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

M. Bakooie¹, E. Pourjam¹*, S. B. Mahmoudi², N. Safaie¹, and M. Naderpour³

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. maritime (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 MseI 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 Lakanpaul, 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 Lakanpaul, 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 Lakanpaul, 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 SI pollinator lines.

**MATERIALS AND METHODS**

**Plant Materials and Pathogen**

Sugar beet genotypes used in this study were SB34 and SB33 populations, the F1 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 cm³ 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±2°C 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 Sassar, 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>
<thead>
<tr>
<th>Marker</th>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>Position(^a)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nem06</td>
<td>Nem06FWD</td>
<td>TGCGAGCTGCTTGACGGGTTGTC</td>
<td>1-24</td>
<td>Weiland and Yu, 2003</td>
</tr>
<tr>
<td></td>
<td>Nem06REV</td>
<td>GTTTGCTTCCTCAAGATTTGCTGAAG</td>
<td>577-553</td>
<td></td>
</tr>
<tr>
<td>nem06</td>
<td>nem06FWD1</td>
<td>TGACGGGTTTGCTCAATATGC</td>
<td>3-21</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>nem06REV1</td>
<td>TCCATTTCTGACCTCAATTATT</td>
<td>126-103(^b)</td>
<td></td>
</tr>
<tr>
<td>NEM06</td>
<td>NEM06FWD2</td>
<td>AAAGAAAGGGAACCTAAAAATGTAG</td>
<td>80-103(^c)</td>
<td>This work</td>
</tr>
<tr>
<td></td>
<td>NEM06REV2</td>
<td>TCAGAATTTGCTGAAGGTGCATT</td>
<td>557-537</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) 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 Paulettta, 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 F1 generation [(7112×SB36)×SB33], only one seedling with one gall was observed. Therefore, F1 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) F1 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.

<table>
<thead>
<tr>
<th>Plant phenotypes</th>
<th>Genotype</th>
<th>Plant number</th>
<th>Average number of galls</th>
<th>SNP marker (555, 478 bp)</th>
<th>(555, 478, 124 bp)</th>
<th>(555, 125 bp)</th>
<th>Percent association</th>
</tr>
</thead>
<tbody>
<tr>
<td>Susceptible</td>
<td>SB33 7112×SB36</td>
<td>5</td>
<td>&gt;200&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Panletta</td>
<td>11</td>
<td>&gt;100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Jolgeh 4</td>
<td>4</td>
<td>&gt;100&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>Resistant&lt;sup&gt;a&lt;/sup&gt;</td>
<td>(7112×SB36)×SB33</td>
<td>25</td>
<td>0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22</td>
<td>3</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.1</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.2</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>100</td>
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<tr>
<td></td>
<td>33.HSF.3</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.4</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.5</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.6</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>100</td>
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<tr>
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<td>33.HSF.7</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>0</td>
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<td>100</td>
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<tr>
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<td>33.HSF.8</td>
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<td>1</td>
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<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.9</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>33.HSF.10</td>
<td>5</td>
<td>0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
<td>3</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

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

**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.HSF₁ 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.HSF₂, 33.HSF₃, 33.HSF₄, 33.HSF₅ 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 F1 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 F1 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 (Muchuro 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, Rc4 (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.

ACKNOWLEDGEMENTS

The authors thank of the scientific, research and technology BASIJ organization, Iranian National Science
Foundation (INSF), and Tarbiat Modares University (Iran) for financial support of this study.

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**طراحی نشانگر جنگ شکلی تک نوکلونیدی برای غربال مقاومت/حساسیت زنوتیپ های چندنژدی نسبت به نماد مولد گره ریشه**

م. باکویی، ا. پورجم، س. ب. محمودی، ن. صفاپور و م. نادرپور

چکیده

استفاده از نشانگرهای مولکولی برای تشخیص ارقام حساس و مقاوم در برنامه‌های اصلاحی انتخاب در جنگ‌نژدی با یک از اکثر مسیرهای محقق شده. با استفاده از آغازگرهای اختصاصی آلت (MAS) که با پاسخ‌های خاصی مانند محدود کردن عدد‌یابی (R6m-1) یا نشانگر مولکولی جنگ شکلی تک نوکلونیدی (A/G) پیش‌بیانه با یک از اکثر مقاومت، جهت تغییر زندگی‌های مقاوم طراحی شده است. تمایز زنوتیپ‌های مقاوم و حساس با استفاده از واکنش تکنیقالای پیلمرز و

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نیاز به فراهم کردن منابع فراوانی شکل‌بندی و انتخاب دو روش برای این منجر شد. نتایج حاصل از داده‌ها، مربوط به نشانگر شکل‌بندی تک نوکلوتیدی با نتایج آزمایش‌های ریس مناسب در گلخانه، برای 100 زنوتیب چندتراکم کامل همکاری دارد. این داده‌ها نشان می‌دهد که نشانگر حاضر امکان غربال گیری تر، حساب‌رسی ارزش‌های وسیع زنوتیب‌های چندتراکم را در برنامه‌های اصلاح برای مقاومت به نهایت مولد گره ریشه فراهم می‌کند.