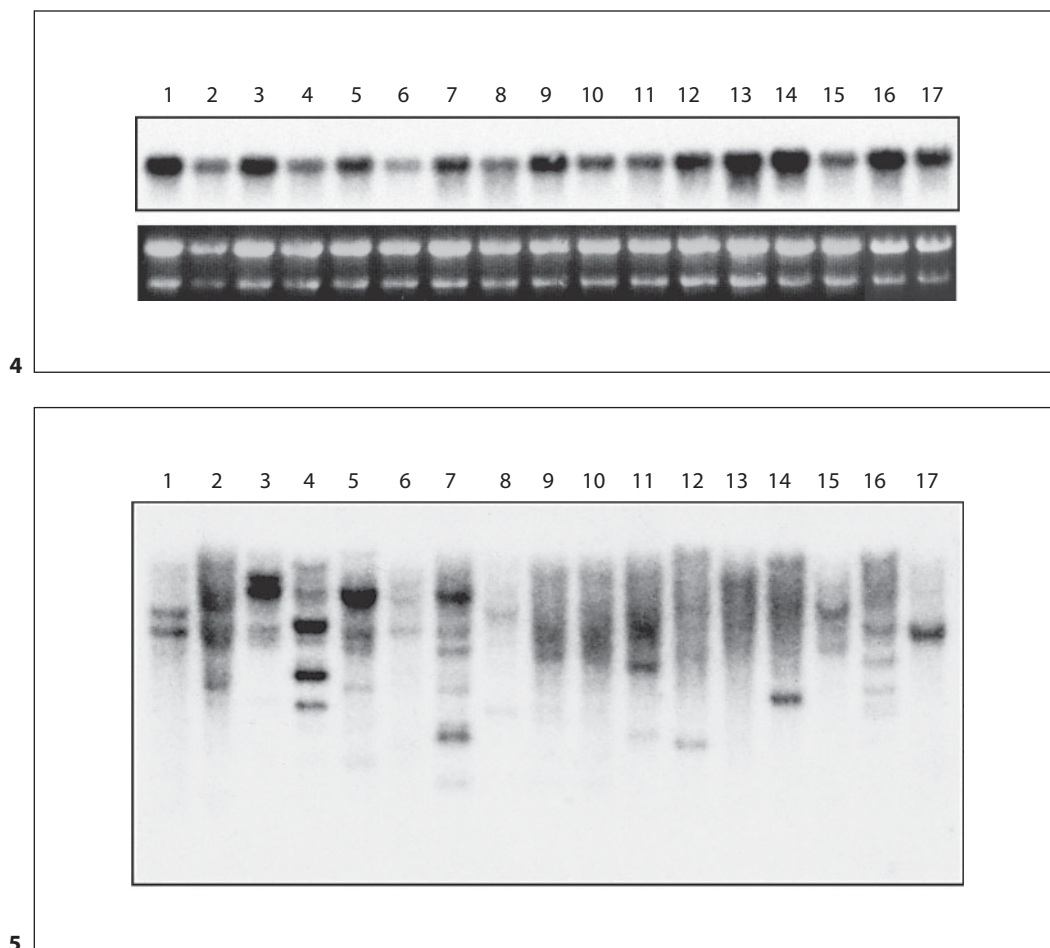


Fig. 3. Sequences of the *Cry j 2* promoters from Kuji 13 and Takasaki 4. The 5'-nucleotide immediately adjacent to the putative site of initiation of translation is designated -1. Identical nucleotides are indicated in white on black. Dots indicate gaps introduced to maximize the extent of homology between sequences. Putative CAT and TATA boxes are indicated by underlining. Elements found in both sequences that might be involved in pollen-specific expression are boxed and labeled, and + and - designate the direct and the reverse orientation of the elements, respectively. LAT52 = The *cis*-regulatory element of LAT52; 52/56 box = the core motif of the LAT52/56 box; 56/59 box = the core motif of the LAT56/59 box; NPT303 = the *cis*-acting element of NPT303; Q = the Q element. The nucleotide sequences corresponding to the sense and antisense primers used for examining the diversity of promoter sequences are underlined by horizontal arrows and indicated by F1 and R1, respectively. The primers used for the construction of the reporter gene are indicated by horizontal arrows and designated F2 and R2.

Fig. 4. Variations in levels of expression of transcripts of *Cry j 2* in mature pollen from 17 specimens of plus trees. Five micrograms of total RNA, prepared from the mature pollen of each of 17 specimens of plus trees, were loaded on an agarose gel that contained formaldehyde and were fractionated by electrophoresis. Af-

ter transfer of bands of RNA to a nylon membrane, the membrane was allowed to hybridize with a ^{32}P -labeled 1,432-bp fragment of *Cry j 2* cDNA. The lower panel shows the results of staining of the gel before blotting with ethidium bromide to confirm that the amounts of RNA in different lanes were equal.

Fig. 5. DNA gel blot analysis of *Cry j 2* genes using various coniferous species. Total DNA prepared from each of 17 coniferous species was digested with *EcoRI* and subjected to electrophoresis on an agarose gel. After transfer of DNA fragments to a nylon membrane, the membrane was allowed to hybridize with a ^{32}P -labeled 1,432-bp fragment of *Cry j 2* cDNA. Lane 1, *C. japonica*; lane 2, *S. sempervirens*; lane 3, *C. lanceolata*; lane 4, *C. obtusa*; lane 5, *C. pisifera*; lane 6, *T. dolabrata*; lane 7, *T. standishii*; lane 8, *S. verticillata*; lane 9, *A. firma*; lane 10, *P. jezoensis* var. *hondoensis*; lane 11, *P. thunbergii*; lane 12, *P. densiflora*; lane 13, *T. diversifolia*; lane 14, *P. japonica*; lane 15, *T. cuspidata*; lane 16, *T. nucifera*, and lane 17, *C. harringtonia*. The amount of DNA from each species is given in Materials and Methods. The species analyzed in lanes 1 through 3 belong to Taxodiaceae; the species in lanes 4 through 7 belong to Cupressaceae; the species in lane 8 belongs to Sciadopityaceae; the species in lanes 9 through 14 belong to Pinaceae; the species in lanes 15 and 16 belong to Taxaceae; and the species in lane 17 belongs to Cephalotaxaceae.

Fujimura et al. [9] detected three spots that corresponded to isoforms of *Cry j 2* on two-dimensional immunomaps. The three isoforms had different frequencies of 32.5–40% with IgE in sera from 40 patients. The relationship between polymorphisms in the amino acid sequence and allergenicity remains to be clarified. Tamura et al. [36] revealed that ¹⁶⁹KWVNGREI¹⁷⁶ is the core of IgE-binding epitope with which 10 of 20 pollinosis patients exhibited IgE reaction. Changes in the amino acid sequence of this region could not be detected among *Cry j 2* isoforms that we identified (fig. 1). However, it has been proposed that specific human IgE antibodies to *Cry j 2* bind to conformational epitopes [15], and the general importance of carbohydrate moieties for binding to IgE is known [37]. The differences among amino acids in *Cry j 2* proteins might affect the modification of these proteins with carbohydrate chains. For example, an asparagine residue at position 394 of *Cry j 2.1* generates a potential site of N-glycosylation (N-X-S/T) that other isoforms do not possess (fig. 1). It is likely that each *Cry j 2* isoform has a different allergenicity. The characterization of the immunochemical properties of each isoform of *Cry j 2* remains to be performed.

The *Cry j 2* mRNA is dominantly expressed in pollen [20], but we found variations in levels of expression of *Cry j 2* among plant specimens at the transcriptional level (fig. 4). Levels of *Cry j 2* protein in extracts of pollen collected in various geographic regions and from different clones were reported to vary dramatically, ranging from 29 to 140 and from 92 to 1,145 µg per gram pollen, respectively [15, 16]. Our results indicate that the variations in levels of *Cry j 2* protein are due to differences at the transcriptional level. We also detected polymorphisms in the promoter region of the *Cry j 2* gene (fig. 3). However, there was no obvious relationship between polymorphisms in the promoter sequence and the variations in levels of expression of *Cry j 2* (data not shown).

The promoter sequences of all *Cry j 2* genes contained a number of the *cis*-elements that are known to be responsible for pollen-specific expression (fig. 3). We examined the activities of two *Cry j 2* promoters in transgenic *Arabidopsis* plants but failed to detect any activity in the transgenic pollen (data not shown). By contrast, the promoter sequence of the *Cry j 1* gene is associated with expression in the pollen of transgenic tobacco plants [38]. The reasons for the difference between the promoter activities of *Cry j 1* and *Cry j 2* remain to be determined since common *cis*-elements are present in the promoter sequences of both genes. The absence of promoter activity in transgenic *Arabidopsis* plants might have been due to

our heterologous system that involved two evolutionarily distant species [39]. The 5'-untranslated region of the pollen-specific *LAT59* gene has a strong inhibitory effect in somatic cells, as well as in pollen [40]. It is possible that the 5'-untranslated region of *Cry j 2* might inhibit the expression of a reporter gene in transformed *Arabidopsis*.

The cross-allergenicity between *C. japonica* and *C. obtusa* has been demonstrated with both human and monkey IgE antibodies [41, 42]. Conservative allergens are assumed to have common epitopes, with strong cross-reactivity existing among species of Cupressaceae [43, 44]. Our DNA gel blot analysis suggested that *Cry j 2* homologs might be widely distributed in Taxodiaceae and Cupressaceae, while *Cry j 2* is not very similar to homologs in Pinaceae (fig. 5). These observations are consistent with the previous studies by Chaw et al. [45] and Gugerli et al. [46], who identified the evolutionary split of Pinaceae from other conifers by molecular phylogenetic analysis [45, 46]. The low level of homology among allergens from Taxodiaceae and Pinaceae suggests one explanation for the fact that allergy to *Pinus* pollen is rarer than that to *C. japonica* and *C. obtusa* pollen.

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