

Cry j 1 isoforms derived from *Cryptomeria japonica* trees have different binding properties to monoclonal antibodies

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Summary

Background We identified five *Cryptomeria japonica* trees producing Cry j 1 isoforms that cannot be detected in a sandwich ELISA using two monoclonal antibodies, J1B01 and J1B07, suggesting that the binding affinity of these isoforms for both monoclonal antibodies is low.

Objectives The binding properties of the Cry j 1 isoforms produced by five trees to J1B07 and J1B01 were examined. The complementary DNA (cDNA) sequences of the Cry j 1 isoforms were also determined.

Methods To clarify the binding properties of these Cry j 1 isoforms to J1B01 and J1B07, Cry j 1 was quantified in pollen samples collected from each of the five trees, by sandwich ELISAs using polyclonal antibodies and either J1B01 or J1B07. The cDNA sequences of isoforms with different binding properties were determined. To test the assumption that amino acid substitutions affect the binding affinities of Cry j 1 isoforms for monoclonal antibodies, cleaved amplified polymorphic sequences (CAPS) markers representing the putative polymorphisms were used to analyse additional trees.

Results Four of the five trees produced Cry j 1 isoforms with extremely low binding affinity for J1B07, whereas the other tree produced two different isoforms with low binding affinity for either J1B01 or J1B07. Cry j 1-encoding cDNA sequences for one of the four trees and for the exceptional fifth tree indicate that amino acid substitutions at positions 55 and 352 in mature Cry j 1 affect its binding to J1B01 and J1B07, respectively. This was supported by the results of CAPS analysis.

Conclusion The existence of Cry j 1 isoforms with low binding affinity for either J1B01 or J1B07 was established. Furthermore, a single amino acid substitution is involved in this difference in binding affinity for each monoclonal antibody.

Keywords allergen, CAPS, Cry j 1, *Cryptomeria japonica*, isoform, Japanese cedar, monoclonal antibody, pollinosis, Taxodiaceae

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Introduction

Monoclonal antibodies are widely used for various purposes, including the quantification and *in situ* detection of specific substances with enzyme immunoassays. In the clinical aspects in allergic diseases, *in vitro* standardization of the allergenic extracts used in skin prick tests and immunotherapy is often performed by monoclonal-antibody-based ELISA. Alternatively, polyclonal antibodies (pAbs) can be used for the standardization of allergen extracts, but monoclonal antibodies have the significant advantage of consistent quality compared with pAbs. When pAbs are used, there can be cross-reactivity with contaminants of the purified antigen used for immunization or variation in quality among different serum pools. In contrast, once a monoclonal antibody with high avidity and specificity is developed, there is a limitless supply. Therefore, high repeatability and sensitivity can be

expected of sandwich ELISA systems based on monoclonal antibodies. Conversely, monoclonal antibodies are sometimes too specific and fail to recognize isoforms [1–3]. The existence of isoforms that do not bind to the monoclonal antibodies used causes overestimation or underestimation in the quantification of allergens. To avoid inaccurate estimations, it is essential to use monoclonal antibodies with the same avidity for various isoforms.

Recently, several monoclonal antibodies have been established against Cry j 1, a major allergen of *Cryptomeria japonica* (Japanese cedar) pollen [4–6]. *C. japonica* pollinosis is one of the most severe allergic diseases in Japan, and more than 10% of the Japanese population suffers from it. Two major allergens, Cry j 1 and Cry j 2, have been isolated and characterized [7–12], and it has been reported that more than 90% of patients with *C. japonica* pollinosis express specific IgE directed against both of them [13]. Therefore, the development of accurate quantification methods for Cry j 1 and Cry j 2 is important so that allergen extracts can be standardized for both research and clinical use. The Japanese

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Society of Allergology adopted a sandwich ELISA using two monoclonal antibodies, J1B01 and J1B07, for *in vitro* standardization of allergen extracts [14]. With this ELISA, variation in the Cry j 1 content of the pollens from different trees was investigated, and Cry j 1 content was found to vary from 16 to 1320 µg/g pollen among 158 *C. japonica* plus trees [15]. 'Plus' trees are selected from natural stands and man-made forests for their superior characteristics of straight boles and fast growth. Such trees with low allergenicity would be preferable to others, not only in reducing pollinosis but also for timber production. The selection of trees with lower Cry j 1 content has been attempted to reduce pollinosis by replanting current *C. japonica* varieties with low allergenic trees. Using this method in a preliminary screening for trees with lower Cry j 1 content, we identified five *C. japonica* trees with extremely low Cry j 1 content. These trees might produce Cry j 1 isoforms with low binding affinity for either J1B01 or J1B07 monoclonal antibody. Therefore, we investigated the binding affinities of the Cry j 1 isoforms produced by these five trees for J1B01 and J1B07. According to the result, the complementary DNA (cDNA) sequences encoding Cry j 1 isoforms that had different binding affinities to the monoclonal antibodies were investigated to deduce amino acid substitutions, which affected the binding affinities.

Materials and methods

Plant materials

Fifteen *C. japonica* plus trees (FT03, FK04, GM02, IY15, IY17, KA04, KI01, KJ17, KN07, KW29, KZ29, MS01, NT05, NT10 and ST23) growing in the crossing garden at the Forest Tree Breeding Center (Juo, Ibaraki) were used. To investigate the binding properties of the Cry j 1 isoforms for monoclonal antibodies, pollen samples were collected from the 15 trees in March 1995, 1999, 2002 and 2003. They were stored at -30°C until Cry j 1 extraction after desiccation with silica gel at 4°C . The Cry j 1 cDNA sequences of samples from three trees, KJ17, KN07 and NT05, were examined. Pollen samples were collected from these trees for RNA extraction in March 1999, and stored at -80°C until use.

Inheritance analysis with a specifically developed cleaved amplified polymorphic sequences (CAPS) marker was carried out using the F_1 population (38 individuals) from a cross between two cultivars, Yabukuguri and Kumotooshi. Total DNA samples were isolated from the young leaves of these plants by the cetyltrimethyl ammonium bromide method [16]. Using the CAPS marker, trees that produced isoform(s) with low binding affinity for J1B01 were identified among 855 *C. japonica* plus trees. Total DNA from these trees had been prepared in a previous study [17].

Complementary DNA cloning and sequencing

cDNA cloning and sequencing was performed as described in a previous report [18]. Briefly, poly A⁺ RNA was purified using Oligotex-dT30 (Super) latex beads (JSR Corp., Tokyo, Japan) from the total RNA extracted from pollen samples as described by Sone et al. [10]. Cry j 1-encoding cDNA was specifically amplified from the poly A⁺ RNA sample using a

primer pair designed on the basis of the Cry j 1 cDNA sequence previously reported by Sone et al. [10] (forward: 5'-TCATAATCATAGCATAGCCG-3'; reverse: 5'-CAACATGCTAGAATATATGC-3'). For reverse transcription and the following PCR, a TaKaRa RNA LA PCR Kit (AMV) vs. 1.1 (TaKaRa Bio Inc., Ohtsu, Japan) was used. Reverse transcription was performed at 55°C for 30 min. PCR amplification was carried out for 2 min at 94°C , followed by 40 cycles of 30 s at 94°C , 1 min at 55°C , and 3 min at 72°C with a final incubation for 2 min at 72°C . Cry j 1-encoding cDNA was cloned using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA, USA). DNA sequencing was performed using the dideoxynucleotide chain termination method with a 373 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Pollen extraction

Crude extract containing Cry j 1 was obtained from 100 mg of pollen samples with 5 mL of 0.125 M NaHCO₃ (pH 8.3) [19]. The extraction was performed at 4°C , for 2 h.

Sandwich enzyme-linked immunosorbent assay

Sandwich ELISA was performed with pollen extract using two kinds of monoclonal antibodies, J1B01 and J1B07, and rabbit anti-Cry j 1 pAb. Combinations of antibodies were denoted as *capture antibody-detector antibody*, i.e., J1B07-J1B01, J1B01-pAb, and J1B07-pAb. J1B07-J1B01 and J1B07-pAb ELISAs were performed as described in a previous paper, with some modifications [20]. The extract from the pollen sample of KN07 in 0.125 M NaHCO₃ was used as the standard, the Cry j 1 concentration of which was determined as described by Yasueda et al. [14]. The standard curve was drawn from 0.195 to 12.5 ng/mL and the dilution rates of the pollen extracts were 1:800, 1:1600, 1:3200, and 1:6400 in the J1B07-J1B01 and J1B07-pAb assays. Because the sensitivity of the J1B01-pAb assay was lower, it was performed at twofold higher concentrations. In addition, ELISAs with dilution rates of 1:40, 1:80, 1:160, and 1:320 (lower dilution series) were performed for samples that showed absorbances below the limit of detection at a dilution rate of 1:800.

Cleaved amplified polymorphic sequences analysis

Two CAPS markers namely CRYJ1-55 and CRYJ1-352 were used. For CRYJ1-55, a forward primer (5'-GCATAGCCG-TATAGAAAGAAAT-3'), a reverse primer (5'-AGTGCG-CAGAGTAAGAGC-3'), and a restriction endonuclease *Mva*I (Toyobo Co. Ltd, Osaka, Japan) were used. CRYJ1-352 was performed with a forward primer (5'-ACGATATGGACTTG-TAC-3'), a reverse primer (5'-ACAACAATATACATCAT-GAT-3'), and a restriction endonuclease *Psp* 1406I (Takara Shuzo Co. Ltd, Otsu, Japan). The PCR condition was as described for the amplification of Cry j 1-encoding cDNA. The reaction mixture (100 µL) contained 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of each primer and 1.0 U of *Taq* DNA polymerase (Invitrogen Corp.). Template DNA (25 ng) was added to 15 µL of the reaction mixture. Digestions with restriction endonucleases were performed following the manufacturers' instructions. The PCR products and digested PCR products were separated by electrophoresis on 1.5% agarose gels.

Results

Measurement of Cry j 1 by sandwich enzyme-linked immunosorbent assays

J1B07–J1B01, *J1B01–pAb*, and *J1B07–pAb* ELISAs were performed with extracts from pollen samples from FT03, KJ17, KA04, KI01, and NT05. The results are shown in Fig. 1. For comparison, the absorbance curve for the extract from pollen collected from KN07 (control) is also shown. The Cry j 1 content of KN07 estimated by *J1B07–J1B01*, *J1B01–pAb*, and *J1B07–pAb* ELISAs were 443, 436, and 440 µg/g pollen, respectively (Fig. 2). In the *J1B07–J1B01* assay, the absorbances for all five trees were below the limit of detection that indicated the value of 15.6 µg/g pollen. With the lower dilution series, the absorbances for FT03, KA04, KI01, and NT05 were also below the limit of detection, indicating that their Cry j 1 contents were less than 3.125 µg/g pollen, whereas the absorbance for KJ17 gave a calculated value of 11 µg/g pollen. In the *J1B01–pAb* assay, all five trees produced concentration-dependent curves, and the Cry j 1 contents of FT03, KJ17, KA04, KI01, and NT05 were estimated to be 156, 98, 284, 345, and 236 µg/g pollen, respectively, which are within the normal range. On the other hand, in the *J1B07–pAb* ELISA, the absorbances for four trees (not KJ17) were below the limits of detection, indicating that the Cry j 1 isoform(s) produced by these four trees have low binding affinity for J1B07. KJ17 also produced a concentration-dependent curve in the *J1B07–pAb* ELISA, and the Cry j 1 content was estimated to be 96 µg/g pollen. This result demonstrates that KJ17 produces Cry j 1 isoform(s) with binding properties for the two monoclonal antibodies that differ from those of the other four trees.

Cry j 1 complementary DNA sequences

Cry j 1-encoding cDNA sequences derived from KJ17, KN07, and NT05 were determined. Eight to 11 cDNA clones were sequenced for each tree. Two, one, and two major Cry j 1-encoding cDNA sequences were isolated from KJ17, KN07, and NT05, respectively, and in total, four different Cry j 1-encoding cDNA sequences were identified from these trees (Fig. 3). The putative amino acid sequences deduced from these cDNAs also differed (Fig. 4). Each of the four amino acid sequences consisted of a 21-residue signal peptide and a 353-residue mature protein. Two of the four were identical to those encoding Cry j 1a and Cry j 1b, which have been reported previously [18, 21]. The Cry j 1a-encoding cDNA sequence is recorded in GenBank under accession number D343639. The two new isoforms are designated Cry j 1c and Cry j 1d. Only Cry j 1a was detected in KN07. Cry j 1b, and Cry j 1c were detected in NT05, and Cry j 1b and Cry j 1d were detected in KJ17.

Relationship between amino acid substitution at position 352 and binding affinity for J1B07

From the result of the ELISAs and the Cry j 1-encoding cDNA sequences, we deduced that the amino acid substitution from Arg to His at position 352 changed the binding affinity to J1B07 (see details in Discussion). The relationship between the decrease in binding affinity for J1B07 and the

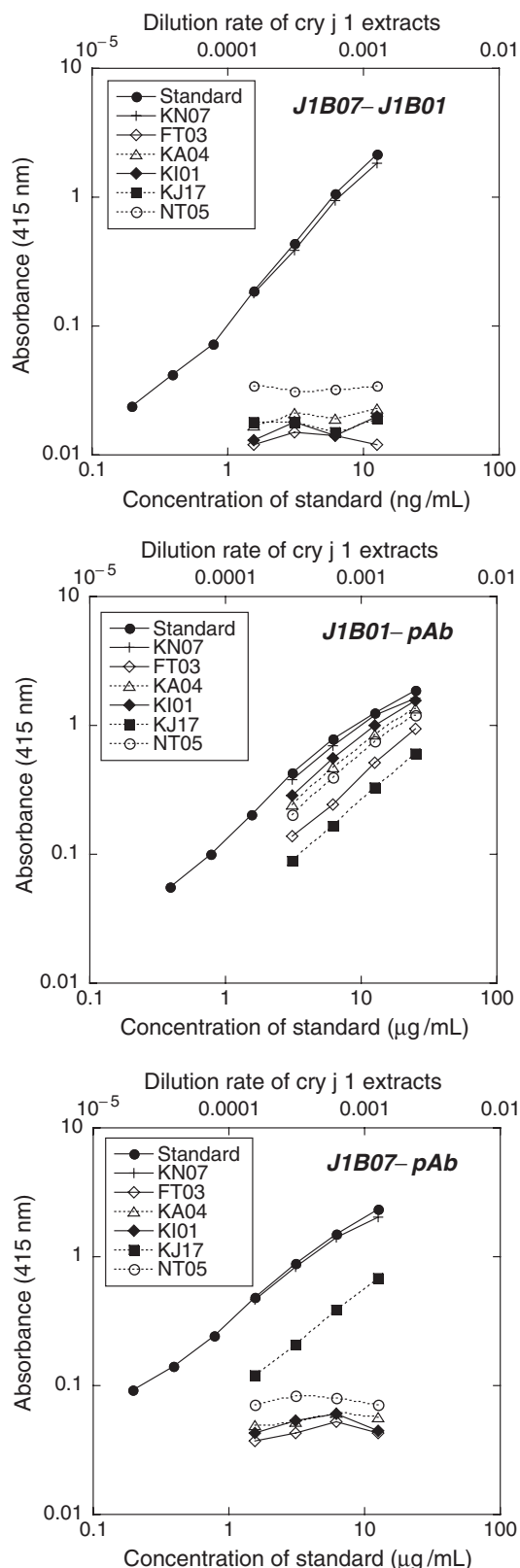


Fig. 1. Binding of Cry j 1 isoforms derived from six *Cryptomeria japonica* trees in three sandwich ELISAs. Reactivity of increasing concentrations of FT03, KJ17, KA04, KI01, NT05, and KN07 extracts were compared with the Cry j 1 standard.

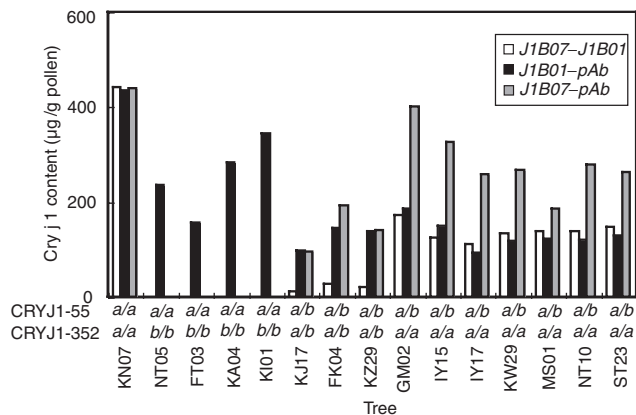


Fig. 2. Cry j 1 content of the studied trees estimated by three different ELISAs, *J1B07-J1B01*, *J1B01-pAb*, and *J1B07-pAb*. CRYJ1-55 and CRYJ1-352 genotypes are given together.

amino acid substitution at position 352 was confirmed with FT03, KA04, and KI01. CRYJ1-352, a CAPS marker associated with the corresponding amino acid substitution, was used to analyse these three trees. The primer positions and recognition site for *Psp1406 I*, the restriction endonuclease used in CRYJ1-352, are shown in Fig. 3. The codon that encodes Arg at position 352 is included in the recognition sequence of *Psp1406 I*, AACGTT, and is changed to AACATT in Cry j 1b and Cry j 1c. Therefore, we anticipated that the 440-bp fragment PCR amplified from the Cry j 1a- and Cry j d-encoding genes would be digested to yield two fragments of 360 and 80 bp, whereas the fragments amplified from the genes encoding Cry j 1b and Cry j 1c would remain uncut. Because it had already been demonstrated that CRYJ1-352 identifies different alleles at a single locus [18], we designated the allele represented by two cut fragments *352a* and that represented by a single fragment *352b*. Accordingly, CRYJ1-352 genotypes are denoted as follows: *352a* and *352b* homozygotes, *352a/a* and *352b/b*, respectively; and the heterozygote of *352a* and *352b*, *352a/b*. In CRYJ1-352 analysis, NT05, KJ17, and KN07 produced band patterns corresponding to *352b/b*, *352a/b*, and *352a/a*, as expected from the Cry j 1-encoding cDNA sequences determined for each tree (Fig. 5). In the subsequent analysis of FT03, KA04, and KI01, all three trees produced a single band at 440 bp, the band pattern corresponding to *352b/b* (Fig. 5). This result further supports the proposition that the amino acid substitution at position 352 affects isoform binding to J1B07.

Relationship between amino acid substitution at position 55 and binding affinity to J1B01

From the result of the ELISAs and the Cry j 1-encoding cDNA, we inferred that the amino acid substitution from Pro to Leu affected the binding affinity to J1B01 (see details in Discussion). A CAPS marker that associates with the amino acid polymorphism at position 55 was developed and designated CRYJ1-55. The primer set was designed to amplify a fragment of 600 bp that includes position 55. The restriction endonuclease *MvaI*, which recognizes the sequence CCAGG that encodes Pro (CCA) at position 55, was selected. It was anticipated that Cry j 1a, Cry j 1b, and Cry j 1c would produce two digested fragments of 320 and 280 bp, whereas

an uncut fragment of 600 bp would be produced by Cry j 1d. After digestion of the PCR products with *MvaI*, KN07 and NT05 presented two fragments of 320 and 280 bp, whereas KJ17 produced a single fragment of 600 bp, together with two fragments of 320 and 280 bp, as expected (Fig. 5).

The co-dominant inheritance of CRYJ1-55 conforms to a Mendelian pattern, as revealed by segregation analysis using the F_1 generation from a cross between two *C. japonica* cultivars, Yabukuguri and Kumotooshi ($P > 0.05$, χ^2 test), indicating that CRYJ1-55 identifies different alleles at a single locus. Therefore, as for CRYJ1-352, the allele represented by the two cut fragments was designated *55a* and that producing a single uncut fragment was designated *55b*. The CRYJ1-55 genotypes of KJ17, KN07, and NT05 were denoted *55a/b*, *55a/a*, and *55a/a*, respectively. In addition, the genotypes of the combination of two CAPS markers were denoted as the CRYJ1-55 and CRYJ1-352 alleles. Following this, KJ17, KN07, and NT05 were denoted as *55a-352b/55b-352a*, *55a-352a/55a-352a*, and *55a-352b/55a-352b*, respectively (Table 1a).

To confirm the relationship between the amino acid substitution at position 55 and the binding affinity of Cry j 1 for J1B01, we attempted to find *55b/b* trees, from 855 *C. japonica* plus trees using CRYJ1-55. None were found, but 20 *55a/b* trees were identified. CRYJ1-352 and *J1B07-J1B01*, *J1B01-pAb*, and *J1B07-pAb* assays were performed on nine of the 17 trees (FK04, GM02, IY15, IY17, KW29, KZ29, MS01, NT10, and ST23) to determine the binding properties of the isoforms produced by these trees. The CRYJ1-352 genotypes of two trees, FK04 and KZ29, were *352a/b*, whereas those of the other seven trees were *352a/a* (Fig. 5). The Cry j 1 content of each tree estimated with the three ELISAs is given in Fig. 2.

Like Cry j 1 of KJ17, the Cry j 1 contents of FK04 and KZ29 were quantified with the *J1B01-pAb* and *J1B07-pAb* assays, whereas the absorbances for these trees were too low to estimate the Cry j 1 content when the *J1B07-J1B01* assay was used. With the lower dilution series, FK04 and KZ29 produced concentration-dependent curves and their Cry j 1 contents were estimated to be 27 and 20 µg/g pollen, respectively. These results indicate that only *55a* isoforms were detected in samples from FK04, KJ17, and KZ29 in the *J1B07-J1B01* assay, and the binding affinity of *55b* was reduced to approximately 10–15% of that of *55a*. Moreover, the estimated Cry j 1 content was similar in the *J1B01-pAb* and *J1B07-pAb* assays. In other words, these trees produced similar amounts of *55a* and *55b* isoforms. In addition, it was revealed that both two trees produced *55a-352b* and *55b-352a* isoforms, not *55b-352b* and *55a-352a*. If they produced *55a-352a* isoform, Cry j 1 content estimated in *J1B07-J1B01* would be within the normal range because *55a-352a* could bind to both J1B01 and J1B07.

The seven trees with *55a/b* or *352a/a* genotypes showed similar Cry j 1 contents with the *J1B07-J1B01* and *J1B01-pAb* assays. Furthermore, the Cry j 1 contents estimated with the *J1B07-pAb* assay were approximately twofold greater than those estimated with the *J1B01-pAb* or *J1B07-J1B01* assays. This result can be explained as follows: only the *55a* isoforms are detected with *J1B01-pAb* and *J1B07-J1B01* assays, whereas total Cry j 1 content was measured by *J1B07-pAb*. The binding affinities of the isoforms produced by each

		CRYJ1-55	
Cry j 1a	1	TCATAATCATAGCATAGCCGTATAGAAAGAAATTTCTACACTCTGCTACCAAAAAATGGATTCCCCTTGCTTAGTAGCATTACTGGTTTTCTCTTTTGAATTGGATCTTGCTTTTCTGAT	
Cry j 1b	1	C.....	
Cry j 1c	1	T.....	
Cry j 1d	1	T.....	
Cry j 1a	121	AATCCCATAGACAGCTGCTGGAGAGGAGACTCAAACCTGGGCACAAAAAGAAATGAAGCTCGCAGATTGTGCAGTGGGCTTCGGAAGCTCCACCATGGGAGGCAAGGAGGAGATCTTTAT	
Cry j 1b	121	T.....G.....T.....	
Cry j 1c	121	T.....A.....C.....	
Cry j 1d	121	T.....A.....C.....	
		MvaI	
Cry j 1a	241	ACGGTCACGAACTCAGATGACGACCTGTGAATCCTGCAACAGGAACCTCTGCGCTATGGAGCAACCCGAGATAGGCCCTGTGGATAATTTTCAGTGGGAATATGAATATAAAGCTCAAA	
Cry j 1b	241	..A...A...T...T...C.....	
Cry j 1c	241	..A...A...T...T...C.....	
Cry j 1d	241	..G...G...C...A...T.....	
Cry j 1a	361	ATGCCTATGTACATTGCTGGGTATAAGACTTTTGATGGCAGGGGAGCACAAGTTTATATTGGCAATGGCGGTCCCTGTGTGTTTATCAAGAGAGTTAGCAATGTTATCATACACGGTTTG	
Cry j 1b	361	A.....G.....	
Cry j 1c	361	A.....G.....	
Cry j 1d	361	G.....T.....	
		CRYJ1-55	
Cry j 1a	481	TATCTGTACGGCTGTAGTACTAGTGTGTTGGGAATGTTTGGATAACGAGAGTTTGGGGTGGAGCCTGTTATCCTCAGGATGGCGATGCTCTTACTCTGCGCACTGCTACAAATAT	
Cry j 1b	481	C...T.....	
Cry j 1c	481	C...T.....	
Cry j 1d	481	C...G.....	
Cry j 1a	601	TGGATTGATCATAATCTTTCTCCAATCTCTGATGCTGCTGATGCTACTCTTACTTCGACTGGAGTTACTATTTCAAACAATCTTTTTTCAACCATCATAAAGTGATGTTGTTA	
Cry j 1b	601	C...T.....	
Cry j 1c	601	C...T.....	
Cry j 1d	601	A...C.....	
		CRYJ1-352	
Cry j 1a	721	GGGCATGATGATGCATATAGTGATGACAAATCCATGAAGGTGACAGTGGCGTTCAATCAATTTGGACCTAAGTGTGGACAAGAATGCCAGGGCACGATATGGACTTGATGTTGCA	
Cry j 1b	721		
Cry j 1c	721		
Cry j 1d	721		
Cry j 1a	841	AACAATAATTATGACCCATGGACTATATATGCAATTGGTGGGAGTTCAAATCCAACATTCTAAGTGAAGGGAATAGTTTCTACTGCACCAATGAGAGCTACAAGAAGCAAGTAACCAT	
Cry j 1b	841		
Cry j 1c	841		
Cry j 1d	841		
Cry j 1a	961	CGTATTGGATGCAAAACATCATCTTGTCAAATTTGGGTGTGGCAATCTACACAAGATGTTTTTATAATGGAGCTTATTTGTATCATCAGGGAATATGAAGGGGTAATATATA	
Cry j 1b	961		
Cry j 1c	961		
Cry j 1d	961		
		Psp1406 I	
Cry j 1a	1081	ACAAAGAAAGAAGCTTTCAATGTTGAGAATGGGAATGCAACTCTCAATTGACAAAAATGCTGGGTTTTAACATGCTCTCTCTAAACGTTGTTGATGATGCATATATTCTAGCATG	
Cry j 1b	1081	C.....A.....	
Cry j 1c	1081	C.....A.....	
Cry j 1d	1081	T.....G.....	
		CRYJ1-352	
Cry j 1a	1201	TTGTACTCTAAATTAACATCAACAAGAAATATATCATGATGTATATTGTTG	
Cry j 1b	1201	... pCCI-2-2	
Cry j 1c	1201	...	
Cry j 1d	1201	...	

Fig. 3. Four different complementary DNA sequences derived from KJ17, KN07 and NT05. Underlined regions are derived from clone pCCI-2-2, reported by Sone et al. [10]. Cry j 1a, Cry j 1c, and Cry j 1d were detected specifically from KN07, NT05, and KJ17, respectively, and Cry j 1b was detected from both KJ17 and NT05. The primer positions and restriction endonuclease recognition sites used in CRYJ1-55 and CRYJ1-352 are indicated in the highlighted boxes.

tree were deduced from the results of the ELISAs and CAPS genotypes (Table 1a).

Estimation of allele frequencies of 55b and 352b

The allele frequencies of 55a-352a, 55a-352b and 55b-352a, which were associated with the isoforms that had different binding affinities to the monoclonal antibodies, were estimated from 94 plus trees as 77.7%, 18.6% and 3.7%, respectively (Table 1b).

Discussion

From the results of ELISAs and the Cry j 1-encoding cDNA sequences, we inferred the binding properties of each isoform to J1B01 and J1B07. Because the Cry j 1 produced by NT05 was not detected by J1B07-pAb but was detected by J1B01-pAb, we inferred that both Cry j 1b and Cry j 1c have low binding affinity for J1B07 but bind to J1B01. On the basis of this assumption, we postulated that Cry j 1d would have low binding affinity for J1B01. In the assays with KJ17, Cry j 1b

Cry j 1a -20 MDSPCLVALLVFSFVIGSCFSDNPIDSCWRGDSNWAQNRMKLADCAVGFSGSTMGKGGLYTVTNSDDDPVNPAPGTLRYGATRDRPLWIIIFSGNMNIKLMKPMYIAGYKTFDGRGAQV
Cry j 1b -20 S.....V.....P.....
Cry j 1c -20 F.....A.....P.....
Cry j 1d -20 F.....A.....**L**.....

Cry j 1a 99 YIGNGGPCVFIKRVSNVHGLYLYGCSTSVLGNVLINESFGVEPVHPQGDALTLRTATNIWIDHNSFSNSDGLVDVLTSTGVTISNNLFFNHHKVMLLGHDDAYSDDKSMKVTVAF
Cry j 1b 99 H.....
Cry j 1c 99 H.....
Cry j 1d 99 H.....

Cry j 1a 219 NQFGPNCQRMPRARYGLVHVANNNDPWTIYAIGGSSNPITLSEGSFTAPNESYKKQVTIRIGCKTSSSCSNWVWQSTQDVFYNGAYFVSSGKYEGGNIYTKKEAFNVENGATPQLT
Cry j 1b 219
Cry j 1c 219
Cry j 1d 219

Cry j 1a 339 KNAGVLTCSLSKRC
Cry j 1b 339**H**.....
Cry j 1c 339**L**.....
Cry j 1d 339 R.....

Fig. 4. Amino acid sequences deduced from complementary DNA sequences derived from KJ17, KN07, and NT05. The amino acid substitutions associated with the polymorphisms represented by CRYJ1-55 and CRYJ1-352 are highlighted. The signal peptide was underlined.

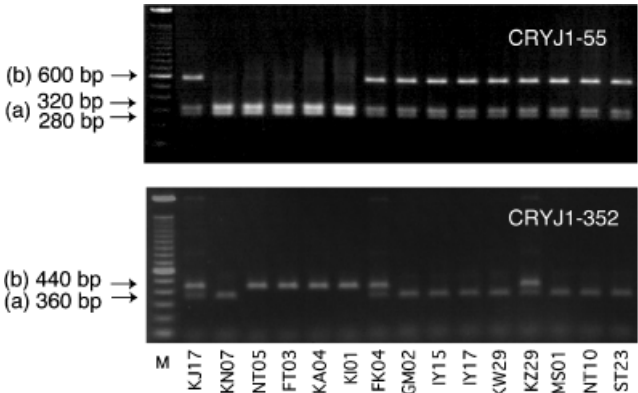


Fig. 5. CRYJ1-55 and CRYJ1-352 band patterns for the studied trees. The 80 bp bands were too faint to be indicated. M, 100 bp ladder marker.

Table 1a. The binding affinity of isoform(s) produced by each tree was deduced from the CRYJ1-55 and CRYJ1-352 genotypes.

Tree	Genotype	Binding affinity	
		J1B01	J1B07
KN07	55a-352a/55a-352a	+/+	+/+
NT05	55a-352b/55a-352b	+/+	-/-
FT03	55a-352b/55a-352b	+/+	-/-
KA04	55a-352b/55a-352b	+/+	-/-
KI01	55a-352b/55a-352b	+/+	-/-
KJ17	55a-352b/55b-352a	+/-	-/+
FK04	55a-352b/55b-352a	+/-	-/+
KZ29	55a-352b/55b-352a	+/-	-/+
GM02	55a-352a/55b-352a	+/-	+/+
IY15	55a-352a/55b-352a	+/-	+/+
IY17	55a-352a/55b-352a	+/-	+/+
KW29	55a-352a/55b-352a	+/-	+/+
MS01	55a-352a/55b-352a	+/-	+/+
NT10	55a-352a/55b-352a	+/-	+/+
ST23	55a-352a/55b-352a	+/-	+/+

+, high binding affinity; -, low binding affinity.

and Cry j 1d were detected with J1B07-pAb and J1B01-pAb, respectively, but both of these isoforms were scarcely detected with J1B07-J1B01. In both Cry j 1b and Cry j 1c, Arg is replaced by His at position 352, indicating that this substitution affects their binding affinity for J1B07. On the other hand, the amino acid substitution specific for Cry j 1d, Pro to Leu at position 55, changed the binding affinity of the isoform for J1B01. Relationships between the amino acid substitutions at positions 55 and 352 and the low binding affinity to J1B01 and J1B07, respectively, were further determined on additional trees.

One of the major aims of this study was to select the monoclonal antibody that identifies various isoforms to establish an accurate Cry j 1 quantification system using ELISA. Although some isoforms bound to both J1B01 and J1B07, we identified other isoforms that were only recognized by one or other of these monoclonal antibodies. To select the monoclonal antibody that binds more frequently to Cry j 1 isoforms, the allele frequencies of 55b-352a and 55a-352b were compared using the CAPS markers, CRYJ1-55 and CRYJ1-352. CAPS markers allow the detection of a nucleotide mutation at a particular position without time consuming and expensive processes such as the preparation of cDNA, cloning, and sequencing. In CAPS analysis, if any mutations occur in the endonuclease recognition site, the PCR-amplified fragments are not digested. Thus, by analysis with the CAPS markers alone, without sequencing, we cannot exclude the possibility that substitutions at position 56 and those at position 351 or 353 might also affect the binding of Cry j 1 to J1B01 and J1B07, respectively. However, the results presented here demonstrate that these CAPS markers are useful in determining the binding affinities for the monoclonal antibodies of the Cry j 1 isoforms produced by the trees analysed. As a result, the allele frequency of 55a-352b was much higher than that of 55b-352a and not negligible. Accordingly, the J1B01-pAb ELISA would be preferable for the selection of trees expressing less Cry j 1. After candidate trees have been screened with a J1B01-pAb assay, CRYJ1-55 could help the accurately quantification of total Cry j 1 in the pollen. When 55a/b trees have been identified

Table 1b. Relationships between the binding affinity of isoforms and CRYJ1-55 and CRYJ1-352 polymorphism.

Allele	Binding affinity		Frequency (%)
	J1B01	J1B07	
55a-352a	+	+	77.7
55a-352b	+	–	18.6
55b-352a	–	+	3.7

The allele frequency is shown together
+, high binding affinity; –, low binding affinity.

among the candidate trees, the Cry j 1 content of their pollen could be measured with a J1B07-*pAb* assay.

Interestingly, only one or two Cry j 1-encoding cDNA sequences were detected from the pollen samples of each tree used in this study. This indicates that the alleles for Cry j 1 isoforms occur at a single locus, as was suggested in the previous studies [18, 22]. In our previous study, CRYJ1-352 displayed Mendelian segregation and the location of the *Cry j 1* gene was determined on the linkage map of Iwao-sugi, a cultivar of *C. japonica* [18]. Similarly, CRYJ1-55 also displayed Mendelian segregation in another F₁ population. Tani et al. [22] also mapped the Cry j 1-encoding gene, but used different F₁ families and genetic markers from those we used. Wang et al. [21] suggested that Cry j 1 is encoded by a small multi-gene family that consisted of five to eight copies per diploid. Cry j 1 genes of the same sequence might be repeated in tandem, or pseudogenes might exist. Our results indicate that a single tree would produce only one or two Cry j 1 isoforms that were determined by the Cry j 1 genotypes, even if various isoforms existed within the species. In addition, the heterozygote trees produced similar amounts of the different Cry j 1 isoforms. This allowed us to deduce the binding affinity of each isoform for specific monoclonal antibodies by considering the binding properties and cDNA sequence(s) of the Cry j 1 isoforms derived from a single tree. In further studies, homogenous Cry j 1 will be isolated from the pollen collected from a homozygote tree and used to examine its immunochemical character. The fact that a single tree produces one or two isoforms indicates that the quality of allergen extracts will depend on the trees that provided the pollen source. In some cases, trees used for pollen collection should be selected on the basis of their Cry j 1 genotypes, according to the use to which the allergen extract is put. For instance, isoforms that react with both J1B01 and J1B07, such as Cry j 1a, should be used as the Cry j 1 standard for quantification in ELISAs based on these monoclonal antibodies. Furthermore, if immunochemical properties, such as T cell stimulation and IgE reactivity, differ among isoforms, the *Cry j 1* genotypes of the trees providing the pollen would become more important.

Our investigation revealed that a single amino acid difference caused low binding affinity to each monoclonal antibody. Since J1B01 and J1B07 recognize different conformational epitopes (unpublished data), the low binding affinities to the monoclonal antibodies indicate a change in the conformation of the epitopes. Both the 55b and 352b isoforms, especially the 55b isoforms, might have a low IgE-binding affinity. Suzuki et al. [23] reported that J1B01

blocked IgE binding to Cry j 1 in sera collected from 17 patients with *C. japonica* pollinosis much more efficiently than did J1B07. Furthermore, several studies have demonstrated that J1B01 and the monoclonal antibodies that share the same epitope, namely 013, 046, and S14, inhibited human IgE binding to Cry j 1 most strongly of the monoclonal antibodies tested [4, 24–26]. These facts suggest that the B cell epitope that is targeted by J1B01 is absent in the 55b isoforms. The replacement of Pro with Leu at position 55 changes the conformation of the B cell epitope. Comparison of three-dimensional structures of the 55b isoforms, such as Cry j 1d, and the other isoforms might reveal a major B cell epitope in Cry j 1. The immune reactivity of the isoforms associated with 55b might be significantly less than that of the other isoforms, and thus these isoforms may be useful for immunotherapy. However, the amino acid residues at positions 55 and 352 were not included in the T cell epitopes that had been identified previously [27–29]. Sufficient T cell stimulation could be achieved using the isoforms associated with 55b, while avoiding IgE-mediated anaphylactic effects. In contrast, these isoforms might be not useful in the diagnosis of *C. japonica* pollinosis. The variation in the immunochemical properties of the Cry j 1 isoforms will be investigated in future studies.

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