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Allergenicity Assessment of Osmotin, a Pathogenesis-Related Protein, Used for Transgenic Crops

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ABSTRACT: Genetic engineering can enhance abiotic stress tolerance of plants, thereby increasing productivity. The present study investigates allergenicity of osmotin protein used for developing transgenic crops. Bioinformatic analysis of osmotin was performed using SDAP and Farrp allergen databases. Osmotin was cloned in pET22b+ vector, purified to homogeneity, and analyzed for digestibility, heat stability, and IgE binding using atopic patients' sera. Osmotin showed 40−92% and 48−75% homology with allergens in SDAP and Farrp databases, respectively. These cross-reactive allergens were from apple, tomato, peach, capsicum, kiwi fruit, and cypress. Osmotin was resistant to pepsin digestion and heat treatment at 90 °C for 1 h. Osmotin protein showed dose-dependent inhibition with pooled patients' sera. It showed significant IgE binding with 22 of 117 patients' sera who were sensitized to tomato and apple, thus indicating cross-reactivity among tomato, apple, and osmotin allergens. In conclusion, osmotin was identified as a potential allergen and showed cross-reactivity with tomato and apple allergens.

KEYWORDS: Genetic engineering, genetically modified food, food allergy, pathogenesis related protein, osmotin, *Nicotiana tabacum*, cross-reactivity, allergenicity, transgenic crops

INTRODUCTION

Genetic engineering provides crop improvement by transfer of genes conferring resistance to pests, diseases, herbicides, and abiotic stresses, resulting in enhancement of the efficiency and product yield.[1] Foods produced through genetically engineered crops such as corn, soybean, canola, alfalfa, and squash have reached consumers in some countries.[2] However, the newly introduced proteins in transgenic crops may result in an unintentional introduction of a new or cross-reactive food allergen.[3] The potential allergenicity of these novel proteins is required to be assessed as per guidelines for food safety assessment.[4,5]

Osmotin, a 26 kDa cationic protein from tobacco (*Nicotiana tabacum*), accumulates in cells on osmotic stress adaptation.[6] It belongs to the pathogenesis related-5 (PR-5) protein family that provides osmotolerance to plants and is referred to as thaumatin-like protein.[7] Transgenic potato and rice plants overproducing osmotin delay the development of disease symptoms.[8,9] Osmotin has also demonstrated antifungal activity against *Phomopsis viticola* and *Botrytis cinerea* mycelia.[10] The antifungal activity is correlated with plasma membrane permeabilization of sensitive fungi.[11]

Genetic modifications can affect the allergenicity either by introducing new allergens or by changing the level or nature of intrinsic allergens. Transgenic crops such as tobacco, strawberry, wheat, cotton, tomato, and mulberry containing the osmotin gene were developed to improve the yield.[12−17] Randhawa et al. developed molecular diagnosis of transgenic tomato with multiplex polymerase chain reaction (PCR).[18] Furthermore, the transgenic tomato containing the osmotin gene was tested in contained field trials.[19] However, there is a need to address the safety aspect of osmotin protein. The present study aimed to assess the allergenicity of osmotin protein using bioinformatic and immunobiochemical methods.

MATERIALS AND METHODS

Sequence Homology of Osmotin. The amino acid sequence was used for the database search. The sequence homology of osmotin (accession number P14170) was analyzed using Structural Database of Allergenic Proteins (SDAP; 737 sequences; http://fermi.utmb.edu/SDAP/) and Food Allergy Resources and Research Program (Farrp; version 10; 1471 sequences with isoforms; http://www.farrp.org/) to detect the sequence identity with other allergens presenting a 35% amino acid identity through a window of 80 amino acids.

Cloning of Osmotin Gene. For subcloning of the osmotin gene in the expression vector, restriction analysis was done to identify the noncutter enzymes, and *EcoR I* and *Xho I* restriction enzymes were used for cloning. The forward primer 5′-GAATTGAATTCGATGGGCAACTTGAGATCTTCTTTTG-3′ and reverse primer 5′-AGCTCGAGCTCCTTAGCCACTTCATCACTTCCAG-3′ having *EcoR I* and *Xho I* sites, respectively, were selected for PCR amplification. Five nanograms of phagemid (a gift from Prof. K.C. Bansal, Indian Agricultural Research Institute, New Delhi) containing the osmotin gene was amplified using primers that incorporated *EcoR I* and *Xho I* sites in the amplified product. The PCR product was run on the agarose gel and eluted using gel-filtration kit (Qiagen, Germany). The purified product and pET22b+ vector were digested with *EcoR I* and *Xho I* enzymes, ligated at 16 °C for 16 h, and transformed into DH5α cell. Individual colonies were picked and grown overnight at 37 °C to isolate plasmid. It was digested with restriction enzymes, and a positive clone containing the gene was sequenced.

Protein Expression and Purification. The plasmid containing the insert was transformed into BL-21 *Escherichia coli* cells. A clone containing the plasmid was grown until 0.6−0.8 o.d. induced with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h and harvested. The pellet

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Table 1. SDAP Database Showing % Amino Acid Identity of Osmotin with Allergen Homologues[a]

S no.	allergen	species	accession no.	sequence identity	*E* score	% identity
1	Lyc e NP24	*Lycopersicon esculentum*	P12670	226/246	1.9×10^{-93}	91.87
2	Cap a 1 w	*Capsicum annuum*	CAC34055	217/246	5.5×10^{-91}	88.21
3	Cap a 1	*Capsicum annuum*	AAG34078	148/246	6.6×10^{-65}	60.16
4	Act c 2	*Actinidia deliciosa*	P81370	149/246	1.1×10^{-59}	60.57
5	Mus a 4.0101	*Musa acuminata*	1Z3Q_A	136/246	6.2×10^{-57}	55.28
6	Jun a 3	*Juniperus ashei*	P81295	117/246	2.5×10^{-42}	47.56
7	Cup s 3.0101	*Cupressus sempervirens*	AAR21073	111/246	1.3×10^{-41}	45.12
8	Cup a 3	*Cupressus arizonica*	CAC05258	107/246	7.3×10^{-41}	43.50
9	Pru p 2.0301	*Prunus persica*	ACE80955	100/246	6.2×10^{-23}	40.65
10	Pru av 2	*Prunus avium*	P50694	99/246	1.6×10^{-21}	40.24
11	Pru p 2.0101	*Prunus persica*	ACE80959	101/246	7.2×10^{-21}	41.06
12	Mal d 2	*Malus domestica*	CAC10270	100/246	7.7×10^{-10}	40.65

[a] Homology of the amino acid sequence was performed with different allergens in SDAP.

Table 2. Sequence Homology Search Using Farrp Allergen Database[a]

S no.	allergen	accession no.	species	*E* score	% identity
1	Act d 2	CAI38795.2	*Actinidia deliciosa*	4.5×10^{-60}	75.00
2	Act d 2	ABQ42566.1	*Actinidia deliciosa*	5.5×10^{-59}	73.80
3	Jun a 3	P81295.1	*Juniperus ashei*	1.4×10^{-42}	63.79
4	Jun r 3.2	AAR21072.1	*Juniperus rigida*	1.5×10^{-42}	63.70
5	Jun v 3	Q9LD79.2	*Juniperus virginiana*	4.2×10^{-18}	62.51
6	Jun r 3.1	AAR21071.1	*Juniperus rigida*	2.7×10^{-42}	62.51
7	Cry j 3.8	BAF51970.1	*Cryptomeria japonica*	4.6×10^{-43}	62.51
8	Cup s 3.1	AAR21075.1	*Cupressus sempervirens*	6.9×10^{-42}	61.30
9	Cup s 3	AAR21074.1	*Cupressus sempervirens*	6.9×10^{-42}	61.30
10	Cup a 3	CAC05258.1	*Cupressus arizonica*	4.3×10^{-41}	58.80
11	Mal d 2	AAX19848.1	*Malus* × *domestica*	6.5×10^{-10}	49.97
12	Mal d 2	AAX19851.1	*Malus* × *domestica*	6.5×10^{-10}	49.97
13	Mal d 2	Q9FSG7.1	*Malus* × *domestica*	6.5×10^{-10}	49.97
14	Pru av 2	AAB38064.1	*Prunus avium*	1.1×10^{-21}	47.80

[a] The amino acid sequence of osmotin was blasted against the Farrp allergen database for 80 mer window. The cut off for sequence identity was taken as ≥35% homology with allergens.

was suspended in binding buffer containing 50 mM NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole (pH 8.0), sonicated, and centrifuged. The supernatant was incubated for 2 h with 2 mL of Ni-NTA (nickel-nitrilotriacetic acid) equilibrated with binding buffer. The column was washed with buffer containing 20 mM imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4, and bound proteins were eluted using buffer containing 250 mM imidazole, 300 mM NaCl, and 50 mM NaH_2PO_4. The purified protein was resolved on 12% SDS-PAGE (sodium dodecyl sulfate−polyacrylamide gel electrophoresis), transferred to nitrocellulose membrane, and probed with antibodies raised in mice against osmotin as described in ref 20.

Digestibility Studies. The digestibility of osmotin protein was determined by incubation in simulated gastric fluid (SGF), that is, 3.2 mg/mL pepsin in 0.03 M NaCl at pH 1.2.[20] Osmotin protein (400 μg) was dissolved in 400 μL of SGF. Digestion was carried out at 37 °C, and 30 μL of the samples was aliquoted at 0, 5, 15, 30, 45, and 60 min. The reaction was stopped by adding 26 μL of sample buffer. The mixture was then boiled for 5 min and was analyzed by SDS-PAGE and Western blotting in comparison with untreated protein.

Heat Stability Studies. The effect of temperature on stability of osmotin protein was determined by incubating it in a buffer at 90 °C.[21] Osmotin protein was dissolved at a concentration of 0.025 mg/mL. The protein was examined for stability at 90 °C for 15, 30, 45, and 60 min in a temperature-controlled heating block. The assay was terminated by ice cooling of sample, and the reaction was stopped by adding sample buffer. The treated protein was analyzed by SDS-PAGE.

Allergen Extracts and Sera Collection. Food extracts for allergy diagnosis were procured from All Cure Pharma Private Limited, New Delhi, approved by Drug Controller. Patients with allergic rhinitis and asthma aged 15−50 years were skin prick tested with various pollens, fungi, insects, and food allergen extracts to detect sensitization. The study protocol was approved by human ethics committee of the institute. The diagnosis of asthma was made following guidelines of the American Thoracic Society.[22] The patients having any two of the symptoms, viz. sneezing, rhinorrhea, nasal blockage, postnasal drip, etc., for most of the time in the last 2 years were diagnosed with rhinitis.[23] Blood samples (sera) were collected from patients showing marked positive skin reactions to different food extracts at the outpatient department, V.P. Chest Institute, Delhi, a referral chest hospital.

Specific IgE Estimation. Specific IgE against purified osmotin protein was determined by ELISA (enzyme-linked immunosorbent assay).[20] Briefly, 250 ng of osmotin protein was incubated in carbonate buffer (250 ng/100 μL per well) overnight at 4 °C in a microtiter plate (Nunc, Roskilde, Denmark). After they were washed with PBST (phosphate-buffered saline−Tween 20) (0.05%), the nonspecific sites

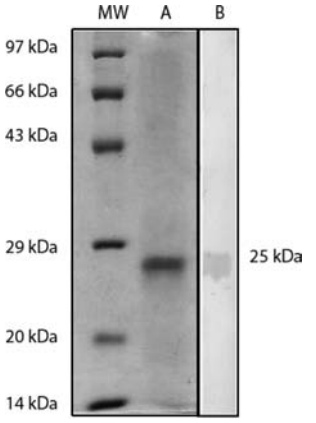

Figure 1. SDS-PAGE profile of purified osmotin protein. The protein was resolved on 12% gel and stained with Coomassie brilliant blue (Lane A). SDS-PAGE resolved protein was transferred on to nitrocellulose membrane and probed with osmotin antibodies raised in mice (lane B). Lane MW, molecular weight marker.

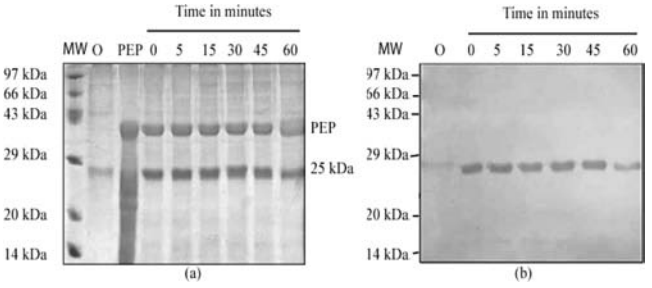

Figure 2. (a) SDS-PAGE profile of osmotin treated with SGF. Osmotin was treated with SGF for 0, 5, 15, 30, 45, and 60 min and resolved on 12% gel and stained with Coomassie brilliant blue. (b) Treated osmotin was transferred on to nitrocellulose membrane and probed with osmotin antibodies. Lanes: MW, molecular weight marker; O, purified osmotin protein; and PEP, pepsin.

were blocked with 3% defatted milk for 3 h at 37 °C. The plate was washed again and incubated with diluted (1:10 v/v) patients' sera showing positive SPT to food extracts overnight at 4 °C. Normal human sera ($n = 4$) were used as a negative control. The plate was washed and incubated with antihuman IgE−horse radish peroxidase (1:1000 v/v, Sigma) for 3 h at 37 °C. The plate was washed with PBS-Tween 20 and followed by PBS, and color was developed with orthophenylene diamine. The reaction was stopped by adding 5 N H_2SO_4, and the absorbance/o.d. (optical density) was read at 492 nm.

ELISA Inhibition. ELISA inhibition was performed to determine the cross-reactivity of the osmotin protein.[24] Briefly, a microtiter plate was coated with purified osmotin protein (100 ng/100 μL per well) and blocked with 3% defatted milk. The plate was incubated with a preincubated mixture of 1:10 v/v osmotin positive pooled patients' sera ($n = 22$) and graded amounts of purified protein, that is, 0.1, 1, 10, 100, and 1000 ng as inhibitor. The remaining steps were the same as followed for ELISA. The percent inhibition in IgE binding was calculated using equation given below

$$1 - \frac{\text{o.d. of the sample with inhibitor}}{\text{o.d. of the sample without inhibitor}} \times 100$$

Statistical Analysis. Statistical analysis for the specific IgE values of the patients showing positive SPT to food extracts and normal human sera (taken as control) against osmotin was performed using Epiinfo. Analysis of variance revealed a significant difference in concentration of IgE between the patients and the control sera samples. A P value ≤ 0.05 was considered as statistically significant.

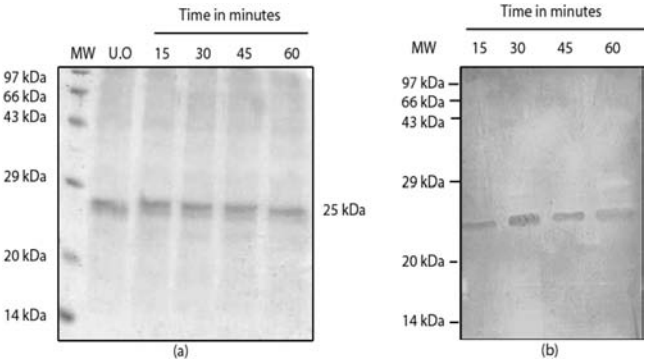

Figure 3. (a) SDS-PAGE profile of heat treated osmotin. Osmotin was given heat treatment for 15, 30, 45, and 60 min at 90 °C and resolved on 12% gel and stained with Coomassie brilliant blue. Lanes: MW, molecular weight marker; U.O, untreated osmotin; and 15, 30, 45, and 60, time in minutes. (b) SDS-PAGE resolved protein was transferred on to nitrocellulose membrane and probed with osmotin antibodies raised in mice.

RESULTS

Homology of Osmotin with Different Allergens. Allergen homologues of osmotin protein were identified using allergenic proteins' databases. In the Structural Database of Allergenic Proteins (SDAP) database, osmotin showed more than 35% identity with 12 predicted/validated allergens (Table 1). In the Farrp allergen database, osmotin showed maximum identity (75%) with kiwifruit allergen Act d 2 followed by Jun a 3 (64%) of *Juniperus ashei* and Cry j 3.8 (63%) of *Cryptomeria japonica* (Table 2). The 80 amino acid sliding window search showed 14 allergens from different plant species having 35% or more identity with osmotin.

Expression of Purified Osmotin. Osmotin was subcloned in pET22 b+ vector having a histidine tag for protein purification. The yield of the purified osmotin protein was approximately 1 mg/L of culture. The recombinant protein appeared at a molecular mass of 25 kDa on SDS-PAGE and reacted with antibodies raised in mice against osmotin on immunoblot (Figure 1).

Stability of Osmotin in SGF. On treatment with SGF for 60 min, the osmotin protein was detectable on SDS-PAGE without any degradation (Figure 2a). The immunoblot with antibodies against osmotin showed an intact band at 25 kDa (Figure 2b). This indicates that osmotin is stable in SGF.

Stability of Osmotin upon Heat Treatment. The untreated osmotin protein was visualized at 25 kDa on SDS-PAGE. On heat treatment at 90 °C for 60 min, osmotin protein was visualized at 25 kDa on immunoblot using osmotin antibodies (Figure 3a). Thus, the immunoreactivity of protein was also intact on heat treatment (Figure 3b).

IgE Binding with Patients' Sera (SPT Positive to Food Extracts). A total of 117 patients' sera were screened against osmotin protein by ELISA. Of these, 22 serum samples from patients positive to different foods showed significant IgE binding to osmotin protein (Table 3). Here, o.d. ≥ 3 times of normal control was taken as cutoff to define ELISA positive results.

Table 3. Specific IgE Binding of Osmotin against Patients' Sera[a]

		specific IgE	
patients no.	allergen (history + SPT positive)	osmotin ($OD_{492\ nm}$)	corresponding allergen ($OD_{492\ nm}$)
1		0.483	
2	apple	0.602	0.549
3		0.551	
4	tomato	0.496	0.312
5	tomato, apple	0.401	0.386, 0.459
6	apple	0.462	0.448
7	mustard	0.552	0.408
8	tomato	0.513	0.561
9	cowpea	0.475	0.368
10	apple	0.510	0.451
11	tomato	0.485	0.357
12	tomato	0.47	0.315
13	mustard	0.461	0.449
14		0.414	
15	tomato	0.432	0.318
16		0.606	
17	apple	0.528	0.548
18		0.625	
19	gram	0.679	0.645
20	lemon, orange	0.457	
21	citrus	0.529	
22	soya	0.484	0.492
23	control	0.114	0.112[b]
24	control	0.098	0.101[b]
25	control	0.087	0.095[b]
26	control	0.102	0.107[b]

[a] IgE binding was measured by ELISA using patients' sera (1−22). The control serum samples (23−26) were from healthy individuals. [b] IgE binding of the control serum samples was measured against apple extract.

Allergenic Cross-Reactivity of Osmotin Protein. ELISA inhibition was performed to determine the cross-reactivity of osmotin with patients' pooled sera (Figure 4). A dose-dependent inhibition of IgE binding to osmotin protein was observed, when serum pool was incubated with increasing concentrations (1−1000 ng) of osmotin. A maximum of 63% inhibition was observed with 1000 ng of osmotic protein. The purified osmotin protein required 8 ng of self-protein for 50% inhibition of IgE binding (EC_{50}).

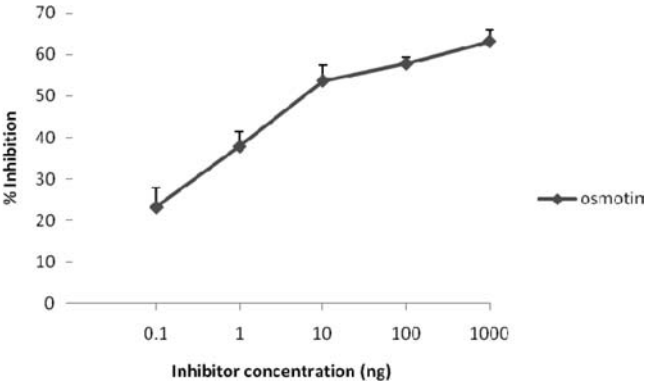

Figure 4. ELISA inhibition for allergenic cross-reactivity of osmotin. For IgE inhibition, osmotin positive patients' pooled sera were incubated with 0.1−1000 ng of osmotin protein as a self-inhibitor. The assay was carried out with purified osmotin protein coated on a microtiter plate.

DISCUSSION

Agricultural biotechnology offers enormous benefit toward improvement of the crops that are used for food and forage. Foods obtained from conventional or genetically engineered crops can be a potential source of allergens. Studies detected a few hundred proteins as food allergens.[25] The foods from transgenic crops can be a risk factor as allergens because of the introduction of new genes (proteins) into crops. Hence, these proteins require safety assessment before genetically modified (GM) foods are released into the market.[26] In the present study, the allergenicity of osmotin was assessed by weight of evidence approach (Codex, 2003).

Many plant proteins belonging to the PR-5 family have been described as food allergens.[27] In the present study, osmotin protein showed significant sequence identity with known allergens of PR-5 family, for example, tomato, kiwi fruit, and apple allergens. This indicates potential cross-reactivity between osmotin and the allergens of PR-5 family. Furthermore, the antigenic regions (epitopes) PRGTKMARVWGRT and NFNA AGRGTCQTG of NP24-I and Jun a 3 allergens are present in the osmotin sequence, suggesting cross-reactivity between these allergens.[28] Thus, osmotin appears to be a potential allergen based on sequence identity and presence of similar epitopes with known allergens.

It is difficult to obtain native purified proteins from plant sources; hence, *E. coli* synthesized proteins were used for allergenicity evaluation. This also facilitates high yield of protein with low batch to batch variation.[29] Many food allergens, for example, ovomucoid, Mal d 2, rAct d 8, etc., have been expressed as recombinant proteins.[30−32] These recombinant allergens have similar structure and immunologic properties as that of native counterparts. The recombinant proteins (allergens) have been recommended for diagnosis and therapy of IgE-mediated allergy.[33] In the present study, osmotin was expressed in *E. coli*, and the purified protein was used for in vitro experiments including IgE binding assay. *E. coli*-expressed osmotin showed IgE binding on immunoblot with antibodies raised in mice.

Several food allergens (proteins) were stable to heat and SGF treatment.[34] Ber e 1, Ses I 1, and Vit v 1 showed limited unfolding at 90 °C.[35] Food allergens like Pru av 1 (Bet v 1 homologues), Gly m 1, Ara h 1, and Ara h 2 also showed little stability in SGF.[36,37] A previous study reported that Mal d 2 was resistant to both thermal treatment and SGF digestion,[38] but the analysis of Mal d 2 sequence by ExPASy cutter tool showed pepsin cleavage sites. In the present study, osmotin was stable to SGF digestion and heat treatment. Bioinformatic analysis by ExPASy peptide cutter showed the presence of pepsin cleavage sites in osmotin (data not shown), but it demonstrated stability to SGF digestion and heat treatment. The experimental results correlate with an earlier study, which showed stability of osmotin to proteolysis.[6]

IgE binding of protein is a prerequisite for clinical reactivity. Here, osmotin demonstrated significant IgE binding with patients' sera, showing positive SPT to food extracts by ELISA.

Among 22 patients' sera showing IgE binding with osmotin, 10 were allergic to apple and tomato. Both apple and tomato allergen showed significant sequence identity with osmotin. It appears that the IgE antibodies specific to these allergens cross-react with osmotin. The *E. coli*-expressed osmotin protein has no glycosylation but shows IgE binding, indicating no cross-reactive carbohydrate determinants interference. Moreover, the ELISA inhibition also showed cross-reactivity with patients' sera. Thus, the results obtained by bioinformatics approach match with the experimental analysis.

GM crop plants, for example, tomato, wheat, and strawberry, using osmotin are at different stages of development. The transgenic tomato containing osmotin gene was further tested in contained field trials.[19] Osmotin belongs to the thaumatin-like protein family described as food allergens. Hence, the concern for allergeniciy of osmotin needs to be addressed on priority because of its application as transgene. Mutations in the IgE binding epitopes of the protein may lead to reduced allergenicity while maintaining its desired function for transgenic crops. In conclusion, osmotin seems to be a potential allergen based on bioinformatics, SGF, heat stability, and immunobiochemical studies.

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ABBREVIATIONS USED

GM, genetically modified; PR, pathogenesis related; SDAP, Structural Database of Allergenic Proteins; Farrp, Food Allergy Resources and Research Program; Ni-NTA, nickel-nitrilotriacetic acid; SGF, simulated gastric fluid; ELISA, enzyme-linked immunosorbent assay

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