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- Note -

A Gene-based dCAPS Marker for Selecting *old-gold-crimson* (*og*^{c}) Fruit Color Mutation in Tomato

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The *old-gold-crimson* (*og*^{c}) fruit color mutation produces deep red tomato fruit with high lycopene content. *og*^{c} is a null mutation allele of lycopene β-cyclase (*Crt-b*) gene (*B* locus) that converts lycopene to β-carotene in the cartenoid biosynthesis pathway in tomato. Breeding of high lycopene tomato cultivars can be accelerated by marker-assisted selection (MAS) for introgression of *og*^{c} allele by using a gene-based DNA marker. In order to develop a marker, single nucleotide deletion of adenine(A) within a poly-A repeat that has been known to be responsible for frame-shift mutation of *og*^{c} was confirmed by resequencing mutant allele and wild-type allele at *B* locus of several tomato lines. For allele discrimination and detection of *og*^{c}, derived CAPS (dCAPS) approach was used by designing a primer that artificially introduced restriction enzyme recognition site of *Hin* fI in PCR products from *og*^{c} allele. This dCAPS marker is co-dominant gene-based PCR marker that can be efficiently used for MAS breeding program aiming the development of high lycopene tomato.

Key words : Tomato, derived CAPS (dCAPS), marker-assisted selection (MAS), lycopene, *old-gold-crimson* (*og*^{c})

Introduction

Tomato (*Lycopersicun esculentum* Mill.) is an economically important vegetable crop that is widely grown around the world including South Korea. Tomatoes are important nutrient source for vitamin A and C, minerals, and phenolic antioxidents, like carotenoids [3]. In the group of pigments comprising the carotenoids in tomato fruits are antioxidant lycopene and β-carotene which accumulate dramatically with fruit color changes during fruit development [1,7,8].

Two distinguishable mutant phenotypes of Beta (*B*) and *old-gold-crimson* (*og*^{c}) have been identified relating to the pigmentation of tomato fruit. Beta phenotype is conferred by partially dominant single allele mutation that causes orange color of fully ripened fruit because of the accumulation of β-carotene at the expense of lycopene. *og*^{c} phenotype is controlled by a recessive allele and produces deep red fruits that lack β-carotene but contain relatively higher amount of lycopene compared to wild-type tomato. The gene responsible for both phenotypes is allelic and single locus mutation of lycopene β-cyclase (*Crt-b*) at *B* locus, which is an enzyme that converts lycopene to β-carotene in the cartenoid biosynthesis pathway in tomato [7].

Breeding of tomato cultivars with increased lycopene content can be accomplished by introgression of the *og*^{c} allele into other cultivated tomatoes [3,6]. Generally, introgression of specific genes can be facilitated by using molecular markers in marker-assisted selection (MAS) breeding program, given that markers tightly linked to the gene of the trait are available. *og*^{c} allele has been cloned, and it has been elucidated that an in/del of single nucleotide A (Adenine) and resulting frame-shift mutation was attributed to the null function of the *B* gene and *og*^{c} phenotype. This gene sequence information can be greatly useful for the development of gene-based DNA marker for the selection of *og*^{c} phenotype.

Allelic discrimination and detection of single point mutations [single nucleotide polymorphisms (SNPs)] can be easily conducted by cleaved amplified polymorphic sequence (CAPS) marker when SNPs create or disrupt a restriction enzyme recognition site, or by designing probes used in hydrolysis or melting curve analysis for automated SNP allele detections [2,4,6]. However, in the case of *og*^{c} allele, neither approach can be successful because the in/del of single adenine resulting in the frame-shift mutation locates at a poly-A region (tendomly repeated 9-bp adenines in wild-type allele and 8-bp adenines in *og*^{c} allele) of the coding sequence. A modification of the CAPS, called derived CAPS (dCAPS) technique does not need for the SNP to exist within an restriction enzyme recognition site, but an artificial restriction site which includes the SNP is introduced into the

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PCR product by a primer containing one or more mismatches to template DNA [5,9].

In this report, we demonstrate a successful case of applying dCAPS technique in effort to develop a gene-based, co-dominant DNA marker for og^c allele. This marker can be efficiently used in MAS breeding programs for high lycopene trait in tomato.

Materials and Methods

Plant materials

Two mutant types and wild-type tomato lines were used for resequencing and confirmation of the lycopene β-cyclase gene (*B* locus) and identification of SNP variation for dCAPS marker development. Line FL8044 and LA3311 were used for the source of og^c mutant allele. F0653307 and hybrid Bella Rosa were used for *B* mutant allele phenotype and control and wild-type control, respectively. The plant seeds were provided from Sakata Seed America, Inc (CA, USA).

DNA sequence conformation of each allele

Genomic DNA from each tomato line was extracted from young leaves by using Plant DNasey kit following the manufacturer's instructions. PCR primer sequences used for resequencing each allele of lycopene β-cyclase gene are listed in Table 1. All PCR amplification was performed in a total volume of 20 μl containing 20 ng of genomic DNA, each forward and reverse primer at 0.3 uM, 1× PCR buffer, 0.2 mM dNTPs, and 0.6U of Taq polymerase (Qiagene, Valencia, CA, USA) with the following cycling profile: 1 cycle of 5 min at 95°C, 35 cycles of 30 sec, at 95°C, 30 sec, at 60°C, and 30 sec at 72°C, and 1 cycle of 7 min at 72°C for an extension step. PCR products were separated on a 1.5% agarose gel containing TBE at 80 V for 1 hr, and visualized under UV light after staining with ethidium bromide.

PCR products were purified by using QIAGEN MinElute PCR purification kit (Qiagene, Valencia, CA, USA) following the manufacturer's instruction. Purified PCR fragments were directly sequenced by dye termination method using ABI3730 capillary DNA sequencer. DNA sequences of the PCR fragments from each tomato line were aligned using ClustalW sequence alignment software.

Development of dCAPS marker for ogc allele

The conversion of SNP into dCAPS by artificial introduction of restriction sites was computationally conducted using a program called, SNP2CAPS. Three candidate dCAPS primers found by the program and their restriction enzymes are listed in Table 1. PCR for allele discrimination by dCAPS markers was conducted under the same condition described above, except that annealing temperature of 55°C was employed.

Restriction enzyme digestion of PCR products was conducted by adding 1 U of each enzyme to 10 μl of PCR products in a total volume of 20 μl and incubating at 37°C overnight Electrophoresis was conducted on a 2.5% regular agarose gel for at 100 V for 3 hr.

Results and Discussion

To confirm the existence of SNP among the three different *B* alleles of og^c type, *B* type, and wild-type tomato line, each allele was sequenced and aligned together with Genbank sequence of wild-type lycopene β-cyclase gene (AF254793). The sequence alignment clearly showed that og^c type carried deletion of an adenine (A) at an poly-A repeat in a coding region, while other types did not (Fig. 1). Poly-A repeat of og^c contained 8 bp adenines, while *B* and wild-type allele

Table 1. Polymerase chain reaction (PCR) primers used in the development of dCAPS marker for og^c allele detection

Purpose[a]	Primer name	Primer sequence (5′-3′)[b]	Restriction enzyme[c]
B locus resequencing	BF	GATGGCCGTGCCCTTCTTCATCCTCG/	-
	BR	CATGGCCACGGCGATGAGGATGC	-
dCAPS forward primers	Apo-BF	CTAAGTCCCACCACCAAAA**AAA<u>T</u>**/	*Apo* I (R,AATTY)
	Hin-BF	CTAAGTCCCACCACCAAAAA**<u>G</u>AA**/	*Hin* fI (G,ANTC)
	Sau-BF	CTAAGTCCCACCACCAAAAAA**<u>G</u>A**/	*Sau* 3AI (,GATC)
dCAPS reverse primer	Hin-BR	GACCAGCCACACTGGGAC/	-

[a]dCAPS forward primers were designed using SNP2CAP program to introduce artificial restriction enzyme recognition sites in PCR products from og^c allele.
[b]Artificially introduced mismatch nucleotides were underlined.
[c]The name of restriction enzymes used for allele discrimination and their recognition sequence.

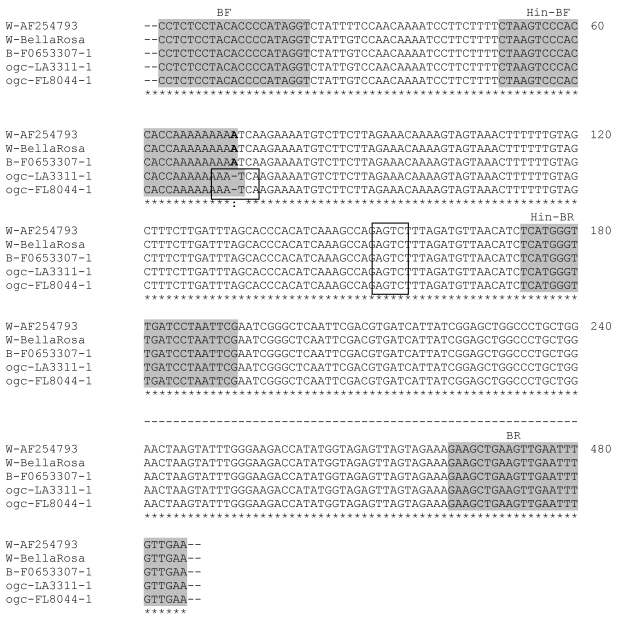

Fig. 1. DNA Sequence alignment of PCR amplicons produced by B locus-specific primers from different tomato lines, Bella Rosa (wild-type allele), 'F0653307-1' (*B* mutation allele), and 'LA3311-1' and 'FL8044-1' (og^c mutation allele) with Genbank sequence (AF254793) of wild-type allele. Each primer binding site is indicated by closed gray boxs and the name of each primer is located on top of the boxes. The restriction enzyme recognition site of *Hin* fI was indicated by transparent boxes.

contained 8 bp adenines [7].

By using SNP2CAPS [9], three possible primers were found generating derived CAPS at poly-A SNP site: first primer sequence had T instead of A at 8th A of poly-A and introduced *Apo* I (R,AATTY) restriction enzyme recognition site in the PCR products from og^c allele, but not from wild-type or *B* allele. Second primer sequence had G instead of A at 6th A of poly-A and introduced an *Hin* fI (G,ANTC) site in PCR product from og^c allele, but not from wild-type or *B* allele. Third primer sequence had G instead of A at 7th A of poly-A and generated *Sau* 3AI (,GATC) site in PCR product from og^c allele, but not from wild-type or *B* allele. The dCAPS primer sequences, PCR-extended sequences and artificially generated restriction site are shown in Table 1 and Fig. 1.

Among these three primers, primer 2 carrying *Hin* fI restriction site was chosen for the development of dCAPS marker. Enzyme cutting at the primer site results in only 18 bp length difference of PCR products between og^c and wild-type or *B* type allele as shown in Fig. 2. Therefore, a revere PCR primer needed to be designed to produce PCR

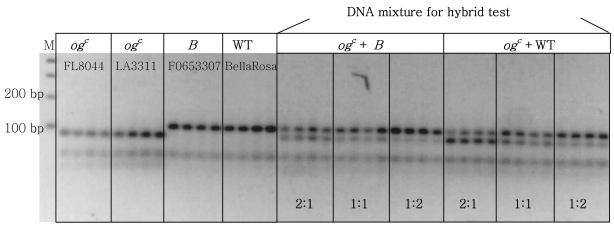

Fig. 2. Agarose gel image showing the genotyping results of Hin fI dCAPS marker for og^c mutation allele; 103 bp and 39 bp for *B* and wild-type (WT) allele, 83 bp, 39 bp, and 20 bp for og^c allele. Marker genotype and phenotype matched well, and hybridity test using DNA mixture indicated availability of the dCAPS for detecting heterozygotes. M=100 bp DNA ladder.

fragments of minimum size for effective separation of DNA fragments on a regular agarose gel. Moreover, we found an additional *Hin* fI restriction recognition site about 100 bp downstream of the primer 2. This *Hin* fI site was conserved in all alleles of the *B* gene sequenced in this study, and reverse primer was designed very next to this site so that the primer pair amplify 142 bp-long DNA fragment (Fig. 2).

This primer pair was evaluated for dCAPS genotyping. Two og^c type tomato lines, FL8044 and LA3311, one beta(B) type, F0653307-1, and one wild-type cultivar, Bella Rosa were included for the genotyping test of the dCAPS marker. Since plant material carrying heterozygous ogc allele was not available in this study, artificial hybrid DNA samples were created by mixing genomic DNA sample of og^c type (FL8044) with B type (F0653307) or wild-type (Bella Rosa) in three different ratio of 2:1, 1:1, and 1:2.

Electorphoresis of the PCR products by primer 2 after *Hin* fI digestion clearly showed the separation of PCR bands of expected sizes: 103 bp and 39 bp for B and wild-type allele, 83 bp, 39 bp, and 20 bp for og^c allele. In addition, the presence of PCR bands for each allele was observed from DNA mixture samples for hybridity test, indicating the prime pair being an efficient marker for heterozygous allele detection.

In conclusion, dCAPS technique used in our study was an effective approach to develop a PCR-based marker from a SNP for which CAPS or probe-based SNP technique can't be easily applied, as implied by the adenine SNP within a poly-A repeat of og^c allele. The dCAPS developed in this study is co-dominant PCR-based marker that targets for SNP directly responsible for null function mutation of lycopene β-cyclase. This marker can be useful in MAS breeding of og^c phenotype and in improving lycopene content in tomato fruit.

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초록 : 토마토 과색 돌연변이 유전자(old-gold-crimson) 선발을 위한 dCAPS 분자표지 개발

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old-gold-crimson (og^c) 과색 돌연변이는 라이코펜의 함량이 증가된 진붉은색 토마토 과실을 생산한다. 이러한 돌연변이는 토마토의 carotenoid 생합성경로에 관여하여 라이코펜을 β-carotene으로 전환시키는 라이코펜 β-cyclase (*Crt-b*) 유전자(B)에 point mutation을 일으켜 정상적인 효소생성을 저해한다. 높은 함량의 라이코펜을 생성시키는 토마토 품종개발은 유전자 연관 DNA 마커를 이용한 분자표지이용선발(MAS)을 통해 가속화 될 수 있다. og^c 돌연변이는 라이코펜 β-cyclase(*Crt-b*) 유전자 내 poly-A 서열반복 지점에서 adenine (A) 단일 뉴클레오티드의 결손에 의한 frame-shift mutation에 의해 일어나며, 이러한 대립유전자의 분별과 검증을 위해 og^c 대립유전자로부터 합성되는 PCR 산물에 *Hin* fI 제한효소 인식부위가 인위적으로 생성되도록 PCR 프라이머에 단일 뉴클레오티드 mismatch 부위를 만들어 dCAPS 마커를 개발하였다. 본 dCAPS 마커는 유전자 유래의 공우성 PCR 마커로서 고함량 라이코펜 토마토개발을 위한 육종 프로그램의 MAS에 효과적으로 사용될 수 있다.