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Identification of molecular markers linked to sugar beet cyst nematode resistance gene(s)

N. Rahmani[(1)*], M. Mesbah[(2)], P. Norouzi[(2)]

[(1)]*M.Sc. of Agriculture Biotechnology, Islamic Azad University, Sciences and Research Branch, Tehran, Iran.*
[(2)]*Professor, Seed and Plant Certification and Registration Institute, Karaj, Iran.*
[(3)]*Associate Professor, Sugar Beet Seed Institute, Karaj, Iran.*

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ABSTRACT

Beet cyst nematode (Heterodera schachtii Schmidt). Spreads extensively in different regions of sugar beet cultivation, and therefore many efforts are accomplished to prevent decrease of sugar and root yield caused by the disease. The most appropriate control method of this disease is improvement of resistant varieties. Wild species of procumbentes in Beta are very important because they contain resistance genes against different diseases including beet cyst nematode. Ten-mer primers and specific sequences were selected to identify DNA molecular markers linked to beet cyst nematode resistance genes. For this purpose, at first, segregating populations for resistance genes were selected and after seed sowing in pots in greenhouse, seedlings were inoculated with 1000 nematode larva several times. Resistant plants (with lower than 10 cysts) and susceptible plants (with less than 10 cysts) were identified and divided to two groups. In the next step, RAPD-PCR, finger printing of the samples and comparison the DNA banding pattern of the two groups was done by using selected primers such as: OP-X-02, OP-X-15, OP-G-02, OP-D-13, OP-B-11, OP-Y-10 and Sat-121 specific primers and the oligonncleotides TGAACACCTTTCAAAT (forward) and CGTAAGAGACTATGA (Reverse) and one hundred primers from the operon kits. After calculating of correlation between resistance plants and the presence of special DNA band, OP-D-13 and Sat-121 molecular markers were identified to be linked to resistant genes against beet cyst nematode. In conclusion, these two molecular markers can be used for screening of resistant plants in laboratory conditions.

Keywords: sugar beet, resistance, cyst nematode, molecular markers

INTRODUCTION

Sugar beets are traditionally affected by the losses caused by various pests and diseases including cyst pathogenic nematode of sugar beet (*Heteroderaschachtii* Schmidt). The first symptoms of the disease are the weakness, yellowing and stunted growth. The infected plants become dwarf, their roots become small and deformed with scattered hairy roots, and the white cysts are visible on hairy roots (Mohamadigoltape et al. 1998). The root yield loss of sugar beet by the nematodes attack is estimated to be about 10% from which nearly 90% is associated with cyst nematode. Therefore, it is known as an important pathogenic agent of sugar beet throughout the world (Sandal et al. 1997). Given the fact that the classical evaluation methods for selection for disease resistance are phenotypic and depend upon environmental conditions and the uniformity of pathogenic agent, they have be conducted in specific seasons, and some infected plants may escape in some ways and appear to be resistant, the application of molecular methods as complementary or alternative methods can help identifying the plants containing the resistance gene at genotypic level. Therefore, DNA markers can be useful tools for selecting resistant genotypes and can save the evaluation time and improve the preci-

*Corresponding author's email: rahmani_nassim@yahoo.com

sion (Norouzi 2003).

Within wild species of beet, at least three nematode-resistance gens are located on different chromosomes of Procumbentes including the *Hs1* gene on chromosome1 of all three Procumbentes species, *Hs2* gene on chromosome7 of *Beta procumbens* and *B. webbiana*, and *Hs3* gene on chromosome8 of *B. webbiana*. The genes of the resistance to sugar beet cyst nematode have been introduced from gene pool of wild *Beta* species into bred lines (Kleine et al. 1998).

Salentijn et al. (1994) found recurrent sequences of Sat-121 with tight linkage with beet cyst nematode (Mesbah 1997). In addition, they identified some random primer sequences with linkage to this gene. Hallden et al. (1997) used RAPD technique by making use of a number of primer sequences for the identification of the gene(s) of the resistance to beet cyst nematode. Mesbah et al. (1997) identified three recurrent DNA sequences called OPX2, PB6-4 and Sat-121 in the genomes of wild beet species from Procumbentes with genetic linkage with nematode resistance gene(s). Cai et al. (1997) used genome-specific satellite markers to isolate the first gene responsible for nematode resistance known as $Hs1^{pro-1}$. The comparison of the sequence of *Hs1* gene decoding locus in three species of Procumbentes showed 96% sequence similarity between *B. procumbens* and *B. webbiana* and approximately 93% sequence similarity between *B. procumbens* and *B. patellaris* (Kleine et al. 1998). *Hs1* originated from *procumbens* species is known as $Hs1^{pro-1}$ which has been used more than other resources for transferring to sugar beet cultivars. This gene generates a single-gene, dominant resistance (Kleine et al. 1998). However, among markers which have been determined for this gene, it is sometimes observed that the plants are resistant to cyst nematode but they do not exhibit the relevant marker (Salentijn 1995). Therefore, it seems necessary to look for new markers in addition to test some previously-recognized markers. The present study is aimed at studying and identifying molecular markers by the aid of RAPD random primers and selected oligonucleotides in order to use them in *in-vitro* screening of nematode resistant genotypes.

MATERIALS AND METHODS

Plant materials

Genotype W1009 is a translocation line originated from *B. procumbens* which has vertical resistance to cyst nematode. The seeds of plants produced by self-pollination, the hybrids of the cross of translocation line (W1009) with sugar beet inbred lines including 231 × (MSC2×W1009)-1, 231 × (9801×W1009), 231 × (20447×W1009) and 231 × (20314×W1009), and the commercial cultivars of Nemakil and Rasoul were used as the resistant and sensitive control cultivars, respectively, for the evaluation of the resistance of the selected sugar beet genotypes to cyst nematode.

The resistance of the selected sugar beet genotypes to cyst nematode was studied in two separate experiments (in 2005) on the basis of a Randomized Complete Block Design under greenhouse conditions. In each experiment, 50 seedlings of the genotypes were inoculated with 1000 active second-age larvae of the nematode at several stages. Nine weeks after the final inoculation, the number of cysts on the roots of and in the sand surrounding the seedlings was counted and used as the basis for the comparison of the resistance of the genotypes. The collected data were analyzed with SAS statistical software.

Genomic DNA extraction

Nine plants from the most resistant and nine plants from the most sensitive plants of 231 × (20314×W1009) cross were selected for preparing genomic bulk. Furthermore, all resistant plants of the crosses 231 × (9801×W1009), 231 × (20447×W1009), 231 × (20314×W1009), and 231 × (MSC2×W1009)-1 as well as the most sensitive plants of these crosses were selected as single plants for molecular marker test. In addition, out of the plants produced by self-pollination the resistant single plants of the translocation line were used for validation of the tested marker.

After selecting the resistant and sensitive bulk plants, their DNA was extracted by the method of Norouzi (2003) and after their marker test, the DNAs of all resistant and sensitive plants of the crosses 231 × (9801×W1009), 231 × (20447×W1009), 231 × (20314×W1009), and 231 × (MSC2×W1009)-1 were extracted to determine the linkage of the intended marker to the resistance gene on the single plants.

RAPD-PCR test

Genomic DNA was conducted with PCR buffer, 200 µmold NTP, 25 nanogram primers and one unit of polymerase Smartaq enzyme in total volume of 25 µL.

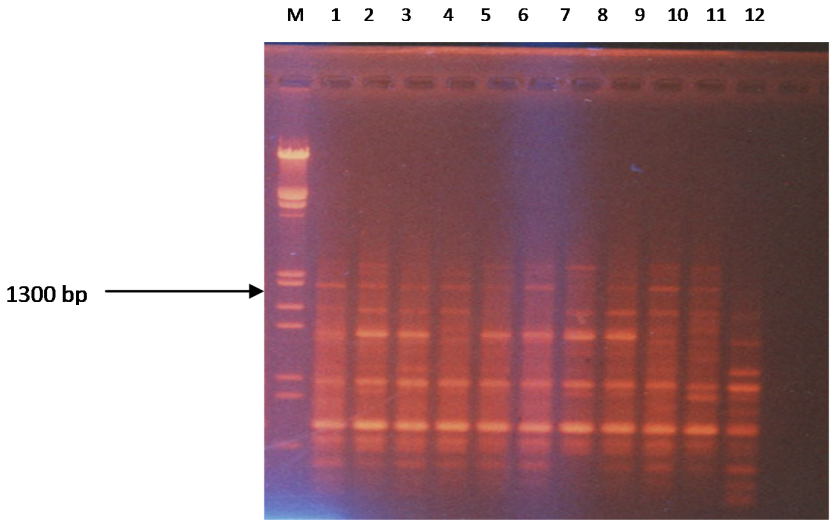

Fig. 1. Banding pattern of RAPD OP-D-13 primer from left to right; M = molecular weight marker (EcorRI/Hind III+DNAλ); 1 = resistant bulk resulted from the cross 231*(20314*W1009); 2,3,4,…,9 = single resistant plants resulted from the cross 231*(20314*W1009); 10 = single sensitive plant resulted from the cross 231*(20314*W1009); 11 = sensitive bulk resulted from the cross231*(20314*W1009); and12=Master mix without DNA.

Tested primers

Genomic pieces were amplified by random primers OP-Y-10, OP-B-11, OP-D-13, OP-G-02, OP-X-15, OP-X-2 and oligonucleotides TGAACAC-CTTTCAAAT and CGTAAGAGACTATGA related to reciprocal sequence of Sat-121 and 100 random primers from the kits C,D,F,G,M (Apron Co.). The amplified pieces were separated byelectrophoresison1.2% Agar gel and the gels were stained in 1 mg l^{-1}ethidium bromide solution for 10-20 minutes. DNA bands were made visible by ultraviolet lamp and photographed by camera.

Data analysis

The results for primer OP-D-13 and primer Sat-121 were statistically analyzed by MS-Excel software to find their correlation for which the matrix of 0 and 1 was formed in which 0 and 1 show the absence and presence of the intended band, respectively.

RESULTS

It was found that the genotypes had significant differences in the studied traits in both experiments. Duncan's multiple range tests at 1% level categorized the genotypes into three groups: resistant, sensitive and very sensitive. The resistant sources W-1010 and W-1009 and the resistant commercial cv. Nemakil with the lowest number of cysts were categorized in the resistant group whereas the commercial cv. Rasoul and some of the studied hybrids were categorized as the most sensitive genotypes. Among the studied random primers, OP-D-13 showed polymorphism in the region of about 1300 bp suggesting that the resistant plants, though not all of them, had a band around 1300 bp, but this band was not observed in sensitive plants. Fig. 1 shows the banding pattern of the samples tested by OP-D-13 primer.

With respect to specific primers, repeat sequences of Sat-121 were subjected to PCR test on two sensitive and resistant bulks, sensitive and resistant single plants and self-pollinating plants of translocation line. The presence of smear and the band of about 160 bp in resistant bulk and its absence in sensitive bulk showed the presence of polymorphism. The results of the polymorphism study on resistant and sensitive single plants revealed that no sensitive plant produces the band of 160 bp and smear, i.e. where there is the band of 160 bp and smear, the plant is resistant. However, this band and smear were not observed in all resistant plants. Fig. 2 presents the pattern of the banding of samples by Sat-121 primers.

DISCUSSION

Repeated sequences primers Sat-121 were undergone PCR test on two sensitive and resistant bulks, single sensitive and resistant plants, and self-pollinating plants. The presence of smear and band of about 160 bp in resistant bulk and its absence in sensitive bulk was the difference in banding pattern. Although this pattern was not repeated in all single resistant plants, the plant was resistant wherever the smear and band status

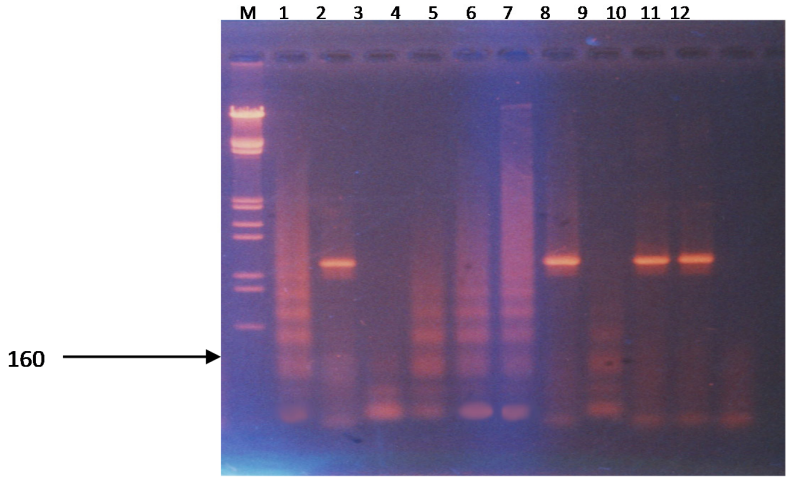

Fig. 2. Banding pattern of samples by Sat-121 primers from left to right; M = size marker (EcoRI/Hind III+DNAλ); 1 = resistant bulk resulted from the cross 231*(20314*W1009); 2,3,4,…,9 = single resistant plants resulted from the cross 231*(20314*W1009), 10 = sensitive sample resulted from the cross 231*(20314*W1009); 11 = sensitive bulk resulted from the cross 231*(20314*W1009), and 12=Master mix without DNA

of 160 bp was observed.

Lange et al. (1994) used Sat-121 primer and reported that in plants to which one fragment of or whole chromosome number 1 of *B. patellaris* or *B. procumbens* had been added the sequence can be used as the marker of the locus of the genes $Hs1^{pro-1}$ and $Hs1^{pat-1}$. The correlation of Sat-121 repeated sequence with $Hs1^{pro-1}$ locus was tested on two different back-crossed populations by Salentijn et al. (1994). The examination of banding pattern showed that the resistant plants had 159-bp band, while the sensitive plants did not. However, three resistant plants out of 187 tested plants of the first population and eight resistant plants out of 174 tested plants of the second population lacked Sat-121 sequence. Mesbah et al. (1997) successfully identified Sat-121 repeated fragment which was devoted to the genome of Procumbentes species and was not linked to *B. vulgaris* species.

Salentijn et al. (1994) used RAPD random primers called OP-X-15, OP-X-02, OP-B-11, and OP-Y-10 among which OP-X-02 primer showed polymorphism at about 1100 bp in banding pattern in plants containing $Hs1^{pro-1}$ chromosome fragment and other primers revealed difference in banding pattern in plants containing $Hs1^{Pat-1}$chromosome fragment. Despite the fact that there was about 93% sequence similarity between $Hs1^{pro-1}$ and $Hs1^{Pat-1}$, $Hs1^{Pat-1}$ markers were not able to identify plants containing $Hs1^{pro-1}$ which is probably related to the difference in the regions around these two genes, that is, the difference in primer annealing site.

The present study put the RAPD random primers including OP-B-11, OP-Y-10, OP-X-15 and OP-X-02 under PCR test but no differences were observed in banding pattern.

Mesbah et al. (1997) confirmed that the repeated sequence OP-X-02 in the genome of Procumbentes species was linked to the gene(s) of resistance to cyst nematode. This was not confirmed in the present study. The lack of the difference between sensitive and resistant plants in the present study might be associated with the fact that Mesbah et al. (1997) used a plant with a monosomic addition line of Procumbens but in the present PCR test, translocation line plants with a fragment of Procumbens genome and/or the progenies of the cross between translocation line and sugar beet lines were used in which the primer annealing site may not be a part of the transferred fragment.

In order to identify the genes of sugar beet cyst nematode resistance with bulk segregant analysis, Hallden et al. (1997) used RAPD random primers including OP-D-13, OP-AH-09, OP-AA-09, OP-L-10, OP-K-20, OP-H-15, OP-H-03 and OP-G-02. They found that the markers OP-D-13, OP-G-2, OP-H-03, OP-H-15, OP-K-20, OP-L-10, OP-AA-09 and OP-AH-09 were linked to the resistance gene. The distance of the OP-D-13, OP-AH-09 and OP-G-02 from *Hs1* gene was 0.2, 1.2 and 1.5 cM.

In the present study, RAPD random primers were used including OP-H-15, OP-H-03, OP-G-02, OP-D-13, OP-AH-09, OP-AA-09, OP-L-10 and OP-K-20 among which the primers OP-D-13 and OP-G-02 created bands with the length of about 1300

bp and 850 bp in resistant bulk, respectively, while these bands were not observed in sensitive bulk. This difference in banding pattern was not repeated in primer OP-G-02 in single plants and was caused by random polymorphism. However, the difference in banding pattern was repeated in the primer OP-D-13.

Sandal et al. (1997) reported that among plants to which *Hs1* gene was transferred there were observed resistant plants which have lost this gene and that the band of about 159 bp of Sat-121 repeated sequence was not observed in these resistant plants. They hypothesized that at least one another gene is involved in the resistance to sugar beet cyst nematode. Out of the studied back-cross plants, there were resistant plants which neither had Hs1 gene nor showed linkage with the markers linked to *Hs1* gene. The *Hs1* gene transferred to the susceptible plant might have been removed in the first meiosis division due to the generation of loop.

However, given the fact that these plants were shown to be resistant in greenhouse experiments, it is likely that more than one gene is responsible for the resistance in addition to the assumption of disease escape, so that some resistant plants lack *Hs1* gene. In the present study, two molecular markers linked to the gene(s) of resistance to sugar beet cyst nematode were identified. The presence of either marker was fully correlated with the phenotypic resistance of the plant. Furthermore, neither one was observed in susceptible plants.

The correlation between the results of OP-D-13 primer and Sat-121 primer was statistically analyzed by MS-Excel software in which a 1-0 table was created. Numbers 1 and 0 marked the presence and absence of the intended band. The correlation was found to be 88%. The correlation between the results of greenhouse evaluation of the resistance to sugar beet cyst nematode and the results of molecular markers was also analyzed by MS-Excel software; again by creating a 1-0 table in which 1 represented the resistant plant and 0 represented the susceptible plant. The correlation between the results of greenhouse evaluation of the resistance to sugar beet cyst nematode and the results of Sat-121 molecular marker was found to be 66.64% and the correlation between the results of the greenhouse evaluation and the results of OP-D-13 molecular marker was found to be 66.91%. The approximate distance of OP-D-13 molecular marker from the resistance gene was estimated by using the ratio of the number of recombinant plants (44) to total number of plants (133). It was found to be 0.33.

Thus, it is concluded that the markers obtained from the breeding of nematode-resistant cultivars can be used for genotypic screening of resistant plants.

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