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Peach (*Prunus persica* L. Batsch) Allergen-Encoding Genes Are Developmentally Regulated and Affected by Fruit Load and Light Radiation

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The fruits of Rosaceae species may frequently induce allergic reactions in both adults and children, especially in the Mediterranean area. In peach, true allergens and cross-reactive proteins may cause hypersensitive reactions involving a wide diversity of symptoms. Three known classes of allergenic proteins, namely, Pru p 1, Pru p 3, and Pru p 4, have been reported to be mostly involved, but an exhaustive survey of the proteins determining the overall allergenic potential, their biological functions, and the factors affecting the expression of the related genes is still missing. In the present study, the expression profiles of some selected genes encoding peach allergen isoforms were studied during fruit growth and development and upon different fruit load and light radiation regimens. The results indicate that the majority of allergen-encoding genes are expressed at their maximum during the ripening stage, therefore representing a potential risk for peach consumers. Nevertheless, enhancing the light radiation and decreasing the fruit load achieved a reduction of the transcription rate of most genes and a possible decrease of the overall allergenic potential at harvest. According to these data, new growing practices could be set up to obtain hypoallergenic peach fruits and eventually combined with the cultivation of hypoallergenic genotypes to obtain a significant reduction of the allergenic potential.

KEYWORDS: ***Prunus persica*; peach allergenic potential; gene expression; Pru p 1; Pru p 2; 3D modeling; Pru p 3; Pru p 4; fruit load; light radiation**

INTRODUCTION

In the Mediterranean area, fruits from species belonging to the Rosaceae family (i.e., peach, apple, plum, cherry, apricot, etc.) are often responsible for food-induced allergies (*1*). Such hypersensitive responses may be caused both by specific reactions toward allergenic proteins and by the cross-reactivity existing within and among several classes of allergens, present in the Rosaceae family and in other taxonomically unrelated species (*2*). Peach is the fresh fruit most frequently involved in allergic reactions both in adults and in patients older than 3 years of age, causing a wide variety of symptoms, such as urticaria and oral allergy syndrome (OAS), but also anaphylaxis even in the absence of OAS (*3*). Therefore, the severe symptoms induce allergic patients to avoid the consumption of both fresh and transformed (i.e., juices, jams, yogurt, etc.) peaches, because the major allergens are often stable and highly resistant to heat treatment and pepsin digestion (*4, 5*), and reject their well-known nutraceutical properties.

Known peach allergens belong to three main classes, namely, Pru p 1, Pru p 3, and Pru p 4, with different geographical patterns of prevalence. Allergic reactions caused by Pru p 1, similarly to apple Mal d 1, belonging to the class II allergy, mainly affect the northern and central European populations and are often associated with birch pollinosis due to a cross-reactivity with Bet v 1 (*6*). Both are 17−18 kDa allergens belonging to group 10 of the pathogenesis-related proteins (PR-10), for which many different biological functions have been proposed (*7*−*9*). Pru p 1 allergenic protein was shown to differentially accumulate in diverse nectarine varieties (*10*), but no information about the expression of the related gene is so far available.

Pru p 3 is a nonspecific lipid transfer protein (nsLTPs) causing class I food allergy, mainly in the Mediterranean area (*11, 12*). Plant nsLTPs form large multigene families encoding 9 kDa proteins, with eight conserved cysteines forming four disulfide bonds, and belong to the PR-14 family. Many different biological functions have been suggested for plant nsLTPs (*11, 13*−*19*), also supported by the presence of several isoforms showing moderate levels of amino acid sequence identity and

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differential gene expression patterns, as previously reported in peach by Botton et al. (*20*). Nevertheless, changes in Pru p 3 protein accumulation in fruits of different peach cultivars, and a differential expression of two LTP genes, namely, *Pp-LTP1* (encoding Pru p 3) and *Pp-LTP2*, in reproductive organs of peach were previously demonstrated (*10, 20−22*). LTPs are considered to be panallergens, because they belong to a family of structurally highly conserved proteins that are also present in non-Rosaceae vegetable foods (*12*).

As far as Pru p 4 is concerned, a strong involvement in pollen−fruit cross-reactions may be postulated for this class of peach allergens belonging to the profilin family. Profilins from many different species were recently reported to cause allergic reactions, with a strong cross-reactivity to birch pollen profilin Bet v 2 and grass pollen, the latter being more common in the Mediterranean area (*23, 24*). Profilins are small actin-binding cytosolic proteins of 12−15 kDa, present in all eukaryotic cells and involved in diverse steps of plant growth and development (*25−27*). Plant profilins are usually encoded by small gene families. In *Arabidopsis* five profilin-encoding genes with distinct but overlapping expression patterns have been characterized (*28*). Indeed, individual members of the profilin gene family play essential and redundant roles during plant development (*29*). In peach, two cDNAs encoding profilin have been isolated and named *Pru p 4.01* and *Pru p 4.02* (*23*).

The total allergenic potential of ripe peaches may be determined by the presence of both major and minor allergens and, given that the final amount of allergenic proteins was often shown to be positively correlated with the transcription rate of the respective genes, at least in apple (*30*), it is crucial to determine the factors affecting their expression. In this view, the present study was focused on the identification and characterization of expression profiles of genes encoding allergens (Pru p 1, Pru p 2, Pru p 3, and Pru p 4) during peach fruit growth and development. Nevertheless, because fruit load and light radiation may significantly affect the final quality of peaches, the effects of such factors on the allergenic potential were assessed and possible implications on peach tree cultivation critically discussed.

MATERIALS AND METHODS

Sequence Analysis and 3D Modeling. CLC Sequence Viewer v. 5.0 (CLC Bio, Aarhus, Denmark) was used for multiple alignments of deduced amino acid sequences by using the built-in algorithm with default parameters, for obtaining the neighbor-joining phylogenetic tree and its bootstrap analysis, and for molecular mass and isoelectric point (p*I*) calculations. In silico predictions of signal peptide and allergenicity were performed with SignalP 3.0 (*31, 32*) (www.cbs.dtu.dk/services/SignalP/) and EVALLER (*33*) (bioinformatics.bmc.uu.se/evaller/), respectively. The software Sequence Analysis v. 1.6.0 (Informagen Inc., Greenland, NH) was used for pairwise alignment and identity calculation.

Three-dimensional structures were obtained by means of homology modeling, and PDB files were retrieved with Phyre Web server v. 0.2, developed from 3D-PSSM (*34*). The 3D models were drawn and rendered by means of MacPymol for Mac OS X (DeLano Scientific LLC, Palo Alto, CA).

Plant Material. For the gene expression studies throughout fruit growth and development, fruits were sampled in two subsequent seasons, 2005 and 2006, from trees of cv. Fantasia at S1 (first exponential growth phase), S2 (endocarp lignification), S3 (second exponential growth phase). and S4 (climacteric stage), as defined by Tonutti et al. (*35*). However, because the results collected in 2005 were confirmed in 2006, at least in terms of general trends, only the former are reported in the present study. At each developmental stage, 50 fruits were harvested from five different homogeneous trees to prevent effects due to the lower fruit load caused by the sampling. Mesocarp and epicarp were excised separately, immediately frozen in liquid nitrogen, and stored at −80 °C.

Fruit load and light radiation trials were carried out in two consecutive seasons, 2006 and 2007, on nectarines of cv. Stark Red Gold, starting when the fruit cheek-to-cheek diameter was 15−18 mm. Because the results collected in 2006 were confirmed in 2007, at least with regard to general trends, only the former are herein reported. Three levels of fruit load expressed as fruit number per square centimeter of trunk sectional area were imposed as equal to 1.76, 0.88, and 0.53 for high, medium, and low loads, respectively. The different loads were distributed in 30 trees on turf random blocks (Ctrl) and on blocks in which the ground was covered with reflecting mulches (Light; Extenday, www.extenday.com), each block consisting of 15 trees. The reflecting mulches achieved a 7-fold enhancement of the ground-reflected light, an increase of 4 °C in the intracanopy average maximum temperature, and no significant changes of relative humidity. Fifty fruits were sampled separately for each trial at commercial ripeness, from five homogeneous trees, assessed as indicated by Botton et al. (*21*). Mesocarp and epicarp were excised from sampled fruits, immediately frozen in liquid nitrogen, and stored at −80 °C.

RNA Extraction and cDNA Synthesis. Total RNA was extracted following the method of Ruperti et al. (*36*), starting from 6 g of mesocarp and 1 g of epicarp tissue. Minor adaptations to the protocol were brought about by adding 50 (mesocarp) and 300 μL (epicarp) of a calcium hydroxide suspension just before the first centrifugation step to enhance the precipitation of contaminating pectins. cDNA was synthesized from 2 μg of DNA-free total RNA in a final volume of 25 μL containing 200 units of MMLV Reverse Transcriptase (Promega, Madison, WI), 1× MMLV buffer, 25 units of RNasin (RNase inhibitor, Amersham Biosciences, Piscataway, NJ), 1 μg of Random Hexamers (Invitrogen, Carlsbad, CA), and 2 mM dNTPs. The reaction was carried out for 1 h at 37 °C in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA).

Real-Time PCR. Real-time PCR relative quantification was performed in a total volume of 10 μL using the Power SYBR Green PCR Master Mix (Applied Biosystems) with 3 pmol of every primer and 2 μL of a 1:10 dilution of cDNA. The gene-specific primers (**Table 1**) were designed with Primer3plus software v. 0.4.0 (*37*) (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) according to the instructions reported in the SYBR Green PCR Master Mix protocol (Applied Biosystems). The specificity of amplification was assessed by following subcloning and sequencing of the PCR products obtained under the same conditions adopted in the real-time experiments. The reaction mix was amplified in a 7500 Real-Time PCR System (Applied Biosystems) as described by Botton et al. (*38*). After every PCR cycle, a data acquisition step was introduced to record the fluorescent signals at the optimum temperature, previously determined by melting point analysis of every specific amplification product (**Table 1**). Data were acquired and elaborated as previously described (*38*). Gene expression values were normalized to the internal transcriber spacer (*PPITS*, AF179562, **Table 1**) and reported as arbitrary units (AU) of the mean normalized expression, using eq 2 of Q-Gene. The correct size of the amplification products was checked by

Table 1. Sequences of Gene-Specific Primers Used To Quantify Allergen-Related Transcripts in Peach Fruit Tissues[a]

gene	acc no.	forward	reverse	size (bp)	T_m (°C)
Pru p 1.01	DQ251187	5′-CTGTGATGCTTGGGTCTTGC-3′	5′-GCCCAACAACCCTATGGCTA-3′	106	76.0
Pru p 1.02	AM290651	5′-GGCAAAGAGAAGGCAACTGG-3′	5′-TGGGTTGGCCACAAGGTAGT-3′	59	75.0
Pru p 2.01	AF362988	5′-TCATCACATTCTGCCCATAAGC-3′	5′-TGACATCAACAATGGGTCAAGA-3′	119	68.5
Pru p 2.02	AF362987	5′-GCACTCCGCCTCAAGAAACT-3′	5′-GGTCCACCACTGCACGTAAA-3′	132	76.5
Pru p 3.01	AY620230	5′-CCACCGTGAAGTGAGCTTGA-3′	5′-TCGTGAGGAATCCCTAAGTGG-3′	106	74.0
Pru p 4.01	AJ491881	5′-CAGTACGTCGATGACCACT-3′	5′-AAATCTTTCAGAATGGCAGCTATC-3′	148	83.5
Pru p 4.02	AJ491882	5′-CAGTTGAAGCCTGAAGAAGTGA-3′	5′-ATGCCAATCAGCAAAGCAA-3′	193	79.0
PPITS	AF179562	5′-TGAATTGCAGAATCCCGTGA-3′	5′-TGACCTGGGGTCGCGTTGAA-3′	313	78.5

[a] For each gene, the accession number of its nucleotide sequence, the couple of primers used, the size of the PCR product, and its melting temperature are reported. The oligos used to amplify the reference gene (*PPITS*) are also listed.

running each reaction in a 1.5% agarose gel stained with ethidium bromide and viewed under UV light. All PCR experiments were carried out in triplicate and using RNA from two independent extractions.

Statistical Analysis. Statistical analyses were performed using the CoStat v. 6.311 software package (CoHort Software, Monterey, CA). A three-way completely randomized ANOVA was performed both for each factor (fruit load, light enhancement, and tissue) and by calculating the interactions between the three factors ($P \leq 0.05$). Means were compared with LSD tests at the 0.05 significance level.

RESULTS

Bioinformatic Characterization of New Candidate Allergens. Several cDNAs encoding yet unknown candidate allergens were identified among the publicly available peach ESTs (the procedure is detailed in the Supporting Information). The most interesting sequences encode a new Bet v 1-like isoform (*Pru p 1.02*) and two thaumatin-like isoforms (*Pru p 2.01* and *2.02*) and were named according to the guidelines of the International Union of Immunological Societies (*39, 40*) (IUIS, www.allergen.org). Although the immunological properties of these proteins have not been tested in vivo, an allergenicity index was calculated by means of a bioinformatic approach (*33*), and similarity as well as phylogenetic analyses were carried out along with homology modeling on Pru p 1.02, Pru p 2.01, and Pru p 2.02 deduced proteins to support their structural similarity with known allergens (**Figures 1−3**). The main calculated biochemical properties (molecular mass and p*I*), the presence of a signal peptide, and the allergenicity scores are reported in tables (see also the Supporting Information for an extended version), for both known and candidate peach allergens.

Multiple alignments of known Bet v 1-like amino acid sequences belonging to Rosaceae species pointed out a close similarity of Pru p 1.01 and Pru p 1.02 with Pru av 1.01 and Pru av 1.02 isoforms, respectively (**Figure 1A**). Nevertheless, the neighbor-joining phylogenetic tree (**Figure 3A**) showed a separate clustering of the two proteins, Pru p 1.01 being grouped with the respective isoforms of cherry, apricot, pear, and apple and Pru p 1.02 with the closely related cherry Pru av 1.02 and apple Mal d 1. According to the in silico predictions, Pru p 1.02 showed an allergenic score as high as 24.478, which is very close to that of Pru p 1.01 (24.616), and a predicted three-dimensional structure almost identical to that of Pru p 1.01 (**Figure 1B**,**C**).

Concerning the two candidate allergenic proteins belonging to the thaumatin-like protein (TLP) family, an allergenicity index equal to 6.285 was found for Pru p 2.01, whereas that of Pru p 2.02 was as low as 4.722 (**Table 2**). According to the multiple alignments with known TLP allergens (**Figure 2A**), Pru p 2.01 and 2.02 showed only 4 and 5 unique amino acids of 32, respectively, in correspondence with the dominant epitope as previously mapped in other species (*41, 42*). As far as the phylogenetic analysis, Pru p 2.01 clustered close to almond Pru du 2.01, whereas Pru p 2.02 was more similar to the Pru av 2 allergen of cherry (**Figure 3B**). Moreover, with regard to the 3D structures, a close similarity with apple Mal d 2 was observed (**Figure 2B**), pointing toward comparable allergenic properties.

Gene Expression during Fruit Growth and Development. Gene expression profiles during peach fruit growth and development were studied only for genes encoding isoforms belonging to the four main allergen classes except for *Pp-LTP2* (herein named *Pru p 3.02*), which was shown to be expressed at almost undetectable levels in the ripe fruit tissues (*20*) (data not shown).

The two genes encoding Pru p 1 isoforms showed slightly different expression patterns during fruit growth and development (**Figure 4A**). *Pru p 1.01* expression in the mesocarp followed the fruit growth pattern, increasing its transcript accumulation from S1 to S2 phase, decreasing to the initial level in S3, and finally rising at its maximum expression in S4, paralleling the second exponential fruit growth and the onset of the ripening syndrome. In the epicarp, a gradual increasing trend was observed from S1 to S4, reaching a maximum in the last stage as equal as in the mesocarp. *Pru p 1.02* expression in the mesocarp gradually decreased by 1 log throughout the experiment from S1 to S4, whereas its transcripts in the epicarp remained at almost constant levels from S1 to S3, slightly peaking at S4. The levels of expression of the two Pru p 1-encoding genes were quite similar at S4 in the epicarp, but in the mesocarp *Pru p 1.01* was more expressed.

With regard to the two newly identified TLP-encoding genes, *Pru p 2.01* and *Pru p 2.02*, the patterns of expression were completely different (**Figure 4B**). As far as the former is concerned, its transcripts remained quite constant in the epicarp from S1 to S3 and peaked at S4 following a 3 log increase during fruit ripening. In the mesocarp the gene was expressed almost constantly throughout the experiment with a maximum in S4, differing by 3 logs with respect to its lower expression level in the epicarp. *Pru p 2.02* transcripts accumulated in the mesocarp at detectable levels in only S1 and S4, whereas in S2 and S3 the detected amplification products were most likely aspecific due to the absence of specific targets. The same pattern was observed in the epicarp, although the transcripts were still detectable in S2 and S3. The maximum expression level of *Pru p 2.02* was reached in S4, increasing by 5 logs from the S3 phase. Pru p 2-encoding genes were mostly expressed in the fruit skin, with a 3 log difference in both cases with respect to the pulp, whereas *Pru p 2.01* was in general the most expressed.

As far as the two profilin-encoding genes are concerned, quite constant patterns were observed, although for *Pru p 4.01* the

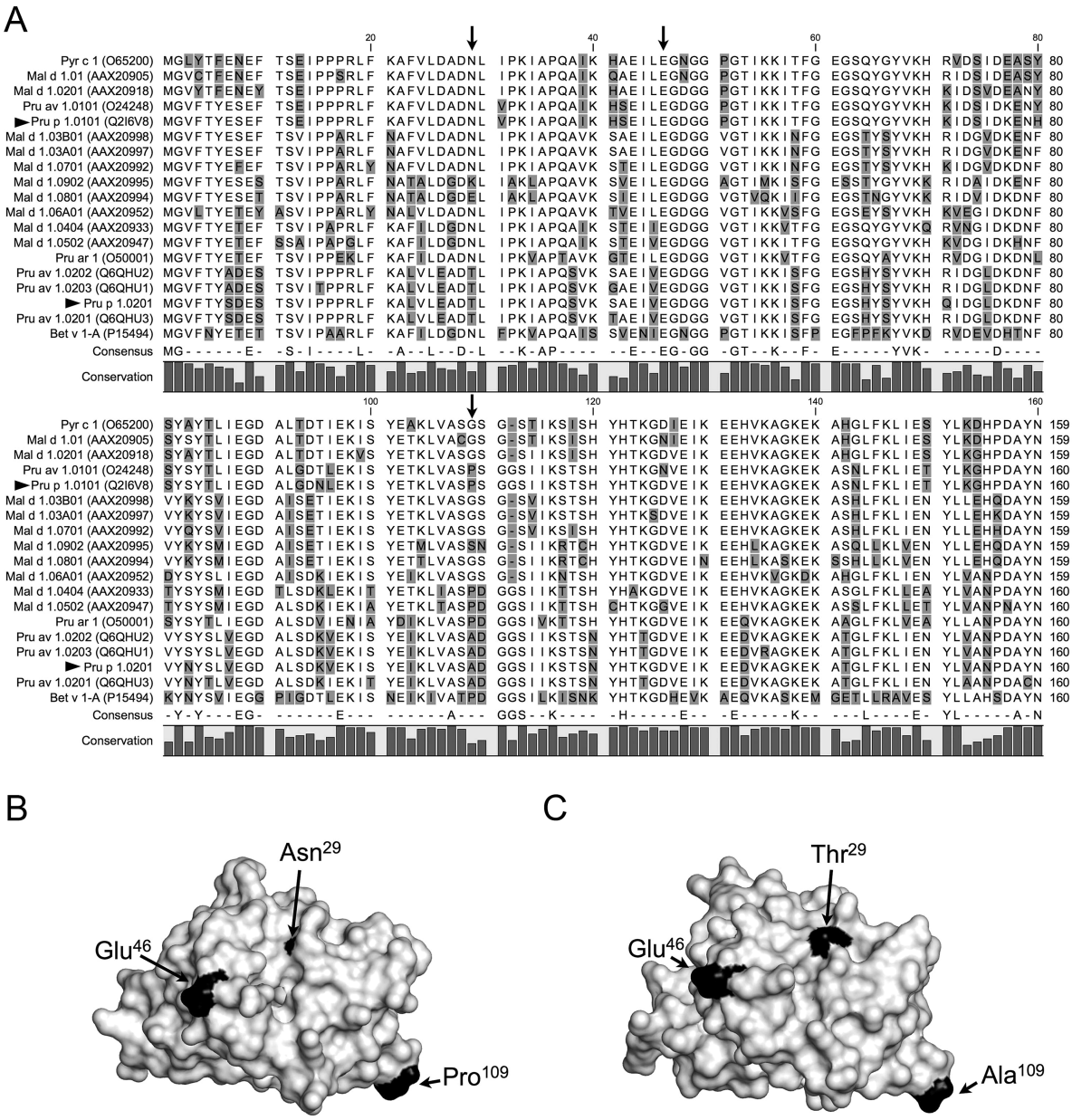

Figure 1. (**A**) Multiple alignments of birch Bet v 1 and Rosaceae Bet v 1-like amino acid sequences as included in the official list of allergens (www.allergen.org). Both the known (Pru p 1.01) and the newly identified (Pru p 1.02) peach Bet v 1-like allergens are included (indicated with arrowheads). The bar chart below the alignment indicates the amino acid conservation. Nonconserved amino acids are shaded, and the consensus sequence displays the 100% conserved residues. The black arrows above the alignment and the box indicate the IgE epitope. Accession numbers are reported when available. (**B**) Three-dimensional model of Pru p 1.01 allergen (PMDB id: PM0075482). (**C**) Three-dimensional model of Pru p 1.02 allergen (PMDB id: PM0075483). The putative epitopes are black. The corresponding amino acid residues are also indicated.

overall expression levels were about 6 logs higher than those of *Pru p 4.02* (**Figure 4C**). With regard to the former, the transcripts accumulated at almost equal values and with overlapping trends in both mesocarp and epicarp. However, the range of expression was always restricted to 1 log. For *Pru p 4.02* the same range of variation was observed but at lower relative values. In the epicarp the gene expression slightly decreased throughout the experiment, reaching a minimum in the last developmental stage, whereas in the mesocarp a pattern similar to *Pru p 4.01* was observed.

For the first time the expression pattern of the *Pru p 3.01* gene was studied by means of real-time PCR (**Figure 4D**), largely confirming the results obtained by means of northern hybridization (*20*). However, in the present study, changes in expression of the LTP-encoding gene were pointed out also in the mesocarp, in which *Pp-LTP1* (i.e., *Pru p 3.01*) transcripts were previously detected only during the S1 phase (*20*). A pattern paralleling fruit growth was pointed out in the pulp for *Pru p 3.01*. With regard to the expression in the epicarp, a constant increasing trend was detected throughout the whole fruit growth and development, reaching the maximum level in S3 and S4. The difference of expression existing between mesocarp and epicarp was quantified as high as 4 logs.

Effect of Fruit Load and Enhanced Light Radiation. Results are summarized in **Figure 5** and **Table 3**. Fruit load and light radiation had no significant effect on the expression of the *Pru p 1.01* gene, as pointed out by statistics in **Table 3**. However, a decreasing trend in the transcript accumulation was observed in both mesocarp and epicarp in control fruits concurrent with the increase in fruit load. The light enhancement emphasized the trend in the epicarp, whereas the opposite effect was observed in the mesocarp (**Figure 5A**). The transcript

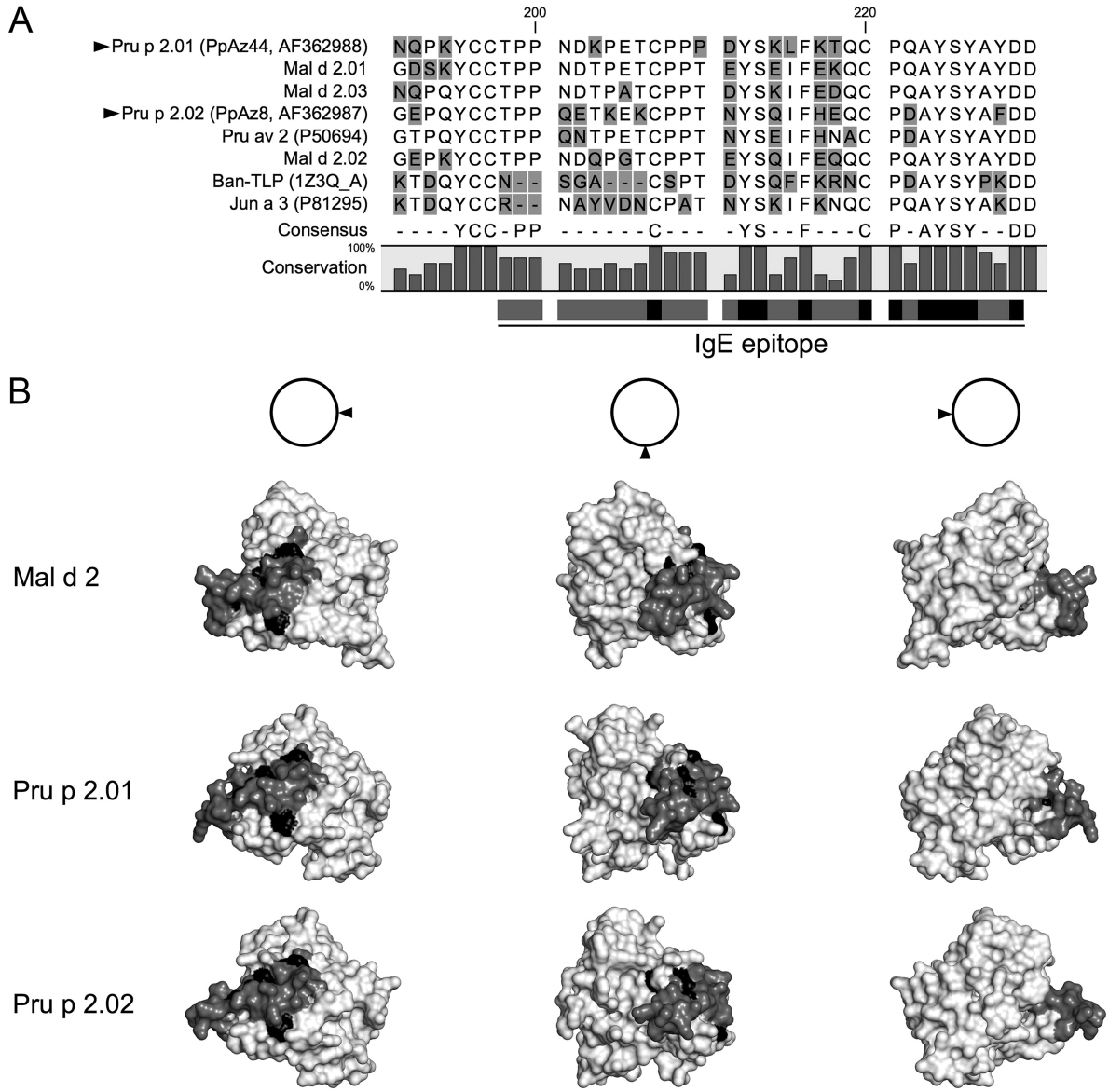

Figure 2. (**A**) Multiple alignments of Rosaceae TLP amino acid sequences including both Pru p 2.01 and Pru p 2.02 from peach (indicated with arrowheads), as well as Jun a 3 from the mountain cedar *Juniperus ashei* and Ban-TLP from *Musa acuminata*. Only the putative epitope region is shown. The bar chart below the alignment indicates the amino acid conservation. Nonconserved amino acids are shaded, and the consensus sequence displays the 100% conserved residues. The dominant IgE epitope is reported below the sequence (gray, nonconserved residues; black, conserved residues), according to the indications of Leone et al. (*41*). (**B**) Three-dimensional models of Mal d 2, Pru p 2.01 (PMDB id: PM0075484) and 2.02 (PMDB id: PM0075485) allergens. The epitope pointed out in (**A**) is reported on the molecular surfaces with the same colors. Proteins are represented in three different orientations (as indicated by the symbols above the models) for a better delineation of the surface exposed epitopes.

accumulation of *Pru p 1.02* was affected by both fruit load and light radiation. The fruits harvested from trees with a high fruit load always showed the highest level of *Pru p 1.02* expression, whereas no significant difference was detected in the fruits from the medium- and low-load trees. Nevertheless, the medium-load control trees carried the fruits with the lowest level of *Pru p 1.02* transcripts both in the pulp and in the skin, although not significantly different from the low-load ones. Light enhancement was shown to significantly decrease the expression of this gene, with the highest extent in the epicarp of high-load fruits. With regard to this differential effect of light, stronger in the epicarp, a clear interaction between this factor and the tissue was pointed out (**Table 3**). On average, a 1 log *Pru p 1.02* lower transcript accumulation was assessed in the pulp than in the skin. As far as *Pru p 2.01* expression is concerned, fruit load was shown to slightly affect the transcriptional rate, with minimum levels of specific mRNA found in the samples from the medium-load trees. Light enhancement itself had no effect on *Pru p 2.01* transcript accumulation, but was shown to interact both with fruit load and with tissue, although to a low and almost undetectable extent (**Table 3**). In epicarp an average 4 log higher expression was observed than in the mesocarp (**Figure 5B**). The *Pru p 2.02*-specific mRNA accumulation was significantly affected neither by fruit load nor by light enhancement, but a generally higher expression was observed in the low-load samples of control fruits. As far as the two profilin-encoding genes are concerned, significant effects were observed mainly in the less expressed gene, namely, *Pru p 4.02*, whereas *Pru p 4.01* expression levels remained almost constant, at least from a statistical point of view (**Figure 5C**). Light enhancement was found to slightly reduce the latter gene transcription, regardless of the tissue. With regard to the general trends, two opposite patterns of expression were observed for *Pru p 4.01* in relation to the fruit load effect, with positive and negative correlations

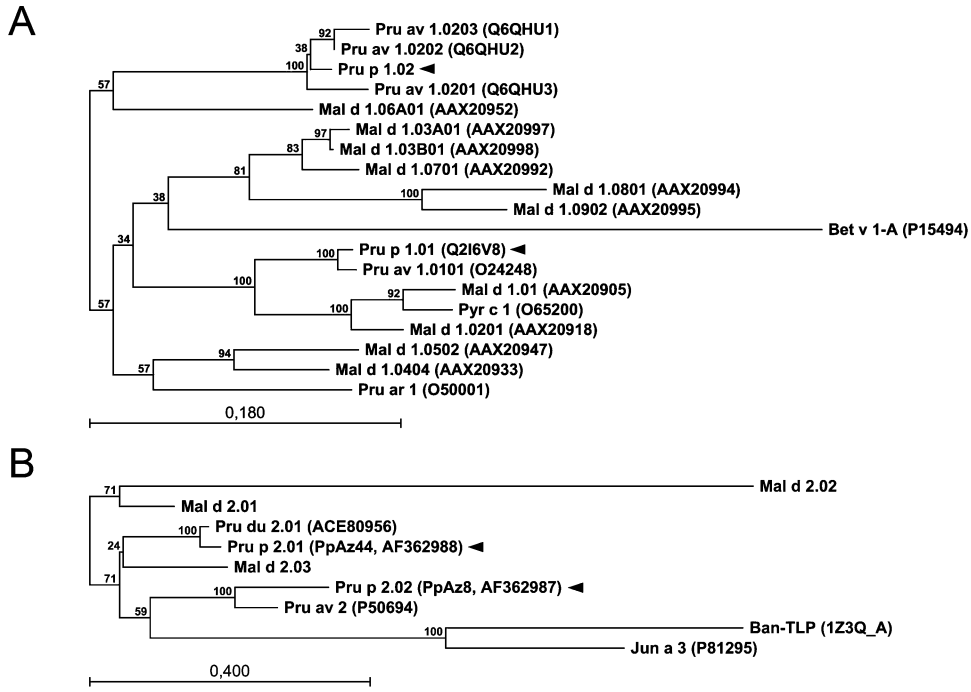

Figure 3. Neighbor-joining phylogenetic trees of the sequences aligned in **Figures 1A** (**A**) and **2A** (**B**). The arrowheads indicate the peach allergens. Bootstrap values are reported at each node, and accession numbers are displayed in parentheses when available.

Table 2. Classification of the Main Peach Allergens Performed According to Radauer and Breiteneder (*52*)[a]

protein superfamily	allergen class	allergen/isoallergen	synonym	nucleotide acc no.	EVALLER score	mol mass (kDa)	p*I*	signal peptide
Bet v 1	Pru p 1	Pru p 1.01	*ypr-10*	DQ251187	24.616	17.6	6.02	no
		Pru p 1.02		AM290651	24.478	17.4	5.26	no
thaumatin-like proteins	Pru p 2	Pru p 2.01	*PpAz44*	AF362988	6.285	25.8	8.64	no
		Pru p 2.02	*PpAz8*	AF362987	4.722	23.3	4.87	yes
prolamin type 1 nsLTPs	Pru p 3	Pru p 3.01	*Pp-LTP1*	AY620230	11.053	9.1	9.57	yes
		cPru p 3.02	*Pp-LTP2*	AY093699	5.442	9.5	9.20	yes
profilins	Pru p 4	Pru p 4.01		AJ491881	13.047	14.2	5.14	no
		Pru p 4.02		AJ491882	13.414	14.1	5.13	no

[a] The allergenicity scores according to EVALLER are also reported (for an extended version see the Supporting Information). All parameters were calculated for the mature proteins. Accession numbers are given as a reference and do not always refer to full-length sequences ("c" before the name means "candidate").

in mesocarp and epicarp, respectively. Nevertheless, the gene was less expressed in the skin than in the pulp, with a 2-fold difference. Finally, with regard to *Pru p 4.02*, fruit load and light radiation were shown to positively and negatively affect, respectively, the accumulation of specific transcripts in the mesocarp. Interestingly, the lower the fruit load, the higher was the down-regulation obtained by increasing the light radiation. In the epicarp, fruit load did not significantly influence the expression level, whereas light enhancement down-regulated *Pru p 4.02* in all of the samples, although to a lower extent than in the mesocarp. The expression of this gene did not differ significantly in the two tissues analyzed.

In the case of *Pru p 3.01* gene (**Figure 5D**), a clear positive correlation was shown in both pulp and skin of control fruits between fruit load and transcript accumulation, the high-load samples being those with the highest gene expression. Interestingly, light enhancement was shown to significantly affect *Pru p 3.01* transcription, reversing its expression pattern in the mesocarp. On the one hand, a strong up-regulation of 3 log was evidenced in the low-load fruits along with a slight increase of 1 log in the medium-load ones. On the other hand, the opposite effect was found in the high-load samples, in which *Pru p 3.01* transcript accumulation was negatively affected by light enhancement. In the skin, the same effect was observed, although to a lower extent. As a matter of fact, the higher the fruit load, the lower was the up-regulation by light. Moreover, *Pru p 3.01* was expressed on average 4 logs more in the epicarp than in the mesocarp, confirming the above results.

DISCUSSION

The results obtained by means of the bioinformatic analysis point toward a close structural similarity between the newly identified candidate allergens and the corresponding proteins found in other Rosaceae species such as apple, cherry, apricot, and almond. Therefore, we can assume to have identified a new Bet v 1-like (Pru p 1.02) and two TLP-like (Pru p 2.01 and Pru p 2.02) allergens in peach.

The expression of Pru p 1-encoding genes during fruit growth and development correlated well with the onset of ripening, as demonstrated in apple for some Mal d 1 isoforms (*43*), with the only exception of *Pru p 1.02* in mesocarp, for which the expression level decreased throughout development and ripening (**Figure 4A**). Moreover, because the overall amounts of transcripts of the two genes were comparable, the related proteins might accumulate in similar quantities, therefore

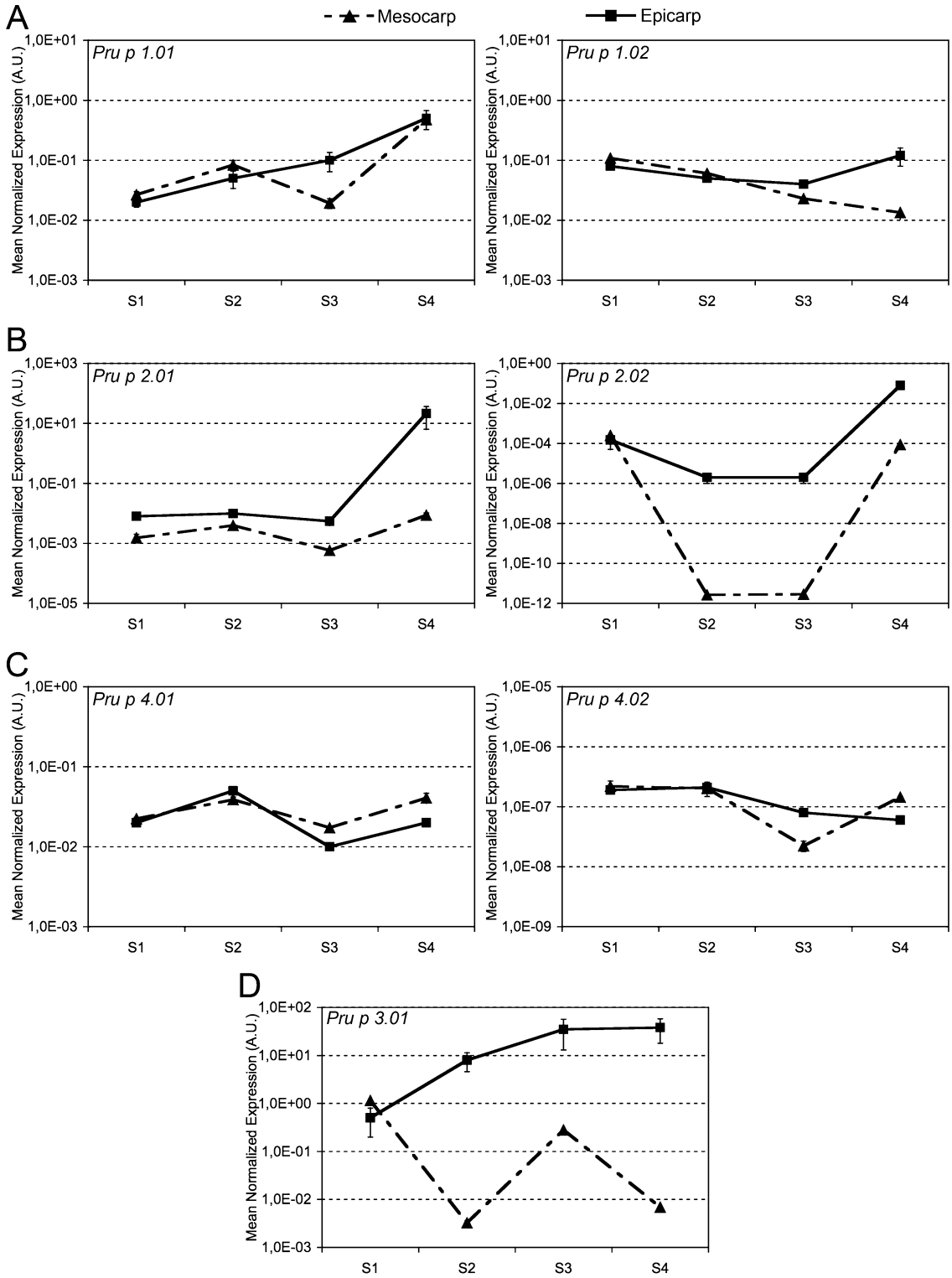

Figure 4. Expression profiles of Pru p 1.01, Pru p 1.02 (**A**), Pru p 2.01, Pru p 2.02 (**B**), Pru p 4.01, Pru p 4.02 (**C**), and Pru p 3.01 (**D**) genes in mesocarp (triangle, broken line) and epicarp (square, continuous line) in peach fruits collected at S1 (first exponential growth phase), S2 (endocarp lignification), S3 (second exponential growth phase), and S4 (climacteric stage). The bars show the standard error when detected.

representing two relevant allergens both in fruit pulp and in skin. With regard to the physiological function of Pru p 1, a putative involvement of Bet v 1-like proteins in binding and transport of plant steroids and in intracellular signaling was previously suggested (*8*). Moreover, because brassinosteroids may be good candidate ligands of Bet v 1-like proteins (*44*) and given that such molecules have been shown to regulate fruit ripening in both climacteric and nonclimacteric fruits (*45, 46*), the involvement of Pru p 1 isoforms in peach fruit ripening could be speculated.

As far as Pru p 2 genes are concerned, the expression studies pointed out a strong correlation between the amount of transcripts and the onset of ripening. Both *Pru p 2.01* and *2.02* transcripts were shown to strongly increase at S4 (**Figure 4B**), as already observed for the former, but not for the latter, with northern analysis previously carried out by Ruperti et al. (*47*).

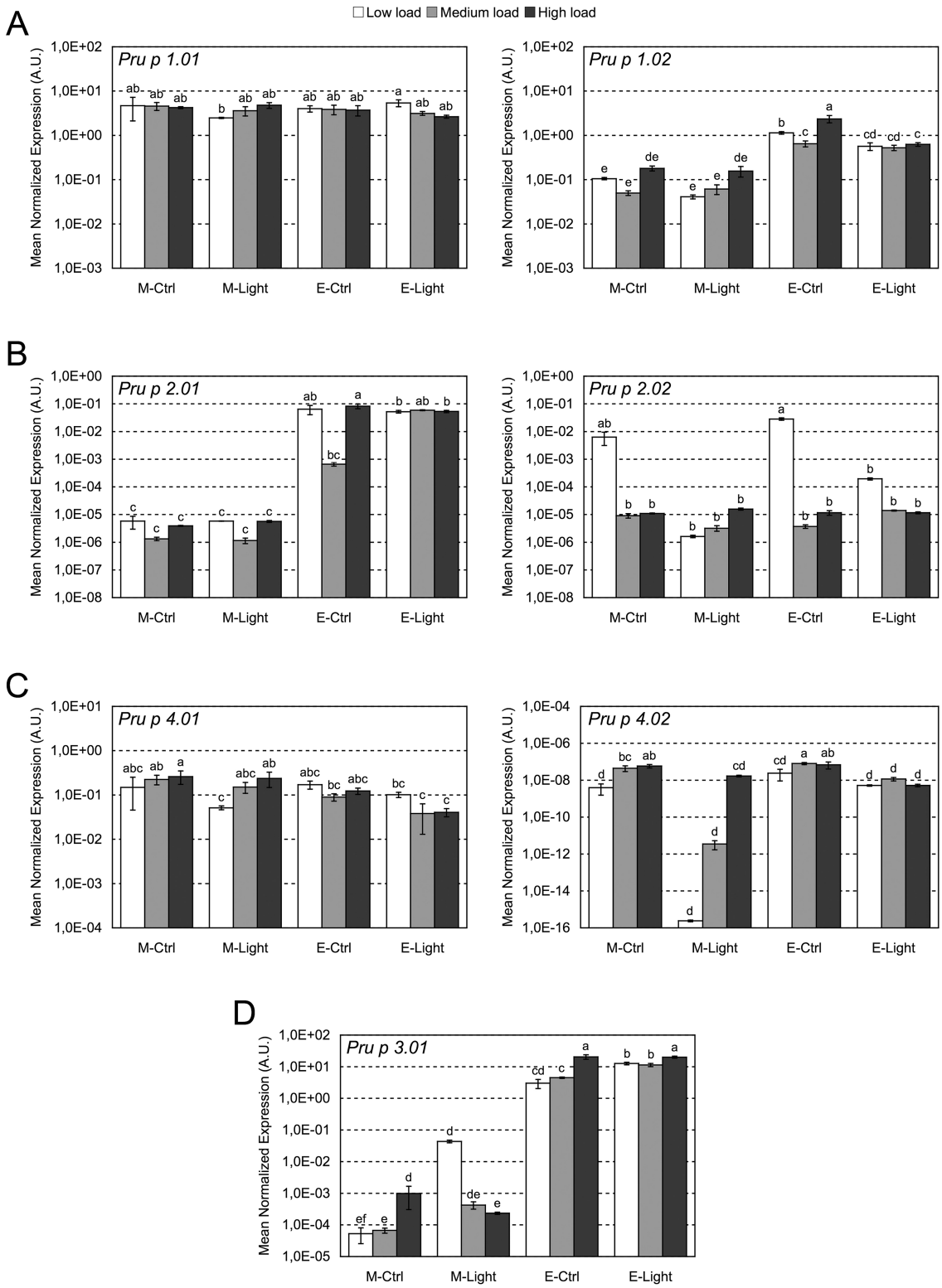

Figure 5. Expression profiles of *Pru p 1.01*, *Pru p 1.02* (**A**), *Pru p 2.01*, *Pru p 2.02* (**B**), *Pru p 4.01*, *Pru p 4.02* (**C**), and *Pru p 3.01* (**D**) genes in mesocarp (M) and epicarp (E) of fruits grown in standard conditions (Ctrl) or with enhanced light radiation (Light). Three fruit load levels were also assessed: low (white bars), medium (light gray bars), and high (dark gray bars). The letters represent the nonsignificant ranges according to the LSD test ($P \leq 0.05$), whereas the line bars show the standard error.

Nevertheless, several other authors reported an increased transcription of TLP-encoding genes in ripening apple and tomato fruits, hypothesizing a specific biological role of TLPs during this developmental stage (*48*−*50*).

As far as the profilin genes are concerned, fairly constitutive gene expression levels were pointed out (**Figure 4C**), similar to those observed in apple (*38*). Profilins are 14−17 kDa proteins found in all eukaryotic phyla that bind to actin and to phosphatidylinositol 4,5-bisphosphate, thus likely being an important link between signal transduction cascades and cytoskeleton organization, covering essential cellular functions (*51*). In this view, the cellular level of such important proteins and the related gene transcription rates have to be almost constant, to allow the cell to maintain its shape and the full

Table 3. Statistics Summarizing the Effects of Fruit Load (F), Light Enhancement (L), Tissue (T, Mesocarp and Epicarp), and Their Interactions (F × L, F × T, L × T, and F × L × T) on the Transcript Accumulation of Peach Allergen-Related Genes[a]

				interaction			
gene	fruit load	light enhancement	tissue	F × L	F × T	L × T	F × L × T
Pru p 1.01	ns ($P = 0.8577$)	ns ($P = 0.3720$)	ns ($P = 0.6363$)	ns ($P = 0.8999$)	ns ($P = 0.2019$)	ns ($P = 0.5097$)	ns ($P = 0.1745$)
Pru p 1.02	*** ($P = 0.0001$)	*** ($P = 0.0000$)	*** ($P = 0.0000$)	** ($P = 0.0014$)	** ($P = 0.0020$)	*** ($P = 0.0001$)	** ($P = 0.0017$)
Pru p 2.01	* ($P = 0.0115$)	ns ($P = 0.5497$)	*** ($P = 0.0000$)	** ($P = 0.0030$)	** ($P = 0.0100$)	ns ($P = 0.5205$)	** ($P = 0.0026$)
Pru p 2.02	ns ($P = 0.2451$)	ns ($P = 0.2386$)	ns ($P = 0.4434$)	ns ($P = 0.2518$)	ns ($P = 0.5530$)	ns ($P = 0.4516$)	ns ($P = 0.5641$)
Pru p 3.01	*** ($P = 0.0000$)	*** ($P = 0.0005$)	*** ($P = 0.0000$)	* ($P = 0.0173$)	*** ($P = 0.0000$)	*** ($P = 0.0005$)	* ($P = 0.0181$)
Pru p 4.01	ns ($P = 0.4090$)	* ($P = 0.0396$)	* ($P = 0.0102$)	ns ($P = 0.9163$)	* ($P = 0.0296$)	ns ($P = 0.9671$)	* ($P = 0.8113$)
Pru p 4.02	** ($P = 0.0017$)	*** ($P = 0.0000$)	ns ($P = 0.0745$)	* ($P = 0.0131$)	ns ($P = 0.3138$)	ns ($P = 0.1244$)	ns ($P = 0.9578$)

[a] P values of the ANOVA statistical tests ($P < 0.05$) are also provided (ns, nonsignificant).

functionality of the intracellular transport. Therefore, the expression patterns herein reported are consistent with the cellular role of profilins and confirm our data concerning apple (*38*).

In the present research, the expression of the gene encoding the major allergen Pru p 3 was studied for the first time in the pulp. Interestingly, the maximum level in this tissue was detected during the two exponential growth phases, S1 and S3, when cell division and cell enlargement occur, respectively (*35*). In the epicarp, the expression of *Pru p 3.01* reached its maximum at S4, confirming the results of Botton et al. (*20*). Because the patterns of transcript accumulation are quite different in epicarp and mesocarp (**Figure 4D**), diverse physiological roles might be postulated for Pru p 3 in peach.

Fruit load and enhanced light radiation were shown to significantly affect the expression of some allergen-related genes and, because several interaction effects were reported, the results have to be considered as a whole (**Table 3** and **Figure 5**). The enhancement of intracanopy light radiation negatively affected the transcription of the genes analyzed, often dependent on the fruit load as observed for *Pru p 3.01* and *4.02*. This is in agreement with a reduced competition among fruits for assimilates partitioning, light being the major factor affecting the leaf functioning as a carbon source. The only exceptions were *Pru p 3.01* in the mesocarp of low-load (LL) fruits and *Pru p 1.01* in the epicarp, both up-regulated by light. On the other hand, fruit load showed a positive correlation with allergen-related gene expression, except for *Pru p 2.02* in both tissues and *Pru p 4.01* only in the epicarp. Considering the tissue specificity, higher levels of transcripts were more frequently reported in the epicarp, with the only exception of *Pru p 4.01*. Taken together, these data would indicate that the overall allergenic potential might represent an index of the nutritional sufferance of the fruit population within the tree.

As concluding remarks, the expression studies herein reported might be useful for obtaining hypoallergenic fruits by means of innovative agricultural practices. In fact, it has been shown that a reduction of the transcription rate of the majority of peach allergen-encoding genes can be achieved by reducing the competition for assimilates partitioning. Therefore, light enhancement and fruit thinning might allow the harvest of less-stressed fruits with a reduced allergenic potential. Indeed, it is worth noting that the comprehensive approach adopted in this study might be useful to pursue the setting up of innovative "allergomic" tools that may improve the general knowledge of the overall allergenic potential of foods as well as allergy diagnosis and therapies. Nevertheless, such an approach, along with the previous one adopted in apple (*38*), was shown to be effective in isolating the most relevant factors affecting the fruit allergenic potential, thus allowing future allergological in vivo studies to be focused just on the most significant aspects.

ABBREVIATIONS USED

OAS, oral allergy syndrome; PR, pathogenesis-related; nsLTPs, nonspecific lipid transfer proteins; ESTs, expressed sequence tags; TLP, thaumatin-like protein.

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Supporting Information Available: Identification of candidate allergen-encoding genes and classification of known and candidate peach allergens. This material is available free of charge via the Internet at http://pubs.acs.org.

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Identification of candidate allergen-encoding genes

Peach allergen-related sequences were searched both in ESTree (Lazzari et al. *BMC Bioinformatics* **2005**, *6*, S4-S16) (www.itb.cnr.it/estree/) and GenBank (www.ncbi.nlm.nih.gov/Genbank) databases, by using a simple keyword-based search with terms related to known *Rosaceae* allergens, their function, and classification. In particular, the following terms and/or combinations of them were used to retrieve allergen-related sequences: 'allergen', 'PR', 'pathogenesis-related', 'Bet v 1', 'Mal d 1', 'steroid', 'Bet v 2', 'Mal d 2', 'TLP', 'thaumatin, 'Mal d 3', 'Pru p 3', 'LTP', 'lipid transfer protein', 'profilin', 'Mal d 4'.

The nucleotide sequences were clustered and contigs assembled by means of CAP3 server of the GDR (Genome Database for Rosaceae) website (Huang and Madan, *Genome Res.* **1999**, *9*, 868-877) (www.bioinfo.wsu.edu/cgi-bin/gdr/gdr_cap3) with the default parameters. The assembled contigs were checked manually and BlastX (Altschul et al. *J. Mol. Biol.* **1990**, *215*, 403-410) analysis was performed to find the open reading frames (ORFs) in order to deduce protein sequences.

Table S1 - Known and candidate (with a 'c' before the conventional nomenclature) peach allergens, as classified according to Radauer and Breiteneder (*52*). The allergenicity scores according to EVALLER (*33*) (bioinformatics.bmc.uu.se/evaller/) are also reported. All parameters were calculated for the mature proteins determined by SignalP (*31,32*). Accession numbers are given as a reference and do not always refer to full-length sequences.

Protein superfamily	Allergen class	Allergen/Isoallergen	Synonyms	Nucleotide Acc. No.	EVALLER score	M.W. (kDa)	pI	Signal peptide
Bet v 1	Pru p 1	Pru p 1.01	*ypr-10*	DQ251187	24.616	17.6	6.02	No
		Pru p 1.02	-	AM290651	24.478	17.4	5.26	No
Thaumatin Like Proteins	Pru p 2	Pru p 2.01	*PpAz44*	AF362988	6.285	25.8	8.64	No
		Pru p 2.02	*PpAz8*	AF362987	4.722	23.3	4.87	Yes
Prolamins Type 1 nsLTPs	Pru p 3	Pru p 3.01	*Pp-LTP1*	AY620230	11.053	9.1	9.57	Yes
		cPru p 3.02	*Pp-LTP2*	AY093699	5.442	9.5	9.20	Yes
Profilins	Pru p 4	Pru p 4.01	-	AJ491881	13.047	14.2	5.14	No
		Pru p 4.02	-	AJ491882	13.414	14.1	5.13	No
Ole e 1-family	cPru p 5	cPru p 5.01	-	DW359139	5.541	15.5	7.31	Yes
Class I chitinases	cPru p 6	cPru p 6.01	*CHI*	AF206635	14.020	-	-	-
	cPru p 7	cPru p 7.01	-	AJ822121	6.709	-	-	-
Class IV chitinases	cPru p 8	cPru p 8.01	-	BU047007	3.231	-	-	-
Hydrolases, β-1,3-glucanases	cPru p 9	cPru p 9.01	*Gns1*	PPU49454	8.100	35.1	9.01	Yes
		cPru p 9.02	*Gns2*	AF435088	8.100	35.4	9.01	Yes
		cPru p 9.03	*Gns3*	AF435089	7.665	34.1	9.10	Yes
WIN-like proteins	cPru p 10	cPru p 10.01	*PpAz89*	AF362989	7.253	13.2	5.13	Yes
Cupins, Auxin Binding Proteins	cPru p 11	cPru p 11.0101	*ABP19*	PPU81163	11.294	19.7	6.57	Yes
		cPru p 11.0102	*ABP19A*	PPU79114	11.666	19.8	8.59	Yes
		cPru p 11.02	*ABP20*	PPU81162	12.581	19.5	5.47	Yes