Electrophoretic and Immunological Analyses of Almond (*Prunus dulcis* L.) Genotypes and Hybrids

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Aqueous extracts from sixty almond samples representing various genotypes and interspecies hybrids of almond, including almond-peach, were analyzed for protein and peptide content using electrophoresis, Western immunoblotting, and enzyme-linked immunosorbent assay (ELISA). Nondenaturing nondissociating polyacrylamide gel electrophoresis (NDND-PAGE) of the aqueous extracts indicated that a single major storage protein (almond major protein − AMP or amandin) dominated the total soluble protein composition. Denaturing SDS−PAGE analyses of the aqueous extracts revealed that the AMP was mainly composed of two sets of polypeptides with estimated molecular masses in the ranges of 38−41 kDa and 20−22 kDa, regardless of the source; however, distinct variations in the intensity and electrophoretic mobility of some bands were noted between samples. In addition to AMP, several minor polypeptides were also present in all the genotypes, and variations were seen in these as well. Regardless of the genotype, AMP was recognized in Western blots by rabbit polyclonal anti-AMP antibodies, mouse monoclonal anti-AMP antibodies (mAbs), and serum IgE from patients displaying strong serum anti-almond IgE reactivity. As with protein staining results, antibody reactivity also revealed common patterns but displayed some variation between samples. An anti-AMP inhibition ELISA was used to quantify and compare aqueous extracts for various samples. All samples ($n = 60$) reacted in this assay with a mean ± standard deviation (σn) = 0.82 ± 0.18 when compared to reference aqueous extract from Nonpareil designated as 1.0.

Keywords: *Almond; ELISA; AMP; amandin; electrophoresis; Western immunoblotting; allergy*

INTRODUCTION

Food-induced allergies are on the rise in western countries. IgE-mediated type I hypersensitivity to foods affects approximately 2% of the adult population and up to 8% of the pediatric population (*50*). Allergies to milk, egg, wheat, and soy in infants are usually outgrown by age three, but allergies to fish, crustaceans, peanut, and tree nuts are frequently permanent and are sometimes life-threatening (*41, 50*). Although the fatal food allergic reactions that occur in the U.S. are difficult to determine accurately, several estimates suggest the number to exceed 100 fatalities per year (*34, 35*).

Almonds belong to the *Rosaceae* family that also includes apples, pears, prunes, and raspberries (*23*). It has been suggested that almonds are native to the temperate, desert areas of western Asia from where they gradually spread to the warm, dry regions of the Mediterranean. In addition to the cultivated almond, *Prunus dulcis*, more than thirty wild or minor cultivated almond species are known to exist. Domesticated almonds have been documented from Bronze Age sites in Greece and Cyprus and were common in Palestine by 1700 BC (*31*). Some botanists believe that cultivated almond is a hybrid of prehistoric origin tracing its roots to several wild almond species still found in warmer parts of Asia. Several of these related almond species continue to be harvested and marketed as almond in Asia and southern Europe. Wild species related to almond freely cross-hybridize with cultivated almond and have been implicated in hybridizations leading to current almond cultivars including *Prunus fenzliana* Fritsch; *P. bucharica* (Korsh.) Fedtsch.; *P. kuramica* (Korsh.) Kitam; *P. triloba* Lindl.; and *P. ulmifolia* (*4, 7, 19, 31*). It is believed that the first plantings of edible almonds in North America were introduced by the Spanish missions between San Diego and Santa Barbara, California. These attempts were not successful because of unfavorable climatic conditions. Not until the 1850s were almonds successfully planted and grown in north-central California. Today, most of the U.S. almonds are grown in California in an area that stretches over 400 miles from Bakersfield to Red Bluff (*31*). The major almond varieties do not self-pollinate and there-

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Table 1. Genotypes Examined, Their Origins, and Expected Genomic Constitution.

no.	genotype	origin[a]		percentage of genome derived from cultivated almond (*P. dulcis*)
1	SB1,16−75	Nonpareil x P. fenzliana		50
2	SB1,16−76	Nonpareil x P. fenzliana		50
3	F5,10−2	(Almond x P. fenzliana) BC1 x Sonora		88
4	F5,4−4	P. webbii x (Nonpareil x P. persica) BC1		38
5	F5,4−10	P. webbii x (Nonpareil x P. persica) BC1		38
6	F5,6−22	(Mission x P.fenzliana) BC1 x Sonora		88
7	F5,6−13	(Mission x P. fenzliana) BC1 x Sonora		88
8	F5,6−1	(Mission x P. fenzliana) BC1 x Sonora		88
9	F5,13−54	(Mission x P. fenzliana) BC1 x Sonora		88
10	F5,10−9	(Mission x P. fenzliana) BC1 x Sonora		88
11	F5,20−38	Padre x F5,4−10	(see no. 5)	69
12	F5,20−42	Padre x F5,4−10		69
13	F5,20−44	Padre x F5,4−10		69
14	F5,20−52	Padre x F5,4−10		69
15	F8n,6−68	F5,4−10 X Solano		69
16	F5,19−82	Padre x F5,4−10	(bitter seed)	69
17	F8N,7−4	F5,4−10 x Sonora		69
18	F8N,7−16	F5,4−10 x Sonora		69
19	F8,7−13	F5,4−10 x Sonora		69
20	F8N,7−11	F5,4−10 x Sonora		69
21	Padre	Almond variety		100
22	Thompson	Almond variety		100
23	Trusito	Spanish almond variety		100
24	Sonora	Almond variety		100
25	Mission	Almond variety		100
26	Nonpareil	Almond variety		100
27	Carmel	Almond variety		100
28	Fritz	Almond variety		100
29	Ruby	Almond variety		100
30	Ne Plus Ultra	Almond variety		100
31	Butte	Almond variety		100
32	Price	Almond variety		100
33	Peerless	Almond variety		100
34	Livingston	Almond variety		100
35	LeGrand	Almond variety		100
36	SB13,45−8	(Nonpareil x P. persica) BC3		94
37	SB13,28−21	(Nonpareil x P. persica) BC3		94
38	SB13,54−39E	(Nonpareil x P. persica) BC2		94
39	8010−22	Nonpareil x F5,4−10		69
40	SB3,56−89	[(Nonpareil x P. persica) BC1] F1		75
41	F10C,12−28	(Nonpareil x P. Persica) F2		50
42	F10C,9−22	(cv Jordanolo x P. persica) F2		50
43	F10C,15−1	(cv Jordanolo x P. persica) F2	{bitter seed}	50
44	F10C,25−10	(Nonpareil x P. persica) F2	{bitter seed}	50
45	F10C,20−51	(Nonpareil x P. persica) F2	{bitter seed}	50
46	F8S,51−12	P. persica x Carmel	{bitter seed}	50
47	F8S,51−36	P. persica x Butte	{bitter seed}	50
48	SB16,14−16	Almond x P. persica	{bitter seed}	50
49	SB16,14−13	Almond x P. persica	{bitter seed}	50
50	LG-OP1	(Nonpareil x P. persica) BC3	{bitter seed}	94
51	40A-17	Peach (P. persica)	{bitter seed}	0
52	Andross	Peach (P. persica) variety	{bitter seed}	0
53	Nemared	P. persica rootstock	{bitter seed}	0
54	F5,22−5	(Nonpareil x P. persica) F2		50
55	SB13,25−75	Nonpareil x F5,4−10		69
56	F5,14−75	(Mission x P. argentia) x Sonora		75
57	F5,16−60	(Mission x P. argentia) F2		50
58	F5,8−33	(Mission x P. argentia) x Sonora		75
59	F5,8−29	(Mission x P. argentia) x Sonora		75
60	F5,8−31	(Mission x P. argentia) x Sonora		75

[a] BC1, 2, and 3 indicate backcrosses to almond for 1, 2, and 3 consecutive generations, respectively. F2 and F3 indicate selfing for 1 and 2 consecutive generations, respectively. Almond refers to cultivated Californian almond.

fore it is a common practice to plant a few other almond varieties in otherwise monotypic orchards.

On a global basis almonds rank number one in tree nut production, and the U.S. is the largest almond producer. On an average, over the past 10 years (1990−1999), the U.S. almond production has accounted for approximately 33% of global almond production. In 1999, the U.S. almond production was 360,000 metric tons, of which 210,000 metric tons ($500 million value) were exported (*3*). In California, the Nonpareil variety thrives and accounts for about half of the total domestic almond production. Carmel, Mission, Neplus, and Peerless are the other major commercial varieties which, together with Nonpareil, account for over 90% of total almond production in the U.S.

As almond consumption continues to increase, the potential for unintended exposure of sensitive (almond-allergic) individuals to almonds and almond products also increases. A recent survey suggests that 0.5% of the U.S. population has some degree of hypersensitivity to tree nuts (*24*). All of the commonly used tree nuts, including almond, walnut, pecan, and cashew, are

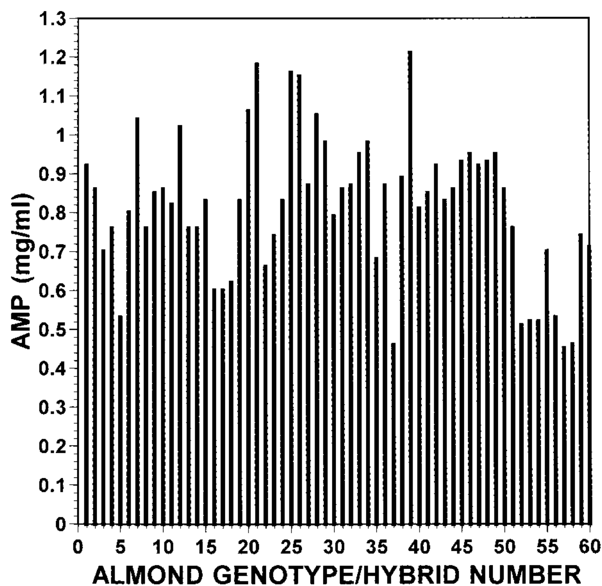

Figure 1. Inhibition ELISA for almond genotypes and hybrids using rabbit polyclonal antibodies. Genotypes and hybrid numbers are the same as those in Table 1. Data are expressed in relation to a reference aqueous extract from Nonpareil (different from genotype 26 listed in Table 1) designated as 1.0.

associated with food-induced allergies (*5, 8, 13, 40*). The clinical reactions to ingested allergens can include hives; nausea; vomiting; facial, tongue, or throat swelling; laryngeal edema; asthma; and hypotension. In severe cases, anaphylaxis can result in death (*41, 50*). Currently, there is no treatment for IgE-mediated food allergies; and therefore, complete avoidance of the offending food is recommended. Avoidance of the offending food is, however, not always possible because the implicated food allergen may be present in trace amounts in a processed food because of contamination via shared equipment or mislabeling (*35, 40*).

To minimize unintended exposure to almonds, it is important to have a sensitive, reproducible, specific, and inexpensive assay to detect the presence of almond in foods and food products. We have recently shown that rabbit polyclonal antibodies raised against the major storage protein in almonds (AMP) can be used in an enzyme-linked immunosorbent assay (ELISA) to detect the presence of almond in a sensitive manner (*2, 33*). Using these antibodies in an inhibition ELISA assay, we could detect AMP at levels as low as 300 ng/mL. The assay could quantitatively detect AMP in each of the major marketing varieties (Carmel, Mission, Neplus, Nonpareil, and Peerless) tested. In this current report we tested the global applicability of this assay by testing aqueous extracts from sixty almond genotypes and inter-species hybrids with our ELISA assay. In addition, we investigated the peptide composition (by gel electrophoresis) and antigenic profile (by Western blot) of the AMP in each sample.

MATERIALS AND METHODS

Almond seeds were obtained from the experimental almond germplasm collection at University of California, Davis, and are listed in Table 1. Sources of chemicals used for ELISAs and electrophoreses have been reported earlier (*2*). All other chemicals were typically of reagent or better grade.

Preparation of Seed Flour. All seed samples were manually powdered using a mortar and pestle, while taking care to ensure no cross-contamination between samples. The powdered samples were defatted using a Soxhlet apparatus (Fisher Scientific Co., Orlando, FL). Ten volumes (w/v) of petroleum ether (boiling point range 38.2−54.3 °C) were used for defatting seed flours. All samples were defatted for 6 h. The defatted flours were then air-dried in a fume hood, homogenized using a Sorvall Omni-Mixer (Sorvall Inc., Newton, CT) with speed setting at 8, and stored in screw-capped plastic vials at −20 °C until further use.

Protein Solubilization and Determination. As the majority of almond proteins are soluble in aqueous media (*37*), 20 mM Tris-HCl pH 8.1 buffer was used. Typically, defatted

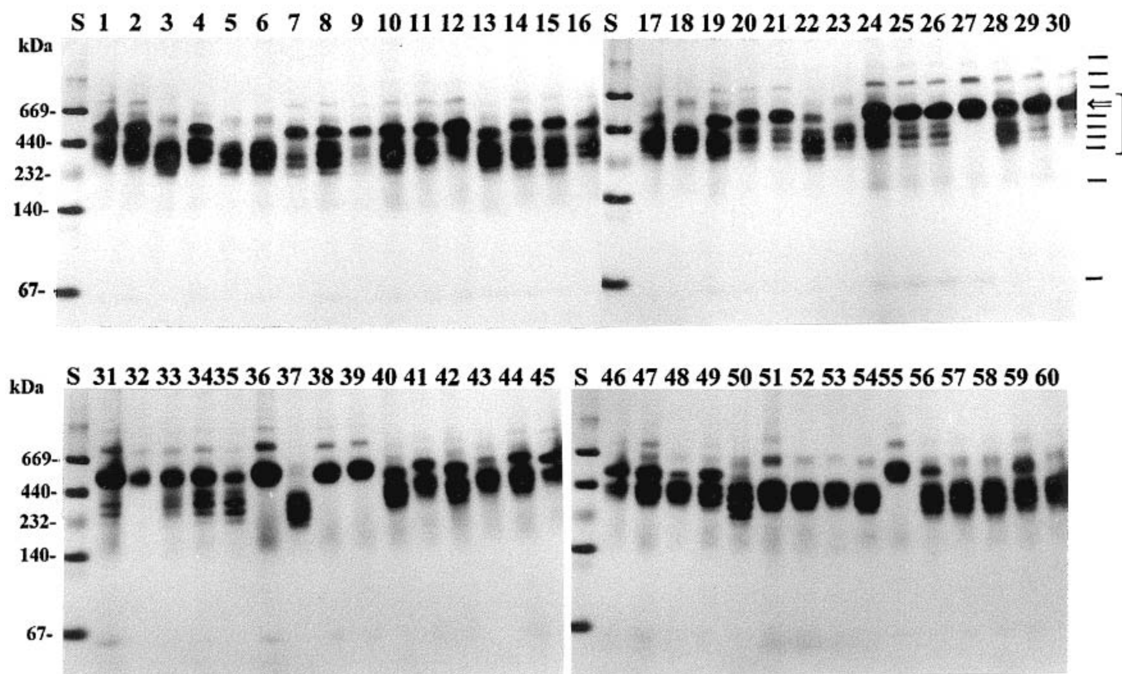

Figure 2. NDND-PAGE of almond genotypes and hybrids. The figure is a composite of four 3−30% monomer acrylamide linear gradient gels with 3% monomer acrylamide stacking gel. S = molecular mass standards thyroglobulin (669 kDa), ferritin (440 kDa, catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa). Protein load in each lane was 20 μg. The numbers on top of the lanes are the same as those used in Table 1.

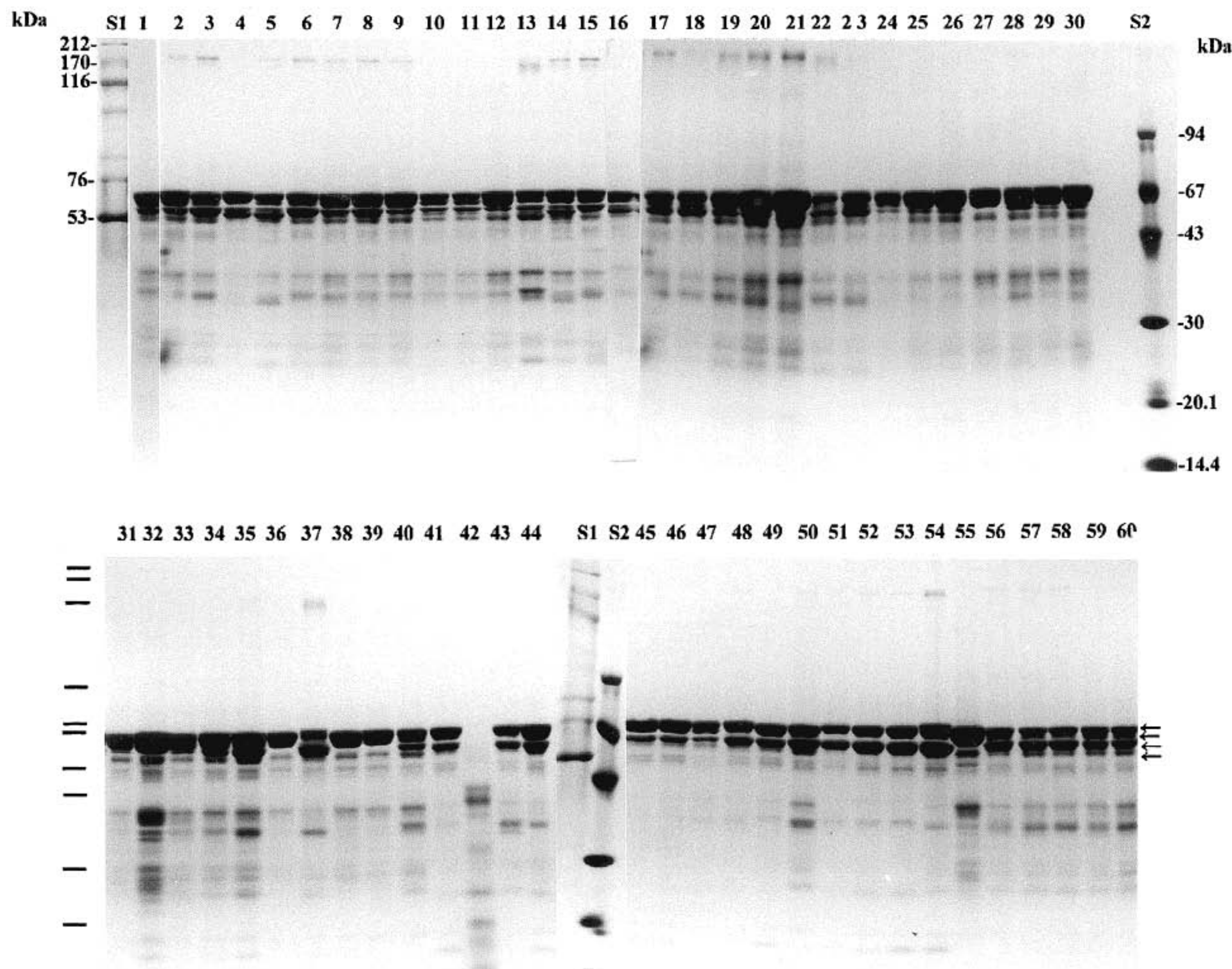

Figure 3. SDS–PAGE in absence of β-ME for almond genotypes and hybrids. The figure is a composite of four 8–25% linear monomer acrylamide concentration with 4% monomer acrylamide stacking gel. S_1 = high-molecular-mass standards myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa); S_2 = low-molecular-mass standards phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Protein load in each lane was 20 μg.

flour was extracted with the buffer (flour-to-buffer ratio 1:10 w/v) at room temperature (25 °C) for 1 h with vortexing at 10 min intervals. Following extraction, samples were centrifuged in a tabletop microcentrifuge for 10 min (13,600*g*) and the supernatants were used for analyses. Soluble protein in the supernatant was determined by the method of Lowry et al. (*20*). Bovine serum albumin (BSA) standard curve in 20 mM Tris-HCl buffer (pH 8.1) was simultaneously prepared for each assay. Extracts were aliquoted and stored at −20 °C.

Preparation of Antibodies. Rabbit polyclonal antibody production has been described earlier (*2*). Mouse monoclonal anti-AMP antibodies (mAbs) were raised using standard techniques (*22*) in the core Hybridoma Facility at Florida State University (FSU). Briefly, pairs of mice were each immunized with 40 μg of AMP in RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT), boosted with 20 μg of AMP in RIBI adjuvant at three week intervals, and given a final injection of 25 μg of AMP in saline equally split between the intravenous and subcutaneous routes. Following fusion, the resultant hybridomas were screened and assayed for relative strength and specificity by direct-binding ELISA (*16*). Human blood serum from patients showing strong IgE reactivity with almonds was collected and analyzed by the procedures described in Teuber et al. (*44*).

ELISA. Competitive ELISAs were performed as previously described (*2*) with some modifications. A 96-well polyvinyl microtiter ELISA plate was coated with 50 μL/well of the indicated AMP solution in coating buffer (48.5% of 0.1 M citric acid and 51.5% of 0.2 M Na_2HPO_4, pH 5.0), and incubated for one h at 37 °C. Wells were washed, blocked with 0.1% bovine serum albumin in 0.05% Tween-20 and 1 mM EDTA in PBS (pH 7.2) for 0.5 h at room temperature and rewashed. A dilution of rabbit anti-AMP antibodies in 0.1% BSA-BSB (buffered saline borate, 0.1 M H_3BO_3, 0.025 M $Na_2B_40_7$, and 0.075 M NaCl, pH 8.2) previously determined to give 50% of maximum binding in a direct-binding assay (typically 1:10,000) was added to each well of a second uncoated plate. Soluble inhibitor was serially diluted into the antiserum and incubated for 1 h at 37° C, whereupon the contents of the plate were transferred to the AMP-coated plate and incubated for an additional 1.0 h at 37 °C. The plates were washed and developed using alkaline phosphatase-labeled goat anti-rabbit IgG (secondary antibody) for 1 h at 37 °C, and phosphatase substrate (50 μL of *p*-nitrophenyl phosphate, 1 mg/mL). Reactions were stopped with the additions of 3 M NaOH (15 μL). Plates were read at 405 nm using an ELISA reader (Power Wave 200, Bio-Tek Instruments Inc. Riverton, NJ). All experiments were performed in duplicate and averages are reported.

Electrophoresis. Nondenaturing nondissociating (NDND) polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE in the absence and presence of a reducing agent (2% v/v) β-mercaptoethanol (β-ME) were performed as described earlier (*37*).

Immunoblotting. Proteins from electrophoresis gels were transferred on to 0.2-μm nitrocellulose (NC) paper according to the method described in Towbin et al. (*46*). For initial screening, 3- to 4-mm wide strips, containing 25 μg of protein per 4-mm strip, were blocked for 1 h at room temperature in

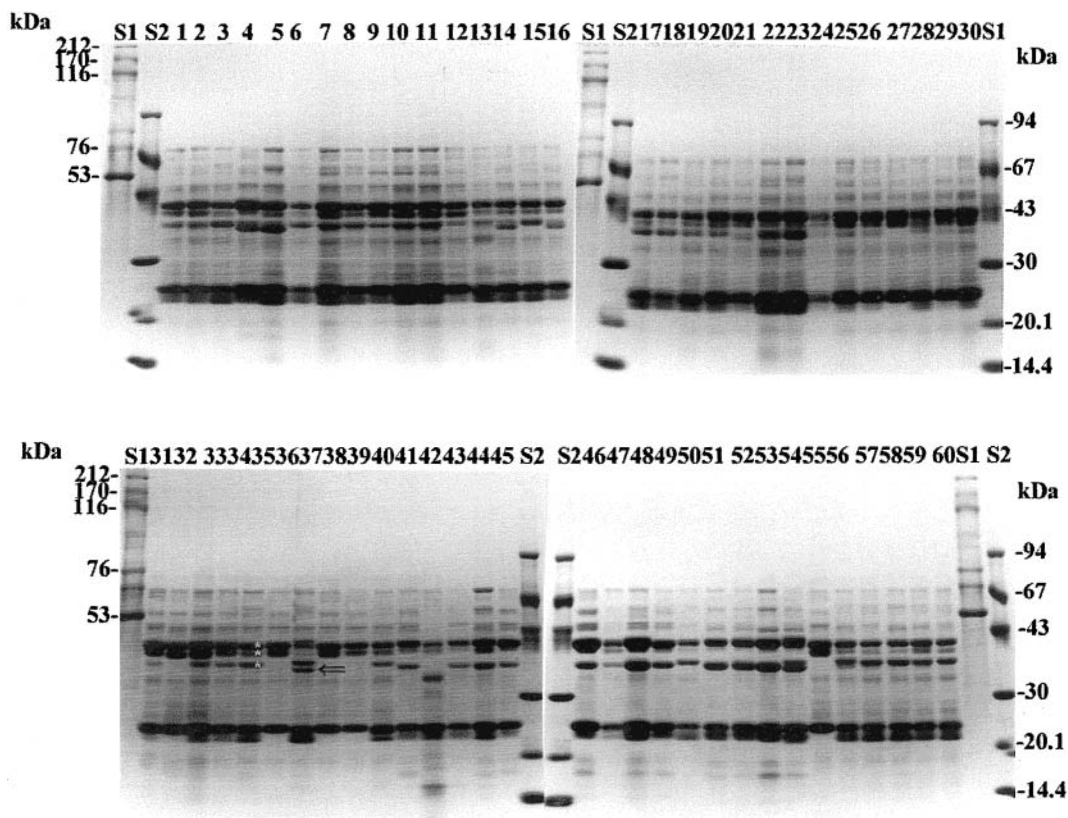

Figure 4. SDS−PAGE in the presence of β-ME for almond genotypes and hybrids. Details are the same as those in Figure 3.

phosphate buffered saline (PBS)/3% nonfat dry milk/0.2% Trition X-100 (TX-100). Pooled sera from five patients was diluted 1:5 in the same buffer and incubated overnight with the strips at room temperature. Additional strips were incubated with PBS/3% nonfat milk/0.2% TX-100 (to check for nonspecific binding of the anti-human IgE polyclonal antibodies) or atopic control sera (1:5 dilution). The strips were then washed for 20 min, 3×, in PBS/0.01% TX-100, and incubated overnight at room temperature with equine polyclonal ^{125}I-anti-human IgE (Sanofi Diagnostics Pasteur, Chaska, MN) diluted 1:5 in the nonfat milk buffer. The strips were washed with PBS/0.01% TX-100 for 20 min, 3×, and exposed to X-ray film at −70 °C for 48−72 h with an intensifying screen.

For comparisons between human IgE reactivity and rabbit polyclonal antibody reactivity or mouse monoclonal antibody (mAb) reactivity, an enhanced chemiluminescence (ECL) system was utilized as follows. Unbound sites on the NC paper strips were blocked using Tris-buffered saline {10 mM Tris, 0.9% (w/v) NaCl, 0.05% (v/v) Tween-20} (TBS-T) containing 0.1% (w/v) BSA for one h at room temperature with gentle rocking. The NC strips were then incubated with rabbit, or patient antisera (a slightly different pool), or mAb in TBS-T at 1:16,000, 1:5, and 1:5,000 dilution (v/v), respectively, at room temperature for one, two, and two h, respectively, with rocking. NC sheets were rinsed twice with TBS-T and washed, with rocking for 15 min. This wash process was repeated 3×. The blots were then incubated at 25 °C for 1 h with peroxidase-labeled goat anti-rabbit or goat anti-human antibody diluted in TBS-T. NC strips were washed 3× with TBS and then reacted with ECL reagents. Western immunoblotting using rabbit and human serum and mouse mAbs was carried out on nitrocellulose using chemiluminescence (ECL-Plus, Amersham, Piscataway, NJ) as described by the manufacturer and exposed to X-ray film.

RESULTS AND DISCUSSION

Almond Genotypes. The almond germplasm examined in this report includes the major Californian cultivars and almond genotypes resulting from controlled crosses to the Central Asian germplasm thought to have contributed to the origin of *P. dulcis*, the cultivated sweet almond (*4, 7, 19*). This wild germplasm, which includes *P. fenzliana* from Turkey, *P. webii* from Romania, and *P. argentia* from Iran, is still harvested and utilized in its native countries. Peach (*P. persica*) is also included because peach and almond−peach hybrids are commonly used for almond rootstocks, and because this germplasm is also sometimes used to supply the bitter almond market (for marzipan and other products). This germplasm collection represents the extensive genetic variability available to almond improvement programs, and includes promising genetic sources for improved kernel oil quality, more productive tree architectures, flower self-compatibility, and improved disease and pest resistance (*1, 12, 18*). The proportion of *P. dulcis* genes in genotypes derived from interspecies crosses was estimated based on the assumption of unencumbered introgression of wild genes, as no evidence of hybrid sterility or other breeding barriers was observed in these crosses (*17*). Earlier findings of changes in kernel chemical composition within this germplasm, which affects both processing and storage quality (*1*), and susceptibility to fungal diseases (*12*), suggested the potential danger of inadvertently introducing higher levels of food allergens in new cultivars. Conversely, a careful screening of allergen levels in the source stock might suggest potential hypoallergenic crosses. However, results from this study indicate a high uniformity for the allergen profile trait suggesting the genetic control is conserved at relatively low levels within the extended germplasm evaluated.

ELISA. We assayed the almond genotypes and hybrids to determine if there was a significant difference in the level of AMP detected by the rabbit antisera in our quantitative ELISA. Any significant differences

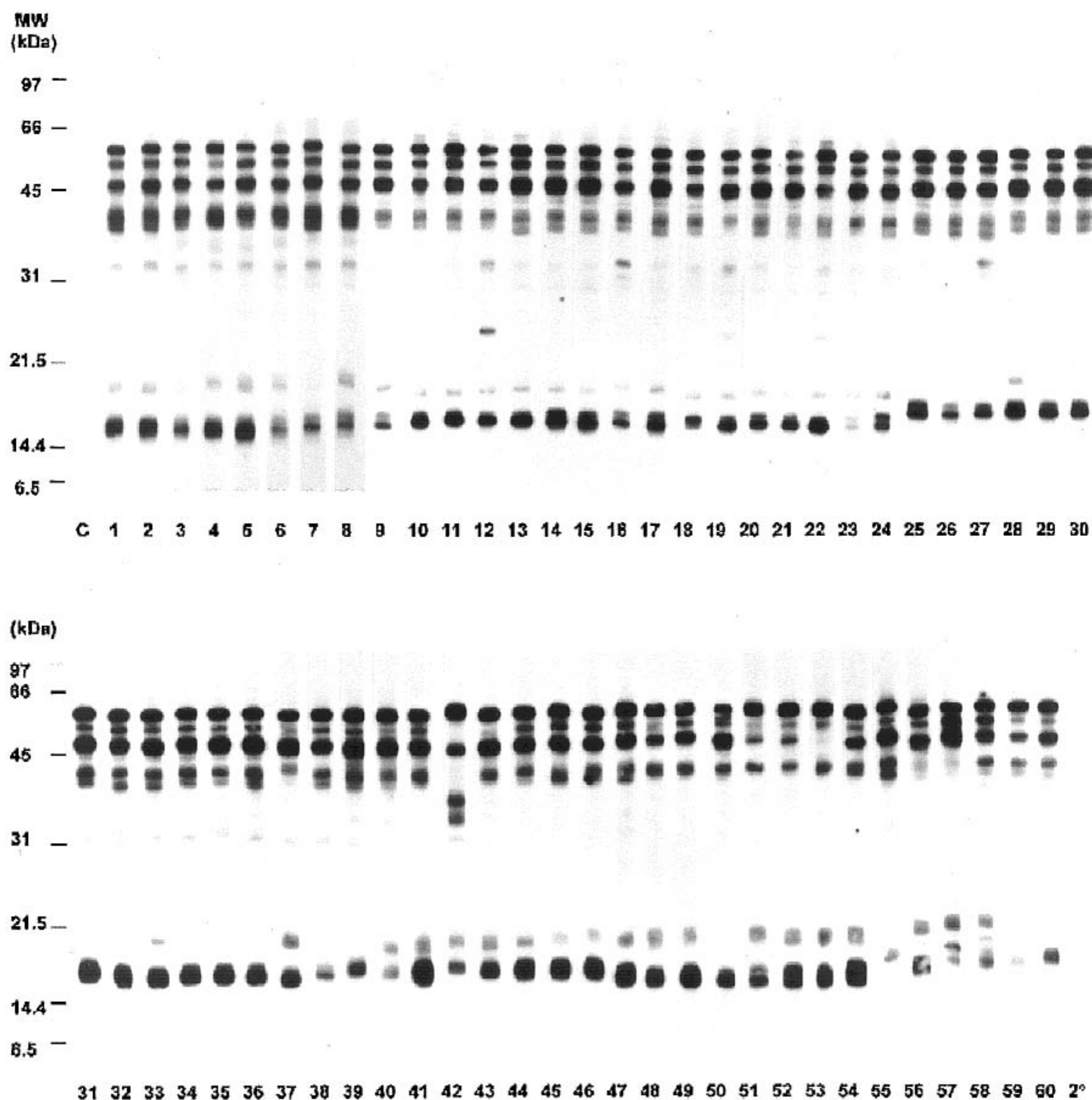

Figure 5. Western immunoblotting for almond genotypes and hybrids using human IgE. Thirteen percent monomer acrylamide gels (SDS−PAGE in the presence of β-ME) were used. The numbers below the lanes are the same as those used in Table 1. C, control atopic serum, not allergic to almonds; 2°, secondary antibody alone.

could adversely affect the reliability of an assay based on the quantification of AMP in foods because the source of almond used is rarely indicated. Our results show that the ELISA could readily detect AMP in all genotypes tested (Figure 1). When normalized to 1.0 mg/mL protein content, the mean $\pm$ standard deviation for activities of sixty samples tested was 0.82 ± 0.18. The difference between the samples with the greatest and least AMP signal was less than 3-fold. These data suggest that our ELISA procedure can be used to quantitatively detect presence of AMP regardless of the almond genotype.

Electrophoresis. A review of literature indicates that there are few studies in which the biochemical composition of almond cultivars has been investigated. Rikhter and Pyzhov (*29*) analyzed 12 almond cultivars grown under uniform soil and climatic conditions in the piedmontane zone of the Crimea for the amino acid composition of whole seeds as well as different protein fractions. These investigators reported the molecular mass of albumin (2S) and globulin (11S or 12S) fractions to be 150,000 and 300,000, respectively. The globulin content varied significantly (globulin N range, 2.13 to 4.29% of the dry seed mass; that is 13.21 to 26.81% globulin [N $\times$ 6.25]) depending on the cultivar. Seron et al. (*38*) analyzed 19 almond cultivars and found that Spanish cultivars could be distinguished from other cultivars (American, Italian, Australian, Caucasian, and French) based on their free amino acid composition analyzed using multivariate analysis (ANOVA, principal component, cluster, and linear discriminant function). Soler et al. (*42*) quantitatively analyzed the almond cultivar Pons grown on the Spanish island of Mallorca for carbohydrate and protein content during seed development and maturation and found that the seed free amino acid content was significantly affected by the development stage. In none of these studies was the protein polypeptide composition investigated.

Traditional almond culture has mainly used open-pollinated seedlings. This fact, taken together with self-incompatibility, has resulted in a fruit/nut that is considered one of the most polymorphic species (*4*). Recent studies (*47*) comparing almond S-RNAse sequences with that of Solanaceae and Scrophulaiaceae

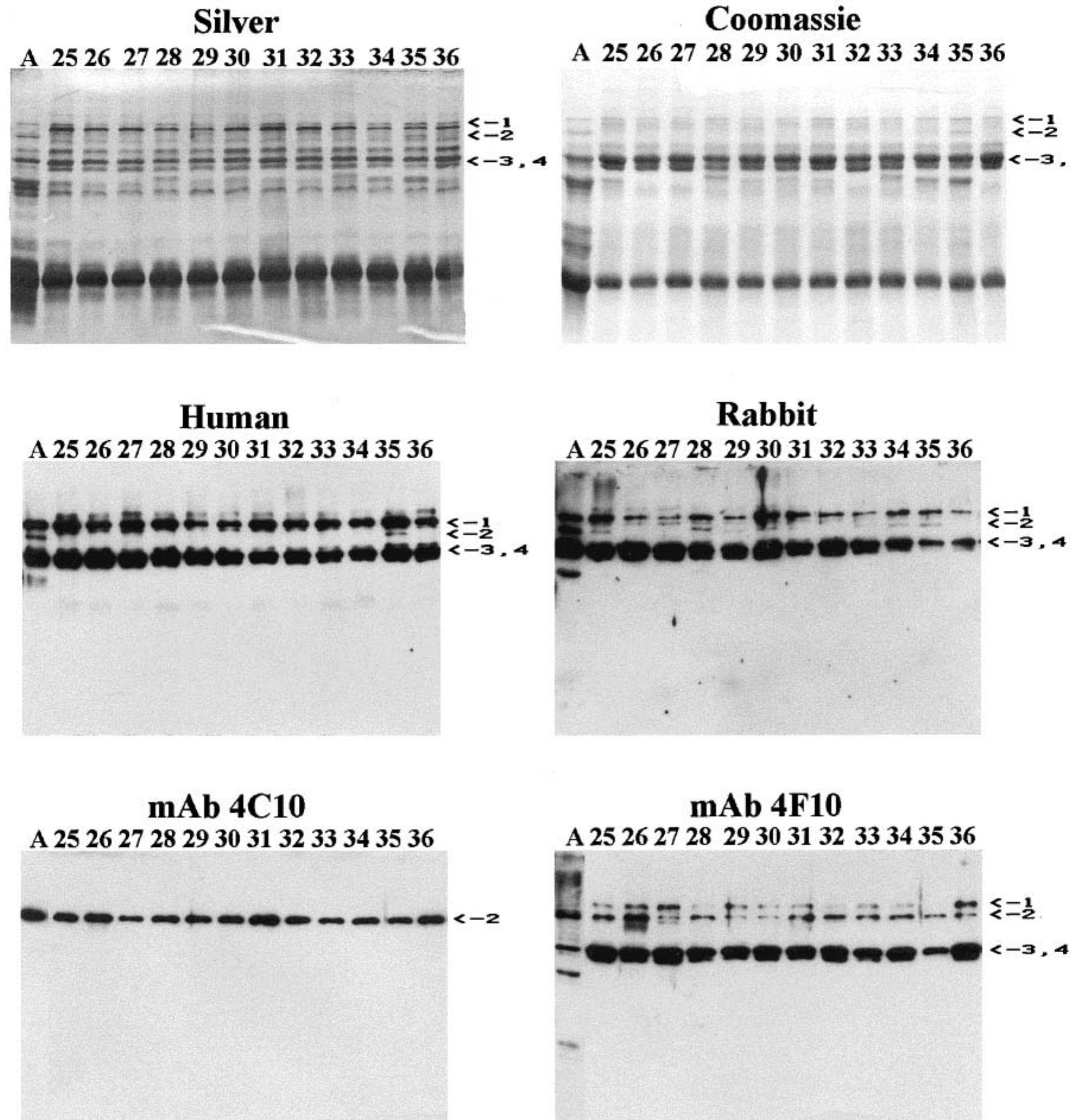

Figure 6. Comparative evaluation of representative almond genotypes and hybrids. Silver, Coomassie Blue, and Western blots using various antibodies. The numbers on top of the lanes are the same as those used in Table 1.

also suggest genetic diversity in almond. On the other hand, Sathe (*37*) has shown that several almond marketing varieties have similar protein and polypeptide compositions. The main source of protein variability was found to be charge heterogeneity. It was therefore important to learn whether the almond cultivars used in the current study had significant differences in protein and polypeptide composition, reactivity with the rabbit or mouse antibodies which could be used in the quantitative assays, and whether such differences, if detected, were recognized by human anti-almond IgE.

NDND-PAGE (Figure 2) of almond genotypes had a complex pattern typically characterized by a total of ≤ 10 proteins (see right-hand side margin of the upper panel for positions of these proteins). It should be noted here that the migration in NDND-PAGE is influenced by both innate electrical charge on protein as well as molecular mass. The predominant proteins in all genotypes migrated in a limited region (area between 232 kDa and 669 kDa) and most of the variability was explained by differences in only five proteins indicated by the bracket (]). More than half (37 out of 60) of the genotypes displayed a pattern in which a single protein (indicated by an arrow) dominated the total protein profile, an observation consistent with earlier results on major marketing varieties of almonds (*37*). Protein migrating slightly faster than bovine serum albumin protein (67 kDa) was faintly visible in most genotypes. In some genotypes, we observed additional weakly staining proteins of lower electrophoretic mobility in the gel region above the 669 kDa marker. Perhaps higher protein loads may reveal their presence in other genotypes as well.

SDS–PAGE gels in the absence of a reducing agent revealed that most genotypes were composed of two sets of major polypeptides (Figure 3), each set consisting of at least two polypeptides (see the arrows on the right side of lower panel). In certain genotypes, additional prominent polypeptides were also present in this same size range. Several other polypeptides, in varying amounts (subjectively judged on the basis of bandwidth and staining intensity), were apparent in the range of

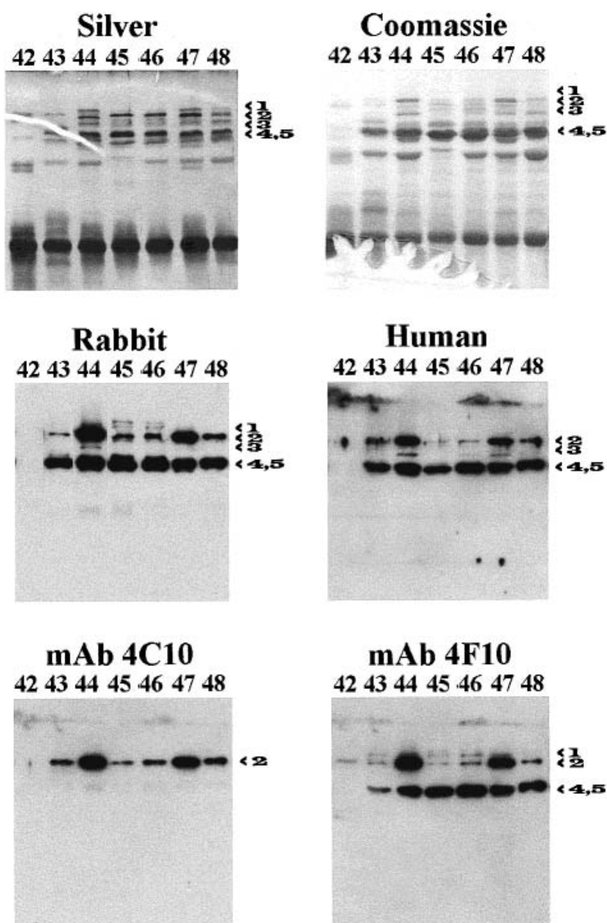

Figure 7. Comparative evaluation of representative almond genotypes and hybrids. Silver, Coomassie Blue, and Western blots using various antibodies. The numbers on top of the lanes are the same as those used in Table 1

20 to 50 kDa in most genotypes. Among these latter peptides, two prominent ones that migrate between the 43 kDa and 30 kDa markers, show considerable variation (for example, compare lanes 21, 22, 27, 37, 55, and 60).

SDS−PAGE in the presence of the reducing agent β-ME, revealed a complex pattern of polypeptide subunits (Figure 4) that was consistent with earlier analysis of marketing almond cultivars important for the U.S. almond growers (*37*). All genotypes had essentially similar polypeptide patterns with regards to the two major polypeptide sets linked via disulfide bond(s) that constitute the AMP. These two polypeptide sets have estimated molecular masses in the ranges of 38−41 kDa and 20−22 kDa. The relative proportion of individual polypeptides in each set varied considerably with respect to polypeptide quantity. For example, there are three major polypeptides in the 38−41 kDa range in genotype #35 (indicated by *), whereas #36 has only two, and #37 has four. Of these four peptides in #37, the uppermost has a slightly higher molecular mass when compared with that in genotype #36. Genotypes #24, 31, 32, 36, 38, 39, and 55 lacked the lower-molecular-mass polypeptide (indicated by ⇐) in the doublet (range 38−41 kDa), a peptide which predominates in several other genotypes. Genotype #54 has an overall banding pattern that is the same as that of the cultivar #37 and distinct from that of the other genotypes.

Plant seed storage proteins are believed to have originated from a few common ancestral genes (*6*). Molecular heterogeneity of storage protein polypeptides within plant species is not uncommon and has been observed in soybean proteins (*9, 10, 26, 36, 39, 49*), sesame 11S globulin (*43*), and several other plant seed storage proteins (*11, 14, 15, 21, 25, 27, 28, 30, 45*). Although precise structural differences underlying such polypeptide micro-heterogeneity are not fully established, glycosylation and proteolytic processing (co- and/or posttranslational) appear to be the main sources of variability (*6, 25, 26*). On the basis of results of electrophoretic analyses of the almond genotypes, AMP (or its charge variants) appears to be a major storage protein in all almond genotypes tested.

Immunoblotting. Earlier studies have shown AMP to be a major storage protein (*48*), as well as a major allergen (*32*), in the major almond cultivars. Results from current investigations indicate that AMP predominates in most, if not all almond types. Because the charge and size heterogeneity observed in the various electrophoretic analyses likely reflects, at least in part, amino acid sequence heterogeneity, we also investigated whether there were significant differences in immunogenecity between the almond varieties. Such differences, if detected, would have profound implications with respect to the utility of our anti-AMP assay and could suggest almond usage or breeding strategies that could lead to the production or development of less allergenic almonds.

Western blotting experiments using human anti-almond antiserum and ^{125}I-labeled secondary antibody clearly show that several of the AMP polypeptides are recognized by the IgE (Figure 5). Subtle variation in polypeptide recognition in different genotypes was noted and these generally correlated with differences in the staining intensity of the corresponding bands in the reducing SDS−PAGE gels. For example, when genotypes 8 and 9 were compared, both had four major bands recognized by IgE in the approximate molecular mass range 40−60 kDa. However, the intensity of the fastest moving band (in this cluster of four bands) in genotype 9 was less than that of the corresponding band in genotype 8. Genotype 42 had two bands (above 31 kDa but below 45 kDa) that distinguished it from the rest of the genotypes. Genotypes 55 and 59 had low levels of low-molecular-mass polypeptides (in the range of 18−22 kDa) when compared with the rest of the genotypes.

To further clarify the correlation between rabbit anti-AMP antisera and the human IgE, we performed a six-way comparison of the 60 samples using similarly run silver-stained and Coomassie-stained gels as well as Western blots probed with our rabbit anti-AMP antisera, pooled human IgE-containing sera from allergic patients, and two mouse mAbs against AMP. Representative data from the six-way comparison experiments are shown in Figures 6 and 7. The Western blot probed with human antisera differed from those shown in Figure 5 in that a different pool of allergic antisera was used and the blots were developed using chemiluminescent reagents rather than radiolabeled second antibody as in Figure 5. The overall pattern, as seen in Figure 6, for many of the samples was remarkably similar to those in Figure 5. Of the many peptides detected with the silver- and Coomassie-staining, only a few were detected by the various antibodies. Four major bands were indicated by the rabbit reagent and each is also detected by the human IgE. This correlation is important in helping to strengthen the usefulness of

the rabbit reagent to detect and quantify the almond proteins that are actually recognized by IgE from humans.

The bands in Figure 6 labeled 2 and the 3, 4 doublet are the most pronounced in both the rabbit and human blots, though some other minor bands are also recognized. Interestingly, mAb 4F10 also recognizes four of the five major bands recognized by human IgE, suggesting that either there are several cross-reactive epitopes on the various peptides or that the same epitope(s) is(are) shared between peptides. In the latter case, the recognized peptides probably represent variants of the same peptide resulting from differential mRNA splicing, posttranslational modification, or both; or are derived from several members of a multigene family. Interestingly, mAb 4C10 recognizes only band 2 and therefore probably binds a different epitope than the one recognized by mAb 4F10 on the band 2 peptide. It would be of interest to determine whether the various antibodies are recognizing any epitopes in common (i.e., immunodominant epitopes). Although there are some differences between the different samples (25−36) in Figure 6, they are not nearly as pronounced as seen between other samples. For example, as seen in both the stained gels and in the four probed gels in Figure 7, band 2 is very strong in samples 44 and 47. Sample 42 is rather anomalous in that the higher mass bands are weakly stained and of different electrophoretic mobilities compared to the other samples and the only band detected on Western blots for sample 42 was a weak signal for band 2 by mAb 4F10. However, the human serum pool used in Figure 5 did recognize proteins from sample 42. These variations are likely due to the influence of the nonalmond gene products introduced by crossing with peach and other genetic material used for the development of different almond cultivars.

CONCLUSIONS

Our results demonstrate that AMP or amandin is a major component of almonds irrespective of their genotype and even when out-crossed with nonalmond species (i.e., peach), and that AMP, the major storage protein in almonds, is recognized by human IgE, rabbit polyclonal antibodies, and mouse mAbs in all genotypes tested. These data, when taken together with the fact that AMP is a major allergen in almond, makes AMP an excellent allergy-relevant target molecule for the purpose of detecting the presence of almonds and almond contamination in food products. Additionally, this survey reveals that human IgE bound similar proteins in all protein extracts of almond species and cultivars; i.e., no potentially hypoallergenic germplasm sources were discovered.

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