Genomics & Informatics Vol. 1(2) 108-112, December 2003

Combined Genome Mapping of RFLP-AFLP-SSR in Pepper

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Abstract

We have constructed a molecular linkage map of pepper (*Capsicum spp.*) in an interspecific F_2 population of 107 plants with 320 RFLP, 136 AFLP, and 46 SSR markers. The resulting linkage map consists of 15 linkage groups covering 1,720 cM with an average map distance of 3.7 cM between framework markers. Most RFLP markers (80%) were pepper-derived clones and these markers were evenly distributed all over the genome. Genes for defense and biosynthesis of carotenoids and capsaicinoids were mapped on this linkage map. By using 30 primer combinations, AFLP markers were generated in the F_2 population. For development of SSR markers in *Capsicum*, microsatellites were isolated from two small-insert genomic libraries and the GenBank database. This combined map provides a starting point for high-resolution QTL analysis, gene isolation, and molecular breeding.

Keywords: AFLP, *Capsicum*, mapping, RFLP, SSR

※ Abbreviations: AFLP, amplified fragment length polymorphism: LG, linkage group: LOD, logarithm of odds ratio: PCR, polymerase chain reaction: QTL, quantitative trait loci: RAPD, random amplified polymorphic DNA: RFLP, restriction fragment length polymorphism: SSR, simple sequence repeat

Introduction

Pepper (*Capsicum spp.*) is an economically important crop worldwide; it is the second most cultivated crop next to rice in Korea. Pepper consists of 12 chromosome pairs with a variable genome size from 3,200 to 5,600 Mb (Moscone *et al.*, 2003). During the last decade, plant genomic researches have become an essential tool for plant molecular genetics and breeding, and its first and indispensable step is the construction of a molecular linkage map (Tanksley *et al.*, 1992; Thorup *et al.*, 2000; Huh *et al.*, 2001). All of the published genetic maps of *Capsicum* so far have been based on either interspecific populations (Livingstone *et al.*, 1999; Grube *et al.*, 2000; Ben Chaim *et al.*, 2001; Kang *et al.*, 2001) or intraspecific populations (Lefebvre *et al.*, 2002). These studies employed RFLP, AFLP and RAPD molecular markers. Nagy *et al.* (1998) and Huang *et al.* (2000) reported the development of SSR markers from a pepper small-insert genomic library on EMBL database.

Here we report the construction of an integrated molecular linkage map of pepper using RFLP, AFLP, and SSR, based on a population of 107 interspecific F_2 individuals. The objective of this report was to add AFLP markers into a map of RFLP and SSR markers for high-resolution mapping.

Materials and Methods

A total of 107 F_2 plants, derived from the interspecific cross *Capsicum annuum* cv. TF68 × *C. chinense* cv. Habanero, were used as a mapping population. These parental plants were chosen by analysis of genetic distance and hybridizing ability (Nahm *et al.*, 1997). DNA was extracted from leaf tissue of each individual plant following a method described in Kang *et al.* (2001). RFLP and AFLP methods were described in Vos *et al.* (1995) and Kang *et al.* (1997, 2001) and the SSR method in Lee *et al.* (2003). Pepper, tomato, and tobacco clones were used as probes for the construction of an RFLP map. With 9 out of 64 *Eco*RI/*Mse*I primer combinations, AFLP analysis was performed and 136 AFLP markers were scored.

Linkage analysis of RFLP, AFLP, and SSR loci was performed using MAPMAKER 3.0/EXP (Lander *et al.*, 1987). To identify linkage groups, pairwise comparisons and grouping of markers were performed using the "group" command at a maximum recombination fraction of 20 cM and a minimum LOD score of 4.0. To establish the most likely order within each linkage group, the "order" command was used and the remaining markers were added into frame map using the "try" command. The order of markers was confirmed using the "ripple" command.

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Accepted 8 December 2003

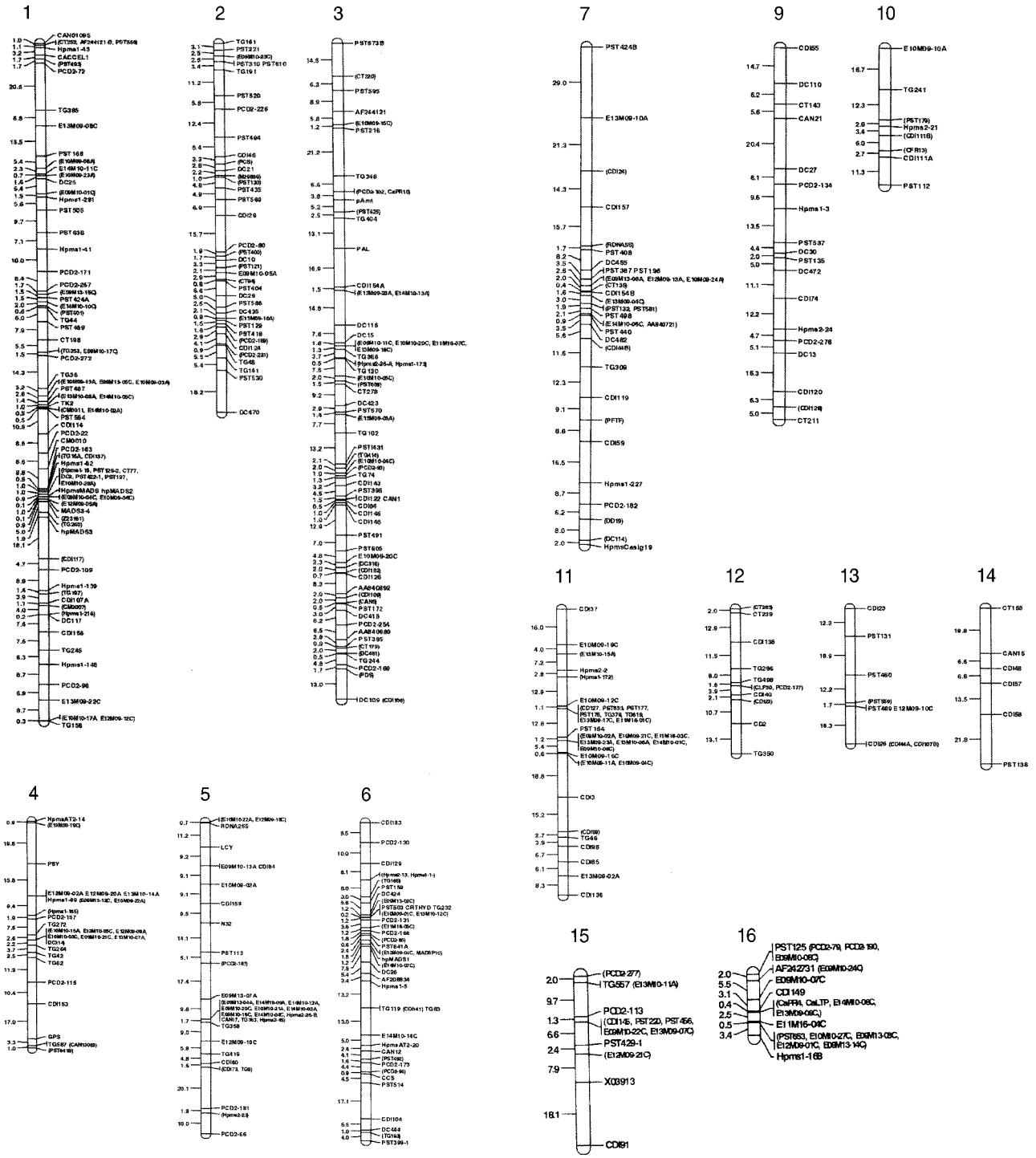

Fig. 1. Combined map with RFLP, SSR and AFLP of pepper. The linkage groups (LG) (1-12) were labeled according to synteny with tomato markers described in Livingstone *et al.* (1999) and Ben Chaim *et al.* (2001), and other groups were arbitrarily labeled according to total map distance of each linkage group. LG 8 is not present in the pepper map anymore as a result of matching corresponding LGs between tomato and pepper. On the left of the vertical double lines are map distances in cM calculated by the Kosambi function and on the right are DNA markers. Markers are framework markers ordered at LOD>3 and markers in the parenthesis were placed between framework markers at LOD<3 or located using the "try" command. Uppercase letters at the end of the marker names indicate that the marker is one of at least two segregating loci detected by a single assay.

Alternatively, a framework of markers was generated using the "compare" command and the best order was confirmed. Recombination fractions were converted to map distances in centiMorgans (cM) using the Kosambi mapping function (Kosambi, 1944).

Results and Discussion

RFLP analysis

To perform RFLP analysis, 550 pepper clones and 108 tomato clones were assayed for polymorphism on southern blot survey filters containing parental total DNA fragments digested with five restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I). Of the 550 pepper clones assayed, the number of probes which revealed RFLPs with at least one enzyme was 255 (46%). When tomato clones were assayed, 63 out of 108 clones (58%) showed polymorphism. Of the 318 polymorphic clones, 287 were usable for RFLP linkage analysis, the other markers could not be scored clearly for F_2 individuals. The addition of 33 RFLP markers resulted in a total of 320 RFLP markers for this map (Table 1).

AFLP analysis

Reliability and reproducibility of AFLP analysis was evaluated and it was found that the technique is both reliable and efficient for marker selection and mapping in hot pepper.

With 9 out of 64 *Eco*RI / *Mse*I primer combinations, AFLP analysis for 106 F_2 individuals was performed and 136 AFLP markers were scored (Kang *et al.*, 1997). All markers were scored as dominant although some bands showed intensity differences between putative homozygous and heterozygous alleles. Per primer combination, there were on average 15.1 markers for *Eco*RI / *Mse*I combination. Although many more polymorphic fragments were observed, not all of them could be registered, due to the dense and sometimes overlapping band patterns.

The usefulness and applicability of AFLP markers in genetic linkage mapping was evaluated by examining all 136 markers with χ^2 test for goodness of fit. This statistical analysis revealed that 22 markers out of 136 (16.2%), deviated from the expected segregation ratio.

SSR analysis

Microsatellites or simple sequence repeats are highly variable DNA sequences that can be used as informative markers for the genetic analysis of plants and animals. For development of microsatellite markers in *Capsicum*, microsatellites were isolated from two small-insert genomic libraries and the GenBank database (Lee *et al.*, 2003).

Table 1. List and number of markers registered on this pepper map.

Marker	Description	Number
SSR	Small-insert and GenBank	46
RFLP		
DC	Pepper SA-induced cDNA Clone (SNU)	28
PCD	Pepper Leaf cDNA Clone (SNU)	37
PST	Pepper Genomic DNA Clone (SNU)	81
CD/CT/TG	Tomato Clone (Cornell U)	63
CDI	Pepper Disease-Related EST Clone (KRIBB)	60
CAN/CLF/CFR	Pepper EST (Sogang U)	10
SB, etc.	Carotenoid & capsaicinoid related genes	27
Tob	Tobacco Disease-Related EST Clone (KRIBB)	2
Others		12
AFLP	*Eco*RI / *Mse*I primer combination	136
Total		502

Using five types of oligonucleotides, $(AT)_{15}$, $(GA)_{15}$, $(GT)_{15}$, $(ATT)_{10}$, $(TTG)_{10}$, as probes, positive clones were isolated from the genomic libraries and sequenced. Out of 130 positive clones, 77 clones showed microsatellite sequences, from which 40 reliable microsatellite markers were developed. $(GA)_n$ and $(GT)_n$ sequences were found to occur most frequently in the pepper genome, followed by $(TTG)_n$ and $(AT)_n$.

Isolated pepper microsatellites were examined for marker development. Out of the 77 microsatellite clones, 29 clones were unsuitable for designing primer sequences because of partial sequence data (19 clones), the location of microsatellite sequences near the cloning site (8 clones), and a sub-optimal melting temperature detected during PCR primer selection (2 clones).

The remaining 48 clones containing complete and suitable microsatellite sequences produced products ranging from 100 to 300 bp. Primers that amplified products were given serial numbers following designation *Hpms*. In addition, 32 microsatellite sequences from the GenBank accession were used for primer design.

Combined mapping

A total of 502 markers (320 RFLP , 136 AFLP, and 46 SSR markers) were placed in 16 groups using a minimum LOD score of 3.0 and maximum recombination value of 0.25 (Fig. 1). The resulting linkage map consists of 10 large (206 - 60.3 cM) and 5 small (32.6 -10.3) linkage groups covering 1,750 cM with an average map distance between framework markers of 3.7 cM. LG 8 is not present in the pepper map anymore as a result of matching corresponding LGs between tomato and pepper. This map consists of 15 linkage groups although pepper has 12 sets of chromosomes. However, more recent data and an

additional intraspecific map we are developing suggest that both LG2 and LG15, and LG12 and LG13 are syntenic, thus reducing the number of linkage groups to 13 (unpublished data). The numbering of LGs is consistent with other pepper and tomato maps.

The total of 320 RFLP markers represens the addition of 179 new RFLP markers onto the SNU map which contained 141 RFLP markers (Kang *et al.*, 2001). New additional RFLPs included pepper genomic clones, tomato clones, defense-related pepper clones, pungency-related clones, and other pepper genes. In the combined map, 63 markers are from tomato and the rest from pepper, which would serve as a good reference for pepper comparative genome studies.

In this study, most AFLP markers could not be placed unambiguously (multiple equivalent LOD scores) within the framework map. This may be due to incomplete information about homozyous and heterozygous genotypes of the AFLP markers in the F_2 mapping population.

The 46 polymorphic SSR loci have been assigned to the *Capsicum* RFLP linkage map. Of these, 13 SSR markers were obtained from GenBank and 29 SSR markers from genomic DNA libraries. Detection of discrete loci, segregation in a Mendelian fashion, and codominance of these SSR markers made them ideal genetic markers.

This map combines the strengths of different marker systems and provides new opportunities for mapping in the large pepper genome.

Our group has also constructed a pepper BAC library, containing about 15 genome equivalents (Yoo *et al.*, 2001, 2003). The combined genetic map and physical map along with this BAC library are very powerful resources to construct a precise integrated map of the pepper genome.

Acknowledgments

This work was supported by a grant from the Korea Science and Engineering Foundation (KOSEF) to the Center for Plant Molecular Genetics and Breeding Research (CPMGBR).

References

Ben Chaim, A., Paran, I., Grube, R. C., Jahn, M., van Wijk R., and Peleman, J. (2001). QTL mapping of fruit-related traits in pepper (*Capsicum annuum*). *Theor. Appl. Genet.* 102, 1016-1028.

Grube, R.C., Radwanski, E.R., and Jahn, M. (2000). Comparative Genetics of Disease Resistance Within the Solanaceae. *Genetics* 155, 873-887.

Huh, J.H., Kang, B.C., Nahm, S.H., Kim, S., Ha, K. S., Lee, M.H., and Kim, B.D. (2001). A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). *Theor. Appl. Genet.* 102, 524-530.

Huang, S., Zhang, B., Milbourne, D., Cardle, L., Yang, G., and Guo, J. (2000). Development of pepper SSR markers from sequence databases. *Euphytica* 117, 163-167.

Kang, B.C., Nahm, S.H., Huh, J.H., Yoo, H.S., Yu, J.W., Lee, M.H., and Kim B.D. (2001). An Interspecific (*Capsicum annuum x C. chinense*) F_2 Linkage Map in Pepper Using RFLP and AFLP Markers. *Theor. Appl. Genet.* 102, 531-539.

Kang, B.C., Yu, J.W., Lee, M.H., and Kim, B.D. (1997). Applicability of AFLP on hot pepper genetic analysis. *J. Kor. Soc. Hort. Sci.* 38, 698-703.

Kosambi, D.D. (1944). The estimation of map distance from recombination values. *Ann. Eugenics* 12, 172-175.

Lander, E.S., Green, P., Abrahamson, J., Barlow, A., Daly, M.J., Lincoln, S.E., and Newberg, L. (1987). MAPMAKER: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1, 174-181.

Lefebvre, V., Pflieger, S., Thabuis, A., Caranta, C., Blattes, A., Chauvet, J.-C., Daubeze, A.-M., and Palloix, A. (2002). Towards the saturation of the pepper linkage map by alignment of three intraspecific maps including known-function genes. *Genome* 45, 839-854.

Lee, J.M., Nahm, S.H., Kim, Y.M., and Kim, B.D. (2003). Characterization and molecular genetic mapping of microsatellite loci in pepper. *Theor. Appl. Genet.* (In press).

Livingstone, K.D., Lackney, V., Blauth, J.R., Van Wijk, R., and Jahn, M.K. (1999). Genome mapping in *Capsicum* and evolution of genome structure in Solanaceae. *Genetics* 152, 1183-1202.

Moscone, E.A., Baranyi, M., Ebert, I., Greilhuber, J., Ehrendorfer, F., and Hunzike, A.T. (2003). Analysis of nuclear DNA content in *Capsicum* (Solanaceae) by flow cytometry and Feulgen densitometry. *Ann. Botany* 92, 21-29.

Nagy, I., Polley, A., and Ganal, M. (1998). Development and characterization of microsatellte markers in pepper. X th Meeting on Genetics and Breeding of *Capsicum* and Eggplant pp.235-237.

Nahm, S.H., Yu, J.W., Kang, B.C., and Kim, B.D. (1997). Election of parental lines for hot pepper mapping population using RFLP and AFLP analyses. *J. Kor. Soc. Hort. Sci.* 38, 693-697.

Tanksley, S.D., Ganal, M.W., Prince, J.P., de-Vicente, M.C., Bonierbale, M.W., Broun, P., Fulton, T.M., Giovannoni, J.J., Grandillo, S., Martin, G.B., Messeguer, R., Miller, J.C., Miller, L., Paterson, A.H., Pineda, O., Roder, M.S., Wing, R.A., Wu, W., and Young, N.D. (1992). High Density Molecular Linkage Maps of the Tomato and Potato Genomes. *Genetics* 132, 1141-1160.

Thorup, T.A., Tanyolac, B., Livingstone, K.D., Popovsky, S., Paran, I., and Jahn, M. (2000). Candidate gene analysis of organ pigmentation loci in the Solanaceae. Proc. Natl. Acad. Sci. USA 97, 11192-11197.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Fritjers, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic. Acids Res.* 23, 4407-4414.

Yoo, E.Y., Kim, S.J., Kim, J.Y., and Kim, B.D. (2001). Construction and characterization of a bacterial artificial chromosome library of chili pepper. *Mol. Cells* 12, 117-120.

Yoo, E.Y., Kim, S., Kim, Y.H., Lee, C.J., and Kim, B.D. (2003). Construction of a deep coverage BAC library from *Capsicum annuum*, 'CM334'. *Theor. Appl. Genet.* 107, 540-543.