

Assessment of cross-reactivity between Japanese cedar (*Cryptomeria japonica*) pollen and tomato fruit extracts by RAST inhibition and immunoblot inhibition

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Summary

Background An association between pollinosis and sensitivity to fruits and vegetables has been reported. Although Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most widespread diseases in Japan, there have been no reports demonstrating cross-reactivity between Japanese cedar pollen and other plant food.

Objective The aim of this study was to demonstrate cross-reactivity between Japanese cedar pollen and tomato fruit (*Lycopersicon esculentum*) using RAST inhibition and immunoblot inhibition.

Methods The RAST and immunoblot inhibition were performed using sera from patients with oral allergy syndrome (OAS) after ingesting fresh tomatoes. We identified some proteins that took part in cross-reactive IgE by the determination of N-terminal amino acid sequences and a homology search through the SWISS-PROT database.

Results In the RAST inhibition, the bindings of IgE from the sera from four out of five (4/5) subjects to Japanese cedar pollen discs were inhibited by more than 50% by preincubation of the serum with tomato fruit extracts. Likewise, the IgE bindings to tomato fruit discs were inhibited more than 50% by Japanese cedar pollen extracts in 3/5 sera. In immunoblot inhibition, IgE binding activities of some protein bands on both membranes were decreased by heterologous inhibitors. However, the combinations of these protein bands involved in cross-reactivity were different between patients.

Conclusion We have demonstrated cross-reactivity between Japanese cedar pollen and tomato fruit using RAST inhibition and immunoblot inhibition.

Keywords cross-reactivity, immunoblot inhibition, Japanese cedar, oral allergy syndrome, pollinosis, RAST inhibition, tomato fruit

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Introduction

It is well documented that subjects suffering from pollinosis frequently show hypersensitivity to fruits and vegetables, in particular to apples and hazelnuts [1–4]. Although Japanese cedar (*Cryptomeria japonica*) pollinosis is one of the most common allergic disorders in Japan [5] and Cry j 2, a major allergen of Japanese cedar pollen, has been reported to show 40% identity with polygalacturonase (PG) derived from tomatoes (*Lycopersicon esculentum*) at the amino acid level [6], there have been no articles demonstrating the cross-reactivity of pollen with fruit and vegetables.

We examined a pollinosis patient allergic to Japanese cedar pollen associated with oral allergy syndrome (OAS) after eating fresh tomatoes. She had eaten fresh tomatoes without any symptoms until suffering from the pollinosis. This patient's IgE recognized the cross-reactive determinants between the pollen and tomato fruit extracts. Furthermore, we performed

a statistical survey using more than 100 sera from patients with increased specific IgE antibodies (more than class 2 in the Pharmacia CAP-FEIA System) to either tomato fruit or Japanese cedar pollen, and to either tomato fruit or *Dermatophagoides pteronyssinus*. The IgE antibody titres to tomato fruit showed a significantly positive correlation with those to Japanese cedar pollen, but not with those to *Dermatophagoides pteronyssinus*.

In this study, we assessed the cross-reactivity between tomato fruit and Japanese cedar pollen by RAST inhibition and immunoblot inhibition.

Materials and methods

Extraction of tomato fruit and Japanese cedar pollen

Tomato fruits were obtained from the Wye Research and Education Center of the University of Maryland (Queenstown, MD, USA). Tomato pericarp was dissected and homogenized for 15 s in cold distilled water [7]. After centrifugation at 2400g for 10 min, the pellet was collected and resuspended in 1 M NaCl at pH 6 with 1 M NaOH. After stirring for 3 h at 4°C, the debris

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was removed by centrifugation at 2400g for 10 min. The supernatant was filtered and brought to 75% saturation with ammonium sulphate for 18 h. The precipitate was collected by centrifugation at 10000g for 20 min and suspended in 0.15 M NaCl (pH 6). This extract was dialysed against 0.15 M NaCl (pH 6) at 4°C over two nights. The residues were removed by centrifugation at 10000g for 20 min. The collected supernatant was used throughout a series of experiments.

Japanese cedar pollen was kindly collected by the Aichi Prefectural Forest Research Institute. The extraction was performed as previously reported [8].

RAST experiment

The Japanese cedar pollen and tomato fruit extracts were each coupled with CNBr-activated paper discs (Tokyo Roshi, Tokyo, Japan), 6 mm in diameter, at the ratio of 50 µg of protein/disc. Protein concentrations of these samples were determined by BCA protein assays (Pierce, Rockford, IL, USA). The RAST was performed by incubation of 50 µL of serum with each disc at room temperature (20°C) for 16 h. After three washes with PBS-Tween, 50 µL of ¹²⁵I-labelled anti-human IgE (Dainabot Co. Ltd, Tokyo, Japan), approximately 2,200 Bq, was added to each tube for a second 16-h incubation. After three additional washes, the bound radioactivity of ¹²⁵I-labelled anti-human IgE was determined on a gamma counter (Packard Co. Ltd, Downers Grove, IL, USA). RAST values were expressed as percent binding to the total radioactivity added. To estimate the confidence of these discs, we compared the results of RAST (% binding) with the values of the Pharmacia CAP-FEIA System (UA/mL) using 37 sera from patients with positive specific IgE antibodies (more than class 2 in the Pharmacia CAP-FEIA System) to both tomato fruit and Japanese cedar pollen. Our results significantly correlated with those of the CAP System in the specific IgE antibody titre to tomato fruit ($r=0.83$, $P<0.001$, $n=37$) and Japanese cedar pollen ($r=0.89$, $P<0.001$, $n=37$).

RAST inhibition

The patients' serum (25 µL) was preincubated with solutions (25 µL) containing Japanese cedar pollen or tomato fruit extracts of five different concentrations from 10 to 10000 µg/mL as inhibitors at room temperature for 5 h. The subsequent procedure was the same as the RAST described above. A RAST inhibition assay using unrelated allergens such as *Dermatophagoides farinae* (Torii pharmaceutical Co. Ltd, Tokyo, Japan) was also performed to rule out the possibility of non-specific inhibition by Japanese cedar pollen and tomato fruit extracts. A semilogarithmic plot enabled the inhibitor concentration required for a 50% inhibition to be made.

Electrophoresis and immunoblotting

Sodium dodecylsulphate-polyacrylamide gel electrophoresis was performed in a 16% Tris-glycine precast gel (TEFCO Corporation, Machida, Japan) according to Laemmli [9] under reducing conditions. Proteins were separated at 120 V for 2 h. After electrophoresis, proteins were transferred to Immobilon™-P Membranes (Millipore, Bedford, MA, USA) as previously reported [10]. For detection of bound IgE, we used a phosphatase-labelled goat anti-human IgE (ε) antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) diluted 1:2000 and developed with BCIP/NBT solution

(Kirkegaard and Perry Laboratories). The protein concentrations of these samples were determined by a BCA protein assay (Pierce).

Immunoblot inhibition

After each protein was transferred to Immobilon™-P membrane, sera with high IgE antibody titres to tomato fruit and Japanese cedar pollen preincubated with heterogeneous protein solutions (0, 1, 10 µL) were added as inhibitors. The detection of bound IgE was the same as described above.

Determination of the N-terminal amino acid sequence of each protein

Immobilon™-P membranes after protein transfer were stained with 0.1% amido black (Sigma Diagnostics, St Louis, MO, USA) in 50% methanol and 10% acetic acid, destained with 40% methanol and 10% acetic acid, and air-dried. Each protein band with specific IgE binding inhibited by heterologous protein extracts was excised and subjected to N-terminal amino acid sequencing via automated Edman degradation using an Applied Biosystems model 470A protein sequencer [11]. The resulting phenylthiohydantion (PTH) amino acid derivatives were identified using a model 120A one-line PTH analyser and the standard Applied Biosystems program. The N-terminal amino acid sequences were screened for homology with those in the SWISS-PROT database. Finally, the obtained whole protein sequences were compared with one another using the computer-assisted method of Higgins and Sharp [12].

Human sera

Serum samples were obtained from subjects having OAS after eating fresh tomatoes. The sera were tested to confirm the specific IgE to Japanese cedar and tomato fruit by RAST, as described above. We used five sera in the RAST inhibition and two sera in the immunoblot inhibition.

OAS was diagnosed based on the repeating histories of immediate hypersensitive symptoms such as irritation and swelling of the oral cavity and lips after eating fresh tomato. Subjects with a vague OAS history were excluded from this study. We could not use double-blind food challenge tests for the determination of OAS by tomato fruit, since the allergic activity of tomato fruit was likely to be decreased by the processes.

These sera were kept at -20°C until use.

Results

RAST inhibition with Japanese cedar pollen and tomato fruit antigens

As shown in Fig. 1, the binding of IgE from 4/5 subjects' sera to Japanese cedar pollen discs was inhibited by more than 50% by preincubation of the serum with tomato fruit extracts, while the addition of Japanese cedar pollen extracts to serum inhibited more than 50% binding of IgE to tomato fruit discs in 3/5 subjects. *Dermatophagoides farinae* did not efficiently inhibit the binding of IgE to Japanese cedar or tomato fruit (data not shown).

Immunoblot inhibition and protein identification

The IgE binding to some protein bands of both Japanese cedar pollen and tomato fruit extracts was mutually inhibited, but the

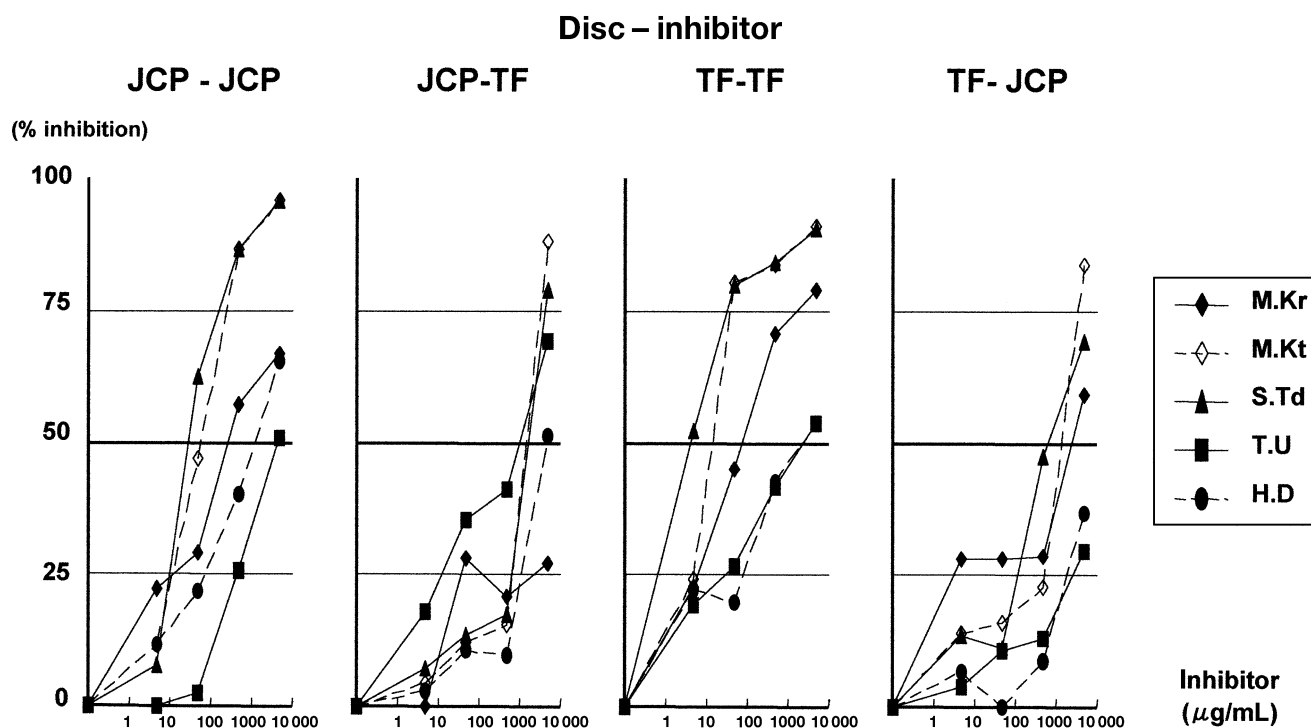


Fig. 1. RAST inhibition between Japanese cedar pollen (JCP) and tomato fruit (TF) using five sera from patients with OAS. A semilogarithmic plot enabled the inhibitor concentration required for 50% inhibition to be achieved.

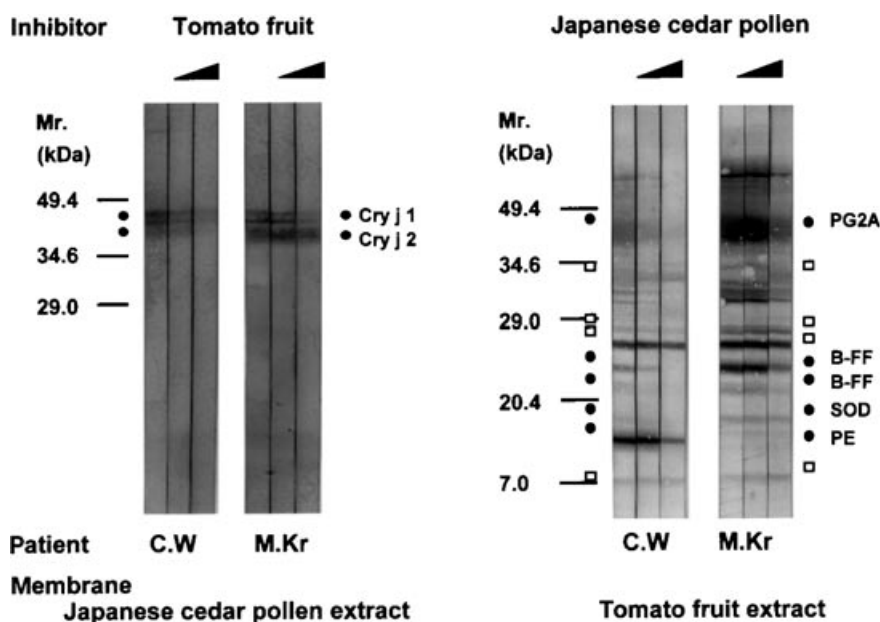


Fig. 2. Immunoblot inhibition with Japanese cedar pollen and tomato fruit extracts. Closed circles indicate allergens. Cry j 1 and Cry j 2 are major Japanese cedar pollen allergens. PG: polygalacturonase, B-FF: β -fructofuranosidase, SOD: superoxide dismutase, PE: pectinesterase. Open squares indicate non-specific binding bands.

protein bands involved in cross-reactivity were different between patients, as shown in Fig. 2.

The determination of the N-terminal amino acid sequences of these proteins and a homology search revealed that the proteins with molecular masses of 45–49 kDa and 41–43 kDa in Japanese cedar pollen extracts were Cry j 1 (accession No. BAA07020) and Cry j 2 (accession No. JC2498) [8,13], respectively. The protein with each molecular mass from tomato fruit was identified as several known proteins and their degraded fragments as follows; 46 kDa as PG2A (accession No. P05117) [14],

22 and 25 kDa as β -fructofuranosidase (B-FF, accession No. S31155) [15], 18 kDa as superoxide dismutase (accession No. AAA34194) [16] and 14 kDa as pectinesterase (accession No. A25010) [17].

Comparison of each amino acid sequence

Possible sequence similarities between these identified Japanese cedar and tomato fruit proteins with IgE cross-reactivity were analysed by the computer-assisted method. Among eight combinations between the cedar and tomato proteins, a significant

Cryj2	248	KNTIGTGDDCVAIGTGSSNIVIEDLICGPGHGISIGSLGRENSEAEVSYV	297
PG2A	263	DTIIGTGDDCISIVSGSQNVQATNITTCGPGHGISIGSLGSGNSEAYVSNV	312
Cryj2	298	HVNGAKFIDTQNGLRIRIKTWQGGSGMAASHIYENVENINSENPILINQFYC	347
PG2A	313	TVNEAKIIGAENGVRIRIKTWQGGSGQASNIKFLNVEMQDVKYPIIDQNYYC	362
Cryj2	348	TSASACQNQRSVQIQDVTYKNIRGTSATAAIIQLKCSDSMPCKDILLSDI	397
PG2A	363	DRVEPCIQQFSVQVKNVVYENIKGTSATKVAIKFDCSTNFPCEGIIMEN	412

Fig. 3. Sequence similarity between Cry j 2 and PG2A. Cry j 2: *Cryptomeria japonica*, accession No. JC2498. PG2A: *Lycopersicon esculentum*, accession No. P05117.

similarity was obtained for only one combination, Japanese cedar Cry j 2 and tomato polygalacturonase, in which 21.8% of amino acid residues were identical on that alignment. Although such an overall identity was low, three conserved stretches consisting of 7–13 residues (251–257, 274–286, and 312–321 of Cry j 2 sequence) are present in the two sequences (Fig. 3).

Discussion

Tomato fruit was reported to be cross-reactive with grass pollen by Petersen et al. [18]. They attributed the cross-reactivity to profilin and N-glycan on the allergens. In this study, we found another cross-reacting pollen with tomato fruit. That was Japanese cedar pollen, which is one of the most common causes of pollinosis in Japan. We first demonstrated cross-reactivity between Japanese cedar pollen and tomato fruit by RAST and immunoblot inhibition.

It is well known that OAS by fruits is accompanied by pollinosis. The association is related with cross-reactivity to IgE antibodies between the pollen and fruit, for example, birch pollen and apple extracts [19]. Halmepuro et al. [20] reported results of RAST inhibition experiments in which binding of IgE from patient sera to apple discs was completely inhibited by preincubation of the serum with birch pollen allergen, while apple allergen partially inhibited the binding of IgE to birch pollen discs. A similar report using Western blot experiments [21] demonstrated that sera preincubated with minimal amounts of birch pollen extracts completely lost activity against apple allergens with a molecular mass of 17–18 kDa, although the reverse was not observed for apple allergens. These facts suggest that all the IgE determinants in apple antigens were present in birch pollen, while birch pollen have IgE determinants other than those in apple antigens.

In the RAST inhibition shown in Fig. 1, the bindings of IgE from five OAS subjects' sera to tomato discs were inhibited (29–83%) by preincubation of the serum with Japanese cedar pollen extracts. On the other hand, addition of tomato extract to serum inhibited 28–87% of the binding of IgE to Japanese cedar pollen discs. These inhibitions in IgE-binding between heterologous combinations were less than those between homologous combinations. These findings suggest that tomato fruit antigens and Japanese cedar pollen antigens have not only cross-reactive IgE epitopes, but also unshared IgE epitopes specific for each allergen.

The sequence similarity was found between Cry j 2 and tomato PG2A, as mentioned also in the original report on the Cry j 2 cDNA cloning [6]. This sequence similarity would support the results obtained in the present study on the cross-reactive IgE against Japanese cedar pollen and tomato (see Figs 1 and 2). Especially, the three regions (251–257, 274–286 and 312–321 of the Cry j 2 sequence) would be a possible epitope recognized by the cross-reactive IgE. Recent reports suggested that seven residues or more were long enough to form an epitope structure [22,23]. Therefore, it will be of special interest to determine whether the synthetic peptides corresponding to these regions inhibit the IgE binding.

As shown in Fig. 2, more than one protein was involved in the competition of IgE binding between the pollen and tomato proteins. However, the sequence similarity was found only between Cry j 2 (41–43 kDa) and PG2A (46 kDa). The possible

cross-reactivity between the other proteins might be ascribed to some carbohydrate epitopes, which had been reported to be common IgE-reactive epitopes shared by many plant and insect allergens [24]. Cry j 1 is known to possess such an IgE-reactive carbohydrate epitopes [25]. Furthermore, Petersen et al. [18] reported that almost all of the IgE-reactive capacity of the components in tomato extract was decreased by destroying the carbohydrate structures with periodate treatment. Indeed, PG2A and B-FF are also known to have N-glycosylation sites. These findings also suggest that IgE binding to Cry j 1 could cross-react with tomato fruit allergens through these carbohydrate structures.

In conclusion, we have demonstrated cross-reactivity between Japanese cedar pollen and tomato fruit extracts in RAST and immunoblot inhibition. The polypeptide portion, as well as the carbohydrate portion reported for some glycoprotein allergens [26], was suggested to be responsible for cross-reactivity between the pollen and tomato proteins. Moreover, there were considerable sequence similarity between Cry j 2 and the PG2A tomato protein. We are planning further studies to clarify that both carbohydrate and sequence similarity are involved in the cross-reactivity between Japanese cedar pollen and tomatoes.

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