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CAPS Marker Linked to Tomato Hypocotyl Pigmentation

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Abstract. Tomato hypocotyl can generally be one of two colors, purple or green. Genetically, this trait is controlled by a single dominant gene. Hypocotyl tissue specific color expression is one of many visible genetic marker sources used to select tomato progeny. However, the visible marker does not show a clear distinction between homozygous genotype and heterozygous genotype from the breeding lines. Therefore, to identify a hypocotyl pigmentation related marker, we screened DNA polymorphisms in thirteen tomato lines showing purple or green hypocotyls. The markers used for screening consisted of primer set information obtained from anthocyanin related genes, conserved ortholog set II (COS II) marker sets localized near anthocyanin related genes, and restriction fragment length polymorphism (RFLP) markers localized near COS II markers, which produce polymorphisms between purple and green tomatoes. One primer from a RFLP fragment resulted in a polymorphism on agarose gel electrophoresis. From the RFLP fragment, a cleaved amplified polymorphic sequence (CAPS) marker was developed to distinguish between purple and green hypocotyls. The genotypes of 135 F_2 individuals were analyzed using the CAPS marker, and among them, 132 individuals corresponded to the phenotypes of hypocotyl pigmentation.

***Additional key words*:** anthocyanin related gene, cleaved amplified polymorphic sequence, conserved ortholog set II

Introduction

Pigmentation of flowers, fruits, leaves, stems, and roots is important for analyzing the phenotypic characteristics and determining the presence of nutritional antioxidants (Noda et al., 2000) in vegetables and ornamentals. Most pigments are regulated by genes encoding most of the enzymes in the anthocyanin biosynthetic pathway. Anthocyanin-related genes, such as *anthocyanidin synthase* (*ans*; Weiss et al., 1993), *rhamnosyltransferase* (*rt*; Brugliera et al., 1994), and *anthocyanin-1* (*an1*; Quattrocchio et al., 1993), have been isolated in petunia and their regulation has been characterized. In tomato, the genes responsible for coloration, such as *chalcone synthase* (*chs*; O'Neill et al., 1990), *dihydroflavonol 4-reductase* (*dfr*; Bongue-Bartelsman et al., 1994), and *anthocyanin1* (*ant1*; Mathews et al., 2003), have been cloned. Thirteen color genes, including *chs*, *dfr*, and *ans*, have been located on the genetic map of tomato (de Jong et al., 2004). In addition, morphological markers related to anthocyanin mutations, such as anthocyaninless (*a*), entirely anthocyaninless (*ae*), anthocyanin free (*af*), anthocyanin gainer (*ag*), and albescent (*alb*), were designated on the tomato map (Tanksley et al., 1992). All of these reported genes and mutations are related to the pigmentation of the fruit or whole plant. However, there is no data indicating which anthocyanin-related gene directly regulates hypocotyl pigmentation in tomato without affecting other organs.

Purple and green coloration has been observed in the tomato hypocotyl tissue. This type of hypocotyl pigmentation may be a unique phenomenon since hypocotyl coloration is not related to the pigmentation of other organs, such as the flower, stem, leaf, or fruit. Based on the observations of breeders at Nongwoo Bio Co., the green hypocotyl trait is linked to important characteristics such as GMS (genetic male sterility) and resistance to bacterial wilt, ToMV (tobacco mosaic virus) and FCRR (fusarium crown and root rot). Additionally, ToMV and FCRR resistance loci were previously reported to be strongly linked to each other

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(Vakalounakis et al., 1997).

Tomatoes with purple hypocotyls have been used for constructing elite lines through selfing and backcross in tomato breeding. Breeders have used hypocotyl color as a visual marker to trace the resistant traits through the introgression of green hypocotyl tomatoes into elite breeding lines of purple ones. However, this visual marker is limited in its use to distinguish between homozygous and heterozygous genotypes. In addition, the identification of hypocotyl color is difficult when the hypocotyl is planted in soil. Therefore, a DNA marker associated with hypocotyl color could be effectively used to select for disease resistant genes and genotypes from progeny, thereby saving time, labor, and the cost of breeding steps.

In this study, we developed a CAPS marker for distinguishing purple and green hypocotyls using the candidate gene approach with anthocyanin-related genes, followed by an analysis of COS II markers and RFLP markers linked to the anthocyanin-related genes. This CAPS marker could be used as a selectable genetic marker in a marker assistant selection (MAS) system for the identification of purple or green genotypes at any stage of tomato breeding.

Materials and methods

Plant Materials

Ten purple hypocotyl and three green hypocotyl tomato lines were used in the development of DNA markers of purple and green color from the information on anthocyanin related genes and linked markers. Purple type (NWp1) and green type (NWg3) tomato lines were crossed, and an F_1 plant from the cross was selfed to produce an F_2 population. The parents, the F_1 plant, an F_2 population, and a BC_1F_1 population, which was produced from a cross of the F_1 plant and NWg3, were used for genetic analysis. One hundred and thirty five plants from the F_2 population were randomly selected to confirm the developed marker. Hypocotyl pigmentation was determined by visually scoring two to three leaves of tomato seedlings.

Primer Design

Primer sets deduced from anthocyanin related genes (de Jong et al., 2004), producing more than 500 bp in size, were used for screening as PCR based markers. The other PCR primers (Table 1) for anthocyanin related genes were designed using Primer3 Input (http://frodo.wi.mit.edu/primer3)

Table 1. Primers designed from anthocyanin related genes.

Gene	Accession no.	Species	Fragment size (bp)	Primer sequence (5'-3')	Chr[z]
chs	U47738	Potato	600	F[y]: GCGACTCCTTCGAACTGTG	5
				R: AAGTTTTTCGGGCTTTAGGC	
tchs1	X55194	Tomato	294	F: CTGAGAACAACAAGGGTGCT	
				R: AGATAAGCCCAGGAACATCC	
tchs2	X55195	Tomato	286	F: CGACTCCTTCGAACTGTGTT	
				R: GGCTGACCCCATTCTTTAAT	
f3h	BG132926 (F)	Tomato	1800	F: GGGCACCTTCAACACTA	2
	BG735030 (R)			R: ATCGGCTCATCCATTAT	
dfr	Z18277	Tomato	1500	F: CACTCTCCTCCGAAGACGAC	2
				R: TCCATTGTCTGCAGTGCTTC	
tdfr	Z18277	Tomato	199	F: AGCTGGATTTATCGGCTCTT	
				R: TCATCAAAGCTTCCTTCCAC	
f3'5'h	AF081575	Petunia	600	F: TTTGTTCACAGCTGGTACGG	11
				R: AGAGGGACAGCTTTCTGCAA	
ans	X70786	Petunia	1000	F: AAGGAGATTCGCGAGAAATG	8
				R: GCCACACTGTTCATCCTCCT	
rt	X71060	Petunia	1500	F: GCTGAGCTTCTCAAGGTTGC	9
				R: ACCATCACCTTTTCCACAGC	
an11	U94748	Petunia	1000	F: TCAATCCCACCCACCACTAT	3
				R: CCCATCATCTCCACCTGAAC	
an1	AF260918	Petunia	1300	F: CGGCCCTAGTTATGATGAATTATC	9
				R: ACCTCCACTTTAAGTTCCCTTAGC	
ant1	AY348870	Tomato	478	F: GGAAGGACAGCTAACGATGTG	10
				R: GTTGCATGGGTGGTAAATTAAG	

[z]Tomato chromosome number for which the gene was mapped.
[y]F, forward primer; R, reverse primer.

Table 2. COS II markers localized near anthocyanin related genes.

Chr[z]	Marker	Primer sequence (5'-3')	Fragment size (bp)
2	C2At2g18030	F[y]: TTGGGCGACCACGCTGAATC	131
		R: TTACCCACATCAGGACCTTGCC	
	C2At2g18050	F: TGCCAAGGCTGTTACTCATCCTCC	221
		R: ATAGTTTGTATGAAGCCTTGATTTTG	
	C2At5g66090	F: ATCTCTCTGAGGGTTCAAGACAGG	-
		R: TATATCAGCTCCATACTTCTTTGC	
	C2At2g17695	F: AGATTTAGAGGAACTACTGAGAAACCTG	176
		R: AACGCCCAATTCAATCCAAAATGC	
	C2At3g51390	F: AGCGTGGACAAAGCTAGTGATGGATATG	109
		R: ATGACAATGTTTAGCGCGTGGAGG	
	C2At3g01160	F: TCTGAAGAAGCTGAAGCAAGTAGAGC	264
		R: TGCCAACTGACGAGCATAAGCTGC	
	C2At4g38630	F: TGGCTGGTAAAGGGGTTCGAG	182
		R: AGCAAAAACAATAATCCTTTGTTG	
	C2At3g51130	F: TACCTCTTGAGTTTCCAGATGGCAC	453
		R: TGTATGAATTAAAATCAGCATGCCC	
	C2At4g36380	F: TCCTCTGCCGTCTGATTTCATCAGTG	159
		R: TGCTGCCTCAATTCCAAATTCTCCTC	
	C2At5g66530	F: TTCAGGAATGGCATTTGCAAGTGTG	181
		R: ACCATTGAATACAGCATCTGGTCGAAC	
	C2At3g54350	F: AGGGGCCTTTGCCATCCTATATG	193
		R: TAGATTCTTCAGACAGAAGGATCCATC	
	C2At4g36530	F: AGATTTGCTAGGCTTTGGTTGGAG	225
		R: AGTGAATCCCCAAACTGTCCTGC	
9	C2At5g02230	F: TATCCCCTAAGTGCTGGTCTGGC	-
		R: TGGATAAAACTATGGTACTCATCATAGTC	
	C2At4g10360	F: TTGATGACCTGAAGAAACTTGAATGG	-
		R: AGTAATAGAAAATCATTGCCAAGTCTG	
	C2At4g33580	F: TTGGAGTTGAACAACCCCATTTGAG	-
		R: TCATTGGTCCCTTCCAGGCTTTC	
	C2At5g02020	F: AGCAAGCTGCACAAAACAAGCAGCC	-
		R: AGACGCACTGCCAGAATCATCTTC	
	C2At4g02730	F: AACCAACGACTCCACCCTACCTTCC	-
		R: TCATGACTACCCGAAACAATCAACG	
	C2At4g12740	F: AATGTGGGCTGGTTTGGGGTATTAT	425
		R: TCCTTCTTCAACCACCTCTTTTGCACC	
	C2At3g51880	F: AAAGAAAGATCCCAATAAGCCTAAG	-
		R: ACTTTTCTCCTCCAGCTTTCCCGAC	
	C2At2g38440	F: AACGGTTGCAACAAGGCCTAGTATTC	-
		R: TCGTCATCCTCTTCATCACTTCCAGC	
	C2At3g07180	F: ATTTCGACCCTAAAACCATGCG	-
		R: TGCCATCTACCGTAGCCATTTCC	
	C2At3g08670	F: ACGTCAAGGGACTTCTTAGAAATTGTC	-
		R: ACGAACTGCAGCTTCTCTTTCTCGTC	
	C2At3g25480	F: ACAAAGTATCCATTCTTTGTTGCTGG	-
		R: TTATCCCACCTCGGATAGCATAAGC	

Table 2. Continued.

Chr[z]	Marker	Primer sequence (5'-3')	Fragment size (bp)
10	C2At3g09740	F: TCCGACATAGAAACTGCTCTTCAG	96
		R: AGTCCTCCGAATCTCAGCATTG	
	C2At2g37510	F: TGGCAATTATGGCATTATTTCGTCG	169
		R: AGGTTTTCATCACCAGTGTATCTTGAAAG	
	C2At3g51840	F: TGATATGCATAAAGAGGCGAAAAGCTC	156
		R: ATGGCTTGCTCCTCAGGAGTCAACAG	
	C2At5g01350	F: TCCAATCTCAGCCATGGCTGG	133
		R: TCTCTTTGATGTCCTCACTGCAAATC	
	C2At2g37330	F: AACTATCCATTATTGGTTTTGTTCTTC	116
		R: ACGTTGTCCAGCAGTATAACCAGCAA	
11	C2At1g51350	F: TCTAGATTCTATCCAGCATGTCTTTGC	287
		R: AACTGCTTGGAAAAGTAAGATTTACTA	
	C2At2g28800	F: TGAAGGATGGACTGCAAGCTGTGC	242
		R: TGCAAGAGGATTGACCCCTGCCTG	
	C2At3g44880	F: ACAAGATTCGTCGTCGAAATTCTC	259
		R: ACCACATCCATTAAATGACCATCC	
	C2At4g01560	F: AGACTGTCTGTTTACCTGATGATGAAGAG	55
		R: TCATCAACATCGTTGCCAGCAAATAG	
	C2At5g22940	F: TATCGTGTAATTTCAGCACTGTTAATGG	285
		R: TCACTATATGCTCTGCTTTTTGACATGG	
	C2At3g44890	F: ATTGGGCAAAGCTCAAATTGTGAC	73
		R: AGCCTCAATTTTCTCGTCTTCCATC	
	C2At3g44600	F: TCCTTTATACCGACTTGAAGCTATTG	356
		R: AGATTCTATGTTTCTTGAAAGCACAGC	
	C2At2g27730	F: TGGAGAAAGAGAAGCTGGAGAAGC	220
		R: TCCTTTGACATTAGGTACCAACCC	
	C2At3g52730	F: ATTATGGCGTTAAAACTCTCTGGG	83
		R: AACAGGCCGCTGTCCAAGAACTG	
	C2At2g27290	F: AATTAGAGCATTGAAGGAGAAAAC	289
		R: TAGCAAGGATTGAACATCAACACC	

[z]Chromosome number.
[y]F, forward primer; R, reverse primer.

after checking if the PCR product contained intron sequences using Intron Finder (http://solgenomics.wur.nl/tools). The anthocyanin related gene sequences originated from potato, petunia, and tomato. The COS II markers (Table 2), which are localized close to the anthocyanin related genes on the tomato genetic map, were selected from the Sol Genomics Network (http://solgenomics.wur.nl). Sequences of the RFLP probe (Table 3), which are localized close to the COS II markers producing polymorphisms, were used for primer design. All designed primers were used to develop a DNA marker for the identification of either a purple or a green hypocotyl in ten purple and three green hypocotyl tomatoes using a high resolution melting (HRM) technology based assay.

Genomic DNA Preparation, PCR, and Distance Calculation

Genomic DNA was extracted as described by Kang et al. (2001). PCR reactions were performed in a 20 μL volume containing 1X buffer, 0.2 mM dNTPs, 0.5 mM each of forward and reverse primer, 20 ng of genomic DNA, and 0.5 units of *Taq* DNA polymerase (Genet Bio Inc., Korea, Cat. No. G1000). A Mastercycler (Eppendorff, USA) was used under the following conditions: denaturation at 94℃ for 5 min; 35 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s; and a final elongation at 72℃ for 5 min. PCR products were separated by 1.5% agarose gel electrophoresis. PCR bands from a Hy primer set (Table 3) were loaded after digestion by *Hpa* II at 37℃ for 2 h.

A HRM technology based assay was performed using a

Table 3. Primers designed from RFLP fragments localized near COS II markers.

Marker	Primer sequence (5'-3')	Fragment size (bp)
CD3	F[z]: GTTCGTGGACGACCAGTTCT	176
	R: AATCTTGCCATTCCATCCAA	
Hy	F: GAAGCCCTCCTCTATAGTTTCC	414
(cLET2D4)	R: GGTCCTGAAATGTCCGATTC	
TG79	F: GGGGAACCCTCTGGAATAAA	223
	R: AAGGAGAAATGGGGAAAACG	
cLED11L12	F: AGGCTTCATAGGAGCAGCAG	195
	R: GAAAATGTGGGAACGGTGTC	
cTOB9B13	F: AAAGTGTATTTGCGGGTTCG	230
	R: ATCGCCGTAAGCACTTGACT	
cTOB1K3	F: CCCAACCGTTCACTCTCCTA	218
	R: TCCATTGACCCGAATATCGT	

[z]F, forward primer; R, reverse primer.

Rotor Gene Q (Qiagen, Hilden, Germany). PCR reactions were conducted in a 20 μL volume containing 1X buffer, 1X LCGreen plus dye (BioChem, Salt lake city, USA), 0.3 mM dNTPs, 0.5 mM of forward and reverse primers, 40 ng of genomic DNA, and 0.5 units of *Taq* DNA polymerase. HRM PCR conditions were as follows: denaturation at 95℃ for 2 min followed by 40 cycles of 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 20 s. HRM was analyzed in the temperature range of 70℃-90℃, at a rate of 0.1℃ every 2 s per step with the Rotor Gene Q software package.

The distance between the marker and hypocotyl color was calculated by CARTHAGENE (Schiex and Gaspin, 1997). The recombination frequency was converted into mapping distances in centiMorgan (cM) using the Kosambi function (Kosambi, 1994). The minimum LOD (logarithm of odds) value and maximum distance were 4.0 and 40, respectively.

Results

Genetic Analysis of Hypocotyl Coloration

Tomato hypocotyls mainly show two colors, purple or green (Fig. 1). In this study, the characteristics of purple and green coloration in tomato hypocotyls were first studied genetically. When a purple hypocotyl line was crossed with a green hypocotyl line, F_1 hypocotyl tissues were subsequently purple. The purple to green ratio was clearly 3:1 in the F_2 population, and 1:1 in the BC_1F_1 population (Table 4). Therefore, this trait is Mendelian and based on a single dominant gene.

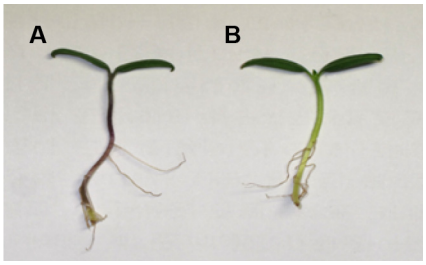

Fig. 1. Hypocotyls showing purple (A) and green (B) color.

Table 4. Segregation of purple or green color of tomato hypocotyls.

Plant material	No. of phenotype		Expected ratio Purple: Green	χ^2	*P*
	Purple	Green			
NWp1	20	0	1:0		
NWg3	0	20	0:1		
F_1 (NWp1 × NWg3)	20	0	1:0		
F_2 (NWp1 × NWg3)	120	40	3:1	0.000	1.000
BC_1F_1 (F1 × NWg3)	40	41	1:1	0.024	0.912

Candidate Gene and COS II Marker Approach

PCR primers (Table 1) designed from anthocyanin related gene sequences did not result in the differential amplification of any specific PCR products in the HRM melting curves between the tomato lines with purple and green hypocotyls. This indicates that the purple and green pigments in the hypocotyl may not be directly associated with the reported anthocyanin related genes. Furthermore, COS II markers did not produce different melting curves on HRM analysis between the tomato lines with purple and green hypocotyls. However, the COS II markers, C2At5g02230 and C2At4g10360, which are located on chromosome 9, produced visible differences in the HRM melting curves (Fig. 2). These different patterns were not converted to PCR based markers, which is convenient and cost effective for a MAS program.

Conversion to a PCR Based Marker from a RFLP Sequence

RFLP markers (Table 3) localized near two COS II markers, which generated polymorphisms between purple and green hypocotyl tomato lines, were used to design HRM primers in order to gain other sequence information for PCR based marker development. These markers did not show any polymorphisms, except for cLET2D4 (Fig. 3). The marker products from the two different colors were sequenced, and the sequence alignment (Fig. 4) showed five nucleotide differences between them in the 414 bp sequence. Among the five nucleotides, a single nucleotide polymorphism (SNP) was found at the 223rd nucleotide, which is in the *Hpa* II enzyme site in green color. When the PCR products from

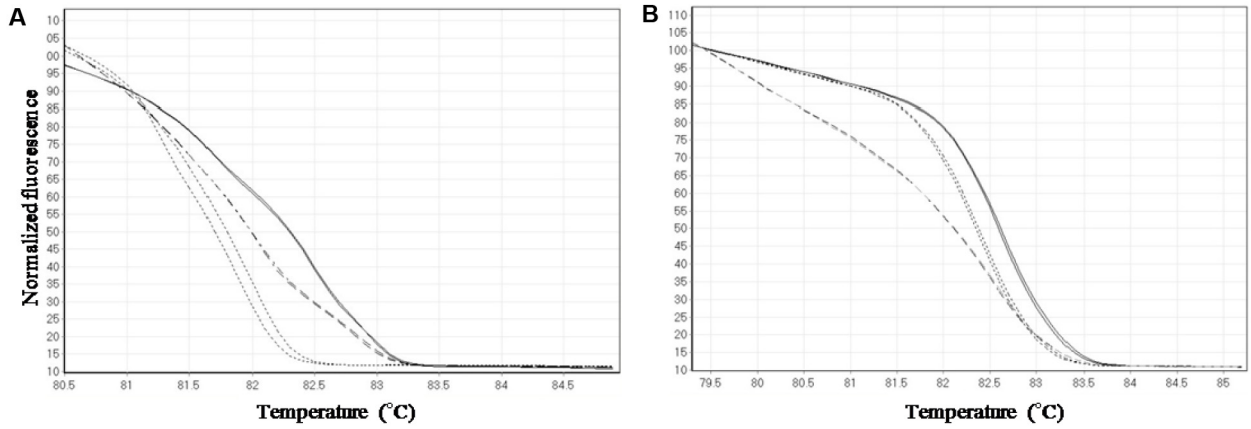

Fig. 2. Melting curves obtained from the HRM assay. The curves were generated using primers of C2At5g02230 (A) and C2At4g10360 (B). Each DNA sequence was replicated twice. Solid, dotted, and dashed lines indicate homozygous purple (NWp1), homozygous green (NWg3), and heterozygous purple (F_1), respectively.

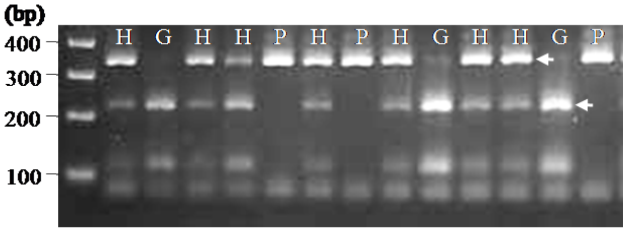

Fig. 3. PCR band pattern using the Hy marker after digestion by *Hpa* II. The upper and lower arrows indicate bands of 337 bp and 224 bp, respectively. The two lower bands are 113 bp and 77 bp in length. P, homozygous purple; G, homozygous green; H, heterozygous purple.

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G GAAGCCCTCCTCTATAGTTTCCATTCTCTCTTTTTCCCTCTTCTTTCTCTCTCAAATCTC 60
P GAAGCCCTCCTCTATAGTTTCCATTCTCTCTTTTTCCCTCTTCTTTCTCTCTCAAATCTC 60
  ************************************************************

G TCCATCTCTTTCAGCTGAAAGATGTCACCCAAATGACAAAAAAGCCCTCCTAGAATTCAA 120
P TCCATCTCTTTCAGCTGAAAGATGTCACCCAAATGACAAAAAAGCCCTCCTAGAATTCAA 120
  ************************************************************

G AAAAGCTCTGGGTAATCCTGATGATTTGGGTAATTGGAATCCCAAAACTGATTGCTGCAT 180
P AAAAGCTCTAGGTAATCCTGATGATTTGGGTAATTGGAATCCCAAAACTGATTGCTGCAT 180
  ********* ************************************************

G TGACTGGTACGGACCTACTCTCGAATGCGATGAAAAATCGAACCGGATTAATCTCATCGA 240
P TGACTGGTACGGACCTACTCTCGAATGCGATGAAAGATCGAATCGGATTAATCTCATCGA 240
  ********************************** ****** ******************

G TTTCTCCAAAATGAATCTCTCTGGATATCTCTCTCCCGCCATCGGAGATCTCACATACCT 300
P CTTCTCCAAAATGAATCTCTCTGGATATCTCTCTCCCGCCATCGGAGATCTCACATACCT 300
  ************************************************************

G TACGAAATTCAGTATCCACAATGTACGTAATCTCACCGGTCCAATTCCATCAACAATTGT 360
P TACGAAATTCAGTATCCACAATGTACGTAATCTCACCGGTCCAATTCCATCAACAATTGT 360
  ************************************************************

G TAAGCTCACGAATCTCAATTTCTTCAGAATTAGTGAATCGGACATTTCAGGACC 414
P TAAGCTCACGAATCTCATTTTCTTCAGAATTAGTGAATCGGACATTTCAGGACC 414
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Fig. 4. Alignment of DNA sequences amplified with the Hy marker. Two PCR products were produced from P (purple parent NWp1) and G (green parent NWg3). The square is the *Hpa* II enzyme site.

the cLET2D4 sequence were digested by *Hpa* II enzyme, the polymorphism was shown to be codominant. We succeeded in the CAPS marker conversion, and named it the hypocotyl

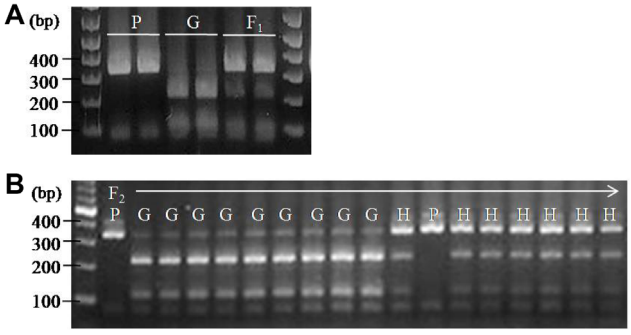

Fig. 5. A. PCR band pattern separated by the CAPS marker; P, purple parent (NWp1); G, green parent (NWg3); F_1, F_1 progeny. B. Separated PCR band pattern in F_2 individuals by the CAPS marker. PCR products were digested with *Hpa* II.

(Hy) marker. When DNA samples from homozygous purple (P), homozygous green (G), and heterozygous purple (H) tomato lines were subjected to PCR analysis with the Hy marker and digested with *Hpa* II, two bands (337 bp and 77 bp) were produced in P, and three bands (224 bp, 113 bp, and 77 bp) were produced in G, and all four bands were produced in H (Fig. 3). Therefore, the 337 bp and 224 bp bands were specific to the P and G characteristics, respectively.

Confirmation of the CAPS Marker Using the F_2 Population

The CAPS marker generated band patterns specific to P and G for the parents and F_1 progeny (Fig. 5A). The genotypes of 135 F_2 individuals obtained were analyzed based on the CAPS marker, and 132 individuals corresponded to the phenotypes of either purple or green hypocotyl pigmentation (Fig. 5B). Three tomato plants belonging to the green hypocotyl type did not have the band pattern corresponding to green color. Instead, they contained the band pattern of heterozygous purple. From this result, it is postulated that the CAPS marker is located at 2.3 cM away from the

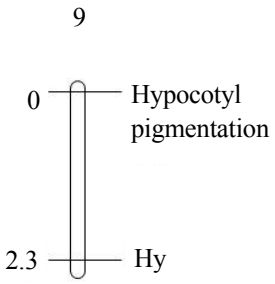

Fig. 6. Locations of hypocotyl pigmentation and Hy (CAPS) marker on chromosome 9.

hypocotyl pigmentation locus (Fig. 6).

Discussion

In most cases, the coloration of organs in plants is a result of complex expression levels of anthocyanin related genes. In tomato, three anthocyanin related genes (*dfr*, Bongue-Bartelsman et al., 1994; *ant1*, Mathews et al., 2003; *chs*, O'Neill et al., 1990) are involved in production of the purple color. Purple coloration is mainly present in the tomato fruit, but in the early stage of a tomato seedling, the hypocotyl is either a purple or green depending on the genotype (Fig. 1). It is not known whether the purple or green hypocotyl color of tomato is related to any anthocyanin related gene.

In the present study, a genetic study of hypocotyl color was conducted using the parental lines, F_2, and BC populations, demonstrating that this trait is controlled by a single dominant gene (Table 4). The segregation result of the tomato hypocotyl color corresponded to the hypocotyl color's in soybean (Choi et al., 1989; Vuong and Harper, 2000) and beet (Boudry et al., 1994). In soybean and beet, the color trait has been reportedly used as a diagnostic genetic marker for developing the breeding lines. In tomato, the hypocotyl color has also been used as a visible genetic marker source for selecting tomato progeny. Indeed, we independently observed the linkage of hypocotyl color with FCRR and GMS, and the MAS program was conducted to develop the lines and varieties that contained multi-characteristics (Kim et al., 2011).

Primer sets from anthocyanin related gene sequences, as well as most of the closely linked COS II markers, did not amplify any polymorphic band (Tables 1 and 2). Furthermore, the genes *rt* and *an1* located on chromosome 9 of tomato map, were originated from Petunia, but not from tomato. So, two *rt*-like EST clones (BM410878 and BM535249) were used to identify the polymorphism between purple and green hypocotyls, but any polymorphic band was not found. From gene sequence information, primer sets amplifying PCR products less than 300 bp, were designed and applied to HRM which could be more reliable to detect the PCR product less than 400 bp in size. However, no polymorphism was found. These results indicate that the hypocotyl coloration may not be directly associated with sequences of anthocyanin related genes. In contrast, a codominant marker was found from the sequence differences in the *A* locus (Borovsky et al., 2004), which controls the purple color of the foliage, flower, and immature fruit of pepper. The *Rt* locus of petunia yielded magenta or blue/purple flower tissue, while an insertion within the coding region (*rt*) inhibited recognition and a coding reaction (Kroon et al., 1994). However, we do not know the gene responsible for anthocyanin accumulation in the tomato hypocotyl, or the functional role of hypocotyl pigmentation, although anthocyanin accumulation in the hypocotyl of an ABA (abscisic acid) overproducing tomato was shown to have a negative correlation with male sterility (Sheoran et al., 2006).

Due to the detection of HRM differences from COS II markers on chromosome 9, COS II linked RFLP sequences were screened in order to distinguish between hypocotyl colors (Table 3). The Hy marker was designed from the RFLP probe sequence of cLET2D4, and was developed as a CAPS marker type for efficient and economical mass selection in breeding steps. The cLET2D4 probe sequence (AW038074) was a cDNA clone produced from a tomato mixed elicitor, and based on the sequence analysis, the Hy marker product was matched to the polygalacturonase inhibiting tomato protein containing a Leucine Rich Repeat on the Sol Genomics Network. This protein, found in the cell wall of many plants, interacts with fungal endopolygalacturonases and inhibits their enzymatic activity (Leckie et al., 1999). Therefore, this gene is not directly associated with hypocotyl coloration; rather, it may be closely localized to the coloration locus.

In this study, the Hy marker was analyzed in 135 tomatoes of an F_2 population. Among them, the color phenotypes of 132 matched the specific band pattern identified from the Hy marker, while three individuals showed a discrepancy between the color and the band. Since the CAPS marker is located at a distance of 2.3 cM away from the hypocotyl pigmentation locus in the 135 F_2 individuals, the application of this Hy marker to the F_2 population was successful. Therefore, the CAPS marker is a closely linked marker for the hypocotyl pigmentation. A better marker could be deduced directly from the hypocotyl pigmentation related gene, which is not known yet. Until then, the Hy marker could help breeders screen for several resistant traits as well as the hypocotyl pigmentation with homozygotes and heterozygotes among tomato breeding lines.

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