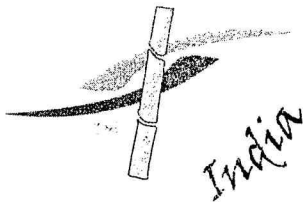
Balancing Sugar and Energy Production in Developing Countries: Sustainable Technologies and Marketing Strategies



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- Breeding
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CLOSE RELATED DNA SEQUENCE TO BOLTING GENE "B" OF SUGAR BEET (*BETA VULGARIS* L.)

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Abstract

Sugar beet (*Beta vulgaris* L.), is a normally biennial crop which completes its life cycle in two years. During the first year, it develops a large succulent roots, in which much reserve food is stored. In the second year, it produces flowers and seeds after vernalization period. Some plants produce seed stalk at the first growing season (bolting). This undesirable character reduces the sugar yield by diminishing the root weight and decreasing the sugar content of the sugar beet root and they can be a possible source of annual wild beets. The pollination of seed crops is affected by contamination of pollens from annual wild beets. The dominant allele of locus "B" causes early bolting without cold treatment. This allele is abundant in wild beets whereas cultivated beets carry the recessive allele. In present study using RAPD and RFLP techniques, we have shown that the former was capable to differentiate between bolting and non-bolting plants, while the latter was not. Six DNA primers were used in this study for RAPD analysis and four restriction endonucleases were used in RFLP. Data showed that (OP1), DNA primer fragment was present in 75% of bolting materials, while they were found in 50% non-bolting ones. Third primer fragment (OP3), was found in 63% of bolting plants, while it was found only in 20% non-bolting materials.

Key words: Sugarbeet; bolting; RAPD; RFLP

INTRODUCTION

Gran(1986) distinguished two types of bolting in sugar beet. The first designated as annual bolting which is controlled by a single dominant gene (B), and genotypes (BB) or (Bb) bolt independently of climatic conditions. The second designated as bolting due to vernalization and was the result of an interaction between the environment and genotype; and characterized by a number of recessive genes. Boudry *et al.* (1994) reported that, the (B) gene seems to remove all necessity for thermal induction, but is affected by the presence of other genes and by the environment. Penetrance of the annual habit (the proportion of Bb individuals exhibiting one annual) was discussed by Owen *et al.* (1940) who stated that heterozygous (Bb) beets are much more complicated to deal with than homozygous (BB) beets. Much of this complexity is probably due to the influence of modifying genes introduced with the (b) gene. Sadeghian and Johansson (1993) mentioned that bolters are beets which produce flowering stalks in the first growing season. Very early bolting plants produce only about half of the root yield in comparison to the normal plants. Early bolters reduce the sugar content and hence the total sugar production. They also cause mechanical difficulties due to the seed stalks at the harvesting time. Early bolters have hard and fibrous roots which can cause problems in processing. This resulted in expensive stoppage, and reduced sugar production. Seeds left from early bolters may create weed beet infestation in the future (O'Connor 1973; O'Connor and Fitzgerald 1987). However, Boudry *et al.* (1994) reported that out crossing of wild beets on seed multiplication plots may introgression the "B" allele into cultivated biennial beets, resulting in varieties contaminated with early bolting plants. As a consequence, root yield and sugar content are reduced. Moreover, bolters cause severe problems during crop harvesting, although breeders are aware of this problem and take strong action to minimize pollen introgression from wild annual beets. Several genetic linkage maps of the 758 Mb sugar beet genome have been developed in the past years including a more recent map covering 664 cM and comprising 315 expressed sequence tag (EST) markers (Schneider *et al.* 2007). These maps have been extensively used for mapping of traits with agronomic importance (Schafer-Pregl *et al.* 1999; Weber *et al.* 2000; Gidner *et al.* 2005; Lein *et al.* 2007; Grimmer *et al.* 2008; Lein *et al.* 2008; Taguchi *et al.* 2009). Further resources are large insert libraries (Hohmann *et al.* 2003; Schulte *et al.* 2006; Lange *et al.* 2008). Gaafar *et al.* (2005) reported that occasionally, bolting plants appear under field conditions, with drastically reduced root yield and sugar content. An early bolting gene, B, was identified from wild beets and it was mapped with RFLP markers to chromosome 2 of sugar beet (Boudry *et al.* 1994).

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Random Amplified Polymorphic DNA (RAPD) technique and PCR-Restriction Fragment Length Polymorphism (RFLP) were employed in this work to differentiate bolting and non-bolting plants.

MATERIALS AND METHODS

Genomic DNA extraction

The leaf samples of bolting and non-bolting plants from Egyptian sugar beet breeding material (Eg.1, Eg.3, Eg.5, Eg.8, Eg.10 and Eg.11) were used for DNA analysis to detect molecular marker in bolting trait. Two to three young leaves were collected from bolting and non-bolting plants for DNA extraction. Modified procedure was used to extract sugar beet nuclear DNA from single individuals according to methods as described by Saghai-Maroof *et al.* (1984).

Determination of DNA concentration

DNA concentration was determined using gel electrophoresis. For each probe, 10 µl (1 µl probe DNA and 9µl 1X bromophenol blue buffer) were loaded on a 1.0% agarose gel. A standard λ-DNA marker of 100, 200 and 300 ng was loaded as a control. After 1.5 h electrophoresis in 1X TAE buffer at constant 60 V, the gel and DNA bands were visualized and photographed using UV light of wave length 302 nm (Video-Document – Equipment, Cybertech, Berlin). DNA concentration was determined by comparison with the standard ë-DNA. Using 1X TE buffer, the DNA concentration of each probe was adjusted to 5 ng / µl for PCR-RFLP and RAPD analysis. Amplification was carried out in a (Thermocycler Eppendorf, Germany) programmed for 35 cycles.

Random amplified polymorphic DNA (RAPD)

Amplification was carried out in a (Thermocycler Eppendorf, Germany) programmed for 35 cycles. Primary denaturation was given 5 min. at 95°C, denaturation occurred for 30 sec. at 95°C. Annealing was allowed 30 sec. and the reaction temperature varied and was dependent on each primer as shown in (Table, 1). Extension was run for 30 sec. at 72°C. For final extension, the mixture was run for 10 min. at 72°C with an applied temperature ramp of 8 at 4°C. The entire reaction mixtures were loaded on 3% agarose gels, and amplified DNA fragments were resolved by electrophoresis followed by staining with ethidium bromide.

RFLP for the 18s rDNA PCR product

Sugar beet DNA samples were digested with four restriction enzymes; *Alu*I, *Bam*HI, *Eco*RI and *Hae*III for 3 hours at 37°C (Table 2). The restriction products were separated on 2% agarose and stained with ethidium bromide.

Table 2. Restriction site *Alu*I , *Bam*HI , *Eco*RI and *Hae*III

Enzyme	Restriction site*
*u*I	5`- AG?CT -3`
*Bam*HI	5`- G?GATCC -3`
*Eco*RI	5`- G?AATTC -3`
Haem	5`- GG?CC -3`

DNA data analysis

Bands of DNA-RAPD pattern were scored visually for all samples studied. Levels of marker polymorphism according to the various molecular techniques used was calculated. A similarity dendrogram for each was produced using software program STATISTICA(R) version 5.0.

Results

DNA extraction and purification

Eighteen plant samples of bolting and bolting resistant plants were used to study bolting behavior at the molecular level. The genomic DNA was extracted from plant samples by CTAB method. Fig. 1 shows separation of DNA extracted from plant samples on 1% agarose gel. These samples were (1BB, 2BB, 3r, 4r, 5BB, 6r, 7r, 8BB, 9BB, 10r, 11r, 12BB, 13r, 14r, 15BB, 16r, 17r and 18BB), (BB) refer to bolting plant while (r) refer to the resistant ones. Table 3 represents the identification of plant samples and age of plants at bolting tendency.

Random primers analysis

RAPD experiments were conducted using four random primers. The primers used in this study were (OP1, OP2, OP3 and OP4). The sequence of these primers and annealing temperatures for each one was presented in

Table 1. List of primer sequences being used and annealing temperatures for every one

No.	Primer	%G + C	Sequence	Annealing Temperature
1	OP1	60	5` GAAACGGGTGGTGATCGCAG3`	54
2	OP2	70	5` AGGCCCCTGT 3`	30
3	OP3	60	5` GGACTGGAGTGTGATCGCAG 3`	54
4	OP4	70	5` ACCGGGAACG 3`	45
5	MS3	66	5` GCAAGTCTGGTGCCAGCAGCC 3`	55
6	MS4	40	5` CCTCCGTCAATTCCTTTAAG 3`	55

Table 3. Identification of 18 plant samples and age of plants at bolting tendency

No.	Plant symbol	Population	Bolting behavior	Age of plant
1	1BB	Eg.1	(BB)	
2	2BB		(BB)	after 120 days
3	3r		(r)	
4	4r		(r)	
5	5BB	Eg.3	(BB)	after 120 days
6	6r		(r)	
7	7r		(r)	
8	8BB	Eg.8	(BB)	
9	9BB		(BB)	after 120 days
10	10r		(r)	
11	11r		(r)	
12	12BB	Eg.10	(BB)	after 120 days
13	13r		(r)	
14	14r		(r)	
15	15BB	Eg.11	(BB)	
16	16r		(r)	after 120 days
17	17r		(r)	
18	18BB	Eg.1	(BB)	after 90 days

r: Resistance to bolting; BB: Bolting plants.

(Table 1). The four examined primers gave between five and eleven fragments.

Primer OP1

Fig. 2 shows the agarose gel separation of RAPD patterns of OP1 primer. The primer gave eleven fragments ranged between 1400 bp and 380 bp. Among all eleven scored fragments produced through this primer there was a single common fragment in all studied materials. This common fragment was shown to be No.8 and had a molecular weight of about 490 bp.

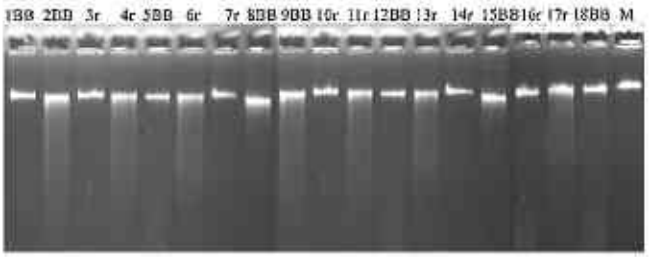

Fig 1. Genomic DNA extracted with CTAB method and separated on 1% agarose gel. Lanes 1-18: Samples 1BB, 2BB, 3r, 4r, 5BB, 6r, 7r, 8BB, 9BB, 10r, 11r, 12BB, 13r, 14r, 15BB, 16r, 17r and 18BB respectively. Lane 19: 100 ng ëDNA marker.

Primer OP2

Fig. 3 illustrates RAPD product of primer OP2, this primer gave five fragments in the agarose gel separation. These fragments ranged from 1400 bp to about 500 bp. The common fragment in the product of this primer was fragment No.4 and had a molecular weight of about 600 bp.

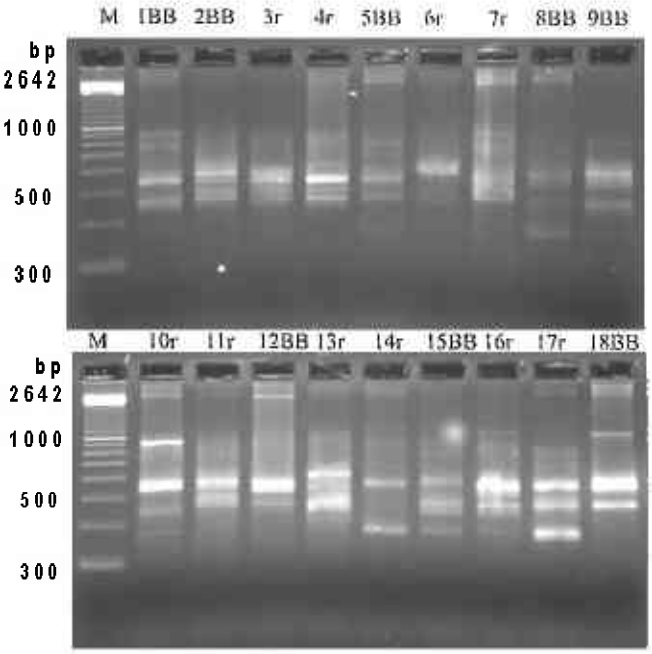

Fig. 2. Primer OP1 RAPD profile of 18 sugar beet samples.

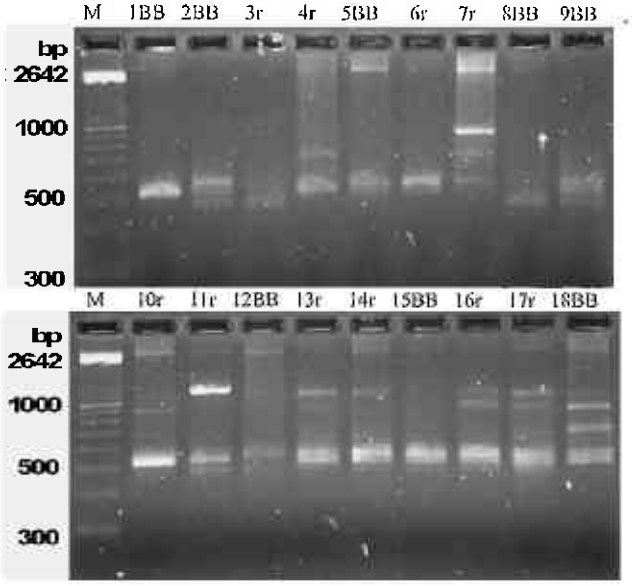

Fig 3. Primer OP2 RAPD profile of 18 sugar beet samples.

Primer OP3

Fig.4 represents the separation of product OP3 primer on the agarose gel electrophoresis. OP3 primer gave six fragments ranged between 850 bp and 380 bp. The band No.5, which had molecular weight about 440 bp, was presented in all studied samples.

Primer OP4

Fig. 5 shows the product of OP4 primer separated on agarose gel. This primer gave five fragments that ranged between 1000 bp and 420 bp. The fragment No.3 had a common presence in all studied materials. The molecular weight of this fragment is about 540 bp.

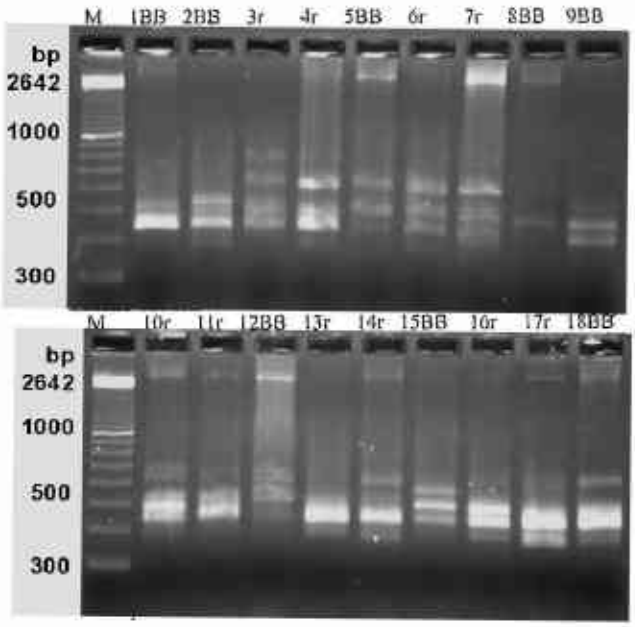

Fig 4. Primer OP3 RAPD profile of 18 sugar beet samples

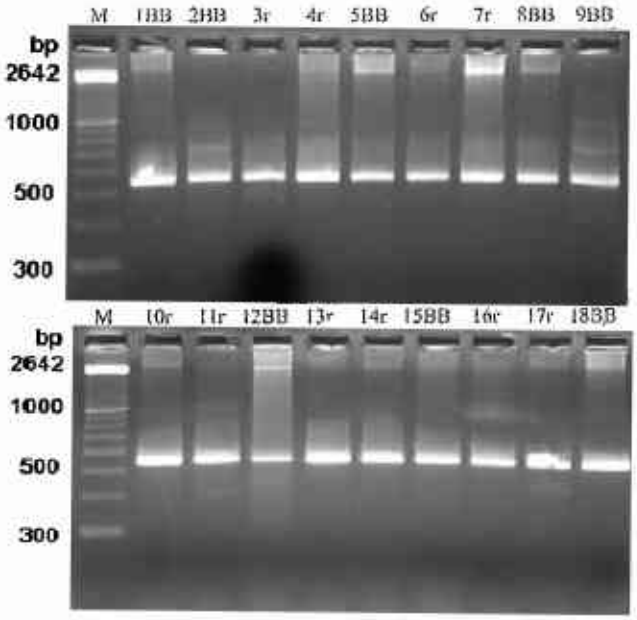

Fig 5. Primer OP4 RAPD profile of 18 sugar beet samples.

PCR-Restriction fragment length polymorphisms (RFLP)

Amplification of 18S rRNA gene (Kuske *et al.*, 1998) was carried out using two specific primers (MS3 and MS4). The sequences of these primers were presented as previously mentioned in materials and methods section (Table 1). Fig.6 shows the agarose gel separation of the product of 18s r-DNA (597bp) amplified with the two primers. The PCR product was digested with four restriction enzymes (*AluI, Bam*HI, *Eco*RI and *Hae*III) the restriction site of these enzymes was shown in (Table 2).

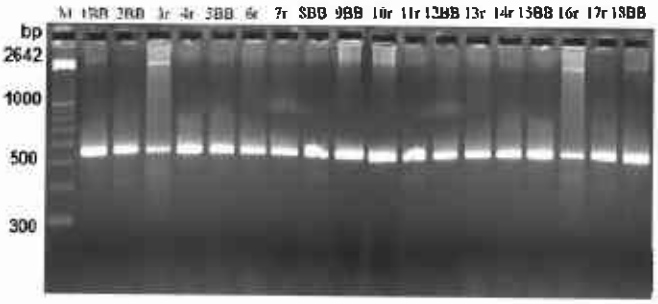

Fig 6. 18s r-DNA (597 bp) amplified with primers MS3 & MS4 and separated on 2% agarose gel. Lanes 1-18: Sugar beet samples 1BB, 2BB, 3r, 4r, 5BB, 6r, 7r, 8BB, 9BB, 10r, 11r, 12BB, 13r, 14r, 15BB, 16r, 17r and 18BB respectively. Lane M: DNA marker.

Fig 7. illustrates an example for the three restriction enzymes (*AluI, Bam*HI and *Eco*RI) which did not give remarkable results. The fourth restriction enzyme *Hae*III gave restriction product in the eighteen leaf samples (Fig. 8). It was worthy to note that there were no such differences between bolting and non-bolting plants resulted in this part of the present investigation.

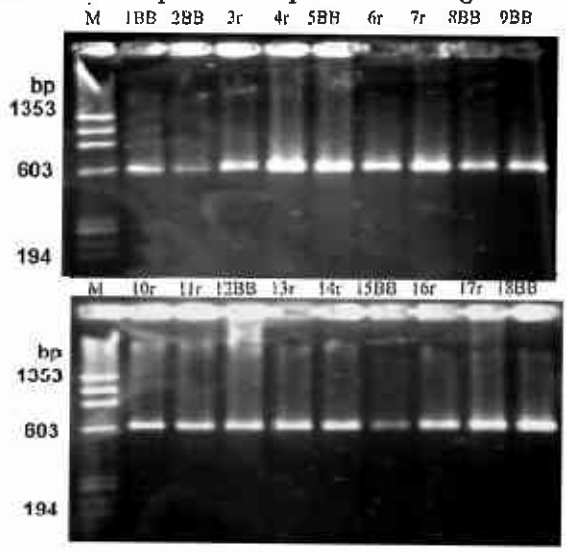

Fig 7. The pattern of 18s r-DNA of 18 sugar beet samples restricted with *AluI, Bam*HI and *Eco*RI.

Cluster analysis using RAPD markers:

Fig.9 illustrates the dendrogram of cluster analysis based on 0 and 1 analysis. This figure shows that the analysis was capable to classify eighteen sugar beet plant samples into two main categories. Category No.1 contains the eight bolting sugar beet leaf samples (1BB, 15BB, 5BB, 9BB, 12BB, 8BB, 18BB and 2BB). The second category contains the ten examined non-bolting plants in three small clusters, these non-bolting plant samples were (6r, 11r, 13r, 16r, 17r, 14r, 4r, 3r, 7r and 10r). The analyzed data revealed that primers OP1 and OP3 were responsible for identifying the studied materials into two main categories, whereas fragment No.7 in OP1

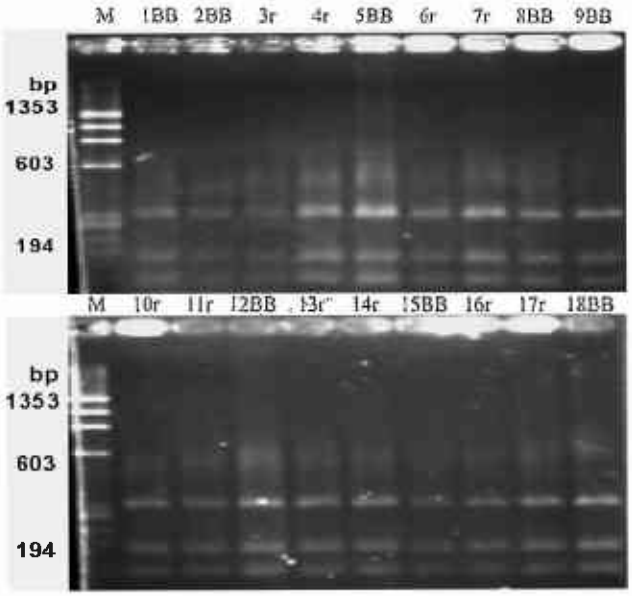

Fig. 8. The pattern of 18s r-DNA of 18 sugar beet samples restricted with *Hae*III.

primer was found in six plant samples from eight studied bolting materials, while they were found in five from ten in non-bolting materials. However, this fragment was presented in 75% of bolting materials, while they found in 50% of non-bolting materials. In the second primer OP3, fragment No.3 was found in five from eight plant samples of bolting plants. Moreover they were found in two from ten non-bolting plants. It was clear that this fragment was found in 63% of bolting plants, while it was found in 20% of non-bolting materials. These two primers were similar in 70% ,of sequence position as shown in (Fig. 10).

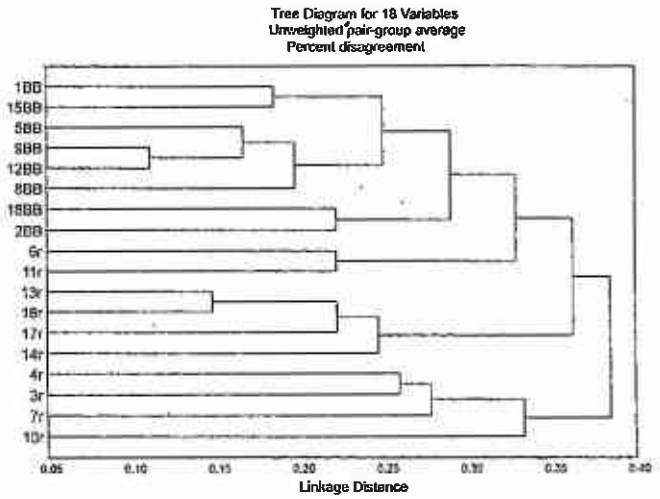

Fig 9. Dendrogram of cluster analysis using four random primers of 18 sugar beet samples based on (0 and 1) data. Whereas: (r): resistance to bolting - (BB): bolting plants.

OP1 5`GAAACGGGTGGTGATCGCAG 3`
OP3 5`GGACTGGAGTGTGATCGCAG 3`

Fig 10. Matching between OP1 and OP3 reveled that there were similarity in 70% of sequence positions.

Discussion

Random Amplified Polymorphic DNA (RAPD) technique and PCR-Restriction Fragment Length Polymorphism (RFLP) were employed in this work to differentiate bolting and non-bolting plants. Pillen *et al.* (1992) have established the first linkage map for sugar beet based on RFLP, isozyme and morphological markers. An extended linkage map of sugar beet (*Beta vulgaris* L.) including nine putative lethal genes and their restorer gene X was established by Pillen *et al.* (1993). Barzen *et al.* (1995) applied RFLP markers to create an extended map of the sugar beet genome containing RFLP and RAPD loci. Heller *et al.* (1996) described the genetic localization of four genes for nematode resistance in different sugar beet lines using RFLP linkage map. Ivanov *et al.* (2002) studied a modified procedure of random amplified polymorphic DNA analysis for searching open reading frames whose expression was different in N (normal) and S cytoplasm's of sugar beet. Hagihara *et al.* (2005) reported that the molecular mapping of a fertility restorer gene (named Rf1) for Owen cytoplasmic male sterility in sugar beet. They found that Eight AFLP and two RAPD markers, tightly linked to the Rf1 locus, were identified using bulked segregant analysis. With the help of RFLP markers, previously mapped on the sugar beet genome, we showed that Rf1 is positioned in the terminal region of linkage group. Physical mapping and sequencing of the whole sugar beet genome is in progress and supported by high resolution FISH (fluorescent *in situ* hybridisation) resolving down to 1 kb. The complete *B. vulgaris* DNA sequence is expected to be available by 2011 and will provide a valuable tool for sugar beet genomics research (Lange *et al.* 2008).

Several authors employed RFLP and/or RAPD techniques in studying bolting phenomenon (El-Mezawy *et al.* 2002; Gaafar *et al.* 2005). The authors mentioned that markers will be the basis for positional cloning of the gene. They present the mapping of 15 AFLP markers and two RFLP markers in close vicinity to the "B" gene. The position of these markers has been verified with sugar beet plants differing with respect to early bolting behavior, and a fine-scale map around the bolting gene "B" of sugar beet has been established. In this work RAPD technique differentiates bolting and non-bolting plants in two distinct groups while PCR-RFLP technique was not capable to give any differences between bolting and non-bolting plants. The available restriction

enzymes used *Bam*HI and *Eco*RI, with restriction sites of six bases were expected not to cut our expected fragment. The cutting sites frequency of a six base cutters according to the formula $(1/4)^n$ is $(1/4)^6 = (1/4096)$; n is the number of bases in the restriction site. On the other hand, four base cutters such as *Hae*ø were expected to cut the expected fragment twice; $(1/4)^4 = (1/256)$. Therefore, we should have used more four base cutters to reveal the polymorphism at the restriction sites variations among the populations under study.

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