식물조직배양학회지 제27권 제5호
Korean J. Plant Tissue Culture
Vol. 27, No. 5, 367~373 (2000)

Linkage Map Construction and Molecular Genetic Approach in *Capsicum* spp.

KIM, Byung-Dong*

Division of Plant Science, and the Center for Plant Molecular Genetics and Breeding Research, Seoul National University, Suwon 441-744, Korea

ABSTRACT We have constructed a molecular linkage map of chili pepper (*Capsicum* spp.) in an interspecific (*C. annuum* cv. TF68 × *C. chinense* cv. Habanero) F_2 population of 107 plants with 150 RFLP and 430 AFLP markers. The resulting linkage map consists of 11 large (206 - 60.3 cM) and 5 small (32.6 - 10.3 cM) linkage groups covering 1,320 cM with an average map distance between framework markers of 7.5 cM. Most (80%) of the RFLP markers were pepper-derived clones and these markers were evenly distributed across the genome. By using 30 primer combinations, 444 AFLP markers were generated in the F_2 population. The majority of the AFLP markers clustered in each linkage group, although *Pst*I/*Mse*I markers were more evenly distributed than *Eco* RI/*Mse*I markers within the linkage groups. Genes for biosynthesis of carotenoids and capsaicinoids were mapped on our linkage map. This map will provide the basis of studying secondary metabolites in pepper.

Key words: *Capsicum*, carotenoids, lankage map

Introduction

Pepper fruits are consumed as food additives for their unique color, pungency, and aroma in many regions of the world, particularly in Asia and South and Central America. Five species of *Capsicum* peppers, including *C. annuum*, *C. chinense*, *C. baccatum*, *C. frutescens* and *C. pubescens*, are cultivated in different parts of the world. Among them *C. annuum* is most widely grown in both Asia and worldwide (Pickersgill 1997). It includes most of the Mexican chile peppers, most of the hot peppers of Africa and Asia, and various cultivars of sweet peppers grown in temperate regions of Europe and North America.

During the last decade, the construction of molecular linkage map has become an essential tool for plant molecular genetics and breeding research. Despite the pepper genome research is being conducted by only a small number of research groups worldwide, development of a linkage map in *Capsicum* has been greatly aided by use of tomato-derived RFLP probes. All of published genetic maps of *Capsicum* so far have been based on either interspecific populations (Tanksley et al. 1988; Prince et al. 1993) or intraspecific populations (Lefebvre et al. 1997) with the use of tomato-derived RFLP probes. Recently, Livingstone et al. (1999) published another pepper genetic map containing nearly a thousand DNA markers. Nevertheless, the linkage map is only moderately saturated and many markers were distinctly clustered. Prince et al. (1993) suggested that sparsely mapped genomic regions may correspond to regions of the pepper genome which have diverged more rapidly from tomato, so are not detectable with tomato probes. Therefore, current pepper genetic maps still need to be completed using pepper-derived probes for comprehensive understanding of pepper genome structure.

Here we report the construction of a molecular linkage map of pepper using mainly pepper-derived probes based on a population of 107 interspecific F_2 individuals. If a certain gene-specific probe shows complete link-

*Corresponding author Tel 031-290-2563
E-mail Kimbd@plaza.snu.ac.kr

age with the fruit color locus, it could serve as an important candidate controlling the level of carotenoid content in fruit color. In addition, quantitative analysis of carotenoids in the ripe fruits of red pepper by HPLC and QTL analysis would provide a relatively accurate description of the relationship between the candidate genes and the carotenoid contents.

Material and Methods

Mapping population

Pepper accessions *C. annuum* cv. TF68 and *C. chinense* cv. Habanero, both available for public scientists, were obtained from Hung-Nong Seed Company. 'TF68' is a Southern Asian type cultivar bearing long, slim, red, and nonpungent fruits. 'Habanero' is originated from Southern America and bears campanulate, orange-colored, and is very well known for its extremely pungent and aromatic flavors. An F_2 population of 107 plants was constructed by selfing an F_1 hybrid (TF68 as the female parent and Habanero as the male parent) to serve as the mapping population (Nahm et al. 1997). We have been conducting a genome research of hot pepper since 1996 as a comprehensive group effort (Kim B. D. et al. 1997; Kim S. H. et al. 1997).

Map construction

Genome mapping by RFLP and AFLP analysis were followed as previously desribed (Kang et al. 2000)

Results and Discussions

RFLP analysis

Out of the 550 pepper DNA clones assayed, the number of probes, which revealed RFLPs with at least one restriction enzyme, was 256 (46%). When tomato clones were assayed, 61 out of 108 clones (56%) showed polymorphism. Of the 317 polymorphic clones, only 153 were usable for RFLP linkage analysis because half of the markers that showed polymorphism on survey filters could not be scored clearly for F_2 individuals. A total of 43 markers (31.4%) out of 153 markers were deviated from the normally expected F_2 ratio of 1:2:1 or 3:1. Of the 147 markers segregating 1:2:1, 22 markers were skewed toward the *C. annuum* allele, 15 markers toward the *C. chinense* allele, and 3 markers toward the heterozygote. One marker, PCD2-171, did not show skewness towards any allele, in other words, it showed an abnormal 1:1:1 segregation ratio. Among the remaining 6 dominant markers (PCD2-66, DC-114, DC478, CAN17, RDNA5S, and RDNA25S), two markers (DC-114 and RDNA5s) showed segregation distortion toward *C. chinense*.

AFLP analysis

Variations of AFLP patterns between the two lines were analyzed. The number of bands generated by the different *EcoRI/MseI* primer combinations revealed a large range of variation, from 23 bands for E8M14 (+ACT/+CCC) to 118 for E1M4 (+AAC/+CAT), with an average of 70. In contrast, the number of amplified fragments with *PstI/MseI* primers ranged between 15 for P8M10 (+GCG/+CGC) and 70 for P2M1 (+GGT/+CAA) with a mean of 40. The number of amplified bands of each primer was dependent on the sequences of selective nucleotides. The primers having +NAA, +NAT, +NTA, and +NTT (M1, M4, M5, M8, E15, E16) selective sequences amplified more bands than other primers. When polymorphism rates were compared, *EcoRI/MseI* primer combinations showed much a higher polymorphism rate than *PstI/MseI* primer combinations, with an average of 59.8% and 49%, respectively.

EcoRI/MseI primer combinations generated about 2 times more scorable AFLP markers than *PstI/MseI* primer combinations. The average number of scorable markers per primer combinations was 17.7 in *EcoRI/MseI* combinations and 10.4 in *PstI/MseI* combinations. Although many detectable polymorphic fragments were observed, many of them were difficult to score, owing to the dense and sometimes overlapping banding patterns. Of 444 AFLP markers, 232 (52.2%) were B/D (dominant markers contributed by female parent), and 212 (48.8%) were A/C (dominant markers contributed by male parent).

The usefulness and applicability of AFLP markers in genetic linkage mapping was evaluated by examining all 444 markers with x^2 test for goodness of fit. This statistical analysis revealed that 129 markers (29%), 82 and

47 for *Eco*RI/*Mse*I and *Pst*I/*Mse*I, respectively, deviated from the expected Mendelian segregation ratio.

The linkage map

A total of 597 markers (444 AFLP and 153 RFLP markers) were used for linkage map construction. Out of the total 597 markers, 585 were placed in 16 groups using LOD score of 3.0 and maximum recombination value of 0.25 (Figure 1). Within each linkage group markers that can be ordered at 3.0 LOD or above were used as framework markers. As a result, 177 out of 597 markers were positioned as framework markers. The current map contains 150 RFLP and 430 AFLP markers. The resulting linkage map is consisted of 11 large (206 - 60.3 cM) and 5 small (32.6 - 10.3 cM) linkage groups covering 1,320 cM with an average map distance between framework markers of 7.5 cM.

In contrast to other pepper maps reported that were based on tomato-derived probes (Prince et al. 1993 and Livingstone et al. 1999), the SNU map contained RFLP markers evenly distributed within each linkage group, likely due to the difference in the origins of RFLP probes. Our map was mainly based on pepper-derived probes.

The AFLP markers were well distributed over the linkage groups except for LG 8 and LG 10. Many of the AFLP markers were clustered in one region of each linkage group (LGs 2, 3, 5, 6, 7, 9, and 11). The *Pst*I/*Mse*I markers were more evenly distributed than the *Eco*RI/*Mse*I markers. There was significant clustering of skewed markers. The middle of LG 1, between PCD2-257 and PCD2-109, was skewed toward the *C. annuum* allele. The upper middle of LG 2, between *PST*172 and COMT, was skewed toward *C. annuum* allele, and all of LG 6 was distorted in favor of *C. chinense* allele. The middle of LG 9, between PST164 and PST 175, was skewed toward the *C. chinense* allele. The distorted regions corresponding to LGs 1, 2 and 6 were the same as those on the other pepper maps (Prince et al. 1993; Livingstone et al. 1999).

Relationship between candidate genes and fruit color

The fruit color of the parents was either red or orange. F_1 plants bore the same red-colored fruits as TF68. Out of 103 F_2 plants, 78 had red color but 25 had orange color with a varying degree of intensity. This ratio of 3:1 fit very well for Mendelian segregation (x^2 = 0.029) of a single locus determining the color with red dominance over orange. This locus for fruit color was assigned on the linkage group 7 after calculating the recombination frequencies between the red and orange color determining locus and other molecular markers (Kang et al. 2000).

The genomic fragments of several genes related to the carotenoid biosynthetic pathway, including FPS, GGPS, PSY, PDS, LCYB, and CCS, were successfully amplified by PCR in both TF68 and Habanero (data not shown). And, there were no differences in size and intensity in the amplified fragments of candidate genes between TF68 and Habanero. The expected size of the DNA fragments was obtained in the cases of GGPS, LCYB and CCS. The size of the PSY genomic DNA fragment was larger by about 1.4 kb than the expected mRNA fragment, which implies that it contained introns. The amplified fragments of the pepper FPS and PDS were the partial genomic regions at the 3' end.

The amplified fragments of candidate genes were cloned and their identity was confirmed by sequencing. They were used as gene-specific probes for Southern blot analysis and showed polymorphism between parents, which enabled us to convert them into RFLP markers and to locate them in the linkage map (Kang et al. 2000). The candidate genes, GPS, PDS, LCYB, CCS and PSY were assigned on the linkage groups 7, 2, 10, 4, and 7, respectively. FPS could not be located in the map due to the absence of polymorphism. Likewise, zeaxanthin epoxidase and ζ-carotene desaturase could not be located in the map, because they did not show any polymorphism in RFLP analysis. Lycopene epsilon cyclase was not tested. In addition to these, transketolase 2, β-carotene hydroxylase, and plastid fusion and/ or translation factor were also cloned and positioned on the linkage groups 1, 4, and 6, respectively.

Interestingly, one of the candidate genes, PSY, revealed polymorphisms between parents and showed complete linkage with the locus determining mature fruit color (Figure 2). They were assigned together on the same locus of linkage group 7. F_2 individuals having PSY alleles, either homozygous for TF68 genotype (AA) or heterozygous for TF68 and Habanero (AC), all demonstrated red fruit color. Segregation ratio for the PSY was 34: 44: 25 (x^2 = 3.757^{ns}) for genotypes AA: AC: CC. It

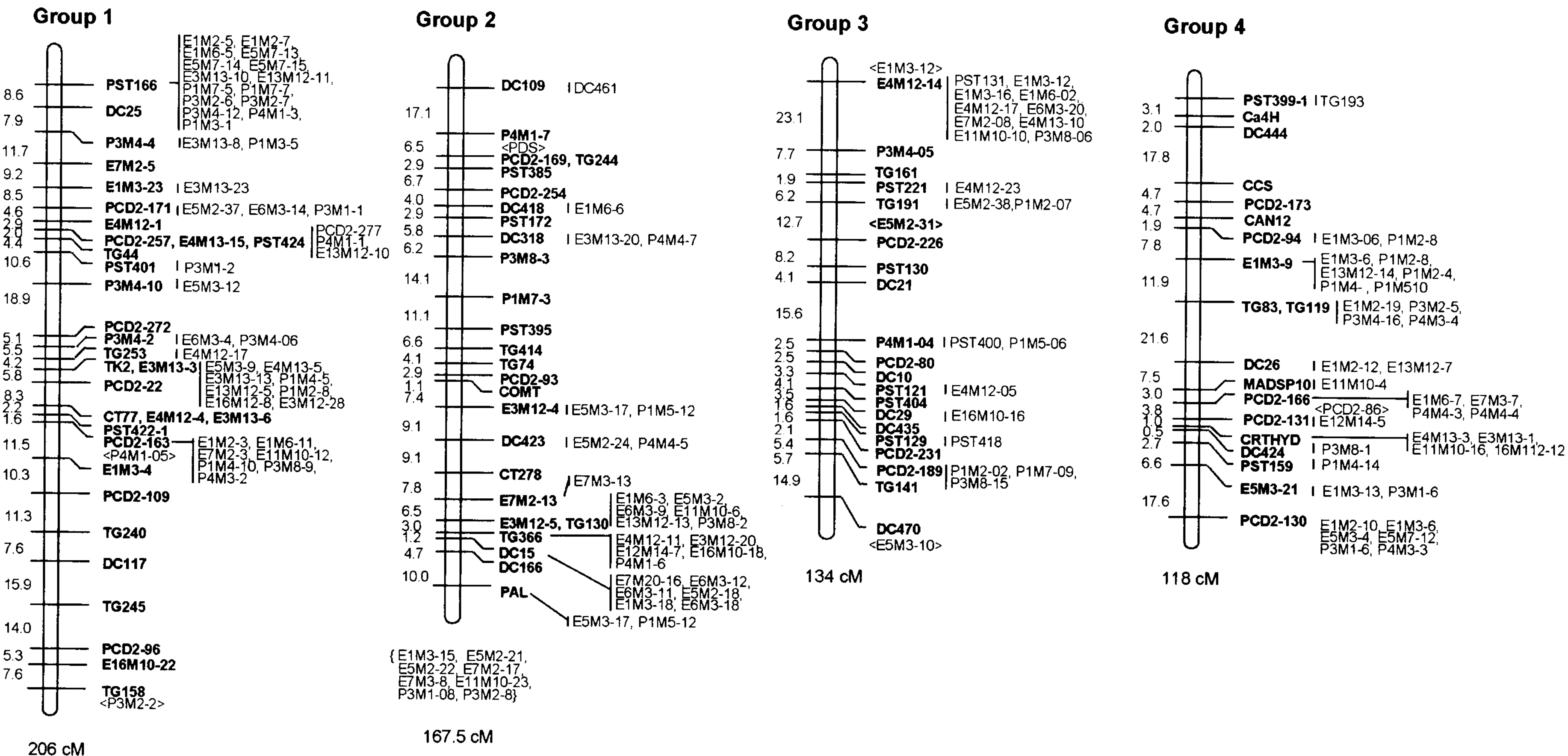

Figure 1. A combined linkage map of hot pepper using 150 RFLP and 430 AFLP markers. The 16 linkage groups were arbitrarily labeled according to total map distance of each linkage group. On the left of the vertical double lanes are map distances in cM calculated by Kosambi function and on the right are DNA markers by identification numbers and names. AFLP markers were designated by the code for *Eco*RI (or *Pst*I) and *Mse*I selective primers followed by the numbers given according to the descending order of molecular weights. Framework markers, in bold, were ordered at LOD>3.0. Markers following the comma were cosegregating markers. Markers to the right of vertical bars were mapped to either side of the closest framework markers at LOD>2.0. Markers in the parenthesis were placed between framework markers at 2<LOD<3. Markers listed below the linkage group showed linkage to that linkage group but specific map position could not be ascertained.

Group 5

DC110
8.9
CAN21
20.1
DC27
5.2
PCD2-134 E1M6-14, E5M3-1, E5M7-3, E7M3-18, E3M13-17, E16M12-13, P1M3-1
0.7
PCD2-78
17.7
<E7M3-4>
E5M2- 5 E7M3-11, E5M3-19, E7M2-6, P1M2-5
11.0
P1M4-8 E5M2-36, E5M3-15, E7M3-3, E3M13-7, E3M13-9, E3M13-14, E3M12-10, E3M12-16, E3M12-18, E12M14-2, E11M10-22, E16M10-20
4.5
PCD2-8
2.7
DC30
1.6
PST135 E16M10-13, E16M10-6, E16M10-5, E11M10-8
4.4
P1M5-13
3.9
<DC472>
PST420 E5M3-20, P1M4-11, P1M7-6
14.7
<E7M2-5>
E4M12-3
7.6
PCD2-276
8.3
<DC13>
P1M5-1

111.3 cM

Group 6

DC114 E1M2-18, E5M3-18, E1M2-14, E1M3-7, E1M3-8, E16M10-3, E16M10-10
26.4
CT135, E1M6-9, E5M3-2, E5M3-14, E5M3-16, E6M3-8, E6M3-13, E7M3-20, E4M13-6, E3M13-24, E11M10-13
RDNA5S
2.3
PST408
6.7
<DC455>
2.0
PST132 E13M12-5, P3M4-9
10.8
PST187, PST196 E16M10-4
12.9
DC482 E5M7-3
14.0
aa/ctg2 E4M13-13, P1M3-10
13.3
PFTF TG216
15.0
PST193 PCD2-216, 212
CaSIG19

103.3 cM

Group 7

GGPS
28.1
PCD2-115
11.3
TG62
3.3
TG42
3.5
TG264
12.2
DC314, E16M112, E11M10-20, E11M10-2 E1M3-14, E1M3-21, E1M6-11, E5M2-8, E5M2-34, E5M2-16, E5M2-35, E4M12-21, E4M13-12, E3M13-22, E3M12-3, E3M12-15, E11M10-9, E16M12-5, E16M12-7, P1M4-5, P3M2--2, P3M8-11, P4M3-1
11.2
PSY
11.9
P3M8-4

96.7 cM

Group 8

TG350
13.6
CD2
0.9
PCD2-177
0.1
E5M2-28
13.3
TG498
26.4
PCD2-27
22.5
PST220
5.9
PCD2-11

82.6 cM

Group 9

E6M3-17 E5M7-19, E7M3-16, E16M12-15
10.4
E3M13-12
11.3
PST164 P1M4-1, P1M5-8
12.9
CD127 E3M13-16, E13M12-1, E4M12-16, E12M14-1, E16M10-17, E16M10-23, E16M12-6
2.4
PST177
0.7
PST175 E1M3-10, E1M6-19, E5M2-33, E4M12-2, E12M14-3, P1M3-8, P1M5-1, P4M3-6
5.4
E3M13-21 E1M2-16, E1M3-3, E7M2-18, E7M3-9, E3M13-4
27.5
TG46
11.6
<P1M4-9 >
E16M10-

82.4 cM

Group 10

<E5M7-7, E7M3-17>
CAN7
12.0
TG363
19.5
PST113
15.0
PCD2-187
20.6
LCY
9.9
RDNA25S

77 cM

Group 11

PCD2-190
13.4
E4M12-10, E11M10-25 E1M2-11, E7M2-9, E4M12-24, E4M13-8, E3M12-27
16.0
PST125 E1M2-6, E1M3-1, E1M3-19, E5M2-2, E5M2-13, E5M2-10, E5M7-10, E4M12-7, E4M12-8, E4M13-14, E12M14-4, E11M10-1, E16M10-9, E13M12-8
15.0
E4M12-7
25.2
PCD2-179

60.3 cM

Group 12

E5M2-15 P3M4-13, P3M4-13
9.4
PCD2-102
6.5
PST 216 P3M8-13
11.0
E3M13-
5.6
CT220 E5M2-4, P1M3-4
<P1M2-6>

32.6 cM

Group 13

<P1M2-1>
PST112
20.7
PST179 P1M7-2
10.8
E1M3-22 E6M3-3, P1M5-4

31.5 cM

Group 14

CAN 15
24.1
PST138

24.1 cM

Group 15

PCD2-181 E1M2-20, E6M3-10
11.8
PCD2-66 P4M4-8, P4M4-9

11.8 cM

Group 16

PCD2-72 P1M5-14
10.1
CT252 P1M5-15

10.1 cM

Figure 1. (continued)

was also confirmed through progeny test with 53 F_3 individuals that plants bearing the red fruit possessed TF68-derived PSY allele(s) and others with orange fruit had Habanero- derived PSY only (data not shown).

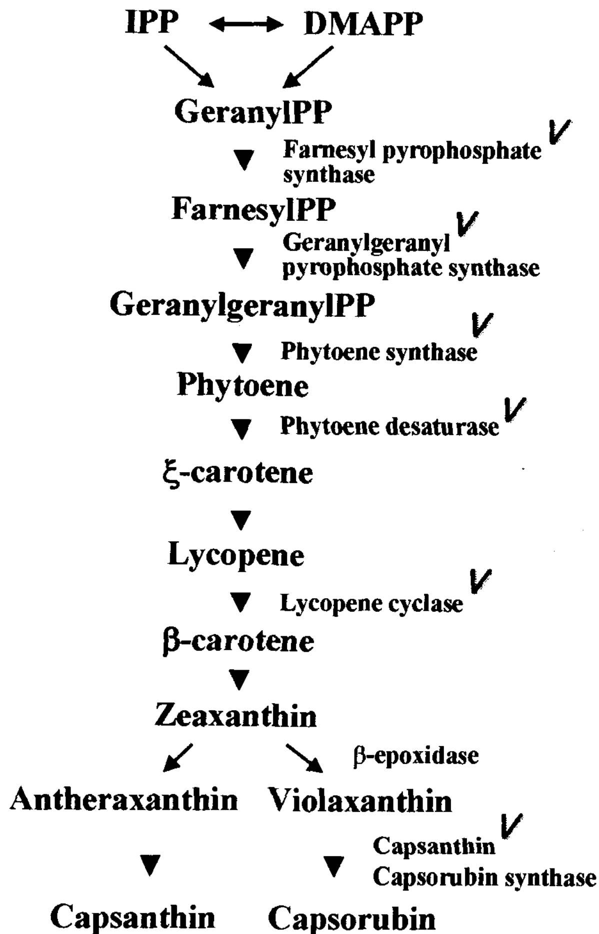

Figure 2. Carotenoid biosynthesis pathway.

Phytoene synthase might be the putative c2 locus

Hurtado-Hernandez and Smith (1985) reported that there were three gene pairs involved in ripe fruit color expression, and that there were eight colors in the F_2 of a cross between the normal red (*y+c1+c2+*) and white (*yc1c2*) fruited peppers. According to them, the orange color results from the highly reduced red pigment due to the presence of the *c2* factors that are in its recessive genetic expression. Kormos and Kormos (1960) reported that the recessive expressions, *c1* and *c2*, reduced the pigmentation of *y+* and *y* by the inhibition of β-carotene. When *c2* was present the pigment reduction was approximately 1/10, and with *c2*, only traces of pigments of *y+* and *y* were expressed.

Another candidate gene, CCS has been known to determine red and yellow color in pepper fruit (Lefebvre et al. 1998) and yellow color in pericarp is due to the deletion of CCS. But primers designed to amplify this genomic fragment successfully generated the same expected length of CCS both in TF68 and Habanero. Southern blot analysis revealed that CCS is present in single copy in both parents. Also, CCS was located in the linkage group 4 whereas the color determining locus and phytoene synthase were both located in linkage group 7. From these results it was concluded that the gene determining mature fruit color for TF68 and Habanero is not the y locus gene for *ccs* but the *c2* locus gene for *psy*.

In this study, orange Habanero fruit had about 1/6 the amount of total carotenoid pigment than TF68. Also, the PSY was not linked to another candidate gene, CCS, which implies that the PSY and CCS segregate independently. This fact is also consistent with the results of the above-mentioned study in which three fruit color loci are known to be unlinked. Further genetic analysis with cultivars with similar phenotypes should be done to confirm that the PSY is responsible for determining red and orange color.

With the aid of molecular markers, breeding of the fruits of different colors will be accelerated since the PSY and CCS specific markers make it possible to select red pepper at the seedling stage without waiting until the mature fruit stage. For example, trying to introgress red color into yellow or orange-fruited pepper with strong pungency such as Habanero will take less time since these markers are very useful in selection of lines with the desirable characters.

Map application

Most immediate application of the molecular linkage map is to locate markers linked to genes of economic and scientific interest on the map. About 110 phenotypic genes have been characterized in pepper (Daksalov and Poulos 1994). Only 3 morphological traits (*fc*, *up*, *Mf*) and 3 disease resistance traits have been placed on the molecular linkage maps (Caranta et al. 1997; Lefebvre et al. 1996; Murphy et al. 1998). With regard to

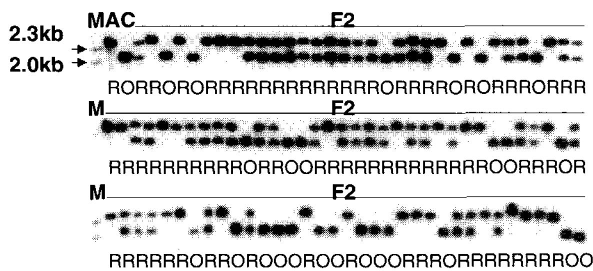

Figure 3. Hybridization of the PSY probe to a Southern blot containing DNA from parents and F_2 plants digested with *Eco*RI. Upper and lower bands represent the PSY alleles from TF68 and Habanero, respectively. The probe was an *Eco*RI-digested, 1.5 kb genomic fragment of the PSY at the 5' end. M,□ //*Hin*dIII size marker; A, TF68; C, Habanero; O, orange; R, red.

fruit quality traits such as fruit color, pungency, and nutritional value, little is known about their structural and regulatory genes. Our map is particularly useful in studying genes related to the biosynthetic pathways of carotenoids and capsaicinoids. Since we used two pepper lines with different fruit colors (red and orange) and pungency levels, we could obtain an F_2 population segregating fruit color and pungency.

Placing expressed cDNA clones (EST clones) for tissue-specific expressed genes on a molecular linkage map is an important tool for clarifying the organization of the plant genome. Furthermore, the EST markers make it possible for direct comparison of gene distributions among different plant genomes. Although we used about 61 EST clones derived from the anther-specific cDNAs of hot pepper, only 5 clones could be mapped on this map. To obtain more detailed information we are currently adding more EST clones of cDNAs derived not only from anther but also from other tissues like fruits and leaves.

Reference

Caranta C, Palloix A, Lefebvre V, Daubeze A-M (1997) QTLs for a component of partial resistance to cucumber mosaic virus in pepper: restriction of virus installation in host-cells. Theor Appl Genet **94**: 431-438.

Daskalov S, Poulos JM (1994) Updated *Capsicum* gene list. Capsicum and Eggplant Newsletter **13**: 15-26.

Huh JH, Kang BC, Nahm SH, Kim S, Ha KS, Lee MH, Kim BD (2000) Candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). Theor Appl Genet. In press.

Hurtado-Hernandez H., Smith PG (1985) Inheritance of mature fruit color in *Capsicum annuum* L. J Hered **76**: 211-213

Kang BC, Nahm SH, Huh JH, Yoo HS, Lee MH, Kim BD (2000) An Interspecific (*Capsicum annuum* × *C. chinense*) F_2 Linkage Map in Pepper Using RFLP and AFLP Markers. Theor Appl Genet. In press.

Kim BD, Kang BC, Nahm SH, Kim BS, Kim NS, Lee MH and Ha KS (1997) Construction of a molecular linkage map and development of a molecular breeding technique. J Plant Biol **40**: 156-163.

Kim SH, Kim YH, Lee ZW, Kim BD, Ha KS (1997) Analysis of chemical constituents in fruits of red pepper (*Capsicum annuum* L. cv. Bugang). J Kor Soc Hort Sci **38**: 384-390

Kormos J, Kormos K (1960) Die Genetischen Typen der Carotinoid-systeme der Paprikafrucht. Acta Bot **6**: 305-319

Lefebvre V, Kuntz M, Camara B, Palloix A (1998) The capsanthin-capsorubin synthase gene: a candidate gene for the y locus controlling the red fruit colour in pepper. Plant Mol Biol **36**: 785-789

Lefebvre V, Palloix A (1996) Both epistatic and additive effects of QTLs are involved in polygenic induced resistance to disease: a case study, the interaction pepper - *Phytophthora capsaici* Leonian. Theor Appl Genet **93**: 503-511.

Livingstone KD, Lackney VK, Blauth JR, Van Wijk R, Jahn MK (1999) Genome mapping in *Capsicum* and evolution of genome structure in *Solanaceae*. Genetics **152**: 1183-1202.

Morgante M, Olivieri AM (1993) PCR amplified microsatellites as markers in plant genetics. Plant J **3**: 175-182.

Murphy JF, Blauth JR, Livingstone KD, Lackney VK, Jahn MK (1998) Genetic mapping of the *pvr1* locus in *Capsicum* and evidence that distinct potyvirus resistance loci control responses that differ at the cellular and whole plant level. Molecular Plant Microbe Interactions **11**: 943-951.

Nahm SH, Yu JW, Kang BC, Kim BD (1997) Selection of parental lines for hot pepper mapping population using RFLP and AFLP analysis. J Kor Soc Hort Sci **38**(6): 693-697.

Prince, JP, Pochard E, Tanksley SD (1993) Construction of a molecular linkage map of pepper and a comparison of synteny with tomato. Genome **36**: 404-417

Prince, JP, Pochard E, Tanksley SD (1993) Construction of a molecular linkage map of pepper and a comparison of synteny with tomato. Genome **36**: 404-417.