Research Article

Identification of SCAR and RAPD markers linked to *Rz1* gene in Holly sugar beet using BSA and two genetic distance estimation methods

Seyed Mohammad Amin Feghhi[1], Peyman Norouzi[2] , Abbas Saidi[3]*, Katayoun Zamani[4] and Reza Amiri[5]
[1]Islamic Azad University, Science & Research Branch, P.O. Box 14778-93855, Tehran, Iran.
[2] Sugar Beet Seed Institute, Karaj, Iran.
[3] Shahid Beheshti University, G.C., Tehran, Iran.
[4] National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.
[5]Department of Agronomy and Plant Breeding, College of Aboureihan, University of Tehran, Iran.
*Email: abbas.saidi@gmail.com

Abstract:
Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV), is the most economically important diseases of sugar beet in Iran. We have identified molecular markers associated with resistance gene(s) to this disease. A F_2 population including of 106 individuals developed at the Sugar Beet Seed Institute, Karaj, Iran, was used to identify molecular markers associated with rhizomania resistance gene from the Holly source. In this study, we used pair combinations of single RAPD primers in bulked segregant analysis (BSA) of two bulks (resistant and susceptible) and F_2 population individuals. Accordingly 397 pair combinations of single RAPD primers were used. However, nine primer pairs showed polymorphism between DNA bulks. The polymorphic markers were tested among the individual plants of the two susceptible and resistant bulks and further were used to analyze the F_2 individuals. Finally, the markers distance from the resistance gene was estimated by using both the Map maker ver.3.0 and the frequency of recombinant plants method. Using frequency of recombinant plants method to tag *Rz1* gene, it was found that R_1, R_2 and R3 markers were 2.3, 8.3 and 16.6 cM apart in repulsion phase and C_3, C_4, C_1, C_2, C_5, and C_6 markers were 20, 21.4, 27.5, 32.9, 43.7, and 51.9 cM apart in coupling phase, respectively. On the other hand, tagging Rz1 gene by Map maker ver.3.0 method showed that R_1, R_2 and R3 markers were 32.4, 44.5 and 60.1 cM apart in repulsion phase and C_3, C_2, C_4 and C_1 markers were 25, 34.7, 46.5 and 57.9 cM apart in coupling phase, respectively. After sequencing the products amplified by MF_1 and MF_2 (Operon primers number have been replaced by these characters) primer pairs, new PCR primers were used to generate the SCAR marker R_1 (this primer sequence is under patent processing and will be shared once it gets patented) which can be readily used for marker assisted selection in breeding programmes.

Keywords: Sugar beet, Rhizomania disease, RAPD marker, primer pair**s**, *Rz1* gene.

Introduction

The causal viral agent of rhizomania disease in sugar beet (*Beta vugaris* L. subs. *vulgaris*) is Beet necrotic yellow vein virus (BNYVV). This virus is transmitted to sugar beet roots by soil-born fungi Polymyxa betae keskin. It has been reported that since BNYVV has serologically similar isolates (Kuszala et al., 1986) and as such they cannot be distinguished from one another (by antibodies taken from them) using ELISA test. RFLP (Kruse et al., 1994) and SSCP (Koenig et al., 1995) molecular markers have been used to identify three major isolates of BNYVVs namely type A, B, and P. Types A and B have four genomic RNA components (Koenig et al. 1995; Kruse et al., 1994). Some BNYVV isolates contains 5th RNA (Koenig et al. 1995; Kruse et al., 1994). The RNA-5 is associated with high virulence and severe disease symptoms (Heijbroek et al., 1999; Link et al., 2005; Schirmer et al. ,2005). Rhizomania severely devastates sugar beet. The sugar content in rhizomania-infected sugar beets can decrease dramatically from 17% to 10% and the root yield can be reduced by 90% (Johansson, 1985). One of the typical symptoms of virus infection is the formation of fibrous roots, resulting in a beard-like appearance (Lein et al., 2007). Dora and Lena were the first varieties conferring partial resistance to rhizomania (Scholten and Lange, 2000). But higher resistance was obtained from Italian germplasm (De Biaggi 1987). Though there are several resistant sources to BNYVV worldwide, however the most famous resistant genes are Rz1 and Rz2, derived from Holly (*Beta vulgaris* L. ssp. *vulgaris*) and WB42 (*Beta vulgaris* L. ssp. *maritima*) sources, respectively (Lewellen et al., 1987; Lewellen, 1995; Scholten and Lange, 2000). Of the two resistance sources, Holly is the most common source represented in most of today's cultivated varieties (Biancardi et al., 2002). However, WB42 based resistant varieties have a lower virus titer in the

presence of more aggressive isolates of BNYVV (Scholten et al., 1999).

Gidner et al. ,(2005) reported a new resistance locus, named *Rz3*, on chromosome three of Beta vulgaris L. ssp. maritima accession WB41. Lein et al. (2007) concluded that it is possible that *Rz2* and *Rz3* are allelic Lennefors (2006) has estimated the distance between *Rz1* and *Rz3* to be less than 5 cM. In a similar study, the distance between *Rz1* and *Rz2* was reported to be 20 cM by Scholten et al. (1999) and 35cM by Amiri (2003).   Thus, based on these findings it cannot be concluded that *Rz2* and *Rz3* are identical genes.

The most effective approach to control the disease is the use of tolerant cultivars (Asher, 1993). Molecular markers can help us to save time and efforts in breeding programs by confirming the presence of resistance in the selected individuals of segregating populations. For instance, Barzen et al. (1992, 1995) developed an AFLP and RAPD map of sugar beet for a resistant gene derived from *B. vulgari.ssp. maritima* which was mapped to linkage group III. Pelsy and Merdinoglu (1996) mapped 12 RAPD markers closely linked to Holly-type resistant gene by use of bulked segregant analysis (BSA) (Michelmore et al.,1991). Barzen et al. (1997) found a different set of 12 RAPD markers by BSA and mapped them on linkage group IV. Two of these markers were tightly linked with recombination values of 1.4 cM for both of them.

In this study 397 RAPD primer pair combinations were used to identify markers that have tight linkage to rhizomania resistance gene in 106 plant individuals of Holly-type (Holly derived) population. As RAPD markers were dominant markers they can not be used directly in marker-assisted selection, hence a sequence characterized amplified region (SCAR) marker was developed and subsequently mapped at a distance of 2.3 cM from the Holly resistance locus.

Material and methods
Plant material: Studies on the inheritance of resistance to BNYVV were performed on the F_2 population (supplied by Amiri, 2003) of a cross between a resistant sugar beet *Beta vulgaris* ssp. *vulgaris* Holly1-4 (a selection from the Holly source (Lewellen et al., 1987)) and a susceptible annual male sterile beet plant.

DNA extraction and RAPD protocol: Genomic DNA was extracted from snap-frozen leaf tissues of the F_2 population individuals and parents using the procedure as described by Murry and Thompson

(1980), with minor modifications. The quantity and the quality of DNA were assessed by agarose-gel electrophoresis, using 0.8% agarose with known concentrations of uncut lambda DNA (Roche). Gel images were captured by UV.DOC system and ACDSee.Pro.2.0.219, Final software.   DNA concentrations were quantified by spectrophotometry. To optimize PCR conditions, suitable density of salt, primers and DNA were determined by density gradient. PCR was performed in the presence of 50 ng each primer (Advance Biotech=AB and Operon kits=OP), 1 unit SmarTaq DNA Polymerase (Smar Taq), 0.2 mM each of dGTP, dTTP, dCTP, and dATP, 2.5µl 10x reaction buffer (100 mM Tris-Hcl, pH = 9; 500 mM KCl), and 50 ng genomic DNA in a 25µl total reaction volume.

DNA amplification was performed in a Biometra T3 thermocycler. The PCR reaction cycles were optimized at one cycle of 5 min at 94^0C, followed by 40 cycles of 40 s at 94^0C, 40 s at 34^0C, 80 s at 72^0C, and one cycle of 10 min at 72^0C for final extension. Amplified products were separated by gel electrophoresis using 1.4% agarose gel with TAE buffer (as in Sambrook et. al., 1989) and staining with ethidium bromide (1µg/ml).

Bulked Segregant Analysis (BSA):To identify molecular markers co-segregating with BNYVV resistance,   BSA was performed as described by Michelmore et al. (1991) with some modification (i.e., seven individuals were pooled to each bulk). The diluted DNA samples (50ng/µl) of the seven most resistant (R) and seven most susceptible (S) individuals of the F_2-A1-110 population were pooled into R and S bulks and were screened with 397 RAPD primer combinations. Mean of healthy plants absorption value in each ELISA plate ($\bar{X}_h$) was calculated and then $\bar{X} + 3S_d$ parameter obtained as resistance threshold (where S_d is standard deviation). Plants were considered resistant if their samples showed an absorption value less than of $\bar{X} + 3S_d$ and considered susceptible if their samples showed an absorption value more than $2\bar{X}_h$. The mean ELISA values of the R and S individuals were 0.137 and 1.258, respectively.   In order to determine candidate markers, primer pair combinations that gave at least one polymorphic band between the bulks were tested on seven R and seven S bulk individuals followed by 26 individuals of F_2 population (a total of 40 plants).. Finally, those primer pair combinations showing at least 30 cM from *Rz1* were tested on the rest of the population to 106 individuals (included the seven most resistant (R)

and seven most susceptible (S) individuals plus the other individuals of F_2 population).

Mapping of the RAPD markers: RAPD markers were mapped by analyzing the segregated families with both the Mapmaker version 3.0 (Lander et al. 1987) and recombined individuals frequency methods (Barzen et al., 1992; Paterson et al., 1991; Amiri et al., 2009). It was assumed that all seven most resistant plants, with a maximum log10 virus concentration of 0.3ng/ml, contained the major gene for resistance, whereas, all seven most susceptible plants, with a minimum log10 virus concentration of 0.6ng/ml, lacked the gene for resistance. Linkage was considered significant if the logarithm of odds (LOD) score was further than 3.0 Yates adjusted chi-square.

Yates test was used to compare observed and expected markers ratios to improve the

$$\chi^2 = \sum \frac{(|\text{observed} - \text{expected}| - 0.5)^2}{\text{expected}}$$

approximation to the χ2 distribution and, thus, be able to obtain a more exact probability value from the χ2 table. Yates proposed this correction for continuity, applicable when the criterion has a single degree of freedom. The purpose of the correction is to make the actual distribution of the criterion more likely to the χ2 distribution based on normal deviations (Steel & Torrie, 1980).

The equations of Barzen et al. (1992) in coupling and Amiri et al. (2009) in repulsion condition were used to calculate the marker distance and location of Rz1 gene. As shown below, the formula suggested by Amiri et al. (2009) has multiplied the susceptible individuals without band by a factor of two in repulsion phase. Since RAPD is dominant marker (Novy and Vorsa, 1996; Haley et al. 1994; Van den Broecke et al. 1998; De Loose and Van Bockstaele 1995; Francis et al. 1998; Hansen et al. 1998; Hallden et al. 1996), polymorphism observed in repulsion phase, may not be useful to determine the recombinant plants in the resistant population (In repulsion phase, the presence of marker in resistant plants might be due to either escape of susceptible individual into resistant ones or heterozygote individuals). Thus, the recombinant plants in the susceptible population are multiplied by two as proposed by Amiri et al. (2009).

Amiri et al. (2009) equation:

$$\text{Distance } \textcircled{R} = \frac{\text{Susceptible without band} \times 2}{\text{Total individuals}}$$

R=repulsion

Barzen et al. (1992) equation:

$$\text{Distance} \textcircled{c} = \frac{\text{Resistant without band} + \text{susceptible with band}}{\text{Total individuals}}$$

c=coupling

Cloning of the selected RAPD fragment: The selected band amplified by MF1 and MF2 primers in susceptible bulk was eluted and purified from an agarose gel by gel extraction kit (Qiagen; Hilden, Germany) and cloned into pTZ57R/T vector (Fermentase) and commercially sequenced by M13 forward/reverse primers (Seqlab Co. Germany).

Design SCAR primers: Primers were designed Oligo 5 software. SCAR primers (20 mer) designated as ZF1 and ZF2 contained the original primers sequence and adjacent internal bases. Conditions for amplification of the SCAR marker included one cycle of 5 min at 94^0C, followed by 40 cycles of 40 s at 94^0C, 45 s at 60^0C, 60s at 72^0C, and one cycle of 10 min at 72^0C for final extension. The reaction mixture was the same as the RAPD reaction.

Results

Identification of RAPD markers linked to rhizomania resistance gene Rz1: To identify RAPD markers linked to rhizomania resistance gene, bulk DNAs were constituted with the most resistant and susceptible plants of the segregated families of Holly 1-4 and screened with 397 primer pair combinations. About 2.5 per cent of primer pair combinations didn't amplify the bulk DNA. Aonther 34 primer pair combinations amplified at least one band either in resistant or in susceptible bulk. Further investigation showed that only nine primer pair combinations produced polymorphic bands that appeared to be related to resistance and repeatable. The nine primer pair combinations were named MF1,MF2-r-(765bp); MF3,MF4-r-(~920bp); MF2,MF3-r-(~950bp); MF5,MF6-c-(~1580bp); OP-P13-c-(~1360bp); MF9,MF10-c-(~800bp); MF7,MF8-c-(~1220bp); MF8,MF11-c-(~1250 bp), and MF12,MF7-c-(~1475bp). Their respective markers were named R1, R2, R3, C3, C4, C1, C2, C5, and C6. Using the recombined individuals frequency method, the distances of these markers from Rz1 were measured as 2.3 cM, 8.3 cM, 16.6 cM, 20 cM, 21.4 cM, 27.5 cM, 32.9 cM, 43.7 cM, and 51.9 cM, respectively. Three primer pairs had a polymorphic band in susceptible bulk (repulsion phase) and six primer pairs had a polymorphic band in resistant bulk (coupling phase).

However, two of the primer pairs (43.7cM for C5 and 51.9 cM for C6) in coupling did not exhibit tight linkage due to long distance of the marker from Rz1 and therefore were not included in the Mapmaker analysis.

As shown in Figure 1, the R1 marker produced a 765bp band in the susceptible bulk, as well as the individual plants comprising the bulk. No PCR products were observed in sample lane number 10. In figure 2, the R2 marker produced a 920bp band in the susceptible bulk and the individuals comprising the bulk. No PCR products were observed in sample lane number 1 and 10. Genetic distances and marker arrangements of R1, R2, R3, C3, C4, C1 and C2 also were determined by mapmaker version 3.0 (table 1).

These markers are in one linkage group. The χ^2 for R1, R2, C4, and C1 markers was non-significant, thus, conforming to 3:1 ratios. However, the χ^2 for C3 marker was significant at five per cent probability level and for C2 and R3 marker was significant at one per cent probability level, indicating that these markers did not conform to 3:1 Mendelian ratio. Linkage analysis of the markers linked to the resistance gene was performed to determine the genetic map. Linkage analysis is based on simultaneous segregation-distortion test of two marker locations. The distortion may be due to either physical linkages between two genetic locations, or due to one by one individual locations. The C2 and R3 markers were significant at one per cent probability level and thus were not included in further analysis. To increase the precision of markers' distances from Rz1, the C3 was excluded in further analysis as well. Removal of these markers did not affect the linkage group but reduced the other marker's distances related to the resistance gene (table 2).

SCAR marker: Two SCAR markers (designed viz., ZF1 and ZF2 primers) produced one 765bp fragment in susceptible bulk and its individuals, but this fragment was not amplified in resistant bulk and its individuals after amplification by PCR (Fig 3). The SCAR primers were tested on both the parents and produced a single 765bp fragment.

Discussion
In this study, we used 397 primer pair combinations that were more effective than when used singly. Comparing the two systems, only about 3% of the paired primers did not amplify the resistant and susceptible bulks, whereas, 18% and 24% of the same primers used singly did not amplify the resistant and susceptible bulks of the same population (by Amiri et al. (2009) and Mesbah (2008),

respectively). The single primers of these primer pair combinations have been used on this population by Nouhi et al. (2008). The best tight-linkages reported were for OP-X9-1120 with 27 cM apart from Rz1 in coupling phase and for OP-AN9-600 with 13.7 cM apart from Rz1 in repulsion phase.

Nine primer pair combinations were found to show polymorphic bands in this population, however two of them (C5 and C6) were ignored due to their distance of more than 40 cM from Rz1. In addition, R3 and C2 markers were removed from further analysis since their χ^2 was significant at one per cent probability. The distortion of segregation could be due to the selective deletion of male gametes as a result of pollen grains sterility or self–incompatibility (Subudhi and Huang, 2002; Wagner et al. ,1992), selective deletion of female gametes (Subudhi and Huang, 2002) or the differences of selective capability advantages of zygotes (Wagner et al., 1992).

In figure 1 and 2, the repulsion band (R1 and R2) is presented in all individuals comprising the susceptible bulk (lanes 8-14) and is absent in individuals comprising the resistant bulk (lanes 1-7), except lane 5 which is resistant which may be due to heterozygous nature of the individual plant in representing this lane. Since the distance of marker R1 is less than 5cM from Rz1 it can be concluded that this marker is more reliable.

RAPD are dominant markers and unless they are converted to SCAR markers, they can not be used in MAS. Hence, R1 marker was converted into SCAR marker. The ZF1 and ZF2 primers amplified one intensive band (765bp) in homozygous recessives or heterozygous of F2 population. The new PCR primer used to generate the SCAR marker R1 can readily be used for marker assisted selection in breeding programs after confirmation in other segregating population for Rz1 gene. Currently, this primer sequence is under patent processing and will be shared once it gets patented.

Comparing the two methods for estimation, genetic distances obtained from the Mapmaker software are too far from those obtained from the frequency of recombinant plants method (figure 4).

Similar findings have also been reported by Amiri & Sarafraz (2007). They showed that if the linkage between the marker and the gene is in coupling phase, the distances obtained from the frequency of recombinant plants would fairly be in agreement with those obtained from the Mapmaker ver.3. On the

other hand, if the marker and the gene have linkage in repulsion phase, the results obtained from the software and the likelihood function will be in close agreement but would differ widely with genetic distance obtained from the frequency of recombinant plants. Their results showed that when one is concerned with a dominant marker in an F2 population, the distance estimated by likelihood function and Mapmaker software will be biased for repulsion phase, but not so when using the frequency of recombinant plant (Amiri & Sarafraz 2007). On the other hand, to calculate the frequency of recombinant plants, Mapmaker differentiates from likelihood function and equates it to zero, and when the marker and the gene are linked in repulsion phase, the frequency of the homozygous recessive phenotype (susceptible plants without band or homozygous recessives for both the marker and genetic loci) greatly affects the estimation of the frequency of recombinant plants. Thus, a decrease in frequency of recessive phenotypes increases the bias in estimation of the frequency of recombinant plant by Mapmaker (Amiri et al. 2009).

Because of the low frequency of susceptible plants without the marker in repulsion (one plant), the distances calculated from the software are too biased and estimations obtained from the frequency of recombinant plants are correct and so in dominant markers such as RAPD when the marker and the gene are linked in repulsion phase, it is recommended to estimate the genetic distance by frequency of recombinant plants.

References

Amiri, R. 2003. Identification of molecular marker linked to rhizomania resistant gene(s) for rapid germplasm-estimation. Final report of research at Sugar Beet Seed Institute, Karaj, Iran. 1- 32.

Amiri, R. and Sarafraz, E. 2007. Comparison of recombinant plant frequency, likelihood function and mapmaker software for estimation distance of AFLP and RAPD markers in F2 population. Abstract book of the 5th national biotechnology congress of Iran. 24-26 Nov. 2007, summit meeting conference hall, Tehran-Iran. PP: 832. (in persian)

Amiri, R., Mesbah, M., Moghaddan, M., Bihamta, M. R. , Mohammadi, S. A. and Norouzi, P. 2009. A new RAPD marker for beet necrotic yellow vein virus resistance gene in Beta vulgaris. *Biologia Plantarum.,* **53 (1):** 112-119.

Asher, M.J.C. 1993. Rhizomania. In: Cooke, D.A. & Scott, R.K. (eds) The sugar beet crop, Science into practice. Chapman & Hall, London, pp 311-346.

Barzen E., Mechelke W., Ritter E., Schulte-Kappert E. and Salamini F. 1995. An extended map of the sugar beet genome containing RFLP and RAPD loci. *Theor. Appl.Genet.,* **90**: 189–193.

Barzen, E., Mechelke, W., Ritter, E., Seitzer, J.F. and Salamini, F. 1992. RFLP markers for sugar beet breeding: chromosomal linkage maps and location of major genes for rhizomania resistance, monogermy and hypocotyl colour. *Plant J.,* **2:** 601–611.

Biancardi, E., Lewellen, R.T., de Biaggi, M., Erichsen, A.W. and Stevanato, P. 2002. The origin of rhizomania resistance in sugar beet. *Euphytica*, **127:** 383-397.

De Biaggi, M. 1987. Methodesde selection, un cas conceret. Proceedings of the 50th IIRB Congress, Brussels. PP. 157-163.

De Loose, M., and Van Bockstaele,E.1995. RAPD markers, combined with gelcompar analysis allow to identify sugar- and fodderbeet varieties and populations. Proceedings of the 58 th IIRB Congress, Beaune, PP. 129-132.

Francis, S. A., Redfearan, M. , Chwarszczynska, D. M., and Asher, M. J. C. 1998. Use of molecular markers in breeding for disease resistance in sugar beet (*Beta vulgaris* L.). *Aspects Appl. Biol.,* **52:** 279–285.

Gidner S., Lennefors B.T., Nilsson N.O., Bensefelt J., Johnsson E., Gyllenspetz U. and Kraft T. 2005. QTL mapping of BNYVV resistance from the WB41 source in sugar beet. *Genome,* **48:** 279-285.

Haley, S.D., Afanador , L. and Kelly, J.D. 1994. Selection for monogenic pest resistance traits with coupling and repulsion phase RAPD markers. *Crop Sci.,* **34:** 1061-1066.

Hallden, C., Hansen, M. , Nilsson, N. O., Hjerdin, A. and Sall, T. 1996. Competition as a source of errors in RAPD analysis. *Theor. Appl. Genet.,* **93:** 1185-1192.

Hansen, M., Hallden , C. and Sall, T. 1998. Error rates and polymorphism frequencies for three RAPD protocols. *Plant Mol. Biol. Rep.,* **16:** 139-146.

Heijbroek, W., Musters, P.M.S. and Schoone A.H.L. 1999. Variation in pathogenicity and multiplication of beet necrotic yellow vein virus (BNYVV) in relation to the resistance of sugar beet cultivars. *Eur. J. Plant. Pathol.,* **105:** 397-405.

Johansson, E. 1985. Rhizomania in sugar beet—a threat to beet growing that can be overcome by plant breeding. *Sver Utsädes Fören Tidskr,* **95:** 115–121

Koenig, R., Lüddecke, P. and Haeberlé, A.M. 1995. Detection of beet necrotic yellow vein virus strains, variants and mixed infections by examining single-strand conformation polymorphisms of immunocapture RT-PCR products. *J. gen. Virol.,* **76:** 2051-2055.

Kruse, M., Koenig, R., Hoffmann, A., Kaufmann, A., Commandeur, U., Solovyev, A.G., Savenkov, I. and Burgermeister, W. 1994. Restriction fragment length polymorphism analysis of reverse transcription-PCR products reveals the existence of two major strain groups of beet
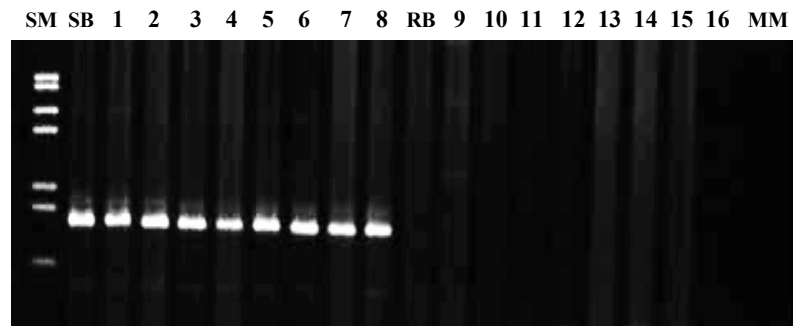
necrotic yellow vein virus. *J. gen. Virol.,* **75:** 1835-1842.

Kuszala, M., Ziegler, V., Bouzoubaa, S., Richards, K., Putz, C., Guilley, H. and Jonard, G. 1986. Beet necrotic yellow vein virus: different isolates are serologically similar but differ in RNA composition. *Ann. Appl. Biol.,* **109:** 155-162.

Lander, E. S., Green, P., Abrahamson, I., Barlow, A., Daley, M. J. , Lincoln, S. E. and Newburg, L. 1987. MAPMAKER: An interactive computer package for constructing primary genetic maps of experimental and natural populations. *Genomics,* **1:** 174-181.

Lein, J. C., Asbach, K., Tian, Y., Schulte, D., Li, C., Koch, G., Jung, C. and Cai, D. 2007. Resistance gene analogues are clustered on chromosome 3 of sugar beet and cosegregate with QTL for rhizomania resistance. *Genome,* **50:** 61-71.

Lennefors, B.L., 2006. Molecular breeding for resistance to rhizomania in sugar beets. Doctor's dissertation. Swedish University of Agricultural Sciences versity *Uppsala,* **106:** 1-41.

Lewellen, R.T. 1995. Performance of near-isolines of sugarbeet with resistance to rhizomania from different sources. In: Proc 58th IIRB cong, pp 83-92.

Lewellen, R.T., Skoyen I.O. and Erichsen A.W. 1987. Breeding sugar beet for resistance to rhizomania: Evaluation of host-plant reactions and selection for and inheritance of resistance. In: Proc 50th IIRB cong, Brussels, Vol. II: pp 139-156.

Link, D., Schmidlin, L., Schirmer, A., Klein, E., Erhardt, M., Geldreich, A., Lemaire, O. & Gilmer, D. 2005. Functional characterization of Beet necrotic yellow vein virus RNA-5- encoded p26 protein: evidence for structural pathogenicity determinants. *J. gen. Virol.,* **86:** 2115-2125.

Mesbah M. 2008. Identification of molecular markers linked to rhizomania resistance genes for rapid screening of sugar beet germplasm. *Final report. Sugar Beet Seed Institute, Karaj, Iran.* 44p (in Persian).

Michelmore, R.W., Paran, I., and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in speciWc genomic regions by using a segregating population. *Proc. Natl. Acad. Sci. USA,* **88:** 9828–9832.

Murry, M. G. and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.,* **8:** 4321-4325.

Nouhi, A., Amiri, R., Haghnazari. A., Saba, J. and Mesbah, M. 2008. Tagging of resistance gene(s) to rhizomania disease in sugar beet (*Beta vulgaris* L.). *African J. Biotech.,* **7** (4): 430-433.

Novy, R. G. , and Vorsa, N. 1996. Evidence for RAPD heteroduplex formation in cranberry: implication for pedigree and genetic-relatedness studies and a source of co-dominant RAPD markers. *Theor. Appl. Genet.,* **92:** 840-849.

Paterson, A. H., Tanksley , S. D. and Sorrells, M. E. 1991. DNA markers in plant improvement. *Advan. in Agron.,* **46:** 39-90.

Pelsy, F. and Merdinoglu, D. 1996. Identification and mapping of random amplified polymorphic DNA markers linked to a rhizomania resistance gene in sugar beet (Beta vulgaris L.) by bulked segregant analysis. *Plant Breed.,* **115:** 371-377.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning. A Laboratory Manual2. Cold Spring Harbor Laboratory Press. New York.

Schirmer, A., Link, D., Cognat, V., Moury, B., Beuve, M., Meunier, A., Bragard, C., Gilmer, D. and Lemaire, O. 2005. Phylogenetic analysis of isolates of beet necrotic yellow vein virus collected worldwide. *J. G.Virol.* ,**86:** 2897-2911.

Scholten, O.E. and Lange, W. 2000. Breeding for resistance to rhizomania in sugar beet: A review. *Euphytica,* **112:** 219–231.

Scholten, O.E., De Bock, T.S.M., Klein-Lankhorst, R.M. and Lange, W. 1999. Inheritance of resistance to beet necrotic yellow vein virus in Beta vulgaris, conferred by a second gene for resistance. *Theor. Appl. Genet.*, **99:** 740-746.

Steel, R. G. D. J. and Torrie, H. 1980. Principles and procedures of statistics, a biometrical approach. Mc Graw- Hill, Inc, New York.

Subudhi, P. K. and Huang, N. 2002. Identification of genes responsible for segregation distortion in a doubled haploid population of rice by using molecular markers. *Rice Genet. Newsl.,* **12:** 239-240.

Van den Broecke, A., Riek, J. D. , Roldan-Ruiz, I. , Wauters, A. , Van Bockstaele , E. and De Loose, M. 1998. DNA markers: a tool for plant variety description, recognition and stability testing. Proceedings of the 61 st IIRB Congress, Brussels, PP. 151-165.

Wagner, H., Weber, W. E. and Wricke, G. 1992. Estimating linkage relationship of isozyme markers and morphological markers in sugar beet (Beta vulgaris L.) including families with distorted segregations. *Plant Breed.* ,**108:** 89-96.
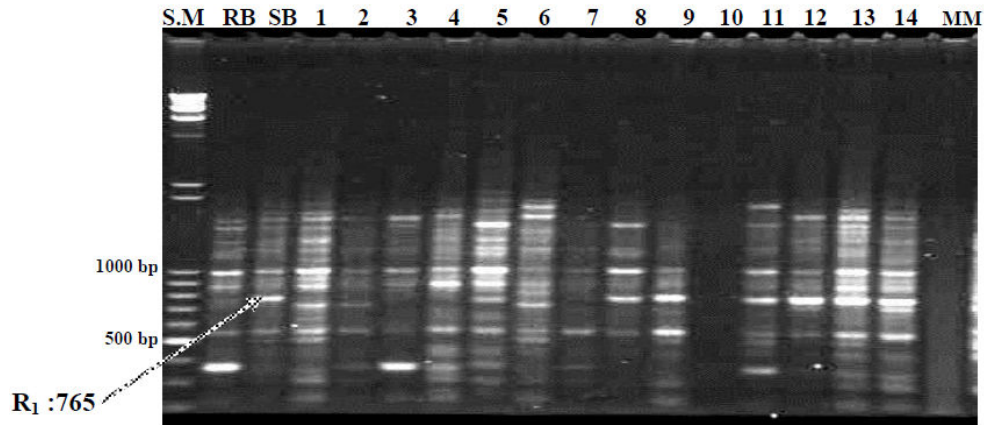
S.M :100 bp DNA Ladder+*Hin* III   MM : Mastermix without DNA

Feghhi figure 1. The repulsion band shown in 765bp. The sample number 10 has not amplified. RB=Resistant Bulk, SB=Susceptible Bulk. 7 resistant bulk & 7 susceptible bulk respectively.
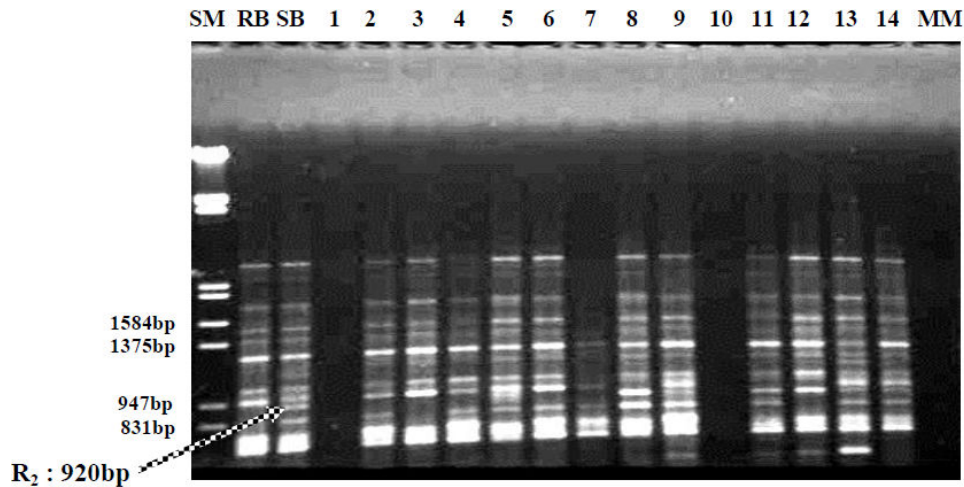
S.M: Lambda DNA/*Eco*RI+*Hin*III   MM: Mastermix without DNA

Feghhi figure 2. RAPD marker present in resistant bulk (RB), susceptible bulk (SB), and their individual resistant (lanes 1-7) and susceptible (lanes 8-14) plants.

SM: Lambda DNA/*Eco*RI+*Hin*dIII .MM: Mastermix without DNA

Feghhi figure 3. The results of SCAR marker (765 bp fragment) in susceptible bulk (SB), their susceptible individuals (lanes 1-8), resistant bulk (RB) and their resistant individuals (lanes 9-16) plants.
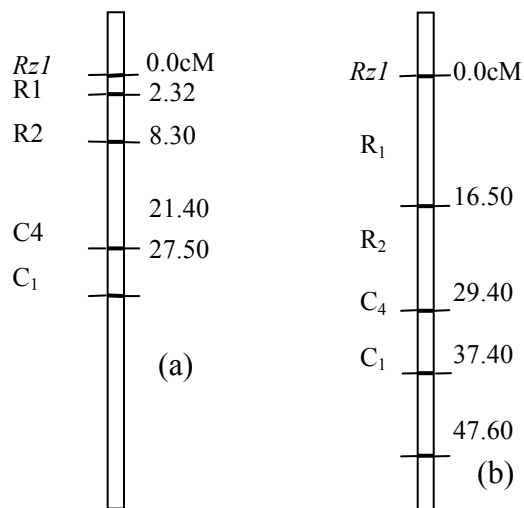
Feghhi figure4. Distances (cM) between markers by
frequency of recombinant plants (a) & map maker
v.3.0 (b).

Table1. Arrangement and distance of seven markers from each other and *Rz1* by mapmaker version 3.0

Markers	Distance		
3 C2	34.7 cM		
1 gene	……..		
4 C3	25.0 cM		
7 R1	7.4 cM		
6 R2	12.1 cM		
5 C4	2.0 cM		
2 C1	11.4 cM		
8 R3	2.2 cM		
	94.7 cM	8 markers	log-likelihood= -134.29

Table2. Arrangement and distance of four markers from each other and *Rz1* by mapmaker version 3.0

Markers	Distance		
1 gene	………		
4 R1	16.5 cM		
5 R2	12.9 cM		
3 C4	8.0 cM		
2 C1	10.2 cM		
	47.6 cM	5 markers	log-likelihood= -85.6