

The *PpLTP1* Primary Allergen Gene is Highly Conserved in Peach and Has Small Variations in Other *Prunus* Species

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Abstract Peach lipid transfer protein (LTP1), Pru p 3.01, is a major allergen causing severe systemic reactions in peach allergic patients in Mediterranean countries and China. Significant expression variability has been reported among peach cultivars at both transcript and protein level. In this study, the allele diversity of the LTP1-encoding gene was assessed in a large set of peach cultivars by direct sequencing of the gene and its upstream region. Evolution of the LTP1-encoding genes in peach (*Prunus persica*) and three other *Prunus* species (*Prunus kansuensis*, *Prunus mira*, and *Prunus davidiana*) were also inferred. Sequence analysis revealed that LTP1-encoding genes are highly conserved among peach cultivars and wild peach. Three different allele sequences were obtained from 50 *Prunus* accessions on the basis of the upstream region of the LTP1-encoding gene and three allele specific markers were derived according to the

polymorphic sites. These markers were used to test 316 peach cultivars, most of which clustered within the three main subpopulations of peach, ‘Yu Lu’, ‘Hakuho’ and landraces. The genotypic frequencies and allele frequencies in the oriental peach cultivars in these subpopulations except for ‘Yu Lu’ were found to be in Hardy-Weinberg equilibrium ($P < 0.05$). The dominant alleles were upLTP1-a in the ‘Yu Lu’ subpopulation and upLTP1-c in the ‘Hakuho’ subpopulation, allele upLTP1-b and allele upLTP1-c had the highest rates in the landraces, and the dominant allele in the three peach wild relatives was allele upLTP1-b. Furthermore, we found many light-responsive elements in the upstream region. Most of the polymorphic sites in *Prunus* species are located in the intron region. Phylogenetic analysis suggests that *Prunus kansuensis* KC311794 and *Prunus mira* KC311791 are related more closely to *Prunus persica* KC311795 than to *Prunus*

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dauidiana KC311792 and KC311793. The allele sequences we derived on the basis of this variability were distributed unevenly, indicating the need to study allergenicity in different subpopulations and the association between allele sequences and allergenicity.

Keywords *Prunus persica* · Lipid transfer protein · Genetic diversity · Allele frequency · Peach subpopulation

Abbreviations

ORF	Open reading frame
LTP	Lipid transfer protein
AS	Allele-specific markers
SNAP	Single nucleotide amplified polymorphisms
SPT	Skin prick test

Introduction

Food allergy is a problem worldwide and is on the increase. Plant-food allergies, especially fruit allergy, have become a major threat to health due to the dramatic increase in production and consumption of various fruits (Wang et al. 2012). Peach (*Prunus persica*) originated in China and is still one of the most widely cultivated and consumed fruits in China. As a fresh fruit, peach is also a major cause of food allergy in the Mediterranean area (Pastorello et al. 1998; Rodriguez et al. 2002), especially in Spain (Fernandez-Rivas et al. 2003; Hernandez et al. 1985; Kivity et al. 1994) and Italy (Ortolani et al. 1989), where severe allergic reactions to peach are prevalent. Studies on food allergy diagnosis emphasize the utility of allergen component testing (Sicherer and Leung 2013). In China, peach allergy is also frequently reported, especially in northern China (Gao et al. 2013). The major allergens of peach belong to two different families of pathogenesis-related (PR) proteins: PR-10 proteins of approximately 18 kDa, Pru p 1, which results in oral allergy syndrome (OAS), prevalent mostly in central and northern Europe (Wensing et al. 2002), and the 9-kDa lipid transfer protein (LTP) (Pastorello et al. 1999), Pru p 3, which can cause severe systemic reactions including gastrointestinal (GI) symptoms, urticaria and anaphylaxis. In the Mediterranean area, LTP (Fernandez-Rivas et al. 2003; Gamboa et al. 2007; Rodrigues-Alves et al. 2009; Sánchez-Monge et al. 1999) is the primary allergen involved. Peach LTP1 (Pru p 3) has also been shown to be the major factor involved in most Chinese peach allergy cases (Gao et al. 2013).

LTP has been identified as a pan-allergen that can cause cross-reactive allergy with many botanically unrelated foods, such as peach, apple, tomato, rape, carrot, barley, corn, wheat, rice, sorghum, broccoli, onion and grape. The high

allergenicity is derived from its extreme resistance to digestion (Asero et al. 2002, 2001) and thermal processing (Scheurer et al. 2004). In Europe, cross-reactivity of mugwort, peach, apple and chestnut have been reported (Diaz-Perales et al. 2000; Rodrigues-Alves et al. 2009). In China, extensive cross-reaction between LTP from peach and mugwort has been reported and mugwort pollen LTP has been identified recently as the primary sensitizer in most peach allergy cases (Gao et al. 2013; Ma et al. 2013).

Previous studies have shown that three members of the peach LTP family encode the peach lipid transfer proteins Pru p 3.01, Pru p 3.02 and Pru p 3.03 (Chen et al. 2008); however, the major allergen, expressed at high levels in peach fruit, is LTP1 or allergen Pru p 3.01 (Yang et al. 2011). This is a 9 kDa protein with an isoelectric point (PI) greater than 9 (Pastorello et al. 1999), accumulating particularly in the downy hairs covering the peach fruit (Barre et al. 2009). Significant variability in the expression pattern of the *LTP1* gene has been detected in different apple cultivars (Bolhaar et al. 2005; Borges et al. 2006; Botton et al. 2008; Carnes et al. 2006; Sancho et al. 2008). Botton has also demonstrated that the expression pattern of peach *LTP1* and the accumulation of the encoded allergenic protein (Pru p 3.01) differ markedly among peach cultivars (Botton et al. 2006), which has been further confirmed by Brenna et al. (2004). In addition, most US peach cultivars have higher levels of Pru p 3 than Spanish peach cultivars (Ahrazem et al. 2007).

Our previous study also showed differences in gene expression among diverse peach cultivars (Yang et al. 2011). However, to date, information on the factors causing these differences has been very limited. This study aims to clarify whether, among peach cultivars, they are a result of differences at the genomic DNA level, more specifically in the promoter region.

Materials and Methods

Cultivars Used for Cloning and Sequencing

A total of 50 *Prunus* accessions, 47 *Prunus persica* (L.) Batsch including the ancestral peach ('Mao Tao'), three peach wild relatives (one *P. kansuensis*, one *P. mira* and one *P. dauidiana*) were chosen for cloning and sequencing of the LTP1-encoding gene (Table 1). Of the 47 *Prunus persica* (L.) Batsch accessions, 27 along with the three wild relatives were also studied for the upstream region of the LTP1-encoding gene as marked in Table 1. The 47 peach cultivars chosen were highly diverse, covering different geographic areas (North and South China) and with a different domestication history (wild, landraces and breeding), and were considered to be the core germplasm resources and represent the genetic diversity of Chinese peach. There were also abundant

Table 1 Detailed information on the peach cultivars chosen for cloning and sequencing of *Pru p 3.01* including subpopulations, main fruit traits, origin, collection and geographic area^a

Subpopulation	Cultivar	Pedigree	Traits ^b	Origin	Collection ^c	Geographic area ^d	Breeding/Landrace ^e	GenBank
Yu_Lu	Asama Hakuto	Gaoyangbaitao offspring	PWR	Japan	FHPRI	J	B	
	Chi Yu Lu ^f	Yu Lu selfing	PWR	Zhejiang	FHPRI	SE	B	KC311810 KC31181
	Xing Yu	-	PWR	Zhejiang	FHPRI	SE	B	
	Hua Yu Lu	Yu Lu selfing	PWR	Zhejiang	NPGR(Jiangsu)	SE	B	
	Chang Ling	-	PWR	Zhejiang	NPGR(Zhengzhou)	SE	L	
	Zao Yu Lu ^f	-	PWR	Zhejiang	NPGR(Zhengzhou)	SE	L	
Hakuho	ZhengZhou 8 ^f	Hakuho × Bi Tao	PWR	Zhengzhou	NPGR(Zhengzhou)	N	B	KF179638
	Okubo ^f	Seedling selection of Hakuto	PWR	Japan	NPGR(Jiangsu)	J	B	KC311807, KC311808
	Dixon ^f	-	PWR	USA	SAAS	O	B	KF179616
	FengBai	Seedling selection of Okubo	PWR	Dalian	NPGR(Jiangsu)	NE	B	
	GanXuan 2 ^f	-	PYR	Gansu	NPGR(Zhengzhou)	NW	L	KF179621, KF179622
	Hakuho	Hakuto × Tachibanawase	PWR	Japan	NPGR(Jiangsu)	J	B	
	Hong Bu Ruan-August ^f	Local Cultivar	PWR	Shanxi	SAAS	N	L	KC311803
	Hong Shan Hu ^f	Qiu Yu × NJN76	NWR	Beijing	SAAS	N	B	KF179623
	Hui Yu Lu ^f	Zhaohui × Yuhualu	PWR	Jiangsu	NPGR(Jiangsu)	SE	B	KF179624
	Hu Jing Mi Lu ^f	One parent is Hahuho	PWR	Jiangsu	NPGR(Jiangsu)	SE	B	KC311804 KC311805
	Jin Xia	Okubo × Okitsu	NWR	Shanxi	SAAS	N	B	
	Jin Xiu Huang Tao ^f	BaiHua × Yunshu1	PYR	Shanghai	FHPRI	SE	B	KC311806
	Mei Gui Lu	SunagoWase × Yu Hua Lu	PWR	Zhejiang	NPGR(Zhengzhou)	SE	B	
	OhdamaHakuho	Tachibana × Hakuto	PWR	Japan	FHPRI	J	B	
	Yue165	-	PWR	Guangdong	NPGR(Jiangsu)	S	L	
	Yue192 ^f	-	PWR	Guangdong	NPGR(Jiangsu)	S	L	KF179631 KF179632
	Yu Hua Lu ^f	Bai Hua Shui Mi × Shang Hai Shui Mi	PWR	Zhejiang	NPGR(Jiangsu)	SE	B	KC311809
	Yu Lu Pan Tao ^f	-	PWF	Zhejiang	FHPRI	SE	L	KC311812, KC311813
	Zao Mei	Qing Feng × Zhao Xia	PWR	Beijing	NPGR(Jiangsu)	N	B	
	Zao Shang Hai Shui Mi	Unknown	PWR	Shanghai	NPGR(Jiangsu)	SE	L	
Or.Yellow	Zhangbai 2 ^f	Local Cultivar	PWR	Gansu	NPGR(Zhengzhou)	NW	L	KF179636
	Zhao Hui	Bai Hua Shui Mi × Tachibanawase	PWR	Jiangsu	NPGR(Jiangsu)	SE	B	
	Feng Huang ^f	Seeding Selection of Zao Sheng Huang Jin	PYR	Dalian	NPGR(Zhengzhou)	NE	B	KF179617 KF179618
Or.Nectarine_	Zheng15-1 ^f	-	PYR	Zhengzhou	NPGR(Zhengzhou)	N	B	KF179634 KF179635
	Feng Guan 2 ^f	Guan Tao5 × LianHuang	PYR	Zhejiang	NPGR(Zhengzhou)	SE	B	KC311801 KC311802
	Feng Guang You Tao	Unknown	NWR	Japan	SAAS	J	B	
	Jing Chun ^f	Seedling Selection from Zao Sheng Huang Jin	PWR	Beijing	SWU	N	B	KF179625
	Nan Fang Jin Mi	-	NYR	unknown	NPGR(Zhengzhou)		L	
	Pan Tao Huang Hou ^f	Zaohong2 × ZaoLuPanTao	PWF	Zhengzhou	NPGR(Zhengzhou)	N	L	KF179628
	Zheng Huang 3 ^f	Zao Shu Huang Gan × Feng Huang	PYR	Zhengzhou	NPGR(Zhengzhou)	N	B	KC311814 KC311815

Table 1 (continued)

Subpopulation	Cultivar	Pedigree	Traits ^b	Origin	Collection ^c	Geographic area ^d	Breeding/Landrace ^e	GenBank
Landrace	Zhong You 4 ^f	25–17 × May Fire	NYR	Zhengzhou	NPGR(Zhengzhou)	N	B	KC311817
	Bai Lu Tao ^f	-	PWR	Shanxi	SAAS	N	L	KC31179, KC311799
	Chang Chi Huang Mi	-	PWR	Jiangsu	NPGR(Jiangsu)	SE	L	KF179614, KF179615
	Da Hong Pao ^f	-	PRR	Hubei	NPGR(Zhengzhou)	N	L	KC311800
	Mei Guo Pan Tao	-	PWF	USA	FHPRI	O	B	
	Qiu Mi	-	PWR	Shan'xi	NPGR(Zhengzhou)	NW	L	
	Wu Yue Xian	-	PWR	Beijing	SAAS	N	L	
	Xiao Bai Tao ^f	-	PWR	Henan	NPGR(Zhengzhou)	N	L	KF179629, KF179630
	Xin Jiang Huang Rou Tao	-	PYR	Xingjiang	NPGR(Zhengzhou)	NW	L	
	Zhong You 5_FH	25–10 × May Fire	NWR	Zhengzhou	NPGR(Zhengzhou)	N	B	
	Zhong Hua Shou Tao ^f	-	PWR	Shandong	SAAS	N	B	KF179637
	Mao Tao ^f	Common root stock in south China	PWR	Unknown	NPGR(Jiangsu)	-	WL	KF179626, KF179627

^a Information on these cultivars and subpopulation classification are taken from Li et al. (2013)

^b The first letter indicates fruit shape: peach (*P*), nectarine (*N*), the second letter indicates flesh color: white (*W*), yellow (*Y*), red(*R*), the third letter indicates shape: flat peach (*F*) and round peach (*R*)

^c NPGR National Peach Germplasm Repositories; SAAS Shanxi Academy of Agriculture Sciences; FHPRI Fenghua Peach Research Institute; SWU Southwest University

^d Different zones located in China: *N* north; *NW* northwest; *NE* northeast; *S* South; *SE* southeast; *SW* southwest

^e *B* Breeding; *L* Landrace; *W* wild relative species

^f Cultivars chosen for *LTP1* promoter region cloning and sequencing

differences in the physical properties as described in Table 1. Additionally, these cultivars are widely planted and consumed in China and therefore were the best choice to investigate the genetic variation of the *Pru p 3.01* allergen gene in Chinese peach. A very recent publication (Li et al. 2013) demonstrated that the 47 peach cultivars can be clustered into five peach subpopulations: ‘Yu Lu’, ‘Hakuho’, ‘Oriental Yellow peach’, ‘Oriental Nectarine’ and ‘Landrace’. These accessions were collected from 5 germplasm collections: 14 from the National Peach Germplasm repository at Jiangsu Academy of Agriculture Sciences (Southeast Region), 7 from the Fenghua Honey Peach Research Institute (Southeast Region), 8 from Shanxi Academy of Agriculture Sciences (Central Region), 1 from Southwest University (Southwest Region) and 17 from the Zhengzhou Fruit Research Institute (Chinese Academy of Agriculture Sciences, Central Region). The one wild peach species ‘Mao Tao’ and the three wild relatives were investigated to trace back the evolution of the *LTP1* gene. Fresh leaves of these accessions were collected and frozen in liquid nitrogen and stored at −40 °C prior to nucleic acid isolation.

DNA Isolation

Total genomic DNA extraction was based on an improved cetyltrimethylammonium bromide (CTAB) protocol as described previously. Minor modifications (Roche et al. 1997; Zhang et al. 2009) were as follows: 1 g leaves was ground in liquid nitrogen to a fine powder prior before addition of 4 mL CTAB buffer (2 %, w/v, CTAB, 100 mM Tris–HCl, 50 mM EDTA, 1.4 M NaCl, 0.5 %, v/v, β-mercaptoethanol, pH 8.0).

The mixture was incubated at 65 °C for 45 min, followed by three extractions with an equal volume of chloroform/isopropanol (24:1). The supernatant was decanted and precipitated with cold isopropanol, and the pellet washed three times in 75 % ethanol before dissolving in TE buffer. RNA was removed by digestion with deoxyribonuclease-free ribonuclease A, followed by chloroform/isopentanol extraction and ethanol precipitation. Finally, the DNA pellet was re-dissolved in TE buffer. The DNA concentration was measured using an ultraviolet spectrophotometer (Beckman coulter DU800) at 260 nm, and adjusted to 20 ng/μL.

PCR Primers, Genome Cloning and Sequencing of the Intron-Containing *LTP1* Gene

For each cultivar, the intron-containing *LTP1* was cloned and sequenced. The primer pair used for cloning *LTP1* has been published previously (Chen et al. 2008). The primer pair used for cloning the upstream region of the *LTP1* gene was designed using Primer Premier 5.0 README based on the whole peach genome sequence available online (http://www.rosaceae.org/gb/gbrowse/prunus_persica/). PCR amplification, cloning and sequencing procedures have been described previously (Chen et al. 2008). For all 50 accessions, six to eight clones of the *LTP1* gene were sequenced in both directions.

DNA Sequence Analysis

DNA sequences were aligned and putative single nucleotide polymorphisms (SNPs) were identified using the Seqman program (DNASTAR, Madison, WI). One *LTP1* sequence (EU424264) derived from GenBank was chosen as the reference sequence to search for mutant sites in the *LTP1* encoding region of the peach cultivars and *Prunus* species. Its upstream region in GenBank was used as reference for comparison of the upstream region of the *LTP1* sequences. Phylogenetic and molecular evolutionary analyses of the *LTP1* gene among different *Prunus* species were conducted using MEGA version 5 (Tamura et al. 2011).

Development of Allele-Specific Markers

To test the authenticity of the polymorphisms in the upstream region revealed by cloning and to discriminate the derived allelic sequences, polymorphisms in the sequences were used to develop single nucleotide amplification polymorphism (SNAP) markers (Drenkard et al. 2000) using the software programme PRIMER DE-SIGNER ver. 2.0 (Scientific and Educational Software, Cary, NC). The methods used were described previously (Gao et al. 2005): the 3' end in one or both of the forward and reverse primers was located exactly at polymorphic nucleotides found in the upstream sequences. A designed mismatch was added at the second or the third position at the 3' end of the primer to reduce the chance of primers annealing to the untargeted template. For a much stronger effect, the designed mismatches were usually complementary to the matching nucleotide. The candidate allele-specific (AS) primer pair was tested on the gDNA of four representative cultivars from which the sequence was obtained and four negative cultivars with other allelic sequences.

Results

Diversity of *LTP1* Genes and Deduced Proteins

The peach lipid transfer protein (PpLTP1 or Pru p 3.01) gene sequence has two exons, 344nt and 10nt, and one intron. Aligning the *LTP1* gene sequences derived from 46 different peach cultivars and 1 ancestral peach ('Mao Tao') resulted in only one sequence (accession no.KC311795): no mutant site was found at the amino acid level.

With the same primer pairs, *LTP1*-f/r (Table 2), we obtained two allele sequences from *Prunus davidiana* Franch denoted *Pru da 3.01a/b* (accession no. KC311792 and KC311793), one from *Prunus kansuensis* Rehd, *Pru k 3.01* (accession no.KC311794) and one from *Prunus mira* Koehme, and *Pru m 3.01* (accession no. KC311791). Alignment of the four sequences with *Pru p 3.01* gave 18 SNP sites including two indels, ten of which were located in the first exon region, including one in the signal-peptide-encoding region. The second exon is very short and highly conserved. In the intron region, eight SNPs (including two indels) were found (Fig. 1). Comparison of the deduced amino acids revealed ten amino acid changes, including one in the signal peptide (Fig. 2). *Pru da 3.01a* and *Pru da 3.01b* were diverse at the DNA level but identical at the protein level, and most of the polymorphism found in the two allele sequences was in the intron region. *Pru k 3.01* and *Pru m 3.01* share high identity with *Pru p 3.01* at the protein level, as only one amino acid variant between *Pru k 3.01* and *Pru p 3.01* and two between *Pru m 3.01* and *Pru p 3.01* were found. A total of seven amino acid variants were detected between *Pru da 3.01a/b* and *Pru p 3.01*, including one in the signal peptide encoding region. However, the typical characteristics of lipid transfer proteins, such as the eight conserved cysteines and the lipid binding motifs were identical (Fig. 2)

Evolution of the Non-specific Lipid Transfer Protein (*LTP1*) Encoding Gene in Different *Prunus* Species

Pru p 3.01 sequences derived from several peach cultivars and three *Prunus* species *LTP1* gene sequences from our own cloning and sequencing work, along with other *Prunus* species *LTP1* genes from the NCBI GenBank, are available. This puts us in a position to gain insight into the evolution of the *LTP1* gene among different *Prunus* species. A neighbor-joining (NJ) tree of *LTP1* genes from *Prunus* species, including *Prunus dulcis*, *Prunus sargentii*, *Prunus armeniaca*, *Prunus avium*, *Prunus kansuensis*, *Prunus mira*, *Prunus davidiana* and *Prunus persica*, was constructed using *Mal d 3.01* (*LTP1* from apple) as an outgroup (Fig. 3). Generally, these *Prunus* species fell into four clades. *Prunus kansuensis*, *Prunus mira* and *Prunus persica* were clustered within the same clade, while *Prunus davidiana* was grouped in another clade. As expected, two *Prunus davidiana* sequences (*Prunus da 3.01a* and *Prunus da*

Table 2 PCR primer pairs used for genomic cloning of *LTP1* genes and their upstream region in different peach cultivars and *Prunus* species

Primer name	Primer sequence(5'-3')	Tm °C/cycles ^{a,b}		Product (nt)	Gene
		Pfu	Taq		
LTP1-f	ATCATAGTCAAGAGAGATGG	54/30	56/2	645	<i>Pru p 3.01</i>
LTP1-r	CCCTAAGTGGATCACATAGC				
ProLTP1-f	TGTACTTGTACGTGATGATGGCTCT	58/35	60/2	2297	
ProLTP1-r	CTGCGGCATTGTTAGGGTTGACT				

^a Tm, annealing temperature; cycles, number of PCR cycles

^b Pfu and Taq columns refer to the Tm and number of cycles in the step using Pfu DNA polymerase and Ex-Taq DNA polymerase in the two-step clone, respectively

3.01b) were clustered in one subgroup. However, an unexpected feature from the NJ tree was that the *LTP1* sequences of *Prunus sargentii*, *Prunus dulcis* and *Prunus davidiana* fitted in a single group, indicating a higher similarity than *Prunus persica* and *Prunus davidiana*. Additionally, compared to *Prunus armeniaca* and *Prunus avium*, *Prunus dulcis* and *Prunus sargentii* were clustered more closely with *Prunus persica*.

Sequence Diversity Analysis of the Upstream Region of the *LTP1* Gene from Different Peach Cultivars

To further investigate the genetic diversity of the *Pru p 3.01* gene, we also cloned and sequenced 1,670 bp upstream from

the translation initiation codon (Fig. 4). From the 26 peach cultivars, we obtained 39 sequences, which clustered in three different consensus sequences on the basis of the SNPs found in these DNA regions. These were named upLTP1-a, upLTP1-b and upLTP1-c. Aligning the three sequences, 24 SNPs were detected, including 20 transitions, 2 transversions and 2 indel (Supplementary Fig. 1). At this DNA locus, 13 varieties were homozygous while the other 13 were heterozygous. The results showed that allele upLTP1-c was distributed within 23 of the 26 cultivars and allele upLTP1-a distributed within 9 cultivars. However, only six peach cultivars had allele upLTP1-b, all six cultivars originating from northern China, and all of which also had allele upLTP1-c. Sequencing of the

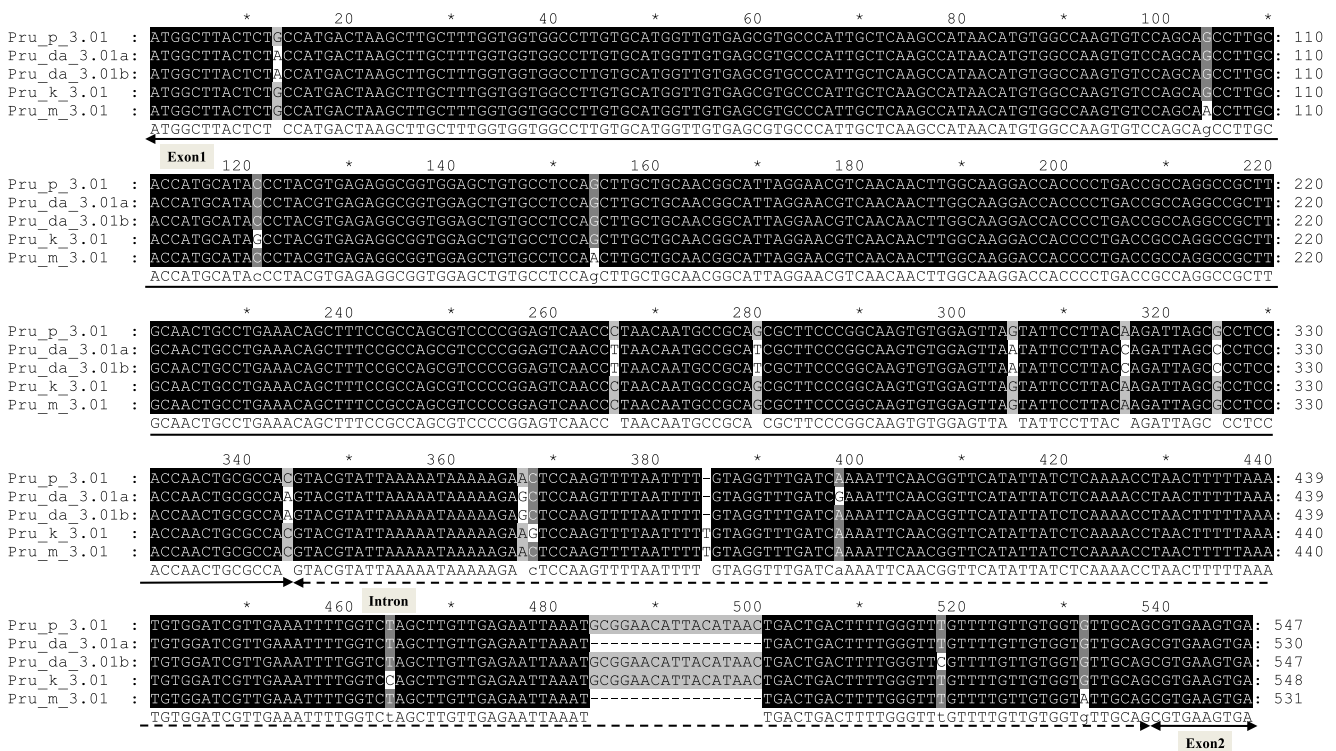


Fig. 1 Alignment of *LTP1* genes derived from different *Prunus* species. *Pru p 3.01*-*Prunus persica* (GenBank accession no. KC311795); *Pru m 3.01*-*Prunus mira* (GenBank accession no. KC311791); *Pru k 3.01*-*Prunus kansuensis* (GenBank accession no. KC311794); *Pru da 3.01a*/

b-*Prunus davidiana* (GenBank accession no. KC311792 and KC311793). Exon 1 and exon 2 are underlined with solid lines and the intron is underlined with dashed lines

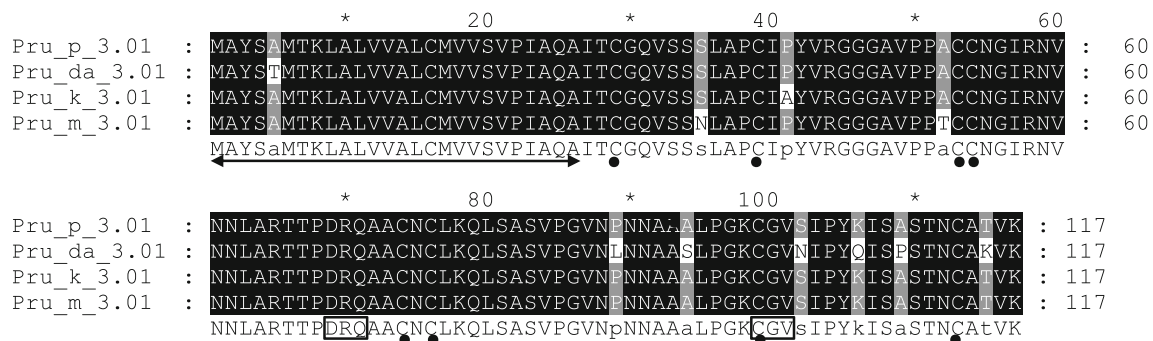


Fig. 2 Alignment of the deduced amino acid sequences of LTP1 protein variants derived from different *Prunus* species. Pru p 3.01-*Prunus persica*; Pru m 3.01-*Prunus mira*; Pru k 3.01-*Prunus kansuensis*; Pru

da 3.01A/B-*Prunus davidiana*. • Cysteine residues; signal peptides are underlined, and lipid-binding motifs are boxed

one ancestral peach ('Mao Tao') wild peach showed that 'Mao Tao' is heterozygous, having upLTP1-b and upLTP1-c alleles, and the results for the three peach wild relatives (*P. kansuensis*, *P. mira* and *P. davidiana*) showed that all three relatives have, and only have, allele upLTP1-b.

Analysis of the consensus sequence of the *LTP1* upstream region gave several putative TATA and CAAT boxes in the region close to the transcription start site, highlighted in Fig. 4. In the more distal region, we also found a number of cis-acting elements, especially light-responsive elements and MYB-like motifs.

Development of Allele-Specific Markers and Their Allele Frequency in Peach Subpopulations

Polymorphisms in the promoter region of the *Pru p 3.01* gene were used to create allele-specific markers. On the basis of the

24 SNPs (including 2 indels), three AS markers were developed as shown in Supplementary Fig. 1 and tested in cultivars with these polymorphisms in order to demonstrate their authenticity (Fig. 5). The results showed that the primer pair upLTP1-a F/R can target the fragment in the cultivars from which upLTP1-b was derived as well as primer pairs upLTP1-b F/R and upLTP1-c F/R. The SNAP marker sequences, PCR conditions and product sizes are given in Table 3.

The Hardy-Weinberg equilibrium (HWE) of locus upLTP was evaluated based on the allele frequency (AF) in different peach subpopulations (Table 4). In all subpopulations except 'Yu Lu', the upLTP allele was in HWE ($P < 0.05$). Allele upLTP1-a was the dominant allele in the 'Yu Lu' subpopulation and allele upLTP1-c in the 'Hakuho' subpopulation, while allele upLTP1-b and allele upLTP1-c had the highest rates in

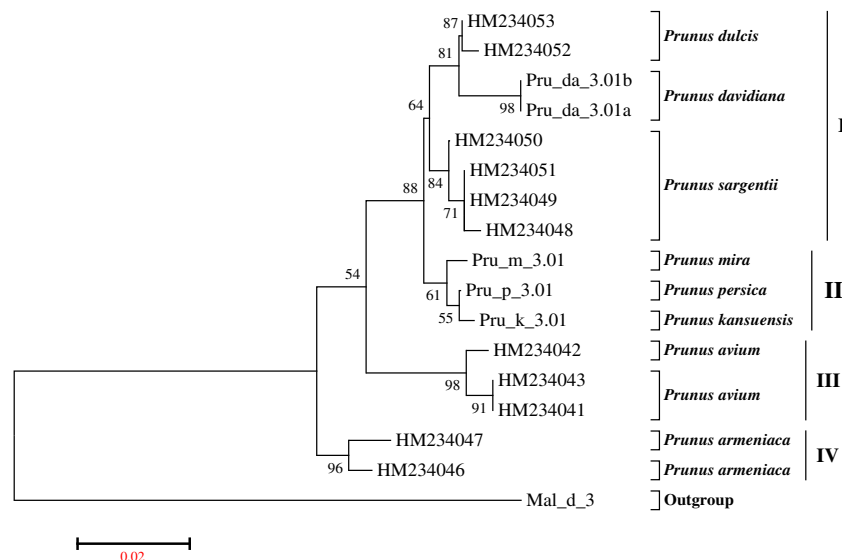


Fig. 3 Phylogenetic tree of LTP1 genes from different *Prunus* species. Phylogenetic analysis based on the complete LTP1 gene (beginning with the TSS 'ATG' and including two exons and one intron) and using *Mal d 3.01* (accession no. AY572516) as the outgroup. Numbers on branches correspond to the bootstrap support value >50 % from 500 bootstrap replicates. *Pru p 3.01* (accession no. KC311795), *Pru m 3.01* (accession

no. KC311791), *Pru k 3.01* (accession no. KC311794) and *Pru da 3.01a/b* (accession no. KC311792 and KC311793) are newly derived genes. GenBank: HM234041~HM234053 were retrieved from the DNA NCBI Database. *Pru da 3.01(a/b)* denotes the two allele gene sequences in *Prunus davidiana*

Fig. 4 Consensus sequence of gDNA and deduced amino acids of PpLTP1 derived from peach cultivars. SNPs within alleles identified from different peach cultivars are indicated: Y=C or T; W=A or T; R=G or A; M=A or C; S=C or G. Putative CAAT and TATA boxes, light responsive elements and myb binding site are highlighted in *purple*, *green*, *red* and *yellow*, respectively. The poly-T region is *underlined*. Deduced amino acids are indicated in *italics*. The consensus sequence was derived from GenBank (accessions no. KC311796–KC311817)

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GGCTGTATCTAACGGTCAAGAACTTAAATAAAATTAATGAAATG
-1624 TATGATCAGTACCAAAAAATAATTTCTGTTTTAAAAGTTGAGGTATTCAAAGAAGATATTCTAACAAATTAATAATGCAATATTGAATAAAAGTTT
-1524 TTGTCACATCTCGGATCGGCTCCGCCGTTCATGATATTTCCGTTTGGAGTCCCTGCGCATCAGGTTTGTCTATGGGAATTCACGAGCAACTTCCAG
-1424 TGGGTCACCCATTCTGCGATTACTCTAGCTCAAACTTACTTAAGTTCGGAATTCCTATAATTCAAAGCCAGTAAGTCTCAAAGGCCCTCGTGTAGAT
-1324 GGAAGTAAAGTATGACATATAAGACATATCACCCCTCTCCATTGATCGATGTGGATGTTACACTTTTGTGTAAGAGAGTGGGTATGAACAAATAG
-1224 TTGGAAGCTGAAAGTCGGTCCGATTTACCATGAAGAGATGGTTCTTAATAATTTGCCTCGTTTACAAACATAAAACAATAGATTTTGATGACCAGA
-1124 AGTTTTTTTGTACCTGTTTTTGGAGCAAAACCGAATGGCAGGGGAGACACGGGTCTGGTGTACTGCAAGCTCAACAAGAAACATTTAAGTGAACAAAT
-1024 CAATTATGCAAGTACGACCATTTTACATTAATATCATGGGACCCGACCTTGATTGTGCACCTAACGTTGCCAGCAAGCCCTGTTTGAACAGAGAT
-924 AAGTTTGGGTACCAATTATAATTTTTTTTTTTTTCAGCCAAACTGGGGGAAGACATTATCCAAGTTAATATGTTATTCCTGGTTTTACCTTTTGGG
-824 ATAGTCATGATCCATGGGAAATTAAGTTTAACTCAAGTGTGACAATTTCTGTAATGACACAATAACATAAGATCTACATTATAATATATGCTGTGTC
-724 TTCATAACGGGATTTTTTGGTTCGATATATAACATGAGTTAGCTTGTACAAAAATTTGATACGAAAAATCTCAAAGAACATGATTCTCTTTGTTTT
-624 GGCCTTCTCTTTCATGTAGGTAGTAAAGTTGGGGATCATTTATGTGATTAGGCTAAGTTTGTGCTTAAGCGCATTTAAGGGATCAACTCAATCTTAAA
-524 ACTTGAACATAGTACCCCAAGCATTAATCCACCCCACTACAACTGATTCTCTCCAACTCCACACATATTTGGGGCCATGTGTAATATTTTTTG
-424 TTCACAATAACAGCTTGTGTGAAAGGTTAACTGCTGGGAAATTTAATTTTACCTATCTAATTTATGTTTCCAGCTGTGAATTTAAGACAAAA
-324 ATTAGTATGATGAATAATTCAATTTTATAATATGAATAAAAAATTTTACTTACATCTATTAGCATTTTATAGATTTTGTATGTATAAAT
-224 ATGATGTTAAAAATGAATTTATTTATAAAATCTATTATTTTGAATCTGACAGTTAATAAATGATGAATAAGTATGATCAATAATTCACCTCA
-124 TTTCTCTCATAACACAGAGCATGGTGCATACTACTACTCTCAAGTTCTCAAAATCAAGTGCTTCAAAATCCGTTTCAAACTCCCTTTTTT
-24 CTTCGATCATCATAGTCGAGAGATAGGCTTACTCTGCCATGACTAAGCTTCTTGGTGGTGGCTTGTGATGTTGTGAGCGTGCCCATTTGCTCAAG
H A Y S A M T K L A L V V A L C H V V S V P I A O
77 CCATAACATGTGGCCAAAGTGTCAGCAGCTTGACCATGCAATCCCTACGTGAGAGCGGTGGAGCTTGCCCTCCAGCTTGCTGCAACGGCATAGGAA
A I T C G Q V S S S L A P C I P Y V R G G G A V P P A C C N G I R N
177 CGTCAACAACCTTGGCAGGACCCCTGACCCGAGGCGCTTGCAACTGCCTGAACAGCTTTCCCGACGCTCCCGGAGTCAACCCATAACATGCC
V N N L A R T T P D R Q A A C N C L K O L S A S V P G V N P N A
277 GCAGCGCTCCCGGCAAGTGTGGAGTTAGTATTCTTACAAAGATTAGCGCTCCACCACTGCGCCAGGTACGTATTAAAAATAAAAGAACCTCAAGTT
A A L P G K C G V S I P Y K I S A S T N C A T
377 TTAATTTGTAGGTTGATCAAAATTCAGCGGTTCATATTATCTCAAACTAACTTTTAAATGTGGAGCTGTGAAATTTGGTCTAGCTTGTGTAGAA
477 TTAATGCGGAACATTACATAACCTGACTGCTTTTGGGTTTGTTTTGTGTGGTGTGACAGGTGAGTGAGCTGATATGGGAAGTATTTTCGGCCG
V K
577 CAGATAATATTAGAGAGTCGAATAAAATGTGGCTATGTGATCCACTTAGGG

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landrace subpopulations. The AF of allele upLTP1-b was only 0.086 in the ‘Hakuho’ subpopulation.

Discussion

Differences in *LTP1* Encoding Genes and Their Promoter Regions Among *Prunus* Species

Here we report, for the first time, a comparison of *LTP1*-encoding gene sequences derived from different peach

cultivars. Although it has been demonstrated that expression patterns among peach cultivars differ (Yang et al. 2011), the reason behind these differences remains unknown. It has been suggested that a difference in the coding region is involved (Ahrazem et al. 2007). However, we found no mutant sites in the *LTP1* coding region of geographically and genetically different peach cultivars, which suggests that different expression levels of *Pru p 3.01* are not associated with the coding region itself.

As for the three genotypes we derived from the promoter region of peach and peach wild relatives, we found that all three

Fig. 5 Test of the three allele specific markers on gDNA of ten cultivars. Lanes (peach cultivars): 1 Jin Xiu Huang Tao, 2 Chang Chi Huang Mi, 3 Qiu Fen, 4 Zheng Huang 3, 5 Mao Tao, 6 Yan Xia, 7 Okubo, 8 Chi Yu Lu, 9 Da Hong Pao, 10 Hakuho; *M* standard markers. The upLTP1-a, upLTP1-b and upLTP1-c bands amplified with the three allele specific markers are indicated

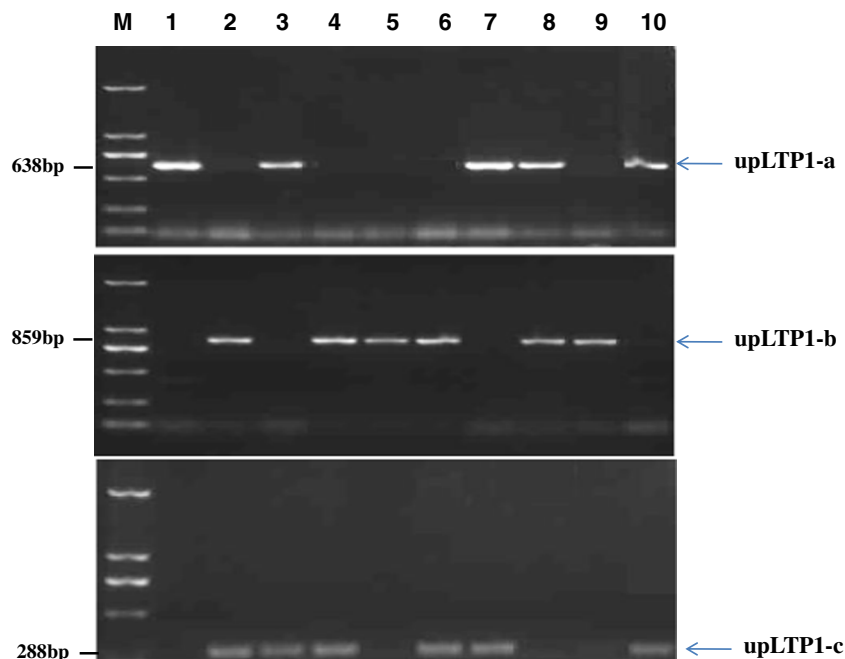


Table 3 Description of allele-specific (AS) markers for the upstream region of the *LTP1* gene

Marker names ^a	Primers ^b (5'-3')	SNPs detected ^c	Product (bp)	Tm ^d (°C)	Cycles ^e
upLTP1_a	F:TGTACATATAAGACATATCACC <u>G</u> T R:GACAAGCTAACTCATGTTATAC	–1,289 T	638	58	42
upLTP1_b	F:TAAGCAGTACGCACCA <u>TTTC</u> R:TATTATTATTGACTGTCAGATTC	–1,006 CATTTC	859	56	42
upLTP1_c	F:TGATATTATCCGTTTCG <u>T</u> GT R:CCGACTTTTCAGCTTTCCT <u>A</u>	–1,473 T –1,224 T	288	58	35

^a Marker upLTP1-a, upLTP1-b and upLTP1-c were used for target sequences upLTP1-a, upLTP1-b and upLTP1-c

^b In bold, allele-specific SNPs; underlined, designed mismatch nucleotide

^c Position refers to the genomic sequence counted from the ATG starting codon

^d PCR annealing temperature

^e Number of cycles

relatives only have one allele, upLTP1-b, while the one ancestral peach ('Mao Tao') has both the upLTP1-b and upLTP1-c alleles. This suggests that upLTP1-b exists mainly in old peach cultivars, as further demonstrated by the fact that, among the peach cultivars, this allele is present mainly in the north China peach populations, which were always considered to be old accessions. We conclude that alleles upLTP1-a and upLTP1-c have evolved from upLTP1-b.

We sequenced and aligned the corresponding genes from *Prunus davidianan* (ST), *Prunus kansuensis* (GS) and *Prunus mira* (GH). There are eight *Prunus* species in the NJ phylogenetic tree we derived: peach (*Prunus persica*), apricot (*Prunus armeniaca*), cherry (*Prunus avium*), almond (*Prunus dulcis*), Sargent's cherry (*Prunus sargentii*), Gansu (*Prunus kansuensis*), Guanghe (*Prunus mira*), and Shantao (*Prunus davidiana*). Among these species, peach, apricot and cherry are commonly consumed as fruit, almond for seed kernel; Sargent's cherry is an ornamental while

Gansu, Guanghe and Shantao are used mainly as *Prunus* rootstocks. The interspecific cross compatibility of *Prunus* genus varied with cross combination and peach was cross-compatible with almond, Gansu, Guanghe and Shantao. The NJ phylogenetic tree showed that Shantao (*Prunus davidiana*) clustered more distantly to peach (*Prunus persica*) than Guanghe (*Prunus mira*) did, which is consistent with the previous study of Roche et al. (1997). The result might be explained by the evolution of *Prunus* species, which indicates that *Prunus mira* (GH) is the closest ancestor of *Prunus*. In addition, *Prunus kansuensis* (GS) was also found to be closely related to *Prunus persica*, which suggests that, in the process of evolution, *Prunus kansuensis* (GS) and *Prunus mira* (GH) might have an ancestor in common with *Prunus persica*. However, it is not sufficient to use just the one gene, *LTP1*, to clarify the relationship of the different *Prunus* species. The introduction of the evolutionary biology of allergens might be helpful in the study of the evolution of allergenicity. According to Radauer and Breiteneder, allergenicity might be an intrinsic molecular property of ancestral members of certain protein families, distributed within the present-day allergen sources of the plant kingdom through domestication (Radauer and Breiteneder 2007). Alternatively, allergenicity might have emerged only as a result of random mutation in certain proteins, inherited by their progeny. Both theories need further experimental analyses for a better understanding of allergenicity.

The Promoter Region of *PpLTP1*

Our previous study (Yang et al. 2011) found that the expression of *Pru p 3.01* in peach peel paralleled peach growth and development, reaching a maximum level during the ripening stage. This is in agreement with publications by others (Botton et al. 2009; Brenna et al. 2004). Fruit load and light irradiation has been shown to correlate with expression of the *LTP1*-

Table 4 Allele frequency (AF) of *PpLTP1* in oriental peach subpopulations

Peach subpopulations	Sample number	Allele frequency ^f			
		<i>a</i>	<i>b</i>	<i>c</i>	
'Hakuho'	97	0.355	0.145	0.5	$\chi^2=58.77, P=0.000$
'Yu Lu'	17	0.412	0.294	0.294	$\chi^2=10.96, P=0.089$
Landraces ^d	36	0.069	0.486	0.444	$\chi^2=24.31, P=0.000$
Admix ^e	166	0.223	0.241	0.536	$\chi^2=31.82, P=0.000$
Total	316	0.255	0.244	0.502	$\chi^2=110.79, P=0.000$

^d Landrace indicates the local peach varieties

^e Admix indicates peach cultivars not clustered within the 'Hakuho' 'Yu Lu' and Landraces subpopulations

^f *a*, *b* and *c* refer to the three allele sequences upLTP1-a, upLTP1-b and upLTP1-c respectively

encoding gene (Botton et al. 2009). Concerning the corresponding LTP1 protein in apple fruit (Mal d 3.01), several external (elevation, light, water, time of harvest and storage) and internal (genotype and tissue) factors are involved in determining the allergenic potential (Botton et al. 2008). Shade, in particular, may reduce apple peel allergenicity (Botton et al. 2008, 2006). It has been found that expression of *BpLTP1* from *Betula platyphylla* can be upregulated by exogenous abscisic acid and salicylic acid, downregulated by exogenous methyl jasmonate, while it is not altered significantly by exogenous gibberellin (Guan et al. 2013). Tian et al. (2013) even revealed that gender was involved in the expression of *LTP1* from floral buds of Chinese cabbage *pak choi*. Taking all the above factors into account, it is likely that expression of allergen-related genes is regulated by several potentially interacting factors.

Regarding the effect of light, we found several light-responsive elements in the upstream region of *Pru p 3.01*, which is in line with the previous study of Botton et al. (2006), who also identified that the promoter contains many elements with organ-, tissue- and cell-specific expression and is hormone-sensitive. Based on these findings, we hypothesize that light plays a crucial role in regulating expression of the peach allergen, Pru p 3.01, in fruit peel. Interestingly, some myb-like and myc-like sites were also found in the putative promoter region. These results suggest that the expression pattern of the *Pru p 3.01* gene in peach fruit may also be affected by myb-like and myc-like factors, which should be further studied.

Tracking the upstream region of the *LTP1* encoding gene to identify genotype-specific effects on allergenicity, we were able to distinguish three different genotypes. From our study we can also now group most Chinese peach cultivars into subpopulations based on the polymorphisms in the upstream region of the *LTP1* gene. This is a preliminary demonstration that peach cultivars in these subpopulations have different expression patterns of the *LTP1* gene which may, together with the current results, give us an additional tool to identify peach cultivars with low allergenicity.

Pru p 3.01 Protein Accumulation Level in Peach Cultivars

Using polyclonal antibodies against lipid transfer proteins, the mean Pru p 3 levels of 15 cultivars in Spain have been found to be 132.86, 0.61, and 16.92 $\mu\text{g/g}$ fresh weight of peel, pulp, and whole fruit, respectively (Ahrazem et al. 2007). It seems that most US cultivars have higher levels of the Pru p 3 allergen than Spanish local cultivars. These Spanish yellow and non-melting cultivars are from very early introductions from China and have very close relationships with current Chinese landraces in north China (Li et al. 2013). We are now producing monoclonal antibodies against Pru p 3.01 and aiming to develop sandwich ELISA kits to quantify Pru p



Fig. 6 Skin prick test (SPT) of three cultivars on one patient. The area of the rash indicates the extent of allergic reaction. A Peach cultivar ‘Bailu’, rash diameter 5 mm; B peach cultivar ‘Qiufen’, rash diameter 3.75 mm; C nectarine cultivar ‘Zhongyou 8’, rash diameter 2 mm; H positive control to histamine, rash diameter 6 mm; I negative control, normal saline. This experiment was approved by the local ethics committee (Authorization No. 2011-R-1, the Second Affiliated Hospital, School of Medicine, Zhejiang University, China) and the patient gave written consent

3.01 content. Preliminary results from eight peach cultivars showed large variation from 14.1 $\mu\text{g/g}$ to 86.2 $\mu\text{g/g}$ fresh weight of peel, but much smaller for pulp from 0.57 $\mu\text{g/g}$ to 5.15 $\mu\text{g/g}$ fresh weight (data not shown). We are currently working with more peach cultivars with a much wider genetic background to analyze the variability of Pru p 3 content in fruit and any link to *PpLTP1* allelic diversity and will perform genome-wide association analysis using the recently developed 9 k SNP chip (Verde et al. 2012).

Differences in the results of the skin prick test (SPT) in one peach allergy patient, using varying concentrations of peach extract, have previously demonstrated the higher content of allergen, inducing larger reaction wheals (Wang et al. 2012). To show the allergenicity difference among peach cultivars by clinical SPT is complex and it is difficult to screen a large number of cultivars with limited Pru p 3 sensitive patients. Our preliminary results with two peach and one nectarine variety, on a peach-allergic patient showed that the allergic reaction provoked by the peach extracts was different (Fig. 6). We aim to select some cultivars with a significantly lower content of Pru p 3 in peel, pulp or both using monoclonal antibodies, and further test them on peach allergy patients by SPT or even oral challenges to confirm their allergenicity.

Conclusions

We have shown that differences in the *LTP1*-encoding gene expression pattern are not associated with the gene coding region, which is fully conserved among peach cultivars. The abundant variability in the upstream region, which can result in three different allelic sequences, might affect expression of

the *LTPI*-gene. Our findings show that the dominant allele sequence in different peach subpopulations is variable, which may explain the diversity and evolution of this allergenic protein. It is also apparent that there is a need to study the allergenicity of peach in different subpopulations, and the association between allele sequences and allergenicity. These results will be directed towards understanding allergenicity caused by peach and in breeding hypoallergenic peach varieties.

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upLTP1_c_R

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upLTP1_b_F

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upLTP1_a_R

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Article Title: The *PpLTP1* primary allergen gene is highly conserved in peach and has small variations in other *Prunus* species

Journal name: Plant Molecular Biology Reporter

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Figure legend:

Supplementary Figure 1 Alignment of the allelic sequences (upLTP1-a, upLTP1-b and upLTP1-c) derived from the upstream region of peach *LTP1*

The location of the forward and reverse primers of the three AS markers are shown. TSS indicates the 'ATG' transcript start site.