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Original Article

Immunologic determination of the major allergen, Cry j 1, in *Cryptomeria japonica* pollen of 117 clones in Toyama prefecture: Some implications for further forestry research in pollinosis prevention

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Abstract

Background: A number of recent studies have clarified the characteristics of major allergens of *Cryptomeria japonica*, such as the locality, molecular weights of Cry j 1 and Cry j 2 and cross-reactivity with some other species of pollen. However, in order to plant *C. japonica* forests with low allergen levels, there is still a shortage of fundamental data for forest breeding.

Methods: To obtain the some fundamental data concerning Cry j 1, three items were investigated: (i) variations in weight and Cry j 1 content per single pollen grain among 117 clones; (ii) environmental factors affecting Cry j 1 content; and (iii) genotype variations of Cry j1.

Results: Although the variation in weight per single pollen grain among 117 clones was relatively small (mean (±SD) 10.2 ± 0.1 ng), the Cry j 1 content was found to vary widely from 0.26 to 7.54 pg. The expression level of Cry j 1 was higher in samples collected from lower altitudes. Using western blotting, it was shown that there were two Cry j 1 genotype variations.

Conclusions: From these results, we have concluded that immunologic determination of Cry j 1 by individual pollen grain levels provides useful data, especially for forest breeding to prevent pollinosis.

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Key words: Cry j 1, *Cryptomeria japonica* D. Don, environmental factor, genotype variation, Japanese cedar pollinosis.

Introduction

Japanese cedar pollinosis is a type I allergy that is caused by two kinds of major allergens, Cry j 1[1] and Cry j 2[2] in the pollen. The number of patients increases yearly. So, it is of importance to reduce allergen quantity in the air by forest-managing efforts. A number of recent studies have clarified the characteristics of major allergens of *Cryptomeria japonica*, such as the locality,[3] molecular weights[1] of Cry j 1 and Cry j 2 and cross-reactivity[4] with some other species of pollen. However, in order to plant sugi forests with low allergen levels, there is still a shortage of fundamental data for forest breeding. Therefore, the present study was performed to obtain some fundamental data, especially regarding the variation of Cry j 1 content per pollen grain among a variety of sugi plus trees. Environmental factors influencing Cry j 1 content and the genotype variation of Cry j 1 were also studied.

Methods

Measurement of Cry j 1 content per pollen grain and comparison of individual variations of two cultivars

Two kinds of cultivars (Mio and Masuyama) were used. They were planted in the Toyama forest experiment station. Pollen samples were collected from eight individuals.

Collected pollens were dried using Silica gel and stored at 4°C until use. After 0.1 g pollen had been dissolved in 10 mL water and mixed by vortexing, 1 μL was taken and the number of pollen grains was counted using the blood corpuscle calculation board (Fox Rosenthal board) after a 10-fold dilution (Fig. 1). The weight of one pollen grain was obtained by calculations based on these numerical values. In addition, 0.1 g pollens was separately suspended in Coca solution for 3 h for extraction and the Cry j 1 content was determined by the fluorescent sandwich ELISA method.[5] Then, values were converted into Cry j 1 content per pollen grain.

Comparison of Cry j 1 content per pollen grain among clones

Pollen samples were collected from 117 clones of the plus trees selected in Toyama prefecture. The weight and Cry j 1 content per pollen grain were measured by the same method (ELISA).

Comparison of Cry j 1 content by age and gibberellin processing

Bokasugi trees planted in Toyama forest experiment station were used. One sample was collected from a 14-year-old tree that underwent the gibberellin processing.

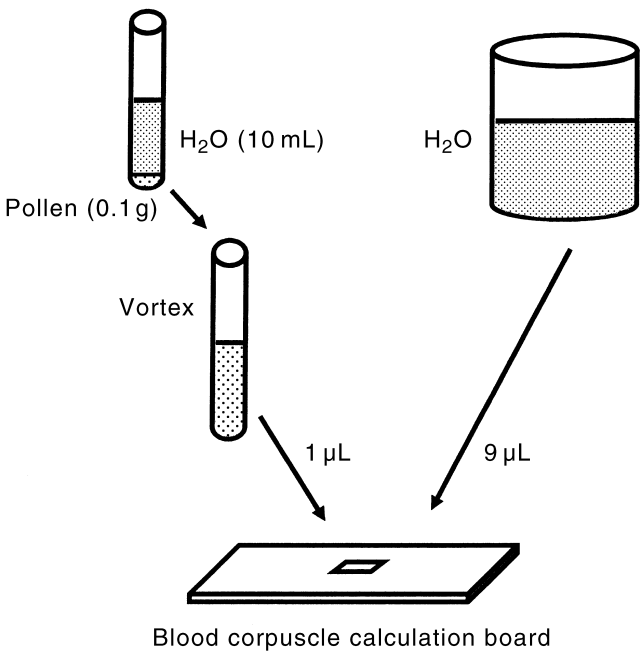

Fig. 1 The method of counting pollen grains.

Another sample was taken from a 51-year-old tree that had not undergone the processing. The Cry j 1 content per pollen grain was measured by the same method (ELISA).

Comparison of Cry j 1 content at different altitudes

The pollen of Bokasugi was collected from eight individuals planted at heights of 9 and 240 m (four samples from each altitude). The Cry j 1 content per pollen grain was measured by the same method (ELISA).

Investigation of genotype variations of Cry j 1

Genotype variations were investigated by western blotting. Pollen was collected from 103 clones of plus trees selected in Toyama prefecture. Pollen extracts were prepared by suspension in Coca solution for 3 h. Electrophoresis of the pollen extract was performed on a sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE). Protein components separated by SDS-PAGE were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk solution dissolved in Tris-buffered saline (TBS). After washing, the membrane was reacted with anti-Cry j 1 IgG (Cosmo Bio, Tokyo, Japan). After washing with TTBS (TBS added to 0.05% Tween 20), the membrane was reacted with antirabbit IgG labeled with alkaliphosphatase (Dakopatts, Glostrup, Denmark). Allergen bands were detected using a 5-bromo-4-chloro-3-indolyl phosphate potassium salt and nitro blue monotetrazolium chloride (BCIP/NBT) kit (Vecter Laboratories, Burlingame, CA, USA) and the mobility position was compared. Results are presented as the mean ± SD.

RESULTS

Variations in the weight and Cry j 1 content per pollen grain among individuals of the same cultivar

The weight per pollen grain of two cultivars is shown in Fig. 2. It was clarified that there was an approximate 1.2-fold difference between Mio (10.09 ± 0.26 ng) and Masuyama (8.59 ± 0.23 ng). However, there was no significant difference among individual samples from the same cultivar. On the basis of the calculation of this result, it was found that there was significant difference of approximately 1.8-fold between Mio and Masuyama in Cry j 1 content per pollen grain (Fig. 3).

Variations in weight and Cry j 1 content per pollen grain among clones

The distribution of the weight and Cry j 1 content per pollen grain among 117 clones is shown in Fig. 4. There was an approximate 1.9-fold difference between the lightest and heaviest clone. However, 72 of 117 clones were 10–11 ng in weight and the mean weight was 10.2 ± 1.16 ng. From this result, it was clarified that the variation in weight per pollen grain among clones was relatively small. Based on the calculation of this result, the Cry j 1 content per pollen grain varied from 0.26 to 7.54 pg and the mean Cry j 1 content per pollen grain was 3.7 pg. It was clarified that there was an approximate 29-fold difference between clones with the lowest and highest Cry j 1 content planted in Toyama prefecture.

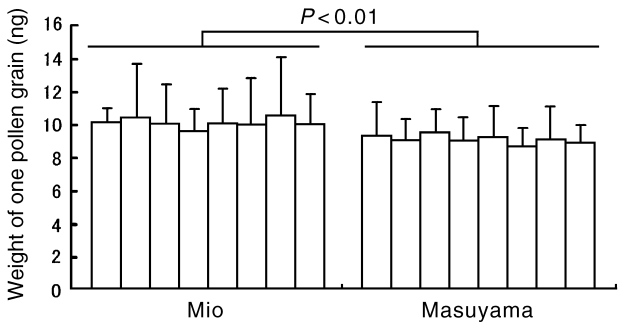

Fig. 2 Comparison of the weight and variations per pollen grain between two cultivars.

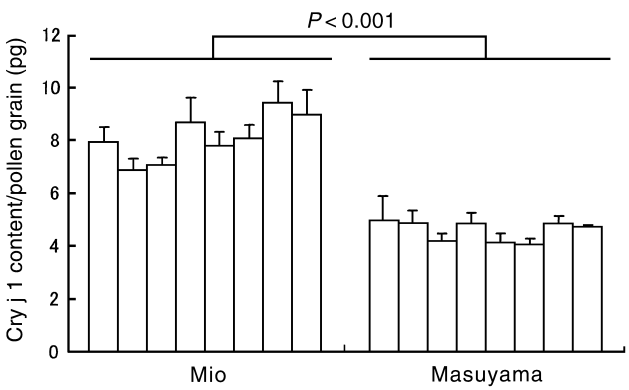

Fig. 3 Comparison of Cry j 1 content and variations per pollen grains between two cultivars.

Difference in Cry j 1 content by age and gibberellin processing

No significant difference was observed in Cry j 1 content between the 14-year-old tree that underwent gibberellin processing (1.97 ± 0.21 pg) and the 51-year-old tree that did not undergo the processing (1.95 ± 0.13 pg).

Difference in Cry j 1 content at different altitudes

The results of the Cry j 1 content of individuals planted at different altitudes (9 and 240 m) are shown in Fig. 5. The Cry j 1 content (3.93 ± 0.32 pg) of individuals planted at 9 m was greater than for those planted at 240 m (1.92 ± 0.14 pg; $P < 0.01$).

Investigation of genotype variations of Cry j1

The result of western blotting is shown in Fig. 6. Two bands were detected by western blotting using anti-Cry j 1 antibody at approximately 40 kDa, as reported by

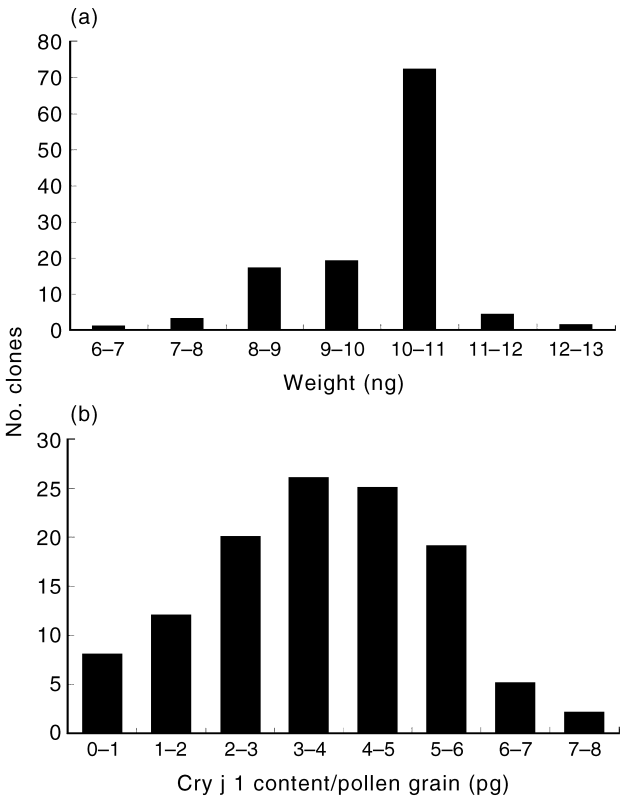

Fig. 4 Variations in (a) weight and (b) Cry j 1 content per pollen grain among clones.

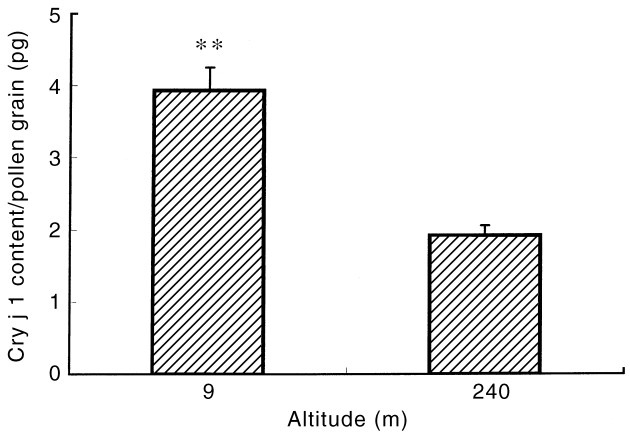

Fig. 5 Differences in Cry j 1 content at different altitudes. $^{**}P < 0.01$.

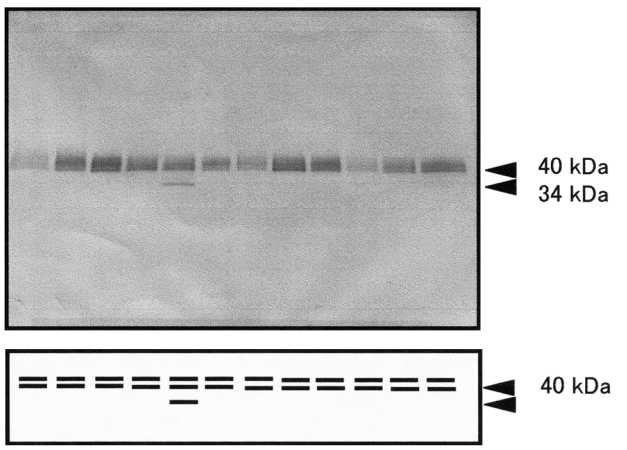

Fig. 6 The band pattern of Cry j 1 in the clone with genotype variation.

Yasueda *et al*.[1] However, a new band was detected at 34 kDa in nine clones. There were no significant differences in Cry j 1 content between clones that possessed the new band (3.68 ± 1.65 pg) and those that did not (3.64 ± 1.45 pg).

Discussion

It is a new challenge for pollinosis prevention that sugi (*C. japonica* D. Don) trees that emit low allergen pollen and produce excellent wood are used. For this purpose, we must undertake some basic investigations in order to select sugi with low allergens. The variation of Cry j 1 content per 1 or 100 g pollen among clones has been reported previously.[6–9] However, in this case, the difference in weight per pollen grain was not considered. So, we started to calculate the weight per pollen grain. In the present experiments, it was clarified that there was a difference among the clones, although the variation was small. There are many reports stating that the size and the form of the pollen become abnormal[10–12] when there is an abnormality in the chromosome number, such as triploids. Triploids and aneuploids were found in the plus tree of sugi.[13] So, it was decided that the Cry j 1 content should be measured based on calculations of the weight per pollen grain. It is also necessary to investigate whether the quantity of the allergen is influenced by the environment. From these experiments, it was clarified that the expression level of Cry j 1 was higher in samples collected at 9 m compared with samples collected at 240 m. It has been reported that birch pollen allergen (Bet v 1) content was higher in places where the daily mean temperature was higher during the growing season.[14] So, the Cry j 1 expression level may also be affected by temperature because there is a temperature difference of approximately 1.5°C between altitudes at 9 and 240 m throughout the year. In addition, it will be necessary to examine the soil conditions etc., in the future. From these facts, when we select clones with low allergen level, we should collect the pollens from the clone accumulation place under the same conditions, such as seed orchard and cutting orchard. In addition, it has been reported that there was an approximate 1.2-fold difference in Cry j 1 content when the pollen was collected from the same tree for 5 years.[15] So, we should investigate pollens as much as possible in the same year.

It was clarified that the variation in Cry j 1 content among the clones was very large and there was a 29-fold difference between clones with the highest and lowest Cry j 1 content. Although the mean Cry j 1 content determined in the present study (3.7 pg) was less than that of a previous report (5.53 pg),[15] this may be explained by the different kinds of sugi in each region. Taking these facts into consideration, it is necessary to convert sugi pollen information from the dispersion number of pollens into the concentration of the allergen in the air. In order to plant sugi forests with low allergen generation, we have to investigate the inheritance. There was no difference in Cry j 1 content among individuals from the same cultivar and Cry j 1 content was not influenced by gibberellin processing and aging. So, it was thought that the

heritability of Cry j 1 is comparatively high. After this, we will investigate more details of inheritance effects in the progeny.

It was clarified that there was a genotype variation in Cry j 1 and it was divided into two types. The ratio of clones with a band at 34 kDa was approximately 9% in Toyama prefecture. Further research will be necessary to determine whether there is a geographic tendency that would be applicable throughout Japan.

Although further study would be necessary, especially on Cry j 2, our method to determine Cry j 1 in individual pollen grains will provide useful information for forestry research, especially for forest-breeding methods of sugi.

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