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Construction of a Molecular Linkage Map and Development of a Molecular Breeding Technique

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Peppers are originated in South America and introduced into Korea in early 1600s. Hot peppers became a very important constituent of almost every food and are therefore, indispensible in Korea. Hot peppers are also economically very important vegetable crop in Korea. Although hot pepper breeding programs for commercial variety were started in early 1970s, which is relatively late compared with other vegetable crops, most of open-pollinated varieties were replaced by F_1 hybrids during last two decades. And moreover, several seed companies are exporting F_1 hybrids pepper seed to Southeast Asia and the U. S.A. (Park, 1992).

Nowadays, the traditional agriculture is rapidly being converted into techno-agriculture in the developed countries. Developing genetic resources and new varieties of plants and animals through genome research is one of such examples. In case of the U.S.A., numerous researchers are engaged in Rice Genome project which has been supported by Rockefeller foundation since 1985. Besides, genome research projects of maize (Ahn *et al.*, 1993), tomato (Tanksley *et al.*, 1992), wheat (Bonierbale *et al.*, 1988), lettuce (Kesseli *et al.*, 1994), sorghum (Hurbert *et al.*, 1990) are being supported by USDA research grants. Map-based cloning has been successfully used in a number of instances to isolate genes from plants like *Arabidopsis* (Arondel *et al.*, 1992), rice (Song *et al.*, 1995), and tomato (Martin *et al.*, 1993) of which high-density molecular map has been already constructed. Molecular linkage maps have now been constructed for more than 30 plant species and work is under way to construct maps for the other species. To breed internationally competitive pepper varieties we should start hot pepper genome research in a full-scale keeping pace with conventional breeding.

The basic chromosome number of pepper, like most *Solanaceous* species, is n=12, and haploid genome size is 2.7×10^9, which is much larger than other *Solanaceous* species. In contrast to tomato and potato, pepper genome research is being conducted by only small research groups and is in infant stage. The objectives of our research is: (1) to construct molecular linkage maps based on RFLP and AFLP, (2) to isolate DNA markers tightly linked to several major important traits, (3) to breed plant materials for genome research, and finally, (4) to isolate genes controlling major traits through map-based cloning. To perform research efficiently, we have organized our program into two sectors, one sector for breeding genetic materials and the other for genetic and chemical analyses. Breeding sector which consists of Hung-Nong Joong-bu Breeding and Research Station and Kyung-pook National University is responsible for breeding NILs and RIL. Analysis sector which consists of laboratories from Seoul National University, Kangwon National University and Korea Basic Science Institute is responsible for molecular linkage map-construction by RFLP and AFLP, FISH analysis, and secondary metabolite analysis. The research results up to date are as follows.

CONSTRUCTION OF MOLECULAR LINKAGE MAP BY USING RFLP AND AFLP

To date, there are three molecular linkage maps of pepper. Tanksley group have established two molecular linkage maps at interspecific level from 46 backcross and 46 F_2 individuals (Prince *et al.*, 1993). Le-

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febvre (1995) reported an integrated molecular linkage map using doubled haploid population obtained from intraspecific F_1 hybrids. Now their research foci are moving to isolating DNA markers around pathogen-resistance genes.

To construct molecular linkage maps a fairly well-defined series of steps are followed. These include 1) selecting the parent plants, 2) producing mapping populations, 3) selecting probes from cDNA or genomic libraries, 4) scoring polymorphisms in the mapping population, and 5) linkage analysis. To select parents for molecular mapping interspecific and intraspecific genetic variations were examined in the genus *Capsicum* based on restriction fragment length polymorphism (RFLP). Four distinct clusters depending on species (*C. annuum, C. baccatum, C. pubesence* and *C. chinense*) were delineated among 15 accessions of pepper (Fig. 1). Accession numbers 2002 (*C. annuum*) and 1679 (*C. chinense*) were selected as mapping parents because they were shown to be genetically polymorphic and yet interfertile (Nam *et al.*, 1997). Characteristic differences between two parental lines are listed in Table 1. The F_2 population of 100 plants was constructed by selfing a F_1 hybrid of the lines 2002 and 1679. To obtain a high proportion of single and low copy number probes a cDNA library was constructed from cDNAs of young leaves, flowers, and immature fruits. The constructed cDNA library size was estimated to be 2×10^6 pfu. To gain an understanding of organization and evolution of plant chromosomes of *Solanaceae* we also obtained tomato clones from Cornell University and used for construction of a genetic map of pepper. The survey filters were prepared from parental DNA digested with 5 different restriction enzymes (*Eco*RI, *Dra*I, *Eco*RV, *Hin*dIII, *Xba*I). Up to now 63 polymorphic clones were selected from total 176 clones which include both tomato and pepper clones. We are constructing an RFLP linkage map using the clones and an F_2 population of 86 plants (Fig. 2, Table 2).

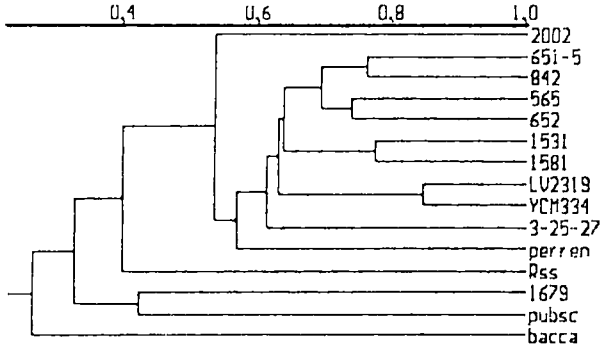

Fig. 1. Dendrogram obtained from UPGMA cluster analysis program based on Jaccard similarity coefficient by using 95 RFLP bands of 15 pepper accessions.

Table 1. Several different characters between mapping parents.

Traits	*C. annuum*	*C. chinense*
Plant growth habit	erect	compact
Leaf surface shape	smooth	wrinkled
Leaf pubscence	glabrous	abundant
Stem color	purple	green
Stem pubscence	abundant	glabrous
Flower/node	1	>2
Petal shape	sickled	straight
Stigma length	long	short
Fruit setting temperature	medium	high
Fruit shape	long/slim	campanulate
Fruit color (immature)	dense green	light green
(mature)	red	orange
Fruit pungency	mild	hot

Previously, most of molecular linkage maps were constructed by using RFLPs. But the technique requires a great deal of time and efforts, and has some limitations in applying to plant breeding. AFLP technique is a combined system of RFLP and PCR technique and has both the reliability of RFLP and the power of PCR. Recently, several successful reports regarding marker selection and mapping by using AFLP were published (Becker *et al.*, 1995; Ballvora *et al.*, 1995; Meksem *et al.*, 1995; Thomas *et al.*, 1995; Cervera *et al.*, 1996; Sharma *et al.*, 1996; Folkersma *et al.*, 1996). We adopted AFLP to detect and map polymorphic DNA markers in hot pepper. With the three *Pst*I+3 and eight *Mse*I+3 primers, 14 different primer combinations could be analyzed with two parents of mappping population (data not shown). This

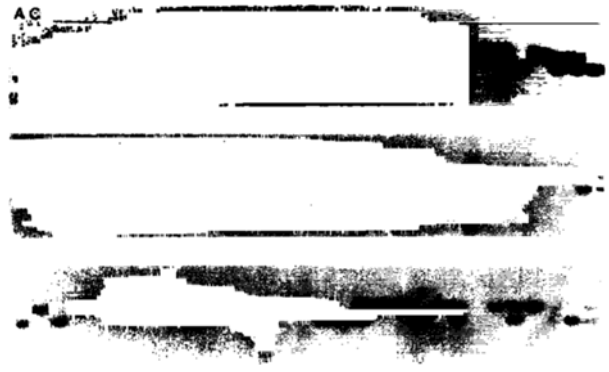

Fig. 2. Autoradiograph of RFLP segregation using pepper cDNA clone PCD2-102 and blot from F_2 population of (A) *C. annuum cv.* T.F68 and (C) *C. chinese cv.* Habanero.

Table 2. Segregations and *chi*-square goodness-of-fit tests for 25 RFLP markers in a F_2 population derived from a single cross between T.F68 (*C. annuum*) and Habanero (*C. chinense*).

Locus (clone) name	Restriction enzyme	Homozygous T.F68 allele	Heterozygous	Homozygous Habanero allele	χ^{2a}	Skewed towards
PCD2-18	*Hin*dIII	41	32	13	23.860*	T.F68
PCD2-22	*Hin*dIII	24	38	24	1.163^{ns}	
PCD2-66	*Hin*dIII	24	0	62	0.326^{ns}	
PCD2-78	*Eco*R I	21	39	26	1.791^{ns}	
PCD2-79	*Eco*R I	15	42	27	3.400^{ns}	
PCD2-80	*Hin*dIII	22	43	22	0.024^{ns}	
PCD2-82	*Xba* I	14	41	30	5.730^{ns}	
PCD2-86	*Eco*R I	21	47	18	0.675^{ns}	
PCD2-93	*Eco*R I	27	51	7	12.812*	Hetero
PCD2-94	*Hin*dIII	21	43	22	0.024^{ns}	
PCD2-96	*Hin*dIII	25	44	17	1.535^{ns}	
PCD2-100	*Dra* I	42	31	13	21.495*	T.F.68
PCD2-102	*Xba* I	22	41	23	0.210^{ns}	
PCD2-109	*Xba* I	34	43	9	13.441*	T.F.68
PCD2-113	*Xba* I	19	45	22	0.396^{ns}	
PCD2-115	*Xba* I	26	33	27	4.675^{ns}	
PCD2-125	*Xba* I	22	43	21	0.024^{ns}	
PCD2-127	*Hin*dIII	20	49	17	1.884^{ns}	
PCD2-130	*Hin*dIII	24	44	18	0.884^{ns}	
PCD2-131	*Xba* I	19	46	21	0.512^{ns}	
PCD2-131	*Xba* I	27	41	18	2.070^{ns}	
PCD2-132	*Xba* I	22	35	29	4.076^{ns}	
PCD2-134	*Hin*dIII	21	41	24	0.396^{ns}	
TG194	*Hin*dIII	3	44	39	31.838*	Habanero
TG366	*Hin*dIII	21	45	20	0.210^{ns}	

[a]Chi-square values to test segregation ratio; χ^2-test value is each 3.841 (d.f. 1) and 7.815 (d.f. 2) at significance level 0.05
*Significant at significance level 0.05
^{ns}No significant at significance level 0.05

Fig. 3. F_2 segregation pattern of AFLP markers for the primer pair *Pst*-GGA/*Mse*-CAG. Genomic DNAs from the parental lines TF68(A) and Habanero (C), and 60 random F_2 plants were prepared for segregation analysis of AFLP. Numbered arrows indicate AFLP markers.

survey revealed that for each primer combination the number of visible bands on gels ranged 41 and 81 (mean 56.6). When all 14 primer combinations were taken into account, 185 out of 792 AFLP marker bands were polymorphic (23.2%). In order to make linkage analysis markers were scored either presence or absence (Fig. 3) and all markers were tested for the expected 3:1 segregation by *chi*-square test at the P=0.05 level. Linkage analysis were performed using MAPMAKER (version 2.0) with a Macintosh computer. All pairs of linked markers were identified using group command with LOD score 7.0>0 and recombination fraction 0.25. We selected 174 AFLP markers that showed 3:1 Mendelian segregation. Linkage analysis revealed 12 linkage groups and AFLP markers seem to be distributed evenly over the linkage group (Fig. 4). Since their distribution throughout the genome is largely even, AFLPs could serve as a powerful and fast tool for estimating the genetic diversity of breeding lines.

DEVELOPMENT OF GENETIC MATERIALS FOR GENOME RESEARCH

In order to proceed mapping research on a full scale

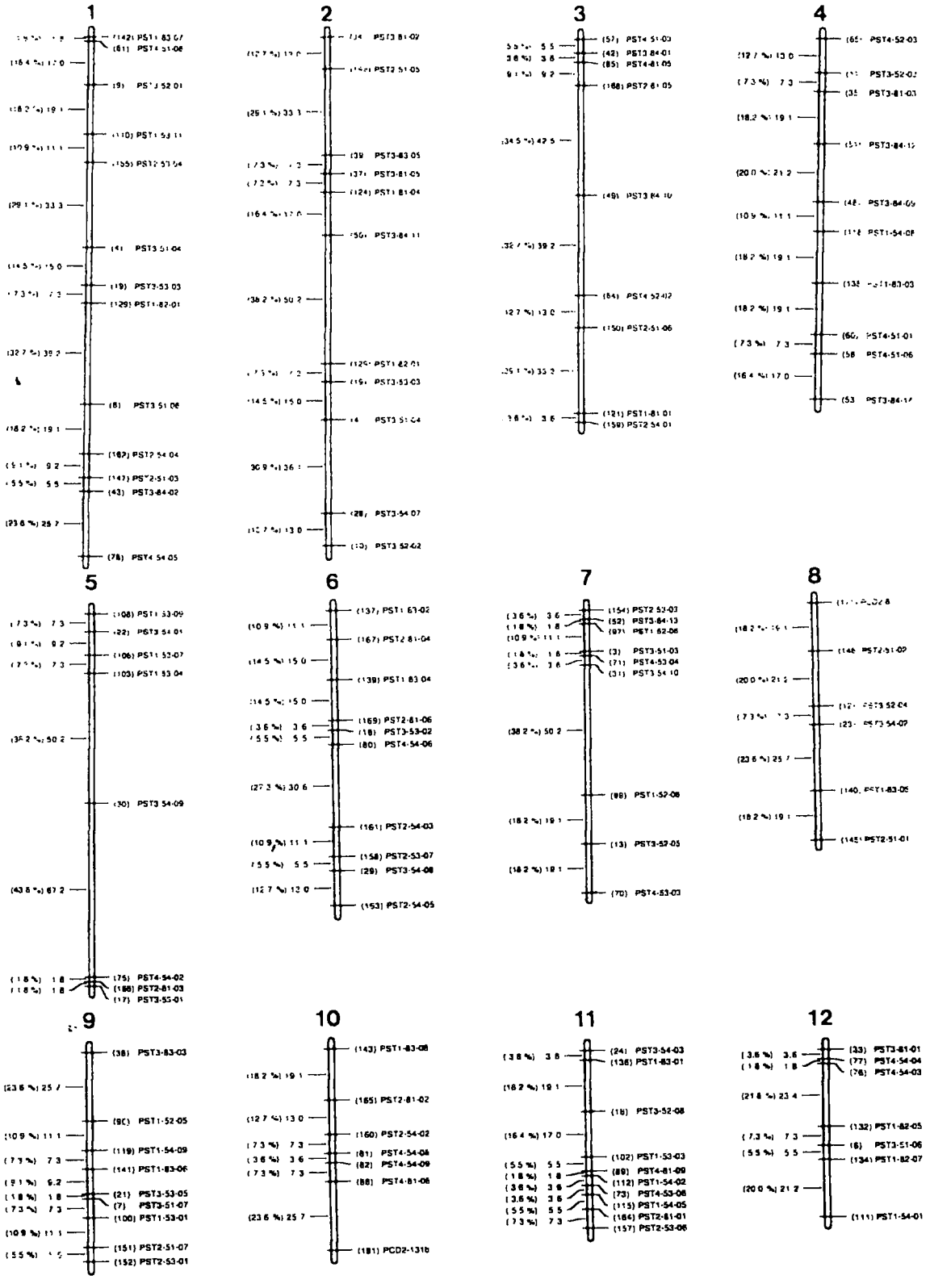

Fig. 4. A tentative AFLP linkage map of hot pepper. Total 174 AFLP markers are designated by code for the *Pst*I+3 and *Mse*I+3 selective primers followed by numbers.

Table 3. Polymorphism, origin and distribution of AFLP markers in F_2 mapping population.

Primer pair	Visible band (no.)	Polymorphic bands (no.)	Origin of amplification		Polymorphism (%)	Linkage group covered
			C. annuum	*C. chinense*		
Pst-GGA/Mse-CAC	50	10	6	4	20	6
Pst-GGA/Mse-CAG	65	15	8	7	23	3
Pst-GGA/Mse-CTA	73	16	7	9	21	5
Pst-GGA/Mse-CTC	64	24	15	9	37.5	3
Pst-GGA/Mse-CAG	52	10	9	1	19.2	2
Pst-GGA/Mse-CAA	81	13	8	5	16.0	3
Pst-GGA/Mse-CAG	67	16	8	8	23.8	3
Pst-GGA/Mse-CAT	73	19	10	9	26.0	3
Pst-GGA/Mse-CTA	55	15	8	9	27.3	2
Pst-GGA/Mse-CTC	44	7	2	5	15.9	3
Pst-GGA/Mse-CAA	41	9	7	2	22.0	3
Pst-GGA/Mse-CAG	45	11	8	3	24.4	3
Pst-GGA/Mse-CAC	42	6	3	3	14.2	3
Pst-GGA/Mse-CAT	41	14	6	8	31.1	5
Total	792	185	105	80	-	-
Average	56.6	13.2	-	-	23.3	-
Range	41-81	6-24	-	-	14-2-37.5	-

genetic materials such as recombinant inbred lines (RILs), near isogenic lines (NILs), and trisomics are indispensible. We therefore are developing genetic materials in conjunction with genome analysis research.

To construct an RI population, F_1 plants are selfed to generate F_2s, individual F_2 plants are selfed and each plant is individually harvested, creating F_3 families. The process, called single-seed descent, is repeated until the level of heterozygosity is negligible (F_8 or beyond). At this point, selfing will produce progenies which are essentially identical to previous generations. Thus, RIs constitute permanent populations which can be propagated for mapping. This materials can be used as a starting point of intra- or international collaboration of mapping project. As mentioned above we generated F_2s by selfing F_1 hybrids of *C. annuum* and *C. chinense*. At this point we are creating F_3 families.

NILs are a pair of two plant lines that are theoretically identical to each other except for a small portion of genome which contains introgressed gene. NILs are generated by repetitively backcrossing a line carrying a gene of interest (donor plant) to a line having otherwise desirable properties (recurrent parent). Genetic mapping using NILs has been suggested as an effective way for gene tagging. As one of our research objectives are tagging several important traits, we also are developing NILs for two major disease resistance genes and four morphological genes. Target genes for NILs, and their donor and recurrent parents are summarized in Table 4.

As for disease resistance NILs we are targeting

Table 4. Crosses made for disease resistance near isogenic lines.

Genetic backgroud	Source of resistance	Remark
KC201 (Chilsung)	Inbred line +KC294 (AC311) +KC263 (AC2258 +B14 (PI201234)	*Phytophthora* blight resistant
KC201 (Chilsung)	Inbred line +KC79-1-5 (PI271322) +KC297 +KC177-7-1	Bacterial spot resistant (Bs_1) (Bs_2) (quantitative)

Phytophthora and *Xanthomonas* resistance. *Phytophthora* blight of pepper plants caused by *Phytophthora capsici* Leonilan is a serious soil-borne disease in the major pepper growing areas of Korea. To generate NILs for this disease resistance we selected accessions PI201234, AC2258 and CM334, which are reported to have the highest level of resistance, as donor parents of resistance. These lines were crossed to a Korean local cultivar Subi and the classical backcross method is being used to obtain the NIL. Until now two times of backcrossing were made and BC_3 families were obtained. Bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* is not a major disease in Korea but the relationship between avirulence genes of pathogen and resistance genes of plants provides very good model system for studying pathogenesis and resistance mechanism in pepper. Single dominant genes, Bs1, Bs2, and Bs3, which render

pepper plants resistance (through hypersensitivity) to race1 (Bs2 or Bs3), race2 (Bs1 or Bs2) and race3 (Bs2) of bacterium are known. KC79 (Bs3), K297 (Bs2), and KC177-7-1 (non-hypersensitive, quantitative resistance) were used as sources of resistance for NILs. Crosses were made between these resistant lines and Subi which was also used as recurrent parent in the NILs for *Phytopthora* resistance. BC_2 seeds are now being prepared by crossing BC_1 with the recurrent parent Subi.

The labor cost comprises 69.7% of the total production cost of hot pepper in Korea. To decrease the labor cost, new varieties which can be harvested once-over by a machine, should be bred in the near future. Determinate flowering, cluster fruiting, pod-separation genes are considered as major genes for once-over harvesting variety. We are placing the research focus on cluster-fruit setting and pod-separation genes regarding this matter. To tag these genes we also are developing the NILs.

Aneuploids are plants having the chromosome set in not an exact multiple of the typical haploid set for the species. Among them trisomics, with one extrachromosome, have been routinely used for genetic analysis. The phenotype of trisomics depends on which chromosome is extra in addition to the diploid complement. Trisomics have been used to assign linkage groups to the chromosome. In order to produce and identify trisomics, we first induced autotetraploid by treating pepper seeds with colchicine. To generate autotriploids crosses are being made between induced autotetraploids and diploids. Trisomics will be generated from both the self-fertilization of autotriploids and their cross-pollinations with diploids. The chromosome identification should precede in order to identify trisomics. We performed karyotyping of five *Capsicum* species (Fig. 5). The chromosomes of these species were morphologically very similar and therefore could not be unequivocally identified using standard technique. Hence, we are trying to identify *Capsicum* chromosomes by using fluorescent *in situ* hybridization (FISH) technique.

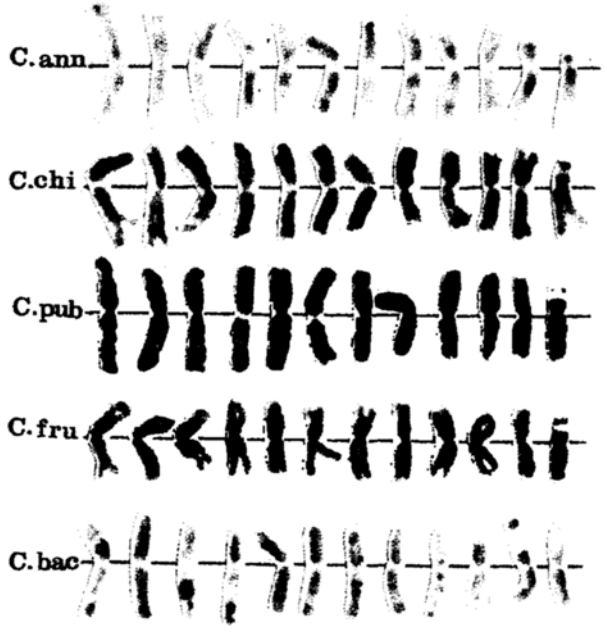

Fig. 5. Karyotypes of five *Capsicum* species.

CHEMICAL FINGER PRINTING

Capsaicinoids, amino acids, carotenoids, and vitamins are major components of the pepper fruit that determine taste, and pepper quality is determined by the amounts and kinds of them. Accurate measurement of these secondary metabolites of hot pepper fruit is a prerequisite for a high quality pepper breeding. But standardization of separating and quantifying these component still need to be fixed. Besides, current method regards one quality component as one compound as a whole. But, taking capsaicinoids as an example, there are seven different capsaicinoids (Fig. 6), and the hot flavor is generally made up of at least two and perhaps all of these compounds. Hence we have to analyze all of each compound separately to get a precise data. This conduct enables the genetic analysis of these secondary metabolites. We defined profiles of these compounds of each individual plant as a "chemical fingerprint." Chemical fingerprints will be integrated into our molecular linkage map. To achieve this goal, tech-

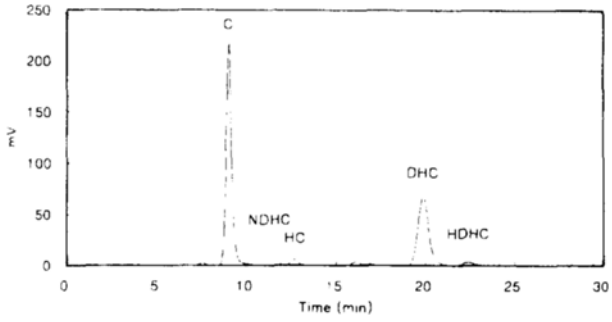

Fig. 6. High-performance liquid chromatography of capsaicinoids from red pepper, cv. Bookwang, Key: C, capsaicin; NDHC, nordihydrocapsaicin; HC, homocapsaicin; DHC, dihydrocapsaicin; HDHC, homodihydrocapsaicin.

niques for separating and quantifying carotenoids, amino acids, vitamins, and capsaicinoids, have been established (Kim *et al.*, 1997). Chemical fingerprinting analysis is currently being conducted on F_2 population.

CONCLUSION

Although this research program is in the state of beginning, it seems like that the progress will be made more rapidly than expected. The reason is because new techniques and facilities derived from human genome research and other advanced genome program will overcome the current limitations and difficulties.

Genome research cannot be accomplished by one or two research groups. That is why the program should be well organized and planned by scientists in various fields with common interest. Considering the tremendous amounts of time, money, and efforts required for performing genome research, the genome research programs should aim at concrete goals, such as getting new solutions that cannot be obtained by conventional approach, rather than remain as research for research is sake.

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