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Genetic Mapping of Beta-carotene QTLs in Tomato fruits

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Genetic Mapping of Beta-carotene QTLs in Tomato fruits

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Index

ACKNOWLEDGEMENT ..... **V**

ABSTRACT ..... **VI**

ABBREVIATIONS ..... **VII**

1 INTRODUCTION **1**

1.1. BETA–CAROTENE AND TOMATO 1
1.2. INTROGRESSION LINES AND QUANTITATIVE TRAITS LOCI 1
1.3. MARKERS ASSISTED SELECTION 2
1.4. GENETIC MAPPING 2
1.5. PROJECT BACKGROUND 2
1.6. GOAL OF THE THESIS PROJECT 3

2. MATERIAL AND METHODS **4**

2.1. TOMATO GENOMIC DNA EXTRACTION 4
2.2. PRIMER DESIGN AND MARKERS DEVELOPMENT 4
2.3. PCR AMPLIFICATION 5
2.4. RESTRICTION ENZYME DIGESTION 5
2.5. RECOMBINATED PLANTS SELECTION 6

3. RESULTS **7**

3.1. RECOMBINANT PLANTS SELECTION 7
3.2. MAPPING RESULTS 7

4. DISCUSSION AND CONCLUSION **9**

4.1. LOW NUMBER OF RECOMBINANT PLANTS 9
4.2. PCR AMPLIFICATION 9
4.3. SELECTED RECOMBINANT PLANTS 10
4.4. CONCLUSION 10

REFERENCES **11**

APPENDIX **13**

Index of Figures

Figure 1 represents HPLC figure from Dr. Je Min Lee of analyzing b-carotene content in tomato IL2-1 and IL2-2 that grown in different location and different years. M82 is using as control group. As figure 1 shows that both IL2-1 and IL2-2 contain higher b-carotene compare with M82 without location and year limitation. 2

Figure 3 represents part of tomato IL2-2. A. represents IL2-2 separated into 3 regions: 2-C that is overlapped with IL2-1, 2-D and 2-E which is overlapped with IL2-3.; B .represents the location of SCAR markers 2A and U572717 and CAPs marker C2At1g60640 on IL2-2, 2-C region. 3

Figure 4 represents different polymorphisms of 4 tomato plants (Number 160, 161, 162 and 163) by using SCAR marker 2A (A) and CAPs marker C2At1g60640 (B). Tomato plant number 161 identified as recombinant plant due to its heterozygote genotype on chromosome 2-2 2-D, and with M82 genotype on chromosome 2-2 2-C 6

Figure 5 represents the genetic map of molecular markers and the genotype of recombinants plants. Marker SSR40 and CT196 located on IL2-2 2-E, marker U569822 and 2A locate on IL2-2 2-D, and the rest markers are locates IL2-2 2-C. The red color represents M82 and green color represents IL2-2 which contains entire chromosome2-2 segment of *S.pennellii*. The Grid brown represents F1 population which means the genotype is heterozygote. 8

Figure 6 represents recombinant plant samples with SSR marker 248G15-1 and CAPS marker CT276. Number above each band indicates the number of individual recombinant plants. 9

Figure 7 represents the chromosome 2 of tomato introgression line 13

Index of Tables

Table 1. 12 Molecular markers that used for recombinant plant samples, with the location on IL2-2 and the PCR annealing temperature. 5

Table 2 Restriction Digestion Enzyme that used for recombinant plant samples with the corresponding marker and the reaction temperature. 6

Table 3 the genotype of recombinant plants that selected among 983 F2 IL2-2 populations using SCAR marker 2A and CAPS marker C2At1g60640. 7

Table 4 the genotype of recombinant plants that identified by molecular markers 14

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Abstract

This thesis project's aim is to select Beta-Carotene (β-Carotene) synthesis related recombinant plants using molecular markers in a tomato *Solanum Pennellii* introgression line (IL) 2-2 in 2-C region. Tomato public sequence of contig 46485-3 was used as source for the development of a cleaved amplified polymorphic sequences (CAPS) marker on IL2-2 at 2-C area, together with other molecular markers that were designed by Dr. Je Min Lee, in total 12 markers were used for genetic mapping. Possible recombinant tomato plants with recombination on IL2-2 at 2-C region were first identified by CAPS marker C2At1g60640 and SCAR marker 2A that are located on IL2-2 at 2-C and 2-D regions, using F2 populations of IL2-2 which originated from a cross between tomato cultivar M82 (LA3475) and wild green-fruited species *Solanum Pennellii* (LA716). After that, 6 CAPS markers, 3 SCAR markers and 1 SSR marker were applied on recombinant plant samples to analyze the genotype. In total, 12 molecular markers were applied on recombinant plants and a genetic map was constructed. 8 recombinant plants were selected among 983 F2 populations, 2 of them introgressed with *S. pennellii* on chromosome 2-2 within 2-C region, 5 were recombinant with M82 on IL2-2 in 2-C area and 1 recombinant with M82 on IL2-2 in 2-D and 2-E regions. The identified plants that contain recombinants on IL2-2 within 2-C region will be selfed to obtain sub-introgression lines of IL2-2 with shorter introgressions for future research such as to define QTLs that involved to increase β-carotene on IL2-2 within 2-C region, and to select candidate gene(s) by looking at gene expression profiling.

Abbreviations

CAPS	Cleaved Amplified Polymorphic Site
IL	Introgression Line
PCR	polymerase chain reaction
QTL	Quantitative Trait Loci
SCAR	Sequence Characterized Amplified Region
SSR	Simple Sequence Repeats

1 Introduction

1.1. Beta–Carotene and Tomato

Beta-carotene (β-carotene) is a member of the carotenoids that is involved in fruit ripening, has antioxidant properties, and is the main dietary precursor of vitamin A. During plant development, carotenoids play dual roles as essential photoprotectants in green tissues and as dispensable colorants in flowers and fruits (Grotewold.E. 2006). In tomatoes, carotenoids such as β-carotene and lycopene are the primary components of pigment of ripen fruit, and genes that co-response to the regulation of β-carotene synthesis has been studied for many years (Römer et al., 2000, Giuliano et al., 2000.Ronen et al., 2000. Bramley., 2002)), .

1.2. Introgression lines and Quantitative Traits Loci

Solanum Pennellii (S.pennellii) and *Solanum Lycopersicum* (*S.lycopersicum*) can produce viable progeny that expose the variation that has driven evolutionary change and provided the raw material for crop domestication and breeding. Introgression lines (ILs) are a set of nearly isogenic lines developed through a succession of backcrosses between *S.pennellii* and *S.lycopersicum*, where each line carries a single genetically defined chromosome segment from a divergent genome (Zamir D. 2001), and representing whole-genome coverage of *S.pennellii* in overlapping segments in genetic background of *S.lycopersicum* cultivar M82 presently consist of 76 genotypes (Lippman et al., 2007). The phenotypes of the ILs are not observed in the parents because of a mechanism called transgressive segregation, and it results from novel interactions between *S.pennellii* alleles and the independently evolved molecular networks of *S.lycopersicum* (de Vicente and Tanksley, 1993).

Quantitative trait loci (QTL) affect plant morphology, metabolism and gene expression, and some QTLs associated with β-carotene have been identified (Rousseaux et al., 2005). It is known that Beta-carotene are elevated in chromosome 2-5, 11-1 and 12-3, however, it is at or below tomato M82 levels in chromosome 2-2 and 12-2. Combining ILs elevated in each of these genes, might lead to increased carotenoid levels by altering expression in multiple steps in synthesis pathway.

Populations of ILs are very effective in identifying and stabilizing QTL, because any phenotypic difference between an IL and the recurrent parent is attributed solely to one or more donor parent genes within the introgressed chromosomal segment (Zamir D. 2001).

1.3. Marker Assisted Selection

Marker assisted selection enables to select specific segments of DNA that are associated with different measurable differences and effects on a complex trait. It can identify alleles of a QTL via association between the markers and phenotype. Different types of DNA markers have been developed and evolved such as Simple sequence repeats (SSR) markers which have the properities of genetic co-dominance, high reproducibility and multialleic variation (He et al., 2003); Cleaved amplified polymorphic site (CAPS) markers that rely on differences in restriction enzyme digestion patterns of PCR fragments caused by nucleotide polymorphism between two different genotype (Baumbusch et al. 2001); and Sequence characterized amplified regions (SCARs) marker. CAPS markers together with sequence characterized amplified region (SCAR) markers allowcross-mapping in related species (Bai et al, 2004).

1.4. Genetic Mapping

Genetic mapping refers to the use of molecular markers that genetically link on chromosome and arrange by frequency to construct a linkage map as a forerunner to identifying QTLs associated with important traits (Hackett et al, 2007).

1.5. Project Background

Giovannoni's laboratory is focused on research of fruit ripening and related signal transduction system, and one of the experimental approaches is to isolate candidate ripening regulatory genes. This thesis project is part of a project from Dr. Je Min Lee (Boyce Thompson Institution) which aims to select candidate genes that evolved in increasing carotenoid levels on IL2-2. Figure 1 shows that tomato IL2-1 and IL2-2 contain higher β-carotene when compared to tomato cultivar M82. Therefore, this thesis project focuses on the IL2-2 at 2-C region, aiming to select recombinant plants using molecular markers and to represent a genetic map.

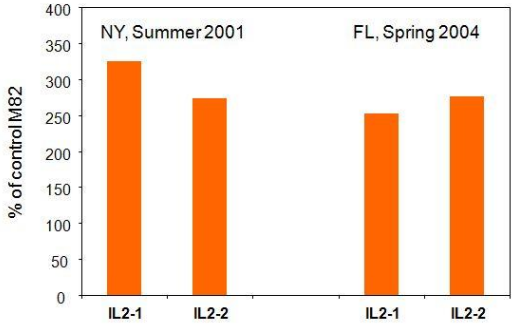

Figure 1 represents HPLC figure from Dr. Je Min Lee of analyzing β-carotene content in tomato IL2-1 and IL2-2 that grown in different location and different years. M82 is using as control group. As figure 1 shows that both IL2-1 and IL2-2 contain higher β-carotene compare with M82 without location and year limitation.

Plant materials in this project are using genomic DNA that extracted from F2 population of an introgression line (IL) 2-2, which originates from a cross between

tomato *Solanum Lycopersicum* M82 (LA3475) and wild green-fruited species *Solanum Pennellii* (LA716). In total, DNA was extracted from 983 individual plants.

We focus on IL 2-2 at 2-C region that partially overlaps with IL2-1 and IL2-2 (Fig1.A). Two markers (SCAR marker 2A and CAPS marker C2At1g60640) that were proposed by Dr. Je Min Lee were used for pre-selection to select recombinant tomato plants among 983 populations.

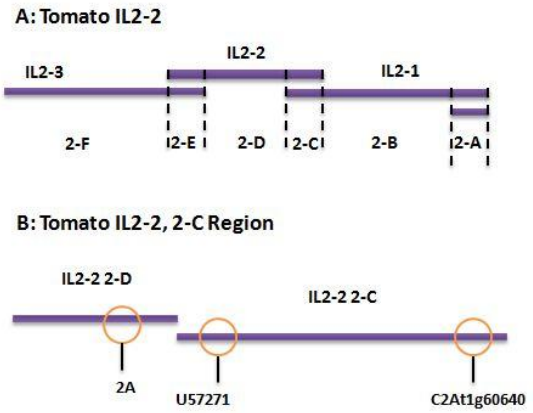

Figure 2 represents part of tomato IL2-2. A. represents IL2-2 separated into 3 regions: 2-C that is overlapped with IL2-1, 2-D and 2-E which is overlapped with IL2-3.; B .represents the location of SCAR markers 2A and U572717 and CAPs marker C2At1g60640 on IL2-2, 2-C region.

SCAR marker 2A is located on the end of IL2-2 at 2-D region, and CAPs marker C2At1g60640 is located on the end of IL2-2 at 2-C. These two markers were used to identify the recombinant plants that contain possible recombinant on IL2-2 in 2-C region. The genetic distance between 2A and C2At1g60640 is about 3.5cM. New markers need to be developed because the sequence of ends of IL2-2 of 2-C did not defined.

After this, 10 molecular markers (6 CAPS markers, 3 SCAR markers and 1 SSR marker) are applied on recombinant plant samples to analyze the genotype for genetic mapping.

1.6. Goal of the thesis Project

The goals of this thesis project are 1) to develop new CAPS markers which lay in between SCAR marker 2A and U572717on IL2-2 at 2-C region, and to select recombinant plants from 983 IL2-2 F2 populations together with other molecular markers; and 2) to build a genetic map.

2. Material and Methods

2.1. Tomato Genomic DNA Extraction

Tomato leaf material was collected from F2 population resulting from IL2-2 crossed with M82. 1 cotyledon per plant was harvested in 1.5ml Eppendorf tubes with 2 glass beads at the bottom. 300µl extraction buffer with Na bisulfite (3.8g/ml) was added, and samples were homogenized using Fast Prep at speed 4.5 for 45 seconds. After this, 300µl nuclear buffer and 150µl of 5% sarcosine were added and samples were incubated at 65℃ for 30 minutes. After incubation, 750µl CH_3Cl was added into each tube. Samples were inverted by hand and centrifuged at 10'000rpm for 15 minutes. 500µl supernatant was transferred into new 1.5ml tubes and mixed with 500µl isopropanol. Tubes were inverted 30 times by hand and centrifuged at maximum speed for 10 minutes. The supernatant was discarded and 1ml 60% Ethanol was added. After 5 minutes centrifuging at maximum speed, the liquid was discarded and the pellet was resuspended in 50µl sterilized distilled water, mixed with RNase. Samples were stored at room temperature overnight, and finally stored at -20℃.

2.2. Primer Design and Markers Development

Primer design is based on the public sequence of contig46485-3 that locates on IL2-2 at 2-C region; between SCAR markers 2A and U572717. A set of polymerase chain reaction (PCR) primers for PCR amplification was synthesized. 1 pair of primer (5'-GGAGATAACTCGAGGGCTCAG, 3'-CGCCAACTTTATACCCAATGAATGTGC) was chosen as CAPs marker that named 46485-3, and yielding a PCR fragment of approximate 1415 nucleotides. All primers were evaluated using PCR with DNA from M82 and *S.pennellii*, and finalized with electrophoresis analysis. Together with other markers designed by Dr. Je Min lee, in total 12 molecular markers (Table 1) were used on recombinant plant samples.

Table 1. 12 Molecular markers used for recombinant plant samples, with the location on IL2-2 and the PCR annealing temperature.

Marker Type	Name	Annealing Tm (℃)	Restriction Enzyme	Digestion Tm (℃)	IL2-2
CAPS	C2At1g60640	55	EcoRV	37	2-C
CAPS	TG276	60	AluI	37	2-C
CAPS	26D15-3	60	HinfI	37	2-C
SCAR	2B	65			2-C
CAPS	T1238	60	XhoI	37	2-C
SCAR	T0888	60			2-C
SCAR	U572717	60			2-C
CAPS	46485-3	60	HealII	37	2-C
SCAR	2A	60			2-D
CAPS	U569822	60	AciI	37	2-D
CAPS	CT196	60	DpnII	37	2-E
SSR	SSR40	55			2-E

2.3. PCR Amplification

The reaction mixture (25μl) contained 2.5μl 10×PCR reaction buffer, 2.0μl dNTPs, 1.0μl primer mix that contains both forward and reverse primers (1μM/μl), 1.0μl DMSO, 0.5μl M_gCl_2, 0.25μl Taq DNA Polymerase, 5μl DNA and distilled water. The PCR reaction was programmed for 3 minutes at 94℃, 35 cycles of 1 minute at 94℃, 1 minute at annealing temperature, and 2 minutes at 72℃ with a final elongation for 7 minutes at 72℃.

2.4. Restriction Enzyme Digestion

Restriction enzyme digestion was performed in order to develop CAPS. The restriction enzyme digestion reaction was a 25μl reaction mixture containing 8.3μl distilled water, 1.5μl buffer, 0.2μl restriction enzyme and 5μl PCR products. The reaction mixture was incubated at 37℃ for 3 hours, and subsequently resolved in a 2.0% agarose gel. Restriction with 12 enzymes was assayed for CAPs marker 46485-3 and HealII (Table 2) were the enzymes used to generate polymorphisms. Together with other 6 CAPS markers that were developed by Dr. Je Min Lee, in total 7 CAPs (Table 2) markers were applied in this thesis project.

Table 2 Restriction Digestion Enzyme that used for recombinant plant samples with the corresponding marker and the reaction temperature.

CAPS Marker	Restriction Enzyme	Digestion Temperature (℃)
C2At1g60640	EcoRV	37
CT279	AluI	37
26D15-3	HinfI	37
T1238	XhoI	37
46485-3	HealII	37
U569822	AciI	37
CT196	BsaWI	60

2.5. Recombinated Plants Selection

Recombinant plants were identified first by SCAR marker 2A and CAPs marker C2At1g60640 which give different polymorphisms of the recombinated plants on gel (Figure 3). After that, selected recombinant plants were detected by using SCAR marker U57271 to identify if the recombination happened on IL2-2 within 2-C region, and whereas the rest molecular markers were used to recognize the genotype.

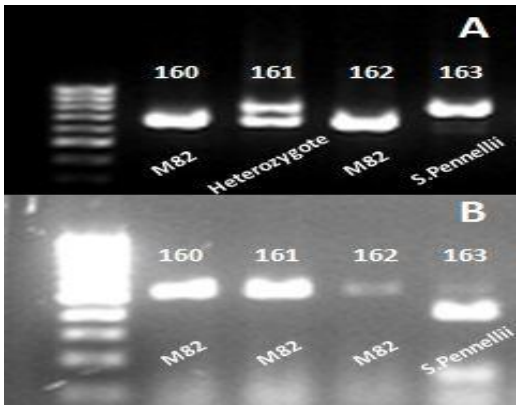

Figure 3 represents different polymorphisms of 4 tomato plants (Number 160, 161, 162 and 163) by using SCAR marker 2A (A) and CAPs marker C2At1g60640 (B). Tomato plant number 161 identified as recombinant plant due to its heterozygote genotype on chromosome 2-2 2-D, and with M82 genotype on chromosome 2-2 2-C

3. Results

3.1. Recombinant Plants Selection

After the first round selection using SCAR marker 2A and CAPS marker C2At1g60640, 8 recombinants plants that contain possible recombination on IL2-2 within 2-C region were selected. Table 3 represents the genotype of the recombination on the two marker regions. Plant number 19 and 156 recombinated with *S.pennellii* and the rest were containing M82 segments.

Table 3 the genotype of recombinant plants that selected among 983 F2 IL2-2 populations using SCAR marker 2A and CAPS marker C2At1g60640.

Plant Number	2A	C2At1g60640
19	Heterozygote	*S.pennellii*
55	Heterozygote	M82
110	Heterozygote	M82
156	Heterozygote	*S.pennellii*
161	Heterozygote	M82
778	Heterozygote	M82
794	Heterozygote	M82
908	M82	Heterozygote

3.2. Mapping Results

DNA was re-extracted from the 8 recombinant plants, and in total 12 molecular markers were used to analyze the genotype. As figure 4 shows, 2 recombinant plants contain segments from *S.pennellii* on IL2-2 at 2-C area: plant number 19 and 156. The recombinant region in plant number 19 was between Marker TG276 and C2At1g60640 where the genetic distance was only 0.5cM, and the genetic distance of recombinant region in plant number 156 was about 2.5cM. 5 plants (number 55, 110, 161, 778 and 794) were recombinant with M82. The genetic distance of recombinant region in plant number 161, 778 and 794 was 0.5cM, and with 2.5cM in plant number 55 and 110. All the 7 described recombinant plants only had recombination on IL2-2 at 2-C area and with heterozygote genotype on IL2-2 at 2-D and 2-E regions. Plant number 908 was the only recombinant with M82 partly on IL2-2 at 2-C region and the recombination mostly happened on IL2-2 at 2-C and 2-D regions.

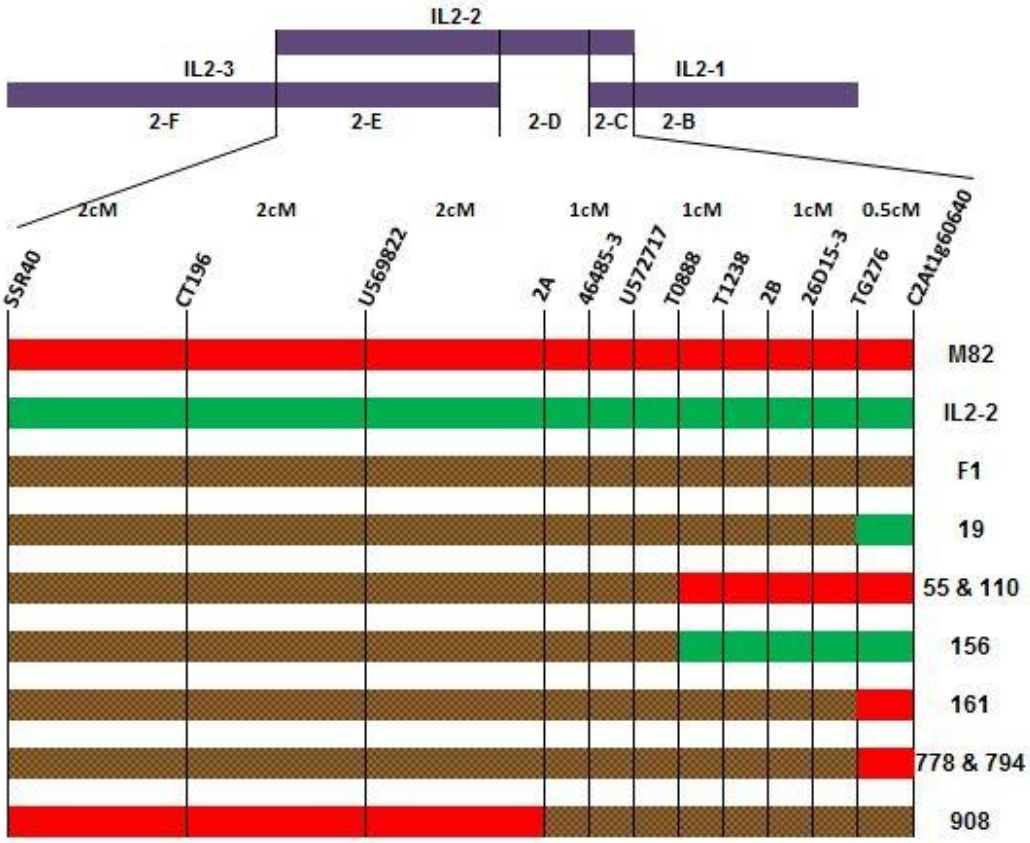

Figure 4 represents the genetic map of molecular markers and the genotype of recombinants plants. Marker SSR40 and CT196 located on IL2-2 2-E, marker U569822 and 2A locate on IL2-2 2-D, and the rest markers are locates IL2-2 2-C. The red color represents M82 and green color represents IL2-2 which contains entire chromosome2-2 segment of *S.pennellii*. The Grid brown represents F1 population which means the genotype is heterozygote.

4. Discussion and Conclusion

4.1. Low Number of Recombinant Plants

The genetic distance between marker 2A and C2At1g60640 is 3.5cM which indicate that the recombinant frequency between the two markers on IL2-2 is 3.5%, therefore 34 recombinant plants are expected among the 983 F2 populations. However, we only observed 8 recombinant plants, 4 times lower than the expected numbers. There are two explanations for this phenomenon: one is due to the variation of each generation, the other can be that homologous recombination frequency is influenced by several environmental conditions such as temperature, day length, water availability and salinity, which may have a significant effect on plant genome stability (Boyko et al, 2005).

4.2. PCR Amplification

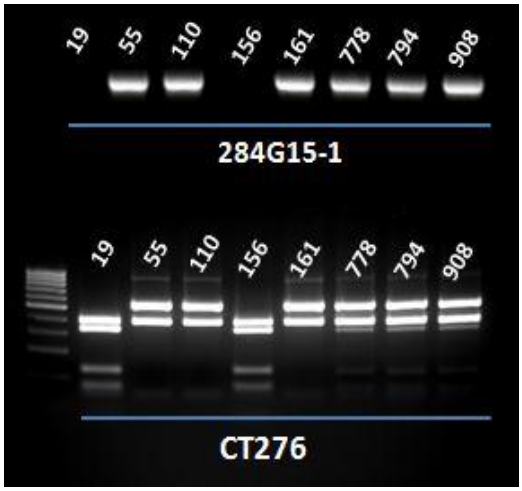

Figure 5 represents recombinant plant samples with SSR marker 248G15-1 and CAPS marker CT276. Number above each band indicates the number of individual recombinant plants.

SSR marker 284G15-1 that genetically close to TG276 was used to *analyze* the genotype of recombinant plants together with other markers. The genotype of recombinant samples that are given by SSR marker 284G15-1 should be the same as analyzed by CAPS marker TG276 due to the small genetic distance between these two markers. However, SSR marker 284G15-1 cannot distinguish the genotype as well as CAPS marker CT276 did. It shows black on gel of recombinant plants number 19 and 165 which are recombinants with *S.pennellii*, and it did not distinguish heterozygote genotype of plant number 778, 794 and 908 with M82 recombinant plants number 55, 110 and 161. The explanation for this could be that there are oligonucleotides variations between *S.pennellii* and M82 at region of SSR marker 284G15-1, therefore, only DNA template of M82 has been amplified which lead to no genotype differences between M82 and Heterozygote, and it is also the reason for no PCR amplification of recombinants with *S.pennellii.*

4.3. Selected Recombinant Plants

Except plant number 908, the phenotype of the other 7 recombinant plants will be record, and using HPLC to analysis β-carotene content in its fruits. All the 7 recombinant plants only contain recombination on IL 2-2 2-C and with heterozygote genotype IL2-2 2-D and 2-E, which is a good foundation to form sub-introgression lines (sub-IL) of IL2-2 for future QTLs research and candidate genes selection. Plant number 19 and 156 contain *S.pennellii* segments on IL2-2 within 2-C region, however, plant number 19 has more preference than other recombinant plants due to the small genetic distance of the recombination which was only 0.5cM between marker TG276 and C2At1g60640. This can help to define QTLs that related to increase β-carotene synthesis on IL2-2 in 2-C region, and to select candidate gene(s) by looking at gene expression profiling using different approaches such as quantitative real-time PCR (qRT-PCR) (Huis et al, 2010), microarray, and expressed sequence tag (EST) that allows to discover functionally associated genes on a large scale (Wu et al, 2005).

4.4. Conclusion

Plant material in this project was genomic DNA extracted from F2 population of IL2-2. 8 recombinant plants were selected among 983 F2 populations. Except plant number 908, the remaining 7 recombinant plants all only contain recombination on IL2-2 within 2-C region. Plant number 19 and 156 introgressed with *S.pennellii* segments, and the genetic distance of the recombination between marker TG279 and C2At1g60640 was only 0.5cM in plant number 19. Except recombinant plants number 908, the identified recombinants will be selfed to obtain sub-introgression lines of IL2-2 with shorter introgressions for future research to define QTLs that involved to increase β-carotene on IL2-2 in 2-C area, and to select candidate gene(s) by looking at gene expression profiling.

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Appendix

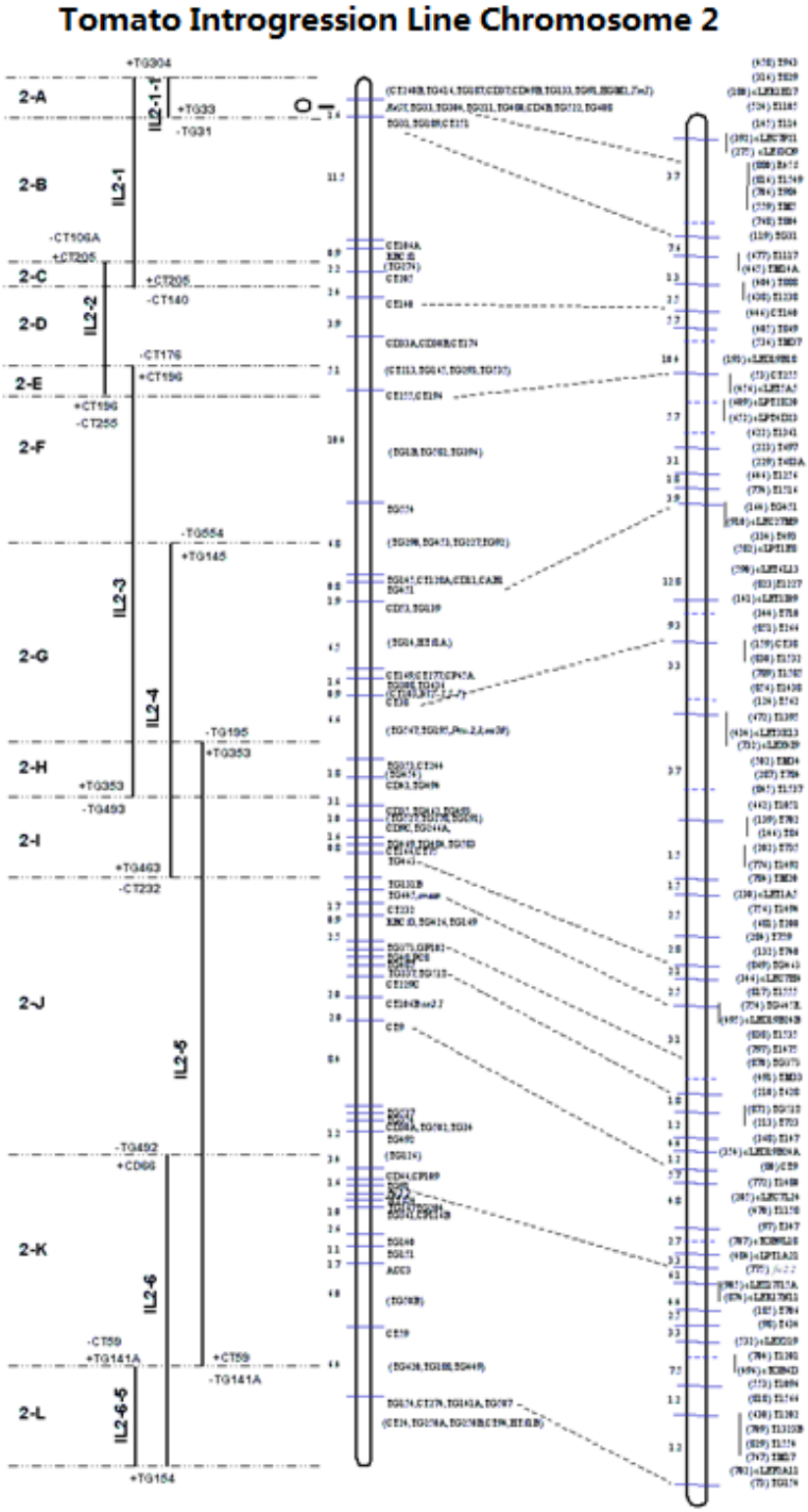

Figure 6 represents the chromosome 2 of tomato introgression line

Table 4 the genotype of recombinant plants that identified by molecular markers.

Plant Number	SSR40	CT196	U569822	2A	46485-3	U572717	T0888	T1238	2B	26D15-3	TG276	C2_At1g60640
19	H	H	H	H	H	H	H	H	H	H	B	B
55	H	H	H	H	H	H	A	A	A	A	A	A
110	H	H	H	H	H	H	A	A	A	A	A	A
156	H	H	H	H	H	H	B	B	B	B	B	B
161	H	H	H	H	H	H	H	H	H	A	A	A
778	H	H	H	H	H	H	H	H	H	H	H	A
794	H	H	H	H	H	H	H	H	H	H	H	A
908	A	A	A	A	H	H	H	H	H	H	H	H

H = Heterozygote; A = M82; B = *S.penne*

Contig of 46485-3

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