

Natural variability of restriction profiles in non-coding part of *Prunus persica* (L.) Batsch. Pru p 3 gene

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Peach is a popular sweet fruit with a very good climate adaptation and high production. It offers many health benefits determined by its biochemical composition. However, sensitive people can be sensitised by Pru p 3 non-specific lipid transfer protein family directly or through cross reaction as a member of Bet v 1 homologues. The majority of research is focused on the protein, less data can be found in genomics or transcriptomics. This study performed RFLP analysis by chosen restricted enzymes (*Bfal*, *Msel*, *Nlalll*) for non-coding region of Pru p 3 (NCBI: KC311811.1) as a tool to distinguish closely related isoforms of the allergen. *Bfal* cut amplicon into 5 fragments corresponding to the Yulu variety *in silico* cleavage and polymorphism was not detected. For *Msel* and *Nlalll* polymorphisms were found in the cleavage sites, two types of restriction profiles were created for both. None of the *Nlalll* profiles corresponds to the restriction profile of *in silico* cleavage. The study confirms the varietal differences in Pru p 3 gene and supports a hypothesis that allergenicity depends on both qualitative and quantitative factors that are different and specific to each variety.

Keywords: Pru p 3, nLTP, non-coding region, restriction variability, peach

1 Introduction

Prunus persica (L) Batsch is world-known popular fruit tree of the *Spiroideae* subfamily with a very good climate adaptation and high production in cultivation regions. It originated in China and for Chinese cultivars, higher diversity is reported in literature when compared to other peach germplasm collections (Zhang et al., 2006). Peach is a self-pollinated species with high degree of self-compatibility and homozygosity (Baird et al., 1996), but genetics and genomics analyses provided effective tools for its marker-assisted selection. Microsatellites and simple sequence repeat markers were reported to be suitable as DNA markers to evaluate genetic relationships between individuals, marker-assisted selections and for population genetic studies in different *Prunus* species (Aranzana et al., 2010; Wünsch et al., 2005; Cheng and Huang, 2009; Bouhadida et al., 2010; Xie et al., 2010). Actually, more than 500 simple sequence repeats have been mapped in the reference map, and many other microsatellites are available from the peach genome sequence data produced by the International Peach Genome Initiative (IPGI). Actual genomic knowledge

of peach is collected in eight bioinformatic screened supercontigs that represents the sequential data of eight peach chromosomes with the numbering of appropriate tights. Proceeded genomic data cover near 99% of its genome with the relevance higher than 92% (Verde et al., 2013).

Peach fruit contains many of health benefits determined by its biochemical composition. The fruit is a rich source of elements as potassium, sodium, calcium, iron, silicon, zinc, phosphorus, manganese, cadmium, magnesium, copper, and vitamins as niacin, riboflavin, β -carotenes and vitamin C, and the content of these elements is affected by many factors, mostly by cultivar (Wills et al., 1983; Ashhamary and Al-Horayess, 2013; Mitic et al., 2019).

Beside the beneficiary characteristics, peach is a fruit that may be harmful for sensitive people suffering by allergy. Peach allergy was reported to have two different patterns. In Central Europe, it is connected mainly with oral allergy syndrome related to a primary sensitization to birch pollen Bet v 1 allergen and profilins. In Southern

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Europe, it is connected mainly with systemic symptoms, in many cases due to sensitization to lipid-transfer protein Pru p 3 (Gamboa et al., 2007). Pru p 3 is a non-specific lipid transfer protein of peach fruit and is proposed to be a model of true food allergens (Salcedo et al., 2008), because of its resistance to proteolytic digestion, oral sensitisation and severe clinical symptoms (Salcedo et al., 2007). It belongs to pan-allergens that are involved in IgE-mediated reactions to both plant food and pollens (Zuidmeer and Ree, 2007). Pru p 3 is well characterized on its biochemical and immunological level (García-Casado et al., 2003; Pasquato et al., 2006; Cubells-Baeza et al., 2017), but many unusual geographical profiles of LTP sensitization were reported across Europe in the clinical practice (Fernández-Rivaz et al., 2003, 2006; Reuter et al., 2006). These are explained by different dietary habits, specific exposure to pollens and natural differences in Pru p 3 content in peach varieties (Duffort et al., 2002; Ahrazem et al., 2007; Salcedo et al., 2008). Up to date, Pru p 3 gene and its expression is characterized only a few in the individual peach varieties (Carnés et al., 2002; Brenna et al., 2004) and no specific description of natural restriction variability of the gene exists. The aim of the study is to analyse the existing natural variability in restriction patterns of a non-coding part of the Pru p 3 gene of *Prunus persica* (L) Batsch.

2 Material and methods

2.1 Biological material and DNA extraction

Young healthy leaves of seven different undefined peach varieties, planted in gardens in south part of Slovakia, were collected in the region of Komárno, more specified Harčáš (figure 1). All of them were surface sterilized by ethanol, rinsed by distilled water and transported to laboratory immediately where were kept frozen until further processing. Total genomic DNA was

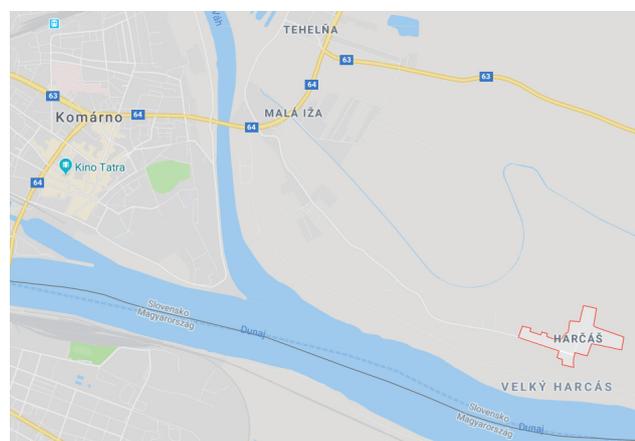


Figure 1 The location of accessions origin (GPS coordinates: lat. 47.747; long. 18.180)

extracted from 100 mg of frozen leaves following the manufacturer's instruction for the Nucleospin Plant II kit. Quantity and quality of extracted DNA was checked by Nanophotometer P360 (Implen) and all the samples were diluted to 50 ng/μl.

2.2 PCR analysis and restriction cleavage

A non-coding region of Pru p 3 gene was subjected to the PCR amplification. Primers were designed to match a region of nucleotides 23 – 1050 of the NCBI accession number KC311811.1 corresponds to Pru p 3 sequence of peach variety Yulu (Figure 2). PCR thermal profile used in the analysis was as follows: 95 °C for 3 min followed by 35 cycles of 95 °C for 45 sec, 60 °C for 45 sec and 72 °C for 1 min, ended by last elongation step at 72 °C for 5 min and 55 sec. A specificity of obtained PCR amplicons was checked by agarose gel electrophoresis. Restriction endonucleases used in the restriction variability analysis were selected in a manner to meet the criteria – cleavage must be processed throughout the sequence, every endonuclease used must cleave at least three positions in the amplicon, different types of expected polymorphism – none, length polymorphism and presence of restriction site polymorphism. Based on the criteria, three different restriction endonucleases were used: *Bfal*, *MseI* and *NotI*. Restriction cleavage of PCR amplicons was performed as the manufacturer's protocols recommended. The restriction fragments were separated in the 8% PAGE gels and stained by GelRed™.

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1  tgtacttcta cgtgatgatg gccttccca tcttctcag gcaatcata tcgtctgtt
61  attttccaca tgctagaaca atcttaatc tcgtctgtt cttttccatt tgcaagaaca
121 atcttaatta atctagtctg ttgtttaaa tatataatt tttttctca ttgtttgaa
181 ggctcagat cctcgaagg ataggccca tatacataa tatcccaag tcacacact
241 attgatatt ttgttaatt tcatagagat ggaccatatt cattcatgca aaattttat
301 gacaaagtaa aaaattatc ttggaagaat attatgggtt ggatcccatc caaggctgtg
361 tctaacgctc aagaaacta aataaaataa aatgaaatg catgatcagt accaaaaata
421 aatttctgt ttaaaaagt gaggtatca aagaagat tctaacaat tataaattgc
481 aatattgaa taaagcttt tgcacatcc tggatcgct ccgccgtagc atgatattat
541 ccgtttgag cccccgccca tcaggtttg cttatgggaa ttcacgagca actttccagt
601 gggtcacca ttctgggatt actctagccc aaacttactt aagtcggaa ttcctataat
661 tcaaaagcca gtaagcttc aaaaggctc gtgctagat gaggtaaagta tgcacatata
721 agacatatca cccccctcc attgatcag tggaaatgtt acactttttg tgaagagag
781 tgggtgatg aacaaatagc tggaaagctg aaaagtcggt cggatttacc atgaaagaga
841 tggttcttaa taatttgctt cgtttacaaa cataaaaca atagattttg atgaccagaa
901 gttttttgt acctggttt ggagcaaac acgaatgcca ggggagacac gggctctggtg
961 tactcgaacg tcaacaagaa acatttaagt gaaacaatc aattatcgag tacgcacat
1021 ttacattaa tatcatggga ccccgacctt gattgtgcac ctaactaagt tggcagcaag
    
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Figure 2 A fragment of non-coding sequence stored in NCBI under the accession KC311811.1

3 Results and discussion

Virtual cleavage profiles were designed for used endonucleases the non-coding segment of Pru p 3 gene. Those were compared further with the seven randomly chosen peach varieties to define the possible sequential polymorphism. Non-coding part of Pru p 3 gene in Yulu variety resulted in four cleavage sites in *Bfal* virtual restriction with the length of restriction fragment as 491 bp, 334 bp, 70 bp, 60 bp and 51 bp. All of the restriction sites were supposed to be without polymorphism, when based on *in silico* data of Pru p 3 genomic sequences stored in the public databases. All of these restriction sites were found in the analysed peach varieties with the correspondent restriction fragments and no changes of restriction profiles were observed. The only difference was presented in the three shortest fragments are of a low abundance and poor visibility (Figure 3).

Virtual restriction by *MseI* resulted eleven fragments in total with following length: 208 bp, 207 bp, 138 bp, 124 bp, 108 bp, 63 bp, 54 bp, 43 bp, 39 bp, 18 bp and 4 bp for peach variety Yulu, with two couples of non-separable fragments (208/207 bp and 43/39/18/4 bp) in the 8% PAGE gels, therefore a 15% PAGE gel was used. Here, polymorphism is described based on *in silico* data of Pru p 3 genomic sequence stored in the public databases. In the group of the shortest fragments, a deletion of 6 nucleotides exists among the stored genomic sequence of Pru p 3 for most of the compared peach varieties. Length polymorphism based on described deletion was obtained in the samples 1, 3 and 4 and confirmed the natural variability in the restriction profile of non-

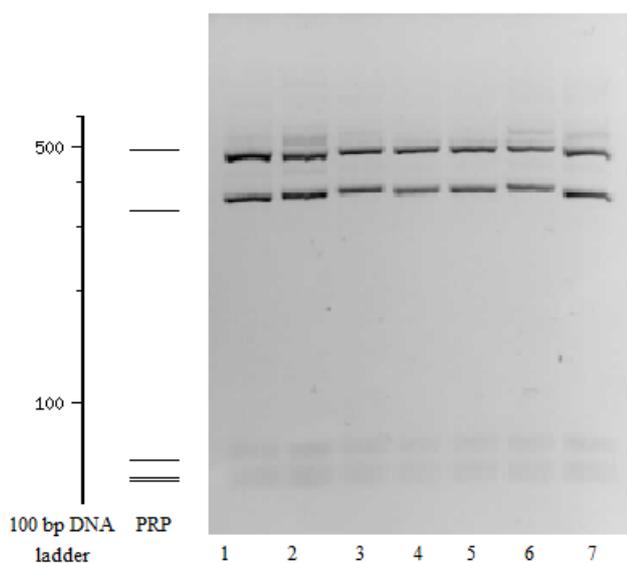


Figure 3 Obtained *Bfal* restriction profiles of analysed peach samples compared to the predicted restriction profile (PRP)

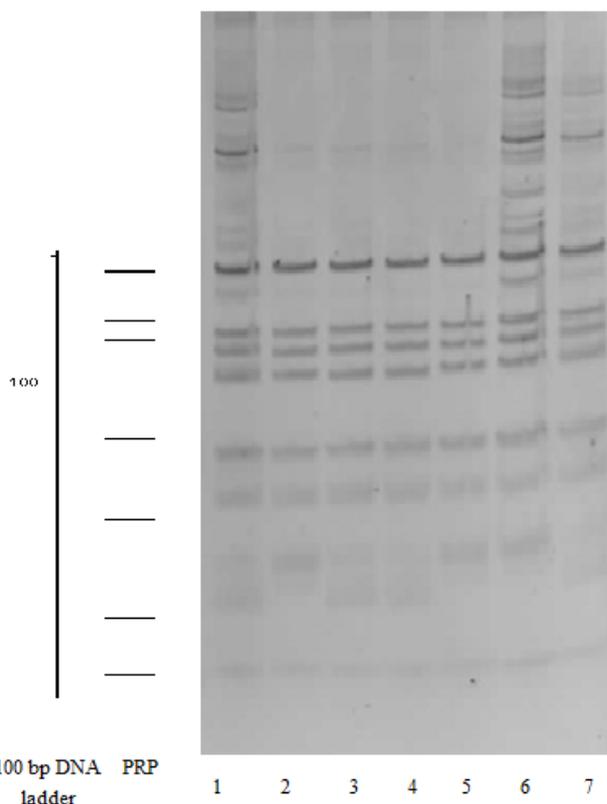


Figure 4 Obtained *MseI* restriction profiles of analysed peach samples compared to the predicted restriction profile (PRP)

coding part of Pru p 3 gene. In the case of this restriction endonuclease, other type of polymorphism was found in the cleavage sites of analysed peach genotypes and two types of restriction profiles were obtained in the analysed samples. In three peach varieties, different restriction profiles exist as the virtual one and nine other restriction fragments were generated (Figure 4).

Non-coding part of Pru p 3 gene resulted in six cleavage sites in *NlaIII* virtual restriction with the length of restriction fragment as 300 bp, 216 bp, 195 bp, 129 bp, 116 bp and 50 bp for peach variety Yulu. For this restriction endonuclease, a substitution-based polymorphism is described in the *in silico* data of Pru p 3 genomic sequences stored in the public databases. A substitution C/T exist in the cleavage site of *NlaIII* in the 69th nucleotide of analysed amplicon and the same substitution is in the 401st nucleotide of analysed amplicon. This results in the length polymorphism where two different restriction profiles can be obtained – 68 + 216 bp and 115 + 122 bp fragments or, if this restriction site absent, restriction fragments of 286 bp and 246 bp appear. Restriction profile of 115 + 122 bp fragments was obtained in samples 2,3 and 6. The restriction cleavage in the 69th and 286th nucleotide was obtained in none of the analysed samples. The same situation was observed for

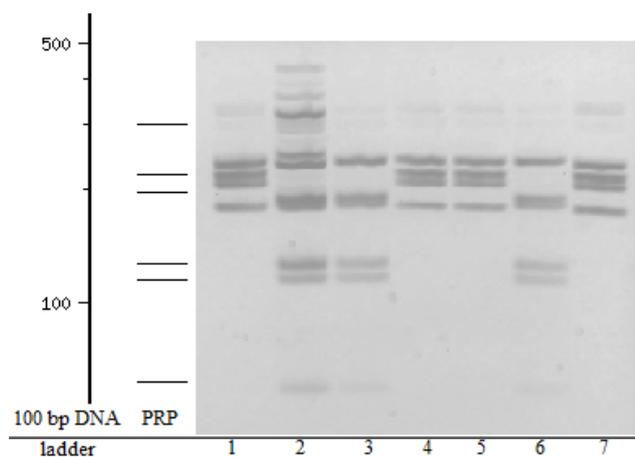


Figure 5 Obtained *NlaIII* restriction profiles of analysed peach samples compared to the predicted restriction profile (PRP)

the restriction fragment of 300 bp, which was obtained in none of the analysed samples, too (Figure 5). We suppose, that other type of nucleotide substitutions exists in this restriction cleavage sites in peach varieties. In all of the analysed samples, the shortest fragment is not present, too. In sample 2, restriction site of 286th nucleotide of analysed amplicon is missing and cytosines of both *NlaIII* sites are present in the 68th and 401st positions what resulted in the fragment with the length of 332 bp. In a summary, none of the analysed peach varieties correspond to the restriction profile of a virtual cleavage of Yulu peach variety. Samples 3 and 6 generated a completely different restriction profiles, where only a restriction fragment of 197 bp was identified from the prediction cleavage of the Yulu variety sequence and two other restriction fragments were generated as completely different. For *NlaIII*, the differences exist for the length of the generated restriction fragments, too.

Food allergy has an increasing prevalence, that is, why different useful tools are being developed for the strategy of selecting suitable varieties for breeding of hypoallergenic fruit (Hoffmann-Sommergruber et al., 2005). A similar approach was initiated in peach recently. It is assumed that genotypic variability of 4 allergen-coding gene families (Pru p 1–4) and different level of transcription could be responsible for different allergenic response (Zhong-Shan et al., 2016). The hypothesis is based on the assumption that allergenicity depends on both qualitative and quantitative factors that are different and specific to each variety (Chen et al., 2008). The hypothesis supports Gao et al. (2008) suggesting that the final structure of a protein and allele doses are linked to (hypo-) allergenicity. Protein structure is influenced by an origin gene sequence and as Ying-Tao et al. (2014) identified, in peach germplasm are three different allele

sequences with variable frequency in cultivars what could be link to varying Pru p 3 content (Aranzana et al., 2019). Differences in gene sequences are confirmed by this article. However, the identification and description are impeded by the multiplicity of isoallergens of peach allergen families and are not sufficient by traditional immunological techniques. The real-time RT-PCR method, which is also a powerful tool for monitoring and quantifying gene expression, enables specificity and high sensitivity. The specificity given by the primers in RT-PCR is able to distinguish isoallergens at the transcribed mRNA level, allows to work with a single isoform at once and to provide information about isoforms related to peach allergy and shifts them to proteomics (Helsper et al., 2002; Monaci and Visconti, 2009). A preliminary study on peach allergen encoding genes (Botton et al., 2009) did not cover all members identified by genomic research.

Plant non-specific lipid transfer proteins are ubiquitous and encoded by multigene families. They are involved in many biological processes and their physiological functions are not clearly understood (Chae et al., 2009). Peach lipid transfer protein was reported previously as to be highly conserved in its coding sequences in *Prunus persica* (Ying-Tao et al., 2014). The allergen coding gene has three members encoding Pru p 3.01, Pru p 3.02 and Pru p 3.03 lipid transfer proteins (Chen et al., 2008), however LTP1 (Pru p 3.01), 9 kDa protein with an isoelectric point >9 (Pastorello et al., 1999) is expressed at high levels in peach fruit (Yang et al., 2011). Lipid transfer protein genomic variability was described for different *Prunus* species by Ying-Tao et al. (2014) with the results of following substitutions in exon1: G/A in 13th nucleotide, G/A in 104th nucleotide, G/C in 121st nucleotide, G/A in 154th nucleotide, C/T in 266th nucleotide, G/T in 280th nucleotide, A/C in 316th nucleotide, G/C in 325th nucleotide and C/A in 344th nucleotide. These nucleotide variability results in natural variants of signal peptides of LTP1 proteins.

Pru p 3 gene and its expression is characterized only a few in the individual peach varieties (Carnés et al., 2002; Brenna et al., 2004). Expression levels of LTP1 was measured in apples and significant differences were detected among varieties (Bolhaar et al., 2005; Borges et al., 2006; Sancho et al., 2008). Non-specific lipid transfer protein of peach was analysed for its expression previously and was characterized as two expressed isoforms – LTP1 and LTP2. LTP1 is expressed in pollinated flowers preferentially and LTP2 in ovary. In fruit, only LTP1 mRNA was detected (Botton et al., 2002, 2009). These analyses were performed for the peach varieties Springcrest, Royal Gem and Zorzi.

4 Conclusions

The Pru p 3 gene amplicons of the non-coding region were used in the RFLP analysis by three different restriction enzymes *Bfal*, *MseI* and *NlaIII*. The primers were designed for PCR according to the sequence of NCBI database with accession number KC311811.1 in a manner to be capable of capturing multiple Pru p 3 allergen isoforms. The genomic variability was screened in a group of undefined peach varieties. *Bfal* restriction cleavage did not provide varietal specificity, unlike the next two restriction enzymes, where variability exists in restriction sites. For *NlaIII*, none of the analysed peach varieties correspond to the restriction profile of a virtual cleavage of Yulu peach variety. Obtained *in silico* and *in vitro* RFLP profiles do not match each other which points the necessity to obtain sequence records of each isoform, which would facilitate subsequent analysis.

For the future, construction of a phylogenetic dendrograms based on genomic sequences data could bring new insights into the development of allergen isoforms during the historic breeding of peach, find gene mutation sites, and ultimately successfully identify the specific area responsible for protein allergenicity.

Further research in the field could provide a simple and fast screening methodology for determining hypo-/hyper-allergenicity of the variety, which would benefit the final consumer.

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