

Short communication

Comparison of concentrations of Cry j 1 and Cry j 2 in diploid and triploid Japanese cedar (*Cryptomeria japonica*) pollen extracts

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Japanese cedar (*Cryptomeria japonica*) pollinosis has been a serious allergic disease in Japan. There are two kinds of Japanese cedar trees; the popular one is diploid, the less popular is triploid. These trees are not very different morphologically. However, the relative allergenicity of their pollens is unknown, although both major allergens, Cry j 1 and Cry j 2, have been purified and cloned from the diploid line. Triploid trees are sterile and the allergenicity of their pollen may differ. Using Japanese-cedar-allergic patient sera, we compared the concentration of these two major allergens in both kinds of pollen. Pollen collected from different years and regions was also studied. The results indicate that triploid tree pollen extract has lower concentrations of both major allergens; therefore, the pollen may be less allergenic.

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Key words: Cry j 1; Cry j 2; Japanese cedar or sugi (*Cryptomeria japonica*); major allergen; pollinosis; triploid.

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Japanese cedar or sugi (*Cryptomeria japonica*) pollinosis is one of the most important allergic diseases in Japan. Two major allergens in Japanese cedar pollen extract have been purified. One has been designated as sugi basic protein (SBP) (1) and as Cry j 1 (2). The other is Cry j 2 (3). Cry j 1 consists of more than four isoforms when analyzed by two-dimensional electrophoresis (4). These isoforms were shown to have identical allergenicity in immunodiffusion analysis, intradermal skin test, and the radioallergosorbent test (RAST). Cry j 1 showed a doublet in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions (3). However, Cry j 2 has a single band and increases its apparent molecular weight from 37 to 45 kDa in SDS-PAGE under reducing conditions (3). Furthermore, the content of Cry j 1 and Cry j 2 in extracts varies from batch to batch (5). Pollens collected from various regions showed that Cry j 2

ranges from 2.9 to 14 mg per 100 g pollen extracted, whereas the amount of Cry j 1 is nearly constant, about 35 mg per 100 g pollen being extracted.

In Japan, a systematic tree-breeding program for forestry was established in 1957. Both diploid and triploid cedar trees were introduced into various areas, and a survey of cedar tree distribution showed that very few are triploid. A review of recent articles about pollinosis in Japan showed that pollinosis is proportional to the density of diploid cedar trees and that pollinosis declines where there is a higher density of triploid cedar trees (6, 7). Triploid trees are sterile (8) and their pollen may be less allergenic than that of normal diploid trees. If this is true, allergy to cedar pollen may be reduced by substituting triploid trees for diploid ones in the forestry program.

This paper compares the concentrations of the two known major allergens, Cry j 1 and Cry j 2, from triploid and diploid tree pollen extracts.

Pollen collected from different regions of central Japan was also compared.

Material and methods

Japanese cedar (C. japonica) pollens

Pollen of three different clones was collected from 1993 to 1995 by the Aichi Prefectural Forest Research Institute. Another eight different clones of pollen were collected in 1995 and were kindly provided by the Prefectural Forest Research Institute of the Gifu, Shizuoka, Mie, and Niigata prefectures. Of these 11 kinds of clones, three were triploid and eight diploid. The three triploid pollen specimens, one from Aichi, one from Shizuoka, and the third from Niigata, have been previously studied (9–11). All specimens were stored at 4°C until used except that the one collected in 1993 was kept at room temperature.

Extraction

Japanese cedar pollen was extracted (1 : 20 w/v) in 0.125 M NaHCO₃ at 4°C with rotation for 2 h. Extracts were centrifuged at 12 000 g for 10 min at 4°C, and the supernatants were used after filtration with a 0.45-µm Millex-HV unit (Millipore, Bedford, MA, USA). Protein concentrations of these extracts were determined by BCA protein assay (Pierce, Rockford, IL, USA).

SDS-PAGE

SDS-PAGE was performed in a 12% Tris-glycine Novex precast gel (Novex, San Diego, CA, USA) according to Laemmli (12), under nonreducing and reducing conditions. Proteins were separated at 130 V for 2 h.

Transfer and immunoblotting

After SDS-PAGE, proteins were transferred to Immobilon-P™ Membranes (Millipore, Bedford, MA, USA) as previously reported (13).

Human sera

Serum samples were obtained from 100 untreated Japanese-cedar-allergic patients, living in Aichi prefecture, diagnosed on the basis of clinical history. Of 100 patients, 68 had a score of more than 3 (3.5 kU/l) (Pharmacia CAP System) against Japanese cedar pollen extract and were positively considered allergic to Japanese cedar. The control serum was obtained from a healthy volunteer from Aichi prefecture. These sera were frozen until used.

Results

IgE reactivity to proteins of Japanese cedar pollen extract

Japanese cedar pollen extracts from diploid clones were made as previously reported (1). Proteins separated by 12% SDS-PAGE were stained by silver nitrate. Two major allergens, Cry j 1 and Cry j 2, were identified (Fig. 1a). Both allergens migrated as doublets with apparent molecular masses of 45–49 and 41–43 kDa, respectively. These two sets of doublets were confirmed to be similar within sets by N-terminal amino acid sequences determined from blotted membranes (data not shown). Sera of patients who had definite allergic symptoms and high CAP scores were used to determine IgE-binding reactivity to pollen proteins separated electrophoretically. Of 68 patient sera, 35 reacted to doublet bands with apparent masses of 45 and 49 kDa; 32 reacted to the other doublet bands with apparent masses of 41 and 43 kDa; 24 reacted to proteins smaller than 17 kDa; 20 reacted to both doublet sets; seven reacted to a 95-kDa protein; six reacted to a 60-kDa protein; and six reacted to a 160-kDa protein. Representative IgE-reactive protein-binding patterns to diploid Japanese cedar pollen extract are shown in Fig. 1b.

Comparison of the concentrations of Cry j 1 and Cry j 2 in triploid and diploid Japanese cedar pollen extracts

Since the extractable amount of Cry j 2 has shown large variation from batch to batch of pollen, the amount of extracted proteins of each batch of

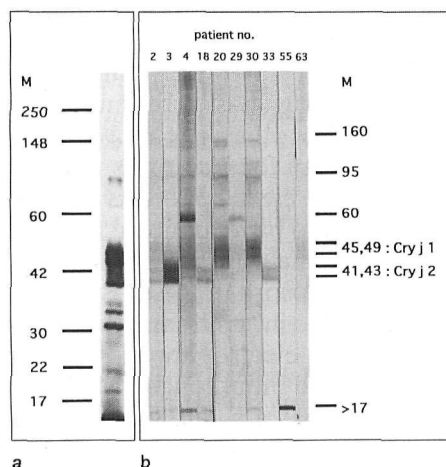


Fig. 1. a) SDS-PAGE of diploid Japanese cedar pollen extract in nonreducing gel with silver staining. M: molecular weight in kDa. b) Serum reactivity to diploid Japanese cedar pollen extract. M: molecular weight in kDa.

pollens was carefully monitored by extracting, at least in triplicate, each three different times, by a uniform extraction method. We studied diploid and triploid pollen from the 1993 collection, two diploid and one triploid from the 1994 collection, and seven diploid and three triploid from the 1995 collection. The average deviation among extracted proteins in mass was about $11 \pm 4\%$. Since triploid cedar pollen extract generally contained less of both allergens, the amount of total protein loaded for triploids was either the same or larger. Both types of tree pollens collected in the same area and same year were extracted. The concentrations of Cry j 1 and Cry j 2 were compared with sera of patients specifically allergic to either of the allergens or both. The results, with different clones indicated, are shown in Fig. 2a. The results indicate less Cry j 1 and Cry j 2 in the triploid pollen extracts, although a larger amount of total protein was loaded for triploid. Pollens collected from 1993 to 1995 were also compared, as shown in Fig. 2b. To study geographic variations among Japanese cedar pollens, we extracted and compared pollens collected from central Honshū (Fig. 3). Pollen collected from three different areas consistently showed that extracts of triploid pollen contained a smaller quantity of allergens than diploid pollen.

Discussion

There are two kinds of Japanese cedar trees; the popular one is diploid, and the less popular is

triploid. Though the trees are distinct genetically, there is little morphologic difference between them. However, since triploid trees are sterile, their allergenicity may be affected. Therefore, we screened 100 patient sera from Aichi prefecture, using electrophoretically separated proteins from diploid cedar tree pollen extracts. Representative electrophoretic patterns are shown in Fig. 1b. Of all the screened sera, nearly half reacted to Cry j 1 or Cry j 2. This finding differs from those previously reported. Sawatani et al. (5) reported that 24/25 and 19/25 sera were positive in skin test with purified proteins to Cry j 1 and Cry j 2, respectively. However, when tested by Pharmacia RAST-RIA kit, 21/25 and 17/25 were positive, respectively. Sakaguchi et al. (3), using the enzyme-linked immunosorbent assay (ELISA) with purified protein, reported that 12/14 reacted to Cry j 1 and 10/14 reacted to Cry j 2. A possible cause of the discrepancy between these findings and ours is the study area, because both previous studies were performed in the Kanto area. The incidence of sugi pollinosis in Kanto is 45.6–47.6% and that in the Aichi (Chubu) area is about 24.6–27.8% (7).

Though different chromosome numbers may affect various properties of the pollen gains, it is assumed that chromosome numbers will not affect properties of Cry j 1 and Cry j 2, such as post-translational modification. We confirmed this assumption by partial amino acid sequence from blotted membranes (data not shown) and SDS-PAGE mobility. It is known that the extract ability of these two

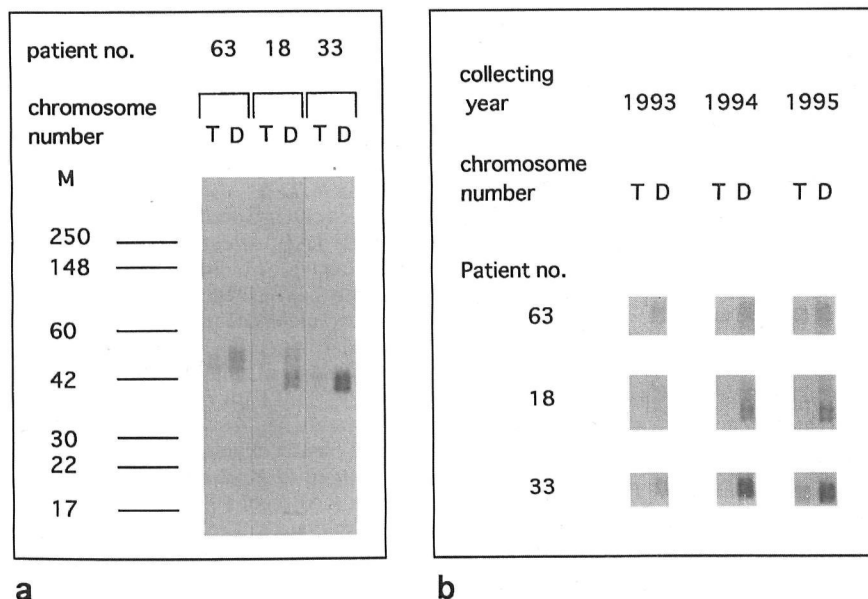


Fig. 2. a) Comparison of Cry j 1 and Cry j 2 in diploid and triploid pollen extracts with patient sera specific to either of allergens or both. T: triploid, 30 μ g; D: diploid, 11 μ g. M: molecular weight in kDa. b) Comparison of Cry j 1 and Cry j 2 between extracts of diploid and triploid pollen collected in different years. T: triploid; D: diploid. Extracts loaded were 1993: (T: 45 μ g, D: 45 μ g), 1994: (T: 30 μ g, D: 15 μ g), 1995: (T: 13 μ g, D: 8 μ g).

area number		1	2	3
chromosome number		T D	T D	T D
patient no.	63			
	18			
	33			

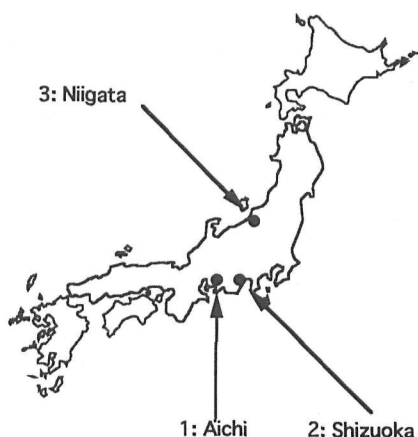


Fig. 3. Comparison of Cry j 1 and Cry j 2 in diploid and triploid pollen extracts from different geographic areas of Japan in 1995. Areas are indicated on map. T: triploid; D: diploid. Extracts loaded are 1) Aichi: (T: 13 μ g, D: 8 μ g), 2) Shizuoka: (T: 15 μ g, D: 15 μ g), 3) Niigata: (T: 10 μ g, D: 10 μ g).

allergens depends on the extraction buffer strength. With a uniform extraction buffer and consistent extraction method, the amount of these two allergens in the extractable proteins can be compared. Using sera of patients allergic to Cry j 1 and Cry j 2, we can show a quantitative difference between the amount of Cry j 1 and Cry j 2 in diploid and triploid cedar tree pollen extracts. The amounts of Cry j 1 and Cry j 2 in the triploid tree pollen extracts seem lower than those in the diploid tree pollen extracts. However, we cannot rule out losses during extraction or differences in extract ability as possible reasons for observed qualitative differences.

The amounts of Cry j 1 and Cry j 2 in extracts of triploid pollens collected from different areas were also less than those of corresponding diploid pollens (Fig. 3). As reported, the percentages of triploid trees in the Niigata, Aichi, and Shizuoka prefectures were 5.94%, 2.38%, and 0.63%, respectively (8). The incidences of Japanese cedar pollinosis in these three prefectures were 9.5%, 24.2–44.1%, and 51.0–71.8%, respectively (7). It seems that the higher the percentage of triploid trees, the lower was the incidence of cedar pollinosis. However, this correlation did not take into account wind

drift in that area. Could this difference lie in the fact that triploid trees are less allergenic? With the skin prick test, the diploid extract did show a stronger reaction than did the triploid extract (data not shown). Since we have observed that the amounts of Cry j 1 and Cry j 2 were less in the triploid pollen extract than those of the diploid trees, the correlation with the incidence of Japanese cedar allergy may be based on differences in extractable allergens.

Pollens collected in different years also demonstrated this difference in quantities of extractable allergens between diploid and triploid forms. The pollen collected in 1993 had a smaller amount of allergens in both forms. This 1993 pollen could have deteriorated during storage at room temperature. The variations in quantity of Cry j 2 were larger than those of Cry j 1 in these pollen extracts. This phenomenon was previously observed in diploid cedar tree pollen extracts (5). Cry j 1 remained at nearly a constant concentration for years, whereas the amount of Cry j 2 varied (Fig. 2b).

Triploid Japanese cedar trees have been grown for many years, but this variety has never been popular in Japan. Since Japanese cedar pollen allergy is rising, it may be necessary to promote the sterile, less allergenic, triploid trees.

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