

Changes in quantity of Cry j1, a major cedar pollinosis allergen, in *Cryptomeria japonica* pollen during spring in Japan

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(Received 15 September 2011; final version received 12 September 2012)

The changes in quantity of Cry j1 in *Cryptomeria japonica* pollen were assessed during the course of spring in Japan. *C. japonica* pollen was collected from the same trees in Hiroshima and Yamanashi Prefectures, and Cry j1 was quantified by enzyme-linked immunosorbent assay. The quantities of Cry j1 were found to gradually decrease, and this trend was similar in both Hiroshima and Yamanashi Prefectures. In Yamanashi Prefecture, no significant differences in the quantities of Cry j1 between the upper and lower parts of the tree were observed in samples collected from March 1 to 14. Furthermore, the quantities of Cry j1 on March 1, 5, 10 and 14 were significantly lower than those on February 1. These results suggest that expression of *C. japonica* pollen levels in terms of particle numbers is not sufficient and that quantities of Cry j1 vary throughout the course of spring

Keywords: *Cryptomeria japonica*; pollen; Cry j1; cedar pollinosis

Introduction

The allergic reaction induced by *Cryptomeria japonica* pollen is a typical type I allergy. The number of *C. japonica* trees in Japan has increased threefold over the last 35 years (Murayama, 2002), and the number of Japanese patients with allergies induced by the pollen of *C. japonica* has thus increased in recent years (Maeda, Akiyama, & Shida, 2008; Sakaguchi, Hirahara, Fujimura, & Toda, 2011; Terada et al., 2009). Approximately 16.2% of Japanese experience cedar pollinosis during spring (Baba & Nakamura, 2003; Dake et al., 2006), while 30% of adults between 20 and 30 years of age possess IgE antibodies against *C. japonica* pollen (Inoue, Sakaguchi, Hori, & Watanabe, 1986; Inoue et al., 1988; Takahashi et al., 2007). Furthermore, about 80% of patients are aged between 20 and 30 years and live in Tokyo (Endo, 2002).

Cry j1 and Cry j2 were identified as the principal allergens in Japanese cedar pollen in 1983 and 1990, respectively (Sakaguchi et al., 1990; Yasueda, Yui, Shimizu, & Shida, 1983), and the immunological profiles of these allergens have since been investigated. Cry j1 and Cry j2 are glycoproteins with of a molecular weight of approximately 40,000 Da. However, the antigenicity of Cry j1 differs from that of

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Cry j2, and Cry j1 is present in Japanese cedar pollen at levels far lower than Cry j2, with Cry j1 being mainly present in the Ubisch body and exine (Nakamura, Sato, & Nakamura, 2004; Saito & Teranishi, 1999).

Pollen counts for *C. japonica* pollen began in 1965 (Murayama, 2002), and since then, airborne pollen advisories have been issued to the public every spring (Takahashi et al., 1993). However, the amount of allergen present is not estimated, as this information is not taken into consideration when assessing *C. japonica* pollen levels. In the present study, the amount of Cry j1, a major allergen present in *C. japonica* pollen (Sakaguchi et al. 1990; Sugata et al., 2007), was assessed and changes in quantities of Cry j1 during the spring were determined.

Materials and methods

Separation of Cry j1 from C. japonica pollen

C. japonica pollen was collected in Hiroshima (four trees) and Yamanashi (three trees) Prefectures and was stored at -80°C until use. Soluble antigen was extracted from *C. japonica* pollen using a fast protein liquid system according to the procedure of Yasueda et al. (1983). One hundred grams of pollen was mixed with 1000 ml of diethyl ether to remove fat and was then added to 1000 ml of 0.125 M ammonium bicarbonate solution (pH 8.0), followed by stirring for 24 h at 4°C . This suspension was then centrifuged at $10,000 \times g$ for 20 min, and the supernatant was used as *C. japonica* pollen antigen.

The soluble components were separated from *C. japonica* pollen by anion exchange chromatography using fast protein liquid chromatography (FPLC) on a Hi Load Q Sepharose column (GE Healthcare UK Ltd.). The components were separated in the non-adsorbed fraction by cationic exchange chromatography using FPLC on a Hi Load SP Sepharose column (GE Healthcare UK Ltd.) subsequently. The components separated from the fractions previously were subjected to gel filtration chromatography on a Superdex G75 column (GE Healthcare UK Ltd.) and the fractions corresponding to molecular weight of Cry j1 were collected.

Extraction of Cry j1 for sample measurement

Pollen was immersed in distilled water for 24 h at 37°C , after which 1 g of pollen was added to 20 ml of 0.125 M ammonium bicarbonate solution (pH 8.0), followed by stirring for 24 h at 4°C . This suspension was then centrifuged at $2150 \times g$ for 25 min, and supernatant was collected as Cry j1 antigen.

Preparation of anti-Cry j1 antibody

Rabbits (body weight, 3 kg; Saitama Experimental Animals Supply Co., Ltd., Saitama, Japan) were used for preparation of anti-Cry j1 antibody. Rabbits were reared individually in cages housed in an animal room maintained at a temperature of $22 \pm 2^{\circ}\text{C}$ and a humidity of $60\% \pm 10\%$ under a 12-h light/dark cycle (07:00–19:00). The air in the room was changed 15 times per hour. Rabbits were fed solid stock food (Oriental Yeast Co., Ltd., Tokyo, Japan) and were allowed free access to water.

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Rabbits were intradermally immunised with isolated Cry j1 mixed (1:1) with Freund's complete adjuvant (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) initially, followed by Cry j1 solution nine times at 7-day intervals. Blood was collected from rabbits immunised 10 times with Cry j1, which were sacrificed under anaesthesia with pentobarbital sodium (Kyoritsu Seiyaku Corporation, Tokyo, Japan), and sera were obtained by centrifugation at $2190 \times g$ for 20 min. Anti-Cry j1 IgG antibody was separated from sera by affinity chromatography using Protein G (GE Healthcare UK Ltd., Little Chalfont, UK).

All experiments conformed to Japanese regulations with regard to animal care and use, as specified in the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, 1987), and protocols were approved by the Animal Research Committee of Azabu University.

Measurement of protein

Concentrations of IgG antibody were measured using a commercial kit (Bio-Rad Protein Assay Kit I; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Concentrations of Cry j1 were measured using the Bradford method with Coomassie Brilliant Blue G-250 (Bradford, 1976).

Conjugation of rabbit anti-Cry j1 antibody with horseradish peroxidase

Rabbit anti-Cry j1 antibody was conjugated with horseradish peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan), according to the procedure of Nakane and Kawaoi (1974).

Enzyme-linked immunosorbent assay for Cry j1

Rabbit anti-Cry j1 antibody in 0.05 M sodium bicarbonate buffer (pH 9.6) was incubated at 100 μl /well at room temperature for 1 h. After blocking with 1% bovine serum albumin (Wako Pure Chemical Industries Ltd.) in sodium bicarbonate buffer (pH 9.6) at 200 μl /well, plates were incubated at room temperature for 1 h, followed by rinsing with phosphate buffered saline (PBS, pH 7.2) containing 0.1% polyoxyethylene (20) sorbitan monolaurate (Wako Pure Chemical Industries Ltd.). Standard solutions with a known Cry j1 concentration or sample were then added at 100 μl /well. Plates were incubated at room temperature for 1 h and were rinsed as described previously. Peroxidase-conjugated rabbit anti-Cry j1 antibodies were added at 100 μl /well, and plates were rinsed as described previously. Substrate was 1% 2,2-azino-di-(3-ethyl-benzthiazoline sulphonic acid-6) (ABTS; Zymed Laboratories, San Francisco, CA, USA), and after incubation for 1 h, absorbance at 415 nm was measured using a microplate reader (Corona Electric Co., Ltd., Ibaraki, Japan).

Statistics

Quantities of Cry j1 in *C. japonica* in the upper and lower parts of trees in Yamanashi Prefecture were assessed using unpaired Student's *t*-test. The significance of differences in quantities of Cry j1 in *C. japonica* in Yamanashi Prefecture at the various sampling dates (February 25 and March 1, 5, 10 and 14) was compared by

two-tailed multiple *t*-test with Bonferroni correction following ANOVA. *P*-values <0.05 were considered to be significant.

Results

Quantity of Cry j1 in C. japonica pollen

The satisfactory linear standard curve of ELISA for measurement of Cry j1 was obtained (Figure 1). The detection limit was 31.3 ng/ml.

The contents of Cry j1 *C. japonica* pollen collected from trees in Hiroshima and Yamanashi Prefectures are shown in Tables 1 and 2, respectively. Quantities of Cry j1 gradually decreased in mid-March in Hiroshima and Yamanashi Prefectures. In Yamanashi Prefecture, no significant differences in the quantities of Cry j1 between the upper and lower parts of the tree were observed in samples collected from March 1 to 14. The quantity of Cry j1 on February 1 was found to be significantly higher when compared to those on March 1, 5, 10 and 14.

Discussion

Areas populated by *C. japonica* occupy about 18% of the forested areas of Japan (Kiyono, Nagao, & Shinohara, 2002). Cases of cedar pollinosis, an allergy to cedar pollen, have been increasing in recent years (Dake et al., 2006; Murayama, 2002; Takahashi et al., 2007). *C. japonica* pollen counts are, therefore, monitored during spring, and the information is provided to the public. However, quantities of Cry j1, the major allergen in *C. japonica* pollen, are not monitored. In the present study, changes in the quantity of Cry j1 in *C. japonica* pollen were measured over the course of spring.

The quantity of Cry j1 in *C. japonica* pollen decreased gradually, and smaller amounts of Cry j1 were seen in mid-March in both Hiroshima and Yamanashi

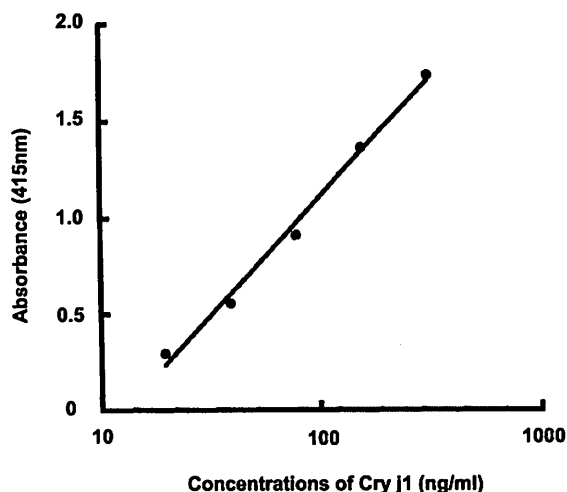


Figure 1. Standard curve of enzyme-linked immunosorbent assay. The horizontal axis indicated concentrations of Cry j1 expressed in ng/ml.

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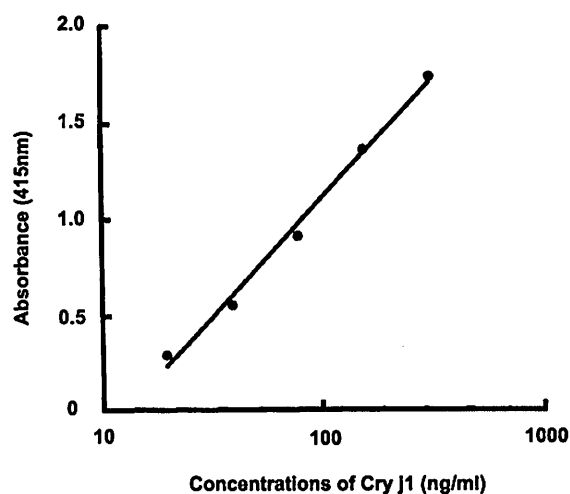


Figure 1. Standard curve of enzyme-linked immunosorbent assay. The horizontal axis indicated concentrations of Cry j1 expressed in ng/ml.

Table 1. Changes in the quantity of Cry j1 in *Cryptomeria japonica* pollen in Hiroshima Prefecture.

Tree no.	Parts of a tree	Quantity of Cry j1 in <i>C. japonica</i> pollen ($\mu\text{g/g}$)								
		26 February	2 March	3 March	4 March	6 March	7 March	8 March	13 March	14 March
1	Upper part	—	154.0	—	120.5	—	—	64.5	—	—
	Lower part	788.4	140.1	—	302.0	—	—	120.5	—	—
2	—	—	234.0	—	222.6	168.3	152.6	213.8	121.3	—
3	—	—	—	—	277.1	132.6	100.1	95.3	—	—
4	—	—	—	242.3	170.1	—	—	146.0	125.8	—
5	—	—	343.8	—	254.2	—	—	196.2	—	51.2

Prefectures. Similar changes in the quantity of Cry j1 were seen in Hiroshima and Yamanashi Prefectures, and the quantity of Cry j1 in *C. japonica* pollen was lower when large numbers of pollen particles were present in the air (Baba & Nakamura, 2003; Endo, 2002; Takahashi et al., 2007). Unfortunately, it was impossible to repeatedly collect cedar pollen from same trees in Hiroshima Prefecture; thus

Table 2. Changes in the quantity of Cry j1 in *Cryptomeria japonica* pollen in Yamanashi Prefecture.

Tree no.	Parts of a tree	Quantity of Cry j1 in <i>C. japonica</i> pollen ($\mu\text{g/g}$)				
		25 February	1 March	5 March	10 March	14 March
1	Upper part	591.4	239.6	335.6	261.4	99.2
	Lower part	723.0	367.8	302.0	244.0	105.8
2	Upper part	445.0	283.0	241.0	213.8	83.8
	Lower part	428.2	241.6	268.4	162.6	90.6
3	Upper part	NT	338.8	317.6	240.0	133.8
	Lower part	NT	343.8	254.2	196.2	51.2
Mean $\pm$ SD	Upper part	—	287.1 $\pm$ 49.7	298.1 $\pm$ 50.2	238.4 $\pm$ 23.8	105.6 $\pm$ 25.6
	Lower part	—	317.7 $\pm$ 67.0	274.9 $\pm$ 24.5	200.9 $\pm$ 40.9	82.5 $\pm$ 28.2
Total		546.9 $\pm$ 138.4	302.4 $\pm$ 55.4*	286.5 $\pm$ 37.6*	219.7 $\pm$ 36.3*	94.1 $\pm$ 27.2*

NT, not tested.

* $p < 0.05$, significance of differences in quantities of Cry j1 in *C. japonica* at the various sampling dates (February 25 and March 1, 5, 10 and 14) was compared by two-tailed multiple *t*-test with Bonferroni correction following ANOVA.

quantities of Cry j1 in *C. japonica* were only evaluated statistically in samples from Yamanashi Prefecture. No significant differences in the quantity of Cry j1 in *C. japonica* pollen were observed between the upper and lower parts of trees, although number of cedar pollen particles was apparently influenced by solar irradiation and rainfall in a previous study (Murayama, 2002). However, the present results suggested that the quantities of Cry j1 in *C. japonica* pollen are not influenced by solar irradiation. The differences in quantities of Cry j1 over time were compared, as no significant differences of quantities were observed between the upper and lower levels of the same tree. The quantities of Cry j1 in cedar pollen in March were significantly lower than in February. Takahashi et al. (2007) reported that the symptoms of Japanese cedar pollinosis began when levels of Cry j1 reach 1–3 pg/m³. Furthermore, symptom scores reflected an elevation in airborne Cry j1 levels in mid-March (Takahashi et al., 2007). Our data suggest that cedar pollen from *C. japonica* contains smaller quantities of Cry j1 in mid-March.

The amount of pollen detected on microscopic examination is frequently used during pollen season (Saito & Teranishi, 1999). Although further studies that monitor the quantities of Cry j1 during the same spring seasons are needed to clarify relationship between quantities of Cry j1 and pollinosis, our data suggest that advisories for allergic reactions caused by *C. japonica* pollen should also consider the quantity of Cry j1 present in the pollen, in addition to particle counts for airborne pollen. Furthermore, although *C. japonica* is indigenous to Japan (Begum, Nakaba, Oribe, Kubo, & Funada, 2010), surveys of pollen in other species, such as hogweed, that grow in deforested areas of Europe and the USA, should also take into account the amounts of allergen.

Acknowledgements

This research was partially supported by a research project grant awarded by the Azabu University.

References

- Baba, K., & Nakamura, A. (2003). Epidemiology of pollinosis and perennial allergic rhinitis in Japan. *Allergology*, 15, 84–91 (in Japanese).
- Begum, S., Nakaba, S., Oribe, Y., Kubo, T., & Funada, R. (2010). Changes in the localization and levels of starch and lipids in cambium and phloem during cambial reactivation by artificial heating of main stems of *Cryptomeria japonica* trees. *Annals of Botany*, 106(6), 885–895. doi:10.1093/aob/mcq185
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254. doi:10.1016/0003-2697(76)90527-3
- Dake, Y., Enomoto, T., Cheng, L., Enomoto, K., Shibano, A., Ikeda, H., ... Yamanishi, E. (2006). Effect of antihistamine eye drops on the conjunctival provocation test with Japanese cedar pollen allergen. *Allergology International*, 55(4), 373–378. doi:10.2332/allergo-int.55.373
- Endo, E. (2002). Clinical epidemiology of Japanese cedar pollinosis. *Igaku No Ayumi*, 200, 371–374 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/journal/download?GoodsID=aa7ayuma/2002/020005/006&name=0371-0374j&UserID=202.209.152.41>
- Inoue, S., Sakaguchi, M., Hori, M., & Watanabe, T. (1986). A seroepidemiological study of *Cryptomeria japonica* (Japanese cedar) pollinosis: measurement of IgE antibody. *Igaku No Ayumi*, 136, 959–960 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/>

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References

- Baba, K., & Nakamura, A. (2003). Epidemiology of pollinosis and perennial allergic rhinitis in Japan. *Allergology*, 15, 84–91 (in Japanese).
- Begum, S., Nakaba, S., Oribe, Y., Kubo, T., & Funada, R. (2010). Changes in the localization and levels of starch and lipids in cambium and phloem during cambial reactivation by artificial heating of main stems of *Cryptomeria japonica* trees. *Annals of Botany*, 106(6), 885–895. doi:10.1093/aob/mcq185
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1–2), 248–254. doi:10.1016/0003-2697(76)90527-3
- Dake, Y., Enomoto, T., Cheng, L., Enomoto, K., Shibano, A., Ikeda, H., ... Yamanishi, E. (2006). Effect of antihistamine eye drops on the conjunctival provocation test with Japanese cedar pollen allergen. *Allergology International*, 55(4), 373–378. doi:10.2332/allergo-int.55.373
- Endo, E. (2002). Clinical epidemiology of Japanese cedar pollinosis. *Igaku No Ayumi*, 200, 371–374 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/journal/download?GoodsID=aa7ayuma/2002/020005/006&name=0371-0374j&UserID=202.209.152.41>
- Inoue, S., Sakaguchi, M., Hori, M., & Watanabe, T. (1986). A seroepidemiological study of *Cryptomeria japonica* (Japanese cedar) pollinosis: measurement of IgE antibody. *Igaku No Ayumi*, 136, 959–960 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/>

- journal/download?GoodsID=aa7ayuma/1986/013612/017&name=0959-0960j&UserID=202.209.152.41
- Inoue, S., Sakaguchi, M., Morita, M., Shoji, T., Kaneda, S., Kimura, E., ... Hirakawa, K. (1988). Regional difference in the prevalence of IgE antibody to Japanese cedar pollen. *Igaku No Ayumi*, 145, 121–122 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/journal/download?GoodsID=aa7ayuma/1988/014502/035&name=0121-0122j&UserID=202.209.152.41>
- Japanese Association for Laboratory Animal Science. (1987). The guideline for animal experimentation. *Experimental Animals*, 36, 285–288 (in Japanese). Retrieved from <http://www.ncbi.nlm.nih.gov/books/NBK25422/>
- Kiyono, Y., Nagao, A., & Shinohara, K. (2002). Sugi pollinosis as a forestry issue. *Igaku No Ayumi*, 200, 447–451 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/journal/download?GoodsID=aa7ayuma/2002/020005/021&name=0447-0451j&UserID=202.209.152.41>
- Maeda, Y., Akiyama, K., & Shida, T. (2008). A clinical study of Japanese cedar (*Cryptomeria japonica*) pollen-induced asthma. *Allergology International*, 57, 413–417. doi:10.2332/allergolint.O-08-543
- Murayama, M. (2002). Year variation of pollen counts and pollen forecast. *Igaku No Ayumi*, 200, 417–421 (in Japanese). Retrieved from <http://mol.medicalonline.jp/library/journal/download?GoodsID=aa7ayuma/2002/020005/015&name=0417-0421j&UserID=202.209.152.41>
- Nakamura, S., Sato, F., & Nakamura, N. (2004). Immunochemical Localization of Cry j1 and Cry j2 – The allergenic proteins of Japanese cedar pollen- in the germinated pollen. *Japanese Journal of Palynology*, 50, 15–22 (in Japanese with English abstract). doi:10.5105/jse.24.15
- Nakane, P. K., & Kawaoi, A. (1974). Peroxidase-labelled antibody. A new method of conjugation. *Journal of Histochemistry & Cytochemistry*, 22, 1084–1091. doi:10.1177/22.12.1084
- Saito, H., & Teranishi, H. (1999). Development of the simple quantitative method for Cry j1 in sugi pollen using microplatereader. *Journal of Japanese Forest Research*, 81, 318–324 (in Japanese with English abstract). Retrieved from https://www.jstage.jst.go.jp/article/jjfs1953/81/4/81_4_318/_pdf
- Sakaguchi, M., Hirahara, K., Fujimura, T., & Toda, M. (2011). Approaches to immunotherapies for Japanese cedar pollinosis. *Aurin Nasus Larynx*, 38(4), 431–438. doi:10.1016/j.anl.2010.12.002
- Sakaguchi, M., Inoue, S., Taniai, M., Ando, S., Usui, M., & Matsushashi, T. (1990). Identification of the second major allergen of Japanese cedar pollen. *Allergy*, 45(4), 309–312. doi:10.1111/j.1398-9995.1990.tb00501.x
- Sugata, Y., Okano, M., Fujiwara, T., Matsumoto, R., Hattori, H., Yamamoto, M., ... Nishizaki, K. (2007). Histamine H4 receptor agonists have more activities than H4 agonism in antigen-specific human T-cell responses. *Immunology*, 121(2), 266–275. doi:10.1111/j.1365-2567.2007.02574.x
- Takahashi, Y., Aoyama, M., Yoshitake, M., Abe, E., Ohta, N., & Sakaguchi, M. (2007). Relationship between airborne Cry j1 and the onset time of the symptoms of Japanese cedar pollinosis patients. *Allergology International*, 56(3), 277–283. doi:10.2332/allergolint.O-06-472
- Takahashi, Y., Nagoya, T., Watanabe, M., Inoue, S., Sakaguchi, M., & Matsushashi, T. (1993). A new method of counting airborne Japanese cedar (*Cryptomeria japonica*) pollen allergens by immunoblotting. *Allergy*, 48(2), 94–98. doi:10.1111/j.1398-9995.1993.tb00692.x
- Terada, T., Akimoto, Y., Hyo, S., Suzuki, D., Tanaka, K., & Takenaka, H. (2009). Allergic potency of Japanese cedar pollen Cry j1 is reduced by a low concentration of hypochlorous acid generated by electrolysis. *Allergology International*, 58(2), 255–260. doi:10.2332/allergolint.08-OA-0043
- Yasueda, H., Yui, Y., Shimizu, T., & Shida, T. (1983). Isolation and partial characterization of the major allergen from Japanese cedar (*Cryptomeria Japonica*) pollen. *Journal of Allergy Clinical Immunology*, 71(1), 77–86. doi:10.1016/0091-6749(83)90550-X