

A NEW MOLECULAR MARKER LINKED TO GENE FOR MONOGERMITY IN SUGAR BEET (*BETA VULGARIS* L.)

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ABSTRACT

Monogermity is a character that is controlled by one pair of recessive allele in sugar beet. This character is used in many breeding projects. If the character is tagged with molecular markers, it will be possible to easily distinguish monogerm and multigerm plants by DNA extraction followed by PCR analysis in seedling stage. Bulked segregant analysis using 297 RAPD primers in an F₂ population of a hybrid between monogerm and multigerm sugar beet accessions was performed for identification of RAPD marker linked to monogerm gene. DNA of 10 plants from multigerm and monogerm plants were separately mixed. At first, the primers were tested on DNA bulks and then on individual plants of the bulks. When the polymorphism of the primers was confirmed, they were tested on other individual plants. Eventually, two coupling and three repulsion markers were identified in F₂ population, which their distances to monogerm gene were lower than 50 cM. OP-S₄-2000r marker was the nearest marker to monogerm gene. The distance of between this marker and monogerm locus was estimated to 6.1 rf.

Key words: BSA, monogermity, RAPD marker, sugar beet, tagging.

INTRODUCTION

In sugar beet (*Beta vulgaris* L.) the ovary is one seeded, but in multigerm genotypes a cluster of flowers is borne on a single axis; their perianths fuse without cleavage walls and form a multigerm seed cluster at maturity. In monogerm plants only a single flower is present on each floral axis. Thus the multigerm-monogerm trait is an inflorescence character determined by the genotype of the seed parent (Savitsky, 1954). Monogermity was discovered in 1934 in Russia (Sneep et al., 1979). The monogermity of modern hybrids has been, however, developed from a source isolated in the USA (Savitsky, 1950) and depends on a single major gene with alleles *MM* conditioning multigermity and *mm* monogermity (Savitsky, 1952). At present the monogerm character is present in all cultivated hybrids because it avoids the manual thinning of seedlings after field emergence (Hecker and Helmerick, 1985; Poehlman, 1987; Sneep et al., 1979).

Use of molecular markers, such as RAPD

(Lawson et al., 1998; Kumar et al., 2006), and cloning and analysis of differential expression fragments (Gu et al., 2008) are common methods to study of resistance genes (Amiri et al., 2009). Also, the RAPD markers have been used for study of genetic diversity (Beharav et al., 2010; Sikdar et al., 2010). The molecular marker gives better discrimination of resistant from susceptible plants than the ELISA, because, there are no disease escaped plants in marker tests (Redfearn and Asher, 1997; Francis et al., 1998; Francis and Redfearn, 1998; Scholten and Lange, 2000).

Barzen et al. (1992) by using molecular markers mapped the gene for monogermity on IX chromosome in sugar beet. The *M-m* locus was mapped to chromosome 4 in a distal position at 4.2 cM from the locus *GS7* of sugar beet. Abe et al. (1993) published linkage map for 9 isozyme and 4 marker loci in sugar beet (*Beta vulgaris*). They showed that monogermity (*m*) was linked to *pgm*, with a recombination value of 35.8%. Barzen et al. (1995) and Schumacher et al. (1997) reported that the monogerm gene, *M-m*, was located in two RFLP linkage maps. In spite of a different

numbering of the linkage groups, both papers reported the gene on the same chromosome. The *M-m* gene is located in the linkage group 2, according to the designation of sugar beet chromosomes (Schumacher et al., 1997).

Recently Russian researchers have described a new recessive gene for monogermity denoted m^2 and mapped the gene to the same linkage group as the *M-m* locus.

The distance between the two loci was estimated to 32.2 centiMorgans (Shavrukov, 2000). If a marker linked to monogerm gene be available, breeding materials can be screened in seedling stage without need to vernalization and bolting the plants, which is a time-consuming and high cost process.

The aim of this study was identification of molecular markers linked to monogerm (*mm*) gene in sugar beet using an F_2 population. The identified marker(s) will increase precision and accuracy of selection programs, reducing the duration and costs.

MATERIAL AND METHODS

Plant materials

The study was performed with a multigerm sugar beet accession *Beta vulgaris* subsp. *maritima*, accession WB42 and a monogerm *Beta vulgaris* subsp. *vulgaris*, germplasm 261. Both accessions are diploid with $2n = 18$. Plants of the multigerm wild beet accession WB42 were crossed in pairs with monogerm sugar beet germplasm 261.

Mapping F_2 population was obtained by selfing F_1 multigerm plants and was used to identify the RAPD marker(s) linked to the *mm* gene of monogermity.

F_2 seeds were sown in the field and the F_2 plants after vernalisation and bolting were investigated to distinguish their monogermity or multigermity.

Bigerm plants were removed from the population and finally 99 plants were used for the study, of which 24 were monogerm plants and the rest were multigerm plants. Fresh leaves of the plants were frozen in liquid nitrogen and stored in -80 degrees Celsius freezer until DNA extraction.

DNA extraction

Genomic DNA was extracted from frozen leaves of individual plants of F_2 population, following the modified procedure of Saghai-Marooif et al. (1984). Quality and quantity of genomic DNA were estimated using 0.8% agarose gel electrophoresis and spectrophotometry. PCR was performed in a total volume of 20 μ l containing 50 ng genomic DNA, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 25 ng primer (*Operon*, Huntsville, USA and *Advanced Biotechnology*, Surrey, UK), 2 μ l 10x reaction buffer (100 mM Tris-HCl, pH = 9; 500 mM KCl) and 1 unit *Taq* Polymerase (*SmarTaq*, *Cinnagen*, Tehran, Iran). DNA amplification was performed in a *Biometra T1* (*Whatman Company*, Göttingen, Germany) thermocycler in PCR reaction tubes. The thermal cycles were: 1 cycle of 5 min at 94 °C followed by 40 cycle of 45 s at 94 °C, 45 s at 34.5 °C and 80 s at 72 °C, then finally 1 cycle of 10 min at 72 °C for final extension. The PCR products were separated by 1 or 1.2 % agarose gel electrophoresis with TAE buffer and stained with 1 μ g cm^{-3} ethidium bromide solution.

Bulk segregant analysis (BSA)

BSA (Michelmore et al., 1991) was performed to identify the RAPD makers linked to monogerm gene. Ten of the multigerm or of the monogerm plants were used in each multigerm or monogerm bulks of DNA in F_2 population. A total of 297 decamer primers (277 primers from *Operon*, Huntsville, USA, and 20 primers from *Advanced Biotechnology*, Surrey, UK) were used to amplify DNA of the bulks. Primers which amplified a DNA fragment in only one of the bulks were confirmed on the individual plants of the each bulk and the parents. The RAPD markers showing linkage to monogerm gene based on individual plants of the bulks were evaluated further on an additional number of individual plants from F_2 population. The sequence of RAPD primers which produced the markers nearest to the monogerm gene is shown in Table 1. The RAPD markers were named by the primer

name, followed by size of the fragment, and c or r for coupling phase and repulsion phase markers, respectively.

Eventually, the nearest marker linked to monogerm gene (a repulsion phase RAPD marker) was examined on two multigerm cultivars Regina (a multigerm cultivar from Novartis Seeds AB, Landskrona, Sweden) and Jam (a commercial multigerm cultivar from Sugar Beet Seed Institute, Karaj, Iran) and six monogerm sugar beet genotypes: Dorothea (a commercial monogerm hybrid from Novartis Seeds AB), Flores (a commercial monogerm hybrid from Danisco, Copenhagen, Denmark), Latitia (a commercial monogerm hybrid from KWS, Einbeck, Germany), SB-201 and SB-301 monogerm O-types and Shirin (a commercial monogerm hybrid) of Sugar Beet Seed Institute.

Table 1. The sequence of primers which amplified bands most associated to the monogerm gene

Primer	Sequence	Primer	Sequence
OP-S4	5'CACCCCCTTG3'	OP-G6	5'GTGCCTAACC3'
OP-B10	5'CTGCTGGGAC3'	OP-F15	5'CCAGTACTCC3'

Statistical analysis and tagging of the RAPD markers

Yates adjusted χ^2 -test (Steel and Torrie, 1980) was used to compare observed and expected ratios in the F₂ population. Yates proposed this correction for continuity, to improve the approximation to the χ^2 distribution and thus be able to obtain a more exact probability value from the χ^2 table, when the criterion has a single degree of freedom. This correction is intended to make the actual distribution of the criterion more nearly like the χ^2 distribution based on normal deviations (Steel and Torrie, 1980). The distance between the markers and monogerm gene on the basis of recombination fraction (rf) was estimated by frequency of recombinant individuals (Paterson et al., 1991; Barzen et al., 1992, 1997; Amiri et al., 2009) and linkage analysis was performed using the *Mapmaker 3.0* program (Lander et al., 1987). LOD score 3.0 with maximum

distance of 50 cM were used as critical points for the analysis.

RESULTS AND DISCUSSION

Identification of RAPD marker linked to the gene for monogermity

To identify RAPD markers linked to gene for monogermity, DNA bulks were composed from the multigerm and monogerm plants of the F₂ population. 29 primers amplified RAPD markers either in a multigerm or in a monogerm bulk. These primers were further examined on individual plants.

The segregation distortion for RAPD markers in the F₂ population was about 28.6 %. It meant that the markers distribution was skewed toward to one of the parents or we observed the presence of a marker less frequently than the expected value. This may be explained by the selective elimination of male gametes as a result of pollen sterility or incompatibility (Wagner et al., 1992; Scholten et al., 1996; Subudhi and Huang, 2002) or selective elimination of female gametes (Subudhi and Huang, 2002). Zygotic selection could be another explanation due to differences in fitness between the zygotes (Wagner et al., 1992). The study of segregation distortion will have practical implications in breeding effort, since the genes closer to the genes with distorted segregation on the chromosome will be less heritable because of limiting the recombination of characters (Subudhi and Huang, 2002). Thus, it is expected that the segregation distortion is confounded with the physical linkage between two markers or genes, because the linkage maps are constructed on the basis of the Mendelian expected ratios in the population being considered (Liu, 1998).

The primer OP-S₄ generated a DNA fragment that was found to be linked to the monogerm locus in F₂ population. So, this primer was examined on individual plants. The primer OP-S₄ amplifies the RAPD marker OP-S₄-2000r linked to monogerm locus in repulsion phase.

Segregation distortion test for OP-S₄-2000r marker fitted the data into a 3:1 ratio in F₂ population ($\chi^2 = 0.55$, $n = 99$, $df = 1$), indicating no segregation distortion for the marker.

Eventually, two coupling and three repulsion markers were identified in F₂ population, for which their distances to monogerm gene were lower than 50 cM. Distances of *mm* locus and the markers were estimated by the frequency of recombinant individuals (Paterson et al., 1991; Barzen et al., 1992, 1997; Amiri et al., 2009) (Table 2). The multigerm plants without the marker and the monogerm plants having the markers were

considered as the recombinant individuals in coupling phase and the monogerm plants without the marker were considered as the recombinant individuals in repulsion phase. The number of recombinant plants in repulsion phase was taken as twice the number of recombinant monogerm individuals, because the RAPD marker was a dominant marker (Beharav et al., 2010) and in a repulsion-phase marker we could not distinguish recombinant multigerm individuals. In fact, we supposed that the recombination frequency in multigerm and monogerm plants was equal. OP-S₄-2000r marker was the nearest marker to monogerm gene (Table 2).

Table 2. The recombination frequency of the markers in multigerm or monogerm plants (%) and distances [rf] between *MM* or *mm* loci and the RAPD markers (c and r refers to the coupling and repulsion phase RAPD markers, respectively)

Plants type	OP-S ₄ -2000r	OP-B10-880r	OP-G6-1550r	OP-G6-1100c	OP-F15-1300c
Monogerm	0.25	0.25	0.29	33.33	33.33
Multigerm	-	-	-	9.27	15.8
Distance	0.061	0.073	0.254	0.309	0.351

The presence of the RAPD marker OP-S₄-2000r was analyzed in the entire F₂ population, because the marker was nearest linked to monogermity and it could be useful in the breeding programs. Other markers in Table 2 were too far away from the gene. OP-S₄-2000r RAPD marker was present in all of the monogerm plants with exception of 3 plants, so that the distance between the marker and *mm* gene was estimated at 6.1 rf ($2 \times 3 \div 99 = 0.0606$). We used two methods to estimate the marker distances. Distances estimated by *Mapmaker 3.0* for coupling markers (c-markers) were in agreement with those obtained by the frequency of recombinant individuals.

The distances of the repulsion markers (r-markers), which were obtained by *Mapmaker 3.0* (cM) were slightly higher than those of the frequency of recombinant individuals (rf). Mapping was performed using Haldane's mapping function in which no interference is supposed (Lander et al., 1987; Liu, 1998). In this condition, some of the parental gametes would be regarded as recombinant gametes due to the presence of double crossing-over.

Thus, the estimation of the distances would slightly be higher than that obtained from the frequency of recombinant individuals, since the latter method does not consider double crossing-over. Moreover, as the distances of the markers increase, the difference between the distances estimated by the two methods will also increase, since more crossing-over will occur at longer distances. In the present study the difference between distances estimated by the two methods was high for r-markers.

Mapmaker 3.0 uses maximum likelihood function for the estimation of the distances (Lander et al., 1987). In the F₂ population, recombination calculation using dominant markers such as RAPD (Narain, 1990) in the r-markers is only influenced by the frequency of the fourth phenotypic group (in this study the monogerm plants which did not have the r-marker). Thus, the estimation of the recombination frequency by maximum likelihood function would be biased and the amount of the bias will increase with decreasing of the frequency of the fourth phenotypic group (Liu, 1998). Therefore, it

can be concluded that the estimations obtained by this software were biased.

Figure 1 shows OP-S₄-2000r repulsion phase RAPD marker in parents, ten multigerm and ten monogerm plants of the F₂ population. As shown, OP-S₄-2000r RAPD marker was present in the monogerm parent (accession 261) and in ten monogerm plants of the F₂ population. This marker was also present in two multigerm plants of the F₂ population

(fourth and tenth multigerm plants of the F₂ population, Figure 1). On this basis, the genotype of these two multigerm plants was considered as *Mm*. The marker was re-amplified several times in different thermocycler (*Bio-Rad*, MJ mini, Hercules, USA) and labs and based on the amplification results the OP-S₄-2000r RAPD marker was highly reproducible. This confirmed good repeatability and usefulness of the marker.

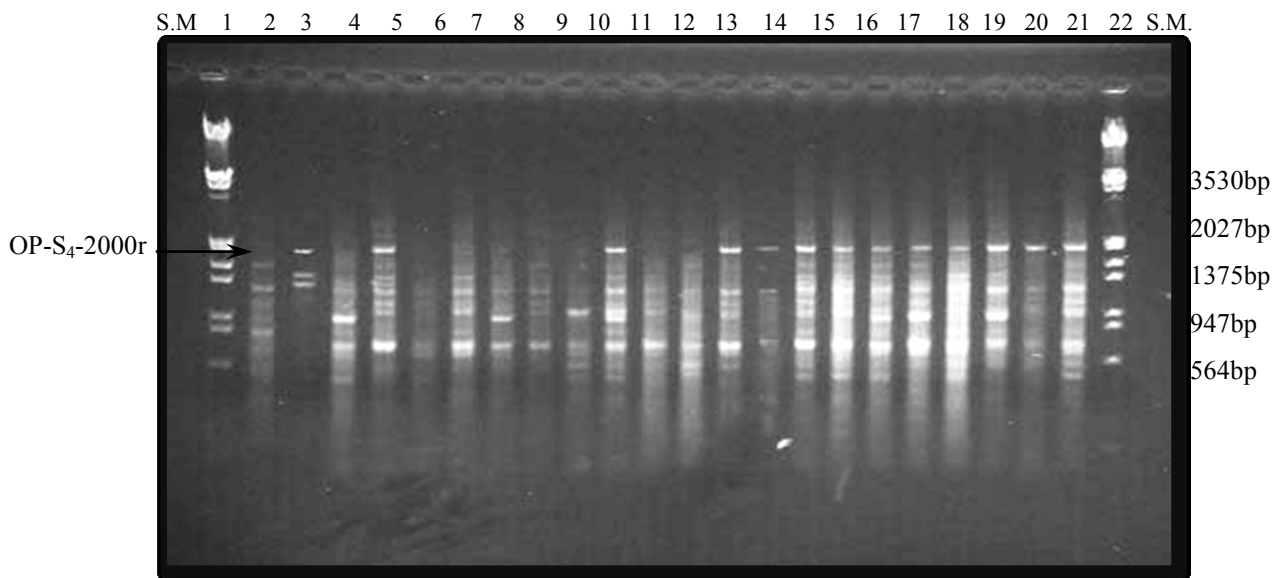


Figure 1. Agarose gel electrophoresis of the repulsion phase RAPD markers in WB42 (lane 1), 261 (lane 2), ten multigerm (lanes 3-12) and ten monogerm (lanes 13-22) plants of the F₂ generation. The arrow points to the segregating marker OP-S₄-2000r. SM = Lambda DNA digested with *ECORI/HindIII*

Eventually, OP-S₄-2000r marker, the nearest marker linked to the gene, was tested on some commercial multigerm and monogerm genotypes. OP-S₄-2000r was observed in all genotypes except SB-301, Regina and Shirin. Existence of OP-S₄-2000r marker in Latitia, Flores, SB-201 and Dorothea corresponded to their monogerm genotypes.

The presence of the marker in Jam cultivar indicated that probably the tested plant was monogerm or heterozygote for monogerm locus. However, lack of amplification of the marker in SB-301 and Shirin genotypes did not match with their genotypes. Thus, four other plants of these genotypes were investigated. Results showed the presence of OP-S₄-2000r in these plants, except fourth plant of Shirin.

Therefore OP-S₄-2000r absence in these two plants of Shirin and in another individual

of SB-301 was explained by their heterogeneity or heterozygosity caused by seed or pollen mixing in the isolated field or by recombination between the gene and the marker.

CONCLUSIONS

The RAPD marker OP-S₄-2000 can be used as a tool for identification and discrimination between dominant homozygous (*MM*) and heterozygous (*Mm*) or (*mm*) genes for monogermity.

However, for identification of homozygous monogerm genotypes from other ones, we need to identify a coupling marker linked to multigerm locus (*MM*), so that the plants without the coupling marker are considered as monogerm genotypes. This subject will be approached in future research.

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