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MOLECULAR CHARACTERIZATION OF ADVANCED MUTANTS FOR EARLY DETECTION OF HIGH β-CAROTENE CONCETRATION IN PEPPER BREEDING PROGRAMMS

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Abstract

The Bulgarian orange-fruited mutant cultivar Oranzheva kapiya is characterized with a high content of the antioxidant β-carotene. To identify the mutation leading to the accumulation of β-carotene and develop a suitable molecular marker for this trait, the genes for geranyl-geranyl pyrophosphate synthase, capsanthin-capsorubin synthase and β-carotene hydroxylase were studied both in the mutant and in the parental cultivar – Pazardzhishka kapiya. Our results suggest that the gene for β-carotene hydroxylase in the cv. Oranzheva kapiya is affected by a mutation, as well as the polymorphism between initial and mutant plants in this locus could be used as a molecular marker in the breeding programmes towards high β-carotene content.

Key words: molecular marker, *Capsicum annuum*, sweet pepper, β-carotene

Introduction. Carotenoids are among the most important metabolites which contribute to the fruit quality of sweet pepper (*Capsicum annuum* Linn.) as a non-nutritional source with antioxidant properties and/or bioactivity. They

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are the main non-enzymatic antioxidants which, thanks to their multiple functions, can improve human health [1]. Since the human organism is not able to synthesize them by itself, the main source of carotenoids is plant food. Daily doses of carotenoids, varying from 180 to 300 mg, can prevent the development of certain types of cancer and a number of severe chronic diseases [2]. Their deficiency is most often associated with protein/calorie malnutrition and affects over 120 million children worldwide [3].

Pepper is a traditionally-important vegetable crop in Bulgaria, which is consumed in large quantities and is an important source of bioactive substances like β-carotene. The expanding "healthy food" market justifies breeding new cultivars with improved quality and high added food value [4, 5]. Breeding programmes based on induced mutations could generate cultivars with improved phytonutrient level. For example, the orange-fruited pepper cultivar Oranzheva kapiya, characterized with an elevated content of β-carotene, was developed in a similar fashion out of the parental red-fruited cultivar Pazardzhishka kapiya 794. Recombinant inbred lines (RILs) through three consecutive back-crosses between initial (parental, wild type, wt) plants with red fruit and mutant plants with orange fruit were developed, followed by self-pollination and development of near isogenic lines (NILs) till M_8 generation, and the mutation was introduced into the pepper breeding programmes [6, 7].

The objective of the present study was to assess suitable breeding markers for early detection of high β-carotene concentration based on molecular characterization of the mutant pepper cultivar Oranzheva kapiya.

Materials and methods. ***Plant material.*** For this study, *Capsicum annuum* Linn. genotypes from the red-fruited local cultivar Pazardzhishka kapiya 794 (PK^{rf}) and the orange-fruited mutant cultivar Oranzheva kapiya (OK^{of}) were used. Seeds from the parent Pazardzhishka kapiya 794 were treated with X-ray irradiation, orange-coloured fruit plants (induced mutant – M) were identified, and a cultivar was developed through subsequent breeding [8].

DNA manipulations. Genomic DNA was isolated from the first true leaves of the PK^{rf} and OK^{of}, using Nucleon PhytoPure Kit (Amersham) following the recommendations of the manufacturer.

PCR reactions were performed using 100 ng DNA template with PuReTaq Ready-To-Go PCR Beads and 5–25 pmol primers (Amersham). Primer annealing temperature was estimated with the online tool: `http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx`

The amplified PCR products were isolated from the agarose by QIAquick Gel extraction kit (Qiagen) according to the original protocol, and then used for A/U cloning by applying Qiagen PCR Cloning Kit. The ligation reactions were mixed with 250 µL freshly-prepared chemically-competent bacterial (*E. coli*-TOP 10 – Invitrogen) cells and the transformation was induced by thermal shock (42 °C for 30 s). Successful transformants were determined by colony PCR and were further

propagated for plasmid preparation by QIAprep Spin Miniprep Kit (Qiagen) sent for sequencing by external service company.

Homology identification of the obtained sequences as well as protein structure analyses were performed using the services at NCBI web site `http://www.ncbi.nlm.nih.gov/`

Results and discussion. Biochemical evaluation performed preliminarily on PK^{rf} and OK^{of} genotypes demonstrated higher β-carotene levels in the obtained orange-fruited mutants [5, 12]. To shed light on the molecular basis of this advantageous mutation, we decided to isolate and characterize key genes of the carotenoid biosynthesis.

Isolation of geranyl-geranyl pyrophosphate synthase (GGPPS) from initial and mutant pepper cultivars. Geranyl-geranyl pyrophosphate synthase catalyzes an important reaction in the acetate pathway for secondary metabolite biosynthesis and serves as a branching point toward biosynthesis of carotenoids, chlorophylls, quinones, etc. Since GGPPS is the first enzyme in the carotenoid biosynthetic pathway, a mutation in its gene can cause dramatic changes of the downstream metabolic flows and can ultimately result in different pigment distribution. Therefore, *Ggpps* was the first target gene to be tested for differences between PK^{rf} and OK^{of} genotypes.

Ggpps was amplified and sequenced from parent and mutant pepper cultivars. The analysis demonstrated amplification of a product with the expected length. The sequenced fragments were homologous to the sequence published in NCBI Gene Bank except for nucleotide 558, where there is a difference (a transversion from G to C) (data not shown). The observed transversion is presented in both the parental and the mutant cultivars and is silent since it is located in the $3'$ end of the alanine codon GCG in the published sequence, which is converted into GCC in PK^{rf} and OK^{of}. For these reasons, we assume that this mutation cannot lead to any functional differences in the GGPPS activity between the red- and the orange-fruited Bulgarian cultivars. This result also strongly suggests that high β-carotene concentration does not result from increased biosynthesis of its precursor.

Isolation of capsanthin-capsorubin synthase (CCS) from parental and mutant pepper cultivars. The enzyme which catalyses the formation of the pepper-specific pigments with red-colour capsanthin and capsorubin from the yellow pigments antheraxanthin and violaxanthin respectively, is called capsanthin-capsorubin synthase (CCS).

Since CCS in pepper is strongly induced at the time of fruit ripening and is able to take on the function of weakly-expressed β-cyclases, it might be considered as an active player in the regulation of the metabolic flow through the carotenoid cycle. Furthermore, it is logical to assume that the colour differences between Pazardzhishka kapiya 794 and Oranzheva kapiya are caused by a mutation leading to an impairment of CCS-activity and subsequent inability of the

mutants to accumulate red pigments. To test this hypothesis, we isolated, cloned and sequenced the *Ccs* from PK^{rf} and OK^{of} cultivars.

Comparison of the *Ccs* sequences of the initial and mutant cultivars revealed one difference between the two genotypes and one difference in the Bulgarian cultivars with the published sequence (Acc.No.GU122933) (Fig. 1).

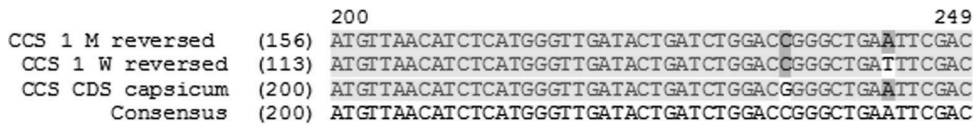

Fig. 1. Alignment of CCS fragments from Oranzheva kapiya (row 1, M) and Pazardzhishka kapiya (row 2, W) compared with the published reference CCS sequence of *C. annuum* (row 3). Row 4 shows the consensus sequence

In-frame translation of the sequences revealed two types of changes in the corresponding CCS amino-acid sequences. The change of G to C at position 235 leads to a replacement (denoted as G79R) of glycine G79 with arginine (R) in the protein sequence. This difference is established in the Bulgarian cultivars when compared to the sequence published in PubMed by Bouvier et al. [9].

The change of A to T at position 243 leads to a conservative replacement (denoted as E81D) of the aspartic acid E81 with the highly-similar glutamic acid (D) in the protein sequence. This is the only detected difference in CCS between the red-fruited parent cultivar and the orange-fruited mutant progeny.

G79R causes the appearance of a strong positive charge in the protein molecule due to the presence of arginine, while E81D is a conservative substitution. Nevertheless, one could hypothesize that the R79/E81 combination in the mutant enzyme might lead to impaired catalytic activity compared to R79/D81 in the parental cultivar gene and G79/E81 in the published sequence. This possibility needs to be further investigated in the future. More detailed analysis will allow the verification or rejection of the putative effect of these replacements on the enzyme structure and activity.

Characterization of polymorphism in CrtZ gene in pepper. Since β-carotene hydroxylase (*CrtZ*) catalyzes the direct conversion of β-carotene to β-cryptoxanthin, a plausible explanation of the accumulation of β-carotene in the mutant orange-fruited pepper cultivar could be the inactivation of this enzyme by a mutation. Therefore, the β-hydroxylase was chosen as the next target gene to compare the sequences in PK^{rf} and OK^{of} cultivars.

In pepper, two β-hydroxylase genes have been described – *CtrZ* and *CrtZ*-2. The encoded proteins show 72% homology across their sequence [10]. PCR amplifications of different fragments and internal regions of β-carotene hydroxylase (*CrtZ*) in both the red- and orange-fruited cultivars were performed with different combinations of specific primers. These primers were based on the published

sequence of the gene (Y09225) and previous cloning reports regarding the organization of the gene in the genome (Table 1) [10, 11].

The *CrtZ*-E and *CrtZ*-E/A primer combinations were chosen to amplify a fragment from the 5′-terminal region of the gene, including the start codon with expected fragment lengths of 847 and 911 bp respectively. Electrophoretic comparison showed monomorphism in the amplified fragments (1500 bp and 2000 bp) of the initial and in most of the mutant.

No amplification was achieved with primers designed to include the stop codon of *CrtZ* both, the initial and the mutant (data not shown on Table 1).

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PCR amplification of *CrtZ* fragments with specific primers for *β*-hydroxylase in pepper

Gene fragments	Minimal length expected according to known cDNA, bp	Primer sequences	Comparison between PK^{rf} and OK^{of}	Obtained length in genomic DNA, bp
CrtZ-A -F	913 – 18–930	TGG CAT GTA CCG ACG ACT TTA	no A	–
CrtZ-A-R	–	TTC CAA CTC TTC AAT TAC CCC		
CrtZ-B-F	516 – 15–530	GCA TGG CAT GTA CCG ACG A	no A	–
CrtZ-B-R	–	CAT CTC GCC CAG TAC TCC AT		
CrtZ-C-F	279 – 607–885	CTC GAC TTG CTA TAA AAA CGG	P	700
CrtZ-C-R	–	TAG GAA CAA GCC ATA TGG GA		
CrtZ-D/C-F	359 – 526–885	TCT ACC CGC GTA TCT CGT GAT	P	800
CrtZ-D/C-R	–	TAG GAA CAA GCC ATA TGG GA		
CrtZ-D-F	341 – 526–866	TCT ACC CGC GTA TCT CGT GAT	P	750
CrtZ-D-R	–	ACC CCA TCA AAT TTG TCC GA		
CrtZ-E-F	847 – 20–866	GCA TGT ACC GAC GAC TTT AA	P	1500
CrtZ-E-R	–	ACC CCA TCA AAT TTG TCC GA		
CrtZ-E/A-F	911 – 20–930	GCA TGT ACC GAC GAC TTT AA	P	2000
CrtZ-E/A-R	–	TTC CAA CTC TTC AAT TAC CCC		
CrtZ-E/B-F	511 – 20–530	GCA TGT ACC GAC GAC TTT AA	no A	–
CrtZ-E/B-R	–	CAT CTC GCC CAG TAC TCC AT		
CrtZ-C/D-F	259 – 607–866	CTC GAC TTG CTA TAA AAA CGG	P	580
CrtZ-C/D-R	–	ACC CCA TCA AAT TTG TCC GA		
CrtZ-D/A-F	404 – 526–930	TCT ACC CGC GTA TCT CGT GAT	no A	–
CrtZ-D/A-R	–	TTC CAA CTC TTC AAT TAC CCC		

Legend: * no A – no specific amplification; P – polymorphism; the start codon – at the 25th nucleotide of the 5′-chain of *CrtZ*; the stop codon – ending at the 972nd bp of the published *CrtZ* sequence

Lack of amplification or no reliable amplifications were noticed with some of the primer combinations *CrtZ*-A, *CrtZ*-D/A and *CrtZ*-E/B (data not shown). We suggest that differences between the studied genomic sequences of parent and mutant cultivars with the published genotype sequence used for the primer design hampered the correct primer design. The *CrtZ*-B primer pair generates a pattern that is generally uniform and monomorphic.

When the reverse primer in *CrtZ*-D and *CrtZ*-E started from the position 866 bp of the known pepper *CrtZ* cDNA, no fragment was amplified in the mutant cultivar.

The *CrtZ*-C primer combination was selected to amplify a fragment from the internal part of the gene. Polymorphism appeared between the initial and the mutant cultivar used for comparison in the investigation. Two bands were amplified in the initial genotype: the first one corresponded to 800 bp and the second one was 700 bp long. In contrast, in the mutant only the 800 bp band was detected (Fig. 2).

BLAST analysis of this primer pair with the available pepper sequences in the GenBank showed that the tested primer pair amplified a fragment from both the hydroxylase-1 (*CrtZ*) (Y09225) and hydroxylase-2 gene (*CrtZ*-2) (Y09722).

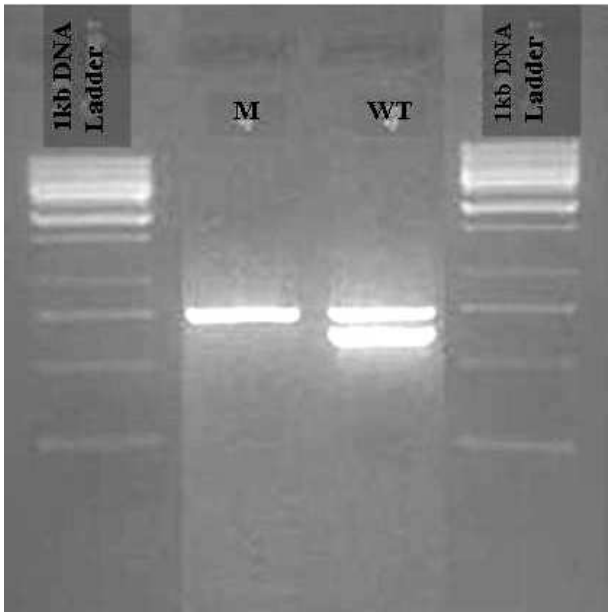

Fig. 2. PCR fragments of sweet pepper accessions from the studied parent and mutant with gene-specific primer pair *CrtZ*-C-F/R. Lanes *1* and *4*: 1 kb DNA Ladder; and PCR amplification patterns of lane *2*: M – OK^{of}, one fragment with ∼800 bp length; lane *3*: WT – PK^{rf}, two fragments with ∼800 bp and ∼700 bp length

Therefore, we suggest that in the mutant a fragment is amplified only from hydroxylase-2 due to a mutation present in *CrtZ*, which impairs proper primer annealing to the template. At the same time, in the parent were amplified fragments from both hydroxylase genes *CrtZ* and *CrtZ*-2. A sequence analysis of isolated *CrtZ*-C fragments from the parent and mutant confirmed this result (data not shown), proving that the amplified fragment in the mutants corresponded to the *CrtZ*-2 gene. On the basis of the DNA sequences, analysis of the protein structure demonstrated that the revealed fragment *CrtZ*-C belonged to the conservative domain of the protein. Therefore, a plausible explanation for β-carotene accumulation in mutant orange-fruited cultivar is that the mutation affects the active centre of β-hydroxylase, which results into the failure of conversion into the next product of the metabolic chain.

Conclusion. PCR investigation with internal gene-specific primers allowed us to suggest changes occurring within the 3'–terminal region of the mutant *CrtZ* gene, involved in pepper carotenoid biosynthesis, which may result in decreased or hampered enzyme activity.

The polymorphism in *CrtZ* revealed through the C and D/C primer combinations could be used as a marker for high β-carotene content. The presence of a fragment from *CrtZ*-2 amplified in the mutant genotypes by primer combination C is very useful as an internal standard (i.e. multiplex) co-dominant allele-specific marker (null). These data obtained on *CrtZ* gene structure can be used in pepper breeding programmes for transferring high β-carotene concentration into pepper genotypes with other valuable traits. It allows early selection of plants from segregating populations saving costs for developing new cultivars and F_1 hybrids.

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