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Established molecular marker in pepper mutants with orange fruit colour and application in crop breeding for high beta-carotene

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ABSTRACT

Advances of plant molecular biology and mutation technologies allow to utilize better the potentialities of mutation lines. The present study concerns the identification of a suitable molecular marker for early detection of high β -carotene content based on characterization of the advanced sweet pepper mutant lines created through irradiation with X-rays. Initial molecular studies achieved through PCR reactions with different internal gene-specific primers conduct to build a hypothesis for genetic changes occurring in the 3'-terminal region of the gene encoding the β -carotene hydroxylase involved in pepper *Capsicum annuum* Linn. carotenoid biosynthetic pathway: this change may result in loosing the enzyme activity.

Key words: *Capsicum annuum* L., molecular breeding, β -carotene hydroxylase, PCR

INTRODUCTION

Carotenoids, the plastid pigments, are among the most important metabolites for fruit quality in pepper being the main non enzymatic antioxidants which improve human health thanks to their multiple functions (Hirschberg, 1998). However the human organism is not able to synthesize them by itself and for this reason the main source of carotenoids are plant foods. Usual doses of carotenoids, varying from 180 to 300 mg/day, can prevent development of certain types of cancer and many severe chronic diseases such as cardiovascular, respiratory, urinary, and intestinal tract diseases (Gilman et al., 1990). Carotenoids affect positively vision, immune function, bone growth, reproduction, cell division and differentiation. Their deficiency is most often associated with protein/calorie malnutrition and affects over 120 million children worldwide (Hirschberg, 2001). Pepper is a traditionally important vegetable crop in Bulgaria, consumed in large quantities, providing human for bioactive substances such as β -carotene. The expanding “healthy food” market justifies strongly breeding new varieties with improved quality and high added food value (Kimura & Rodriguez-Amaya, 2003). Improvement programmes based on induced mutations could generate cultivars with improved phytonutrient level. The objective of the present study was to assess suitable molecular marker for early detection of high β -carotene content based on characterization of the advanced pepper mutant lines created through induced mutagenesis.

MATERIALS AND METHODS

The research was carried out on 11 red and orange fruited *Capsicum annuum* Linn. cultivars (cv) and lines. Physical treatment for induction of mutants was performed on pepper seeds of red fruited local Pazardzhishka kapiya 794 cv through X-rays-120 Gy and mutants (M) with orange colour of the fruit were obtained (Daskalov, 1991; Daskalov & Baralieva, 1992). Advanced orange fruited mutant generations were developed through three back crosses with wild type genotypes (WT) near isogenic lines (NILs) followed by self pollination recombinant inbred lines (RILs) till $M_{1,2-15}$. The altered character, orange fruit, determining higher β -carotene content, was incorporated into two other genotypes with red fruits. The three wild type lines (WT) and the corresponding mutant lines (M) were used in the present molecular study. Biochemical

evaluation performed preliminarily on WT and M lines demonstrated the higher β -carotene levels in the obtained orange fruited mutants (Tomlekova et al., 2007). PCR analyses were performed with different combinations of specific primers (Table 1) corresponding to the structural gene with GenBank accession number Y09225: this gene encodes the enzyme β -carotene hydroxylase (*CrtZ*), responsible for the conversion of metabolite β -carotene into product β -cryptoxanthin in the carotenoid biosynthetic pathway (Bouvier et al., 1998). Genomic DNA was isolated from first true leaf using PhytoPure Kit (Amersham) and CTAB protocol of FAO/IAEA (2001). PCR primers were designed by using GenBank cDNA sequences in The National Center for Biotechnology Information (NCBI). BLAST and protein structure analysis were performed using the web site <http://www.ncbi.nlm.nih.gov/>.

RESULTS AND DISCUSSIONS

The entire *CrtZ* gene was successfully amplified by fragments that pertained to the 5'-terminal, 3'-terminal and internal regions with all the rest of primer sequences shown in Table 1.

Table 1. PCR amplification with specific primers for the gene *CrtZ* in pepper

*A – amplification; M – monomorphism; P – polymorphism; the start codon - localized from the 25th nd at the 5'-chain of *CrtZ*; the stop codon - localized from the 972nd bp of the published *CrtZ* sequence.

The *CritZ-E* and *CritZ-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 847 and 911 bp, respectively. Electrophoretic comparison showed monomorphism in the amplified fragments (1500 bp and 2000 bp) of the WT and in most of the M. In few of the M a DNA fragment was not amplified and a polymorphism was observed.

No amplification was achieved with primers designed to include the stop codon on pepper genomic DNA of the WT and M (data not shown in Table 1). Lack of amplification or no reliable amplifications were noticed with some of the primer combinations after the performed PCR (*CritZ-A*, *CritZ-D/A*, *CritZ-B* and *CritZ-E/B*). We suggest that differences between the studied genomic sequences of WT and M, and the published genotype sequence used for the primer

Gene fragments	Minimal length expected according to known cDNA, bp	Primer sequences	NILs (WT/M)	Obtained length in genomic DNA, bp
<i>CritZ-A</i>	913 - 18-930	F TGG CAT GTA CCG ACG ACT TTA R TTC CAA CTC TTC AAT TAC CCC	no A	-
<i>CritZ-B</i>	516 - 15-530	F GCA TGG CAT GTA CCG ACG A R CAT CTC GCC CAG TAC TCC AT	no specific A	
<i>CritZ-C</i>	279 - 607-885	F CTC GAC TTG CTA TAA AAA CCG R TAG GAA CAA GCC ATA TGG GA	P in all M	700
<i>CritZ-D/C</i>	359 - 526-885	F TCT ACC CGC GTA TCT CGT GAT R TAG GAA CAA GCC ATA TGG GA	P in all M	800
<i>CritZ-D</i>	341 - 526-866	F TCT ACC CGC GTA TCT CGT GAT R ACC CCA TCA AAT TTG TCC GA	M & P in some of the mutants	750
<i>CritZ-E</i>	847 - 20-866	F GCA TGT ACC GAC GAC TTT AA R ACC CCA TCA AAT TTG TCC GA	M & P in some of the mutants	1500
<i>CritZ-E/A</i>	911 - 20-930	F GCA TGT ACC GAC GAC TTT AA R TTC CAA CTC TTC AAT TAC CCC	M & P	2000
<i>CritZ-E/B</i>	511 - 20-530	F GCA TGT ACC GAC GAC TTT AA R CAT CTC GCC CAG TAC TCC AT	no specific A	
<i>CritZ-C/D</i>	259 - 607-866	F CTC GAC TTG CTA TAA AAA CCG R ACC CCA TCA AAT TTG TCC GA	M & P in some of the mutants	580
<i>CritZ-D/A</i>	404 - 526-930	F TCT ACC CGC GTA TCT CGT GAT R TTC CAA CTC TTC AAT TAC CCC	no A	-

design, obstructed the right primer design.

The *CritZ-C* combination was selected to amplify a fragment from the internal part of the gene. Polymorphism was shown between each WT and M included in the investigation. Two bands were amplified in the WT genotypes: the first one corresponded to 800 bp and the second was made up of 700 bp. The 800 bp was amplified in all studied WT and M genotypes while the 700 bp band was amplified in all WT but not amplified in the M genotypes (Figure 1).

In all mutant fragments amplified with reverse primer C, started from the position 885 nd to 866 nd (combinations C, D/C), amplification of a 700 bp fragment for C combination and of a 800 bp fragment for D/C combination, was not observed.

BLAST analysis of primer C with the available pepper sequences in the GenBank showed that the tested primer pair amplified a fragment from the hydroxylase-1 (*CrtZ*) (Y09225), responsible for the conversion of the β -carotene into β -cryptoxanthin but also a fragment from the hydroxylase-2 gene (*CrtZ-2*) (Y09722), responsible for the conversion of the β -cryptoxanthin into zeaxanthin. In testing the segregation in F₂ population of the hybrid *C. annuum* x *C. chinense*, Thorup et al. (2000) found identical polymorphism in genes *CrtZ* and *CrtZ-2* and a high similarity of the sequences. Electrophoretic pattern in WT revealed two fragments while in M only one fragment was observed. Therefore, we suggest that in M a fragment was amplified only from hydroxylase-2, while in WT the fragments were amplified from both hydroxylase genes *CrtZ* and *CrtZ-2*. A sequence analysis of isolated *CrtZ-C* fragments from WT and M confirmed this result. 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 an explanation for β -carotene accumulation and the impossibility of this pigment to be converted into the next product of the metabolic chain could be the result of a probable sequence change of the mutant gene that affected the enzyme active center.

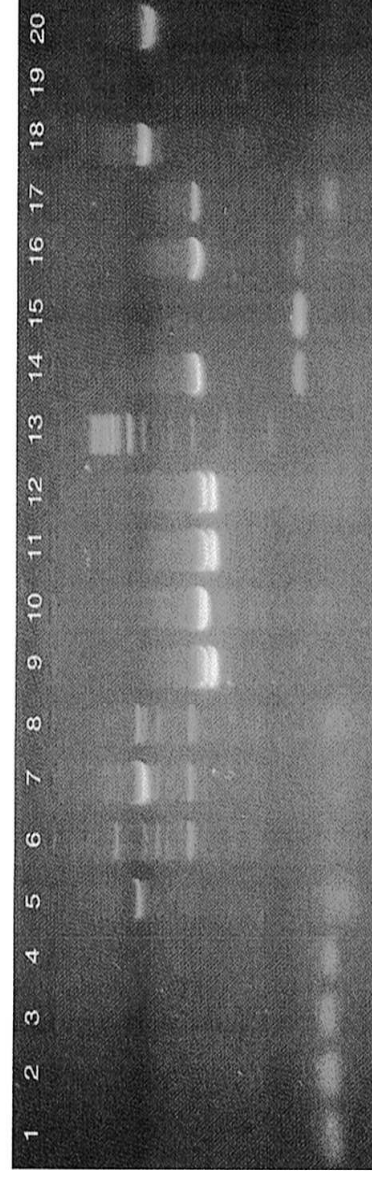


Figure 1. PCR fragments of sweet pepper *CrtZ* with gene specific primer combinations: 1-4 - *CrtZ-A* (WT₁, M, WT₂, WT₃); 5-8 - *CrtZ-B* - (WT₁, M, WT₂, WT₃); 9-12 - *CrtZ-C* - (WT₁, M,

WT₂, WT₃); 13 – 100 bp DNA Ladder; 14-17 – *CrtZ-D* – (WT₁, M, WT₂, WT₃); 18-20 – E-WT₁, M, WT₂)

When the reverse primer in *CrtZ-D* and *CrtZ-E* started from the position 866 nd of the known pepper cDNA in NCBI, a fragment was amplified in some of the M, while in other M no fragment was amplified. This result demonstrated that the breeding material containing high β -carotene levels in which the same mutant character incorporated was not homogeneous. Similarity of hydroxylases sequences was observed in pepper and tomatoes (Thorup et al., 2000), in *Arabidopsis* and in *Lycopersicon* (Hirschberg et al., 1998, 2001), despite of the different loci and number of copies determined for the genes.

CONCLUSIONS

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

The polymorphism in *CrtZ* revealed through the C and D/C combinations could be used as a marker for high beta-carotene. 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). Our initial molecular studies are with direct application in pepper breeding.

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