(original article)

Cross-Allergenicity between *Cryptomeria fortunei* and *Cryptomeria japonica* Pollen

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(Received Sep. 30 1999, Accepted Nov. 15 1999)

The aim of this study is to investigate immunological characteristics of *Cryptomeria fortunei* pollen extract, as well as to study cross-reactivity between *C. fortunei* and *Cryptomeria japonica* pollen extracts.

In ELISA, sera which showed positive against *C. japonica* pollen extract were also positive against *C. fortunei* pollen extract. Specific antibody titers to both pollen extracts showed a high degree of correlation. Binding of IgE antibodies of serum samples from patients allergic to *C. japonica* pollen to the *C. fortunei* pollen extract decreased according to serial dilution of the sera. Using *C. fortunei* pollen extract on the solid phase in ELISA, inhibition reached to 100% with *C. fortunei* or *C. japonica* pollen extract as inhibitor. Same result was obtained when *C. japonica* pollen extract was used on the solid phase. The rate of positive reaction in nasal provocative test using a disk containing *C. fortunei* pollen extract was 70% in subjects exclusively sensitized to *C. japonica* pollen. In SDS-PAGE, the protein profile of the two extracts showed almost the same pattern. IgE immunoblots indicated that molecular mass of the most intensive IgE-bound band was 42kDa in both extracts. These results indicate : 1) *C. fortunei* pollen has allergenic activity, 2) Cross-reactivity is present between *C. fortunei* and *C. japonica* pollen extracts, 3) Molecular mass of the major allergen of *C. fortunei* pollen is 42kDa.

Key words : allergen, cross-allergenicity, *Cryptomeria fortunei*, *Cryptomeria japonica*, pollen

Introduction

Cryptomeria japonica (*Sugi* in Japanese, a family of Taxodiaceae) is the major cause of pollinosis in Japan. The prevalence of *C. japonica* pollinosis in a densely cultivated area is about 10%[1].

On the other hand, *Cryptomeria fortunei* (*Yanagi-sugi* or *Ryuzan* in Japanese) is plentifully planted in China. To our knowledge, it is present in Kyoto, Okayama and Kumamoto prefectures at least. Flowering period and shape such as leaf, male flower (Fig. 1) and pollen (Fig. 2) of *C. fortunei* are

Fig. 1 : Male flower of *Cryptomeria fortunei*

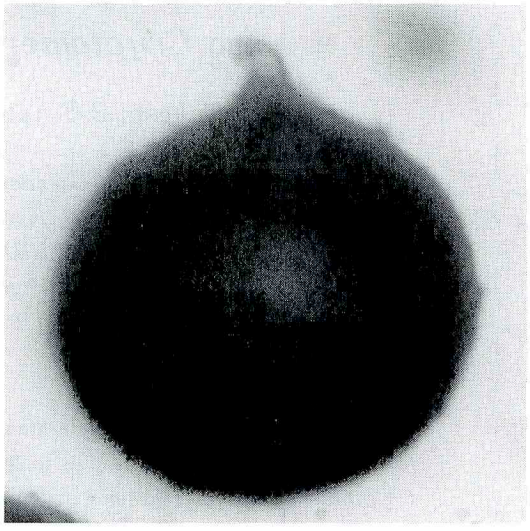

Fig. 2 : *C. fortunei* pollen grain
diameter ~35μm. Original magnification × 1,000

very similar to those of *C. japonica*. So, it is difficult to differentiate those pollen grains in airborne pollen sampling. These facts suggest *C. fortunei* pollen may contribute to the generation of pollinosis.

In this context, we intended to investigate whether *C. fortunei* pollen extract has IgE-binding capacity and whether cross-reactivity is present between *C. fortunei* and *C. japonica* pollen extracts.

Materials and methods

Pollen

Pollen from *C. japonica* was collected as follows :

Several male inflorescences just a few days before dehiscence were placed on water in a 500ml flask. Water-supply was led by a vinyl tube. The inflorescences were wrapped with a parchment paper bag with holes at the top for ventilation. After full dehiscence, holes on the bag were sealed with plastic tape. The inflorescences with wrapped bag were pulled up from a flask and they were shaken vigorously to release pollen. Thus, pollen without any contaminants was obtained. Collected pollen was stored at −80℃ until use.

C. fortunei pollen was obtained from Kansai Breeding Office, National Forest Tree Breeding Center, Okayama.

Pollen extracts

C. fortunei and *C. japonica* pollen were separately suspended in 0.1 M Tris-HCl, pH 8.0 (20ml / g pollen). The samples were shaken in a glass flask at 4 ℃ for 16hr and centrifuged at 10,000rpm for

15min. The supernatant was dialyzed against distilled water and lyophilized.

Protein content of lyophilized samples of *C. fortunei* and *C. japonica* pollen was 16% and 20%, respectively. These were determined using bicinchoninic acid [2].

Human sera

Serum samples were obtained from patients who showed nasal allergic symptoms during *C. japonica* flowering season and were positive in RAST to *C. japonica* pollen. A serum pool was prepared from equal volumes of sera from 10 individuals having RAST-class 3 or more against *C. japonica* pollen. Control serum samples were obtained from three healthy volunteers with no history of allergies. These sera were stored at −80℃ until use.

Enzyme-linked immunosorbent assay (ELISA)

IgE-ELISA was performed on flat-bottom plates coated with 50 μl aliquots of *C. fortunei* or *C. japonica* pollen extract. The plates were prepared as follows : The pollen extract (10 μg protein / ml) dissolved in 0.01 M sodium phosphate buffer containing 0.9% sodium chloride (PBS, pH 7.6) were placed in the wells and incubated overnight at 4℃. Then the wells were blocked with 100 μl of 3% bovine serum albumin (BSA, SIGMA) dissolved in PBS. The assay was carried out with 50 μl of serum at 30 ℃ for 1hr. After the incubation, the wells were washed and incubated with affinity purified peroxidase labeled goat anti-human IgE $_{(\varepsilon)}$ (KPL, 1 : 1,000 v / v, diluted in PBS containing 1% BSA) at 30℃ for 1hr. After washings, 100 μl of freshly prepared substrate solution, *o*-phenylenediamine, was added and 15min later, the reaction was stopped by the addition of 100 μl of 1 N sulphuric acid and then the absorbance at 490nm was measured using a microplate reader (BIORAD).

ELISA titration

ELISA titration of specific IgE for pollen allergen was carried out with a serial dilution of serum pool with PBS containing 1% BSA. Specific IgE-binding to *C. fortunei* or *C. japonica* pollen extract was determined as described above.

ELISA inhibition

Fifty microliters of serum samples were pretreated with an equal volume of serially diluted each pollen extracts for 1hr at room temperature. Then the concentration of residual IgE antibodies was determined by ELISA. Percent inhibition was calculated based on the values obtained in the absence of inhibitor in the system.

Nasal provocative test

Lyophilized extract from *C. fortunei* pollen was dissolved in distilled water at the concentration of 20mg / ml. This was kept as stock solution. Five microliters of stock solution were dropped on a 3mm disk (No. 8 filter paper, TOYO ROSHI) and then dried under vacuum. In this way, allergen disks containing 100 μg of extract per disk were prepared. Allergen disks containing 300 μg protein of extract were prepared by repeating three times this procedure.

Nasal provocative test was carried out according to the method of Okuda [3] using the disk as described above.

After informed consent was obtained, control disk without allergen was initially placed on the surface of the anterior part of the inferior turbinate on one side of the nose. If no reaction developed within 5min, the disk was removed. Then a disk containing 100 μg of extract was put on the same

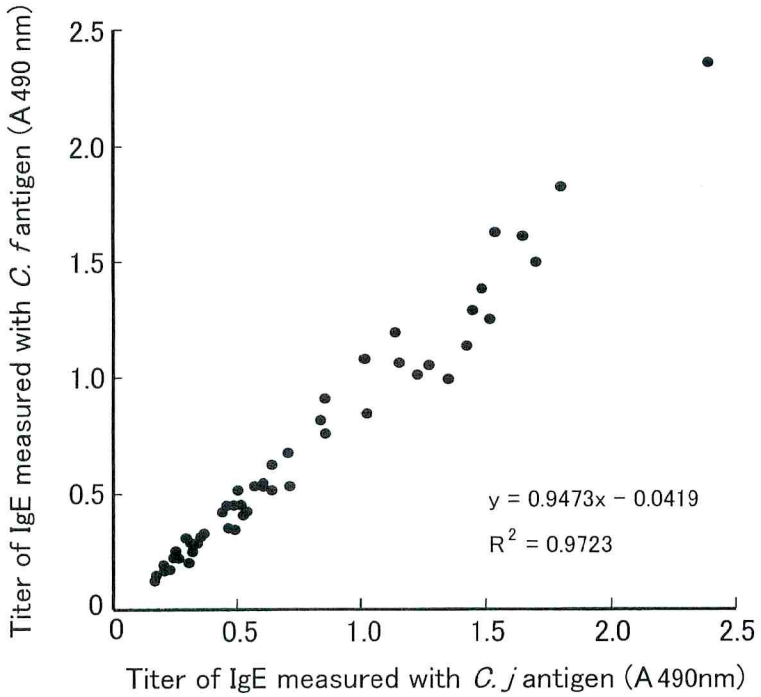

Fig. 3 : IgE-binding activity of *C. fortunei* and *Cryptomeria japonica* pollen extracts

The correlation coefficient of IgE-binding activity between *C. fortunei* and *C. japonica* pollen extracts was calculated to be 0.986.

portion. When it was negative, subject was finally provoked with a disk containing 300μg of extract. When 2 or more of symptoms such as nasal itching, sneezing attacks, watery discharge and nasal obstruction appeared, the test was regarded as positive.

This test was performed out of *C. fortunei* flowering season.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out essentially by the method of Laemmli [4] in a mini slab gel apparatus by using a discontinuous buffer system. The running gel contained 10% acrylamide. Samples containing 50μg of extract were prepared under non-reducing conditions and applied to sample wells.

IgE immunoblots

After SDS-PAGE, the *C. fortunei* or *C. japonica* pollen extract components were electroblotted to a polyvinylidene difluoride (PVDF) membrane (Immobilon P®, Millipore), essentially as described by Towbin et al. [5]. The PVDF membrane was blocked with 3% BSA dissolved in PBS, cut into strips and incubated with a serum pool. After washing, the strips were incubated with horseradish peroxidase labeled anti-human IgE (KPL). The allergens were visualized by ECL Western blotting detection system (Amersham) on X-ray film.

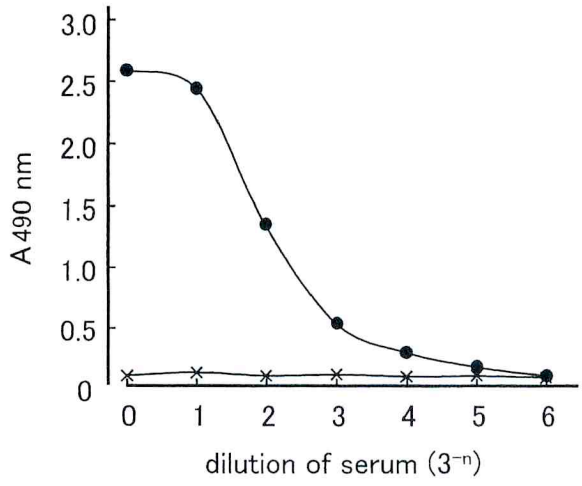

Fig. 4 : ELISA titration

Titration of specific IgE antibodies reactive with *C. fortunei* pollen extract was carried out by serial dilution method using a serum pool obtained from subjects sensitized to *C. japonica* pollen (●) and a non-allergic subject (×).

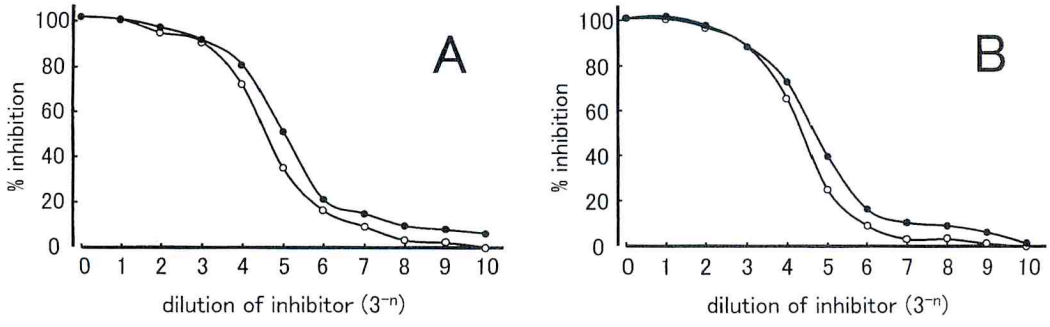

Fig. 5 : ELISA inhibition curves with a serum pool obtained from subjects sensitive to *C. japonica* pollen

C. fortunei (A) and *C. japonica* (B) pollen proteins were coated to flat-bottom plates before addition of serum preincubated with *C. fortunei* (○) or *C. japonica* (●) pollen proteins.

Results

IgE-binding capacity of *C. fortunei* pollen extract

Fifty-two sera which showed positive against *C. japonica* pollen extract in ELISA were tested for reactivity to *C. fortunei* pollen extract. As shown in Fig. 3, IgE titer measured using two antigen samples showed a close relation. The correlation coefficient was calculated to be 0.986.

ELISA titration

Titration curve was shown in Fig. 4. Binding of IgE antibodies in a serum pool decreased according

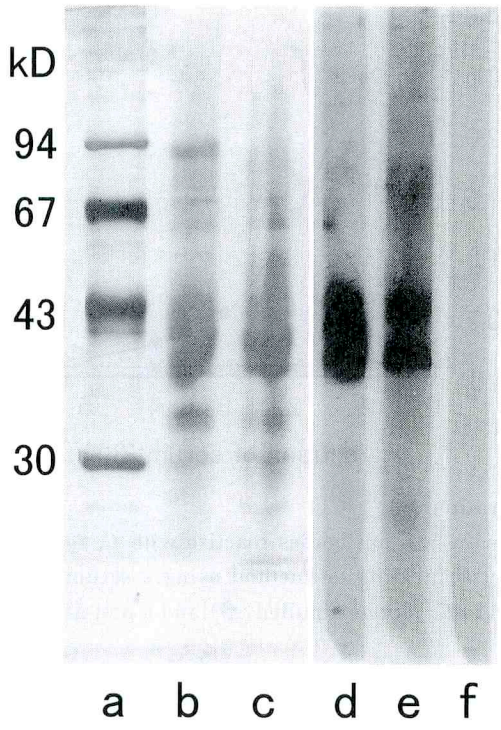

Fig. 6 : *C. fortunei* (b, d, f) and *C. japonica* (c, e) pollen extracts after separation by SDS-PAGE under non-reducing conditions and IgE-immunoblots

Molecular mass marker (a), silver staining (b, c), immunoblot analysis of binding of IgE antibodies in a serum pool from subjects allergic to *C. japonica* pollen (d, e) and immunoblotting developed with a serum from non-allergic subject (f).

to serial dilutions of the serum.

ELISA inhibition

The inhibitory capacities of different concentrations of both pollen extracts were shown in Fig. 5. Using *C. fortunei* or *C. japonica* pollen extract on the solid phase, inhibition reached to 100% with *C. fortunei* or *C. japonica* pollen extracts as inhibitor at the highest concentration used.

Nasal provocative test

Ten subjects exclusively sensitized to *C. japonica* pollen were tested. Six out of ten subjects showed a positive response to a disk containing 100μg of extract. One out of ten was positive to a disk containing 300μg of extract. Three out of ten patients were negative. No responses were elicited in non-allergic individuals.

SDS-PAGE / immunoblots

The protein profile of both pollen extracts was demonstrated by SDS-PAGE and subsequent silver staining. Immunoblots of allergen were carried out with a serum pool. As shown in Fig. 6, several

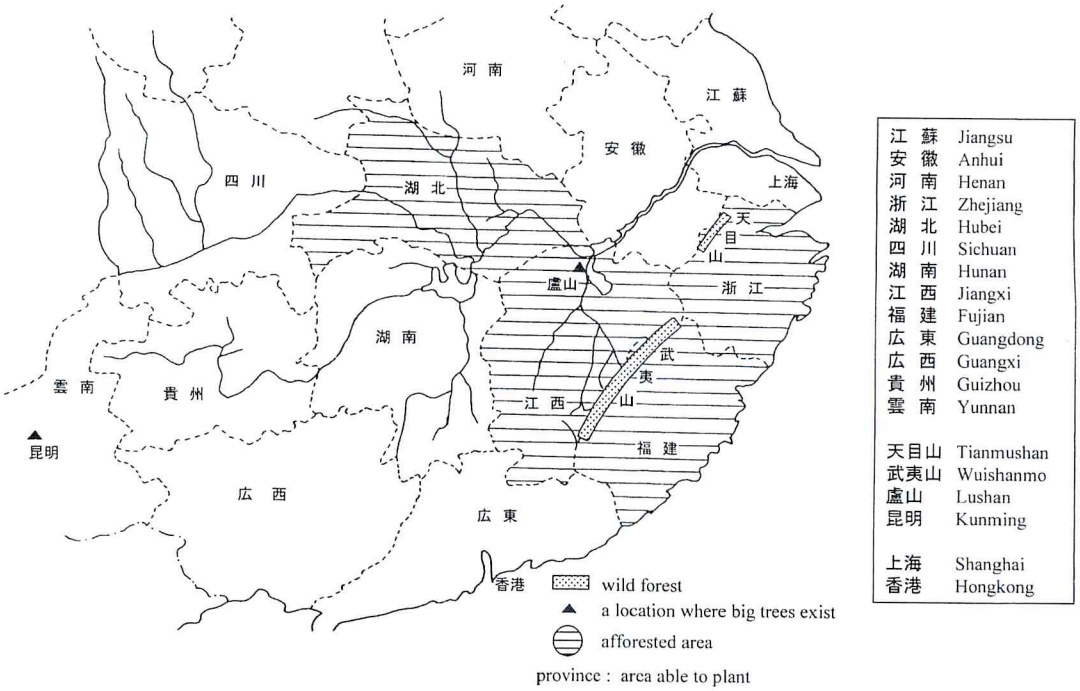

Fig. 7 : Distribution of *C. fortunei* in China (adapted from Hahimoto, Y. [6])

immunoreactive bands were detected. The most intensive band was recognized at around 42kDa in both extracts.

Discussion

C. fortunei seeds were brought from Beijing, China in 1979. The seeds were sown and grown to seedlings at Kamigamo Farm, Kyoto University. A part of the seedlings were transfered to Kansai Breeding Office, National Forest Tree Breeding Center in 1982. *C. fortunei* pollen used in this study was obtained from these trees.

Fig. 7 shows a distribution of *C. fortunei* in China [6].

ELISA was performed with use of sera from 52 individuals sensitive to *C. japonica* pollen. The IgE titer reactive with *C. japonica* pollen extract showed a close correlation with that assayed with *C. fortunei* pollen extract. The correlation coefficient was calculated to be 0.986. In ELISA titration, *C. fortunei* pollen extract showed dose-dependency upon varying concentration of a serum pool indicating that the observed binding of IgE to the antigen is a specific one. However, this fact does not always mean that *C. fortunei* pollen has allergenic activity. Because, the term "allergens" is defined as substances in general which induce the state of hypersensitivity or elicit hypersensitivity reactions in sensitized subjects. We carried out nasal provocative test against subjects exclusively sensitized to *C. japonica* pollen. Seventy percent of the subjects revealed positive reaction to *C. fortunei* pollen extract. These results clearly show that *C. fortunei* pollen extract has allergenic activity.

Cross-reactivity between those allergens was further confirmed by inhibition experiment using *C. fortunei* pollen extract as solid phase. *C. japonica* pollen extract at the protein concentration of 5 μg /

ml inhibited the binding of *C. japonica* pollen-specific IgE antibodies, vice versa. Same results were obtained in homologous system. These results represent that cross-allergenicity is present between *C. fortunei* and *C. japonica* pollen.

C. fortunei pollen proteins were separated by SDS-PAGE, and IgE-binding proteins were detected using a serum pool. Applying IgE-immunoblots, IgE antibodies in the serum were found to be reactive to a 42kDa protein most intensively. This molecular mass was in good agreement with the major allergen of *C. japonica* pollen, Cry j 1 [7]. From these results, it was considered that this protein was to be the major allergen of *C. fortunei* pollen.

This is the first report on cross-allergenicity in the same genus, *Cryptomeria*, although there are some reports concerning cross reactivity among Taxodiaceae and taxonomically related plants [8-12].

To characterize the major allergen of *C. fortunei* pollen in detail and to investigate cross-reactivity among *C. fortunei* and taxonomically related plants, it is necessary to construct cDNA library encoding for the protein.

Acknowledgements

We are much obliged to Kansai Breeding Office, National Forest Tree Breeding Center for supplying *C. fortunei* pollen. We thank Mr. Akira Okazaki, Nara Prefectural Forest Experiment Station for sharing his knowledge on *C. fortunei*. We sincerely appreciate the technical assistance of Mr. Hideaki Kondo. This work was supported by a grant from the Science and Technology Agency, Japan.

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ヤナギスギ花粉とスギ花粉の共通抗原性

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中国に多数植林されているヤナギスギ（中国名：柳杉）は，少数ながら日本にも存在する．ヤナギスギの樹形，雄花，花粉の形や開花期はスギのそれらと相似している．スギ花粉症患者血清を用いて，ヤナギスギ花粉の免疫学的特徴を検討した．両花粉エキスは，ELISA の吸光度で強い相関を，SDS-PAGE でほぼ同じ挙動を示した．ELISA 希釈試験で吸光度は血清濃度依存的であり，ELISA 吸収試験ではどちらのエキスを固相，液相に用いても同じように吸収された．スギ花粉単独感作症例を対象としてヤナギスギ花粉鼻ディスクによる鼻粘膜誘発試験を施行すると 70% が陽性であった．以上の結果から，ヤナギスギ花粉にはアレルゲン活性が存在し，これと IgE 抗体との結合は特異的であり，両花粉エキスの間には強い共通抗原性が存在することが確かめられた．IgE immunoblots では，ヤナギスギ花粉エキスが IgE 抗体ともっとも強く結合したバンドの分子量は，スギ花粉エキスの場合と同じ 42kDa であり，これがヤナギスギ花粉の主要アレルゲンと考えられた．