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Research Report

Sequence Variation in *SlMYB12* is Associated with Fruit Peel Color in Pink Tomato Cultivars

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Abstract. The peel of pink-colored tomato is transparent due to the lack of accumulation of the flavonoid naringenin chalcone during ripening. A strong correlation was found between flavonoid expression and the function of SlMYB12, which is a transcriptional regulator of flavonoid biosynthesis. Thus, S*lMYB12* is a strong candidate gene underlying the pink phenotype. Three allelic variants, a 603 bp deletion, a nucleotide substitution (C > T), and a 1 bp insertion (TG > TAG) in the *SlMYB12* gene have been previously reported. We performed PCR genotyping based on these three allelic variations in 47 tomato cultivars displaying either a pink or red phenotype. However, the genotype did not match with the expected phenotype in one pink cultivar "Prime Alexander". This cultivar was therefore self-pollinated to produce 20 progeny plants. To identify new mutations in *SlMYB12*, the sequence of genomic DNA and CDS were compared between the progeny 17 and the reference line, Heinz 1706. A novel G > T nucleotide substitution was found in the 2nd intron. This SNP leads to a deletion of 7 bp (GTAACAG) from the end of the 2nd exon, resulting in a premature stop codon. The presence of this SNP associates the pink phenotype with the genotype. This novel SNP will be useful as a genetic marker for marker-assisted breeding of pink tomato.

***Additional key words*:** flavonoid, genotype, phenotype, SNP, tomato peel

Introduction

In widely consumed vegetable crops like tomato (*Solanum lycopersicum*), fruit color is an important consumer trait. In Asia, consumption of pink-colored tomatoes is very popular (Ballester et al., 2010). Fruit color is produced mainly by plant pigments such as carotenoid and flavonoids. Carotenoids are 40-carbon isoprenoid compounds that accumulate in flowers and fruits as orange, yellow, and red colored pigments. As accessary pigments, they serve important photoprotective functions during photosynthesis and function in attracting pollinators and seed dispersers. Tomato fruit contains the red pigment lycopene as the major carotenoid, along with β -carotene, phytoene, violaxanthin, and lutein (Ronen et al., 2000; Grotewold, 2006).

Flavonoids are another large group of plant pigments with wide-ranging functions including in pollination, pathogen protection, and seed dispersal. Flavonoids are widely distributed as red, purple, and blue pigments (anthocyanin), with some as yellow pigments (chalcones and aurones) (Harborne, 1986; Harborne and Williams, 2000; Tanaka et al., 2008). Although carotenoids and flavonoids both play a role in determination of tomato fruit color, flavonoids accumulate predominantly in the fruit peel. Naringenin chalcone is the most abundant yellow pigment flavonoid that accumulates in the peel during ripening (Hunt and Baker, 1980). Pink tomato lacks naringenin chalcone in the peel, which leads to a transparent appearance and pink fruit color. Naringenin chalcone is produced by chalcone synthase (CHS) from p-coumaroyl-CoA and malonyl- CoA and subsequently converted into naringenin (Nar) by chalcone isomerase (CHI) (Muir et al., 2001). The observation that the expression of many flavonoid genes is unaffected in the pink tomato line suggests that the gene underlying the pink phenotype encodes a regulatory protein of the flavonoid pathway rather than a biosynthetic enzyme (Bovy et al., 2002; Verhoeyen et al., 2002; Willits et al., 2005; Schijlen et al., 2007).

SlMYB12 is a transcription factor regulating flavonoid

Table 1. Primers and probe used in this study

Name	Sequence (5'-3')	Remarks
MYB12-603del-aF1	GTGACGAACAACCGACCTAGAATAA	SCAR marker for deletion
MYB12-603del-aR6	GCGGACAAAGTTAATTGGTCACTCA	
MYB12-603del-aR5	ATTCTAGCGTTATCAGTCGGCATACA	
MYB12F3	ATGCCGGTACGATTACCTACTAATCT	HRM primer for TG > TAG
MYB12R3	TCTTCCTCTTGAGAAGTAATGTTCCC	
MYB12-1P7	ATGCGGAAAGAGTTAGTAGACTACGAT	
MYB12F4	TATTTCGAAGGATTATTGAGATGCGGAAAGAG	HRM primer for SNP(C > T)
MYB12R4	AAGAAACAAAATGAGTGGTTTAATTAGCAAGCTAA	
MYB12Probe2P1	AACATTACTTCTCAAGAGGAAGATAT	
MYB12CDSF	TCATTGCCTTTTGCTTCTCCATTTTGT	CDS cloning
MYB12CDSR	CTAAGACAAAAGCCAAGATACAATGGTAC	
MYB12F3	ATGCCGGTACGATTACCTACTAATCT	gDNA cloning
MYB12-i2-cR2	CTAGCTCGAATCCATTACACTATGTTA	
SlMYB12-i2-aF1	GATTATTGAGATGCGGAAAGAGTTGT	HRM primer for SNP (G > T)
SlMYB12-i2-aR1	ACAAAATGAGTGGTTTAATTAGCAAGCT	
SlMYB12-i2-pF2	CTTTGGGTAACAGTTAATTAGTCAATTA	

biosynthesis in tomato, and its expression has been reported to be suppressed in the pink lines (Adato et al., 2009; Ballester et al., 2010). The pink trait was the result of a recessive *y* locus present on the short arm of chromosome 1 (Lindstrom, 1925; Rick and Butler, 1956). Genetic mapping, segregation analysis, and virus induced gene silencing results strongly suggest that a mutant allele (*slmyb12*) of *SlMYB12* gene is the genetic determinant of the *y* locus that underlies the pink phenotype (Ballester et al., 2010). Sequence analysis of *slmyb12* by Lin et al. (2014) revealed a 603 bp deletion in the upstream region (-4,865 bp from start codon) and two SNPs causing nonsense mutations (a nucleotide substitution (C > T) and a 1 bp insertion (TG > TAG)) in the 2nd exon. The deletion was hypothesized to cause a transcriptional repression in *SlMYB12* expression that would lead to decreased accumulation of naringenin chalcone in the peel, while the SNPs would introduce premature stop codons predicted to result in the pink phenotype (Lin et al., 2014).

In the current study, to explore allelic variation in the *SlMYB12* gene, we screened 47 tomato cultivars that displayed a pink or red phenotype. We found a novel SNP in the *SlMYB12* gene sequence which could be used as a genetic marker in aiding marker assisted selection of pink tomato plants for breeding.

Materials and Methods

Plant Material

The 47 tomato cultivars were obtained from the Tomato Life Science & Research Center-Korea, Nongwoobio Seed Co. Ltd-Korea, Bunong Seed Co. Ltd-Korea, and PPS Seed Co. Ltd-Korea. The information for cultivar names are given in Supplementary Table 1. The pink cultivar "Prime Alexander" was self-pollinated to produce F2 progeny and 20 plants were selected randomly for further study.

Genomic DNA and RNA Extraction

gDNA and total RNA were extracted from tomato fruit and leaf using the Plant DNA Isolation Kit (Qiagen) and RNeasy Plant Mini Kit (Qiagen), respectively, according to the manufacturer's instruction. High-quality gDNA and RNA was eluted in 30 μL nuclease and RNase free water, respectively. The extracted total RNA was treated with RNase-free DNase I (Qiagen) treatment before cDNA synthesis.

cDNA Synthesis

cDNA was synthesized from 1 μg RNA from all samples using the Superscript® III First Strand Synthesis Kit (Invitrogen) in a 20 μL reaction according to the manufacturer's instructions and utilized for PCR amplification. Briefly, 2 μg (8 μL) total RNA and 1 μL oligo dT (500 $\mu g \cdot mL^{-1}$) were mixed in a reaction tube and then heated at 65°C for 10 min. The tube was quick chilled on ice. The enzyme was then added into the tube and the tube was incubated at 42°C for 50 min. Finally, the tube was incubated at 70°C for 15 min to inactivate the enzyme. The cDNA was stored at -20°C until use.

PCR Amplification

For detection of the 603 bp deletion in 47 cultivars and 20 F2 progeny, PCR amplifications were carried out using a

Takara PCR Thermal Cycler in reaction mixtures (total volume of 20 μL) containing 1 × buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primers, 5 ng template DNA, and 0.5 units *Taq* polymerase (PROMEGA, Madison, USA). The PCR cycle conditions included initial denaturation at 94°C for 5 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 45 s, and a final elongation at 72°C for 5 min. The specific primers covered the full length deletion region. Primers are described in Table 1. The PCR product was separated using 1.5% agarose gel containing Tris-boric acid EDTA (TBE) buffer for 1 h and visualized under ultraviolet light.

SNP Detection using Hybprobe

For detection of single nucleotide polymorphisms (SNPs) in all cultivars and progenies used in this study, HRM analysis combined with 3'-blocked and unlabeled oligonucleotide probe (HybProbe) was used. PCR was performed using saturating dye SyGreen2 to generate melting curves characteristic for the genotype corresponding to the probe. Melting curves were generated and analyzed using a LightScanner® Instrument System. PCR involved initial denaturation at 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s, and then a final extension for 40 s at 72°C. Primer and probe sets for detection of all 3 SNPs are described in Table 1.

Cloning of *SlMYB12* Gene

The gene sequence and CDS were amplified from genomic DNA and cDNA, respectively, by polymerase chain reaction (PCR) using pfuTurbo DNA polymerase (Stratagene, Agilent technologies). The primers (Table 1) used in PCR were designed based on the sequence information available from Sol genomics network homepage (http://solgenomics.net). PCR amplification was carried out under the following conditions: an initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 2 min followed by a final extension at 72°C for 7 min. The PCR product was introduced into a blunt cloning vector using the Topcloner™ Blunt kit (Enzynomics, EZ002S). The ligation products were transformed into *E. coli DH5-alpha* competent cells. The recombinant plasmids were purified using the Plasmid Mini kit (Qiagen, 12125), and sequenced at Macrogen Corp. (Korea). Biological replicates of three colonies were sequenced.

Sequence Alignment

The obtained sequence was aligned with reference sequences (Solyc01g079620) from the Sol genomics network homepage (http://solgenomics.net).

Results and Discussion

SlMYB12 encodes the key transcriptional regulator for accumulation of the flavonoid naringenin chalcone in tomato peels, producing yellow peel color during ripening. The lack of naringenin chalcone results in transparent peel and pink-fruited tomato (Fig. 1A). *SlMYB12* is a candidate gene located

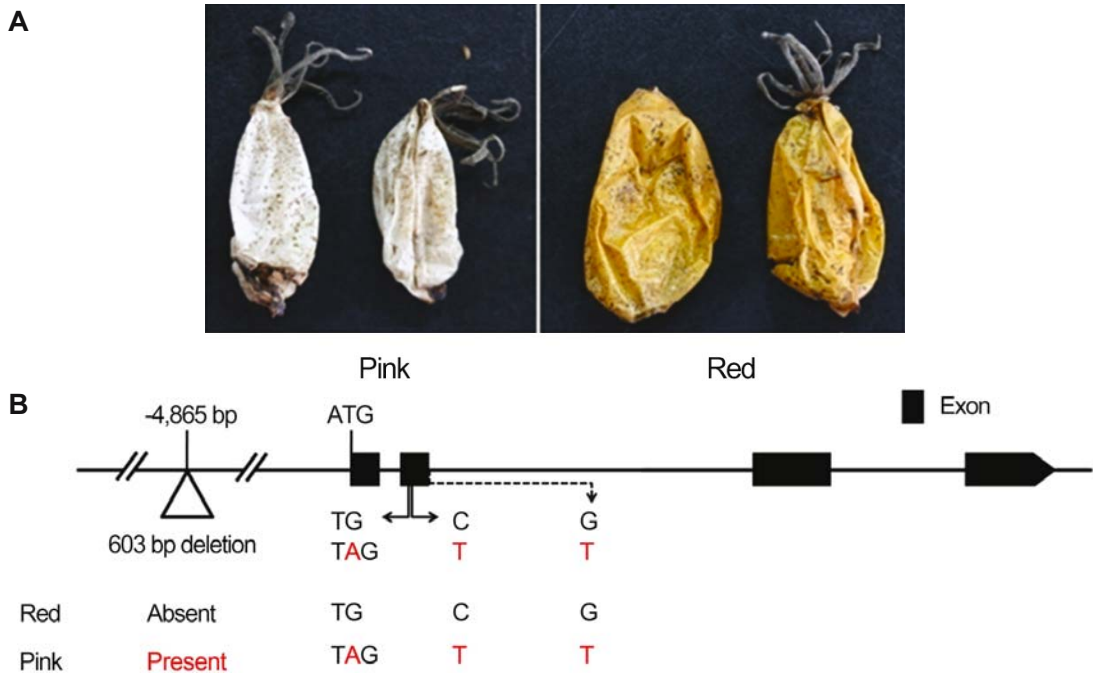

Fig. 1. (A) The peels of pink and red tomato, (B) The structure of *SlMYB12* showing the location of the 603 bp deletion and the SNPs in the 2nd exon (TG > TAG, C > T) and 2nd intron (G > T).

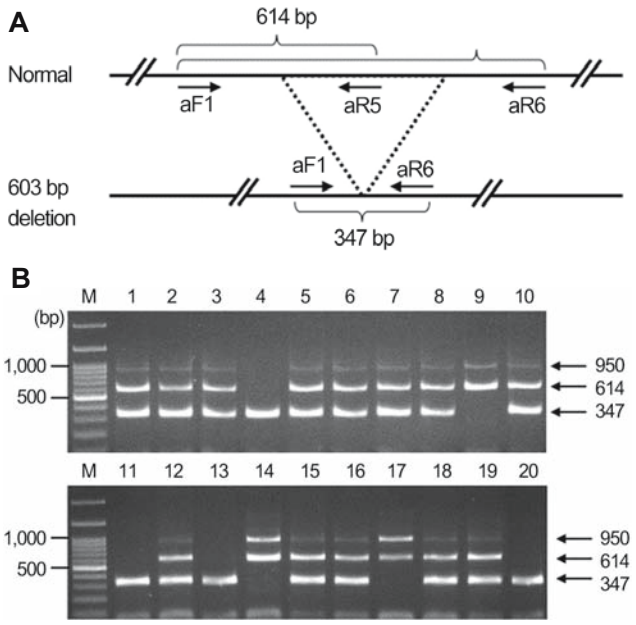

Fig. 2. (A) Representation of primer positions and product sizes for identification of the 603 bp deletion, (B) Representative gel image showing the corresponding genotype of 20 progeny plants. Two primer sets (aF1/aR6 and aF1/aR5) were used in independent PCR reactions and the products were then mixed and separated on a 1.5% agarose gel. Amplification with primer set aF1/aR6 produce 2 amplicons of 347 bp and 950 bp, corresponding to the presence or absence of the deletion respectively. The primer set aF1/aR5 is designed to identify the deletion and thus produce a single band of 614 bp in its absence. The presence of 2 bands (950 bp and 614 bp) confirms no deletion. A single band of 347 bp confirms the presence of deletion. Heterozygous plants display all three bands.

in the *y* locus of chromosome 1 responsible for the pink trait (Adato et al., 2009; Ballester et al., 2010). Lin et al. (2014) reported the presence of three allelic variants in the *slmyb12* gene, including a 603 bp deletion and two SNPs (C > T and TG > TAG) (Fig. 1B), in pink-fruited accessions. In the present study, we studied the association of these sequence variants with the fruit peel color of 47 tomato cultivars that displayed either a pink or red phenotype. Two sets of primers (aF1/aR6 and aF1/aR5) spanning the deletion region were designed to monitor the 603 bp deletion (Fig. 2A). After PCR amplification with the two sets of primers individually, PCR products were mixed and separated on a 1.5% agarose gel. The primer set aF1/aR6 produces a 950 bp product when no deletion is present and a 347 bp product when there is a deletion. Primer aR5 was designed in the deletion region; hence, amplification with the aF1/aR5 primer set produces a single band of 614 bp only if the deletion is absent. The genotypes for the homozygous presence or absence of the deletion were designated "N" and "D," respectively. The genotype "H" was heterozygous for the 603 bp deletion and produced all three PCR products (Fig. 2B). The two SNPs were detected using Hybprobe HRM analysis. All sequence variations we identified were consistent with the previous study (Lin et al., 2014) in both the red and pink cultivars with the exception of one pink cultivar "Prime Alexander". Neither of the two SNPs were found in this F1 hybrid cultivar, whereas we did observe the 603 bp deletion (heterozygous) in the region upstream (-4,865 bp) of the start codon of *SlMYB12* (Supplementary Table 1).

To further analyze genetic variation in *SlMYB12,* the Prime Alexander cultivar was self-pollinated and 20 progeny plants were randomly selected. The genotype for the 603 bp deletion is displayed in Fig. 2B. Four progeny (4, 11, 13, and 20) were homozygous for the deletion while three progeny showed no deletion (9, 14, and 17). The remaining thirteen plants were heterozygous for the deletion (Fig. 2B). Neither of the previously identified SNPs were found in any of the 20 selected progeny (Supplementary Fig. 1A and 1B). Although the upstream 603 bp deletion might be responsible for the pink phenotype, three progeny showed no deletion despite showing a pink phenotype. Therefore, the silencing of *SlMYB12* could be due to the accumulation of other deleterious mutations in the coding sequence (Lin et al., 2014). Thus, we searched for new variations within the gene sequence. Kim et al. (2015) recently reported other SNPs linked to the *SlMYB12* gene in domesticated tomatoes, but these variations were 165,033 bp - 217,966 bp away from *SlMYB12* gene.

We therefore evaluated both the gDNA sequence and CDS of *SlMYB12* in the F2 progeny plants. The gDNA and CDS sequences of *SlMYB12* of progeny 17 (which lacked the 603 bp deletion and both of the SNPs, Table 2) were cloned and sequenced. The alignment of these sequences with the reference line, Heinz 1706 (gene ID: Solyc01g079620 http://solgenomics.net) confirmed the presence of a novel nucleotide substitution (G > T) in the initial base of the 2nd intron, disrupting its 5′ splice site (Supplementary Fig. 2A). *SlMYB12* gene has canonical GT-AG consensus intron borders, where the GT dinucleotide is at the 5′ end and AG dinucleotide at the 3′ end of the intron. The nucleotide substitution (GT > TT) likely results in the use of an alternative splice site within the exon, as evidenced by a 7 bp deletion (**GTAACAG)** in the cDNA sequence at the end of exon 2 (Supplementary Fig. 2B). This 7 bp deletion leads to the production of a non-functional SlMYB12 protein by introducing a premature stop codon, resulting in a truncated peptide (89 amino acids vs the 388 amino acid full-length protein) (Supplementary Fig. 2C).

To validate the novel SNP, high resolution melting curve analysis (HRM), a simple and high throughput method, was used. An unlabeled oligonucleotide probe (Hybprobe) specific to the SNP site was designed (Table 1). This is a more accurate and inexpensive method for detection of SNPs compared to traditional fluorescence-labeled detection. Following PCR in all 20 progeny, post-amplification melting was performed and three different melting curves were observed (Fig. 3). The homozygous G/G (Normal) and T/T (Pink variant) genotypes

Table 2. Phenotype and genotype of the 20 progeny of cultivar "Prime Alexander"

Progeny	Phenotype	Genotype			
		603 bp deletion in the upstream region (-4,865 bp)	2nd exon 'A' insertion (TG > TAG)	2nd exon (C > T) nucleotide change	2nd intron (G > T) nucleotide change
1	Pink	H	-/-	C	T/G
2	Pink	H	-/-	C	T/G
3	Pink	H	-/-	C	T/G
4	Pink	D	-/-	C	G
5	Pink	H	-/-	C	T/G
6	Pink	H	-/-	C	T/G
7	Pink	H	-/-	C	T/G
8	Pink	H	-/-	C	T/G
9	Pink	N	-/-	C	T
10	Pink	H	-/-	C	T/G
11	Pink	D	-/-	C	G
12	Pink	H	-/-	C	T/G
13	Pink	D	-/-	C	G
14	Pink	N	-/-	C	T
15	Pink	H	-/-	C	T/G
16	Pink	H	-/-	C	T/G
17	Pink	N	-/-	C	T
18	Pink	H	-/-	C	T/G
19	Pink	H	-/-	C	T/G
20	Pink	D	-/-	C	G

N: No deletion, H: heterozygous for deletion, D: deletion, -/-: No insertion

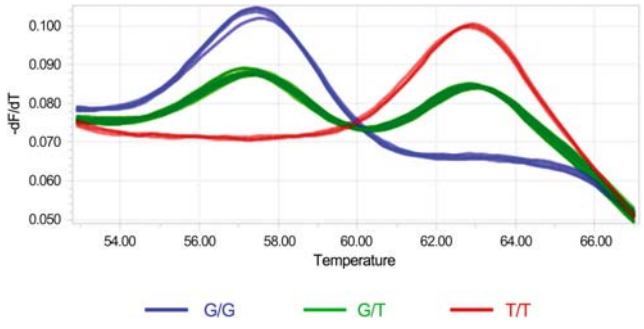

Fig. 3. SNP detection using Hybprobe. Three different melting curves are displayed for the 20 tested progeny. The blue melting curve represents the wild type (G/G) while the red curve displays the presence of the pink variant SNP (T/T). The heterozygous constitution (G/T) is shown as a green curve.

were each represented by a single distinguishable peak (blue and red, respectively) while the heterozygous G/T displayed two peaks (green) (Fig. 3). Table 2 summarizes the phenotype and respective genotypes for the 20 progeny.

Thus, the novel SNP we identified in *SlMYB12* associates the pink-fruit phenotype with the genotype of progeny of the Prime Alexander cultivar. This SNP was found in a homozygous constitution, T/T, in progeny lacking 603 bp deletion, whereas no SNP (G/G) was observed in progeny homozygous for the 603 bp deletion. The thirteen progeny plants heterozygous for the 603 bp deletion were also heterozygous for the SNP (G/T) (Table 2). The 603 bp deletion upstream of the start codon was hypothesized to impair the transcription of the *slmyb12* gene, whereas the SNP leads to a truncated protein (Lin et al., 2014). Overall, the pink trait is recessive; thus plants heterozygous for the deletion (Supplementary Table 1, Table 2) must also contain a SNP on the homologous chromosome. In conclusion, our results show that these two allelic varaints (the 603 bp deletion and the novel SNP in the 2nd intron) of *SlMYB12* could be used as markers for pink tomato breeding.

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