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Development of an SNP Marker for Sugar Beet Resistance/Susceptible Genotyping to Root-Knot Nematode

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ABSTRACT

Linked and/or gene-based molecular markers have been used widely in marker-assisted selection (MAS) to differentiate resistant and susceptible genotypes. Resistance to *Meloidogyne* spp. in *Beta vulgaris* L. is mediated by a single dominant gene (*R6m-1*). Using allele-specific primers (ASPs), an SNP marker harboring a single nucleotide polymorphism (A/G), linked to the resistance gene was developed to differentiate resistant genotypes. The differentiation among the resistant and susceptible genotypes was elucidated in the polymorphic bands of 555, 478 and 124 bp in size, using PCR amplification. The genotyping data using the SNP marker was firmly associated with the bioassay evaluation in the greenhouse for 100 sugar beet genotypes. This data indicated that the present robust marker allowed reliable, sensitive, faster, and cheaper large scale screening of *B. vulgaris* genotypes for nematode resistance breeding programs.

Keywords: *Beta vulgaris*, *Meloidogyne* spp., Selection, SNP marker.

INTRODUCTION

Root-knot nematode (*Meloidogyne* spp.) is one of the most important pathogens of sugar beet in Iran and some other countries that directly and indirectly leads to significant yield loss. Development of sugar beet cultivars resistant to nematodes in the agricultural ecosystems can result in reduction of nematicide applications, which, in turn, reduces the production cost and environmental pollution loads (Zhang *et al*., 2008).

Recognition of disease resistant genotypes in conventional patho-breeding programs demands time-consuming and laborious experiments in greenhouse and is highly affected by environmental conditions. These breeding processes could be replaced simply by taking advantages of the molecular markers through recognition of suitable linked markers and performing marker-assisted selection (MAS) procedure, thereafter. Today, molecular markers are integrated widely in nematode resistance breeding programs, particularly for resistance to root knot and cyst nematodes (Hussey and Janssen, 2002; Young and Mudge, 2002; Xu *et al*., 2013).

The breeding program of sugar beet is efficiently supported by incorporation of DNA marker technology. Several markers including RFLPs (restriction fragment length polymorphisms) (Barzen *et al*., 1992; Pillen *et al*., 1992), RAPDs (randomly amplified polymorphic DNA), AFLPs (amplified fragment length polymorphism) as well as a small number of SSR (simple sequence repeat) markers (Schumacher *et*

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al., 1997) have already been tested for possible linkages to phenotypic, isoenzymes and morphological traits in sugar beet. Resistance to root knot nematode was first identified in sea beet (*B. vulgaris* ssp. *maritima* (L.) Arcang) and introgressed into cultivated sugar beet (*Beta vulgaris* L.). It was demonstrated to be simply inherited by a single dominant gene (Yu, 1995), which is effective against six different *Meloidogyne* spp. (Yu *et al*., 1999). Further studies resulted in the finding of a NEM06 CAPS marker linked to root knot nematode resistance gene (*R6m-1*). Sequence comparison between the fragments amplified from resistant and susceptible genotypes revealed one nucleotide substitution at position 208 was located in the recognition site of *Mse*I restriction endonuclease (Weiland and Yu, 2003). They reported to use PCR-RFLP marker to genotyping resistant/susceptible sugar beet lines that explanatory of single nucleotide polymorphism (SNP) is responsible for the polymorphism.

Single nucleotide polymorphism is the single base pair positions in genomic DNA at which different sequence alternatives (alleles) exist in normal individuals of some population(s). In simple terms, SNPs are the polymorphism occurring between DNA samples with respect to single base (Jehan and Lakhanpaul, 2006). Currently, SNPs are considered as an efficient and robust marker class in genomics. They are the most frequent molecular markers in humans, animals, and plants genomes (Schneider *et al*., 2001, 2007; Jehan and Lakhanpaul, 2006). SNPs represent high potential in pharmacogenomics and excellent applications in association studies, tagging of economic important genes, genotyping, genetic diversity studies and evolutionary investigation of plant species (Jehan and Lakhanpaul, 2006). However, the SNPs studies in plants are only in the early stages.

Single nucleotide polymorphisms analysis using polymerase chain reaction with confronting two-pair primers (PCR-CTPP) generate allele-specific DNA bands with different lengths, while PCR-RFLP requires the use of restriction endonuclease after PCR amplification, as well. This method is inexpensive, time-saving, and reliable for biallelic genotyping (Hamajima *et al*., 2000, 2002; Hamajima, 2001) and appropriate to SNPs where a suitable restriction enzyme is not accessible.

This study was carried out to establish a rapid genotyping assay on the basis of allele-specific primers that is more reliable than the previously published CAPS marker, for identifying resistant homozygous genotypes of sugar beet which could be used in screening of breeding populations and developing resistant S1 pollinator lines.

MATERIALS AND METHODS

Plant Materials and Pathogen

Sugar beet genotypes used in this study were SB34 and SB33 populations, the F_1 plants derived from SB33 population (male parent) crossed with a single cross (7112×SB36) and Half-Sib Families derived from SB33 population. Jolgeh (susceptible variety) and Pauletta (commercial resistant variety to beet cyst nematode and Rhizomania and susceptible to root knot nematode) cultivars were grown as checks.

Root-knot nematode (*Meloidogyne* spp.), which was originally isolated from the infected sugar beet fields in Iran and Azerbaijan and subsequently was purified on Rutgers, Superchief and W. tomato cultivars, was used as inoculum.

Nematode Resistance Assay in Greenhouse

Sugar beet seeds were grown in polyethylene pots containing 450 cm^3 steam pasteurized mixture of 1:1:1 (v/v/v) soil, peat moss and compost in Sugar Beet Seed Institute greenhouse. Two months after germination, seedlings were inoculated with 500 newly hatched second-stage juveniles in

1 mL distilled water. The pots bearing the inoculated plants were arranged in a completely randomized design pattern and maintained at 23±2ºC in the greenhouse. About 70 days after inoculation, all roots were harvested by soaking the pots in water and removing the soil. The number of root-galls per plant were counted using a stereomicroscope. Individual seedling with 10 galls or less and those with more than 10 galls were classified as resistant and susceptible, respectively (Taylor and Sasser, 1978).

SNP Genotyping

Genomic DNA (gDNA) was extracted from leaf tissue of all genotypes according to Dellaporta *et al.* (1983) with some modifications. The quality and quantity of gDNAs were analyzed using spectrophotometer and running on agarose gel, then concentrations were adjusted to 50 ng μL^{-1} for PCR amplifications. Firstly, 600 bp fragment was amplified from genomic DNA of some seedlings of SB33 and SB34 genotypes using the primers pair Nem06FWD and Nem06REV (Weiland and Yu, 2003) (Table 1). Amplified DNA fragment was separated on 1% (w/v) agarose gel, purified by standard techniques (QIAquick, Gel Extraction Kit, Cat. no. 28704) and subsequently sequenced (Alfa Company, USA). A blast search was performed on the amplified sequence and subsequently aligned with previously published marker sequence (accession no. AY210437) using GeneDoc (Gene1) and CLUSTALW (http://www.genome.jp/tools/clustalw/) software.

To develop a marker based on allele specific primers, new set of primer pairs (nem06FWD1, nem06REV1, NEM06FWD2 and NEM06REV2) were designed on the basis of the amplified sequences using the primer 3 program (http://frodo.wi.mit.edu/) and synthesized (AnaSpec Company, Canada) (Table 1). The FWD2 and REV1 possess an allele specific base at the 3' end. The optimal condition for PCR-CTPP was gained to give good correlation with the greenhouse experiment. Finally, the optimum results were gained by PCR reaction containing 2.5 µL of 10X PCR buffer, 1.6 mM $MgCl_2$, 0.4 mM dNTPs mix, 0.2 µM of each FWD1 and REV2 primers, 0.24 µM of each FWD2 and REV1 primers, 2.5 units of Taq DNA polymerase (Cinnagen, Cat. No. PR901650), 100 ng template and double distilled water in a total volume of 25 µL. Negative controls using the PCR reaction mixture excluding DNA were also included for possible contamination. DNA amplification was performed in a Master cycler epgradient Eppendorf thermocycler for 35 cycles of 1 minute of denaturation at 94°C, annealing

Table 1. Oligonucleotide sequences used for sugar beet resistant/susceptible genotyping to root-knot nematode.

Marker	Primer Name	Sequence (5′ -3′)	Position[a]	Reference
Nem06	Nem06FWD	*TGCCGAGCTGCTTGACGGGTTGTC*	1-24	Weiland and Yu, 2003
	Nem06REV	*GTTTCGCTCCTCAGAATTGCTGAAG*	577-553	
nem06	nem06FWD1	*TGACGGGTTGTCAATATGC*	3-21	This work
	nem06REV1	*TCCATTTCCTGACCTACAATTATT*	126-103[b]	
NEM06	NEM06FWD2	*AAAGAAAGGGAACTCAAATGTTAG*	80-103[c]	This work
	NEM06REV2	*TCAGAATTGCTGAAGGTCATT*	557-537	

[a] Positions for Nem06, nem06 and NEM06 markers were predicted on the basis of accession numbers AY210437, KF303133 (and/or KF303134) and KF303135, respectively; [b] Allele specific primer for susceptible genotypes, [c] Allele specific primer for resistant genotypes.

for 1 minute at 59°C, and extension for 45 seconds at 72°C following an initial denaturation at 94°C for 4 minutes. Reactions were stopped after a final extension step of 72°C for 10 minutes. 10-12 µL of PCR products were analyzed by running on 1.5% agarose gel stained with ethidium bromide and photographed with gel documentation system.

The optimized protocol for PCR was examined on DNA extracted from 100 sugar beet genotypes.

RESULTS

Phenotypic Analysis

Resistance screening of sugar beet genotypes to root-knot nematode (Taylor and Sasser, 1978) indicated that the susceptible control varieties, namely, Jolgeh and Pauletta, and 7112×SB36 single cross (female parent) had lots of root galls and were classified as a highly susceptible group. In contrast, all seedlings of SB33 population, except seedlings with 2 galls, and its HSF were gall free. Among the five seedlings tested of F_1 generation [(7112×SB36)×SB33], only one seedling with one gall was observed. Therefore, F_1 generation was classified as a resistant hybrid to root knot nematode (Figure 1, Table 2). These results demonstrated that the resistance to root knot nematode in SB33 population was inherited in a dominant manner.

SNP Marker Analysis

A 600 bp DNA fragment was simply amplified in SB33 and SB34 genotypes

Figure 1. Sugar beet roots 70 days after inoculation with *Meloidogyne* spp. in the genotypes: (a) One of the Half-Sib Family SB33; (b) F_1 generation [(7112×SB36)×SB33], (c) Female parent (7112×SB36); (d) Male parent (SB33); (e) Jolgeh, and (f) Pauletta cultivars.

Table 2. Association between SNP marker and resistance to *Meloidogyne* sp. in sugar beet.

Plant phenotypes	Genotype [c]	Plant number [d]	Average number of galls [e]	SNP marker			Percent association
				(555, 478 bp) [f]	(555, 478, 124 bp) [g]	(555, 125 bp) [h]	
Susceptible [a]	7112×SB36	5	>200[b]	0	0	5	100
	Pauletta	11	>100[b]	0	0	11	100
	Jolgeh	4	>100[b]	0	0	4	100
Resistant [b]	SB33	25	0.08 [a]	22	3	0	100
	(7112×SB36)×SB33	5	0.2 [a]	0	5	0	100
	33.HSF.1	5	0 [a]	3	2	0	100
	33.HSF.2	5	0 [a]	3	2	0	100
	33.HSF.3	5	0 [a]	2	3	0	100
	33.HSF.4	5	0 [a]	4	1	0	100
	33.HSF.5	5	0 [a]	3	2	0	100
	33.HSF.6	5	0 [a]	4	1	0	100
	33.HSF.7	5	0 [a]	5	0	0	100
	33.HSF.8	5	0 [a]	4	1	0	100
	33.HSF.9	5	0 [a]	5	0	0	100
	33.HSF.10	5	0 [a]	2	3	0	100

[a], [b] Plants with more than 10 and those with 10 or less galls were classified as a susceptible and resistant, respectively; [c] SB and HSF stand for Sugar Beet and Half Sib Family, respectively; [d] The total number of roots in each genotype analyzed for phenotypic and SNP marker association; [e] Data average with common letters have not statistical differences ($P \geq 0.05$); [f] All homozygous resistant plants had at least one copy of the marker allele amplified by NEM06FWD2 and NEM06REV2 primers; [g] All heterozygous resistant plants had both copy of the marker allele amplified by NEM06FWD2, NEM06REV2, nem06FWD1 and nem06REV1 primers; [h] All susceptible plants were homozygous for the SNP marker allele amplified by nem06FWD1 and nem06REV1 primers.

using combination of primers Nem06FWD and Nem06REV (Figure 2). Based on the homology search of this fragment with the previously reported sequence (AY210437) showed 98% similarity among the sequences. Alignment of the DNA sequences by CLUSTALW and GeneDoc softwares revealed a point mutation of A to G specifically in resistant genotype SB33 (Figure 3). This "A/G" transition was well conserved among all resistant genotypes tested in this research (Figures 4 and 5). The sequences of these amplified products in the genotypes SB34 a, SB34 b and SB33 have been submitted to the Genbank Database with accession numbers KF303133, KF303134 and KF303135, respectively.

As shown in Figures 4 and 5, using the primer pairs FWD1 and REV2, a monomorph 555 pb fragment was amplified in all tested samples. FWD1 and the susceptible allele specific (REV1) primers amplify a 124 bp DNA fragment only in susceptible genotypes, whereas REV2 and the resistant allele specific (FWD2) primers generate a 478 bp DNA fragment specifically in resistant genotypes.

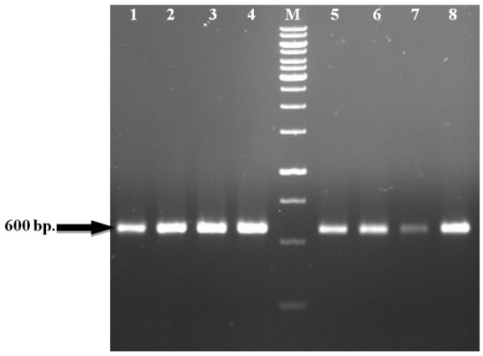

Figure 2. Amplification of 0.6 kb DNA fragment in the different sugar beet genotypes. Lanes (1-4): SB34, and Lanes (5-8): SB33. M lane stands for 1000 bp DNA ladder. The fragment has been amplified using primers Nem06FWD and Nem06REV.

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                    *         20         *         40
AY210437  : GTCTGCTGCCTCAAAAGATCAGCCTAACAATATTTAAAACAAA  43
SB34 a    : GTCTGCTGCCTCAAA-GATCAGCCTAACAATATTTAAAACAAA  42
SB34 b    : GTCTGCTGCCTCAAA-GATCAGCCTAACAATATTTAAAACAAA  42
SB33      : GTCTGCTGCCTCAAA-GATCAGCCTAACAATATTTAAAACAAA  42
            GTCTGCTGCCTCAAA GATCAGCCTAACAATATTTAAAACAAA

                  *         60         *         80
AY210437  : AGAAAGGGAACTCAAATGTTAGATAATTGTAGGTCAGGAAATG  86
SB34 a    : AGAAAGGGAACTCAAATGTTAAATAATTGTAGGTCAGGAAATG  85
SB34 b    : AGAAAGGGAACTCAAATGTTAAATAATTGTAGGTCAGGAAATG  85
SB33      : AGAAAGGGAACTCAAATGTTAGATAATTGTAGGTCAGGAAATG  85
            AGAAAGGGAACTCAAATGTTA ATAATTGTAGGTCAGGAAATG

               *        100         *        120
AY210437  : GAACAAGTTTCAAGATTTTTTACATAAGAAACCATACCTGGTG  129
SB34 a    : GAACAAGTTTCAAGATTTTTTACATAAGAAACCATACCTGGTG  128
SB34 b    : GAACAAGTTTCAAGATTTTTTACATAAGAAACCATACCTGGTG  128
SB33      : GAACAAGTTTCAAGATTTTTTACATAAGAAACCATACCTGGTG  128
            GAACAAGTTTCAAGATTTTTTACATAAGAAACCATACCTGGTG

            *       140         *       160         *
AY210437  : AACAAAGTTCTCTAGTGTGGCAAGCTTGCCCATAGCAATCGCC  172
SB34 a    : AACAAAGTTCTCTAGTGTGGCAAGCTTGCCCATAGCAATCGCC  171
SB34 b    : AACAAAGTTCTCTAGTGTGGCAAGCTTGCCCATAGCAATCGCC  171
SB33      : AACAAAGTTCTCTAGTGTGGCAAGCTTGCCCATAGCAATCGCC  171
            AACAAAGTTCTCTAGTGTGGCAAGCTTGCCCATAGCAATCGCC
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Figure 3. An alignment of a portion of the NEM06 marker sequence amplified by the Nem06FWD and Nem06REV primers from SB34 a, SB34 b, SB33 and the sequence submitted to the Genbank Database (AY210437) by GeneDoc software. Refer to text for more details.

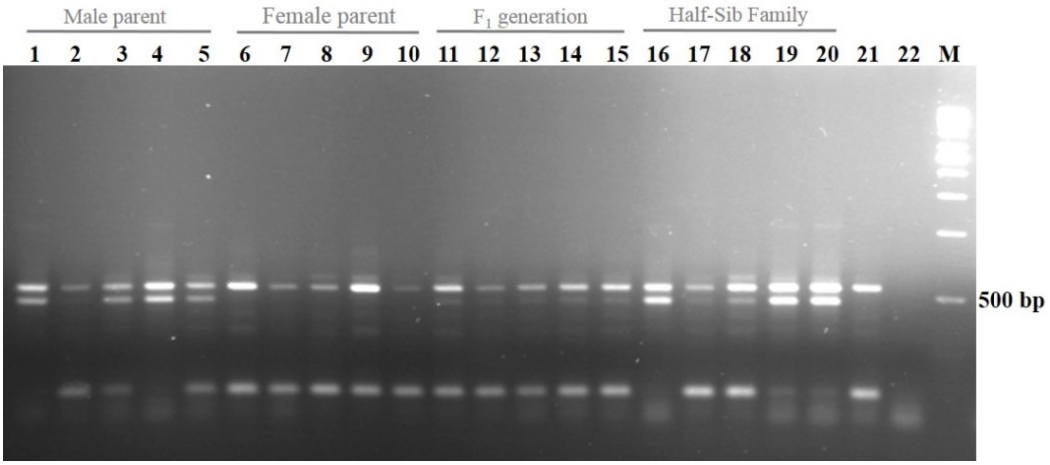

Figure 4. Electrophoretic patterns for resistant/susceptible genotyping by PCR-CTPP in sugar beet genotypes. Lanes 1-5, 6-10, 11-15, 16-20 and 21 belong to genotypes SB33, (7112×SB36), [(7112×SB36)×SB33], 33.HSF.$_1$ and susceptible cultivar Pauletta, respectively. Lanes 1, 4, 16 are homozygously resistant genotypes (555, 478 bp); Lanes 2, 3, 5, 11-15, 17-20 are heterozygously resistant genotypes (555, 478, 124 bp); Lanes 6-10, 21 are homozygously susceptible genotypes (555, 124 bp); Lane 22 is the negative control, and Lane M indicates 1 kb DNA ladder. The fragments have been amplified using primers nem06FWD1, nem06REV1, NEM06FWD2 and NEM06REV2.

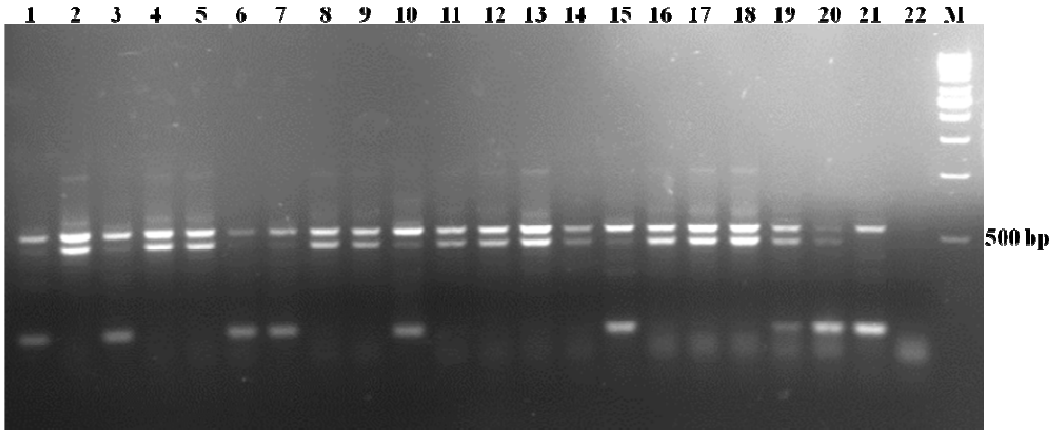

Figure 5. Electrophoretic patterns for resistant/susceptible genotyping by PCR-CTPP in sugar beet genotypes. Lanes 1-5, 6-10, 11-15, 16-20, and 21 belong to genotypes 33.HSF.$_2$, 33.HSF.$_3$, 33.HSF.$_4$, 33.HSF.$_5$ and susceptible cultivar Pauletta. Lanes 2, 4, 5, 8, 9, 11-14, 16-18 are the homozygous resistant genotypes (555, 478 bp); Lanes 1, 3, 6, 7, 10, 15, 19, 20 are the heterozygous resistant genotypes (555, 478, 124 bp); Lane 21 is the homozygous susceptible genotype (555, 124 bp); Lane 22 is the negative control, and Lane M indicates 1 kb DNA ladder. The fragments have been amplified using primers nem06FWD1, nem06REV1, NEM06FWD2 and NEM06REV2.

Genotyping was done using the electrophoretic patterns of the amplified PCR products as follows: 555 and 478 bp for homozygous resistant plants, 555, 478 and 124 bp for heterozygous resistant plants and 555 and 124 bp for homozygous susceptible plants. The size differences among these three fragments were big enough to be distinguished simply by agarose gel electrophoresis.

All seedlings of genotypes SB33, 33.HSF.$_{(1-5)}$ and the F_1 plants [(7112×SB36)×SB33] were grouped in homozygous and heterozygous resistant genotypes based on the new marker (Figures 4 and 5). The female parent (7112×SB36) was also shown to be a susceptible genotype (Figure 4). One seedling of each genotypes 33.HSF.6 and 8 and three seedlings of the genotype 33.HSF.$_{10}$ were heterozygously resistant, but all of the other plants were homozygously resistant (data are not shown). The data obtained in this research by applying the newly developed marker on the genotypes and F_1 hybrids revealed it as a robust marker for resistance or susceptibility genotyping to root knot nematodes in sugar beet. The association between the SNP marker and phenotyping is summarized in Table 2.

DISCUSSION

DNA sequencing allows for detection of single nucleotide polymorphisms (SNPs) between the alleles within a locus. SNP markers have rapidly gained the center stage of molecular genetics during the recent year and have several advantages over other genetic marker types due to their abundance within genomes and wide techniques have been developed for high-throughput SNP analysis, detection formats, and platforms (Zhu *et al*., 2003; Fan *et al*., 2006; Mammadov *et al*., 2012). In this study, one single nucleotide polymorphism was identified by sequence alignment analysis.

SNP markers were developed to identify plant diseases resistance traits in cotton viral blue disease (Fang *et al.*, 2010), loblolly pine pitch canker disease (Quesada *et al*., 2010), tomato nematode, fungi and viral diseases (Arens *et al*., 2010), cowpea *Macrophomina phaseolina* disease (Muchero *et al*., 2011), rice sheath blight disease (Silva *et al*., 2012), *Rice yellow mottle virus* disease (Albar *et al*., 2006), rice tungro disease (Lee *et al*., 2010), soybean rust disease (Kim *et al*., 2012), Asian soybean rust disease (Monteros *et al*., 2010), wheat leaf rust, stripe rust and powdery mildew diseases (Lagudah *et al*., 2009), wheat *Fusarium* head blight disease (Bernardo *et al*., 2012), soybean southern root-knot nematode disease (Ha *et al*., 2007), barley covered smut disease (Lehmensiek *et al*., 2008), *Beet necrotic yellow vein virus* disease for resistance genes, *Rz4* (Grimmer *et al*., 2007) and *Rz5* (Grimmer *et al*., 2008) and in legume viral disease (Naderpour *et al.*, unpublished data).

We developed PCR-based method (PCR-CTPP) instead of PCR-RFLP, to analyze SNP for genotyping resistance to root knot nematode in sugar beet. PCR-RFLP involves three steps; PCR with a thermal cycler, incubation with a restriction endonucleases for PCR product digestion, and electrophoresis for visualizing the fragments. The second step (incubation) takes a long time, depending on the restriction enzyme. PCR-RFLP requires high quality and quantity of genomic DNA to pass successfully the digestion step and they are not amenable to high throughput genotyping application in commercial breeding, also. Polymerase chain reaction with confronting two-pair primers (PCR-CTPP) technique monitors polymorphism at two independent single-nucleotide polymorphisms, at the same time in one tube. Because PCR-CTPP does not need incubation time with a restriction enzyme, the time and costs of the digestion of PCR products can be saved.

We have successfully designed PCR-CTTP conditions for resistant/susceptible genotyping in 100 sugar beet genotypes. In this study, a perfect correlation between the

phenotypes and genotypes was observed (Figures 4 and 5, Table 2). Moreover, the developed marker differentiates homozygous from heterozygous resistant genotypes, which is considered its main privilege over phenotyping. Although more samples of the susceptible and resistant genotypes need to be sequenced to confirm the SNP basis of this marker. Marker assays showed an advantage over biological tests in that the results were clearer, i.e., homozygote/heterozygote presence of the resistance gene could be detected and heterogeneity in seed lots could be identified readily. The PCR-CTPP technique is undoubtedly an inexpensive and time saving method compared to PCR-RFLP, which allowed us to carry out rapid, reliable, sensitive, and cheaper screening of *B. vulgaris* genotypes compared to the previously published markers (PCR-RFLP) for nematode resistance breeding programs. The duplex PCR-CTPP has been used for many human genetic diseases (Hamajima *et al*., 2000; Tamakoshi *et al*., 2003; Itoh *et al*., 2004, 2006) and plant diseases (Albar *et al*., 2006) studies.

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طراحی نشانگر چند شکلی تک نوکلئوتیدی برای غربال مقاومت/حساسیت ژنوتیپ های چغندرقند نسبت به نماتد مولد گره ریشه

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چکیده

استفاده از نشانگرهای مولکولی برای تفکیک ارقام حساس و مقاوم، در برنامه‌های اصلاحی انتخاب به کمک نشانگر (MAS) ، کاربرد فراوانی دارد. مقاومت به گونه‌های *Meloidogyne* در چغندرقند با یک ژن غالب (*R6m-1*) کنترل می‌شود. با استفاده از آغازگرهای اختصاصی آلل(ASPs) ، یک نشانگر مولکولی چند شکلی تک نوکلئوتیدی (A/G) پیوسته با ژن مقاومت، جهت تمایز ژنوتیپ‌های مقاوم طراحی شده است. تمایز ژنوتیپ‌های مقاوم و حساس با استفاده از واکنش زنجیره‌ای پلیمراز و

تشکیل باندهای چند شکلی با اندازه‌های 555، 478 و 124 جفت بازی صورت می‌گیرد. نتایج حاصل از داده‌های مربوط به نشانگر چند شکلی تک نوکلئوتیدی با نتایج آزمایش‌های زیستی در گلخانه، برای 100 ژنوتیپ چغندرقند کاملاً همخوانی دارد. این داده‌ها نشان می‌دهد که نشانگر حاضر امکان غربال معتبرتر، حساستر، ارزانتر و وسیع ژنوتیپ‌های چغندرقند را در برنامه‌های اصلاح برای مقاومت به نماتد مولد گره ریشه فراهم می‌کند.