ORIGINAL ARTICLE

Unraveling the Diagnosis of Kiwifruit Allergy: Usefulness of Current Diagnostic Tests

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Abstract

Objectives: To determine the usefulness of the in vitro and in vivo methods used in the diagnosis of kiwifruit allergy and to specifically assess the impact of seed proteins on sensitivity.
Methods: We performed skin prick tests (SPTs) using various commercial extracts, homemade pulp, and seed extracts and prick-prick tests with kiwifruit on 36 allergic patients. The presence of specific IgE (sIgE) was assessed using the ImmunoCAP (kiwifruit extract), ELISA (Act d 1, Act d 2), ISAC, and FABER assays. Immunoblotting of seed extract was carried out, and a single-blind oral food challenge was performed with whole seeds in seed-sensitized individuals.
Results: The prick prick test with kiwifruit demonstrated the highest diagnostic capacity (81.8% sensitivity and 94.1% specificity) among the in vivo tests. The sIgE levels measured using ImmunoCAP (kiwifruit extract) showed a similar sensitivity to that of global ISAC and FABER (63.9%, 59.5%, and 58.3%, respectively). Act d 1 was the major allergen. Sensitization to Act d 1 was associated with positive sIgE results to whole kiwifruit extract detected by ImmunoCAP ($P<.000$). A positive SPT result to kiwifruit seeds was associated with severe symptoms induced by kiwifruit ($P=.019$) as a marker of advanced disease, but not with clinically relevant sensitization. Challenge testing with kiwifruit seeds performed on 8 seed-sensitized patients yielded negative results.
Conclusions: Sensitization to Act d 1 is associated with a positive result in conventional diagnostic techniques, whereas kiwifruit seed sensitization does not increase the sensitivity of the diagnostic techniques evaluated.

Key words: Allergy. Component-resolved diagnosis. Kiwifruit. Skin test. Storage protein.

Resumen

Objetivos: Determinar la rentabilidad diagnóstica de las técnicas *in vitro* e *in vivo* utilizadas en el diagnóstico de alergia al kiwi y estudiar la influencia de las proteínas alergénicas de las semillas en su sensibilidad.
Métodos: Se seleccionaron 36 pacientes alérgicos a kiwi. Se les realizó *prick* test con cuatro extractos comerciales diferentes y *prick-prick* con kiwi. Se determinó IgE específica mediante ImmunoCAP (extracto de kiwi), ELISA (Act d 1, Act d 2), las micromatrices ISAC y FABER e Immunoblotting de extracto de semilla de kiwi. Se realizó exposición oral simple ciego frente a semilla de kiwi en pacientes sensibilizados a la semilla.
Resultados: El *prick-prick* de kiwi fue la prueba *in vivo* con mayor rendimiento (sensibilidad 81,8%, especificidad 94,1%). El ImmunoCAP de extracto de kiwi mostró una sensibilidad similar a la global del ISAC y del FABER (63,9%, 59,5% y 58,3%, respectivamente). Act d 1 fue el alérgeno mayoritario. Se encontró asociación entre los niveles de IgE específica frente a Act d 1 (ISAC) y el extracto de kiwi mediante ImmunoCAP ($p <0,000$). La prueba cutánea positiva con semilla se asoció con mayor gravedad de síntomas frente a kiwi ($p = 0,019$), como marcador de enfermedad avanzada, pero no como sensibilización clínicamente relevante. La prueba de provocación con semillas fue negativa en los ocho pacientes provocados.
Conclusiones: La sensibilización a Act d 1 se asocia con resultados positivos con las técnicas diagnósticas convencionales. La sensibilización frente a semillas no mejora el rendimiento de las técnicas evaluadas.

Palabras clave: Alergia. Diagnóstico por componentes. Kiwi. Prueba cutánea. Proteína de almacenamiento.

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Introduction

Kiwifruit (*Actinidia deliciosa*) has been one of the most important causes of food allergies in Western countries ever since the first report of hypersensitivity reaction to kiwifruit in 1981 [1-3]. Although kiwifruit allergy has been associated with polysensitization in latex-fruit syndrome [4], as well as in birch and grass pollinosis [5,6], monosensitization is strongly related to Act d 1, a 30-kDa cysteine protease and the major allergen, thus suggesting primary sensitization to kiwifruit [3,7]. The clinical manifestations of this allergy range from oral symptoms to severe systemic reactions, including anaphylaxis [1,3]. Fourteen allergens have been described in green kiwifruit, with marked geographical differences in the molecular profile of sensitized patients [1,8].

The commercial extracts used in skin prick tests (SPTs) have low sensitivity in the diagnosis of kiwifruit allergy (17%-61%) [1,3,7,9], and the results for the ImmunoCAP (Phadia) assay using kiwifruit extract (17%-55%) could be improved [1,3,7,9]. The determination of sIgE by component resolved diagnosis (CRD) using ImmunoCAP testing for pulp allergens has been reported to increase sensitivity to 65%-77% [1,3], whereas the ISAC microarray raises it to 66% [7]. Based on these low values, Sirvent et al [10,11] suggested that the inclusion of the kiwifruit seed allergens Act d 12 (11S globulin) and Act d 13 (2S albumin) in the CRD of kiwifruit allergy might significantly reduce the number of misdiagnosed patients. No studies have been conducted to date to demonstrate this hypothesis, and the clinical relevance of kiwifruit seed allergens in kiwifruit-allergic patients has yet to be established.

The aims of this study were to determine whether sensitization to the kiwifruit seed storage proteins Act d 12 and Act d 13 explains the low sensitivity of currently available techniques and to assess the clinical relevance of kiwifruit seed allergens. We also aimed to analyze the diagnostic performance of the commercial in vivo and in vitro techniques currently used to evaluate sensitization in the diagnosis of kiwifruit allergy.

Material and Methods

Study Population

A sample of 36 kiwifruit-allergic patients (>6 years old) was prospectively recruited throughout 2017 from the Allergology Department of 3 hospitals in Northern Spain: Clínica Universidad de Navarra in Pamplona, Complejo Hospitalario de Navarra in Pamplona, and Hospital Universitario Central de Asturias in Oviedo. Allergy was demonstrated by an open oral food challenge (OFC) with kiwifruit (see Supplement). Patients with a previous history of anaphylaxis or recent clear kiwifruit ingestion–related systemic symptoms (generalized acute urticaria or gastrointestinal symptoms or respiratory symptoms) and positive test results (SPT or specific immunoglobulin E [sIgE] against kiwifruit as determined by ImmunoCAP assay) were excluded from the OFC. Anaphylaxis was defined following the 2014 European Academy of Allergy and Clinical Immunology anaphylaxis guideline [12]. The exclusion criteria are defined in the Supplement. All patients completed the study questionnaire with their clinical data, recording the symptoms experienced following kiwifruit intake and tolerance to other foods. Symptom severity was measured according to a scale of symptoms (contact rash, oral allergy syndrome, systemic symptoms, and anaphylaxis).

Additionally, 31 atopic adult controls sensitized to pollen or plant food allergens were prospectively recruited for the analysis of the specificity of the SPT. An OFC was performed in this control group following a 6-month period during which the fruit had not been eaten. All participants signed the informed consent document, which had previously been approved by the Research Ethics Committee of the University of Navarra (2016.052) and supported by the ethics committees of the participating hospitals. The demographic and clinical data of these patients and controls are summarized in Table 1.

In addition, sera from 35 controls (14 atopic controls sensitized to dust mite and 21 nonatopic controls) from a previous multicenter study (FIS PI 11/01634) were used to analyze the specificity of the in vitro techniques.

Skin Tests

All prospectively recruited cases and controls underwent a prick-prick test with kiwifruit and SPTs with 4 different commercial kiwifruit extracts (ALK-Abelló, Bial, Diater, and Leti) and homemade kiwifruit seed and pulp extracts (preparation described in the Supplement). Peach lipid transfer protein extract (0.1 mg/mL) (Bial), profilin extract (ALK Abelló), *Betula verrucosa* extract (ALK Abelló), and mustard extract (Leti) were tested to evaluate possible cross reactivity with other foods. The wheal and flare sizes were measured after 15 minutes, and wheals with a diameter ≥3 mm were considered to be positive [13].

Determination of Specific Immunoglobulin E

The presence of sIgE against Act d 1, Act d 2, Act d 5, and Act d 8 was determined in all 36 patients using microarray

Table 1. Clinical and Demographic Data of Patients and Controls

	Patients (n=36)	Controls (n=31)
Male sex, No. (%)	12/36 (33.3)	6 (19.4)
Mean (min-max) age, y	27 (6-62)	33 (18-59)
Clinical symptoms, No. (%)		
Contact urticaria	1/36 (2.8)	-
OAS	7/36 (19.4)	-
Systemic symptoms	11/36 (30.6)	-
Anaphylaxis	17/36 (47.2)	-
SPT, No. (%)		
Peach LTP (Bial)	7/36 (19.4)	3/31 (9.7)
Betula verrucosa (ALK-Abelló)	15/36 (41.7)	4/31 (12.9)
Profilin (ALK-Abelló)	9/36 (25)	3/31 (9.7)
sIgE, No. (%)		
ISAC Pru p 3 (LTP)	7/36 (19.4)	ND
ISAC Bet v 1 (PR-10)	6/36 (16.7)	ND
ISAC Phl p 12 (profilin)	8/36 (22.2)	ND

Abbreviations: ND, not done. LTP, lipid transfer protein; OAS, oral allergy syndrome; SPT, skin prick test.

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ImmunoCAP ISAC CRD112 (Thermo Fisher Scientific), with sIgE levels ≥0.3 ISU being considered positive. sIgE against Act d 1, Act d 2, Act d 5, and Act d 10 was determined using nanobead-based microarray FABER (CAAM), with sIgE levels ≥0.3 FIU/mL being considered positive. The levels of sIgE against kiwifruit extract (f84) and Act d 8 were measured using fluorescence enzyme immunoassay ImmunoCAP (Thermo Fisher Scientific), with sIgE levels ≥0.35 kU_A/L being considered positive. In addition, the presence of sIgE against Act d 1, Act d 2, and Act d 5 was repeatedly determined using the enzyme-linked immunosorbent assay (ELISA), in which the positive optical density values for each allergen were as follows: Act d 1, ≥0.235 units; Act d 2, ≥0.142 units; and Act d 5, ≥0.162 units (see Supplement for further detail).

The ISAC, FABER, ImmunoCAP (kiwifruit extract), and ELISA (Act d 1) assays were also used to determine the presence of sIgE in sera obtained from the controls of said retrospective sample (n=35). Some of the analyses were limited owing to the reduced availability of serum samples from some prospectively and retrospectively recruited patients.

Immunoblotting with kiwifruit seed extract (20 µg/strip) was performed with sera from the 36 patients comprising the study population. In addition, immunoblotting with kiwifruit seed extract was performed at 40 µg/strip with the sera from the 17 patients who had a positive SPT result against seed extract (see Supplement for methodological details).

Specific IgE inhibition studies with purified Act d 1 were performed to evaluate the presence of Act d 1 in the ImmunoCAP assay (see Supplement for details).

Single Blind Oral Food Challenge with Kiwifruit Seeds

To assess the clinical relevance of sensitization to kiwifruit seed allergens, a single-blind oral food challenge (SBOFC) was performed with kiwifruit seeds in 8 patients sensitized to kiwifruit seeds. Two types of smoothie were prepared to perform the SBOFC (an active smoothie [AS] containing all seeds of 1 kiwifruit and an inactive smoothie [IS] as a placebo). The food products used for the smoothie recipe were evaluated with SPTs (see Supplement for details). Doses of the smoothies were administered progressively, at 30-minute intervals, starting with the IS and continuing with the AS (0.5 g, 3.5 g, 7 g, 15 g, 30 g, and 70 g of AS), until all seeds present in the kiwifruit had been consumed. Whenever patients reported subjective symptoms after taking the AS, they received the same dose of the IS. The challenge was interrupted in the presence of objective or subjective symptoms on 2 occasions after taking the AS and graded with at least 2 more points in the visual analog scale (VAS) compared with the equivalent dose of the IS. The clinical and serological data of these patients are described in Table 2.

Statistical Analysis

The data were analyzed using Stata/IC 12.0. The sample size was calculated for 60 patients (30 cases and 30 controls) to detect a difference of 30% between 2 diagnostic tests (sensitivities of 50% and 80%, respectively) with a power of 70%, assuming a 2-tailed α value of 5% and a proportion of discordant pairs of 40%.

Table 2. Patients Challenged With Whole Kiwifruit Seeds

	Allergy symptoms with kiwifruit before SBOFC	Bands at 51 kDa and/or 12 kDa in IB (20 µg/strip) of kiwifruit extract	SPT kiwifruit seed extract	Result of SBOFC	Allergy symptoms with tree nuts and seeds	Storage proteins sensitization in ISAC and FABER
Patient 17	OAS	(-)	(+)	(-)	Systemic symptoms with almond, walnut, hazelnut, cashew, chestnut, peanut	ISAC and FABER: (-)
Patient 26	Anaphylaxis	(-)	(+)	(-)	(-)	ISAC: (-) FABER: ND
Patient 23	OAS	Band at 51 kDa	(-)	(-)	(-)	ISAC and FABER: (-)
Patient 25	Systemic symptoms	(-)	(+)	(-)	(-)	ISAC: (-) FABER: Ara h 3 (20.67 FUI/mL)
Patient 24	Systemic symptoms	Band at 12 kDa	(+)	(-)	Systemic symptoms with peanut, almond, walnut, chestnut; OAS with pistachio and cashew	ISAC: rJug r 1 (1.8 ISU) FABER: ND
Patient 4	Anaphylaxis	(-)	(+)	(-)	(-)	ISAC and FABER: (-)
Patient 10	Systemic symptoms	(-)	(+)	(-)	(-)	ISAC and FABER: (-)
Patient 15	OAS	(-)	(+)	(-)	(-)	ISAC and FABER: (-)

Abbreviations: IB, immunoblotting; ND, not done; OAS, oral allergy symptoms; SBOFC, single-blind oral food challenge; SPT, skin prick test.

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Quantitative variables were reported as median (IQR), and qualitative variables were reported as frequencies (percentages) and compared using the χ^2 or Fisher exact test. Ordinal and quantitative variables were analyzed using the Mann-Whitney test. The positive concordance between the skin tests evaluated was calculated considering the sum of positive and negative results, respectively. The correlation between the presence of sIgE against Act d 1 using the ISAC array and against kiwifruit extract using the CAP assay was evaluated based on the Spearman ρ. A concordance analysis between the in vitro techniques was performed using the McNemar test. A *P* value of less than .05 was considered to be statistically significant.

Results

Study Population

Thirty-six kiwifruit-allergic patients (12 male [33.3%]; mean age, 27 years [6-62]) were recruited. The 31 controls (6 male [19.4%]; mean age, 33 years [18 59]) were significantly older than the cases (*P*=.043) because only adults were included in this group. Most of the patients (47.2% [17/36]) had experienced anaphylaxis after ingesting kiwifruit, and 30.6% (11/36) experienced nonanaphylactic systemic symptoms, 19.4% (7/36) developed oral allergy syndrome, and 1 patient (2.8%) had a contact rash with kiwifruit. Twenty five percent (9/36) of the patients were sensitized to profilin, 41.7% (15/36) to *Betula verrucosa*, and 19.4% (7/36) to lipid transfer protein in SPT.

Prick-Prick Test With Kiwifruit is the Most Sensitive In Vivo Test

The sensitivity of the prick-prick test using kiwifruit was 81.8% (18/22). The sensitivity of the test using the 4 kiwifruit commercial extracts studied was 52.8% (19/36) for ALK-Abelló, 61.1% (22/36) for Leti, 63.9% (23/36) for Bial, and 66.7% (24/36) for Diater. Comparison by pairs of the kiwifruit extracts used in the prick tests revealed a concordance of 80.6% to 88.9% between the extracts. The sensitivity of the pulp and seed extracts was 61.1% (22/36) and 47.2% (17/36), respectively, and, interestingly, the concordance of positive SPT between pulp and seed extracts was 80.6% (29/36). The specificity of the in vivo tests is summarized in Table 3.

The ImmunoCAP Assay With Complete Kiwifruit Extract Showed a Similar Diagnostic Capacity to That of the ISAC and FABER Microarrays

The sensitivity of ImmunoCAP using kiwifruit extract was 63.9% (23/36) (median [IQR] 1.15 kU_A/L [0.19-3.37]). The pooled results of the 2 platforms analyzed considering all kiwifruit allergens present in the microarrays yielded a similar sensitivity to that of the ImmunoCAP assay with kiwifruit extract (FABER, 55.9% [19/34]; ISAC, 58.3% [21/36]). Regarding the component sensitization profile, the rate of positive results obtained with the ELISA, ISAC, and FABER assays in the detection of major allergen Act d 1 was 100% (36/36; median, 0.394 units [0.355-0.454]), 58.3% (21/36; median, 0.54 ISU [0-1.78]), and only 11.8% (4/34; median, 0 FIU/mL [0-0]), respectively. The frequency of sensitization to Act d 2 detected by the ELISA, ISAC, and FABER assays was 41.7% (15/36; median, 0.144 units [0.113-0.262]), 2.8% (1/36; median, 0 ISU [0-0]), and 0%, respectively. The frequency of sensitization to Act d 5 detected by the ELISA, ISAC, and FABER assays was 0% (0/36), 2.8% (1/36), and 2.9% (1/34), respectively. Finally, the frequency of sensitization to Act d 8 detected by the ISAC and CAP assays was 8.3% (3/36) in both cases, and the percentage of sensitization to Act d 10 detected by the FABER assay was 5.9% (2/34).

The specificity of the ImmunoCAP and FABER tests in the detection of sIgE against kiwifruit extract was 87.5% (14/16) (the ImmunoCAP assay was only performed in 16 patients) and 97.1% (34/35), respectively. The negative rate obtained in the detection of Act d 1 by the ELISA, ISAC, and FABER tests was 100% (16/16), 97.1% (34/35), and 100% (35/35), respectively. The global specificity of the ISAC and FABER assays was 97.1% (34/35) and 100% (35/35), respectively.

To analyze the lack of correlation between the detection of sIgE against Act d 1 measured by the ELISA and ISAC assays, we performed an SPT with purified Act d 1 in 10 patients [14]. sIgE against Act d 1 was detected in all patients with the ELISA, but in only 3 positive cases of sIgE against Act d 1 with the ISAC assay (patients 6, 7, and 24). These 3 patients had a positive SPT result against purified Act d 1, although the result for this allergen was negative in the remaining 7 patients (patients 5, 9, 17, 21, 22, 23, and 25). The results obtained with the ELISA were not finally included in the analysis owing to possible overestimation (Supplement Table I).

Table 3. Results of Skin Prick Tests With Kiwifruit Extracts

Skin prick test	Positive sensitization, No. (%)	Median (IQR), mm	Specificity, No. (%)
Kiwifruit pulp extract	22/36 (61.1)	4.25 (1-8)	24/25 (96)
Kiwifruit seed extract	17/36 (47.2)	2.25 (0-4.25)	25/25 (100)
Commercial kiwifruit extract, Diater	24/36 (66.7)	4.75 (1-7)	28/31 (90.32)
Commercial kiwifruit extract, Bial	23/36 (63.9)	4.75 (0-7.5)	30/31 (96.8)
Commercial kiwifruit extract, Leti	22/36 (61.1)	5 (0-7.75)	30/31 (96.8)
Commercial kiwifruit extract, ALK-Abelló	19/36 (52.8)	3 (0-6)	30/31 (96.8)
Prick-prick kiwifruit	18/22 (81.8)	7.5 (3.5-9.5)	16/17 (94.1)

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The analysis of concordance between the in vitro techniques is shown in the Supplement.

The Kiwifruit Extract Used in the ImmunoCAP Assay Contains a Sufficient Amount of Major Allergen Act d 1

The kiwifruit extract was inhibited on ImmunoCAP using purified Act d 1 at 5 different concentrations in sera from 4 patients who had a positive ImmunoCAP result against kiwifruit extract. Two of them had a positive ISAC result against Act d 1 (patient 6, kiwifruit CAP 10.6 kU_A/L and Act d 1 ISAC 11.62 ISU; patient 29, kiwifruit CAP 40.1 kU_A/L and Act d 1 ISAC 44.6 ISU), and 2 had a negative ISAC result against Act d 1 (patient 22, kiwifruit CAP 2.53 kU_A/L and Act d 1 ISAC 0 ISU; patient 25, kiwifruit CAP 2.21 kU_A/L and Act d 1 ISAC 0 ISU). Inhibition was 86% and 89% for patients 6 and 29, respectively, and 19% and 43%, respectively, for patients 22 and 25. Based on the results of our study, the major allergen Act d 1(15) is sufficiently represented in the whole kiwifruit extract (Supplement, Figure 1). Interestingly, both a good correlation and a significant association were observed between the ISAC result with Act d 1 and ImmunoCAP with kiwifruit extract (Spearman ρ, 0.8896; P<.000).

Sensitization to Act d 1 assessed using ISAC was also associated with a higher frequency of positive SPT results using commercial extracts (Bial, P<.000; Leti, P=.001; ALK-Abelló, P=.002; Diater, P=.01) and pulp extract (P=.014), but not with the kiwifruit prick-prick test (P=.117).

The Role of Sensitization to Kiwifruit Seeds

Immunoblotting with kiwifruit seed extract was performed for all patients (n=36) at 20 µg/strip (Figure). Bands with a molecular mass of 51 kDa (expected for Act d 12) and 12 kDa (expected for Act d 13) were detected in 19.4% of patients (7/36; patients 9, 18, 20, 22, 23, 31, and 32) and 11.1% (4/36; patients 11, 24, 29, and 30), respectively. Both bands were not detected simultaneously in any patients. None of the 7 patients with bands detected at 51 kDa presented positive SPT results with seed extract, and 3 patients out of the 4 with bands detected bands at 12 kDa had positive SPT results with the seed extract.

In order to evaluate the role of the seeds in the diagnosis of kiwifruit allergy, the participants were divided into 2 groups: sensitized and not sensitized to kiwifruit seeds according to the results of the SPTs. Seventeen out of 36 (47.2%) patients had a positive SPT result against kiwifruit seed extract. Severe symptoms following kiwifruit ingestion (anaphylaxis) were significantly more frequent among patients who were sensitized to the seeds (P=.019). Sensitization to seeds was associated with a higher frequency of positive SPT results against commercial extracts (Bial, P<.000; Leti, P=.002; ALK, P<.000; Diater, P=.014) and pulp extract (P<.014), but not in the kiwifruit prick-prick test (P=.293). Sensitization to seeds was also associated with higher levels of sIgE against Act d 1 (ISAC; P=.004) and whole kiwifruit extract (ImmunoCAP; P=.029). The clinical data of these patients are summarized in Table 4.

The addition of the SPT with seed extract did not sufficiently improve the diagnostic performance of the techniques, as sensitivity only increased from 81.8% (18/22) to 86.4% (19/22) for the prick-prick test, from 66.7% (24/36) to 72.2% (26/36) for the commercial extract, from 63.9% (23/36) to 69.4% (25/36) for ImmunoCAP, from 58.3% (21/36) to 66.7% (24/36) for ISAC, and from 55.9% (19/34) to 70.6% (24/34) for FABER.

Eight patients with a positive SPT result against kiwifruit seeds and/or bands at 51 kDa (1/8) and/or 12 kDa (1/8) in immunoblotting underwent a kiwifruit seed SBOFC. All the results were negative.

Given the low amount of kiwifruit seed proteins found in immunoblotting of patients with a positive SPT against seed extract, a new immunoblot was performed using a greater concentration of seed extract (40 µg/strip) in this group (n=17). The immunoblot of 10 of the 17 patients (58.8%) revealed a 25-kDa protein that, when identified by mass spectrometry, corresponded to a seed specific thaumatin-like protein that differed from the Act d 2 of the pulp (see Supplement for further detail).

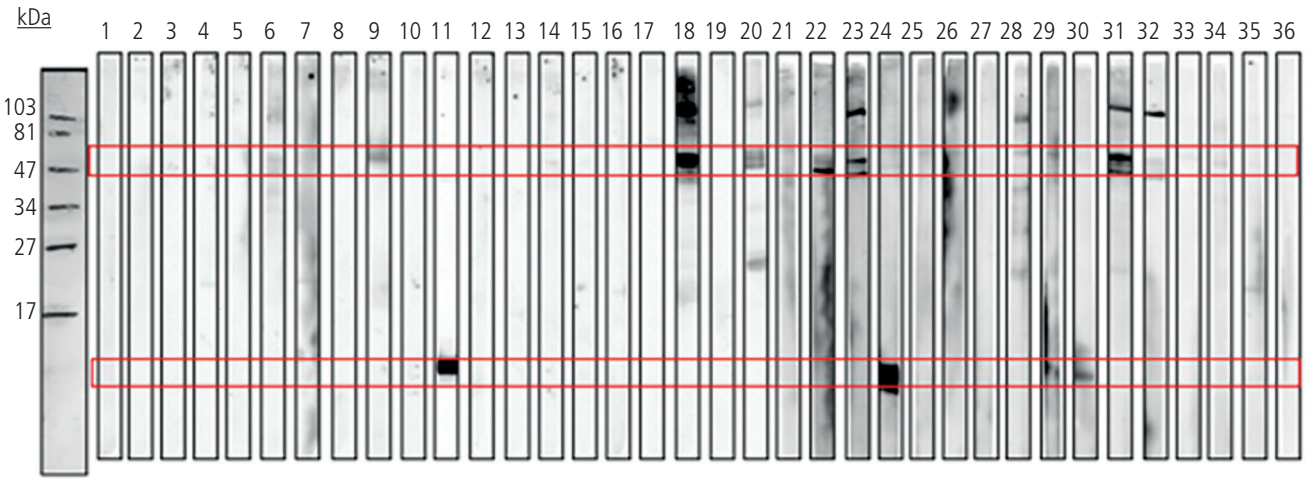

Figure. Immunoblotting of kiwifruit seed extract (20 µg/strip) in patients' sera (n=36).

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Table 4. Clinical Characteristics of Patients Sensitized and Not Sensitized to Kiwifruit Seeds

	Patients with negative SPT result to kiwifruit seed extract (n=19)	Patients with positive SPT result to kiwifruit seed extract (n=17)	*P* Value
Age, mean (min-max)	30.6 (6-62)	22.2 (6-46)	.095
Male sex, No. (%)	5/19 (26.3)	7/17 (41.2)	.483
Clinical symptom severity ranking			
Contact urticaria	1/19 (5.3)	0/17	.019
OAS	6/19 (31.6)	1/17 (5.9)	
Systemic symptoms (urticaria, GI, R)	6/19 (31.6)	5/17 (29.4)	
Anaphylaxis	6/19 (31.6)	11/17 (64.7)	
Median (IQR) age at onset	26 (12-39)	14 (6-23)	.068
Eliciting dose of kiwifruit (n=17), No. (%)			
1/32	4/11 (36.4)	4/6 (66.7)	.302
1/16	5/11 (45.5)	1/6 (16.7)	
1/8	0	1/6 (16.7)	
1/4	1/11 (9.1)	0	
1/2	1/11 (9.1)	0	
sIgE kiwifruit extract CAP, No. (%)	8/19 (42.1)	15/17 (88.2)	.006
Median (IQR) sIgE kiwifruit extract CAP, kU_A/L	0.28 (0.12-2.53)	2.25 (1.05-3.96)	.029
sIgE Act d 1 ISAC, No. (%)	7/19 (36.8)	14/17 (82.3)	.008
Median (IQR) sIgE Act d 1 ISAC, ISU	0.12 (0-0.69)	1.36 (0.59-4.25)	.004

Abbreviations: GI, gastrointestinal; IQR, interquartile rank; OAS, oral allergy syndrome; R, respiratory; SPT, skin prick test.

Discussion

We evaluated a series of in vivo and in vitro tests used to diagnose kiwifruit allergy. According to our results, the best in vivo test is the prick-prick test using fresh kiwifruit, as it yielded the highest sensitivity (81.8%), coinciding with data reported elsewhere [7,16]. In the case of SPT, we found low sensitivity when the test was applied using commercial kiwifruit extracts (52.8%-66.7%). The allergen Act d 1 (actinidin cysteine protease) is thought to comprise about 50% of kiwifruit proteins and degrade other allergen proteins present in the pulp [1,17], thus reducing the presence of other proteins in the commercial extracts and, hence, the capacity to detect patients allergic to other proteins. In fact, 60% (9/15) of the patients with a negative response against Act d 1 in the ISAC assay were misdiagnosed by the SPT using commercial extracts. Moreover, most of the patients not sensitized to Act d 1 in the ISAC assay were also misdiagnosed using other in vitro tests, including ImmunoCAP (13/15; 86.6%), FABER (11/15; 73.3%), and ISAC (15/15; 100%). In contrast, fresh kiwifruit seems to preserve all its proteins, thus ensuring good diagnostic capacity.

Sensitization to Act d 1 (58.3%, measured by ISAC) was more frequent in our study than elsewhere [1,3]. The ImmunoCAP assay with kiwifruit extract also yielded sensitivity that was higher than reported in previous studies [1,3,7] and similar to that of global ISAC and FABER, probably because of the higher prevalence of sensitization to Act d 1 among our population. In addition, we found that sera from Act d 1–sensitized patients were highly inhibited by purified Act d 1 in terms of their capacity to bind to the kiwifruit extract, thus demonstrating high representation of this allergen in this whole extract ImmunoCAP.

The good correlation between the ISAC for Act d 1 and ImmunoCAP for kiwifruit extract (Spearman ρ, 0.8896; $P<.000$) also corroborates this finding.

Based on these results, the ImmunoCAP (kiwifruit extract) approach proved to be similar to CRD in the diagnosis of kiwifruit allergy, showing good ability to detect Act d 1, the major allergen. However, there is room for improvement. The addition of an SPT using seed extract did not sufficiently improve the diagnostic performance of the techniques applied. Therefore, in our opinion, the absence of seed allergens for use in currently available in vitro and in vivo diagnostic tests does not explain their low capacity for diagnosis of kiwifruit allergy. Interestingly, we found that patients sensitized to kiwifruit seeds in the SPT had higher levels of sIgE against the whole kiwifruit extract and Act d 1 and that they also experienced more severe allergic symptoms after ingesting kiwifruit than patients not sensitized to kiwifruit seeds. Therefore, our results suggest that sensitization to kiwifruit seeds could be considered a marker of advanced disease and more severe kiwifruit allergy.

In addition, we were unable to demonstrate the clinical relevance of sensitization to kiwifruit seeds, as none of the 8 patients who were sensitized to kiwifruit seeds experienced symptoms after undergoing an oral challenge with the seeds. A possible limitation of this challenge is the fact that the patients swallowed the seeds in a smoothie, rather than by chewing them; this could have decreased the availability of the seed allergens. However, it has been reported that the seed allergens Act d 12 and Act d 13 can be released from intact

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kiwifruit seeds after 1 hour of exposure to simulated gastric and intestinal fluids [11]; therefore, release of the allergen should not have been affected. Our approach tried to mimic real-life conditions, in which patients swallow the seeds along with the kiwifruit pulp.

In conclusion, we found that the in vivo diagnostic test with the highest capacity was the prick-prick test with kiwifruit. The ImmunoCAP assay using kiwifruit extract showed a similar sensitivity to that of CRD techniques. The addition of an SPT using seed extract did not sufficiently improve the diagnostic performance of the available techniques. However, sensitization to the seed allergens seems to be a marker of advanced disease and more severe kiwifruit allergy. Sensitization to kiwifruit seeds was not clinically relevant in our study population.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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SUPPLEMENTARY MATERIAL

METHODS

Study Population

The exclusion criteria of the study were: patients under 6 years of age, patients in treatment with antihistamine that could not be interrupted, patients in treatment with topical corticosteroids on their arm, patients in treatment with omalizumab in the previous 3 months, patients receiving immunotherapy against peach, patients allergic to placebo components, pregnant women or patients with a physical or mental disability to participate.

Open Oral Food Challenge with Kiwifruit

An open oral food challenge (OFC) with kiwifruit (var. *Actidinia deliciosa*, Zespri®, New Zealand) was performed in 17/36 patients. Oral food challenge was not performed in 15 patients with previous history of anaphylaxis and four patients who had suffered a clear kiwifruit ingestion-related systemic symptom within the previous two years and had positive tests (SPT or specific immunoglobulin E [sIgE] against kiwifruit as determined by an ImmunoCAP assay). The OFC was performed progressively administering the following doses: 2.7 gr, 5.7, 11 gr, 23 gr, and 46 gr, every 15-30 minutes (1). The challenge was positive when objective symptoms of an IgE-mediated reaction or subjective symptoms developed following at least two consecutive doses as assessed by the visual analog scale (VAS ≥2).

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Skin Prick Test

Cow milk (Leti) and banana (Leti) used for the SBOFC recipe were evaluated with a SPT in the 36 cases and fresh lemon, chia seeds, and green food coloring (Dr. Oetker, Bielefeld, Germany) were assessed with a p-p test.

Preparation of Homemade Kiwifruit Extracts (Pulp and Seed)

Briefly, kiwifruit seeds were manually separated from the kiwifruit *(A. deliciosa)* pulp and lyophilized. Powder of the kiwifruit seeds was then suspended in sodium borate buffer, pH 8.0, at a 10% (w/v) ratio and gently stirred for 1 h. After centrifugation at 4 °C, the supernatant was collected, and the pellet was re-extracted twice using the same buffer and under the same conditions. The collected supernatants were lyophilized, and the resulting material was then extracted 3 times with 10% (w/v) acetone to remove the lipid components. The resulting pellets containing the extracted proteins were air desiccated and dissolved in ammonium bicarbonate 0.15 mol/L, pH 8. After centrifugation at 8500 rpm and 4 °C, the supernatants were lyophilized, quantified using the method described by Lowry *et al*. (2), and stored at -20 °C until their use. Pulp kiwifruit extract was prepared as starting material using seedless kiwifruit pulp that was homogenized in 10% (w/v) ammonium bicarbonate (50 mmol/L) and phenylmethylsulphonyl fluoride (1 mmol/L) in a blender. After 1 h of mixing at 4 °C, the homogenate was centrifuged for 30 minutes at 8500 rpm and 4 °C. The pellet was extracted once again using the same solvent and the supernatants were collected, lyophilized, and stored at -20 °C until their use. The lyophilized protein extracts were resuspended in ammonium bicarbonate 0.15 mol/L, pH 8.0, and quantified using the method described by Lowry *et al*. (2)

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Simple Blind Oral Food Challenge with Kiwifruit Seeds

Ingredients used to prepare the active smoothie included natural yogurt, banana, lemon, green food coloring, and the isolated seeds of one whole kiwifruit (approximately 1.2-1.5 g). In the case of the inactive smoothie, chia seeds (0.9 g) were used instead of kiwifruit seeds.

Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with kiwifruit seed extract (20 µg/strip) in 17% polyacrylamide gels. Proteins were visualized by means of Coomassie blue staining or, alternatively, transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The protein concentration was determined using the bicinchoninic acid method (Pierce Chemical, Rockford, Ill). Immunodetection of allergenic proteins was performed with patient sera (n = 36) diluted at a ratio of 1:5. The binding of human IgE was detected using anti-human mouse IgE antibodies diluted at a ratio of 1:5000 (ALK-Abello, Hørsholm, Denmark), followed by horseradish peroxidase-labeled goat anti-mouse IgG antibodies diluted at a ratio of 1:5000 (Pierce). The signal was developed with the ECL-Western blotting reagent (Amersham). Sensitization to the kiwifruit storage proteins Act d 12 and Act d 13 corresponded to bands immunodetected at 51 and/or 12 kDa, respectively, in the kiwifruit extract immunoblotting.

Enzyme-Linked Immunosorbent Assay (ELISA)

Allergens Act d 1, Act d 2, and Act d 5 were purified from *Actinidia deliciosa* fruits using the method published by Palacin *et al.*.(3) Briefly, polystyrene 96-well microtiter plates (Costar 3590, Corning) were coated with 50 µl of purified Act d 1, Act d 2, or Act d 5 at

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a concentration of 5 µg/ml as a solid phase and each individual serum sample at a dilution of 1:6. After washing them with 0.1% phosphate-buffered saline (PBS) with Tween-20, the wells were incubated with a peroxidase-labeled anti-human IgE (FisherScientific; 1:3000 dilution) for 1 hour at 25 °C. The plates were washed again and developed with 50 µl of peroxidase substrate buffer (ThermoScientific). After 30 minutes, the reaction was stopped with 50 µl of 2N HCl, and the optical density was measured at 492 nm. PBS with 1% bovine serum albumin (BSA) was used as a negative control. All assays were performed in triplicate (3,4).

ImmunoCAP Inhibition with Purified Act d 1

Specific IgE inhibition studies with ImmunoCAP were performed using ImmunoCAP FEIA 250 (Thermofisher Scientific, Uppsala, Sweden). Sera from patients showing high levels of sIgE against Act d 1 as detected by ImmunoCAP ISAC or ELISA assays were inhibited with Act d 1 (purified as previously described)(3) at room temperature, for 2 h, at 100 µg/ml and with 5 sequential concentrations diluted at a ratio of 1:10. The inhibitor mixtures (including sera with no inhibitor used as a control) were analyzed to detect sIgE against kiwifruit (f84) in the ImmunoCAP assays. The assays were performed following the manufacturer´s protocol. The percentage of inhibitory IgE binding was calculated using the following formula: ([kUA/L non-inhibited control - kUA/L inhibited sample]/ non-inhibited control kUA/L) x 100.

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RESULTS

Results of the Kiwifruit Extract Immunoblotting with 40 µg/Strip

Immunoblotting of kiwifruit extract at 40 µg/strip (following the same method described above) was performed in patients with a positive SPT against kiwifruit seed extract (n = 17) (Supplement Figure 2). Eleven out of 17 patients (64.7%) had a protein of 51 kDa (expected for Act d 12; patients 6, 10, 13, 14, 15, 24, 25, 26, 28, 34, and 36), 3 out of 17 (17.6%) had a protein of 12 kDa (expected for Act d 13; patients 11, 15, and 24), and 10 out of 17 (58.8%) had a protein of 25 kDa (patients 6, 11, 13, 15, 16, 26, 28, 30, 33, and 34), which, identified by mass spectrometry, corresponded to a thaumatin-like protein of the kiwifruit seed (Supplement Figure 3). This seed thaumatin-like protein was different from the pulp Act d 2, as one patient (patient 34) had it in the seed extract IB but not in the pulp extract IB. Sequence coverage between both proteins was 44%. This seed thaumatin-like protein was not previously described in the literature; nevertheless, further studies are needed to determine its clinical importance. In the literature, sensitization against a 7S-globulin protein of kiwifruit seeds was found in sera from kiwifruit-allergic pediatric patients (5) but, again, we are unaware of its clinical relevance.

Comparison of the Diagnostic Performance of the Different Available *In Vitro* Techniques

In order to increase the sample size for the comparative analysis of the *in vitro* techniques, sera from the 36 prospectively recruited subjects and from 33 kiwifruit-allergic patients of a previous multicenter study (FIS PI 11/01634) were used together. The 35 controls of

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the retrospective study were also used in this analysis. The 33 patients who had been retrospectively selected experienced allergy symptoms after ingesting kiwifruit on at least two occasions (14 developed local or systemic symptoms, a positive SPT against kiwifruit [ALK-Abelló], and positivity for sIgE to kiwifruit [ImmunoCAP, Uppsala, Sweden]; 10 developed local or systemic symptoms, had a positive SPT against kiwifruit, and negativity for sIgE against kiwifruit; and 9 developed systemic symptoms or anaphylaxis and had negative tests). In sera of these 33 patients, the presence of sIgE was determined by the ISAC and FABER assays, ImmunoCAP (kiwifruit extract and Act d 8), and an ELISA (Act d 1 and Act d 2). The diagnostic yield of the CAP and FABER assays did not differ significantly in the detection of kiwifruit extract ($p = 0.118$). The ISAC microarray had a better diagnostic performance than the FABER assay in the detection of Act d 1 ($p < 0.001$) and Act d 2 ($p = 0.031$). The ISAC and CAP assays did not differ in terms of the detection of Act d 8 ($p = 0.5$) (Supplement Table II).

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Supplement Table 1. Prick test with purified Act d 1 extract in ten patients with positivity for Act d 1 in the ELISA

	ISAC Act d 1 (ISU)	ELISA Act d 1 (positive cut point >0.235 units)	Prick test with purified Act d 1 extract (mm)
Patient 5	0.00	0.406	0
Patient 6	12.62	0.434	5.5
Patient 7	6.07	0.472	5.5
Patient 9	0.00	0.401	0
Patient 17	0.00	0.366	0
Patient 21	0.00	0.443	0
Patient 22	0.00	0.38	0
Patient 23	0.00	0.37	0
Patient 24	0.3	0.459	3.5
Patient 25	0.00	0.449	0

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Supplement Table 2. Concordance between the ImmunoCAP, ISAC, and FABER assays in the detection of kiwifruit components using McNemar's comparative test

	Kiwifruit extract	**Act d 1**	**Act d 2**	**Act d 5**	**Act d 8**
Patients: [Positive rate, n (%)]	ImmunoCAP: 34/62 (54.8) FABER: 41/62 (66.1) McNemar *p* = 0.118	ISAC: 24/62 (38.7) FABER: 5/62 (8.1) McNemar *p* = 0.000	ISAC: 6/62 (9.7) FABER: 0/62 McNemar *p* = 0.031	ISAC: 2/62 (3.2) FABER: 2/62 (3.2) McNemar *p* = 1	ISAC: 3/69 (43.5) ImmunoCAP: 5/69 (72.5) McNemar *p* = 0.5
Controls: [falls positive rate, n (%)]	ImmunoCAP: 2/16 (12.5) FABER: 1/35 (2.9) McNemar *p* = 0.5	ISAC: 1/35 (2.9) FABER: 0/35 McNemar *p* = 1	ISAC: 0/35 FABER: 0/35 McNemar *p* = 1	ISAC: 0/35 FABER: 0/35 McNemar *p* = 1	

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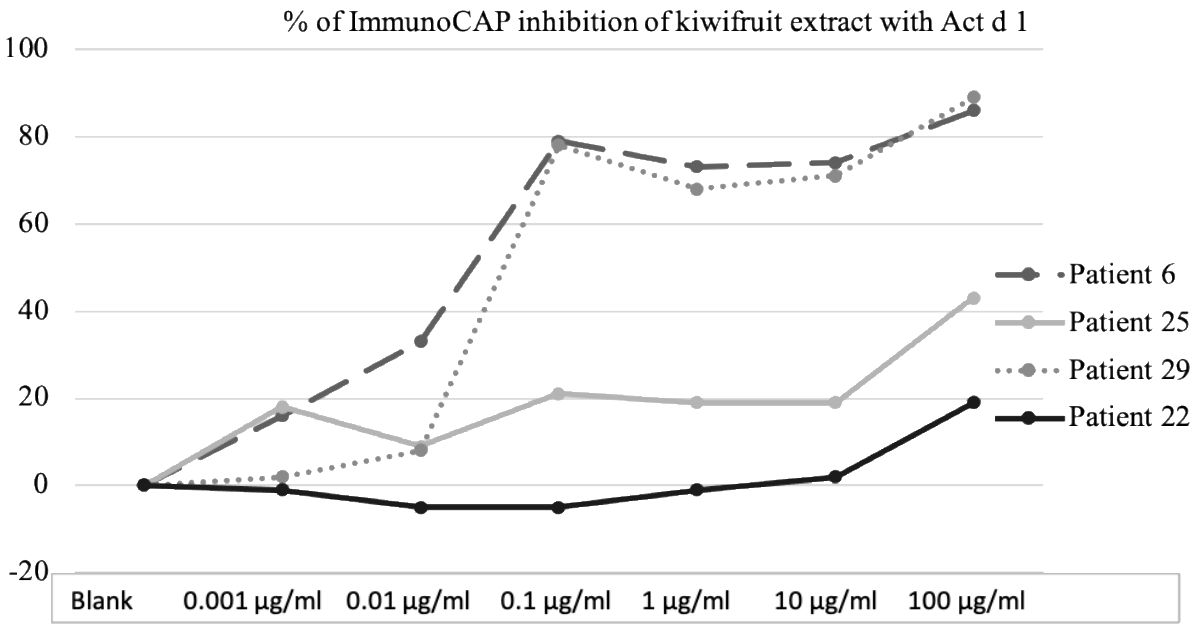
Supplement Figure 1. ImmunoCAP inhibition of kiwifruit extract with Act d 1. Patients 6 and 29, who were positive for Act d 1 in the ISAC assay, showed a high inhibition with purified Act d 1, and patients 25 and 22, who were negative for Act d 1 in the ISAC assay, showed poor inhibition with purified Act d 1.



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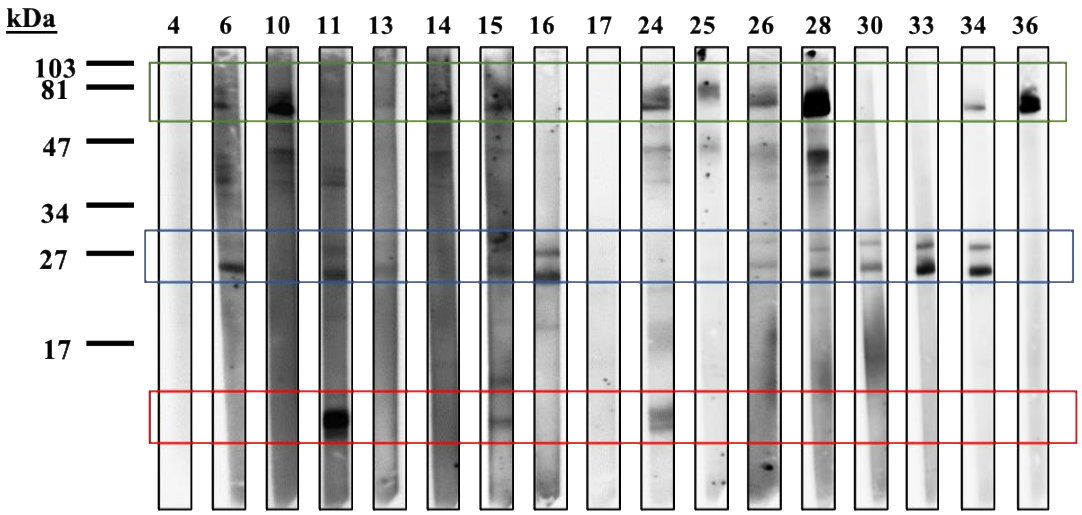
Supplement Figure 2. Immunoblot of kiwifruit seed extract (40 µg/strip) in sera of the 17 patients with a positive skin prick test against kiwifruit seed extract. A band of 25 kDa is identified as a seed-specific thaumatin-like protein.



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Supplement Figure 3. Identification of the 25-kDa kiwifruit seed protein by MALDI-TOF mass spectrometry

Thaumatin-like protein OS = *Actinidia deliciosa* OX = 3627 GN = TLP1 PE = 2 SV =1

Database: UP-Viridiplantae_191001 ***Score***: 107 ***Expect***: 0.00017

Monoisotopic mass (Mr): 25175 Da ***Calculated pI***: 8.29

Protein sequence coverage: 44% Matched peptides shown in **bold.**

1	MSTFKSLSLS	ALLFIAFLFT	CARGATFNI	NNCPFTVWAA	AVPGGGKR**LD**
51	**RGQNWIINPG**	**AGTK**GARVWP	R**TGCNFDGAG**	**RGK**CQTGDCN	GLLQCQAFGQ
101	PPNTLAEYAL	NQFNNLDFFD	ISLVDGFNVA	MEFSPTSGGC	TRGIK**CTADI**
151	**NGQCPNELRA**	**PGGCNNPCTV**	**FKTDQYCCNS**	**GNCGLTNFSK**	FFK**DRCPDAY**
201	**SYPKDDQTST**	**FTCPAGTNYK**	VVFCP		

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