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C09 – Gibberellin-regulated proteins

Pascal Poncet, Tomona Iizuka, H el ene S en echal,
Enrico Scala

Highlights

- Gibberellin-regulated proteins (GRPs) are small, cationic, nonglycosylated monomeric proteins with anti-microbial activity, present in plant foods and pollen.
- GRPs are resistant to heat and proteolysis.
- GRPs are cross-reactive and involved in Pollen Food Allergy Syndromes.
- Main fruits involved: peach and citrus but also apricot, cherry, or pomegranate.
- Cupressaceae is, up to now, the only tree family shown to express allergenic pollen GRP.
- GRPs may induce severe systemic reaction with or without cofactors.

1 – The protein

The very first Gibberellin-Regulated Protein (GRP) allergen was described in 2013 in peach (*Prunus persica*) and was named Pru p 7 (formerly peamaclein).⁵⁵⁹ The sensitization was reported in peach allergic patients negative for the other allergens known in peach, especially the nsLTP Pru p 3 that shares some characteristics with Pru p 7, i.e., low molecular weight (MW) and basic isoelectric point (pI). The characterization was refined and confirmed in 2014.¹⁵⁶¹ Pru p 7 is a nonglycosylated, cationic, monomeric protein with an MW around 7–8 kDa and a pI around 9. It belongs to the cysteine-rich plant antimicrobial peptide families that are involved in plant growth and resistance to bacteria, viruses, or other microorganisms that can cause plant disease.¹⁵⁶² Twelve cysteines involved in 6 disulfide bridges confer the protein stability and resistance to heat and proteolysis.

GRP characteristics

- 6 well-conserved disulfide bridges
- Expressed in pulp and peel of plant food
- Protein present but not synthesized in pollen grain
- Plant defence protein
- The structure displays a cleft likely to bind an unknown ligand
- Pollen/food cross-reactive

2 – The family

The family name GRP is now well accepted in the field of allergy although it may not be the most appropriate since the allergens,

with the associated number 7, rather belong to the Snakin/GASA (Gibberellic Acid Stimulated in Arabidopsis) protein family, a sub-family of GRP. Indeed the phytohormone gibberellin regulates very diverse proteins in plants, nonallergenic ones as well as allergenic such as, besides snakin/GASA proteins, superoxide dismutase, β -1,3-glucanase, calmodulin or oleosin.¹⁵⁶³

Gibberellin is a phytohormone produced by all plants, some fungi and bacteria. It corresponds to a family of tetracyclic diterpenic molecules playing a role in plant growth and breaking dormancy.¹⁵⁶⁴ Gibberellin and GRP have an important role in plant development, host defence and redox homeostasis. Consequently, their concentration is strictly regulated and may be different in specific developmental stages. Furthermore, both biotic and abiotic stresses could influence GRP levels.¹⁵⁶⁵ Nowadays gibberellins are widely used in modern agriculture to increase the yield and/or quality of plant food.¹⁵⁶⁶ Numerous plant foods are submitted to an exogenous gibberellin treatment such as grape, cherry, strawberry, pear, tangerine, plum, orange, blueberry, pineapple, tomato, potato, wheat, rice, barley, hop, sunflower, alfalfa (*Medicago*), chili/red pepper, zucchini, salad, spinach, celery or cotton. By consequence, the utilization of exogenous synthetic gibberellin might affect the concentration of GRPs synthesized in plant foods and even in pollens, therefore influencing also their allergenic potency.

Once produced, GRPs contain a signal peptide of 25 amino-acid that is subsequently removed to obtain the protein mature form of 7 kDa (63 AA). Mature GRPs are structurally characterized by a highly conserved C-terminal region and, as in Pru p 7, by the 12 cysteines at conserved positions. GRPs are water-soluble proteins positively charged at neutral pH with a compact globular conformation, which may result in over-evaluation of its MW depending on the bio- and physicochemical analytical methods used. The protein folding is responsible for conformational epitopes destroyed upon *in vitro* reduction in disulfide bonds.

Snakin-1, the first GRP described in 1999, was isolated from *Solanum tuberosum* from the potato plant tuber allowing extensive studies on its structure and antimicrobial activity.¹⁵⁶⁵ The three-dimensional structure of snakin-1 was obtained by X-ray crystallography.¹⁵⁶⁷ The folding of the protein comprises three alpha-helices and a cleft likely able to accommodate one or more ligands, as yet undetermined (Figure 180).

Snakin-1 is not yet described as an allergen. After the description of Pru p 7, Pru p 7 a GRP from pomegranate (*Punica granatum*) was reported¹⁵⁶⁸ as well as Pru m 7, the GRP from Japanese apricot (*Prunus mume*).¹⁵⁶⁹ In Japan, Japanese apricots are traditionally consumed marinated in salt, they are named umeboshi. More fruits were suspected to contain allergenic GRPs,¹¹³⁰ but convincing data were subsequently obtained only for orange (*Citrus sinensis*) and sweet cherry (*Prunus avium*), Cit s 7¹⁵⁷⁰ and Pru av 7, respectively (IUIS/WHO Pru av 7 descriptions: http://www.allergen.org/viewallerg_en.php?aid=1002). Grapefruit, tangerine and lemon contain cross-reactive GRP with orange (Poncet et al., unpublished results).

A breakthrough was provided by the study of allergenic GRPs when it was demonstrated that an allergen from the Cupressaceae

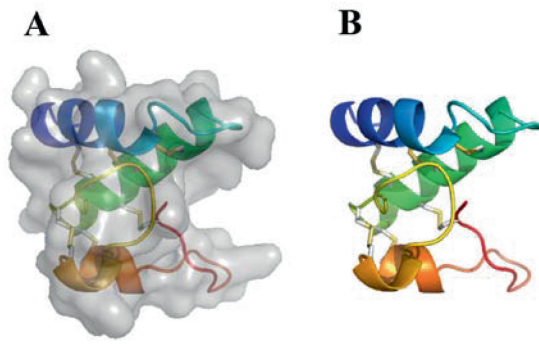


FIGURE 180 Three-dimensional structure of Snakin-1 (PDB 5E5Q). Ribbon representation with (A) or without surface (B).

pollen first reported in 2010,¹⁵⁷¹ the formerly called BP14, was shown to belong to the GRP protein family.¹⁵⁷² The pollen food associated syndrome (PFAS) between peach or citrus and cypress pollen reported in 2006¹⁵⁷³ and 2015¹⁵⁷⁴ was thus explained by the existence of an IgE cross-reactivity between Pru p 7 or Cit s 7 and the allergen BP14.^{230,1575,1576} The gene coding for BP14 was then fully sequenced from common cypress (*Cupressus sempervirens*) strobili by next-generation sequencing and the protein named Cup s 7 (IUIS/WHO Description of Cup s 7, <http://www.allergen.org/viewallergen.php?aid=997>). A homologous allergen, Cry j 7, with similar fruit cross-reactivities, was then described in Japanese cedar pollen (*Cryptomeria japonica*) by studying Japanese patients allergic to Japanese cedar pollen and food.¹⁵⁷⁸ As well the existence of a mountain cedar pollen (*Juniperus ashei*) GRP, Jun a 7, was confirmed.¹¹⁵⁷ We could expect that other trees from the Cupressaceae family such as the Japanese cypress (*Chamaecyparis obtusa*) or the bald cypress (*Taxodium distichum*) also express an allergenic pollen GRP.

Finally, in 2021, an allergenic GRP, Cap a 7, was revealed in bell pepper (*Capsicum annuum*) by studying a Japanese patient allergic to several GRPs, from bell/chili pepper (Cap a 7), from peach (Pru p 7), orange (Cit s 7) and from Japanese cedar pollen (Cry j 7) demonstrating a clinical relevance of the cross-reactivities between different GRPs (IUIS/WHO Description of Cap a 7, <http://www.allergen.org/viewallergen.php?aid=1061>).

Up to now, GRPs from only 9 allergenic sources have been described as allergens. Five from fruits, 1 from a vegetable and 3 from tree pollen, all belonging to the Cupressaceae family (Tables 99 and 100).

Cupressaceae GRPs are very similar with more than 90% sequence identity and share more than 60% sequence identity with fruit and vegetables. Similarly, the percentage of sequence identity between fruit GRPs or vegetable GRPs are high and close to each other. Therefore, all GRP should theoretically be cross-reactive (Table 101). However, the cross-reactivity is not always experimentally observed.¹⁵⁷⁸

The relationships between the different taxa and the 3-dimensional modelling of proteins are depicted in (Figure 181). The pollen GRPs are more distant from plant food-derived ones. Snakin-1 from potato and citrus fruits such as grapefruit (*Citrus*

maxima), tangerine (*Citrus reticulata*) and lemon (*Citrus limone*) are depicted on a yellow background because the GRP allergens are not fully characterized. However, GRP cross-reactivities were shown among citrus fruits and a clementine (*Citrus clementina*) GRP is described in the Uniprot KB database (accession number V4T144) with a 100% sequence identity with orange GRP.

Three-dimensional modelling using potato snakin-1 as a template showed a few structural differences between the various GRPs that could lead to variations in the size of the three epitope regions predicted by the software DiscoTope 2.0 (Figure 181).

3 – Clinical relevance

GRPs are found in both, pulp and peel of fruits, in contrast to nsLTPs, which are mainly present in the peel, and to a lower extent in the pulp. However, bell pepper GRP was only found in the pulp (unpublished results). Interestingly GRPs can be present or absent in different fruit cultivars, even in distinct lots belonging to the same cultivar.^{559,1568}

Very often the sensitization to fruit GRPs is associated with Cupressaceae pollen allergy. This was observed for Mediterranean cypress in Europe^{1576,231} as well as for Japanese cedar in Japan.¹⁵⁷⁷ Forty-six per cent of young Japanese patients allergic to Japanese cedar pollen and fruit are sensitized to GRPs. This observation suggests a possible interdependence of both sensitizations. The association might rely not only on the cross-reactivity between Cupressaceae and fruit GRPs but also on a sensitization process involving some specific ligand-protein interactions common between the two allergenic sources that synergise the allergic response towards GRPs. Interestingly, in the case of allergy to GRPs, sensitization to cypress pollen does not necessarily involve Cup a 1, the major allergen of Cupressaceae pollen. It is not known whether sensitization to GRPs from cypress (i.e., Cup s 7 or Cry j 7), in the absence of recognition of Cup a 1, can generate respiratory symptoms or not. At the same time, it is not known whether sensitization to food GRPs necessarily follows a sensitization to pollen (as in the case of PR-10 or Profilin; (chapters C01, C02) or can be directly caused by fruits, acting as primary sensitizers (as for nsLTPs, in the Mediterranean area). The main fruits involved are peach and citrus. Pomegranate allergy seems much rarer and the only patient allergic to bell/chili pepper was also allergic to Japanese cedar pollen, peach and citrus (see clinical case #4). GRP cross-reactivities that are immunochemically assessed using recombinant protein may not be clinically relevant. This was observed with snakin-1 able to be bound by IgEs from a cypress/peach allergic patient (Cup s 7+/ Pru p 7+) but unable to activate the patient's basophils in agreement with the tolerance of potato consumption by the patient.²³⁰ Differences in antibody affinity probably play a role. At least two pollen food allergy syndromes were previously described between cypress pollen and peach and/or citrus.^{1573,1574} GRPs should be clinically relevant since they can induce *ex-vivo* basophil activation.^{230,231,559} GRP allergies were reported to be more common in adolescents

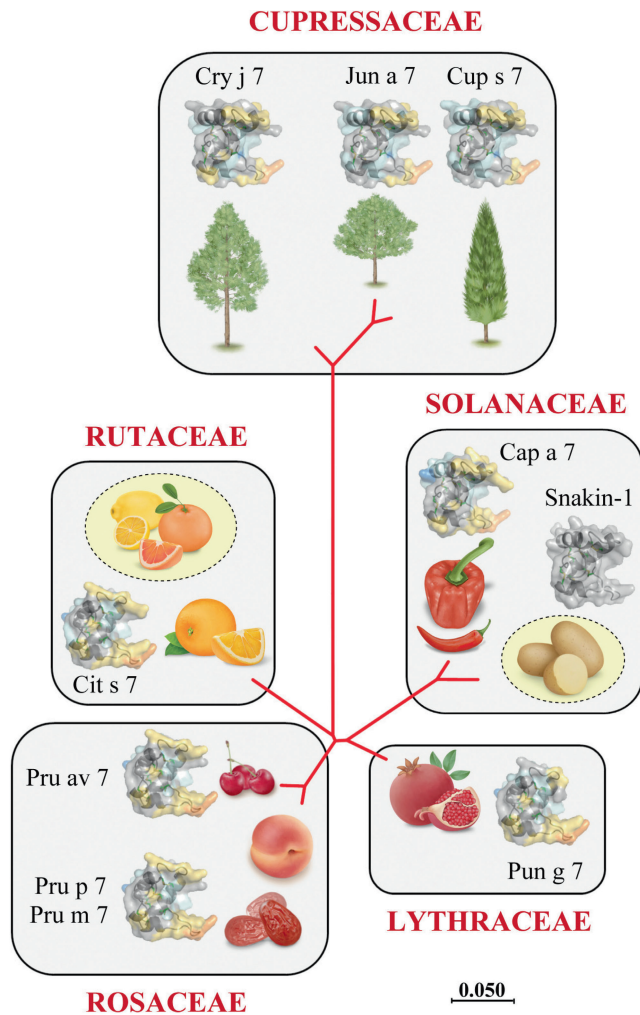


FIGURE 181 Evolutionary relationships of taxa (phylogenetic tree) and 3D modelling of nine allergenic GRP and the prototype reference GRP snakin-1 from potato. The evolutionary history was inferred using the Neighbor-Joining method¹⁵⁷⁹ and evolutionary analyses were conducted in MEGA X¹⁵⁸⁰. Three-dimensional structure modelling of proteins was calculated using snakin-1 as a template. Three conformational epitopic regions were predicted using the software Disco Tope 2.0. They are coloured in yellow and orange.

extracts showed identical performances, while in the case of labile allergen (Pru p 1 or Pru p 4) reactivities, they consistently gave a negative result. This extract evaluation is therefore an excellent approach to identify reactivity to nsLTP or, possibly Pru p 7, during the first screening of the patient.¹⁵⁸³

In another multicenter Italian study, 835 cypress pollen hypersensitive patients were evaluated with a peach extract containing Pru p 7 by SPT. In peach sensitized individuals, IgE to rPru p 3 was evaluated, and only those scoring negative were further studied for IgE reactivity to rPru p 7 by immunoblot and by an, at that time, “experimental” ImmunoCAP Specific IgE test with rPru p 7. Peach SPT reactivity was found in 163 (19.5%) patients, but 127 (77.9%) were excluded because they were also Pru p 3 reactors. On immunoblot, only 3/18 subjects recognized a band at about 7 kDa. Ten/18 (56%)

were Pru p 7 reactors on ImmunoCAP Specific IgE test. Taken together, Pru p 7 allergy seem to occur quite rarely in Italy (less than 3% among cypress reactors⁵⁹²).

GRP sensitization has to be suspected after systemic reactions that could have been associated with well-known cofactors such as physical exercise, NSAID, alcohol, proton pump inhibitors when the fruit has been consumed. Since Cupressaceae pollen allergy is a very frequent association with GRP sensitization, such pollen sensitization should be carefully evaluated even though the association mechanism is, up to now, not well understood. Cypress pollen reactivity, even after skin prick test, in the absence of Cup a 1, polcalcin or CCD IgE recognition may occur in case of GRPs' sensitization. Then, specific IgE against nsLTP is usually negative as well as against profilin. Interestingly sIgE against nsLTP or profilin was not reported in the case of cypress pollen allergy. The recombinant Pru p 7-specific IgE test, commercially available in singleplex and multiplex assays, may help in the diagnosis although a positive GRP immunoassay might not be associated with a clinically relevant IgE reactivity. Therefore, a method evaluating the IgE reactivity to the natural GRP may be helpful to confirm the diagnosis, for instance, immunoblot with total extract in nonreducing conditions. To complete the diagnosis an *ex vivo* basophil activation test could be performed with total extract and with the recombinant GRP since a positive basophil activation test, in contrast to immunoassays, strongly suggests a potential clinical relevance. Because severe reactions such as anaphylactic shock were reported, an adrenaline autoinjector should be recommended to the patient as well as avoidance of the culprit food in both raw and processed forms when the diagnosis is established. An algorithm is presented in (Figure 182).

5 – Clinical cases

Case 1 (original):

Clinical History: Male, Italy, born in 2002. Patient suffering from seasonal allergic rhinitis every year between January and March. He reported three episodes of anaphylactic reaction characterized by hypotension and diffuse urticaria with angioedema during dinner, after the ingestion of (2015) a slice of peeled peach, (2016) pomegranate (2018), and (2020) a few slices of orange. The patient in all cases was brought to the ER, where he received a combination of intramuscular adrenalin and intravenous steroid.

Allergy testing: The patient went through a cutaneous allergic evaluation that gave positive results for cypress pollen (10mm × 7 mm) and a commercial peach extract containing 30 mg/ml of Pru p 3 (12mm × 9 mm). He was then tested for IgE to cypress: 15 kUA/L; peach: 3.5 kUA/L; Pru p 1: <0.1 kUA/L, Pru p 4: <0.1 kUA/L, Pru p 3: <0.1 kUA/L and MUXF3: <0.1 kUA/L. A year later, the patient was further tested, scoring positive for Pru p 7: 14.7 kUA/L.

Conclusion: The serology identifies the patient as genuinely sensitized to Pru p 7. The presence of positive results after SPT to peach extract in the absence of PR-10, Profilin, nsLTP or CCD reactivity indicates a strong suspicion for GRP sensitization. Nowadays

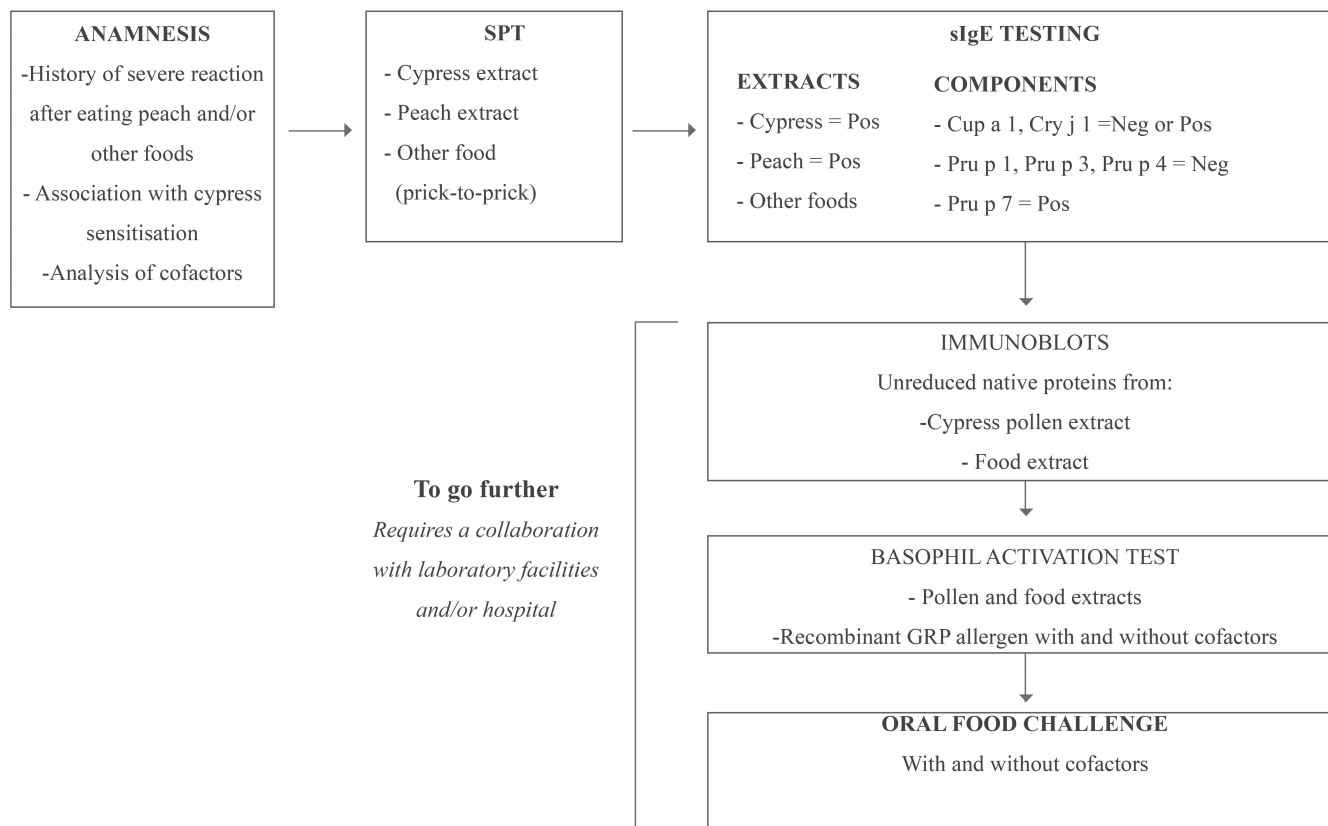


FIGURE 182 Diagnostic algorithm to assess GRP sensitization

it is possible to *in vitro* test Pru p 7, and this analysis should always be included in allergy work-out in patients with severe reactions to *Rosaceae*, pomegranate, or citrus fruits.

Case 2 (original):

Clinical History: Female, Italy, born 1990. The patient has been suffering from seasonal allergic rhinitis between February and March since 2000. After ingestion of a peeled peach and about 30 min running, she had an anaphylactic reaction (low blood pressure, abdominal pain, generalized flushing and swelling, followed by respiratory difficulty due to laryngeal obstruction) and subsequent emergency treatment. Another similar adverse reaction occurred after ingestion of two walnuts associated with moderate physical exercise.

Allergy testing: (A) SPT: Environmental allergens: Cypress pollen (*Juniperus a.*): 10 mm × 6 mm; plane tree (*Platanus a.*): 5 mm × 6 mm; olive tree (*Olea e.*): 3 mm × 2 mm; mugwort (*Artemisia v.*): 7 mm × 4 mm. Food allergens: all negative except walnut (*Juglans r. nut*): 7 mm × 5 mm and peach (*Prunus p.*): 19 mm × 8 mm. (B) *In-vitro* testing: [2015] Total IgE 350.3 kU/L, specific IgE to Cypress pollen (*Cupressus a.*): 12.5 kU/L; plane tree (*Platanus a.*): 0.66 kU/L; Olive tree (*Olea e.*): 0.12 kU/L; Mugwort (*Artemisia v.*): 2.2 kU/L; Walnut (*Juglans r. nut*) 3.82 kU/L; rPru p 3: 1.79 kU/L.

After 6 years, the patient returned to visit reporting a further reaction after physical exertion (bicycle) performed after ingesting an orange. The patient was studied with a multiplex method, which allowed to highlight, in addition to the already known

reactivity to Cypress (Cry j 1: 2.31 kU/L and Cup a 1: 31.93 kU/L) and nsLTP (Ole e 7: 1.26 kU/L; Cor a 8: 0.94 kU/L; Jug r 3: 0.35 kU/L; Art v 3: 0.52 kU/L; Pru p 3: 2.42 kU/L), also the presence of reactivity to Pru p 7 (8.34 kU/L). Interestingly, the 2015 serum stored in our serum bank was also re-tested, and so we were able to demonstrate the presence, since 2015, of a dual reactivity to Pru p 7 and Pru p 3.

Conclusion: Strict avoidance of fruits containing nsLTPs and GRPs fruit before physical exercise. AIT prescribed only for Cypress.

Case 3 (published²³⁰)

Clinical History: The patient is a 40years-old man currently living in Paris (northern France) and born in southwest France. He has suffered since childhood from cypress pollen allergy and also food allergy and he experienced an anaphylactic shock after ingestion of pomegranate (*Punica granatum*, Lythraceae family) and strong oral syndrome after ingestion of *Rosaceae* fruits (apple and peach). He has seasonal rhino-conjunctivitis during the cypress and birch pollen seasons, which overlap in the north of France, relieved by antihistaminic treatment.

Allergy testing: SPT are positive for birch and cypress pollen extracts. Specific IgE antibodies to birch (27.2 kU/L) and cypress (1.42 kU/L) pollen, citrus (1.38 kU/L), apple (2.62 kU/L), peach (1.78 kU/L), strawberry (0.49 kU/L), kiwi (0.43 kU/L) and cherry (1.99 kU/L) extracts were found with singleplex technology (ImmunoCAP Specific IgE test) and also multiplex microchips (ImmunoCAP ISAC). This patient was studied by immunoblot

against cypress pollen, peach, citrus and pomegranate extracts and Pru p 7 and snakin-1, the GRPs of peach and potato, respectively. All immunoblots were positive at low MW corresponding to a GRP-specific IgE reactivity. Moreover, basophil activation test with total allergen source extracts (cypress pollen, peach and pomegranate) and purified allergens (Cup s 7 and Pru p 7) was found positive in contrast to snakin-1 in keeping with the tolerance to potatoes mentioned by the patient.

Conclusion: Strict avoidance of *Rosaceae* fruits and especially pomegranate.

Case 4 (partially published¹⁵⁷⁷)

Clinical History: The patient is a 16-year-old Japanese girl allergic to Japanese cedar (*Cryptomeria japonica*) pollen who suffered from an anaphylactic reaction after consuming chili pepper. She was diagnosed allergic to Japanese cedar pollen when she was 10 years old. She suffered also from an allergy to apple, peach, and orange with symptoms of anaphylaxis exacerbated by physical exercise (or before menstruation) with an onset at the age of 12 years after the consumption of canned peach. At 14 years, consuming a Korean cuisine dish containing beef, bean sprout, spinach, fiddlehead fern, chili pepper, and rice, she developed anaphylaxis with symptoms of facial angioedema, systemic erythema, cough, dyspnea, and cramp. At 16 years, she again experienced a similar reaction after consuming a Chinese cuisine dish containing tofu, minced meat, and chili pepper.

Allergy testing: Specific IgE evaluation showed a high titer to Japanese cedar (220 kU/L) and cypress (31.1 kU/L) pollen extracts

together with other pollen and food sensitizations to peach (4.7 kU/L), apple (2.54 kU/L), orange (4.55 kU/L), potato (1.08 kU/L) and confirmed the sensitization to chili pepper (0.24 kU/L). She has no IgE against nsLTPs and a low titer to PR-10.

Oral food challenges to chili pepper (125 mg) or peach (30 g of canned peach) were positive inducing allergic symptoms that include anaphylaxis and required adrenaline and fluid supplement. In agreement, peach and chili pepper extracts were able to *ex vivo* activate the patient's basophils.

Studied by direct and competitive immunoblot on Japanese cedar pollen proteins, the patient showed IgE reactivities at low MW inhibited not only by Cry j 7, the GRP of Japanese cedar pollen but also by Cap a 7, the GRP from bell pepper.

When tested on bell pepper extracts this patient showed IgE reactivity to a unique cationic LMW *Capsicum annuum* protein from bell and chili pepper pulp extract. The reactivity could be inhibited by Cry j 7, Cap a 7 or Pru p 7. This patient is also allergic to potato, a species from the same family as bell pepper, Solanaceae, and an IgE reactivity was found against recombinant snakin-1, the GRP from potato.

Conclusion: This young patient is shown to be sensitized to another member of the GRP family, an allergen as yet undescribed in *Capsicum annuum*, Cap a 7. Allergy to bell/chili pepper is very rare and IgE reactivity to GRP is exceptionally reflecting a very peculiar mechanism of crossed and reinforced specific sensitization. It seems that this allergy is associated with another very rare allergy to the GRP of potato, snakin-1.