Note

A CAPS marker linked to a genic male-sterile gene in the colored sweet pepper, ‘Paprika’ (Capsicum annuum L.)

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Genic male sterility (GMS) has been widely used as a tool for hybrid seed production in chili pepper (Capsicum annuum L.). Little work has been done on the development of molecular markers linked to pepper GMS genes, which are generally controlled by a recessive nuclear gene. In this study, we developed a DNA marker linked to paprika GMS using bulked segregant analysis (BSA) and an amplified fragment length polymorphism (AFLP) technique. Two F2 populations were made by selfing commercial F1 varieties of ‘Mirage’ and ‘Fiesta’ and subjected to BSA-AFLP using 256 primer combinations. Among five reproducible polymorphic primer combinations, an AFLP marker Egat/Mcgg was converted to a codominant cleavage amplified polymorphic sequence (CAPS) marker. This marker, named PmsM1-CAPS, is located about 2 to 3 cM from the ms locus. Although PmsM1-CAPS was not correlated with GMS in ‘MiniBell’ because it was a different GMS gene, the marker was found to be useful in screening for male sterility, as tested in F2 progenies from ‘Helsinki’ and F3 families derived from the F1 varieties used in this study.

Key Words: pepper, paprika, genic male sterility, GMS, AFLP, CAPS.

Introduction

In European countries, the term paprika implies a blocky type of sweet pepper (Capsicum annuum L.). However, in both Korea and Japan, the word indicates a sweet colored pepper that differs from other bell-type sweet peppers. The seed prices of commercial F1 hybrid paprika varieties are more expensive than those of hot chili peppers. In Korea, most hybrid seeds of hot chili peppers are produced by means of cytoplasmic male sterility (CMS), avoiding the labor-intensive steps of emasculation and hand pollination (Daskaloff and Mihilov 1983, 1998, Peterson 1958, Yoo 1990). Due to these benefits, CMS is considered the most promising tool for decreasing seed production costs in onion, sugar beet, sorghum, sunflower, rice, rapeseed, carrot, and other crop plants (Frankel and Galun 1977). However, it is very difficult to develop a stable S cytoplasm that can be maintained and restored (Lee 2001, Lee et al. 2008, Min et al. 2008). To date, no CMS system useful for seed production in elite lines of paprika has been developed.

In contrast, the use of genic male sterility (GMS) as an alternative seed production tool for paprika is more attractive, as GMS genes can be easily introgressed into diverse genetic backgrounds using simple backcrosses (Rao et al. 1990). This approach may boost the development of new elite lines with stable male sterility. More than a dozen of pepper GMS mutants have been reported to be controlled by each a recessive ms gene, some of which are used commercially (Daskaloff 1968, Meshrham and Narkhede 1982, Shifriss 1973, 1997). In addition, Csillery (1989) and Shifriss and Pilowsky (1993) proposed that a digenic GMS system (ms1ms1ms2ms2 x Ms1Ms1Ms2Ms2) could be used to enhance the number of male-sterile plants by up to 75% in seed production fields. These reports suggest that a GMS system could be a good alternative to the CMS system in paprika hybrid seed production.

Molecular markers associated with interesting traits are helpful in improving the efficiency of breeding programs in diverse crops (Young 1999). One of the limiting factors of the GMS system in chili pepper is the amount of time required to develop a 50% male-sterile line by consecutive sib crossings between male-sterile plants (msms) and heterozygous fertile plants (MsMs), as their genotypes cannot be distinguished from the homozygous fertile (MsMs) and heterozygous (MsMs) plants in the same generation. This limiting factor may be resolved, however, if molecular markers linked to the genes that control paprika GMS can be developed. Several studies for GMS marker development
have been reported in rice, maize, Brassica, etc. (Tang et al. 2006, Xie et al. 2008, Zhang et al. 2008), whereas there is no report on the development of molecular markers for GMS in peppers including paprika.

Here, we report the development of a molecular marker linked to a GMS gene in commercial paprika varieties, and evaluate its usefulness in a paprika breeding program.

Materials and Methods

Plant materials

Open-pollinated F2 populations of seven commercial paprika varieties (Table 1) were kindly provided by Dr. Kweon at the Seed Research and Development Center of the National Agricultural Cooperative Federation, Anseong, Gyeonggi-do, South Korea. All of the F2 progenies were planted in a greenhouse (Anseong, Korea), and their male fertility was continuously evaluated. Among the F2 progenies of ‘Mirage’ and ‘Fiesta’, sixteen fertile plants (expected to be MsMs or Msms) were selected and selfed to obtain their F3 families for further study.

Evaluation of male sterility and DNA extraction

The phenotypes of male fertility (normal pollen grains) and sterility (no pollen grains) in mature anthers were evaluated by visual observation. The commercial F1 varieties and their F2 populations were grown in a greenhouse (Anseong, Korea), and their male fertility was continuously evaluated. Among the F2 progenies of ‘Mirage’ and ‘Fiesta’, sixteen fertile plants (expected to be MsMs or Msms) were selected and selfed to obtain their F3 families for further study.

Amplification fragment length polymorphism and bulked segregant analysis

Amplification fragment length polymorphism (AFLP) analysis was performed as described by Vos et al. (1995) with minor modifications. One μg of genomic DNA from each sample was digested with an EcoRI/MseI combination and then ligated to double-stranded adaptors. The adaptor-ligated DNA was diluted five times with sterilized water, and 2.5 μl were used as template DNA for the preamplification step of the AFLP procedure. After determining which amplifications were successful using 1.0% agarose gel electrophoresis, the preamplified DNA was diluted 1:10 and used for selective amplification or bulked segregant analysis (BSA; Michelmore et al. 1991). Equivalent amounts of BSA dilutions of the preamplified DNA from four fertile and four sterile F2 individuals were pooled to obtain fertile and sterile bulks. Each DNA bulk was constructed from Mirage and Fiesta F2 plants, respectively. The selective PCR product was separated on a 5% denaturing polyacrylamide gel in 0.5× Tris-borate EDTA (TBE) buffer, followed by silver staining using a Silverstar Staining Kit (Bioneer Co. Ltd., South Korea).

Conversion of AFLP markers into CAPS markers

AFLP bands of interest were eluted from silver-stained gels according to the soak method (Sambrook and Russell 2001) and reamplified using the same PCR conditions used for AFLP (please see below). The amplified DNA was separated by electrophoresis on a 1.2% agarose gel. After purification of the fragment from the gel with a Zymoclean Gel DNA Recovery Kit (Kyonshin Scientific Co. Ltd., South Korea), direct sequencing was performed using an ABI3700 DNA sequencer (Applied Biosystems, CA, USA) at the National Instrumentation Center for Environmental Management at Seoul National University.

To sequence the genomic regions flanking the AFLP fragment of interest, primer sets specific to the fragment were designed using Primer 3 software (Genetics Computer Group Inc., Madison, WI, USA) and commercially synthesized at Bioneer Co. Ltd. PCR walking was carried out using a GenomeWalker™ Universal Kit (Clontech, CA, USA) according to the manufacturer’s protocol. Following the PCR walking procedure, candidate fragments were subjected to sequence analysis as described above. CAPS Designer (http://solldb.cit.cornell.edu/tools/caps_designer/caps_input.pl) was used to determine a consensus sequence, and CAPS primers (forward primer: 5′-TGGGCTATCCCCGTAAGCC AGCTCAT-3′ and reverse primer: 5′-GCCGAATCCCTTC ATCTATTTCCTC-3′) were designed and synthesized based on this sequence. The PCR mixture was composed of 1 μl of 10 ng μl−1 DNA, 2 μl of 10× PCR buffer (CoreBio, South Korea), 1 μl of 10 mM dNTP mixture (CoreBio),

<table>
<thead>
<tr>
<th>Variety</th>
<th>Company</th>
<th>Fruit color</th>
<th>Segregation of male fertility in the F2 population</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Mirage</td>
<td>De Ruiter Seeds</td>
<td>Red</td>
<td>75</td>
</tr>
<tr>
<td>Fiesta</td>
<td>Enza Zaden</td>
<td>Yellow</td>
<td>75</td>
</tr>
<tr>
<td>Helsinki</td>
<td>Rijk Zwaan</td>
<td>Yellow</td>
<td>50</td>
</tr>
<tr>
<td>Debla</td>
<td>Rijk Zwaan</td>
<td>Red</td>
<td>75</td>
</tr>
<tr>
<td>Jirisan</td>
<td>Rijk Zwaan</td>
<td>Yellow</td>
<td>74</td>
</tr>
<tr>
<td>Boogie</td>
<td>Rijk Zwaan</td>
<td>Orange</td>
<td>72</td>
</tr>
<tr>
<td>MiniBell</td>
<td>Samsung Seeds</td>
<td>Yellow</td>
<td>74</td>
</tr>
</tbody>
</table>
0.1 μl of 5 U μl⁻¹ Top-Taq polymerase (CoreBio), 1 μl of 10 pmole μl⁻¹ of each primer, and 13.9 μl of distilled water. 

PCR was performed in a programmable thermal cycler (Biometra Co., Germany) using the following parameters: denaturation for 3 min at 94°C, 35 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 60 s; and a final 5-min extension at 72°C. The amplified PCR products were separated on a 1.2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Results

BSA-AFLP

To identify markers linked to a male-sterile (ms) gene in the repulsion phase, four DNA bulks were prepared by mixing equal volumes of the preamplified DNA samples. Each bulk consisted of fertile and sterile F₂ individuals from Mirage and Fiesta F₁ plants. Polymorphisms in the four DNA bulks were screened using 256 AFLP primer combinations. A total of 24 primer combinations produced a single polymorphic band for each primer combination. As expected, polymorphic bands were observed only in the fertile bulks of Mirage and Fiesta F₂ individuals. Five polymorphic bands that were widespread and reproducible in the fertile bulks were subjected to further screening of their segregation in 71 and 73 F₂ individuals per segregating population from Mirage and Fiesta F₁ plants, respectively. Among them, an AFLP band amplified with the Ega/Mcgg primer combination was found to be the marker most tightly linked to a male-sterile gene, at a genetic distance of 2 to 3 cM. The AFLP band, designated PmsM1, showed two recombinant events with a phenotype of male sterility in each segregating population (Fig. 1).

Conversion of the PmsM1 AFLP marker to a codominant CAPS marker

Conversion of PmsM1 to a CAPS marker was attempted using direct sequencing and expanding the flanking regions of the sequence. PmsM1 was found to be 288 bp in size. Using a genomic walking kit with primer pairs specific to each end of the PmsM1 sequence, the flanking regions were expanded to 595 bp from the 5’ end and 491 bp from the 3’ end, respectively. These fragments were sequenced and combined to make a consensus sequence with the sequence of PmsM1, resulting in a sequence contig of 1,374 bp (data not shown). For simple and rapid detection of polymorphisms between fertile and sterile bulks, specific primers based on the consensus sequence were designed, synthesized, and used to amplify PCR fragments in a sterile bulk. PCR analysis of the sterile bulk with the specific primer pairs generated a single fragment corresponding to the expected size (980 bp) which was cut out of the gel, purified with a Gel Extraction Kit (Promega, WI, USA), and sequenced directly. A comparison of the two consensus sequences from homozygous fertile and sterile plants showed that there were ten single nucleotide polymorphisms (SNPs), at positions 170, 304, 320, 361, 446, 530, 532, 546, 890, and 924, and that restriction with the enzyme EcoRI was expected to produce three polymorphic PCR bands able to differentiate between the MsMs, Msms, and msms genotypes (Fig. 2). As expected, the combination of PCR amplification with these primer pairs and EcoRI treatment differentiated each genotype for male sterility when this marker, named PmsM1-CAPS, was applied to F₂ segregating populations from the Mirage and Fiesta F₁ plants that were used to develop the PmsM1 AFLP marker. The segregation pattern of PmsM1-CAPS was coordinated with that of PmsM1.

![Fig. 1](image-url) Segregation analysis of the PmsM1 AFLP marker in F₂ individuals from ‘Mirage’ (A) and ‘Fiesta’ (B) F₁ plants. The numbers on the upper line indicate individual F₂ plants. S and F indicate sterility or fertility in the phenotype, respectively. Arrow(s) indicate the polymorphic band, and asterisk(s) indicate a recombinant event of the phenotype.
Use of the *PmsM1*-CAPS marker in marker-assisted selection

*PmsM1*-CAPS was used to identify male sterility in five *F₂* progenies of the commercial varieties Debla, Jirisan, Boogie, Helsinki (Rijk Zwaan, the Netherlands), and MiniBell (Samsung Seeds, South Korea). This marker was polymorphic in *F₂* individuals derived from Helsinki and MiniBell, but no polymorphism was detected in the other varieties. Further studies were carried out with 50 and 74 *F₂* individuals from Helsinki and MiniBell, respectively. The results showed nearly complete accordance, with the exception of one sample, in the Helsinki *F₂* population (Fig. 3A), whereas *PmsM1*-CAPS did not show accordance with the fertile and sterile phenotypes of the MiniBell *F₂* population (Fig. 3B).

To select further heterozygous (*Msms*) candidates for paprika breeding programs, *PmsM1*-CAPS was used to genotype a total of 311 *F₃* progenies from 18 *F₂* individuals originally derived from Mirage and Fiesta and which were...
heterozygous with respect to both PmsM1-CAPS and male sterility (Fig. 4). PmsM1-CAPS segregated in the F\textsubscript{3} progenies of 16 F\textsubscript{2} individuals, but in remnant populations, this marker was homogenous toward fertility despite the sterile phenotype (Fig. 4).

**Discussion**

Until recently, it has not been advantageous to use genic male sterility (GMS) for hybrid seed production of paprika, as the cost of manual hybrid seed production was only a small portion of the seed selling price (personal communication). This is believed to be due to the fact that worker wages for the production of hybrid seeds by emasculation and hand pollination in some developing and under-developed countries are extremely lower and the F\textsubscript{1} seed production cost is fully compensated by the higher seed price of paprika. Now, with the development of a number of molecular markers associated with agriculturally important traits, the potential for use of genic male sterility in hybrid seed production of paprika and other vegetable crops, including tomato (Tanksley et al. 1984), rape (Lu et al. 2008, Xie et al. 2008), and lettuce (Curtis et al. 1996), has gradually increased.

In this study, we attempted to develop a molecular marker linked to a male-sterile gene in paprika using commercial F\textsubscript{1} varieties from the Netherlands as primary materials. An AFLP marker (PmsM1) from BSA-AFLP analysis of two F\textsubscript{2} populations from the ‘Mirage’ and ‘Fiesta’ F\textsubscript{1} cultivars was successfully converted to a codominant PCR-based CAPS marker (PmsM1-CAPS). Although PmsM1-CAPS proved to be useful in selecting the \(ms\) locus genotype in progenies from F\textsubscript{1} hybrids (‘Mirage’, ‘Fiesta’, and ‘Helsinki’), the marker was not useful in many of the populations tested due to the lack of polymorphism. We can postulate the following: 1) the source of the male-sterile gene for hybrid seed production is different in the paprika varieties; or 2) the recombination event between the marker and the \(ms\) gene occurred. We found the former alternative more likely in the F\textsubscript{2} individuals of ‘MiniBell’, in which the PmsM1-CAPS marker did not co-segregate with the phenotypes (fertile or sterile). This was most probably due to the fact that more than two different reservoirs of male sterility must have been used for commercial paprika F\textsubscript{1} hybrid seed production.

PmsM1-CAPS is believed to be located within 2 to 3 cM of the male-sterile gene, although further screening with a larger number of F\textsubscript{2} individuals is needed for an accurate evaluation of the distance from the \(ms\) gene. The specific gene responsible for GMS in paprika remains unidentified. Allelism tests are needed in order to determine the relationship between paprika GMS and that used in the cultivation of different peppers in other countries.

In this study, we developed the PmsM1-CAPS marker,
which is linked to the paprika GMS gene, and demonstrated the validity of the marker in different populations. These results indicate that our marker is useful for marker-assisted breeding for paprika hybrid seed production using the GMS system.

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Literature Cited


