



Immunological characterization of recombinant Mal d 1, the main allergen from apple (*Malus x domestica* L. Borkh)

Federica Savazzini^a, Stefano Del Duca^{d,*}, Mara Vegro^b, Francesca Cipriani^c, Giampaolo Ricci^c, Alessandro Botton^b, Gabriella Pasini^b, Luca Dondini^a, Stefano Tartarini^a

^a Department of Agricultural and Food Sciences, University of Bologna, Viale Fanin 46, 40127, Bologna, Italy

^b Department of Agronomy, Food, Natural Resources, Animals and Environment – DAFNAE - Agripolis, University of Padova, Viale dell'Università'16, Legnaro, Padova, Italy

^c Pediatric Unit, Department of Medical and Surgical Sciences, University of Bologna, Via Massarenti 11, Bologna, Italy

^d Department of Biological, Geological and Environmental Sciences, University of Bologna, Via Imerio 42, 40126, Bologna, Italy

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ABSTRACT

Apple-related allergy is increasing among European population. Avoidance of apple fruit consumption is the only strategy for sensitized people, with a subsequent shortage of healthy nutrients in the diet. Four different protein families, Mal d 1 to 4, determine the allergy to apple causing syndromes known as oral allergy syndrome (OAS) or gastro-intestinal/generalized syndrome (GS) in sensitized patients. With the aim to highlight the dissimilarities in the immunological response inside the Mal d 1 family, recognized as main determinant for OAS, single purified allergens were tested against sera of paediatric and adult patients suffering for both syndromes. Here, we found that Mal d 1 isoallergens showed a remarkable different allergenicity potential, thus indicating an individual pattern in each patient and a diverse sensitization between age groups. Moreover, two different allergens, Mald 2.01 and Mal d 4.01, were analyzed with the above sera. In addition to patients and allergens variability, differences in amount and localization of the allergens, detected by patient's sera, were revealed among hypo- and hyper allergenic apple varieties, confirming the complexity of this food allergy.

1. Introduction

Apple (*Malus x domestica* L. Borkh), one of the most consumed fruit worldwide, has unique health-promoting properties, due to a high content of phenols, triterpenes, vitamins and fibers (Farneti et al., 2015). Moreover, apple consumption was frequently associated to the decrease of several health disorders, as cardiovascular diseases and type II diabetes (Bazzano et al., 2008). Unfortunately, allergies to apple fruit dramatically increased in the recent years, with estimates of perceived reaction as high as 8.5% among children younger than 3 years of age (Zuidmeer et al., 2008). Two allergic syndromes toward *Rosaceae* fruits were described following a primary sensitization by airborne pollens (Eriksson et al., 1982), fresh fruits or commercial juices (Fernandez Rivas et al., 1997). In details, a) the systemic/generalized syndrome (GS), a Class-I allergy, due to hypersensitivity toward lipid transfer proteins (LTP) in the gastro/intestinal tract (Pastorello and Rivolta, 2004) and b) the oral allergy syndrome (OAS), a Class-II allergy,

triggered by the contact of proteins, homologues to birch Bet v 1 and 2, with the oral mucosa (Ebner et al., 1991).

To date, four allergen classes have been identified in apples: Mal d 1 (Bet v 1-homologues, pathogenesis-related (PR-10)), Mal d 2 (thaumatin-like proteins, PR-5), Mal d 3 (non-specific lipid transfer proteins, nsLTP, PR-14) and Mal d 4 (profilins) (Savazzini et al., 2015). Mal d 1 and, to a lesser extent, Mal d 4 are the main responsible for OAS syndrome, largely affecting the Northern and Central Europe populations, due to cross-reactivity with the homologous birch pollen Bet v 1 and 2. Conversely, Mal d 3 was associated to the GS syndrome in Mediterranean regions, where mugwort/plane tree pollens or the direct contact with *Rosaceae* fruits, primarily peach, was responsible of the primary sensitization (Schmidt-Andersen et al., 2011). Other than geographical and seasonality effects, the different environmental and storage conditions, agronomic practices, apple varieties and ripening stage could affect the allergenic potential, due to the altered transcript profile (Botton et al., 2008) and protein content in fruits (Matthes and Schmitz-

Abbreviations: OAS, oral allergy syndrome; GS, gastro/intestinal generalized syndrome; LTP, lipid transfer protein; TLP, thaumatin-like proteins; PR, pathogenesis-related; Abs, polyclonal antibodies; mAbs, monoclonal antibodies; sIgE, specific IgE; CV, coefficient of variation; CBB, coomassie brilliant blue

* Corresponding author.

E-mail address: stefano.delduca@unibo.it (S. Del Duca).

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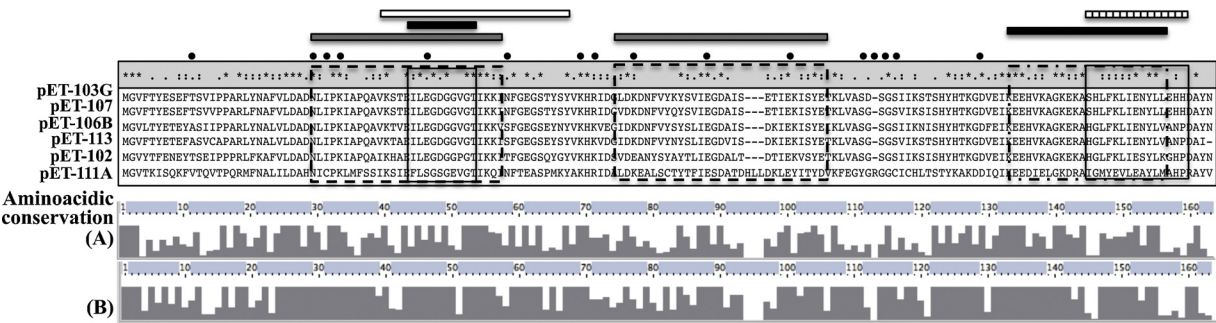


Fig. 1. Kinaciyan et al., 2016 T. Kinaciyan , B. Nagl , S. Faustmann , S. Kopp , M. Wolkersdorfer , B. Bohle . Recombinant Mal d 1 facilitates sublingual challenge test of birch pollen-allergic patients with apple allergy Allergy 2016; 71: 272-274 Alignment and conservation of the deduced protein sequences of the recombinant *Mal d 1* clones. Conservation among (A) all *Mal d 1* and (B) without *Mal d 1.11A*. Fully conserved residues are indicated with (*), amino acids showing strongly (:) and weakly (.) similar properties. PR-10 IgE epitopes as scattered residues (●) and peptide patches (boxes) from literature (Supplementary material File 1). (●) Bet v1/*Mal d 1* /PR-10 residues. (boxes) Bet v1/*Mal d 1* /PR-10 peptide patches: (white) p49-66; (black) p42-52 ‘BV16 Fab’ and p132-154; (dark grey) p29-58 and p73-103; (vertical lines) p142-156 T-cell epitope.

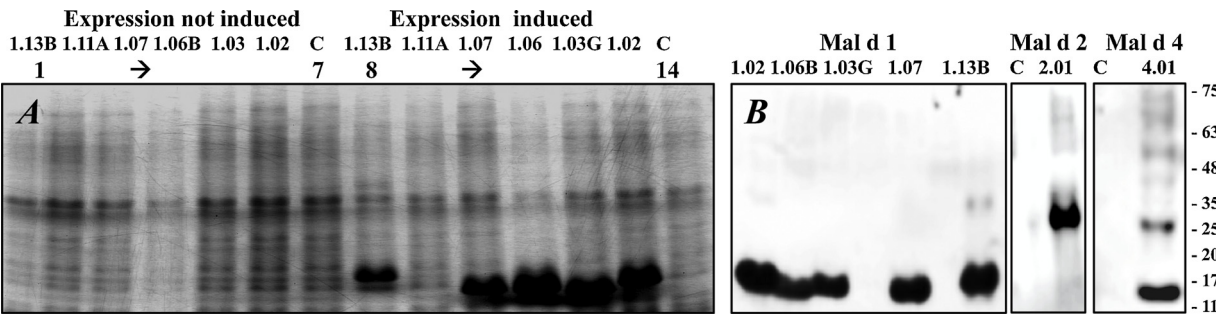


Fig. 2. Expression of recombinant *Mal d* genes. A) Insoluble protein fraction of *E. coli* BL21 bacterial strains, carrying the pET vector with *Mal d 1* genes or empty (C), were stained with CBB, before (lanes 1–7) and after the expression induction (lanes 8–14). B) Purified not-reduced *Mal d* proteins (in sample buffer without reducing agents) were immunodetected by specific anti-*Mal d* IgG Abs. Molecular Mass markers is on the right, in kDa.

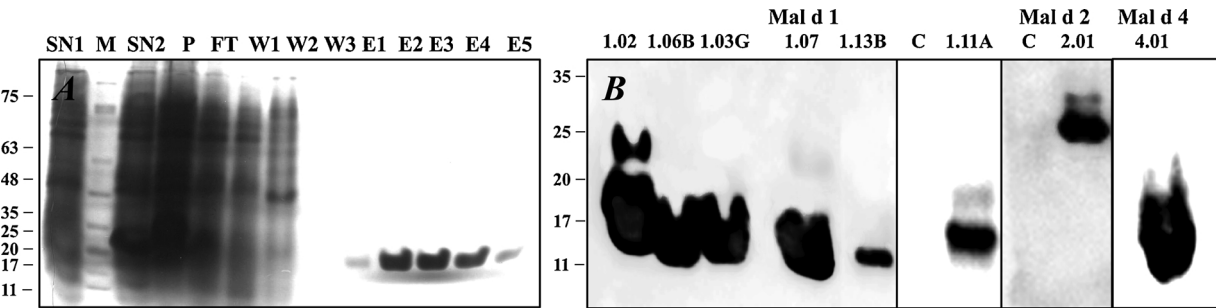


Fig. 3. Recombinant protein purification by Nickel affinity columns. (A) Single purification fractions of the r*Mal d 1.07* stained with CBB From left to right: soluble proteins from *E. coli* bacterial lysate before (SN1) and after (SN2) the expression induction, molecular weight marker (M), cell pellet dissolved in 6 M urea (P), column flow through (FT), column wash steps (W1 to W3), eluted fractions (E1 to E5). B) Western blot analysis of purified and reduced r*Mal d 1, 2* and *4*. Ten (1.02, 1.03, 1.06, 1.07 and 4.01) or twenty (1.11, 1.13 and 2.01) microliters from eluted fractions were immunodetected with anti-*Mal d* Abs. Molecular Mass markers values on the left, in kDa.

Eiberger, 2009). Nevertheless, also processed apples could cause allergic reactions: *Mal d 2* and *3* proteins are highly resistant to proteases of digestive system and also to physical stresses because of their folded structure, and *Mal d 1* was demonstrated to retain some active epitopes even after cooking (Bohle et al., 2006). The immunological characterization of an allergen requires the knowledge of both T- as well as B-cell binding epitopes on the protein surface, which can be of linear or conformational nature (Harrer et al., 2010). A large number of studies on Bet v 1, the major birch allergen involved in the pollen-associated allergy with PR-10 family, demonstrated that IgE epitopes were mainly conformational and discontinuous, and partially overlapped with T-cell epitopes. Due to the high homology in primary and tertiary protein structures, the consensus

sequence analysis was an important tool to hypothesize possible IgE binding sites within homologous cross-allergens in *Rosaceae* family. Based on this, homologous cross-allergens were Bet v 1 and cherry Pru av 1 (Sheurer et al., 1999), pear Pyr c 1 (Karamloo et al., 2001) and apple *Mal d 1* (Vanek-Krebitz et al., 1995), or profilin birch Bet v 2 and apple *Mal d 4* (Radauer et al., 2006). Likewise, *Mal d 1* T-cell epitopes mapping took advantage of the results obtained on Bet v 1 (Kitzmüller et al., 2015). On the other hand, the sequence similarity was demonstrated not to be enough to predict the existence of common epitopes, but specificity and differences among allergens were assessed (Vanek-Krebitz et al., 1995; Haka et al., 2015). All four *Mal d* allergens were proved reactive with patients’ sera, as natural or recombinant proteins expressed in heterologous system

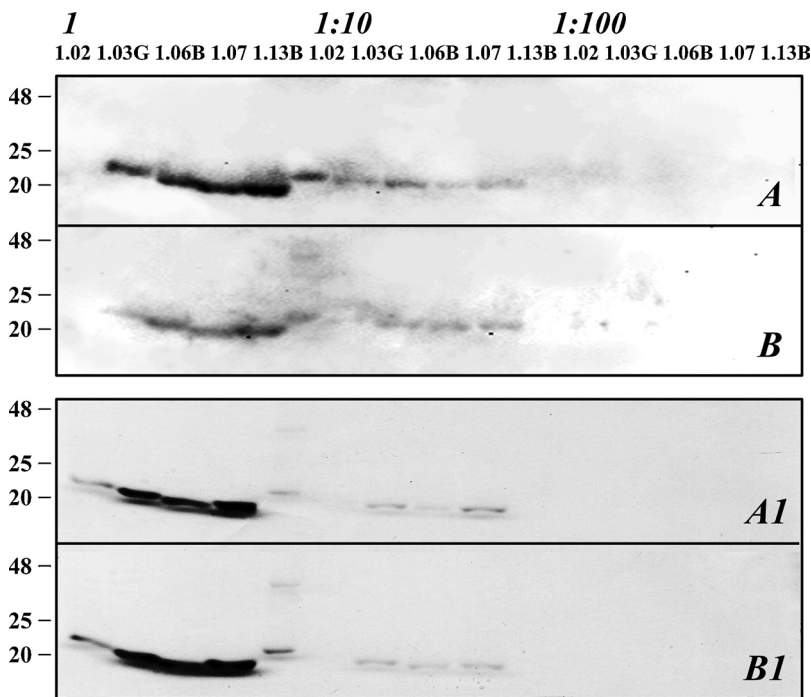


Fig. 4. *Mal d* protein dilution effect on IgE binding. Three dilutions of purified not-reduced rMal d 1.02, 1.03 G, 1.06B, 1.07 and 1.13B were detected with a single paediatric patient serum (at 1:8 serum/blocking buffer). The starting concentration was about 400 ng. Western immunodetection with allergic (A) and negative control Ca sera (B). Both gels were stained with CBB after blotting (A1 and B1). Molecular Mass markers values are on the left, in kDa.

(Oberhuber et al., 2008). Despite the large size of each *Mal d* gene family and the evidence of different allergenicity among Bet v 1 proteins (Wagner et al., 2008), only *Mal d* 1.01 and 1.02 (Son et al., 1999), *Mal d* 2.01 (Krebitz et al., 2003), *Mal d* 3.01 (Diaz-Perales et al., 2002) and *Mal d* 4.01 (Ma et al., 2006) were analysed for affinity with human IgE. Conversely, the production of Bet v 1/*Mal d* 1 chimeric proteins (Klinglmayr et al., 2009), epitope grafting (Holm et al., 2011) and site-directed mutagenesis of the *Mal d* 1.01 isoform (Son et al., 1999), were the preferred method to find IgE-binding sites on protein surface. The mutated variants showed to be hypoallergenic, in comparison to the natural protein (Bolhaar et al., 2005). Based on sequence comparison analysis, other *Mal d* 1 isoforms were hypothesized as allergens, although without any confirmation *in vivo* (Gao et al., 2008). Thus, the contribution of other different *Mal d* 1 isoforms to apple allergy is still controversial and only partially known.

Here we aimed at studying the specific role and the putative IgE binding potential of different apple allergenic proteins, in order to verify the existence of an “individual” or a “syndrome-related” profile for patients suffering for allergy to apples. Other than the proved allergens *Mal d* 1.02, *Mal d* 2.01 and *Mal d* 4.01, five additional *Mal d* 1 genes, encompassing the variety of protein sequence inside this large family, were investigated and evaluated for binding capacity with sera of allergic patients in immunological tests. The differences among *Mal d* 1 isoallergens, in particular for amino acid residues substitutions located at identified epitopes, will be discussed in consideration of their immunological reaction toward the group of patients tested in this study. Finally, sera were also employed to verify the presence and distribution of the allergens in fruits of varieties with different allergenicity potential.

2. Materials and methods

2.1. Sera of allergic patients

Nine sera from adult patients (> 13 years old), suffering for OAS and previously characterized for apple, *Mal d* 1 and *Mal d* 3 specific-IgE (sIgE, KU L⁻¹) by ImmunoCAP (Unicap 1000; Phadia, Uppsala, Sweden), plus one un-sensitized patient's serum, as negative control, were obtained from the Department of Medical and Surgical Science

(University of Bologna). The sera selected for the analysis were named 1 to 15_BO for allergic and Cc_BO for control serum (Ricci et al., 2010).

Eight sera from paediatric patients (< 13 years old), suffering for OAS, were obtained from the same source and named 21 to 26_BO for allergic and Ca and Cb_BO for the two control sera.

The University of Padova (Vegro et al., 2016) kindly provided two pools of sera from adult patients, OAS and GS suffering, along with one negative control.

2.2. Plant material and nucleic acid extraction

Mal d genes were cloned from three apple cultivars, previously evaluated on allergic patients (Ricci et al., 2010): two commercial varieties, ‘Jonagold’ and ‘Florina’, and one old local Italian variety, ‘Durello di Forlì’. ‘Florina’ and ‘Durello di Forlì’ are two important varieties for breeding programs, carrying pathogen-resistance genes (Tartarini et al., 2004). For the tissue printing test, ‘Gala’, ‘Elstar’, ‘Golden Delicious’ and ‘Santana’, the latter known for hypo-allergenicity trait (Vlieg-Boerstra et al., 2013), were also employed. Fruits were harvested at the Bologna University experimental station (Cadriano, Bologna, Italy) at physiological ripening time. Samples of surface layer (peel) and cortex (pulp) were collected, frozen in liquid nitrogen and then stored at -80 °C until use. Total RNA was isolated according to (Dal Cin et al., 2005) and cDNA was synthesized from 1 µg of DNA-free total RNA, following the manufacturer's instructions (DNase from SIGMA Aldrich, St. Louis, MO, USA; SuperScript® II Kit, Invitrogen Thermo Fisher, Waltham, MA, USA). Genomic DNA was extracted from apple leaves according to literature (Mercado et al., 1999).

2.3. *Mal d* genes cloning

Primer sets were designed on seven *Mal d* gene sequences obtained from the *Malus × domestica* ‘Golden Delicious’ genome v 1.0 (Velasco et al., 2010) and from public databases (www.ncbi.nlm.nih.gov; www.allergen.org): five *Mal d* 1 genes (1.02 from subfamily I, 1.06B subf. III, 1.07 subf. IV, 1.11A subf. V and 1.13B subf. III), one *Mal d* 2 (2.01A), and 1 *Mal d* 4 (4.01) (Supporting Information File S1). *Mal d* 1.02, 1.06B, 1.11A and 1.13B were amplified from ‘Florina’ peel cDNA, *Mal d* 2.02 from ‘Jonagold’ pulp cDNA and *Mal d* 4.01 from ‘Durello di Forlì’

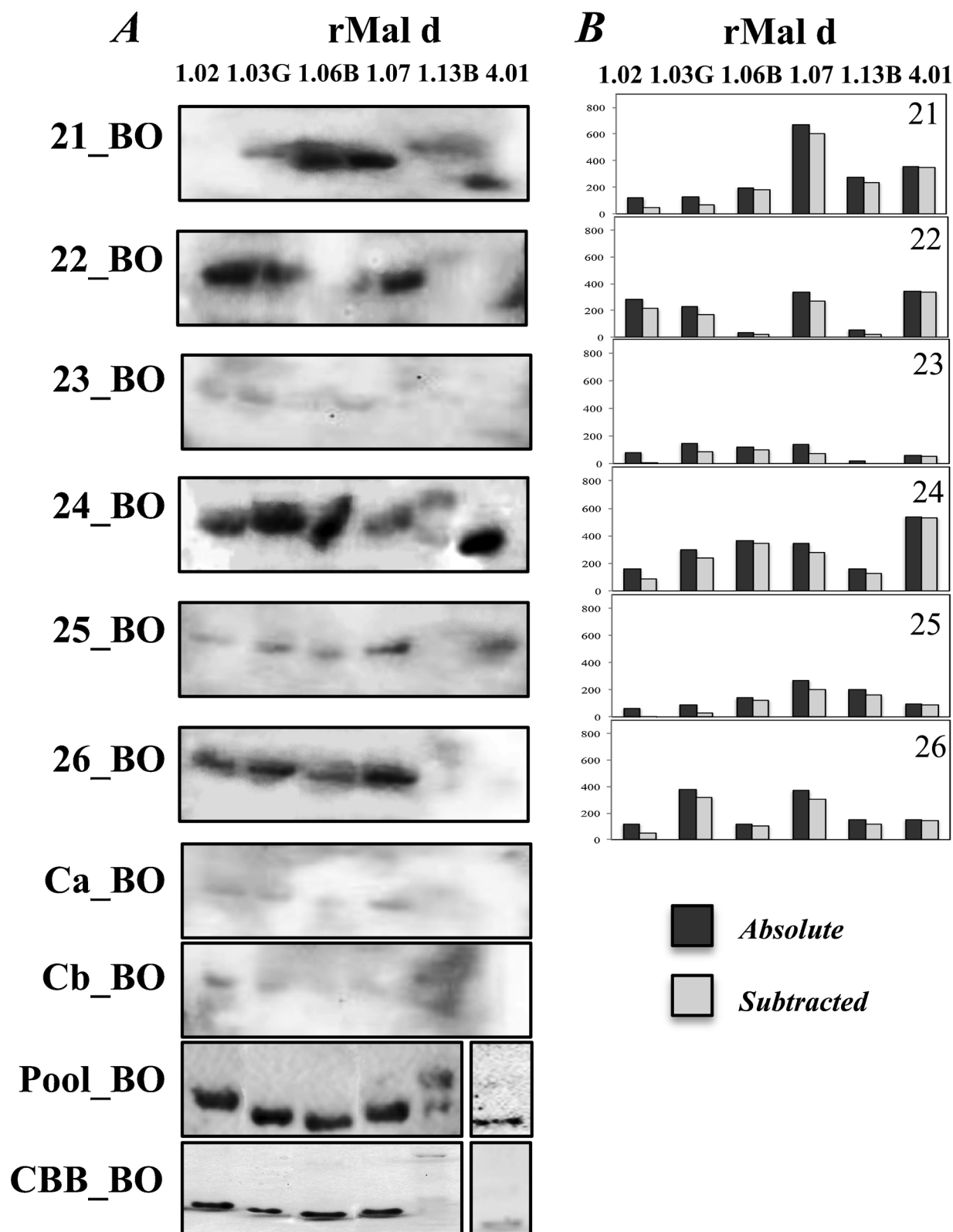


Fig. 5. Binding affinity of rMal d proteins with paediatric sera. (A) Western immunodetection on reduced 100–150 ng rMal d 1 and 20 ng rMal d 4.01. Sera from paediatric allergic patients (21–26_BO) and two negative controls (Ca and Cb_BO) employed at 1:10 dilution in blocking buffer. For pooled sera (Pool_BO), not-reduced 400 ng of rMal d 1 and 100 ng rMal d 4.01 proteins were detected in a 1:10 dilution in blocking buffer. CBB dye staining (CBB_BO). (B) Band intensity quantification, as absolute (dark grey) and controls-subtracted (pale grey) values.

pulp cDNA. Conversely, ‘Florina’ genomic DNA was used to retrieve *Mal d 1.07*, an intronless gene. *Mal d 1.03G* was obtained from ‘Florina’ gDNA by the same amplification of *Mal d 1.07*, due to the high

similarity between primers and the 3’ and 5’ terminal sequences of both genes.

PCR amplifications were performed in Applied Biosystems 2720

Table 1

Band analysis quantification and sera characterization by ImmunoCap. 1A) Mal d protein quantification from immunodetection with patients' sera, calculated as band volumes; 1B) Mal d 1, 3, and apple sIgE, as determined by ImmunoCAP test for adults from Bologna (KU L⁻¹) (Ricci et al., 2010), with the corresponding immunodetection quantification. CV: Coefficient of Variation.

A									
Mal d	Adults			Children			Total		
	Sum	Average	CV %	Sum	Average	CV %	Sum	Average	CV %
1.02	2214	246	68	393	66	64	2607	174	124
1.03	701	78	63	911	152	68	1612	107	101
1.06	1779	198	67	874	146	68	2653	177	120
1.07	1278	142	66	1729	288	69	3007	200	98
1.11	242	27	47	n.d.	n.d.	n.d.	242	27	134
1.13	1170	130	66	647	108	67	1817	121	105
2.01	16	3	40	n.d.	n.d.	n.d.	16	3	224
4.01	1674	186	67	1506	251	69	3180	212	107

B					
Patient	ImmunoCap test			Immunodetection test	
	Apple sIgE	Mal d 1 sIgE	Mal d 3 sIgE	Total Mal d	Total Mal d 1
1_BO	2.47	4.6	0.2	287	271
2_BO	4.79	12.31	2.46	2125	1582
6_BO	45.4	22.65	29.38	1274	585
8_BO	1.52	3	0.2	246	246
10_BO	32.2	56	4.6	1755	1661
11_BO	1.92	10	0.2	1447	1307
12_BO	0.64	2	0.3	272	257
13_BO	32	16	27	1234	1041
15_BO	13	32	0.3	434	434

Thermal Cycler, according the following protocol: a first denaturing step of 94 °C for 4 min, a second step consisting of 35 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 1 min and elongation at 60 °C for 1 min. An additional extension step at 68 °C was added at the end of the 40th cycle for a total 10 min. The amplification mix was composed of 0.5 U of DNA High Fidelity Taq polymerase and 0.3 mM dNTPs mix (Kapa Biosystems, Wilmington, MA, USA), 0.3 µM primers, about 500–800 ng cDNA or 50 ng gDNA. Amplification products were cloned into *Bam*HI-*Xho*I or *Hind*III sites of the plasmid pET28a (Novagen Merck, Darmstadt, DE). Constructs were transformed into *E. coli* DH5alpha and Mal d gene sequences were verified by pairwise alignment with the corresponding reference (Clustalw at <http://www.ebi.ac.uk>).

2.4. In silico sequence analyses

Theoretical molecular weight, isoelectric point, instability index and grand average of hydropathicity were calculated on the deduced amino acid sequences, without His-tags, by ProtParam tool at the ExPASy proteomic server of the Swiss Institute of Bioinformatics (<http://www.expasy.org>).

2.5. Expression and purification of His-tag fusion proteins

Positive clones were expressed in *E. coli* BL21 (DE3) grown in Luria-Bertani (LB) medium, supplemented with Kanamycin (50 mg L⁻¹). Expression and purification were performed according to previous protocols (Wallner et al., 2004), with Nickel affinity-His/tag columns (His select Ni²⁺ affinity gel, Sigma-Aldrich, St. Louis, MO, USA). The different fractions from cell lysates (soluble, insoluble and cell debris fraction) and the purification steps were checked on SDS-PAGE at 15% acrylamide concentration. Purified proteins were stored at -20 °C and their concentration was determined by BCA protein assay kit (Thermo-

Pierce Biotechnology, Rockford, IL, USA) and by Coomassie Brilliant Blue R250 (CBB R250; Sigma-Aldrich, St. Louis, MO, USA) staining on SDS-PAGE.

2.6. Western blot immunodetection and immuno-tissue printing

Before loading on SDS-PAGE samples were denatured for 5 min at 95 °C in sample buffer (SB; 200 mM Tris pH 6.8, 40% glycerol, 8% SDS, 0.04% Bromophenol blue), eventually supplemented with DTT at 100 mM or β-mercaptoethanol at 2% concentration as reducing agents. Western blotting and immunoreactions were carried out as previously described (Vegro et al., 2016; Wallner et al., 2004), using as primary antibodies (Abs): i) purified rabbit polyclonal anti-Mal d 1, 2 and 4 IgG, constructed on shared linear epitopes specific for each Mal d gene family, employed at 1:2000 (v/v) dilution and ii) patient's sera, pooled or single, either diluted 1:8/10 (v/v) or undiluted. The secondary Abs were: i) Goat anti-rabbit IgG-HrP conjugated (Thermo Fisher Scientific, Waltham, Ma, USA) and ii) Goat anti-human IgE-HrP conjugated (Sigma-Aldrich, St. Louis, MO, USA), both employed at 1:2000 (v/v). Enhanced chemiluminescence (ECL, SuperSignal West Dura extended Duration Substrate, Thermo Fisher Scientific, Waltham, Ma, USA) was detected by CCD camera (Kodak Image Station 440cf, Eastman Kodak Company, Rochester, NY) and band volumes were quantified with the GelAnalyzer2010 program (<http://gelanalyzer.com>). Coefficient of variation (CV), defined as the ratio of the standard deviation to the mean, was employed to calculate the precision of the band quantification analysis. Tissue printing and immunodetection were carried out according to the method of Marzban (Marzban et al., 2005).

3. Results

3.1. Mal d genes cloning, synthesis and purification

Eight Mal d genes were amplified and cloned in pET28 vector: 6 Mal d 1 (1.02, 1.03 G, 1.06B, 1.07, 1.11A and 1.13B), one Mal d 2 (2.01A) and one Mal d 4 (4.01). Alignments with the published apple genome sequences (Velasco et al., 2010) and with sequences stored in public databases (www.ncbi.nlm.nih.gov and www.allergene.org) revealed that the cloned genes corresponded to known Mal d. Nevertheless, some sequences showed nucleotide substitutions, some of them leading to amino acid changes in the recombinant Mal d 1.03 G, 1.07, 1.13B, Mal d 2.01A and Mal d 4.01 proteins (Fig. 1 and Supplementary material Fig. S1).

The physico-chemical properties, calculated without His-tags, differed among the recombinant proteins, in particular among the three Mal d families (Supplementary material Fig. S1). The estimated isoelectric point varied from 4.6 to 6, with Mal d 1 grouped in a narrow range from 5.3 to 6. The eight recombinant proteins were also mainly non-polar proteins, as deduced by their negative value of grand average of hydropathicity index. Broader differences were revealed by the instability index with calculated values around 20–25, the *in vivo* half-life of Mal d 1 were longer than Mal d 2.01 and Mal d 4.01 proteins, whose index reached values higher than 40, a threshold indicative of an unstable protein. Mal d 1.11A was clearly separated from Mal d 1 family and with a higher index, indicating that the different amino acid composition brought more instability inside this protein.

Recombinant Mal d 1 and Mal d 4.01 proteins were actively synthesised in *E. coli* insoluble fractions, while rMal d 2 was less expressed. Mal d 1 and 4 recombinant proteins were clearly visible in CBB stained gels as an intense band corresponding to the calculated molecular weight, which was completely absent before the induction of the synthesis (Fig. 2A). Before proceeding to purification, the bacterial lysates were also subjected to immunodetection using anti-Mal d 1, 2 and 4 Abs, which proved that the above-mentioned bands were actually the expressed recombinant allergens (Fig. 2B). Likewise, to confirm the identity of the purified proteins (Fig. 3A), Western blot detection was

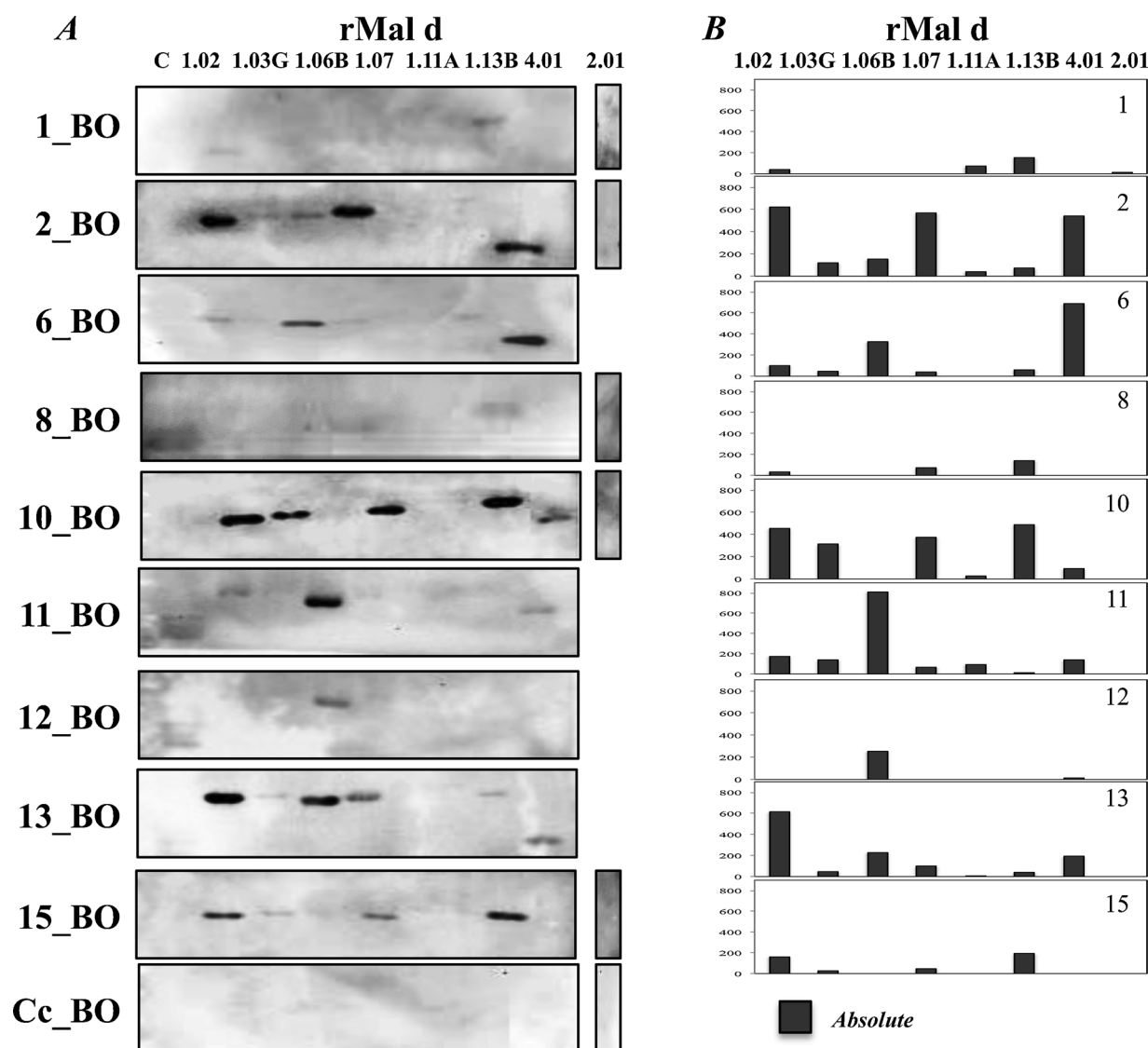


Fig. 6. Binding affinity of rMal d proteins with adult sera. (A) Western immunodetection on 50 ng of reduced rMal d proteins. Sera from adult allergic patients (1 to 15_BO) and negative control (Cc_BO) were employed at 1:8 dilution in blocking buffer. The bacterial insoluble fraction carrying empty pET plasmid was employed as negative control (C, first lane). (B) Band intensity quantification, as absolute values.

performed on the elution fractions (Fig. 3B). The amount of recombinant protein was highly variable, ranging from 3.6 mg per liter of bacterial culture for rMal d 1.02 or 1.07 to 0.1 mg per liter for rMal d 1.11A, Mal d 4.01 or rMal d 2.01A.

3.2. Immunological characterization of the recombinant Mal d proteins with paediatric sera

Purified rMal d 1, 2 and 4 have been detected by the specific IgE from patients allergic to apple in immunoblot experiments. A first test was performed to check five Mal d 1 isoforms at three different concentrations, starting from about 400 ng and decreasing according to a 1:10 scale. The serum of a single paediatric patient, suffering for OAS syndrome, equally recognized all the five isoforms in the highest concentrated samples, with the 1.13B isoform showing a lower intensity (Fig. 4A). At 1:10 concentration, the binding reaction was still faint, while at 1:100 no signal was visible. The negative paediatric control serum Ca showed a faint reactivity until 1:10 concentration, but less intense than allergic serum (Fig. 4B). Thus, unspecific IgE were demonstrated to be present in negative paediatric control serum and they could actively link with the Mal d protein epitopes.

The set of six paediatric sera, with a clinical history for OAS syndrome, and two negative controls (unsensitized sera) were tested individually or as a pool against five rMal d 1 (1.02, 1.03 G, 1.06B, 1.07 and 1.13B) and the profilin rMal d 4.01. About 100–150 ng Mal d 1 was loaded for the single patient's serum test, while about 400 ng for the pooled sera test; in case of Mal d 4.01, 20 and 100 ng were loaded for the single and pooled sera test, respectively. Gels stained with CBB dye confirmed the equal amount of Mal d 1 proteins and the presence of two faint bands for rMal d 1.13B (Fig. 5A, CBB_BO). The pool of sera resulted in a sharp detection of all rMal d 1, with a lower signal for both rMal d 1.13B bands (Fig. 5A, Pool_BO). In single patient's sera, different intensities were shown among the isoforms and each patient showed a specific reactivity profile (Fig. 5A, 21_BO to 26_BO). Both negative controls demonstrated a faint reactivity (Fig. 5A, Ca and Cb); thus, the band intensities were calculated as values subtracted to the negative controls mean (Fig. 5B and Table 1A). Except for the weak signal of 23_BO sample, all sera showed IgE binding capacity toward Mal d proteins. Four patients out of six reacted positively with the profilin rMal d 4.01 and, in one patient (24_BO), the profilin produced a more intense band than any rMal d 1. In the tested paediatric sera, rMal d 1.07 showed the greatest affinity for immunoglobulin, with the highest

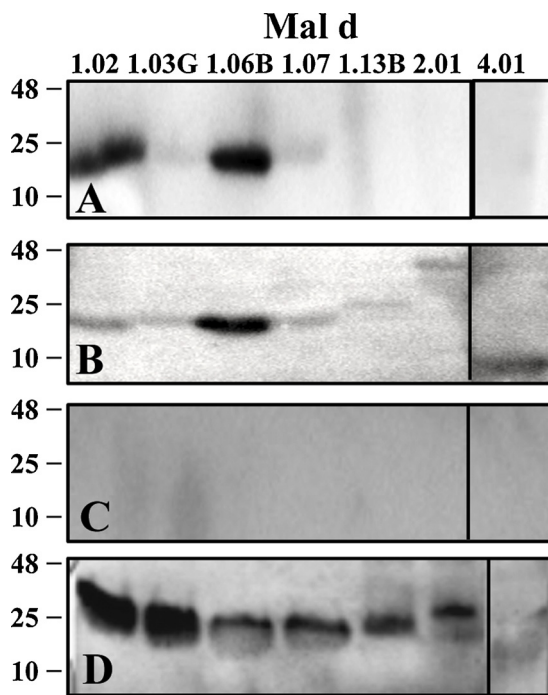


Fig. 7. Binding affinity of rMal d proteins with two adult sera pools. Western immunodetection on 200 ng of reduced rMal d 1, 2.01 and 4.01 with different pools of adult patients' sera, not diluted in blocking buffer. A) OAS syndrome pool, B) GS pool, C) negative control serum, D) CBB protein staining. Molecular mass marker on the left, in kDa.

quantification score in five out of six sera, followed by rMal d 4.01. Mal d 1.02 was the less reactive allergen, with the patient 22_BO showing the highest score. The variability of the binding reaction, calculated as CV of the band intensities, was around 65% and similar for all the Mal d tested (Table 1A).

3.3. Immunological characterization of the recombinant Mal d proteins with adult sera

The nine single adult sera were tested against 50 ng of six rMal d 1 isoallergens (1.02, 1.03 G, 1.06B, 1.07, 1.11A and 1.13B), rMal d 2.01A and rMal d 4.01. All sera reacted positively revealing differences in IgE binding capacity (Fig. 6A). Among the six rMal d 1, the isoform 1.02 had the broader reaction, with eight positive sera out of nine, followed by 1.07, 1.13B, 1.03 G, 1.06B and the less reactive 1.11A. The rMal d 2.01A did not actively bind with any of the samples tested even if employed as reduced form, which generally increased the IgE-binding activity in comparison to the non-reduced form (Herndl et al., 2007). Finally, rMal d 4.01 reacted with the majority of samples (six out of nine). Because the control serum (Cc_BO) was completely negative toward all Mal d proteins, the band analysis did not need any unspecific signal subtraction, as previously reported for paediatric sera; thus, absolute values were used to evaluate the binding capacity of each protein (Fig. 6B and Table 1A). In adults, rMal d 1.02 had the broadest and strongest binding reaction, followed by rMal d 4.01 and rMal d 1.06B; rMal d 1.07 and 1.13B showed a lower but quite similar binding capacity, while rMal d 1.03 G and 1.11A were the weakest against our set of patients. The variability of the immune-binding reaction among adult patients was similar to paediatric ones, around 65% for each Mal d, except for Mal d 1.11A, tested only on adults, with 47%. Concerning Mal d 1, the comparison between immunodetection, calculated as band intensities, and ImmunoCap test produced highly similar and comparable results for the majority of the patients tested (Table 1B). For instance, patients 2, 10, 11 and 13_BO had generally higher values in both tests and patients 1, 8, 12 had lower one. Only two patients showed

dissimilar results after the two tests: 15_BO and 6_BO.

The pool of OAS adult sera from Padova University showed a sharp binding reaction toward Mal d 1.02 and 1.06B, while the other Mal d 1 isoforms were not detected, even if the protein amount was 200 ng (Fig. 7A). Otherwise, the group of patients selected for GS syndrome showed a broad IgE binding activity toward all Mal d proteins, even Mal d 2.01A (Fig. 7B). Mal d 1.02 and 1.06B produced the sharpest band in both sample pools. The negative control serum did not give any visible signal (Fig. 7C).

3.4. Localization of Mal d proteins in apple fruits

The localization of the Mal d proteins in apple fruit was carried out by immune tissue printing on several apple varieties, using two sera from adult patients and Mal d 1 Abs (Fig. 8). Furthermore, three apple varieties ('Jonagold', 'Durello di Forlì' and 'Florina') did not show any visible signal when tested against the negative control serum (Cc_BO).

The two sera 6_BO and 10_BO, belonging to adult subjects suffering for OAS, showed different profiles for Mal d 1 and 4 (Fig. 6). In details, 6_BO had a strong reaction toward rMal d 4.01 and rMal d 1.06B, while 10_BO had a broader reaction to rMal d 1.02, 1.03 G, 1.07 and 1.13B, but weaker toward rMal d 4.01 and did not match with 1.06B. In addition, both patients revealed a high IgE reaction in ImmunoCAP test: 6_BO had equal IgE amount against Mal d 1 and 3, while 10_BO had high IgE score for Mal d 1 and low for Mal d 3 (Table 1B). Both sera equally reacted toward the tested varieties, detecting Mal d 1 as well as Mal d 4 allergens in fruit tissues. Conversely, remarkable differences were noticed among varieties. 'Jonagold', 'Florina', 'Gala', 'Elstar' and 'Golden Delicious' contained a higher amount of allergens in pulp and peel tissues compared to 'Santana' and 'Durello di Forlì', as showed by the reaction with the IgE of the two OAS patients. On the other hand, in the latter two varieties Mal d allergens were mainly located in the peel cell layers, while a very low signal detected inside the pulp; these results were confirmed by the Mal d 1 specific Abs when tested by immune tissue printing (Fig. 8).

4. Discussion

Apple allergenicity is the result of complex interactions involving allergens, patients and apple genotype specificities. This study reports the immunological response of some Mal d allergens toward two groups of patients with different age, mainly suffering for OAS syndrome, with the aim to verify if some allergen isoforms among others could be responsible for a higher immunogenic response. In birch, some naturally occurring Bet v 1 isoforms were confirmed to be hypoallergenic (Wagner et al., 2008). Here, other than the allergens Mal d 1.02, 2.01A and 4.01, new Mal d 1 isoforms have been evaluated: Mal d 1.03 G, 1.06B, 1.07, 1.11A and 1.13B, covering a broad range of protein diversity inside the Mal d 1 family.

The majority of the tested allergens was cloned from the apple fruit tissues, where they were actively transcribed (Botton et al., 2008). The cloned genes corresponded to those retrieved from public databases, with few and generally silent nucleotide substitutions. Surprisingly, the most divergent clones compared to the published isoforms sequences were the intronless Mal d 1.03 G and 1.07, both cloned directly from genomic DNA, carrying three amino acid changes each. These changes are mainly due to the sequence variability among apple varieties in comparison to those available on the 'Golden Delicious' genome (Velasco et al., 2010). The amount of recovered and purified protein was less than that reported in previous studies, in particular for isoforms Mal d 1.11A, 1.13B and Mal d 2.01A (Oberhuber et al., 2008; Wallner et al., 2004).

Dimerization or even polymerization events was previously noted in pollen and in *E. coli* cells for Mal d 1.0108 (Roulias et al., 2014), but not for Mal d 1.0101 (Ahamer et al., 2017). The importance of aggregation was elucidated in birch Bet v 1, where it was firstly supposed to be

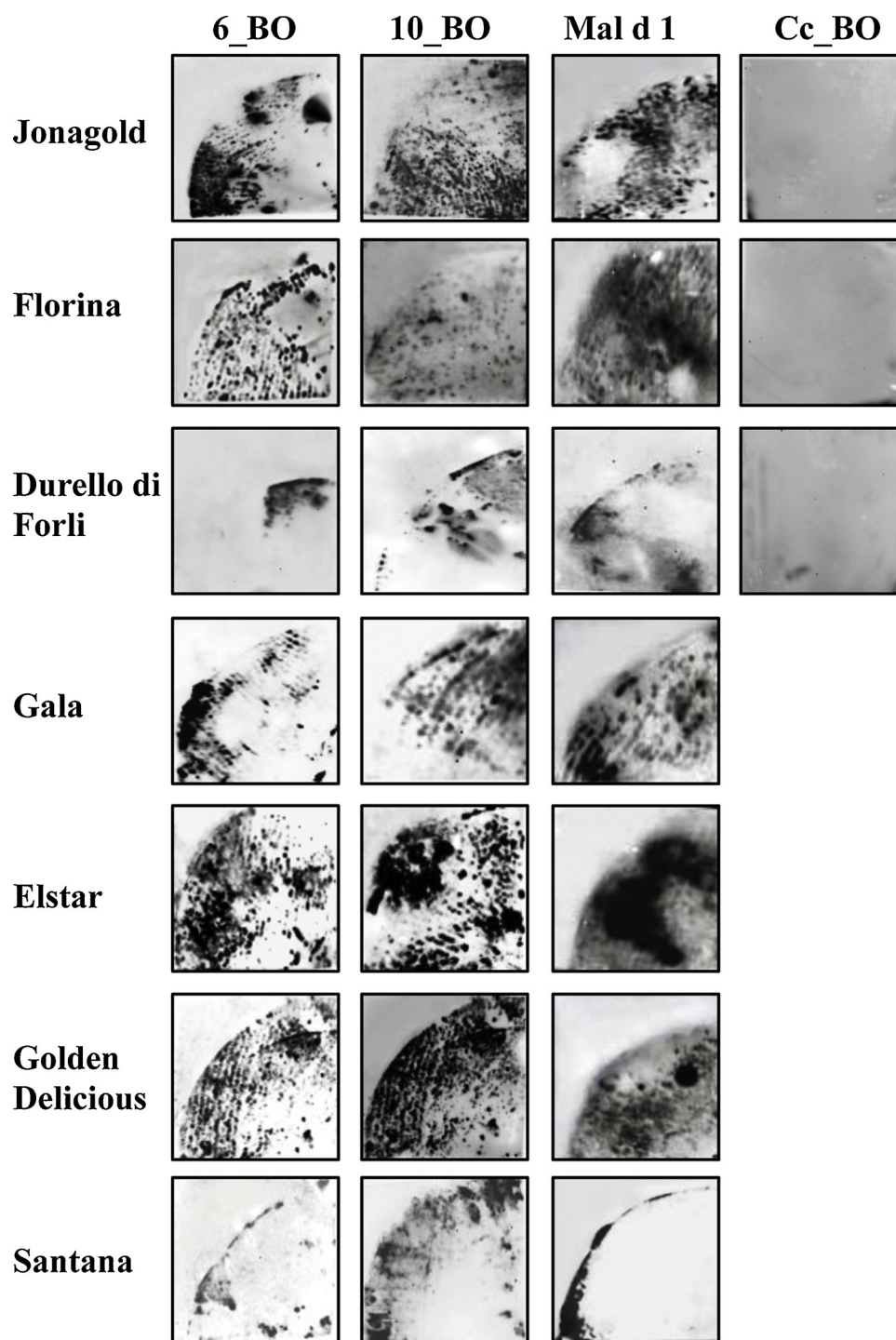


Fig. 8. Tissue printing immunodetection. Mal d proteins were detected on apple slice with: (i) sera of two adult patients (6_BO, 10_BO) and one negative control (Cc_BO) and (ii) anti Mal d 1 Abs.

crucial for allergenicity (Schöll et al., 2005), but then correlated to an increase of the immunization (Zaborsky et al., 2010). Mal d 2.01 contains 16 cysteine residues, which create eight disulphide bridges (Breiteneder, 2004). Mal d 1.11A and 1.13B contain four and one cysteine residues respectively, while Mal d 4.01 has two cysteine residues. For the first time it has been observed that Mal d 1.13B can aggregate into dimers and Mal d 4.01 can produce several homopolymers, recognized by the Mal d 4 antibody as well as by human sera. Therefore, a further immunological characterization will be useful to verify the existence of these aggregate *in vivo* in fresh apples, and if they have some allergenic properties. It has been reported that other post-translational

modifications, such as glycosylation events for Mal d 2 (Herndl et al., 2007; Breiteneder, 2004) may be responsible of introducing new epitopes, thus influencing antibody binding and allergenicity. Despite the difficulties in obtaining large protein amount, all the purified recombinant Mal d 1, 2 and 4 proteins could actively bind to their specific Abs and to human IgE, demonstrating that the protein structures were conserved in respect to those naturally present in apple and the immunogenicity of binding sites was maintained.

In apple, Mal d 1 is the main and most studied allergen: this large gene family has a pool of genes actively expressed in apple fruits, among which *Mal d 1.01* and *1.02* are the highest transcribed genes

(Botton et al., 2008). In the present study, rMal d 1.02 was the most active IgE-binding protein toward OAS adult sera, but it produced a weak reaction towards OAS paediatric sera. Recently, this isoform was silenced and the following tests with transgenic apples on adult patients demonstrated a strong allergenicity reduction, caused also by the lowered expression of other *Mal d 1* genes complementary to the antisense DNA (Dubois et al., 2015). The same research demonstrated that overexpressing this isoform did not increase fruit allergenicity, thus hypothesizing that other Mal d isoforms should be equally important in triggering the allergic reaction. In the present study, many Mal d 1 isoallergens actively bind human IgE with different affinities among patients of the same age, children and adults. The wide IgE repertoire emphasized the unique response that each patient had toward the different Mal d, confirming that the human IgE against PR-10 allergens are polyclonal (Holm et al., 2011). Besides rMal d 1.02, the isoform rMal d 1.06B had a stronger IgE-binding potential among adults than towards children; both isoforms confirmed the high IgE-binding potential in another group of adult patients with OAS from Padova, even if they were evaluated as a pool and not as a single serum. Moreover, it was hypothesized that differences in allergenicity among apple cultivars could be associated with alleles of *Mal d 1.06A* gene (Gao et al., 2008). Conversely, rMal d 1.07 and 1.03 G isoforms were highly reactive in children and less in adults: rMal d 1.07, with the highest score in paediatric patients and the third place in adults, is the highest IgE-binding reactive isoform among all Mal d 1. The rMal d 1.11A isoform has an amino acid sequence very different from all the other Mal d 1 proteins (40% identity), in particular at the level of the major active epitopes. In the present study, this protein had the weakest signal in adult patients and seemed not to be a potential allergen, at least at the low concentration tested. Sera of patients suffering for OAS from Bologna and Padova never or barely detected Mal d 2.01, confirming that OAS syndrome is not caused by an immunological response toward this protein. Only the GS pool from Padova had a faint reaction toward this allergen, as well as with all the other Mal d 1 and 4, endorsing the hypothesis that patients affected by the GS could own a broad spectrum of IgE against different Mal d allergens. Mal d 4.01 reacted actively with the majority (80%) of children and adults sera with the highest intensity among all rMal d (Table 1A), confirming the importance of this pan-allergen in sensitized patients with both OAS and GS syndrome (Wensing et al., 2002). In the present research, we have employed sera from patients of different ages, with the aim to understand differences between adults and children, as frequently observed in clinical reports due to molecular spreading with increasing age (Hatzler et al., 2012). Even if the two sample groups were not large enough for a statistically based test, due to the shortage of sera in particular from younger patients, the differences we have scored could be a starting point for further research on a larger sample of patients.

Protein epitopes were highly conserved and widespread within the Bet v 1 family, as well as among profilins. However, since the first reports, the major apple allergen Mal d 1 demonstrated to contain some, but not all, epitopes of the homologue birch allergen (Vanek-Krebitz et al., 1995). By the use of site-directed mutagenesis, isoforms analysis, synthetic peptides and X-ray analysis, many conformational IgE binding sites were found on Bet v 1/PR-10 allergens. These binding sites are present as single amino acidic residues dispersed along the protein sequence or as conserved amino acid regions; recently, the use of Bet v 1-specific human mAbs allowed to isolate additional and even multiple binding sites on the allergen (Fig. 1 and Supplementary material Fig. S1). Many of the epitopes and key amino acids on Bet v 1 allergen were also confirmed on Mal d 1 (Son et al., 1999; Klinglmayr et al., 2009; Bolhaar et al., 2005), but several studies revealed partial identity and critical differences (Holm et al., 2011). Recently, the protein structure of Mal d 1.0101 was published: according to this study, the residues 42–52, corresponding to the major epitope Bet v 1-BV16, formed a contiguous patch on the surface, similar in shape, size and with highly conserved amino acidic sequence to birch Bet v 1 (Ahamer et al., 2017).

This epitope was largely conserved in the Mal d 1 clones examined here, except for the highly dissimilar sequence of the rMal d 1.11 protein. The high sequence similarity among Mal d 1, could explain that the majority of its isoforms can activate an immunological response (particularly when loaded at high concentration), and be potentially allergens.

On the other hand, human sera variability, which we considered as the second allergenicity factor, could hamper the detection of single IgE binding site (Berkner et al., 2014). Therefore, loading a low and equal amount of each isoallergen was a first attempt to increase the resolution of IgE affinity from a polyclonal serum and hypothesize some correlations between binding sites and patient's type. Even a single amino acid exchange, in particular if this occurs on the protein surface, could influence the secondary structures of epitopes and affinity toward IgE binding, as demonstrated for Bet v 1 isoforms (Wagner et al., 2008). The main differences between rMal d 1.02/1.06B and rMal d 1.03 G/1.07, which have the lower and higher values between the two patients' groups respectively, are located at some residues dispersed on the surface, part of putative conformational IgE epitopes (Thr10- > Ala; Thr57- > Asn) and at Bet v 1₁₄₂₋₁₅₆ T-cell epitope (Ser145-Hist146- > Hist-Glyc). Additional studies will be necessary to confirm the importance of the above amino acid substitutions in determining the different reactivity, by the use of peptide-specific mAbs and T-cell clone lines.

The third factor in apple allergenicity is apple biodiversity: some apple varieties are known for their low allergenic potential, while some others were always reputed as high allergenic (Ricci et al., 2010; Vegro et al., 2016). Our study confirmed that 'Santana' and 'Durello di Forlì', two hypoallergenic varieties, showed a very low signal in comparison to the other tested varieties. In 'Santana', Mal d 1 was well detected in the peel by Mal d 1 Abs as well as by the OAS patients' sera, while it was quite undetectable in pulp tissue. Moreover, the serum 6_BO, selected because of the stronger response toward rMal d 4.01 in comparison to rMal d 1, reacted similarly to 10_BO, indicating that this apple has very low amount of both Mal d 1 and 4 proteins. The reaction of the same serum with the 'Golden Delicious' slices gives a very different result, being the pulp rich of Mal d proteins, as previously confirmed by transcript and protein quantification (Botton et al., 2008; Matthes and Schmitz-Eiberger, 2009). A more comprehensive study should be performed including Mal d 2 and 3, and eventually GS patients on the same varieties.

Natural proteins extracted from apple fruits are frequently used in epidemiological studies on allergies, but they could easily degrade with reduced efficacy (Rudeschko et al., 2005). Recombinant Mal d proteins were also produced with the aim to facilitate the dose-defined evaluation of the short-lived Mal d 1 allergen in clinical tests: the commercially available Mal d 1.0108 (Biomay, Wien, Austria) was recently employed in skin prick and oral challenge tests, instead of apple extract or fruit slices (Kinaciyan et al., 2016). If confirmed in further studies, the different reactivity between adults and children toward specific allergen isoforms could help the choice of the proper clinical test.

One of the major problem for designing a breeding program for OAS-hypoallergenic apple variety is to accurately determine the allergen content in fruits (protein or transcript). Moreover, it is crucial to evaluate which isoform (among many allelic variants) are present in the selected genotypes taking into account, case by case, the variability of the patient reactions. This paper indicates that a few isoforms could be clearly correlated to either OAS or GS syndromes. These achievements could support the design of new breeding program aimed to the selection of hypoallergenic varieties and, in the future, to set up cheaper and faster tests for a relatively low cost apple germplasm screening.

Author contributions

F.S., and S.T. contributed in conception and experiment design; F.S., M.V. and L.D. contributed in experiment performing; F.C. and G.R. collected patient's sera and contributed in manuscript drafting.

A.B. and **G.P.** provided pooled patients' sera. **S.D.D.** took part in data curation and formal analysis. All Authors contributed in result discussion, manuscript editing and revision.

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Conflict of interest statement

The authors declare no competing financial interest.

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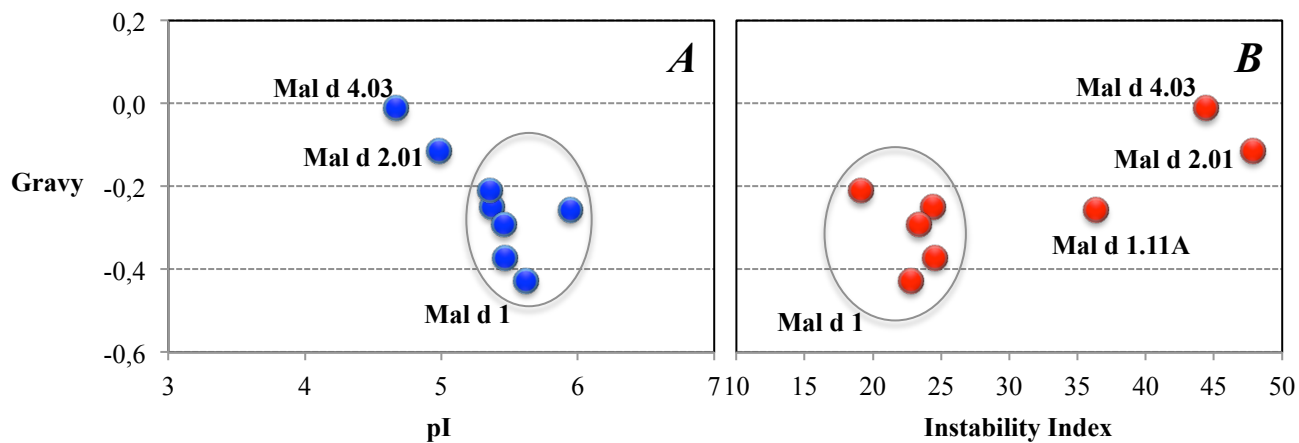
Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.scienta.2019.108926>.

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Suppl. Fig. 1. Physico-chemical properties of the recombinant Mal d proteins. A) Isoelectric point (pI); B) Instability index (x axis) and grand average of hydropathicity (y index) were calculated on the aminoacidic sequence of the cloned *Mal d* genes without the His-tags.

1. Literature for Bet v 1 and Mal d 1 epitopes, as in Figure 1

Bet v1/Mal d 1 /PR-10 amino acid residues:

F. Ferreira, C. Ebner, B. Kramer, G. Casari, P. Briza, A.J. Kungl, R. Grimm, B. Jahn-Schmid, H. Breiteneder, D. Kraft, M. Breitenbach, H.J.R.G. Rheinberger, O. Scheiner, Modulation of IgE reactivity of allergens by site-directed mutagenesis: potential use of hypoallergenic variants for immunotherapy, *FASEB J.* 12 (1998) 231-242.

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Y. Ma, G. Gadermaier, B. Bohle, S. Bolhaar, A. Knulst, Z. Markovic-Housley, H. Breiteneder, P. Briza, K. Hoffmann-Sommergruber, F. Ferreira, Mutational analysis of amino acid positions crucial for IgE binding epitopes of the major apple (*Malus domestica*) allergen, Mal d 1, *Int. Arch. Allergy Immunol.* 139 (2006) 53-62.

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O. Mirza, A. Henriksen, H. Ipsen, J.N. Larsen, M. Wissenbach, M.D. Spangfort, M. Gajhede, Dominant epitopes and allergic cross-reactivity: complex formation between a Fab Fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1, *J Immunol.* 165 (2000) 331-338.

M.D. Spangfort, O. Mirza, H. Ipsen, R.J.J. van Neerven, M. Gajhede, J.N. Larsen, Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis, *J. Immunol.* 171 (2003) 3084-3090.

M. Levin, A.M. Davies, M. Liljekvist, F. Carlsson, H. J. Gould, B. J. Sutton, M. Ohlin, Human IgE against the major allergen Bet v 1 – defining an epitope with limited cross-reactivity between different PR-10 family proteins. *Clin. Exp. Allergy* 44 (2014) 288–299.

C. Ebner, Z. Szepefalusi, F. Ferreira, A. Jilek, R. Valenta, P. Parronchi, E. Maggi, S. Romagnani, O. Scheiner, D. Kraft, Identification of multiple T-cell epitopes on Bet v 1, the major birch pollen allergen, using specific T-cell clones and overlapping peptides, *J. Immunol.* 3 (1993) 1047-1054.

R. Wiche, M. Gubesch, H. Konig, K. Fotisch, A. Hoffmann, A. Wangorsch, S. Scheurer, S. Vieths, Molecular basis of pollen-related food allergy: identification of a second cross-reactive IgE epitope on Pru av 1, the major cherry (*Prunus avium*) allergen, *Biochem. J.* 385 (2005) 319-217.

J. Holm, M. Ferreras, H. Ipsen, P.A. Wurtzen, M. Gajhede, J.N. Larsen, K. Lund, M.D. Spangfort, Epitope Grafting, Re-creating a Conformational Bet v 1 Antibody Epitope on the Surface of the Homologous Apple Allergen Mal d 1, *J. Biol. Chem.* 286 (2011) 17569–17578.

Bet v1/Mal d 1 /PR-10 peptide patches:

p49-66

S. Lebecque, C. Dolecek, S. Laffer, V. Visco, S. Denepoux, J.J. Pin, C. Guret, G. Boltz-Nitulescu, A. Weyer, R. Valenta, Immunologic characterization of monoclonal antibodies that modulate human IgE binding to the major birch pollen allergen Bet v 1, *J. Allergy Clin. Immunol.* 99 (1997) 374-84.

p42-52 'BV16 Fab'

O. Mirza, A. Henriksen, H. Ipsen, J.N. Larsen, M. Wissenbach, M.D. Spangfort, M. Gajhede, Dominant epitopes and allergic cross-reactivity: complex formation between a Fab Fragment of a monoclonal murine IgG antibody and the major allergen from birch pollen Bet v 1, *J Immunol.* 165 (2000) 331-338.

M.D. Spangfort, O. Mirza, H. Ipsen, R.J.J. van Neerven, M. Gajhede, J.N. Larsen, Dominating IgE-binding epitope of Bet v 1, the major allergen of birch pollen, characterized by X-ray crystallography and site-directed mutagenesis, *J. Immunol.* 171 (2003) 3084-3090.

p132-154

J. Hecker, A. Diethers, D. Schulz, A. Sabri, M. Plum, Y. Michel, M. Mempel, M. Ollert, T. Jakob, S. Blank, I. Braren, E. Spillner, An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE, *Allergy.* 67 (2012) 1530–1537.

p29-58 and p73-103

A. Gieras, P. Cejka, K. Blatt, M. Focke-Tejkl, B. Linhart, S. Flicker, A. Stoecklinger, K. Marth, A. Drescher, J. Thalhamer, P. Valent, O. Majdic, R. Valenta, Mapping of Conformational IgE Epitopes with Peptide-Specific Monoclonal Antibodies Reveals Simultaneous Binding of Different IgE Antibodies to a Surface Patch on the Major Birch Pollen Allergen, Bet v 1. *J Immunol.* 186 (2011) 5333-5344.

p142-156 T-cell epitope

B. Jahn-Schmidt, A. Radakovics, D. Luttkopf, S. Scheurer, S. Vieths, C. Ebner, Bet v 1142-156 is the dominant T-cell epitope of the major birch pollen allergen and important for cross-reactivity with Bet v 1-related food allergens, *J. Allergy Clin. Immunol.* 116 (2005) 1, 213-218.

2. Primers for cloning Mal d genes

Primer name	Primer sequence (3'-5')
Md102-pET Fw	CTA GTA GGA TCC ATG GGT GTC TAC ACA TTT GA
Md102-pET Rv	TAC TAG CTC GAG GTT GTA TGC GTC GGG GTG GC
Md106B-pET Fw	CTA GTA GGA TCC ATG GGT GTC CTC ACA TAC GA
Md106B-pET Rv	TAC TAG AAG CTT GTT GTA GGC ATC AGG ATT GG
Md107-pET Fw	CTA GTA GGA TCC ATG GGT GTT TTC ACA TAC GA
Md107-pET Rv	TAC TAG AAG CTT GTT GTA GGC ATC ATG GTGC T
Md111-pET Fw	CTA GTA GGA TCC ATG GGT GTC ACC AAG ATC AG
Md111-pET Rv	TAC TAG CTC GAG GAC GTA GGC GCG AGG GTG CG
Md113A-pET Fw	CTA GTA GGA TCC ATG GGT GTC TTC ACA TAT GA
Md113A-pET Rv	TAC TAG AAG CTT GTT ATA GGC ATC AGG GTT GG
Md202-pET Fw	CTA GTA GGA TCC ATG ACG ATG ATG AAG AGC CA
Md202-pET Rv	TAC TAG CTC GAG TGG GCA GAA TGT AAT GAC GT
Md401-pET Fw	CTA GTA GGA TCC ATG TCG TGG CAG CAG TAC GT
Md401-pET Rv	TAC TAG AAG CTT GAG ACC CTG CTC GAT AAG GT

3. SEQUENCES Mal d

Mal d pET-clones

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>pET_1.06B
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Other Mal d sequences retrieved from NCBI Database

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Mal d 1.02

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Mal d 1.07

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Mal d 1.13

No sequences on NCBI

Mal d 2.01

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Mal d 4.01

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Golden Delicious Genome

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>MDP0000246775-201-GDgenome

MSMMKSQXASLLGLTLAILFFSGAHA AKITFTNNCPNTVWPGTLTGDQKPQLSLTG FELASKASQSVXAPSPWSGRFGWGRTRCSTDA
AGKFTCETADCGSGQVACNGAGAVPPATLVEITIAANGGQDYDVS LVDGFNLP MSVAPQGGTGECKPSSCPANVNKXCPAXLQVKA
ADGSVISCKSACLAFGDSKYCCTPPNNTPETCPPTEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP

>MDP0000218699-201-GDgenome

MSMMKSQAASLLGLTLAILFFSGAHA AKITFTNNCPNTVWPGTLTGDQKPQLSLTG FELASKASXSVDAPSPWSGRFGWGRTRCSTDA
AGKFTCETADCGSGQVACNGAGAVPPATLVEITIAANGGQDYDVS LVDGFNLP MXVAPQGGTGECKPSSCPANVNXXCPAXLQVKA
ADGSVISCKSACLAFGDSKYCCTPPNXTPETCPPTEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP

>MDP0000299245-4.01-GDgenome

MSWQQYVDDHLMCDIDGNSLTAAAILGQDGSVWAQSATFP AVRPSFFPSC LFRCLSSRFTSFTGSDRFRKFKPEEIAAILKDFDQPG
TLAPTGLFLGGTKYMIQGEPGA VIRGKKGSGGITIKKTSQALLIGIYDEPLTPGQCNIVVERLGDYLIEQGL

>MDP0000264119-4.01-GDgenome

MSWQQYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSASFPAFKPEEIAAILKDFDQPGTLAPTGLFLGGTKYMIQGEPGA VIRGKK
VQMFDFVFLFDIXIDVMRL LHQSGSGGITIKKTSQALLIGIYDEPVTGQCNIVVERLGDYLIEQGL

4. ALIGNMENTS OF THE CLONED MAL D 1 PROTEINS

CLUSTAL 2.1 multiple sequence alignment all 6 mald 1 + bet v1

```
Bet_v_1      MGVFNJETETTSVIPAARLFKAFILDGDNLFPKVAPQAISSEVENIEGNGGPGTIKKISFP
pET_102      MGVYTFENEYTSIIPPRLFKAFVLDADNLIPKIAPQAIAKHAEILEGDGGPGTIKKITFG
pET_103G     MGVFTYESEFTSVIPPARLYNAFVLDADNLIPKIAPQAVKSTEILEGDGGVGTIKKINFG
pET_106B     MGVLTYTEYASIIIPPARLYNALVLDADNLIPKIAPQAVKTVEILEGDGGVGTIKKVSFG
pET_107      MGVFTYESEFTSVVPPARLFNAFVLDADNLIPKIAPQAVKSTEILEGDGGVGTIKKINFG
pET_111A     MGVTKISQKFVTQVTPQRMFNALILDAHNICPKLMFSSIKSIEFLSGSGEVGTIKQINFT
pET_113B     MGVFTYTEFEFASVCAPARLYNALVLDADNLIPKIAPQAVKTAEILEGDGGVGTIKKISFG
*** . . . :. . *:::***. *: :. . * :.* * ****:.*
```

```
Bet_v_1      EGFPFKYVKDRVDEVDHTNFKYNSVIEGGP---IGDTLEKISNEIKIVATPDGGSILKI
pET_102      EGSQYGYVKHKIDSVDENYASYATLIEGDA---LTDTEIKVSYETKLVAS-GSGSIIKS
pET_103G     EGSTYSYVKHRIDGLDKDNFVYKYSVIEGDA---ISETIEKISYETKLVAS-DSGSIIKS
pET_106B     EGSEYNYVKHKVEGIDKDNFVYSYSLIEGDA---ISDKIEKISYEIKLVAS-GSGSIIKN
pET_107      EGSTYSYVKHRIDGVVDKDNFVYQYSVIEGDA---ISETIEKISYETKLVAS-GSGSVIKS
pET_111A     EASPMKYAKHRIDALDKEALSCTYTFIESDATDHLDDKLEYITYDVKFEGYGRGGCICHL
pET_113B     EGSEYSYVKHKVDGIDKDNFVYNSVSLIEGDV---ISDKIEKISYETKLVAS-GSGSVIKS
* . *.*::: :.* *::**.. : :.* :: : * : . *.*: :
```

```
Bet_v_1      SNKYHTKGDHEVKAEQVKASKEMGETLLRAVESYLLAHSDAYN
pET_102      ISHYHTKGDVEIKEEHVKAGKEKAHGLFKLIESYLGHPDAYN
pET_103G     TSHYHTKGDVEIKEEHVKAGKEKASHLFKLIENYLLEHHDAYN
pET_106B     ISHYHTKGDVEIKEEHVKAGKERAHGLFKLIENYLVANPDAYN
pET_107      ISHYHTKGDVEIKEEHVKAGKEKASHLFKLIENYLLEHHDAYN
pET_111A     TSTYKAKDDIQIKEEDIELGKDRAIGMYEVLEAYLMAHPRAYV
pET_113B     TSHYHTKGDVEIKEEHVKAGKERAHGLFKLIENYLVANPDAI-
. *::*.* ::* *::: .* : . :.* ** : *
```

Mal d 1 Percent Identity Matrix - created by Clustal2.1

	103G	107	106B	113B	102	111A
1: pET_103G	100.00	95.60	79.87	82.39	76.73	42.77
2: pET_107	95.60	100.00	79.25	82.39	77.99	42.77
3: pET_106B	79.87	79.25	100.00	88.68	75.47	41.51
4: pET_113B	82.39	82.39	88.68	100.00	74.21	42.14
5: pET_102	76.73	77.99	75.47	74.21	100.00	41.51
6: pET_111A	42.77	42.77	41.51	42.14	41.51	100.00

Alignment Mal d 1.02

CLUSTAL 2.1 multiple sequence alignment

```
pET_1.02                MGVYTFENEYTS EIPPPRLFKAFVLDADNLI PKIAPQA IKHAEILEGDGG
CBL94141.1-1.02-Florina MGVYTFENEYTS EIPPPRLFKAFVLDADNLI PKIAPQA IKHAEILEGDGG
AAX20927.1-1.0201-Golden MGVYTFENEYTS EIPPPRLFKAFVLDADNLI PKIAPQA IKHAEILEGDGG
MDP0000942516-1.02-GDgenome MGVYTFENEYTS EIPPPRLFKAFVLDADNLI PKIAPQA IKHAEILEGDGG
*****

pET_1.02                PGTIKKITFGEGSQYGYVKKHIDSVD EANYSYAYTLIEGDALTD TIEKVS
CBL94141.1-1.02-Florina PGTIKKITFGEGSQYGYVKKHIDSVD EANYSYAYTLIEGDALTD TIEKVS
AAX20927.1-1.0201-Golden PGTIKKITFGEGSQYGYVKKHIDSVD EANYSYAYTLIEGDALTD TIEKVS
MDP0000942516-1.02-GDgenome PGTIXKITFGEGSQYGYVKKHIDSVD EANYSYAYTLIEGDALTD TIEKVS
**** *****

pET_1.02                YETKLVASGSGSI IKSISHYHTKGDVEIKEEHVKAGKEKAHGLFKLIESY
CBL94141.1-1.02-Florina YETKLVASGSGSI IKSISHYHTKGDVEIKEEHVKAGKEKAHGLFKLIESY
AAX20927.1-1.0201-Golden YETKLVASGSGSI IKSISHYHTKGDVEIKEEHVKAGKEKAHGLFKLIESY
MDP0000942516-1.02-GDgenome YETKLVASGSGSI IKSISHYHTKGDVEIKEEHVKAGKEKAHGLFKLIESY
*****

pET_1.02                LKGHPDAYN
CBL94141.1-1.02-Florina LKGHPDAYN
AAX20927.1-1.0201-Golden LKGHPDAYN
MDP0000942516-1.02-GDgenome LKGHPDAYN
*****
```

Mal d 1.02 was cloned from 'Florina' cultivar.

Alignment Mal d 1.03G

CLUSTAL 2.1 multiple sequence alignment

```
pET_1.03G          MGVFITYESEFTSVIPPARLYNAFVLADADNLIPKIAPQAVKSTEILEGDGG
FN823235.1-1.03G-Florina  MGVFITYESEFTSVIPPARLYNAFVLADADNLIPKIAPQAVKSTEILEGDGG
AY789274.1-1.03G01-Fiesta  MGVFITYESEFTSVIPPARLYNAFVLADADNLIPKIAPQAVKSTEILEGDGG
AAX21008.1-1.03G01-Golden  MGVFITYESEFTSVIPPARLYNAFVLADADNLIPKIAPQAVKSTEILEGDGG
MDP0000312569-1.03G-GDgenome  MGVFITYESEFTSVIPPARLYNAFVLADADNLIPKIAPQAVKSTEILEGDGG
*****
```

```
pET_1.03G          VGTIKKINFGEGSTYSYVKHRIDGLDKDNFVYKYSVIEGDAISETIEKIS
FN823235.1-1.03G-Florina  VGTIKKINFGEGSTYSYVKHRIDGLDKDNFVYKYSVIEGDAISETIEKIS
AY789274.1-1.03G01-Fiesta  VGTIKKINFGEGSTYSYVKHRIDGLDKDNFVYKYSVIEGDAISETIEKIS
AAX21008.1-1.03G01-Golden  VGTIKKINFGEGSTYSYVKHRIDGLDKDNFVYKYSVIEGDAISETIEKIS
MDP0000312569-1.03G-GDgenome  VGTIKKINFGEGSTYSYVKHRIDGLDKDNFVYKYSVIEGDAISETIEKIS
*****
```

```
pET_1.03G          YETKLVASDSGSIIKSTSHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
FN823235.1-1.03G-Florina  YETKLVASDSGSIIKSTSHYHTKGDVEIKEEQVKAGKEKASHLFKLIENY
AY789274.1-1.03G01-Fiesta  YETKLVASDNGSIIKSTSHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
AAX21008.1-1.03G01-Golden  YETKLVASDNGSIIKSTSHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
MDP0000312569-1.03G-GDgenome  YETKLVASDXGSIIKSTSXYHTKGDVEIKEEXXKAGKEKASHLXKLIENY
*****  *****  *.:*****  *****  *****
```

```
pET_1.03G          LLEHHDAYN
FN823235.1-1.03G-Florina  LLEHQDAYN
AY789274.1-1.03G01-Fiesta  LLEHQDAYN
AAX21008.1-1.03G01-Golden  LLEHQDAYN
MDP0000312569-1.03G-GDgenome  LLEHQDAYN
****.:****
```

Mal d 1.03G was cloned from ‘Florina’ cultivar. Compared to the published *1.03G* from cultivar ‘Fiesta’ (AY789274.1) and ‘Florina’ (FN823235.1 BAC clone, protein id CBL94173.1), the recombinant gene carried 8 or 9 different nucleotides, for a total 3 amino acid substitutions (in ‘Fiesta’ Asn110 ->Ser, Tyr121->His and Gln155->His; in ‘Florina’ Tyr121->His, Gln132->His and Gln155->His).

Alignment Mal d 1.06B

CLUSTAL 2.1 multiple sequence alignment

```
pET_1.06B          MGVLTYETHEYASIIPPARLYNALVLDADNLIPKIAPQAVKTVEILEGDGG
CBL94151.1-1.06B-Florina  MGVLTYETHEYASIIPPARLYNALVLDADNLIPKIAPQAVKTVEILEGDGG
AAx20971.1-1.06B02-Golden  MGVLTYETHEYASIIPPARLYNALVLDADNLIPKIAPQAVKTVEILEGDGG
AAx18302.1-1.06B-Prima    MGVLTYETHEYASIIPPARLYNALVLDADNLIPKIAPQAVKTVEILEGDGG
AAx20967.1-1.06B01-Golden  MGVLTYETHEYASIIPPARLYNALVLDADNLIPKIAPQAVKTVEILEGDGG
MDP0000216907-1.06B-GDgenome  MGVLTYETHEYASIIPPARLYNALVLXADNLIPKIAPQAVKTVEILEGDGG
*****

pET_1.06B          VGTIKKVSFGESEYNYVKHKVEGIDKDNFVYSYSLIEGDAISDKIEKIS
CBL94151.1-1.06B-Florina  VGTIKKVSFGESEYNYVKHKVEGIDKDNFVYSYSLIEGDAISDKIEKIS
AAx20971.1-1.06B02-Golden  VGTIKKVSFGESEYNYVKHKVEGIDKDNFVYSYSLIEGDAISDKIEKIS
AAx18302.1-1.06B-Prima    VGTIKKVSFGESEYNYVKHKVEGIDKDNFVYSYSLIEGDAISDKIEKIS
AAx20967.1-1.06B01-Golden  VGTIKKVSFGESEYNYVKHKVEGIDKDNFVYSYSLIEGDAISDKIEKIS
MDP0000216907-1.06B-GDgenome  VGTIKKVSFGESEYNYVKHKVEGIDKDNFVYSYSLIEGDAISDKIEKIS
*****

pET_1.06B          YEIKLVASGSGSIIKNISHYHTKGDFEIKEEHVKAGKERAHGLFKLIENY
CBL94151.1-1.06B-Florina  YEIKLVASGSGSIIKNISHYHTKGDFEIKEEHVKAGKERAHGLFKLIENY
AAx20971.1-1.06B02-Golden  YEIKLVASGSGSIIKNISHYHTKGDFEIKEEHVKAGKERAHGLFKLIENY
AAx18302.1-1.06B-Prima    YEIKLVASGSGSIIKNISHYHTKGDFEIKEEHVKAGKERAHGLFKLIENY
AAx20967.1-1.06B01-Golden  YEIKLVASGSGSIIKNISHYHTKGDFEIKEEHVKAGKERAHGLFKLIENY
MDP0000216907-1.06B-GDgenome  YEIKLVASGSGSIIKNISHYHTKGDFEIKEEXHVKAGKERAHGLFKLIENY
*****

pET_1.06B          LVANPDAYN
CBL94151.1-1.06B-Florina  LVANPDAYN
AAx20971.1-1.06B02-Golden  LVANPDAYN
AAx18302.1-1.06B-Prima    LVANPDAYN
AAx20967.1-1.06B01-Golden  LVANPDAYN
MDP0000216907-1.06B-GDgenome  LVANPDAYN
*****
```

Mal d 1.06B was cloned from ‘Florina’ cultivar.

Alignment Mal d 1.07

CLUSTAL 2.1 multiple sequence alignment

```
AAX20992.1-1.0701-Golden      MGVFTYEFEEFTSVIPPARLYNAFVLDADNLIPIAPQAVKSTEILEGDGG
AAX18307.1-1.07-Prima        MGVFTYEFEEFTSVIPPARLYNAFVLDADNLIPIAPQAVKSTEILEGDGG
pET_1.07                      MGVFTYEFEEFTSVVPPARLFNAFVLDADNLIPIAPQAVKSTEILEGDGG
AAX18308.1-1.07-Prima        MGVFTYEFEEFTSVIPPARLYNAFVLDADNLIPIAPQAVKSTEILEGDGG
CBL94174.1-1.07-Florina      MGVFTYEFEEFTSVIPPARLYNAFVLDADNLIPIAPQAVKSTEILEGDGG
MDP0000864747-1.07-GDgenome  MGVFTYEFEEFTSVIPPARLYNAFVLDADNLIPIAPQAVKSTEILEGDGG
*****  *****:*****:*****:*****:*****:*****

AAX20992.1-1.0701-Golden      VGTIKKINFGEGSTYSYVKHKIDGVDKDNFVYQYSVIEGDAISETIEKIS
AAX18307.1-1.07-Prima        VGTIKKINFGEGSTYSYVKHKIDGVDKDNFVYQYSVIEGDAISETIEKIS
pET_1.07                      VGTIKKINFGEGSTYSYVKHRIDGVDKDNFVYQYSVIEGDAISETIEKIS
AAX18308.1-1.07-Prima        VGTIKKINFGEGSTYSYVKHRIDGVDKDNFVYQYSVIEGDAISETIEKIS
CBL94174.1-1.07-Florina      VGTIKKINFGEGSTYSYVKHRIDGVDKDNFVYQYSVIEGDAISETIEKIS
MDP0000864747-1.07-GDgenome  VGTIKKINFGEGSTYSYVKHXIDGVDKDNFVYXYSVIEGDAISETIXKIS
*****  *****:*****:*****:*****:*****:*****

AAX20992.1-1.0701-Golden      YETKLVASGSGSVIKSISHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
AAX18307.1-1.07-Prima        YETKLVASGSGSVIKSISHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
pET_1.07                      YETKLVASGSGSVIKSISHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
AAX18308.1-1.07-Prima        YETKLVASGSGSVIKSISHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
CBL94174.1-1.07-Florina      YETKLVASGSGSVIKSISHYHTKGDVEIKEEHVKAGKEKASHLFKLIENY
MDP0000864747-1.07-GDgenome  YETKLVASGSGSVIKSISHYHTXGDVEIKEEHVKAGKEKASHLFKXIENY
*****  *****:*****:*****:*****:*****:*****

AAX20992.1-1.0701-Golden      LLEHHDAYN
AAX18307.1-1.07-Prima        LLEHHDAYN
pET_1.07                      LLEHHDAYN
AAX18308.1-1.07-Prima        LLEHHDAYN
CBL94174.1-1.07-Florina      LLEHHDAYN
MDP0000864747-1.07-GDgenome  LLEHHDAYN
*****
```

Mal d 1.07 was cloned from 'Florina' cultivar. The *rMal d 1.07* clone had 6 nucleotide differences, causing 3 amino acid substitutions in comparison with the published *Mal d 1.07* genes from 'Golden Delicious' (AY822717), 'Prima' (AY789517) and 'Florina' (FN823235.1 BAC clone, protein id CBL94174.1) (Glu8 ->Ser, Ile14 ->Val14 and Thyr20 ->Phe).

Alignment *Mal d 1.11A*

CLUSTAL 2.1 multiple sequence alignment

```
pET_1.11A                MGVTKISQKFVTQVTPQRMFNALILDAHNICPKLMFSSIKSIEFLSGSGE
MDP0000789174-1.11A-GDgenome  MGVTKISQKFVTQVTPQRMFNALILDAHNICPKLMFSSIKSIEFLSGSGE
CBL94148.1-1.11-Florina      MGVTKISQKFVTQVTPQRMFNALILDAHNICPKLMFSSIKSIEFLSGSGE
                             *****

pET_1.11A                VGTIKQINFTEASPMKYAKHRIDALDKEALSCTYTFIESDATDHLLDKLE
MDP0000789174-1.11A-GDgenome  VGTIKQINFTEASPMKYAKHRIDALDKEALSCTYTFIESDATDHLLDKLE
CBL94148.1-1.11-Florina      VGTIKQINFTEASPMKYAKHRIDALDKEALSCTYTFIESDATDHLLDKLE
                             *****

pET_1.11A                YITYDVKFEGYGRGGCICHLTSTYKAKDDIQIKEEDIELGKDRAIGMYEV
MDP0000789174-1.11A-GDgenome  YITYDVKFEGYGRGGCICHLTSTYKAKDDIQIKEEDIELGKDRAIGMYEV
CBL94148.1-1.11-Florina      YITYDVKFEGYGRGGCICHLTSTYKAKDDIQIKEEDIELGKDRAIGMYEV
                             *****

pET_1.11A                LEAYLMAHPRAYV
MDP0000789174-1.11A-GDgenome  LEAYLMAHPRAYV
CBL94148.1-1.11-Florina      LEAYLMAHPRAYV
                             *****
```

Mal d 1.11A was cloned from ‘Florina’ cultivar.

Alignment Mal d 1.13B

CLUSTAL 2.1 multiple sequence alignment

```
pET_1.13B          MGVFITYETEFASVCAPARLYNALVLDADNLIPKIAPQAVKTAEILEGDGG
MDP0000260110-1.13B-GDgenome  MGVFITYETEFASVCAPARLYNALVLDADNLIPKIAPQAVKTAEILEGDGG
*****

pET_1.13B          VGTIKKISFGEGSEYSYVKHKVDGIDKDNFVYNYSLIEGDVISDKIEKIS
MDP0000260110-1.13B-GDgenome  VGTIKKISFGEGSEYSYVKHKVDGIDKDNFVYNYSLIEGDVISDKIEKIS
*****

pET_1.13B          YETKLVASGSGSVIKSTSHYHTKGDVEIKEEHVKAGKERAHGLFKLIENY
MDP0000260110-1.13B-GDgenome  YETKLVASGSGSVIKSTSHYHTKEDVEIKEEHVKAGKERAHGLFKLIENY
*****

pET_1.13B          LVANPDAI-
MDP0000260110-1.13B-GDgenome  LVANPDAYN
*****
```

Six nucleotides were changed in *rMal d 1.13B*, cloned from ‘Florina’ cultivar, compared with the same sequence in ‘Golden Delicious’ genome (MDP0000260110), but they were all silent mutations with the exception of one base, generating one substitution (Glu124 -> Gly).

Alignment *Mal d* 2.01

CLUSTAL 2.1 multiple sequence alignment

```
pET_2.01      MTMMKSQVASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
Q9FSG7.1-Mald2-Golden  --MMKSQVASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
AAx19846-2.01A-Fiesta  --MMKSQVASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
AAx19845-2.01A-Prima   --MMKSQVASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
AAx19851-2.01B-Prima   --MMKSQAASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
AAx19849-2.01B-Prima   --MMKSQAASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
AAx19850-2.01B-Fiesta  --MMKSQAASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
CAT99612.1-Mald2-Gala  -----
CAT99611.1-Mald2-Florina -----
AAx19847      --MMKSQVASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
AAx19848-2.01B-Fiesta  --MMKSQVASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
MDP0000552328-201-GDgenome MSMMKSQXASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
MDP0000218699-201-GDgenome MSMMKSQAASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
MDP0000246775-201-GDgenome MSMMKSQXASLLGLTLAILFFSGAHAAKITFTNNCPNTVWPGTLTGDQKP
```

```
pET_2.01      QLSLTGFELASKASRSVDAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
Q9FSG7.1-Mald2-Golden  QLSLTGFELASKASRSVDAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
AAx19846-2.01A-Fiesta  QLSLTGFELASKASRSVDAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
AAx19845-2.01A-Prima   QLSLTGFELASKASRSVDAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
AAx19851-2.01B-Prima   QLSLTGFELASKASQSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
AAx19849-2.01B-Prima   QLSLTGFELASKASQSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
AAx19850-2.01B-Fiesta  QLSLTGFELASKASQSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
CAT99612.1-Mald2-Gala  -----ASRSVDAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
CAT99611.1-Mald2-Florina -----ASRSVDAPSPWSGRLWGRTRCSTDAAGKFTCETADCGS
AAx19847      QLSLTGFELASKASQSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
AAx19848-2.01B-Fiesta  QLSLTGFELASKASQSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
MDP0000552328-201-GDgenome QLSLTGFELASKASXSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
MDP0000218699-201-GDgenome QLSLTGFELASKASXSV DAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
MDP0000246775-201-GDgenome QLSLTGFELASKASQSVXAPSPWSGRFWGRTRCSTDAAGKFTCETADCGS
                * * * * * : * * * * *
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```
pET_2.01      GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
Q9FSG7.1-Mald2-Golden  GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19846-2.01A-Fiesta  GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19845-2.01A-Prima   GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19851-2.01B-Prima   GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19849-2.01B-Prima   GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19850-2.01B-Fiesta  GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
CAT99612.1-Mald2-Gala  GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
CAT99611.1-Mald2-Florina GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19847      GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
AAx19848-2.01B-Fiesta  GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
MDP0000552328-201-GDgenome GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPMXVAPQGGT
MDP0000218699-201-GDgenome GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPMXVAPQGGT
MDP0000246775-201-GDgenome GQVACNGAGAVPPATLVEITIAANGGQDYYDVSLVDGFNLPM SVAPQGGT
                * * * * * * * * *
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```
pET_2.01      GECKPSSCPANVNKVCAPLQVKAADGSVISCKSACLAFGDSKYCCTPPN
Q9FSG7.1-Mald2-Golden  GECKPSSCPANVNKVCAPLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19846-2.01A-Fiesta  GECKPSSCPANVNKVCAPLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19845-2.01A-Prima   GECKPSSCPANVNKVCAPLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19851-2.01B-Prima   GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19849-2.01B-Prima   GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19850-2.01B-Fiesta  GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
CAT99612.1-Mald2-Gala  GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
CAT99611.1-Mald2-Florina GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19847      GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
AAx19848-2.01B-Fiesta  GECKPSSCPANVNMACPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
MDP0000552328-201-GDgenome GECKPSSCPANVNXXCPAQLQVKAADGSVISCKSACLAFGDSKYCCTPPN
MDP0000218699-201-GDgenome GECKPSSCPANVNXXCPAXLQVKAADGSVISCKSACLAFGDSKYCCTPPN
MDP0000246775-201-GDgenome GECKPSSCPANVNKXCPAXLQVKAADGSVISCKSACLAFGDSKYCCTPPN
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pET_2.01      NTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
Q9FSG7.1-Mald2-Golden  NTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
AAx19846-2.01A-Fiesta  NTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
AAx19845-2.01A-Prima   NTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
AAx19851-2.01B-Prima   DTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
AAx19849-2.01B-Prima   DTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
AAx19850-2.01B-Fiesta  DTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
CAT99612.1-Mald2-Gala  DTPETCPPTHEYSEIFEKQCP-----
CAT99611.1-Mald2-Florina DTPETCPPTHEYSEIFEKQCP-----
AAx19847      DTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
AAx19848-2.01B-Fiesta  DTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
MDP0000552328-201-GDgenome XTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
MDP0000218699-201-GDgenome XTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
MDP0000246775-201-GDgenome NTPETCPPTHEYSEIFEKQCPQAYSAYDDKNSTFTCSGGPDYVITFCP
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The *rMal d 2.01A* from ‘Jonagold’ did not show any sequence difference with respect to the published *2.01A* gene, with the exception of the 20 nt located at 3’ and 5’ of the gene, corresponding to the *Mal d 2.02* primers employed in the PCR

amplification: at 3' the recombinant clone had six additional nucleotides, coding for methionine and threonine, and at 5' one silent nucleotide substitution. The two additional amino acids at 3' are also present in many EST sequences.

Alignment Mal d 4.01

CLUSTAL 2.1 multiple sequence alignment

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Q84RR5-4.0102-Golden      MSWQAYVDDHLMCDIDGYRLTAAAILGQDGSVWSQSASFPAFK-----
MDP0000299245-4.01-GDgenome MSWQQYVDDHLMCDIDGNSLTAAAILGQDGSVWQSATFFPAVRPSFFPSC
pET_4.01                  MSWQQYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSASFPAFK-----
MDP0000264119-4.01-GDgenome MSWQQYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSASFPAFK-----
AAD29414.1-4.0101-Golden   MSWQAYVDDHLMCDIDGNRLTAAAILGQDGSVWSQSASFPAFK-----
                        ****  *****  *****:***:***.:
                        *****

Q84RR5-4.0102-Golden      -----PEEIAAILKDFDQPGTLAPTGLFLGGTK
MDP0000299245-4.01-GDgenome LFRCLSSRFTSFTGSDRFRKFKPEEIAAILKDFDQPGTLAPTGLFLGGTK
pET_4.01                  -----PEEIAAILKDFDQPGTLAPTGLFLGGTK
MDP0000264119-4.01-GDgenome -----PEEIAAILKDFDQPGTLAPTGLFLGGTK
AAD29414.1-4.0101-Golden   -----PEEIAAILKDFDQPGTLAPTGLFLGGTK
                        *****

Q84RR5-4.0102-Golden      YMVIQGEPEGAVIRGK-----KSGGGITIKKTSQ
MDP0000299245-4.01-GDgenome YMVIQGEPEGAVIRGK-----KSGGGITIKKTSQ
pET_4.01                  YMVIQGEPEGAVIRGK-----KSGGGITIKKTSQ
MDP0000264119-4.01-GDgenome YMVIQGEPEGAVIRGKKVQMFDFVFLFDIXIDVMRLLLHQSGGITIKKTSQ
AAD29414.1-4.0101-Golden   YMVIQGEPEGAVIRGK-----KSGGGITIKKTSQ
                        *****:*****

Q84RR5-4.0102-Golden      ALLIGIYDEPVTGQCNIVVERLGDYLIEQGL
MDP0000299245-4.01-GDgenome ALLIGIYDEPLTPGQCNIVVERLGDYLIEQGL
pET_4.01                  ALLIGIYDEPVTGQCNIVVERLGDYLIEQGL
MDP0000264119-4.01-GDgenome ALLIGIYDEPVTGQCNIVVERLGDYLIEQGL
AAD29414.1-4.0101-Golden   ALLIGIYDEPVTGQCNIVVERLGDYLIEQGL
                        *****:*****
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The recombinant profilin 4.01 from ‘Durello di Forlì’ cDNA was shorter than the sequence predicted on apple genome (MDP0000264119): the first 77 nucleotides of the third exon were absent, generating a protein of 131 amino acids instead of 153 (Supporting Information File S1). The evidence that this profilin was correct was given by allergen database sequences (AF129428, *Mal d 4.0101* ‘Golden Delicious’) and EST sequences, all lacking the above sequence.