



# Gene-Based Marker to Differentiate Among B, A, and R Lines in Hybrid Production of Rapeseed Ogura System

Javad Akbari Afjani <sup>1</sup>, Masood Soltani Najafabadi <sup>2\*</sup>, Reza Gholi Mirfakhraei <sup>3</sup>

<sup>1</sup> Department of Biotechnology, Faculty of Agriculture, Tarbiat Modarres University, Tehran, Iran

<sup>2</sup> Seed and Plant Improvement Institute (SPII), Agricultural Research, Education and Extension (AREEO), Shahid Fahmideh BLVRD, Seed and Plant Improvement Institute Campus, Karaj, Iran

<sup>3</sup> Department of Plant Genetics and Breeding, Faculty of Agriculture, Tarbiat Modarres University, Tehran, Iran

\* **Corresponding author:** Masood Soltani Najafabadi, Seed and Plant Improvement Institute (SPII), Agricultural Research, Education and Extension (AREEO), Shahid Fahmideh BLVRD, Seed and Plant Improvement Institute Campus, Karaj, Iran. Tel: +98-2632704982; FAX: +98-2632709403; E-mail: m.soltanin@areeo.ac.ir

## Abstract

**Background:** In plant breeding program to produce hybrid varieties, pair of male sterile and restorer fertility lines are required. Differentiation of lines possessing restorer fertility allele from the lines lacking it remove the need for the progeny test, and thus reducing the time and the cost in the hybrid production procedure. Canola breeding program in Iran has concentrated toward production of domestic hybrid varieties, however, it suffers from lack of molecular information in restore fertility status of lines, and therefore it needs time and tedious activities.

**Objectives:** To design gene-based markers for distinguishing R-, A- lines and hybrids in sunflower breeding programs.

**Material and Methods:** Aligning sequences of locus responsible for male sterility and that of male fertility resulted in finding differences in the loci, which used to define two set of suitable primer pairs. Genomic DNA from 25 R-lines (23 inbred lines and two commercial lines), 9 A-lines (7 inbred lines and two commercial lines), one B-line and two commercial hybrids were extracted and used in PCR as template.

**Results:** Using one-primer pairs, a band of nearly 1500 bp was amplified in restorer lines but not in A-, B- lines. Another primer pair used to distinguish hybrids (heterozygout) from restorer lines. Results of the report is predicted to be used in canola breeding for hybrid production.

**Conclusions:** Although the molecular bases for the male sterility and fertility restoration in rapeseed is not published, taking advantages of gene-based markers, make rapeseed breeding program more efficient regarding time and costs.

**Keywords:** Biomarkers; *Brassica rapa*, Rf Allele

## 1. Background

Canola (*Brassica napus* L.) is one of the most important oil seed crop in the world and Iran. Open pollinated varieties have been the predominant varieties for the crop for several years. Up on discovery of Polimo and later Ogura male sterility system, and the respected restoration fertility genes, hybrid varieties came into the market (1), and commercially hybrids are available worldwide. Attempts towards finding hybrid technology in Iran resulted in production of a plenty of restorer and A-, B-lines, and hybrid varieties are about to be released in the country (2).

Cytoplasmic male sterility (CMS) is a phenotype occurs in higher plants and is characterized by either production of inactive and dead pollen, or no pollen production at all (3). Usually the phenotype is happened due to interruption in mitochondrial function

and metabolism (4). To overcome the phenotype, usually a gene coding by nucleus which is called restorer fertility (Rf) gene is working in concert (3). CMS-restorer interaction systems have been discovered in several plants, and their molecular bases were elucidated (3). At least three male sterile cytoplasm, Ogu, Pol, and Nap have been reported in canola system (5-7). One protein belonging to PPR (pentatricopeptide repeat protein) family has been reported to restore male sterility by Ogu system (5, 8). Ogu (Ogura CMS) is caused by a mutation in mitochondrial locus called *orf138* (9). Molecular analysis in *Raphanus sativus*, a wild relative of canola, revealed that a locus named Rfo, which is considered to be Ogu restorer, contains three tandem genes including PPR-A, PPR-B, and

PPR-C (5, 10). Transformation of male sterile rapeseed with the genes showed complete restoration of fertility only accomplished with PPR-B (8). Thus for, in the Ogura system, PPR-B is to be considered as *Rf* gene.

Hybrid production in commercial level is owed by discovery of cytoplasmic male sterility and the relevant restorer fertility system (11). Through breeding programs, many inbred lines are yearly produced which their cytoplasmic statues and presence of restorer fertility is not clear. To elucidate the fertility statue, a test cross and then progeny test is required, which is time consuming and costly. Thus, seeking a cost effective and fast approach is of interest to improve breeding programs. Molecular markers are effective tools which can reduce duration of breeding programs. PCR-based markers have been used frequently in tagging genes and marker assisted selections (MAS) for plants and human (12-14). Among the methods used for monitoring genes among genetic materials, gene-based markers are the most accurate and informative ones (15, 16).

## 2. Objectives

In this report, we designed gene-based markers to differentiate among R-, A- lines and hybrids to be used in marker assisted selection.

## 3. Materials and Methods

### 3.1. Plant Materials

Plant materials (seeds of *Brassica napus* L.) have been provided by Oil Seed Crop Dep., Seed and Plant Improvement institute (SPII), and were composed of 23 restorer lines, denoted as Rf1 to Rf23, and seven A-line, A-1-1-1, A-1-2, A-3-2, A-5-1, A-7-1, A-8-1, and A-9-1, and a B-line B-4-1. In addition, three commercial hybrids, Hyb1, Hyb2, and Hyb3, and their restorer parent, R1, R2, and R3 were provided by the above-

mentioned institute. Male sterile lines, Ms-1, and Ms-2, plus two restorer lines, RF-1, and RF-2, as parents for commercial exotic hybrids were kindly provided by Aras Bazr Company.

### 3.2. Bioinformatics and Primer Designing

Sequences for *Rf* (accession number: AJ550021) and *rf* (PPR-like B, accession number: FJ455099) were retrieved from NCBI. The similar sequences to the *rf* sequence, out of many CDS in the locus of *Rf*, were found and called as PPR-B. Sequence alignment between PPR-B and reverse complement sequences of PPR-like B was done using MegAlign v3. Two sets of primer pairs were manually designated, one for distinguishing male sterile from male fertile cytoplasm (RF F and RF R), and the other one for distinguishing the hybrids and the restorer lines (H F and H R) (Table 1).

### 3.3. DNA Extraction, PCR and Gel Electrophoresis

DNA was extracted from the young leaves of the plants using CTAB method (17). Integrity of the extracted DNA was checked by running a sample of the extracted DNA on 1% agarose gel electrophoresis. The PCR program was 94 °C for the initial 5 min to denature the genomic DNA followed by 35 cycles of 94 °C for 30 s, T °C for 30 s (T value was dependent to the primer T<sub>m</sub>, usually the T is set to be 5 degrees lower than the primer T<sub>m</sub>) and 72 °C for 45 s with final extension 7 min in 72 °C.

The PCR was accomplished in a volume of 15 µl with the following composition: 2 µl of the 20 ng.µl<sup>-1</sup> DNA sample, 1 µl of 25 mM MgCl<sub>2</sub>, 1 µl of 5 mM dNTPs, 10 pmol of each primer and 1.5 units of *Taq* DNA polymerase (Cinaclone, Iran). The PCR products were loaded either on 1% agarose gel electrophoresis, or 6% poly acrylamide gel electrophoresis. In both cases, the PCR products were visualized by treating the gel with 1XSafeStain™ dye for 15 min.

**Table 1.** Characterization of primers designed for detecting presence of *Rf* vs *rf* alleles (HF and HR) and also for distinguish among the hybrids and the restorer lines (RFF and RF rf R).

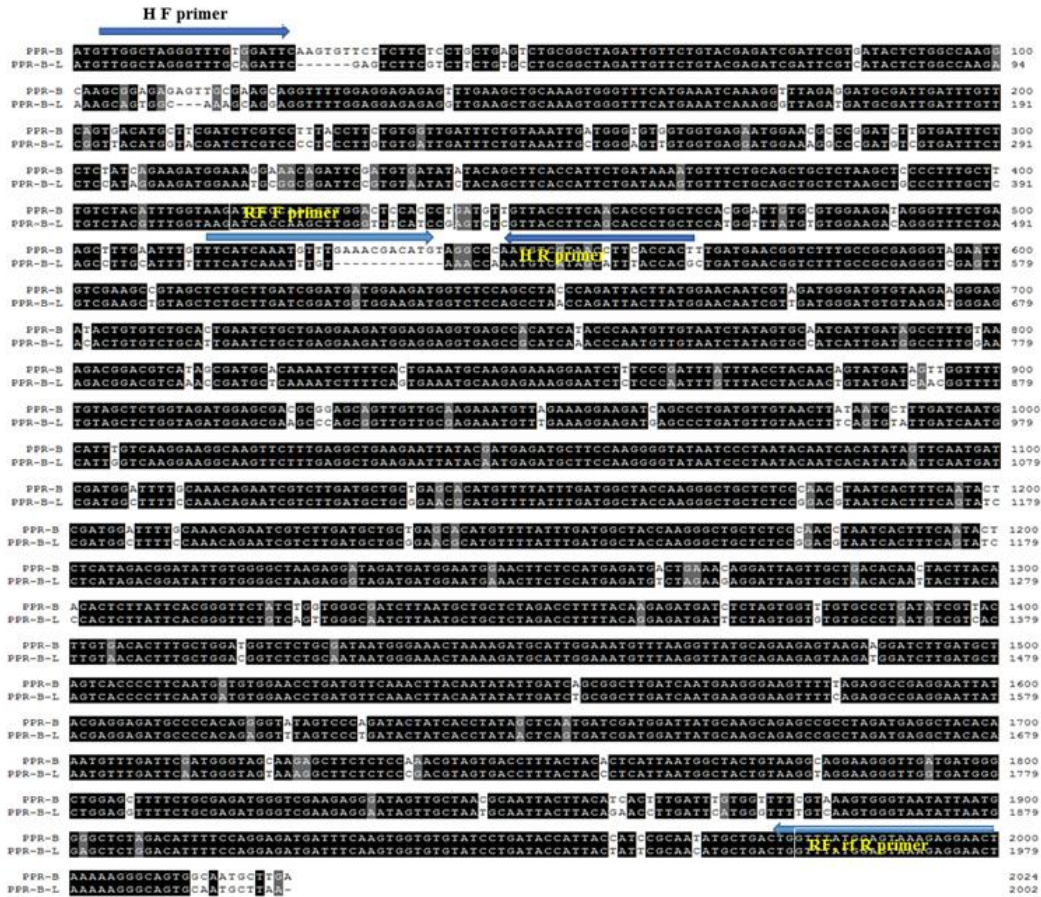
Primer name	Sequence (5' to 3')	Amplicon size (bp)
HF	TTGGCTAGGGTTTGTGGATTC	1680
HR	AGTGGTGAAGGTTACGACATT*	
RFF	TTCATCAAATGTTTGAAACGACATG	565 and 544 (for Hybrids) and 544 (for R lines)
RF rf R	AGTTCCTCTTTACTCCATAAACCCAG*	565 and 544 (for Hybrids) and 544 (for R lines)

\*: Primer sequences are shown as reverse complement of those indicated in Fig. 1.

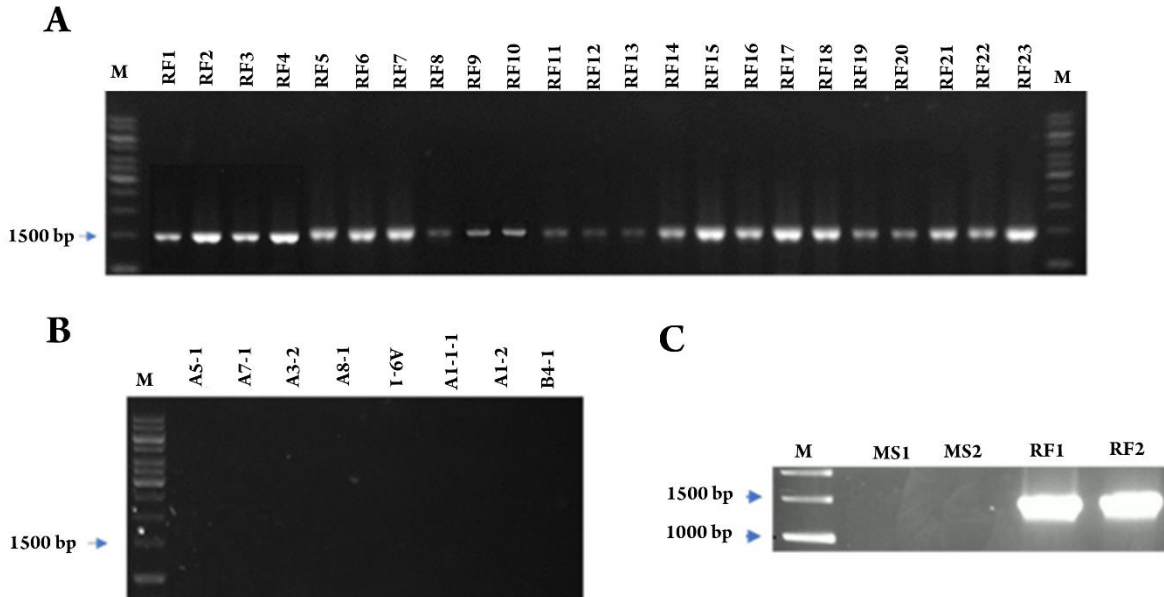
## 4. Results

Sequences for *RF* and *rf* was retrieved from NCBI under accession number of AJ550021 and FJ455099, respectively. The *rf* sequence had been deposited in NCBI as a part of a gene, and *Rf* was presented as a locus with several CDS. Thus, the CDS's were aligned with the reverse complement sequence of *rf* and only the matched sequences was presented here (Fig. 1). Results of e alignment between *Rf* and *rf* sequences indicated that in a region of approximately

2000 bp in length, only 21 bp deletions is presented in *rf* (Fig. 1). A primer pair were designed with forward one landed in a 12 bp deletion as 3' end (Table 1). The forward primer was located in a position able to bind both the *Rf* and the *rf* sequence. As a result, a band of about 1.5 kb was amplified in all the restorer lines (RF) but not in the A- and the B- lines (Fig. 2a and b).

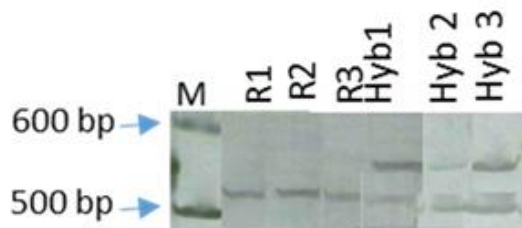


**Figure 1.** Sequence alignment of *PPR-B* (*Rf*) and *PPR-B-L* (*rf*). Arrows show primer positions. HF and HR are used for determining heterozygous and homozygous lines which can amplify in restore lines a fragment of about 665 bp and in hybrids two bands of the length of 665 and 644 bps. Using RF F and RF r R primer pairs a fragment of about 1500 bp is amplifies only in lines harboring RF allele.



**Figure 2.** PCR results for differentiating among R-, A, and B lines. Primer pairs RF F and RF, rf R was used to differentiate among Iranian restorer lines (a), A- and B- lines (b) and two sets of commercial male sterile (MS) and restorer lines (RF) (c). The PCR products were run on 1% agarose gel electrophoresis. RF: restorer fertility lines, A: A- lines, B: B-line, the number for each line is an arbitrary nomenclature, as the lines were derived from Canola Breeding research program in Iran. MS's and RF-1 and RF-2 are parental lines received from a commercial company. M: 1 kb ladder DNA size marker. Position of 1500 bp band is indicated.

The amplification results were loaded on 1% agarose gel. This primer pair was further evaluated in a set of other restorer and A-lines as commercial hybrid parents (18) and the same results were observed (Fig 2c).



**Figure 3.** Heterozygous (hybrid: Hyb) and homozygous (restorer lines: R) differentiation on the gel. Primer pairs H F and H R were applied to produce size-based polymorphisms (see figure 1). The upper band is expected to be 565 bp, and up on 21 bp deletions in the lower band, an amplicon size of 544 bp was produced. The PCR products were run on 6% poly acrylamide gel. M is 50 bp DNA ladder.

In order to design molecular tool for differentiation among the hybrid (heterozygous) and the restorer lines (homozygous), a primer pairs were designed to cover all the deletions in the *rf* allele and, thus, size-based polymorphisms were detected as a molecular marker. Difference of 21 bp in the length could be observed, the bigger band for *Rf* and the smaller band for A-lines. As the different was too small, the PCR products were loaded on 6% poly acrylamide gel containing a 50 bp ladder as molecular size marker (Fig. 3).

## 5. Discussion

Providing low cost facilities and breaking time problem, molecular markers serve as efficient tools in breeding programs, which accelerate selection procedures. They are especially important in selecting traits with recessive inheritance, as without molecular marker tools one more year for selfing or progeny test is required in each generation.

Although investigations in restorer fertility-related loci indicated high level of complexities (19), which undertaken several evolutionary events, however, based on published data and transformation analysis, transformation of male sterile canola lines possessing Ogura cytoplasm with the *Rf* resulted in restoration of fertility (8). The *rf* sequence was identified and cloned in Ogura male sterile canola line using primers designed based on conserved sequences on PPR sequences in radish (8).

While there are lines of evidence indicating complexity in the restorer fertility locus (19), we were able to differentiate genetic materials for restorer and A-, and B lines. On the other hand, for patent and security matters it is really not-known to us how the Ogura male sterile and *Rf* gene was transferred to commercial canola lines. Thus, no matter how the system was created in canola, our results indicate its feasibility in determining and differentiating among lines to be served as hybrid parental lines. The results can be directly entered into applied canola breeding programs.

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