Korean J.Plant.Res. 7(2) 136~140(2004)

Detection of Individuals Restoring Fertility by DNA Fragment Converted into STS (sequence tagged site) in Red pepper

Jang-Soo Lee*, Keun-Hyang Lee[1], Yeo-Song Kim[1], Kwon-Kyoo Kang[1], Ill-Sup Nou[2], and Yutaka Hirata[3]

*Dong Won Nong San Seed Co., Namsa-Myeon 554-5, Yongin, Korea

[1]Department of Horticulture, Hankyong National University, Anseong 456-749, Korea

[2]Faculty of Plant Science and Production, Sunchon National University, Sunchon 540-742, Korea

[3]Graduate School of Agriculture, Tokyo University of Agriculture & Technology, Fuchu, Tokyo 183-8509, Japan

ABSTRACT

Co-segregation of male fertility with DNA markers selected by RAPD analysis as being potentially linked to the restorer gene (Rf) for Cytoplasmic male sterility (CMS) was analyzed using segregating F2 population. One RAPD marker directly linked to the Rf locus was identified. Amplification of OPT-02/570 using the STS primers generated a monomorphic band of each fertile plants randomly selected F2 progenies. From these results, this specific marker would be strongly linked to be restoring gene. The use of STS marker is effective in overcoming the reliability of the RAPD phenotype and improving their utility for MAS, co-dominant STS markers are especially very useful.

Key words **:** bulked segregant analysis, restorer gene (Rf), STS marker

INTRODUCTION

Cytoplasmic male sterility (CMS) is a widespread phenomenon in higher plants, and commercial production of F1 seeds utilizing CMS is now a common practice in many crops. CMS is a maternally inherited trait characterized by the inability to produce viable pollen associated with mitochondrial DNA (mtDNA) rearrangements, mutation, and editing (Dewey et al. 1986; Werner and Frank 1997). Also CMS is often considered to be a nuclear-mitochondrial incompatibility since plants with specific nuclear genes, termed restorers of fertility, are male-fertile when carrying a CMS-inducing cytoplasm (Hanson 1991; Braun et al. 1992). In many cases, restorer genes have been found to alter the expression of mitochondrial gene regions implicated in specifying CMS, but the mechanism through which this is achieved appears to vary among different CMS systems. Until now, genetic markers linked to restorer genes have been identified for various maize CMS system (Sisco 1991; Kamps and Chase 1992; Schnable and Wise 1994; Wise and Schnable 1994), for the *Rfo* restorer of rapeseed *ogu* CMS (Delourme and Eber 1992; Delourme et al. 1994), for a CMS restorer in bean (He et al. 1995), and for the *Rfm*1 restorer of barley (Matsui et al. 2001). In

*Corresponding author : **Jang-Soo Lee,** E-mail:

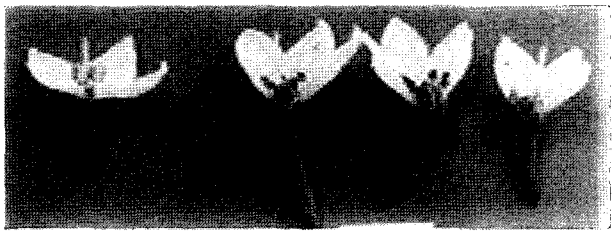
Capsicum, Peterson (1958) firstly reported a CMS plant, which was spontaneously segregated from a cultivated pepper. However few aspects corresponding to the genetic system of CMS and its restoring gene (Rf) of Capsicum have been reported (Shfriss et al. 1993; Kim et al. 2001). The study reported here is focused on restorer genes for the CMS. We report the identification of STS marker linked to fertility restorer gene in order to apply the linked marker to marker-assisted selection and map-based cloning of the relevant gene.

MATERIALS AND METHODS

Plant materials and fertility scoring

Capsicum annuum lines and cultivars used in this studies were kindly provided by DongWon NongSan Seed Company. Cytoplasms and genotypes are designated in italics and between parentheses following the cultivar name. The restorer sources used in the studies are the BC15 breeding line which carries the RfRf restorer gene selected from genetic resources of *C. annuum*.

Plants were grown to maturity in green house under natural conditions. The fertility was assessed by the careful observation of ten flowers per plant at least two times during the flowering period. The overall morphology of the flowers was noted as well as the number of anthers producing pollen (Fig. 1).



MF MS

Fig. 1. Visible variation of male fertility flower(MF) and male sterile flowers (MS).

Isolation of genomic DNA and RAPD

Genomic DNA was isolated from leaf tissue following the procedure of Komatsuda et al. (1998). We are a total of 220 10-mer oligonucleotides primers, corresponding to OPA through OPZ (Operon Technologies, Alameda, Calif.). We used these primers to detect polymorphism between bulked DNA samples (one was the bulked DNA which were mixed with 4 fertile plants randomly selected F2 progenies, and the other was the bulked sample from 4 steriles) in the random amplified ploymorphic DNA (RAPD) profiles. Amplification by polymerase chain reaction (PCR) was performed as described by Park et al. (1997). Amplified fragments were separated by electrophoresis on 1.5% agarose gels prepared in 0.5 × TBE (1 × TBE: 89 mM Tris-borate plus 2 mM EDTA). Approximate size of the amplification products were determined by reference to the DNA molecular weight marker (Promega).

RFLP of mitochondrial DNA

DNA samples (5㎍) were digested with each of for restriction enzymes (BamH I, EcoR I, Pst I and Hind III, electrophoresed, and transferred onto Hybond TM-N plus membranes (Amersham Int Plc, UK)) and analyzed in their RFLP of mitochondrial DNA by Southern hybridization with the DIG system (Boehringer Manheim Germany). Mitochondrial gene clones, atpA, atp6, and cox II of sugar beet were used as the probes, which were supplied by Dr. Kanno, Tohoku Univ., Japan. Their RFLP data were used to generate a clustering tree according to the maximum likelihood algorithm using PHYLIP software program.

Sequence-tagged site (STS) analysis

RAPD fragments were purified from the gel with Geneclean 2 (Bio 101). The fragments were cloned into the PCR II vector using a TA cloning kit (Invitrogen). Nucleotide sequences were determined with Fluoresence Cycle Sequencing Kits and a 373A

Table 1 Genetic analysis of fertility restoration

Female parent (CMS)	Male parent (Restorer)	No. of F2 plants	Number of plants		Ratio
			Male-fertility	Male-sterite	
412	6801	64	47	17	2.76 : 1
413	6810	63	48	15	3.20 : 1
440	10012	65	47	18	2.88 : 1
446	6802	66	49	17	2.54 : 1

automated DNA sequencer (Applied Biosystems, Perkin-Elmer Crop). STS primers designed for OPT-02/570 were 5'GGAGAGACTCACGGATTCAC3' and 5'ACTCGATCGCGCCACTGAA3', and for OPH-04/1320 these were 5'GGAAGTCGCCGACATT ACT3' and 5'AGTAAAGTGTGCTAGCAATG3'. After incubation at 95 for 5 min, the samples were subjected to 30 cycles of 95℃ for 1 min, 60℃ for 2 min, and 72℃ for 2 min. Extension of the amplified product was then allowed to proceed at 72℃ for 10 min. The program Temp Control System TP-3000 (TAKARA) was used for these amplifications.

RESULTS AND DISCUSSION

Fertility segregation in F2 population

Plants of F2 populations were unambiguously classified as ether sterile or fertile, because we observed no partial seed set or variation in anther morphology in the lines. The segregations of F2 populations fitted the expected monogenic segregation ratios of 3 : 1, respectively (Table 1).

Identification of cytoplasmic male sterility

To investigate the difference in RFLP patterns of mtDNAs between intra- and inter-species level, membrane blots were hybridized with the three DNA probes. Southern blot patterns were showed polymorphic among species and identified to CMS lines, which did not appear in normal lines (Fig. 2, arrow). All CMS lines were identical as far as hybridization patterns of 3 mitochodrial genes were concerned. This fact suggests all commercial CMS lines were from a single origin although they have been improved in different breeding fields of seed companies. A cluster analysis of phylogenetic relationship based on the mitochondrial RFLP among *Capsicum* species revealed three major groups; the first group composed of *C. chinense*, the second included *C. annuum* accessions, and the third with *C. baccatum* and *C. frutescens* (Fig. 3). CMS lines were subclustered in the group of *C. annuum*, indicating that CMS might have generated from a variant cytoplasm of *C. annuum*.

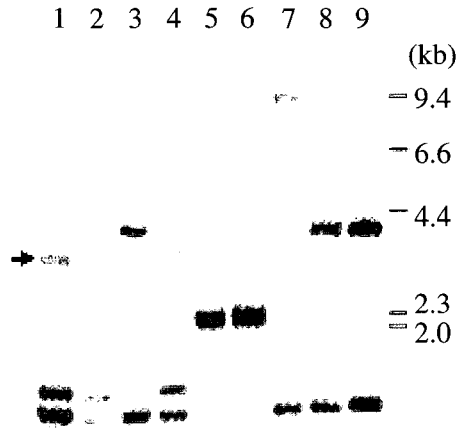


Fig. 2. Southern hybridization of mitochondrial gene, atp6 as a probe. Lane 1; CMS's, Lane 2 to 3; *C. annuum* var. *annuum*, Lane 4; *C. annuum* var. *mininum*, Lane 5 to 7; *C. chinense*, Lane 8; *C. baccatum* var. *baccatum*, Lane 9; *C. baccatum* var. *pendulum*.

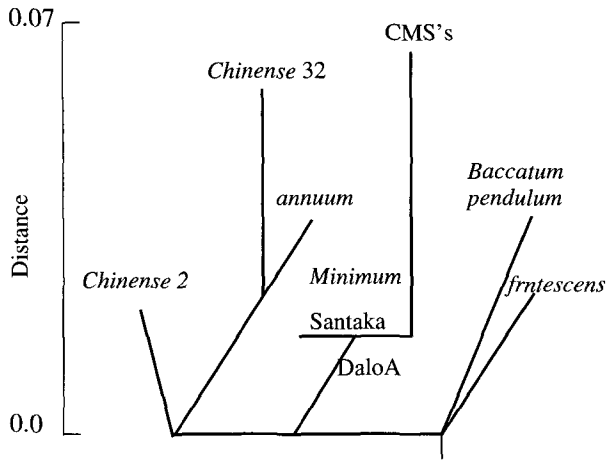


Fig. 3. Phylogenetic tree revealed from RFLP's in mitochondrial DNA of *Capsicum* based on Maximum-Likehood method.

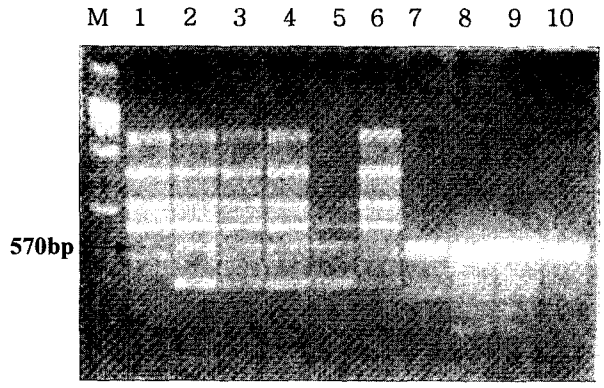


Fig. 4. Identification of the linkage marker to restoring gene by bulked segregant analysis. Lane 1; Female line (CMS), Lane 2; Male line (Restorer), Lane 3; F1, Lane 4 to 5; F2 bulk from 4 fertility plants, Lane 6; F2 bulk from 4 sterile plants. Lane 7 to 10; PCR product from specific band slicing gel of lane 2 to 5, respectively.

Identification of RAPD fragment for fertility restoration

Six 10-mer primers produced a total of seven polymorphic fragments between bulked DNA samples (one was the bulked DNA which were mixed with 4 fertile plants randomly selected F2 progenies, and the other was the bulked sample from 4 steriles) in the random amplified ploymorphic DNA (RAPD) profiles (Fig. 4). The linkage of these fragments to the *Rf* gene was initially tested using 10 F2 individual plants. One fragment generated by OPT-02 (5'GGAGAGACTC3') was linked to the *Rf* locus, while the remaining six primers were not linked to the *Rf* locus. Approximate size of the polymorphic fragment was 570bp, and it was designinated as OPT-02/570.

Converted from OPT-02/570 to STS marker

To obtain additional evidence for specific fertility restorer lines, we attempted to convert RAPD marker to STS marker and tested for polymorphism between normal line and restorer line. Amplification of OPT-02/570 using the STS primers generated a monomorphic band of each fertile plants randomly selected F2 progenies (Fig. 5). Also This STS marker was detected in most of F1 varieties available in Korean market. From these results, this STS marker would be strongly linked to the restoring gene.

Using the RAPD and STS markers flanking the *Rf* locus that we identified in this study, the selection becomes easier. Marker assisted selection (MAS) using RAPD markers is effective because a large number of samples can be handled with easy manipulation at on time. However, there are several problems associated with the reliability of the RAPD phenotype. The use of STS markers developed from RAPDs is effective in overcoming the problems and improving their utility for

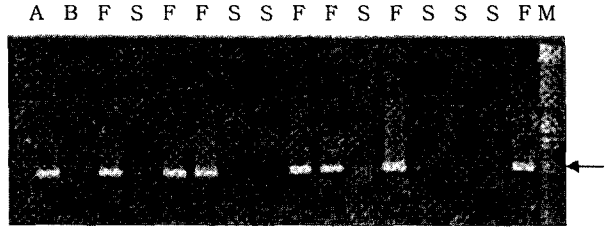


Fig. 5. Detection of individuals restoring fertility by DNA fragment converted in to STS. Lane A; Female line (CMS), Lane B; Male line (Restorer), Lane F; fertile plant of F2, Lane S; sterile plant of F2, Lane M; Molecular marker.

MAS, co-dominant STS markers are especially very useful.

LITERATURE CITED

Braun CJ, Brown GG and Levings CS. 1992. Cytoplasmic male sterility. In: Dennis ES, Hohn B (eds) Advances in plant gene research: cell organelles, vol 6. Springer, Berlin Heidelberg New York, pp 219-245.

Delourme R, Bouchereau A, Hurbert N, Renard M and Landry BS. 1994. Identification of RAPD markers linked to fertility restorer gene for the Ogura radish cytoplasmic male sterility of rapeseed (*Brassica napus* L.) Theor Appl Genet 88:741-748.

Delourme R, Eber F and Renard M. 1991. Radish cytoplasmic male sterility in rapeseed: breeding restorer lines with a good female fertility. In: McGregor DI (ed) Proc 8th Int Rapeseed Conf. Organizing Committee of the Eighth International Rapeseed Congress, Saskatoon, Canada, pp 1506-1510.

Dewey RE, Levings CS and Timothy DH. 1986. Novel recombinations in the mazie mitochondrial genome produce a unique transcriptional unit in the Texas male-sterile cytoplasm. Cell 44: 439-449.

Hanson MR. 1991. Plant mitochondial mutations and cytoplasmic male sterility. Annu Rev Genet 25: 461-486.

He S, Yu Z-H, Vallejos CE and Mackenzie SA. 1995. Pollen fertility restoration by nuclear gene *Fr* in common bean: an *Fr* linkage map and the mode of *Fr* action. Theor Appl Genet 90: 1056-1062.

Kamps TL and Chase CD. 1992. Mapping the restorer gene *Rf3* with RFLPs. Maize Genet Coop Newsl 66: 45.

Kim, D.H., J.G. Kang, S.J. Kim and B.D. Kim. 2001. Identification of cox II and atp6 regions as associated to CMS in *Capsicum annuum* by using RFLP and long accurate PCR. J. Kor. Soc. Hort. Sci. 42(2): 121-127.

Komatsuda T, Kawasaki S, Nakamura I and Takaiwa F, Taguchi-Shiobara F and Oka S. 1997. Identification of random amplified polymorphic DNA (RAPD) markers linked to the v locus in barley, *Hordeum vulgare* L. Theor Appl Genet 95: 637-642.

Masui K, Mano Y, Taketa S, Kawada N and Komatsuda T. 2001. Morecular mapping of fertility restoration locus (*Rfm*1) for cytoplasmic male sterility in barley (*Hordeum vulgare* L.). Theor Appl Genet 102: 477-482.

Schnable PS and Wise RP. 1994. Recovery of heritable, transposon-induced, mutant alleles of the *rf2* nuclear restorer of T-cytoplasm maize. Genetics 136: 1171-1185.

Park JH, YH Kim, IS Nou, JE Choi, IY Shim and KK Kang. 1997. Evaluation of genetic diversity among Korean wild *Codonopsis lanceolata* by using RAPD. Korean J. Plant. Res. 10 (3): 258-264.

Shfriss, C. and M. Pilovsky. 1993. Digenic nature of male-sterility in peper (*Capsicum annuum* L.). Euphytica 67: 111-112.

Sisco PH. 1991. Duplications complicate genetic mapping of *Rf4*, a restorer gene for cms-C cytoplasmic male sterility in corn. Crop Sci 31: 1263-1266.

Werner H and Frank K. 1997. Cell type-specific loss of *atp6* RNA editing in cytoplasmic male sterile *Sorghum bicolor*. Proc Natl Acad Sci USA 94: 11090-11095.

Wise RP and Schnable PS. 1994. Mapping complementary genes in mazie: positioning the *rf1* and *rf2* nuclear-fertility restorer loci of Texas (T) cytoplasm relative to RFLP and visible markers. Theor Appl Genet 88: 785-795.

(Received Jun. 22, 2004)
(Accepted Aug. 3, 2004)