

Kiwifruit Act d 11 is the first member of the ripening-related protein family identified as an allergen

R. D'Avino¹, M. L. Bernardi², M. Wallner³, P. Palazzo², L. Camardella¹, L. Tuppo^{1,2}, C. Alessandri², H. Breiteneder⁴, F. Ferreira³, M. A. Ciardiello¹ & A. Mari²

¹Institute of Protein Biochemistry, CNR, Naples; ²Center for Molecular Allergology, IDI-IRCCS, Rome, Italy; ³Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular Biology, University of Salzburg, Salzburg, Austria; ⁴Department of Pathophysiology, Medical University of Vienna, Vienna, Austria

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Keywords

Act d 11; allergen; Bet v 1-like; kirola; kiwifruit.

Correspondence

Maria A. Ciardiello, Institute of Protein Biochemistry, C.N.R., Via Pietro Castellino 111, I-80131 Napoli, Italy.
Tel.: +39 081 6132712
Fax: +39 081 6132277
E-mail: ma.ciardiello@ibp.cnr.it

The protein sequence of kirola (Act d 11) from green kiwifruit will appear in the UniProt Knowledgebase under the accession number P85524.

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Abstract

Background: Kiwifruit is an important cause of food allergy. A high amount of a protein with a molecular mass compatible with that of Bet v 1 was observed in the kiwifruit extract.

Objective: To identify and characterize kirola, the 17-kDa protein of green kiwifruit (Act d 11).

Methods: Act d 11 was purified from green kiwifruit. Its primary structure was obtained by direct protein sequencing. The IgE binding was investigated by skin testing, immunoblotting, inhibition tests, and detection by the ISAC microarray in an Italian cohort and in selected Bet v 1-sensitized Austrian patients. A clinical evaluation of kiwi allergy was carried out.

Results: Act d 11 was identified as a member of the major latex protein/ripening-related protein (MLP/RRP) family. IgE binding to Act d 11 was shown by all the applied testing. Patients tested positive for Act d 11 and reporting symptoms on kiwifruit exposure were found within the Bet v 1-positive subset rather than within the population selected for highly reliable history of allergic reactions to kiwifruit. Epidemiology of Act d 11 IgE reactivity was documented in the two cohorts. IgE co-recognition of Act d 11 within the Bet v 1-like molecules is documented using the microarray IgE inhibition assay.

Conclusions: Act d 11 is the first member of the MLP/RRP protein family to be described as an allergen. It displays IgE co-recognition with allergens belonging to the PR-10 family, including Bet v 1.

Several IgE-binding proteins have been detected in different species of kiwifruit. The green-fleshed fruit (*Actinidia deliciosa*) is the most studied and commonly available type of kiwifruit. A number of allergenic proteins were identified in this species, and most of them were officially named by the WHO-IUIS subcommittee for allergen nomenclature. Actinidin (Act d 1) (1); a thaumatin-like protein (Act d 2) (2), and

kiwellin (Act d 5) (3, 4) are major allergenic protein components of green kiwifruit extracts. Among other kiwi allergens described so far, recently a member of the pathogenesis-related protein family PR-10 was reported (5).

PR-10 proteins are components of a protein family included in the Bet v 1 superfamily (6), together with the major latex protein/ripening-related protein family (MLP/RRP), the cytokinin-binding proteins from legumes, the norcoclaurine synthases, etc. These proteins share a common fold, whose main feature is the presence of an internal cavity that can accommodate a number of small, mostly hydrophobic ligands (6).

Bet v 1 is the major allergen of birch pollen. Allergenicity of Bet v 1 homologs from many sources, including the

Abbreviations

HSA, human serum albumin; MLP/RRP, major latex protein/ripening-related protein family; RMSD, root mean square deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SPHIAa, single-point highest inhibition achievable assay; SPT, skin prick test.

recombinant homologs from green and yellow (*Actinidia chinensis*) kiwifruit, named Act d 8 and Act c 8, was described (5). Probably due to their low amounts in the fruit, isolation of the natural Act d 8 and Act c 8 has not yet been reported. In contrast, a high amount of a protein different from Act d 8, but showing a molecular mass similar to that of Bet v 1-like proteins, was observed in ripe kiwifruit extracts (7).

The new 17-kDa protein, named kirola, has been identified in green kiwifruit and characterized. The relationships of this new kiwifruit allergen with other Bet v 1-like molecules were explored from both the biochemical and the immunological view points. Clinical relevance as symptom trigger was evaluated. On the basis of the data available, the WHO-IUIS subcommittee assigned the name Act d 11 to kirola.

Materials and methods

The extraction and purification of kirola (Act d 11) from green kiwifruit, as well as the mass spectrometry measurement, the amino acid sequencing, the *in silico* analysis and the computational methods used for statistical evaluation of data are described in Data S1.

Study population, IgE testing

The protocol of the present study was approved by the Institutional Review Board (IDI/CE 2006/192) of IDI-IRCCS (Rome, Italy). All patients gave written informed consent before enrollment in the study.

Thirty-four consecutive patients were selected on the basis of a highly reliable history of allergic reactions to kiwifruit among those attending the outpatient department. After recording preliminary results in this first group, a second selection was set recalling 21 consecutive patients selected among those IgE positive to Bet v 1 by ISAC testing. All the patients included in the study were evaluated by skin prick test (SPT) with a kiwi commercial diagnostic extract (Stallergenes, Antony, France), with an in-house produced solution of kiwi extract (7), by the prick-prick test using fresh kiwifruit, and with the natural purified Act d 11, Act d 1, Act d 2, and Act d 5. For SPT, aliquots of Act d 11, or other natural allergens, at a protein concentration of 1 mg/ml were mixed with sterile glycerin in a 1 : 1 ratio. The solutions were sterilized by membrane filtration through a 0.22- μ m filter (Millipore, Bedford, MA, USA), in a sterile horizontal laminar flow hood. The diluent used to preserve the allergens, and a solution of histamine 10 mg/ml, was used as negative and positive control, respectively. Details on SPT are described in Data S1.

Following the SPT, sera were obtained from patients who consented to blood sampling for *in vitro* procedures.

For immunoblotting, the purified protein was subjected to 15% SDS-PAGE and transferred onto PVDF membranes. IgE immunoblotting was carried out as already described (7) using 50 sera from patients having symptoms on kiwi exposure and a positive SPT to kiwifruit extract.

IgE to kiwi extract was detected by the CAP system (Phadia, Uppsala, Sweden). The prevalence of Act d 11 recogni-

tion by sera from the allergic population was established by spotting the molecule on the ISAC system (Phadia-Multiplexing-Diagnostics, Vienna, Austria) for IgE detection (8). ISAC testing was routinely applied to the whole population accessing the outpatient department for IgE routine testing purposes. This population was already described as exposed to different Fagales rather than to birch pollen (9). Four kiwi allergens were available on the chip: Act d 1, Act d 2, Act d 5, and Act d 11. Patient subset definition was carried out considering Bet v 1-like molecules on the chip. Along with Italian samples, a group of 60 Austrian samples, known to be IgE positive to Bet v 1, were tested on the same ISAC103 microarray. For the aim of this study, ISAC IgE data on Act d 11, Bet v 1, Cor a 1, Dau c 1, and Mal d 1 will be reported.

IgE inhibition assay

IgE inhibition experiments were performed as previously reported (10) and detailed in Data S1. Ten Austrian and ten Italian sera were selected on the basis of their IgE recognition of Bet v 1-like allergens.

Results

Protein purification and primary structure elucidation

The recovery of the pure protein was in the range of 0.5–1 mg/100 g of kiwifruit pulp. Amino acid sequence analysis indicated the absence of PR-10 contaminants, even at very low concentrations as 0.1%. Furthermore, results obtained by inhibition assays using recombinant Bet v 1.0101 diluted in human serum albumin indicated that there was no chance to have any PR-10 contamination above 0.1% into our Act d 11 preparation. Details are given in Data S1.

Amino-terminal sequencing indicated that the N-terminus is blocked. The primary structure was elucidated by sequencing the peptides obtained after digestion with Asp-N protease (Fig. 1). Interestingly, Act d 11 sequence is identical to the tentative consensus sequence deduced from the overlapping EST clones FG437290 and FG440357 obtained from kiwifruit (11). The molecular mass deduced from the amino acid sequence of Act d 11 corresponds to the experimental value obtained by mass spectrometry for the major component, assuming that the N-terminal methionine is acetylated. The sequence obtained from a further EST clone, FG445803, has a substitution S $\rightarrow$ T in position 54, which would give a mass value of 17 460, accounting for one of the minor isoforms.

Amino acid sequence analysis

The homology search performed by NCBI-BLASTP revealed a significant similarity of Act d 11 with members of the Bet v 1-related superfamily. A multiple alignment of Act d 11 with some representative sequences of the MLP/RRP family and Bet v 1-like allergens belonging to the PR-10 family (12) is shown in Fig. 2. The sequence identity between Act d 11 and the MLP/RRPs ranges from 45 to 30%, whereas that

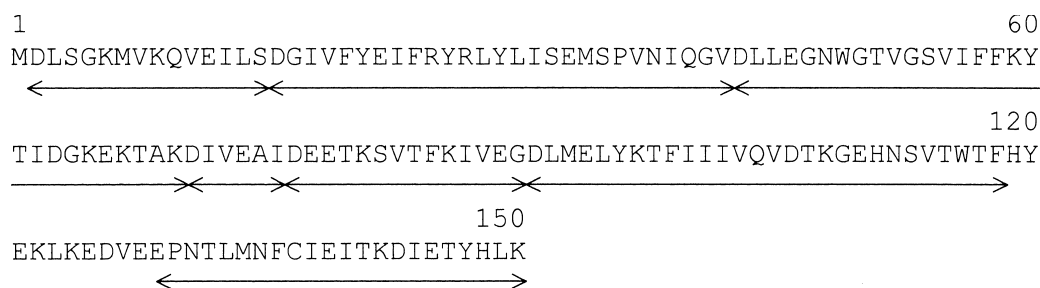


Figure 1 Amino acid sequence of Act d 11. Arrows indicate sequenced Asp-N peptides. The presence of N-acetylated methio-

nine at the blocked N-terminus was deduced from the molecular mass value determined by mass spectrometry.

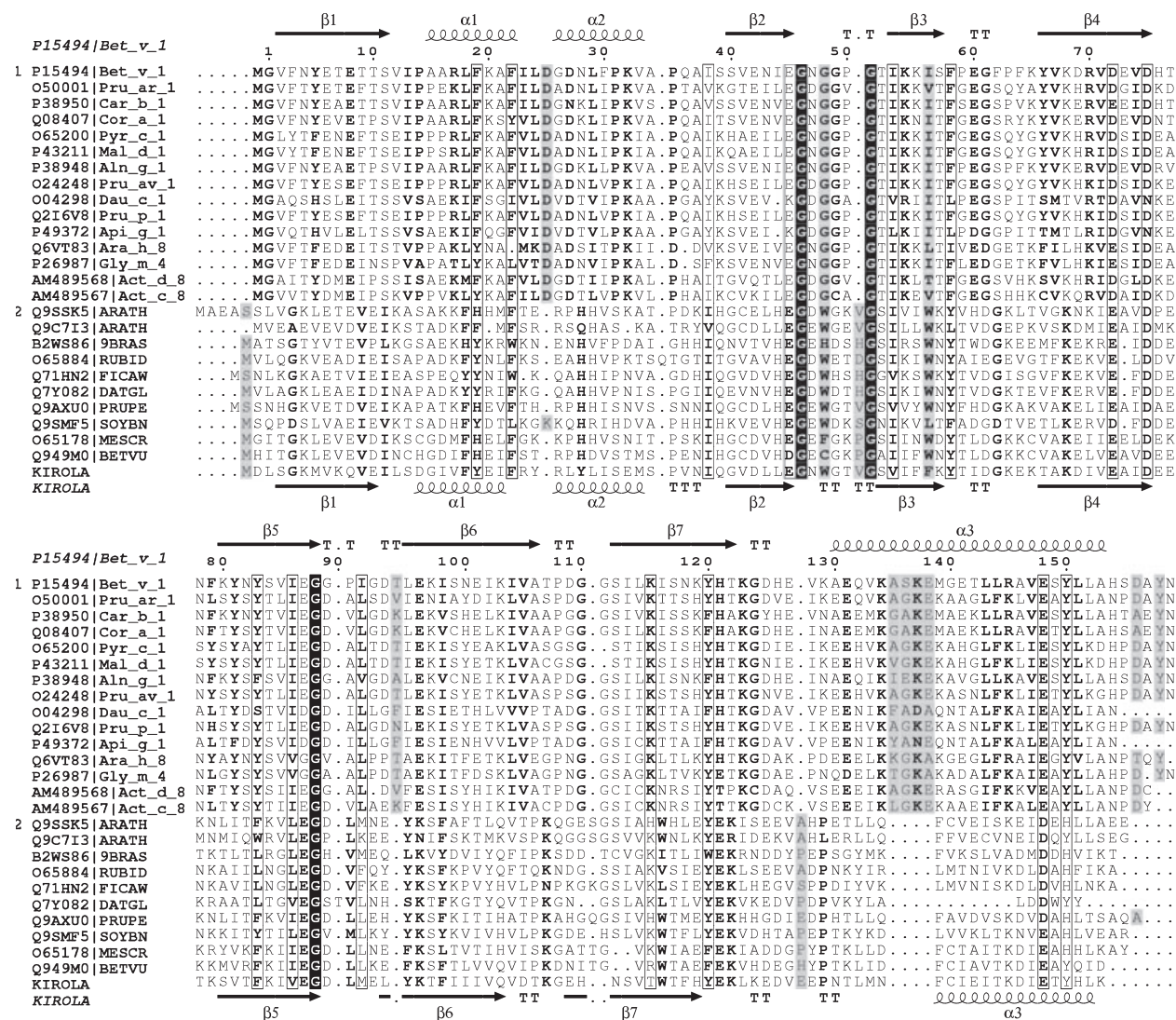


Figure 2 Alignment of Act d 11 with Bet v 1-like allergens (group 1) and MLP/RRPs (group 2). The sequence accession numbers are on the left. The figure has been drawn by ESPript (25). Residues conserved in all aligned sequences are in white and black shaded. The amino acid numbering of the Bet v 1 sequence is

shown on the top. Secondary structure elements of Bet v 1 crystal structure and of Act d 11 model are shown on the top and on the bottom of the figure, respectively. All the sequences shown in the alignment have been used to build the cladogram of Fig E1 (see Data S1).

observed with the PR-10 proteins is never higher than 21%. In line with the sequence identity values, the cladogram built with the same protein sequences includes Act d 11 in the family of MLP/RRP (see Fig E1 in Data S1).

Act d 11 displays an overall identity of 17% with Act d 8 and Act c 8, the recently described (5) Bet v 1-like allergens of green and gold kiwifruit. Almost all the conserved amino acids are clustered in three regions corresponding to the residues 35–51, 65–89, and 142–152 of the Bet v 1 numbering. For instance, in the region of residues 35–51, Act d 11 shows 50 and 38% of sequence identity with Act d 8 and Act c 8, respectively. In the C-terminal region (residues 142–152), 45 and 54% of identities are observed with Act d 8 and Act c 8, respectively. Similarly, the comparative analysis of Act d 11 and Bet v 1 primary structures underlines a very low overall sequence identity and the clustering of most of the conserved positions. For example, the region of residues 35–51 displays 44% of conserved positions.

IgE-binding experiments

Sera of 50 subjects allergic to kiwifruit were analyzed by IgE immunoblotting. Act d 11 was recognized by 11 (22%) of tested sera (Fig E2 in Data S1).

Act d 11 IgE epidemiology using ISAC testing

A total of 12 856 Italian subjects complaining about any allergy-related symptom have been screened by using the ISAC test. Eight thousand two hundred and fifty-six subjects (64.21%) were positive to at least one allergen on the chip and 835 of the 8256 (10.11%) were detected IgE positive to at least one of the kiwi allergens spotted on the microarray. Results of epidemiological data are detailed in Data S1 and in Figs E3 and E4.

Specific IgE value correlations as detected by ISAC testing using the whole Italian cohort are shown in Fig. 3. Although all statistical analysis had $P < 0.0001$ mainly for the number of recorded observations, their r -value varied quite largely among paired allergens under consideration. As expected, the highest correlation was recorded for homologous pollen allergens Bet v 1 and Cor a 1, and for apple allergen Mal d 1, whereas r -values dropped whenever Act d 11 or Dau c 1 was evaluated in relation to the former three allergens and reciprocally, being the r -values ranging between 0.18 and 0.28 (Fig. 3).

IgE inhibition experiments

Figure 4 shows the results of IgE inhibition experiments performed with soluble nAct d 11, rBet v 1.0101, and rCor a 1.0104 on the ISAC-spotted allergens nAct d 11, rBet v 1.0101, rCor a 1.0101, rDau c 1.0101, and rMal d 1.0108.

Among the 10 Italian samples, full homologous inhibitions were achieved by using the three inhibitors (Fig. 4A). Act d 11, not at all or only partially inhibited Bet v 1, and Cor a 1, whereas a broad range of values, from 0 to 100%, were recorded for Mal d 1. Only one of three Dau c 1⁺ sera was

inhibited. Soluble Bet v 1 achieved 100% IgE inhibition on almost all Act d 11⁺ and Mal d 1⁺ sera. Two of 3 Dau c 1⁺ sera were fully inhibited, whereas partial or no inhibition was achieved on Cor a 1⁺. Soluble Cor a 1 achieved an almost full inhibition on almost all Mal d 1⁺ samples, partial inhibition on several Act d 11⁺ and Bet v 1⁺ samples, and three different pattern of inhibition on Dau c 1⁺. Austrian samples behaved similarly with all three inhibitors (Fig. 4B).

Skin testing and kiwi allergy clinical features

As reported in Table 1, none of 34 kiwi allergic subjects selected on the basis of kiwi-related clinical symptoms had positive SPT to Act d 11, whereas when Act d 11 IgE-positive subjects were tested, 7 (33.3%) were recorded positive in SPT. Some Act d 11 SPT-positive/IgE-positive subjects, without sensitization to other kiwi allergens, underwent double-blind placebo-controlled food challenge (DBPCFC) to detect as objectively as possible a clinical reactivity. Some patients were scored positive by the clinical investigator although always reporting mild symptoms after oral mucosa contact with the kiwifruit.

Discussion

Act d 11 is a 17-kDa protein found in variable amounts in extracts of green kiwifruit. This is a ripening-related protein, the amount of which is influenced by natural ripening and postharvesting treatments, including the exposure to the plant hormone ethylene (7). Here, we describe the molecular and immunological characterization of the natural protein purified from ripe green kiwifruit, but a homolog was identified also in the gold species (data not shown).

As far as a major concern was the possible contamination by any known (Act d 8) and unknown PR-10 in our Act d 11 preparation, we set two different experiments to rule out such risk. Both the amino acid sequence analysis and the rBet v 1 inhibition curves showed that any PR-10 below the threshold of the two assays should not lead to any interference with our study results. Furthermore, as the Act d 8 was cloned from the kiwi buds, we are confident that no contamination could occur in our experimental conditions.

Act d 11 displays the highest sequence identity with members of the MLP/RRP family, which belongs to the Bet v 1 superfamily (12). A lower sequence identity is shared with members of the PR-10 protein family including Bet v 1.

Several antigenic regions of the molecular surface of Bet v 1 and of co-recognized allergens were described following studies based on the mapping of conserved residues (13–16), phage-displayed allergen mimotope technology (17, 18), and X-ray crystallography (14, 19). Most of the residues reported to belong to IgE binding or T-cell epitopes in Bet v 1 or in Bet v 1-related allergens (13, 14, 20–22) are conserved in Act d 11 and cluster mainly in the regions comprising the p-loop motif and the protein C-terminal domain, where a local sequence identity significantly higher than the overall one is observed.

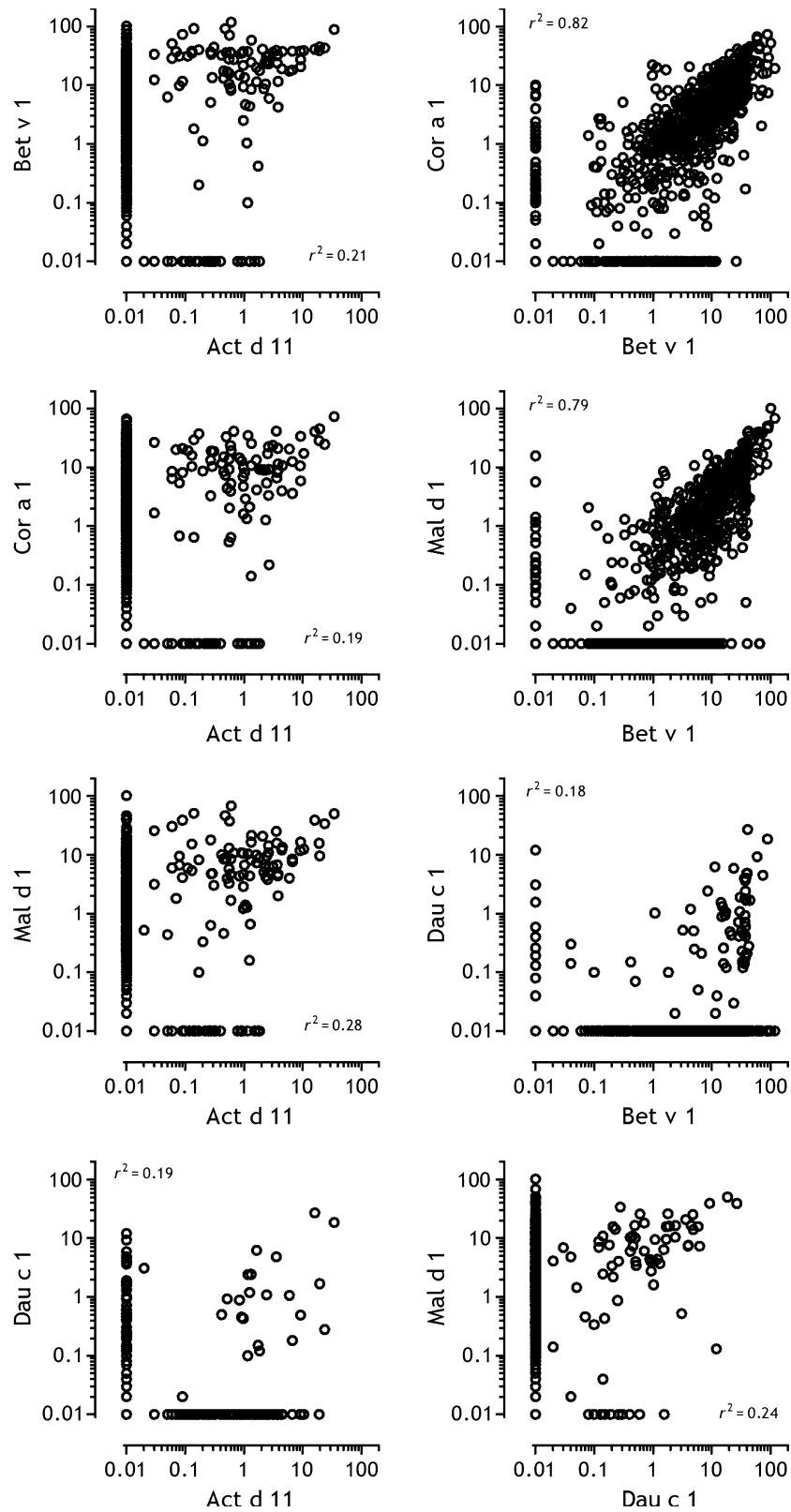


Figure 3 Correlations between IgE values for Bet v 1-like allergens reported in this study measured by the ISAC microarray. Spearman r -values are reported in each panel.

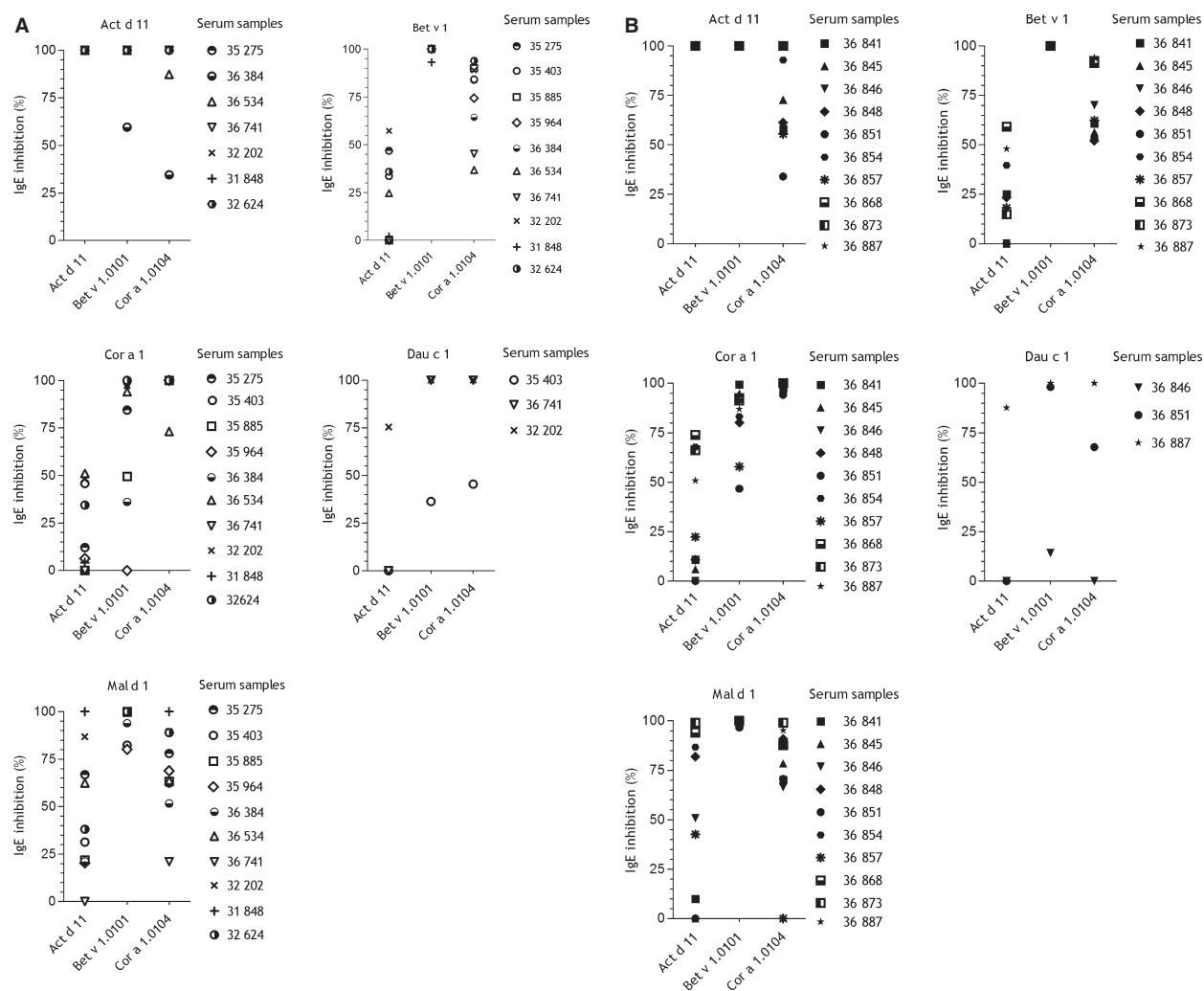


Figure 4 Single-point highest inhibition achievable assay (SPHIAa) results for defining IgE co-recognition of homologous molecule are reported for ten Italian samples (panel A) and ten Austrian samples

(panel B). Percent inhibition values have been calculated by the INTERALL software from data received online from the ISAC system. Further experimental details are reported in Data S1, Results section.

The residue E45 was reported to be critically important in Bet v 1 for IgG and IgE binding (14, 23, 24). Like several MLP/RFPs, Act d 11 shares E45 with Bet v 1 and with most of the homologous allergens. Nevertheless, some of them display a substitution in this position. For instance, Dau c 1 and Api g 1 have a lysine (14) and the non-allergenic Bet v 1-like protein T1 has a glutamine in position 45 (24). The presence of K45 in Dau c 1 may contribute to its lower cross-reactivity with Bet v 1, that is even lower than that displayed by Act d 11, although the former belongs to the same protein family of Bet v 1 and the latter to MLP/RFPs.

The capacity of Act d 11 to inhibit, at least partially, IgE binding to Bet v 1 and to homologs, such as Cor a 1, Dau c 1, and Mal d 1, suggests epitope-sharing regions higher than that inferable from the low overall sequence identity. A value of 35% or more amino acid identity over a sliding window of 80 residues is the criterion used by the FAO/WHO/EFSA/

Codex for the assessment of the allergenic risk of novel proteins. Therefore, on the basis of this criterion, Act d 11 was not expected to show co-recognition with the Bet v 1-like allergens, because of the sequence identity generally not higher than 21%. Nevertheless, the observation that in IgE-binding regions, the residues conserved in Act d 11 are generally more than 35%, and sometimes more than 50%, suggests conservation of epitope regions and may explain the detected co-recognitions. Additionally, these results suggest a revision of the criteria adopted by the FAO/WHO/EFSA/Codex to assess an allergenic risk because they take into consideration only the overall sequence identity and pay no attention to the epitope conservation.

Variability in such IgE co-recognition of Bet v 1-like molecules was quite broadly recorded among single patients analyzed by single-point highest inhibition achievable assay (SPHIAa), regardless whether they were from Austria, an

Table 1 Demographical, clinical, and diagnostic features of patients selected for skin prick test procedure

	Kiwi allergics		Bet v 1-positive	
	N.	%	N.	%
Total	34		21	
Gender: F/M	24/10		17/4	
Age : mean	33.17		33.57	
Age : range	4–59		16–74	
Symptoms*				
Abdominal pain	3	8.8	0	
Anaphylaxis	1	2.9	0	
Angioedema	3	8.8	2	9.5
Asthma	4	11.8	0	
Eczema	2	5.9	0	
Oral allergy syndrome	22	64.7	2	9.5
Urticaria	4	11.8	0	
Vomiting	3	8.8	0	
No symptoms on exposure	0		17	80.9
IgE				
Kiwi extract (Phadia)	23	67.6	8	38.1
nAct d 1	8	23.5	0	
nAct d 11	0		7	33.3
rBet v 1	2	5.9	21	100
rCor a 1	2	5.9	21	100
rDau c 1	0		2	9.5
Skin prick test				
Kiwi extract (in house)†	22	64.7	8	40
Kiwi extract (Stallergenes)	18	52.9	5	26.3
nAct d 1	12	35.3	0	
nAct d 2	4	11.8	3	15
nAct d 5	9	26.5	3	15
nAct d 11	0	0	7	33.3

*Symptoms in kiwi allergics (left column) were recorded as reported by patients in a specific questionnaire. Symptoms in Bet v 1-like-positive subjects were recorded after DBPCFC. †Overlapping results were obtained by using the prick–prick test technique with the fresh kiwifruit.

area with a great exposure to birch pollen, or from Italy, where taxonomically related pollen induces sensitization in patients. In fact, the overall comparison of IgE prevalence for all the Bet v 1-like molecules considered in the present study shows that, regardless the dominating pollen exposure, Bet v 1 seems to act as the best IgE-detecting *in vitro* reagent. It seems to bear almost all the IgE epitopes at least for food allergens, unless IgE binding to Cor a 1 that is not fully inhibited in some patients. This phenomenon appears more pronounced for the Italian subset, but the partial inhibition of some Austrian samples suggests the possible role of other Bet v 1-related molecules as sensitizers in area where birch pollen is considered the primary sensitizer. Furthermore, the different epitope sharing among Act d 11 and Bet v 1-like molecules can be deduced by the different dot plot graph distribution of paired ISAC IgE results. Bet v 1-like molecules displaying the highest structural similarity to the birch pollen

allergen as detected in a large population show greater correlation values, whereas Act d 11 behaves as Dau c 1.

Interestingly, a different prevalence distribution was recorded between the Austrian and the Italian tested samples when kiwi allergens were considered. This is obviously because of the different selection criteria for patient enrollment. Considering the Bet v 1-like-positive cohorts, differences in prevalence were recorded. Correction for the IgE value for Bet v 1 as recorded by the ISAC testing brought no statistically significant differences between the two populations for Bet v 1 but not for Cor a 1, further confirming the leading role of Bet v 1 as IgE detector, but with the need to carefully study epitopes displayed on other Fagales-related pollen whose allergic populations are exposed to.

Kiwi allergic individuals were enrolled on the basis of clear-cut-reported symptoms. This approach could have biased the selection toward the Act d 11 negative condition. Prick-to-prick testing was applied but did not change the results as reported from skin testing with the two extract preparations (commercial and in-house ones) as the patients were either positive for one or the other or to both of them. The lack of Act d 11 positivity in the kiwi allergic group prompted us toward the second group (Bet v 1-positive subjects) where we found the skin test positivity and the IgE reactivity, and, as expected, some minor clinical symptoms escaped at the first patient observation. We overall consider these findings important to define Act d 11 as a low-risk marker of kiwi allergy when this molecule is scored IgE positive.

In conclusion, we herein report the identification of a new kiwi allergen as a MLP/RRP, having a clinical relevance for causing sometimes symptoms in subjects sensitized to Bet v 1-related allergens. Act d 11 primary structure, its higher prevalence in the Bet v 1-like⁺ subsets, and its immunological relation to other Bet v 1-like molecules establish this protein as the first nonPR-10 allergen of the Bet v 1-like superfamily.

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Supporting information

Additional Supporting Information may be found in the online version of this article found at: <http://www.wileyonlinelibrary.com>

Data S1. Repository.

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Kiwifruit Act d 11 is the first member of the Ripening-related Protein family identified as an allergen

Rossana D'Avino^a, Maria Livia Bernardi^b, Michael Wallner^c, Paola Palazzo^b, Laura Camardella^a,
Lisa Tuppo^{a,b}, Claudia Alessandri^b, Heimo Breiteneder^d, Fatima Ferreira^c, Maria Antonietta
Ciardiello^a, Adriano Mari^b

^a Institute of Protein Biochemistry, CNR, Naples, Italy

^b Center for Molecular Allergology, IDI-IRCCS, Rome, Italy

^c Christian Doppler Laboratory for Allergy Diagnosis and Therapy, Department of Molecular
Biology, University of Salzburg, Salzburg, Austria

^d Department of Pathophysiology, Medical University of Vienna, Vienna, Austria

Running title:

Act d 11: a new kiwi allergen related to Bet v 1

Corresponding author:

Maria Antonietta Ciardiello,
Institute of Protein Biochemistry, C.N.R.,
Via Pietro Castellino 111, I-80131 Napoli, Italy.
Phone: +39 081 6132712; Fax: +39 081 6132277;
E-mail: ma.ciardiello@ibp.cnr.it

METHODS

Protein extraction and purification

Ripe kiwi fruits, *Actinidia deliciosa* cv Hayward (green kiwi fruit) were peeled, homogenized with 1 M NaCl (1:1 w/v) and centrifuged. The supernatant was treated with ammonium sulfate (80% saturation) and the precipitate was resuspended and dialyzed against 10 mM Tris/Cl pH 7.5. The sample was loaded onto a DE 52 (Whatman, Brentford, UK) column equilibrated in the same buffer and eluted with a gradient of NaCl. The fractions containing the 17 kDa band upon SDS-PAGE were loaded onto an affinity column containing tomato PME-bound Sepharose and the unbound fraction was collected and further purified by FPLC on a MonoQ HR 10/10 column (Amersham-Pharmacia, Uppsala, Sweden), eluting with a gradient of NaCl in 10 mM Tris/Cl pH 7.5. The fractions containing the protein were loaded onto a Superdex 75 column (Amersham-Pharmacia) equilibrated in 10 mM Tris/Cl pH 7.5, 0.25 M NaCl. Elution from gel filtration provided kirola purified to homogeneity.

Purity of this protein fraction was checked by: (i) SDS-PAGE; (ii) RP-HPLC performed on a Vydac C₈ column (0.21 x 25 cm), using a Beckman (Fullerton, CA, USA) System Gold apparatus; (iii) mass spectrometry on a QStar system (Applied Biosystems, Foster City, CA, USA) equipped with a nanospray source; (iv) automatic amino acid sequencing on a Procise 492 (Applied Biosystems).

Amino acid sequencing

Denaturation and alkylation of the sulphydryl groups of the protein purified from the natural source were carried out as described E1. Digestion of the protein was performed with Asp-N protease (Boehringer-Mannheim, Germany) following manufacturer instructions. The obtained peptides were separated by RP-HPLC, collected and analyzed.

Amino acid sequencing of the entire protein and of peptides obtained by proteolytic digestion was performed with a Procise 492 automatic sequencer (Applied Biosystems).

Mass spectrometry

The mass of the protein was determined by mass spectrometry on a QStar system (Applied Biosystems) equipped with a nanospray source. The protein was desalted by acidification with 0.1% trifluoroacetic acid, adsorption to 10 µl C4 Zip-Tip (Millipore) and elution by 60% acetonitrile containing 0.1% formic acid prior to the introduction in the mass spectrometer by infusion syringe.

In silico analysis

Protein sequences were analyzed using available software on the ExPASy Proteomics Server (<http://www.expasy.org>). Search for potentially matching sequences was performed by NCBI BLASTP. Bet v 1-like allergen sequences were selected from the Allergome web site (www.allergome.org). Multiple sequence alignment was obtained using T-Coffee (www.ebi.ac.uk) on the Expasy Proteomic tools server (www.expasy.ch). Evolutionary tree was calculated by the Neighbour-Joining method by submitting the alignment to CLUSTALW2 Server (www.ebi.ac.uk). The tree was drawn by using the program TreeView. Secondary structure predictions were performed by PsiPred (www.bioinf.cs.ucl.ac.uk).

Clinical questionnaire

Fruit or Vegetable name (i.e. Green Kiwi fruit, the same has been applied to evaluate the Golden kiwi fruit)

1. Do you know this food (If no, do not proceed further)
2. Have you ever eaten this food (If no, give reason for that...)
3. If eaten, was it without any problems? (If no problem, do not proceed further)
4. If problems, please indicate which of the following (one or more can be checked):

- Itching of the lips, or tongue, or palate, or pharynx
- Swelling of the lips, or tongue, or palate, or pharynx
- Rhinitis (running/watery nose, sneezing, itching)
- Conjunctivitis (itching, burning sensation)
- Dyspnoea (shortness of breath, wheezing, coughing)
- Urticaria (skin lesions like when you receive a mosquito bite, same size or larger)
- Gastrointestinal symptoms (nausea, vomiting, abdominal pain, diarrhoea)
- Throat problems (to feel a swelling of the throat making breathing difficult and with strange sounds)
- Anaphylactic shock (falling down because you felt suddenly weak, sweating cold, losing consciousness, needing help from others and carried to the nearest emergency room)
- Any other symptom (please describe...)

If you reported any of the above symptoms:

1. Did the symptoms cleared without medication and medical assistance? (if yes, do not proceed further, state the time required for symptoms to disappear....)
2. If you use or receive medications please report which one (antihistamine, steroids, adrenaline, other)
3. Medication have been (one or more can be checked):
 - Self administered
 - By the family doctor but not in an emergency room
 - In the emergency room
4. In case of medication, how long did it take to have a full resolution of the symptoms?

Skin prick test (SPT)

SPT was performed on the volar surface of the distal forearm by using standardized lancets. Patients were asked to avoid antihistamine intake for at least 7 days before testing. Test evaluation was done after 15 minutes. Wheal areas, transferred on a record sheet by means of a transparent adhesive tape, were calculated by means of computer-aided design software (Autocad 22; Autodesk Inc, San Rafael, CA, USA). Any wheal area was measured only in patients having fully negative control test with saline, and a positive result with histamine. No drop out were recorded for non specific skin test reactions. The widest surface of the two test areas was considered (E2).

Green kiwi fruit challenge procedure

Fully ripe fresh green kiwi fruit is purchased at the local market when needed for challenges. The fruit is manually peeled and once cleaned it is homogenized using a standard mixer. Two different meals were used for the active and placebo preparations not distinguishable in colour, texture, and taste. To mask the kiwi, and make an almost drinkable preparation, the following ingredients have been used: 400 ml of pineapple juice, 60 g white sugar, 40 g coconut flour, 0.2 g ascorbic acid for food preparation to prevent oxidation and, to have the green colour, 5 ml artificially flavoured commercially available peppermint syrup. The mixed kiwifruit preparation was added to the active meal, whereas the same weight/volume of mixed pineapple fruit was added to the placebo meal. Five doses of aprox 1 ml, 10 ml, 20 ml, 50 ml, 100 ml of both preparations were used every thirty minutes, making an overall volume of aprox 190 ml, simulating the exposure to almost half a kiwi fruit in the verum case. To avoid any enzymatic degradation of proteins, the two preparations were ready made soon before the challenges and kept at +4 °C during the challenge procedure.

IgE inhibition assay

Briefly, 20 µl of individual patients' sera have been incubated overnight with 20 µl of a solution containing the highly purified allergen preparation at the concentration of 0.5, 1, and 1 mg/ml, for nAct d 11, rBet v 1, and rCor a 1 respectively. Recombinant Bet v 1 and Cor a 1 were produced as

published with minor modifications (E3) After o.n. incubation, the IgE binding inhibition was evaluated by running the ISAC 103 microarray IgE assay (VBC-Genomics), where, as reported above, recombinant and natural allergens are spotted. A control sample, where only the buffer solution was added, was used as reference value for no inhibition IgE values. The assay was run as a single point highest inhibition achievable assay (SPHIAa) (E4). For control purposes of the specific IgE inhibition obtained on Bet v 1-like molecules several other allergens which were recognized by the IgE of the same patient were used. A no-inhibition value was required to record the experiment as valuable.

rBet v 1 inhibition assay

An inhibition assay has been set using rBet v 1.0101 produced in *Escherichia coli* and available in our laboratory, by diluting the preparation in HSA (Stallergenes, Antony, France). Thirteen two-fold dilutions were obtained starting from a final concentration of 0.5 mg/ml down to 0.000122 mg/ml Bet v 1.0101. A serum sample diluted in buffer was used as the no inhibition reference. A PR-10 strongly positive serum sample has been selected from the sera bank. The main feature was to have IgE to Act d 11 above 5 kUI/l. This cut off was applied to create the curves for all allergens on the ISAC 103, but just Act d 11, Aln g 1.0101, Bet v 1.0101, Cor a 1.0101, Mal d 1.0108, and Pru p 1.0101 exceeded the cut off level, and are reported in *Fig E3*.

Data and Statistics

All the clinical and routine diagnostic data have been saved in an allergy *e*-record as soon as they were made available (InterAll, Allergy Data Laboratories s.c., Latina, Italy). InterAll software is fully interfaced with the ISAC system (VBC-Genomics) allowing storing IgE data in real time. The same data storage, including preliminary statistical evaluation of SPHIAa and the rBet v 1 inhibition assay, have been performed by using the same InterAll software.

Graphpad Prism version 5.02 has been used for advanced statistics and graphs (Graphpad Software Inc., La Jolla, CA, USA). Fisher's exact test has been used to compare prevalence, Mann Whitney test for comparing paired value distributions, and Spearman r for evaluating variance between IgE values. Values at $p < 0.01$ have been accepted as statistically significant.

RESULTS

The homogeneity of the protein preparation was demonstrated through a collection of experimental data obtained using different methodologies. No contaminant proteins were observed when the purified protein was analysed by SDS-PAGE and RP-HPLC. In fact, a single band and a single peak were obtained by SDS-PAGE and RP-HPLC separations, respectively. Furthermore, none of the values obtained by mass spectrometry corresponded to the mass of the PR-10 protein Act d 8 (16,800 Da).

In addition, to exclude the presence of PR-10 proteins in the preparation of pure Act d 11, an amino acid sequence analysis was performed. Unlike the PR-10 proteins, Act d 11 has a blocked N-terminus, therefore blank cycles were recorded when five nanomoles of Act d 11 were loaded on the automatic sequencer and analyzed. The N-terminal sequence of PR-10 proteins was not detected in this analysis. The experiment was then repeated after addition of ten or five picomoles of recombinant Bet v 1, isoform 1.0101, to five nanomoles of Act d 11. The N-terminal amino acid sequence of Bet v 1 (GVFNYY) was detected in the report obtained from the automated sequencer, thus indicating that the presence in the protein preparation of a possible contaminant can be detected, even at very low concentrations as 0.1%.

Act d 11 IgE epidemiology using ISAC testing

As shown in Fig E3/A, 334 subjects had IgE to Act d 1 (40%), 493 (59.04%) to Act d 2, 86 (10.3%) to Act d 5, 115 (13.77%) to Act d 11, being the latter the only IgE positive kiwi allergen in 66 subjects (7.9%). IgE value distributions for the four kiwi allergens are reported in Fig E3/B. Testing by ISAC 103 Austrian samples, different prevalence for the four kiwi allergens were recorded (Fig 3/A). Twenty samples (33.33%) were IgE positive for Act d 11, all of them with IgE positive results on Bet v 1 or any of the other Bet v 1-like molecules. Act d 1 and Act d 2 were IgE positive in 2 and 1 subjects, whereas Act d 5 was never found positive in the Austrian patient group. Act d 11 prevalence was found statistically different between the two groups ($p < 0.0001$). As the Austrian

samples were selected for their Bet v 1 positivity, we evaluated the Act d 11 IgE prevalence in an Italian subset of kiwi allergen sensitized subjects having IgE to Bet v 1 and values on ISAC higher than the lowest Bet v 1 IgE value among the Austrian samples. Fig E3/A reports Act d 11 prevalence within this Italian subset to be significantly higher ($p < 0.0001$) when compared to the whole Italian cohort, but no statistically significant difference was found comparing with the Austrian cohort. The remaining IgE prevalence for kiwi allergens dropped significantly in the selected Italian cohort, but Act d 2 which was still recorded with a higher prevalence compared to Austrians after adjusting for Bet v 1 IgE positivity. Act d 11 IgE value distributions were not statistically different when comparing the three sample subsets (Fig E3/B). The same applies for IgE positive value distribution considering the two Italian subsets for the remaining three kiwi allergens.

Fig E4/A reports data related to the group of 1,084 Italian subjects having at least one IgE positivity to any of the Bet v 1-like molecules on the microarray. The 115 Act d 11 sensitized subjects represented 10.6% of this subset, including 17 individuals (14.78%) having IgE reactivity to Act d 11 not associated with any other Bet v 1-like molecule. In this group IgE values were in the lowest range, between 0.05 and 0.94 kU_A/l, anyhow reproducible (data not shown). As for the kiwi allergens, prevalence comparisons were performed using both the whole Italian cohort and the subset of Bet v 1 IgE positive subjects, selected as reported above. Adjusting for Bet v 1 IgE positive values brought a significant increase of the Italian prevalence for all the Bet v 1-like molecules, being not different when compared to the Austrian ones (Fig E4/A). As it was applied as selection criteria, Bet v 1 IgE values were statistically different ($p < 0.0001$) between the whole Italian cohort and the other two subsets, selected for Bet v 1 IgE positive values. A statistically significant difference for Bet v 1 IgE distribution was recorded also between the two Italian and Austrian selected cohorts ($p < 0.005$) (Fig E4/B). IgE value distributions were statistically significant for Act d 11 IgE when comparing the two Italian subsets ($p < 0.05$), whereas no difference was recorded between Act d 11 IgE values between the Italian subset and the Austrian samples (Fig

E4/B). Statistical results were recorded for the other Bet v 1-like allergens as well. Cor a 1 IgE were markedly different when comparing the whole Italian cohort and the two selected subsets, and had $p < 0.01$ when comparing the Italian subset and the Austrian samples (Fig E4/B). Dau c 1 value distributions were not significantly different among all groups, whereas Mal d 1 IgE value distributions were statistically different ($p < 0.0001$) between the whole Italian cohort and the two selected subsets, but no difference was recorded between the two (Fig E4/B).

IgE inhibition experiments

Single point highest inhibition achievable assay (SPHIAa) results for defining IgE co-recognition of homologous molecule are reported in Fig 5 for ten Italian samples (panel A) and ten Austrian samples (panel B). Single samples have been incubated with the highest concentration available of Act d 11 (0.5 mg/ml), Bet v 1 (0.8 mg/ml), and Cor a 1 (0.4 mg/ml), indicated on X axis. Samples used for SPHIAa had the following features - Italian samples: Act d 11⁺ n = 7, specific IgE mean value = 4.61 kUA/l (range 0.50–19.57 kUA/l); Bet v 1⁺ n = 10, specific IgE mean value = 2.79 kUA/l (range 0.39–8.42 kUA/l); Cor a 1⁺ n = 10, specific IgE mean value = 30.4 kUA/l (range 4.37–43.88 kUA/l); Dau c 1⁺ n = 3, specific IgE mean value = 30.4 kUA/l (range 4.37–43.88 kUA/l); and Mal d 1⁺ n = 10, specific IgE mean value = 14.52 kUA/l (range 4.84–33.38 kUA/l); Austrian samples: Act d 11⁺ n = 7, specific IgE mean value = 4.61 kUA/l (range 0.50–19.57 kUA/l); Bet v 1⁺ n = 10, specific IgE mean value = 2.79 kUA/l (range 0.39–8.42 kUA/l); Cor a 1⁺ n = 10, specific IgE mean value = 30.4 kUA/l (range 4.37–43.88 kUA/l); Dau c 1⁺ n = 3, specific IgE mean value = 30.4 kUA/l (range 4.37–43.88 kUA/l); and Mal d 1⁺ n = 10, specific IgE mean value = 14.52 kUA/l (range 4.84–33.38 kUA/l). Percent inhibition values have been calculated by the InterAll software from data received on line from the ISAC system.

To exclude that the observed co-recognition could be due to PR-10 contamination in the Act d 11 preparation, inhibition assays using solutions of HSA spiked with purified recombinant Bet v 1 at different dilutions, ranging from 0.5 mg/ml to 0.000122 mg/ml, were performed. As

reported in Fig E3 the homologous IgE inhibition, Bet v 1 on Bet v 1, reached inhibition values below 20% starting from 0.002 mg/ml concentration. Bet v 1 preparation was capable to totally inhibit all other allergens including Act d 11 at the highest concentration. Inhibition values below 20% were recorded in a range between 0.004 and 0.001 mg/ml. No IgE inhibition for Act d 11 was recorded at 0.02 mg/ml as in the homologous inhibition. Therefore, results obtained indicated that there was no chance to have any PR-10 contamination of more than 0.1% into our Act d 11 preparation.

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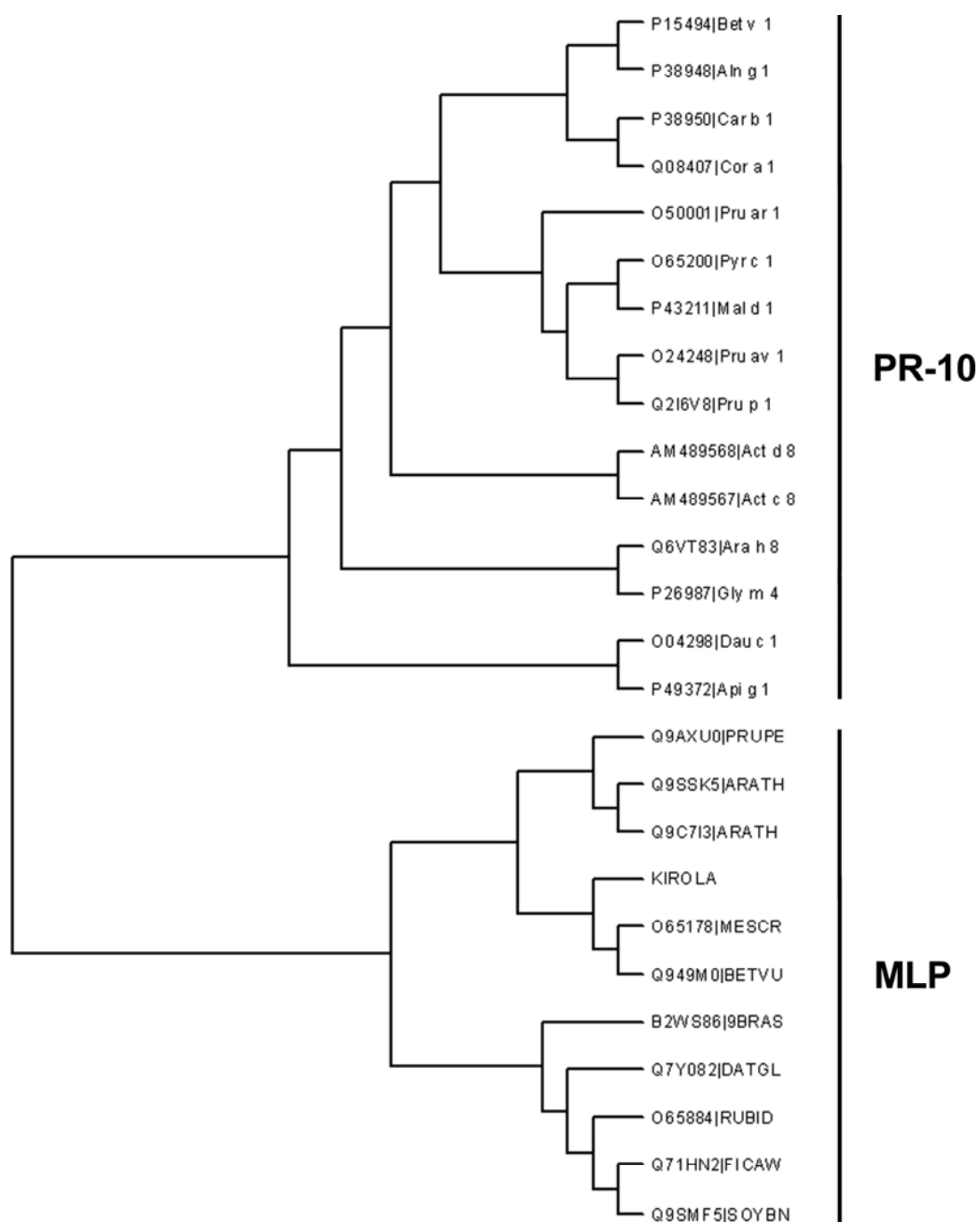


Figure E1. Cladogram showing the relationship among Act d 11 and the homologous proteins shown in the alignment of Figure 2. The evolutionary tree was drawn by using the program TreeView (E5).

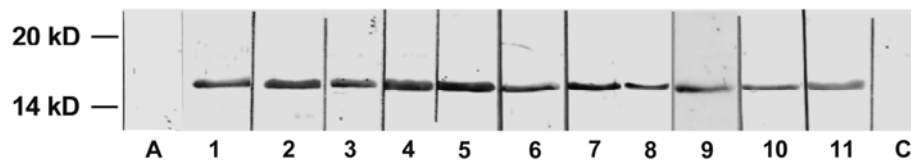


Figure E2. IgE immunoblotting of Act d 11 probed positive with eleven individual sera (lanes 1-11 corresponding to sera AQ49, BK1, BO11, BQ64, BY15, CJ35, 1994, 2001, 4356, 3792, 1176, respectively), with buffer as a negative control for the antihuman IgE antibody (lane C). Lane A reports serum BW11, that is one of the remaining 39 negative samples used as negative control with allergic serum.

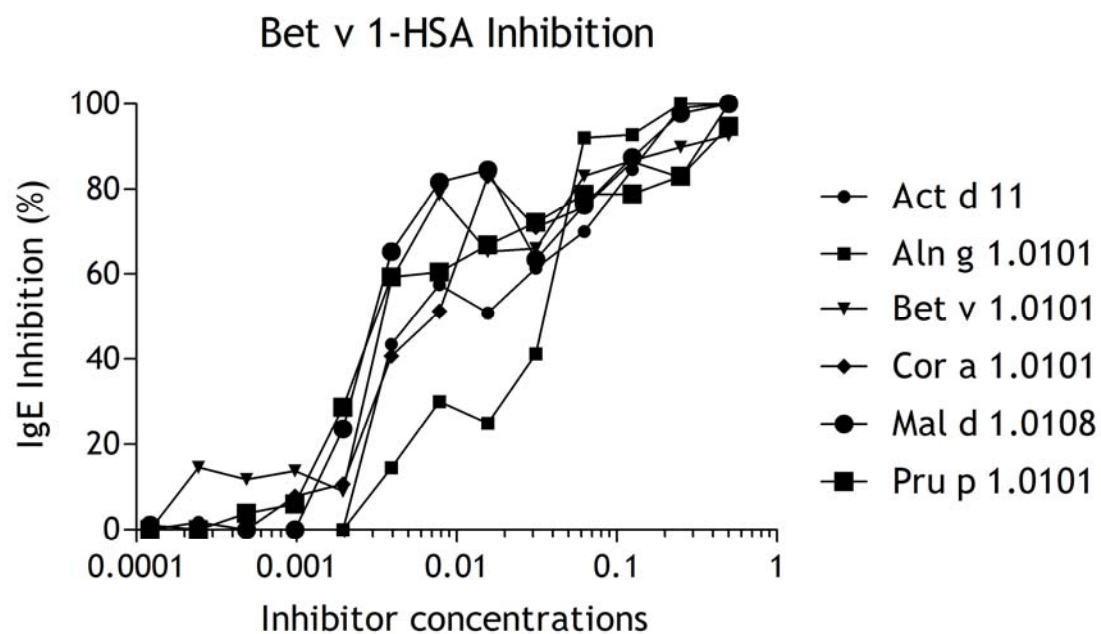


Figure E3. Inhibition curves obtained for Bet v 1 and for Bet v 1-like allergens spotted on the ISAC103 using soluble Bet v 1 as inhibitor at the indicated concentrations.

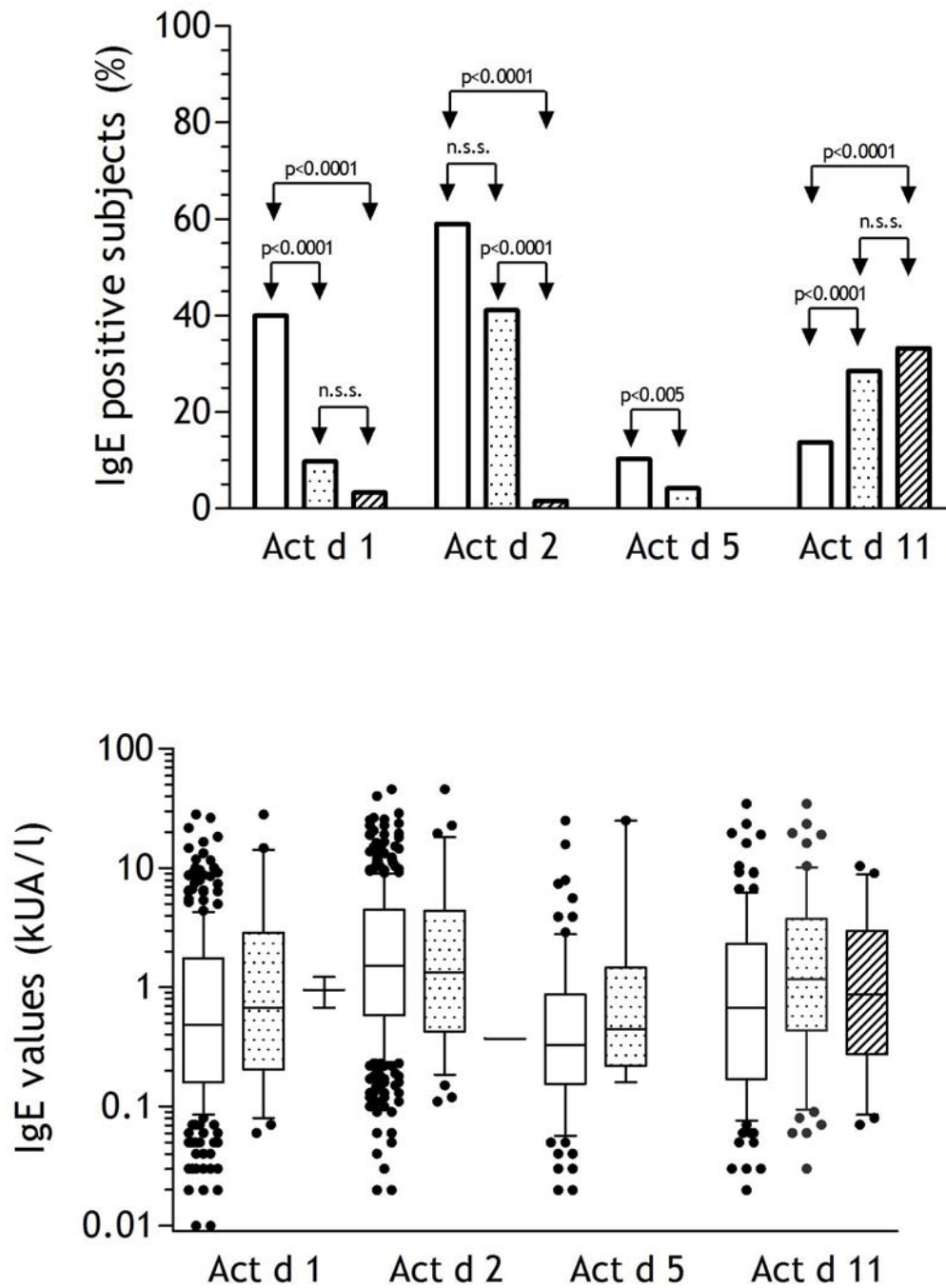


Figure E4. (upper panel) Prevalence of positive IgE to kiwi allergen in the Italian and Austrian cohorts; (lower panel) Value distribution of kiwi allergen IgE in the Italian and Austrian cohorts. In both graphs, white bars show data on 835 subjects having at least one positive IgE to kiwi allergens within the whole Italian tested allergic cohort (n = 8,256); spotted bars show data on 214

Italian Bet v 1 IgE positive subjects with IgE values equal or greater than the lowest value of the Austrian subjects; hatched bars show data on 60 Austrian subjects known to be Bet v 1 IgE positive. Statistically significant differences are reported in right panel whereas none were recorded comparing the three subsets for each allergens for the left panel.

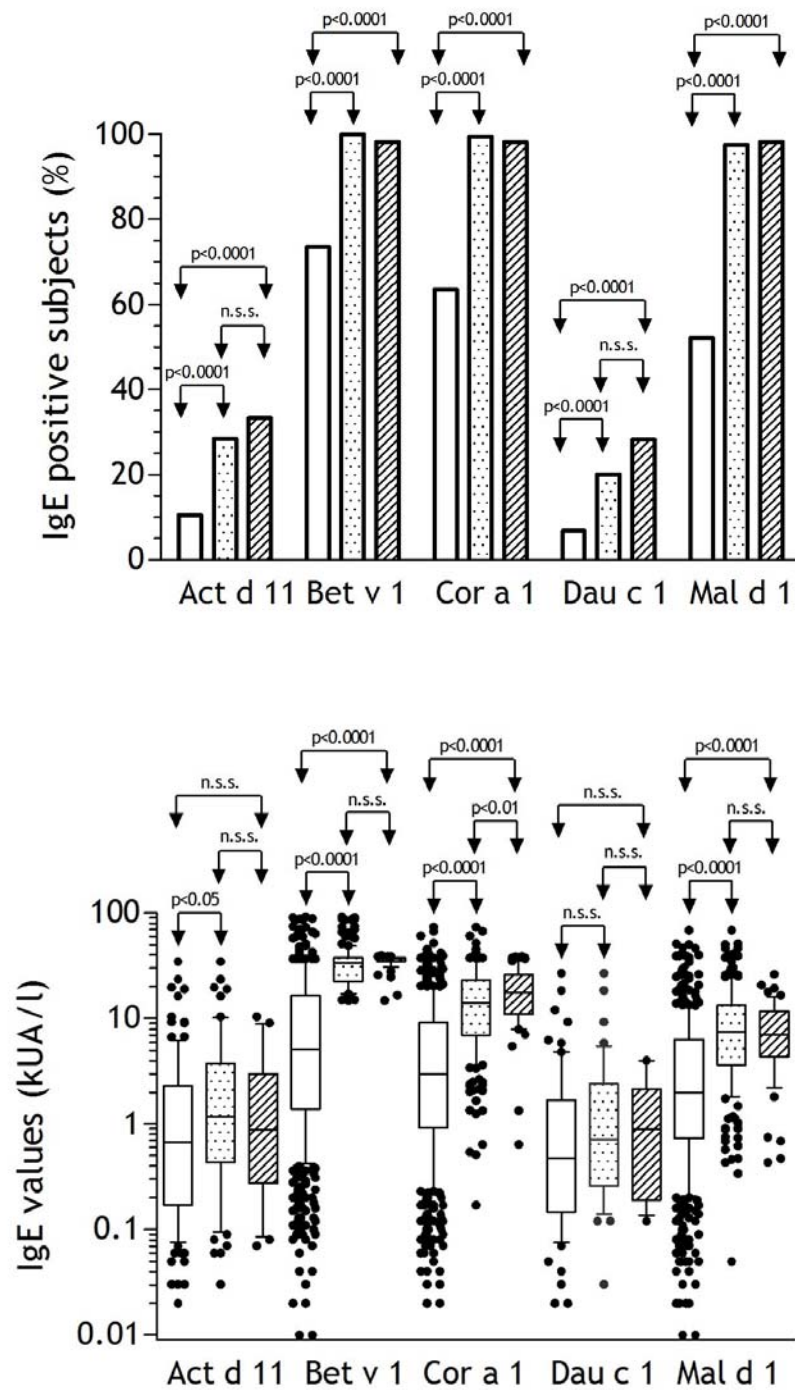


Figure E5. (upper panel) Prevalence of positive IgE to Bet v 1-like allergens in the Italian and Austrian cohorts; (lower panel) Value distribution of Bet v 1-like allergens IgE in the Italian and Austrian cohorts.

In both graphs, white bars show data on 1,084 subjects having positive IgE to at least one Bet v 1-like allergen within the whole Italian tested allergic cohort (n = 8,256); spotted bars show data on 214 Italian Bet v 1 IgE positive subjects with IgE values equal or greater than the lowest value of the Austrian subjects; hatched bars show data on 60 Austrian subjects known to be Bet v 1 IgE positive. Statistically significant differences are reported in right and left panels, respectively.