DEVELOPMENT OF MOLECULAR MARKERS LINKED TO ME1 GENE CONFERRING RESISTANCE TO NEMATODE IN PEPPER

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ABSTRACT

DEVELOPMENT OF MOLECULAR MARKERS LINKED TO ME1 GENE CONFERRING RESISTANCE TO NEMATODE IN PEPPER

Pepper (*Capsicum annuum* L.) belongs to family Solanaceae. It is an agronomically important plant originating from Mexico. Pepper yield, quality and growth are limited by plant nematode parasitism. Root-knot nematodes (*Meloidogyne spp.*) are the most damaging sedentary endoparasites. *M. incognita* race 2 is the most common root-knot nematode found in Turkey. The Me1 gene which confers resistance to *M. incognita* was mapped in pepper to a 28 cM interval on chromosome 9. The aim of this study was to develop molecular markers linked to this nematode resistance gene. Phenotypic characterization of nematode resistance was performed for 200 F2 individuals from the cross between resistant pepper cultivar PM217 and Turkish susceptible cultivar AZN-1. In the F2 individuals, 151 plants (76%) were evaluated as resistant, 49 plants (24%) were susceptible. Markers were tested on parents of the mapping population to identify polymorphisms. A total of 30 standard markers and 548 new marker combinations were tested. Of these 578 markers, 75 (13%) were polymorphic. They were applied to F2 population and 28 (37%) showed clear segregation on F2 population. Eighteen of the markers (64%) segregated dominantly, 10 of the markers (36%) segregated codominantly. Markers located near Me1 on chromosome 9 were used for the construction of a linkage map. Out of three markers, SCAR_CD was the nearest marker to Me1 gene with a distance of 1.1 cM. These markers will provide selection at the genotypic level by marker-assisted selection, which will increase the efficiency and effectiveness of pepper breeding for nematode resistance.

ÖZET

BİBERDE NEMATODA DAYANIKLILIK SAĞLAYAN ME1 GENİNE BAĞINTILI MOLEKÜLER MARKÖRLERİN GELİŞTİRİLMESİ

Biber (Capsicum annuum L.) Solanaceae ailesine aittir. Meksika kökenli olup tarımsal olarak önemli bir bitkidir. Biberin verimi, kalitesi ve büyümesi bitki nematod parazitikliğiyle sınırlandırılmıştır. Kök-ur nematodları (*Meloidogyne spp.*) en zararlı yerleşik endoparazitlerdir. *M. incognita* ırk 2, Türkiye'de en yaygın olarak bulunan kök-ur nematodudur. *M. incognita*' ya dayanıklılık sağlayan Me1 geni, biberde kromozom 9 üzerinde 28 cM aralığında haritalanmıştır. Bu çalışmanın amacı, biberde nematoda dayanıklılık sağlayan Me1 gen'ine bağıntılı moleküler markörler geliştirmektir. PM217 dayanıklı biber çeşidi ve AZN-1 Türk duyarlı çeşidi arasındaki çaprazlamadan oluşan 200 F2 bireyleri için nematoda dayanıklılık fenotipik karakterizasyonu gerçekleştirildi. F2 bireyleri içerisinde 151 bitki (76%) dayanıklı olarak değerlendirildi, 49 bitki (24%) duyarlı olarak değerlendirildi. Markörler, haritalama populasyonunun ebeveynlerinde polimorfizmleri tanımlamak için denendi. Toplamda 30 standart markör ve 548 yeni markör kombinasyonları denendi. Bu 578 markörlerin, 75'i (13%) polimorfiktir. Bu markörler F2 populasyonuna uygulandı ve 28'i (37%) net ayrım gösterdi. Markörlerin 18'i (64%) dominant ayrıldı, 10'u (36%) kodominant ayrıldı. Kromozom 9 üzerinde Me1 yakınında bulunan markörler bağıntılı haritalama çiziminde kullanıldı. Üç markör den, SCAR_CD, 1.1 cM uzaklığıyla Me1 genine en yakın markördür. Nematoda dayanıklılık için biber ıslah verimliliğini ve etkinliğini artıracak olan bu markörler marköre dayalı seleksiyon ile genotipik düzeyde seçimi sağlayacaktır.

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CHAPTER 1

INTRODUCTION

1.1. Pepper (*Capsicum sp.*)

Peppers which are also called garden peppers (Capsicum) belong to the nightshade family of Solanaceae, along with tomatoes, potatoes, and eggplants. Peppers have significant roles in the economy, human diet and pharmaceutical industry. They have the highest vitamin C content among all plants. In addition to vitamin C they are rich in zinc, vitamin A, iron, calcium, magnesium, phosphorus, B-complex vitamins and potassium (Rohami et al. 2010; Masi et al. 2007). The fruit of most species of Capsicum have capsaicin which is a lipophilic chemical that can produce a burning sensation in the mouth. It protects the fruit from consumption by mammals while the bright colours attract birds that spread the seeds (Paran et al. 2007). Capsaicin is an excellent anticoagulant and helps lower the blood pressure as well as cholesterol. Peppers have roles in prevention of heart diseases, increasing blood flow and the neutralization of free radicals through their antioxidant features (Rohami et al. 2010).

All peppers come from the genus, Capsicum, which originated in the American tropics (Pickersgill 1997). They are divided into two categories called sweet and hot peppers. Their pungency is based on having a single gene: cultivars lacking the gene are sweet peppers, those with it are hot peppers. Sweet peppers expanded through regions such as Europe and North America however, hot peppers expanded through the American, African and Asian tropics (Pickersgill 1997). The Capsicum genus contains 30 species. Five of them were domesticated and have been cultivated for use as vegetables and spices for thousands of years (Paran et al. 2007). These domesticated species are *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens*, and *C. pubescens* (Rohami et al. 2010).

1.2. Biology of Pepper (*Capsicum annuum* L.)

C. annuum L. originates from Mexico (Masi et al. 2002). This species contains most of the Mexican chile peppers, hot peppers of Africa and Asia and sweet pepper cultivars grown in temperate countries (Pickersgill 1997). *C. annuum* is a self-pollinating angiosperm. This species and the other domesticated capsicums have a diploid chromosome number of 2n=24 (Rohami et al. 2010). The genome size in nucleotides of *C. annuum* is approximately 3,000 Mbp (Paran et al. 2007).

1.3. World Pepper Production with Emphasis on Turkey

In comparison with other vegetable crops, pepper is ranked third or fourth among all vegetable crops worldwide. According to the statistics of 2009, China is the largest producer of pepper in the world. It produced 14,520,301 tons of pepper and is followed by Mexico and Turkey which produced 1,941,560 and 1,837,000 tons, respectively (Figure 1.1).

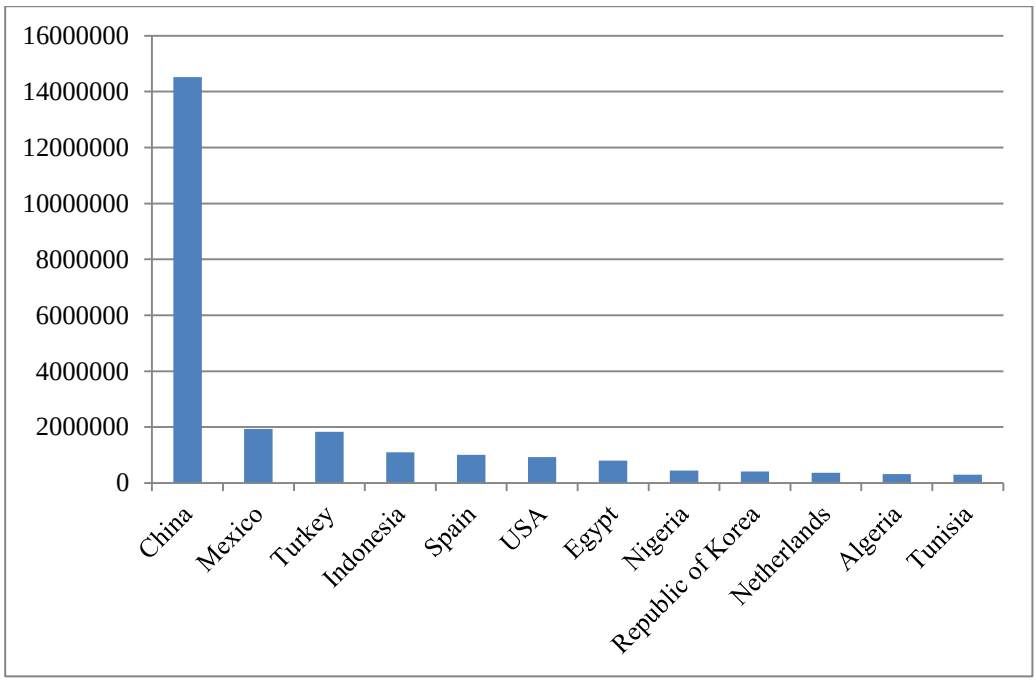

Figure 1.1. World production quantity of pepper.
(Source: FAOSTAT 2009)

1.4. Plant Parasitic Nematodes

The yield, quality and growth of plants are limited by many biotic and abiotic factors. Plant nematode parasitism is one of the most damaging uncontrollable biotic stresses on crops and causes billions of dollars of losses in agriculture each year (Williamson et al. 2003). Parasitism is seldom fatal for plants that are infected. The disruption of water transport and diversion of nutrients to the nematode cause stunted growth and chlorosis. These are the main reasons for poor yields.

Plant parasitic nematodes have small genomes of nearly 100 Mb (Williamson et al. 2006). According to their feeding strategy, they fall into two categories called ectoparasites and endoparasites (Fuller et al. 2008). Ectoparasites use their stylets for feeding. They insert them into epidermal cells so they do not enter the root. However endoparasites enter the root, feed and reproduce within the plant (Fuller et al. 2008). Sedentary endoparasites are considered to be the most sophisticated form of parasites. They form specialized feeding structures that help nematode growth and reproduction by inducing redifferentiation of root cells. Although both root-knot nematodes (*Meloidogyne spp.*) and cyst nematodes (*Heterodera* and *Globodera spp.*) are the most damaging obligate sedentary endoparasites, root-knot nematodes are the major causes of yield losses (Fuller et al. 2008).

1.5. Root-Knot Nematodes

Root-knot nematodes are polyphagous soil-living pests that exist in areas with hot climates or short winters (Williamson et al. 2006). They belong to the genus Meloidogyne (Dijan-Caporalino et al. 2007). This genus contains more than 60 species and some species have several races. *Meloidogyne* spp. can attack and damage more than 2,000 plant species (Sanchez-Puerta et al. 2011). Of more than 70 known species of *Meloidogyne*, only four of them (*M. javanica*, *M. arenaria*, *M. incognita*, *M. hapla*) are major pests worldwide (Sanchez-Puerta et al. 2011; Eisenbach et al. 1991). Among these species, *M. incognita* race 2 is the most common root-knot nematode found in Turkey (Cetintas et al. 2010).

1.6. Life Cycle of Root-Knot Nematodes (*Meloidogyne spp.*)

Root-knot nematodes (*Meloidogyne spp.*) need to form feeding structures to complete their life cycle (Figure 1.2). They start formation of feeding structures by invading a susceptible plant root (Fuller et al. 2008). Infective second stage (J2) larvae are attracted to susceptible plant roots and they usually penetrate roots closely behind the root tip. After invasion, larvae migrate intercellularly towards the root tip and enter the vascular cylinder. They start to feed on three to ten cells which are converted to multinucleated cells called giant cells. With the formation of giant cells, neighbouring cells start to divide and form a gall or root-knot. The J3 larval stage of males and females grow in this gall. The gall contines to swell as males and females are in stage J4. With the last moult, males change their shape, leave the root and fertilize the females. Females keep their eggs outside the root in a gelatinous matrix, then the J2 larvae hatch and are attracted to the roots. Root-knot nematodes complete this life cycle in one to two months depending on environmental conditions (Figure 1.2) (Fuller et al. 2008; Niebel et al. 1994).

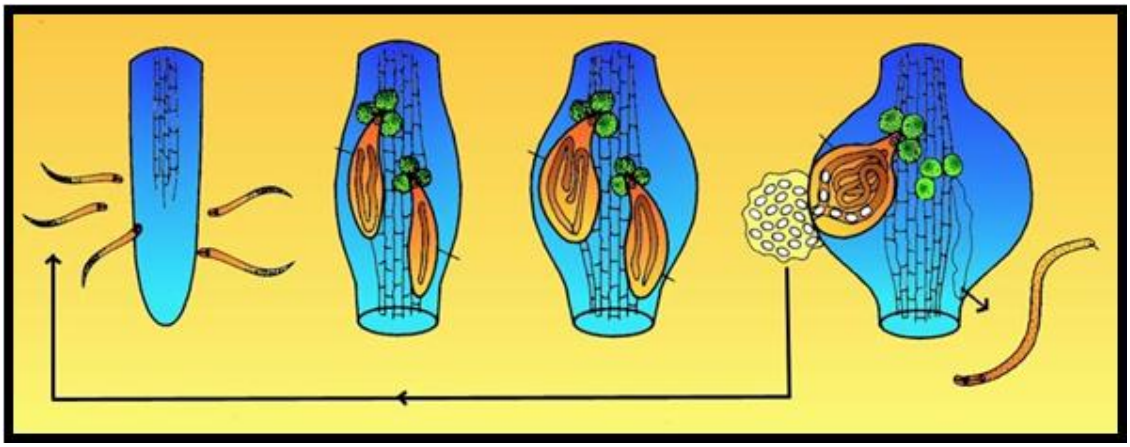

Figure 1.2. Life cycle of a root-knot nematode (*Meloidoyne spp.*).
(Source: Vermaercke et al. 1994)

1.7. Disease Management

Management of root-knot nematodes is very difficult because they are soil-borne pathogens with a wide range of hosts (Mitkowski et al. 2003). Chemical treatments, such as fumigants (1,3-dichloropropene, methyl bromide and dazomet) and nervous system toxins are commonly applied to control nematodes (Mitkowski et al. 2003).

Because of the toxic effect of these chemicals on humans and the environment, they are not preferred (Fuller et al. 2008). Soil solarization controls nematode disease agents in the soil by using solar power. Crop rotation prevents formation of pests and diseases in soil. Thus, rotation is used to limit nematode infestation. But these cultural controls have limited use against nematode species and are, therefore, impractical (Fuller et al. 2008). Biological control, using organisms antagonistic to nematodes such as fungi and bacteria, is another strategy to control nematodes. But developing biological control agents is expensive and this method is not preferred (Mitkowski et al. 2003).

Another strategy to control nematodes is integrated pest management (IPM) (Mitkowski et al. 2003). Complete use of natural resistant crop varieties, chemicals, and cultural and biological controls provides successful management of nematodes (Fuller et al. 2008). However this strategy is still difficult to use for root-knot nematodes (Mitkowski et al. 2003). Natural resistance in plants is the most convenient approach for controlling nematodes. This natural R-gene based approach provides plant improvement by using traditional breeding programmes (Fuller et al. 2008; Wang et al. 2009).

1.8. Natural Resistance Mechanism in Plants

Plants show resistance to nematodes by the expression of specific host genes and those genes prevent or limit nematode multiplication (Fuller et al. 2008). In the case of parasitism, a single dominant resistance gene (R gene) in the host plant interacts with a avirulence gene (Avr gene) in the nematode. This interaction is termed a 'gene-for-gene' interaction and initiates the defence response cascade resulting in resistance (Fuller et al. 2008; Delaney 2009). The pathogen avirulence effector can be detected by the *R* gene by direct or indirect interaction. When the interaction is indirect, the 'guard hypothesis' mechanism occurs. In this mechanism, a plant protein that is not encoded by the *R* gene is targeted by the pathogen *Avr* effector. The *R* protein acts as a guardian of this target and detects the changes in the protein. Thus the R gene initiates the defence response cascade . This cascade often results with the hypersensitive response (HR). The hypersensitive response prevents the spread of infection by rapid death of the cells in the infected region (Fuller et al. 2008). If there is no R gene, the plant target for the Avr effector isn't guarded and plants cannot initiate the defence cascade. A similar

situation occurs if the pathogen lacks the appropriate Avr gene. In such cases, disease results (Figure 1.3) (Delaney 2009).

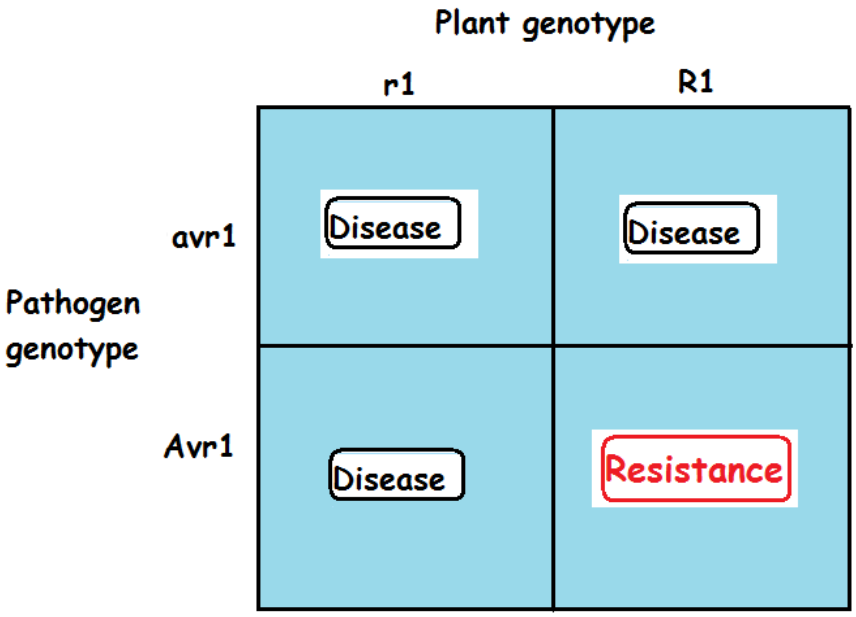

Figure 1.3. Gene-for-gene interaction.
(Source: Drawn from Delaney 2009).

1.9. Nematode Resistance Genes in Solanaceae

Wild plant species such as cotton, wild tomato, sweet potato and pepper show resistance to root-knot nematode *Meloidogyne spp*. Nematode resistance mechanisms including resistance genes (R genes) have evolved in these plant species (Sanchez-Puerta et al. 2011). All resistance genes have conserved elements and they are separated into classes. In plants most R genes include a nucleotide-binding (NB) region, a C-terminal leucine-rich repeat domain (LRR) and encode proteins termed NB-LRR proteins. For NB-LRR proteins, the NB region is the most conserved among R genes (Sanchez-Puerta et al. 2011; Williamson et al. 2006).

In *C. annuum*, resistance to *Meloidogyne spp.* is generated by nine independent resistance genes (N, Me1, Me2, Me3, Me4, Me5, Me7, Mech1 and Mech2). Five of these genes (Mech1, Mech2, Me1, Me3 and Me7) have been mapped (Wang et al. 2009; Djian-Caporalino et al. 2007). Some genes such as Me4, Me2, Mech1 and Mech2 are specific to certain *Meloidogyne spp.* or populations, however Me1, Me3, N and Me7 are effective against a wide range of *Meloidoyne spp.*, including *M. arenaria*, *M. javanica*,

and *M. incognita*. Comparative mapping indicated that the resistance genes are clustered in a 28 cM interval on chromosome P9 (Table 1.1) (Wang et al. 2009; Djian-Caporalino et al. 2007).

Table 1.1. Specific resistance genes against root-knot nematode types and their locations on chromosome P9 (Source: Wang et al. 2009; Djian-Caporalino et al. 2007).

Gene	**Root-Knot Nematode**	**Mapping status**
Me1	*M. incognita, M. arenaria, M. javanica*	Mapped, 27.1 cM of P9
Me2	Restricted resistance	Not mapped
Me3	*M. incognita, M. arenaria, M. javanica*	Mapped, 25.6 cM of P9
Me4	Restricted resistance	Not mapped
Me5	Broad spectrum	Not mapped
Me6	Broad spectrum	Not mapped
Me7	*M. incognita, M. arenaria, M. javanica*	Mapped, 13.5 cM of P9
Mech1	Restricted resistance	Mapped, 19.3 cM of P9
Mech2	Restricted resistance	Mapped, 8 cM of P9
N	*M. incognita, M. arenaria, M. javanica*	Not mapped

A 28 cM region of chromosome P9 of pepper shows colinearity with chromosome T12 of tomato and chromosome XII of potato (Figure 1.4) (Djian-Caporalino et al. 2007). Four nematode resistance genes, Gpa2 and MfaXII in potato, Mi3 and Mi5 in tomato have been identified in this region. Thus, comparative mapping shows that the nematode R-genes are located in orthologous genomic regions of pepper, tomato and potato (Wang et al. 2009; Djian-Caporalino et al. 2007).

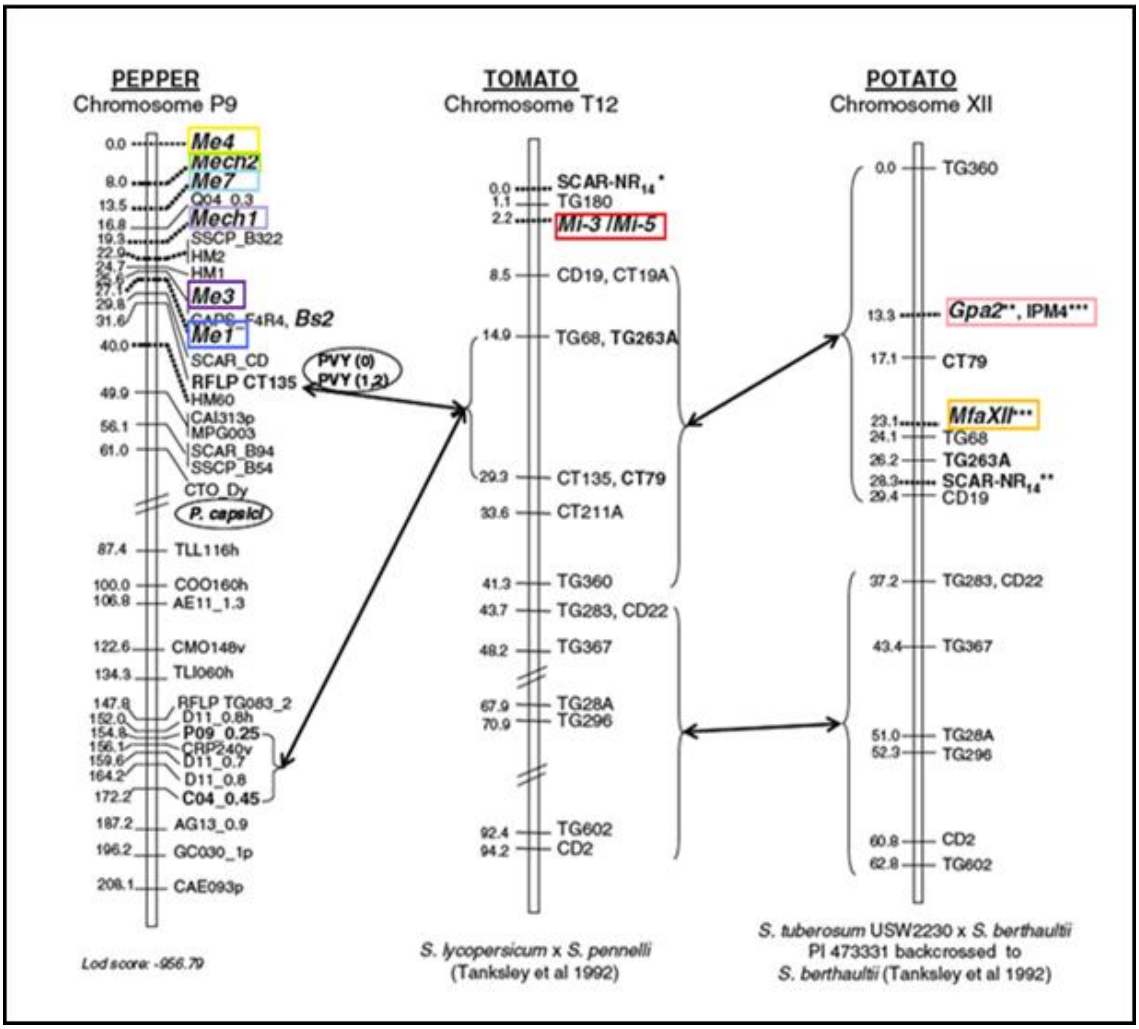

Figure 1.4. Comparative mapping of nematode-R loci of pepper, tomato and potato. (Source: Djian-Caporalino et al. 2007)

Among the Me genes, Me1 gene provides dominant resistance to *M. incognita* in *C. annuum.* Female development is inhibited by the development of defective giant cells by plants carrying Me1. Resistance breeding can be applied to control *M. incognita*. Development of PCR-specific markers linked to the Me1 gene will be useful for breeding cultivars resistant to *M. incognita* (Wang et al. 2009; Djian-Caporalino et al. 2007).

1.10. Genetic Markers

Genetic markers are genes or DNA sequences that have specific locations on chromosomes. Genetic markers are useful in genome analysis and can be easily identified. They associate with a specific locus and they are highly polymorphic.

Genetic markers can be used to develop genetic maps (Kumar 1999; Mohan et al. 1997). Genetic markers have two types: morphological and molecular markers (Kumar 1999).

1.10.1. Morphological Markers

Morphological markers are single genes which have effects on phenotype. There are limited numbers of morphological markers. Environment, epistatic interactions and pleitropic interactions change the expression of these markers (Kumar 1999). Interaction between alleles of these markers are in a dominant or recessive manner so the distinction between heterozygous individuals and homozygous individuals cannot be observed. Therefore, these markers often cannot show genotype (Kumar 1999; Jones et al. 2009).

1.10.2. Molecular Markers

Molecular markers fall into two categories called biochemical markers and DNA markers. Biochemical markers detect polymorphisms at the protein level and isozymes are the most commonly used biochemical markers. Isozymes are alternative forms of the same enzyme (Kumar 1999). DNA markers, also termed genic molecular markers (GMMs), detect polymorphism at the DNA level (Kumar 1999; Varshney et al. 2007). DNA markers can be divided into two groups depending on the detection of polymorphism: hybridization-based markers and PCR-based markers. Both types of markers can be co-dominant or dominant (Kumar 1999; Varshney et al. 2007; Mohan et al. 1997). DNA markers reveal neutral sites of variation at the DNA sequence level which can be detected by many molecular marker techniques including restriction fragment length polymorphism (RFLP), variable number tandem repeat (VNTR), amplified fragment length polymorphism (AFLP), simple sequence repeat (microsatellite) (SSR), inter simple sequence repeat (ISSR), cleavage amplification polymorphism (CAP), random amplified polymorphic DNA (RAPD), sequence-specific amplification polymorphism (S-SAP), sequence tagged sites (STS), sequence characterized amplification region (SCAR), sequence amplification of microsatellite polymorphic (SAMPL), single nucleotide polymorphism (SNP), sequence-related

amplified polymorphism (SRAP), and single copy orthologous genes (COSII) (Kumar 1999; Jones et al. 2009).

Molecular markers allow detection of the genomic structure of various organisms, genotypic changes such as insertions, mutations, deletions and even single nucleotide differences, genome organization and evolution (Jones et al. 2009). These markers are commonly used to track loci and genomic regions in many crop-breeding programmes as they can be tightly linked with a large number of agronomic and disease resistance traits that are found in crop species (Varshney et al. 2007). In this study we applied sequence-related amplified polymorphism (SRAP), simple sequence repeat (microsatellite or SSR), conserved ortholog set II (COSII) and sequence characterized amplification region (SCAR) markers which are described in more detail in the following sections.

1.10.2.1. Sequence-related Amplified Polymorphism (SRAP)

SRAP is a PCR-based molecular marker method that aims at amplification of open-reading frames (ORFs). It is based on a two primer amplification. The forward primer has 17 nucleotides and the reverse primer has 18 nucleotides (Li et al. 2001; Jones et al. 2009). This marker technique is thought be a more powerful technique compared with others because the forward primer's core sequence, CCGG, targets exogenic gene sequences while the reverse primer's core sequence, AATT, binds to the AT-rich sequences of noncoding sequences (Li et al. 2001; Jones et al. 2009).

The SRAP marker system is simple to use. SRAP markers provide large numbers of polymorphic fragments. These markers can be applied to different crops for a variety of purposes such as map construction, gene tagging, genomic and cDNA fingerprinting and map-based cloning. Mainly this marker system targets coding sequences in the genome and results in large numbers of dominant markers (Li et al. 2001; Jones et al. 2009).

1.10.2.2. Simple Sequence Repeat (Microsatellite or SSR)

Plant genomes have large numbers of simple sequence repeats which are repeats that are shorter than 6 bp and tandemly repeated. SSRs can be seen in the genome with

an estimated frequency that ranges from one SSR every 29 to 50 kb (Morgante et al. 1993). These repeats are mainly dinucleotides $(AC)n$, $(AG)_n$, $(AT)_n$; trinucleotides $(TCT)_n$ or tetranucleotides $(TATG)_n$ (Jones et al. 2009). In plant genomes the most common type of SSRs are $(AT)_n$ dinucleotides (Ma et al. 1996). SSRs detect polymorphism based on the number of repeated sequences of the two alleles at a locus (Jones et al. 2009).

SSR markers are useful in plant genetics and breeding because thay are reproducible and transferable to close species. They are multiallelic and codominant. Also they are convenient for marker-assisted selection in many crop species (Varshney et al. 2005; Jones et al. 2009).

1.10.2.3. Conserved Ortholog Set II Markers (COSII)

COSII markers are PCR-based markers that are adapted from a set of single copy conserved orthologous genes (COSII genes) in Asterid species. Each COSII gene matches only one single copy Arabidopsis gene (Fulton et al. 2002).

These COSII genes are useful for detecting synteny between the Solanaceae and Arabidopsis to construct phylogenies and to study genome evolution and genome organization of Solanaceae. Genetic and genomic information can be shared between species in the nightshade family (e.g tomato, pepper and potato). COSII markers are accessible and they allow detection of single copy orthologous genes in a wide array of plant species (Fulton et al. 2002; Wu et al. 2009).

1.10.2.4. Sequence Characterized Amplification Region (SCAR)

SCAR markers are converted RAPD (random amplified polymorphic DNA) markers (Jones et al. 2009; Mohan et al. 1997). The two ends of the RAPD genomic DNA clone are sequenced and oligonucleotides are designed based on the end sequences to develop SCAR markers (Mohan et al. 1997). Primers can be used directly on genomic DNA in a PCR reaction for the amplification of polymorphic regions. SCAR markers are more reproducible than RAPDs however they are more difficult to develop than RAPDs. SCAR markers can be dominant segregation like the original

RAPD or may be converted into codominant markers (Jones et al. 2009; Mohan et al. 1997).

1.11. High Resolution Melting Analysis (HRM)

High resolution melting (HRM) is powerful technique that can detect mutations, polymorphisms and epigenetic differences in double stranded DNA samples. HRM has high sensitivity to single nucleotide polymorphisms (SNPs). At first the DNA region with the mutation of interest is amplified by PCR technique. This amplified region is termed an 'amplicon'. Then HRM analysis begins with heating of the amplicon from nearly 50˚C to nearly 95˚C. When the melting temperature of the amplicon is reached, the two strands of DNA separate (Vossen et al. 2009; Wojdacz et al. 2007). In the HRM technique a fluorescent dye which has the ability to bind double stranded DNA is used. At the begining the fluorescence is high because there are many double stranded amplicons. As the sample is warmed and the two strands of DNA separate, fluorescence is reduced. HRM analysis depends on measurement of this fluorescence. A melt curve is formed depending on the fluorescence of the reaction mixture (Figure 1.5) (Vossen et al. 2009; Wojdacz et al. 2007).

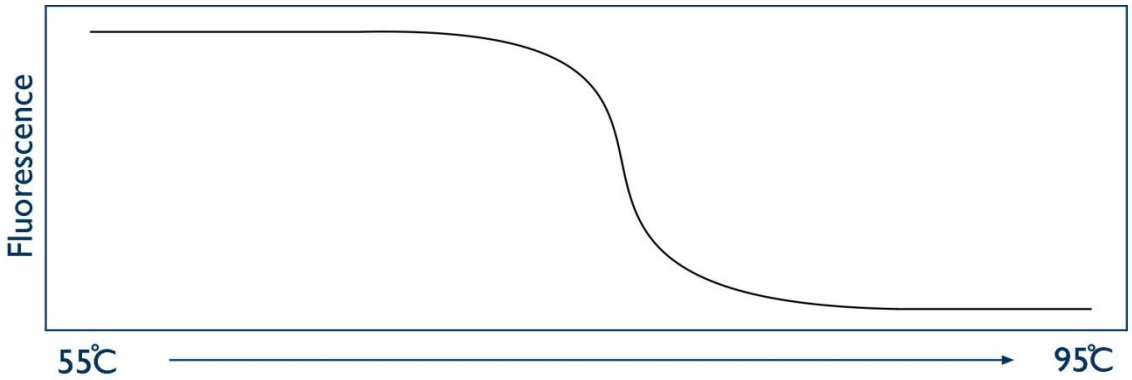

Figure 1.5. Melt curve depending on fluorescence.
(Source: Qiagen handbook for HRM beginners)

DNA with mutations can be easily detected with HRM because of its high resolution. In the case of a diploid organism with a mutation, there are three amplification possibilities: two alleles without mutation (homozygous wild type), one mutant allele and one wild type allele (a 'heterozygote'), or both alleles with the

mutation (homozygous mutant type). With high qulity HRM analysis, each genotype will show a different melt curve (Figure 1.6) (Vossen et al. 2009; Wojdacz et al. 2007).

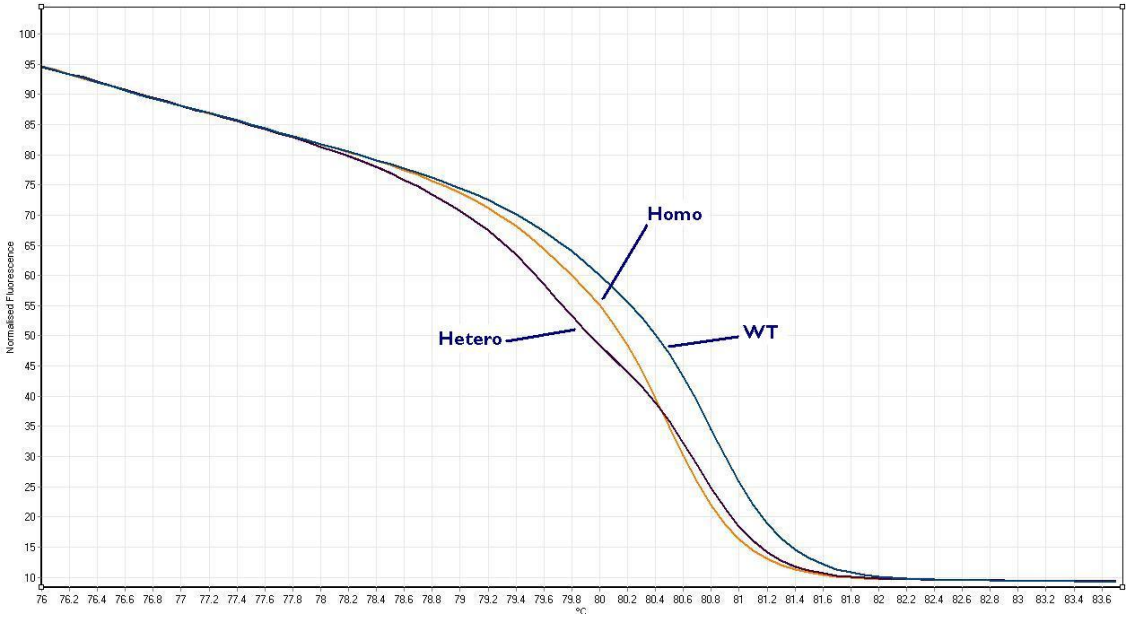

Figure 1.6. Different types of melt curve.
(Source: Qiagen handbook for HRM beginners)

HRM is easy to apply, flexible, low cost and sensitive. Also it is nondestructive and specific. For these reasons, this technique has become preferable for genotyping applications (Vossen et al. 2009).

1.12. Bulked Segregant Analysis (BSA)

Bulked segregant analysis (BSA) is a rapid mapping technique that is convenient for monogenic qualitative traits (Wu et al. 2006). It is generally used for identifying molecular markers linked to a trait of interest. It provides genotyping of two pools (bulks) of DNA samples from individual plants which are assigned to one of the two bulks based on their trait phenotype. The bulks are screened with a large number of markers to identify those that distinguish the bulks. When DNA of ten resistant plants is bulked into one pool, all alleles should be present. Two bulked pools of segregants that differ for one trait will differ only at the locus having that trait (Wenzl et al. 2007). In this way, mapping can be performed more efficiently and quickly.

1.13. Aim of the Study

Pepper (*C. annuum*) has economic, agricultural and pharmaceutical importance in Turkey and throughout the world. The most commonly found root-knot nematode, *Meloidogyne incognita*, has negative effects on pepper production. However the Me1 gene which confers resistance to *M. incognita* was mapped and it is known that this gene is found in a 28cM interval on pepper chromosome 9. The aim of this study was to develop molecular markers which are linked to the nematode resistance gene, Me1, in pepper. For this goal, we phenotyped and genotyped an F2 population derived from the cross between *C. annuum* cv. PM217 and *C. annuum* cv. AZN-1. AZN-1 is a Turkish susceptible cultivar while *C. annuum* cv. PM217 is a resistant cultivar. We applied HRM analysis and used COSII, SRAP, SSR and SCAR markers to saturate the 28cM interval. These markers will provide selection at the genotypic level by marker-assisted selection, which will increase the efficiency and effectiveness of pepper breeding in various ways.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

C. annuum inbred line PM217 (derived from PI 201234) is highly resistant to *M. incognita* (Dijan-Caporalino et al. 2007). *C. annuum* cv. PM217 was crossed with Turkish susceptible cultivar *C. annuum* cv. AZN-1 to generate F1 hybrids. F1 hybrids were self-pollinated to generate a F2 segregating population and 200 individuals from this F2 population were used for nematode tests and genomic studies. Plants were grown in growth chambers at Multi Tarım, Antalya at 24°C during the day, 22 °C at night with nearly 65% humidity.

2.2. Methods

2.2.1 Inoculation with Root-knot Nematode and Evaluation of Disease

The susceptible tomato variety Tueza F1 was inoculated with second stage juveniles (J2) of *M. incognita* (race 2) in the growth chamber at Multi Tarım, Antalya for multiplication of *M. incognita* eggs. Eight weeks after inoculation, *M. incognita* eggs were collected from tomato roots. At the four leaf stage each parent and F2 individuals of the *C. annuum* population were inoculated with 1000 second stage juveniles of *M. incognita* (race 2) in 250 ml pots. The plants were grown in a growth chamber at 24°C during the day, and 22°C at night with 65% humidity. For nematode tests, two isolates (G3, D5) were used. A total of 100 F2 individuals were tested with isolate G3 and another 100 individuals were tested with D5. Parents were tested with both isolates with eight replicates. Eight weeks after treatments, egg masses (EM) and rate of gall formation were calculated. Root systems were rated according to number of egg masses and gall formations. According to egg masses, plant roots which had fewer

than 20 or 20 egg masses were considered resistant and those with more than 20 egg masses were considered susceptible. According to gall formation, roots with two or less than two galls were considered resistant and roots with three to ten galls were considered susceptible.

2.2.2. DNA Extraction

For molecular marker analysis, DNA extraction was performed from fresh leaf tissues of *C. annuum* parents and individuals using the Promega CTAB genomic DNA isolation kit according to manufacturer's instructions. Quantification of DNA was performed with a Nanodrop ND-1000 spectrophotometer and DNA samples were stored at -20 °C in TE buffer.

2.2.3. Molecular Marker Analysis

2.2.3.1. BSA Analysis

Two bulks were constructed, each contained DNA from 10 resistant or 10 susceptible individual plants. First, for the identification of an adequate number of polymorphic markers, surveys were carried out on the two parents *C. annuum* cv. PM217, *C. annuum* cv. AZN-1 and bulks.

2.2.3.2. COSII Analysis

COSII markers C2-At5g06130, C2-At3g09925, C2-At5g58410, C2-At2g37240, C2-At2g29210, C2-At3g09920 are in a 40 cM region on chromosome P9 (Figure 2.1). To find polymorphism, these COSII primers were used to amplify parental DNA which was then digested with 66 enzymes (all except for C2-At5g58410 as only Alu and Taq1 enzymes were used for that marker). In addition to the COSII markers, the CAPS_F4 and R4 primers were used and digested with Tru1l enzyme.

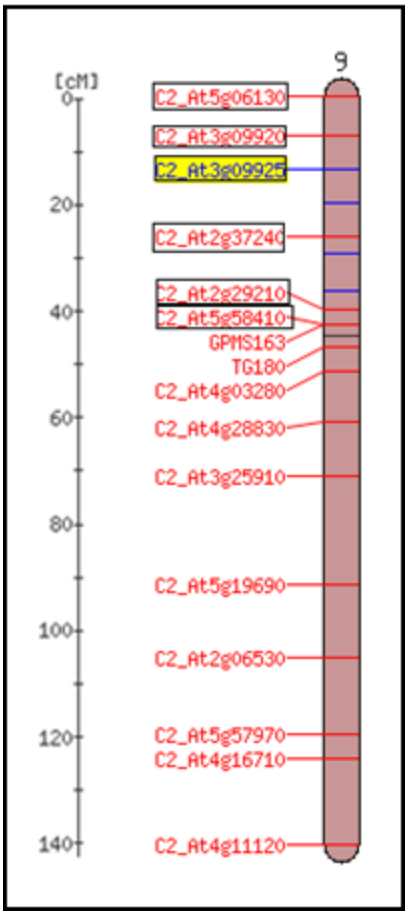

Figure 2.1. CosII markers on chromosome P9.
(Source: Drawn from SolGenomics Network)

DNA amplification was carried out in a 25 µL reaction mixture containing 2.5 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 0.5 µl dNTP (0.2 mM), 0.5 µl forward and 0.5 µl reverse primers (10 pmol), 0.25 µl Taq polymerase (0.25 U), 18.75 µl sterile distilled water, and 2 µl DNA (~55 ng/µl). Samples were amplified in a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems; Authorized Thermal Cycler, Mastercyler epgradientS, Eppendorf; C1000 Thermal Cycler™, BIO-RAD) using the PCR program: one step of 3 min at 94°C, 35 cycles with 30 sec at 94°C, 45 seconds at 55 °C (50°C for CAPS_F4R4) annealing temperature, 45 seconds at 72°C and a final extension step of 10 min at 72°C.

After PCR amplification, samples were digested using different restriction enzymes. The enzyme digestion mixture contained 25 µl PCR product plus 3 µl 10X digestion buffer, 0.5 µl enzyme (10 u/µl) and 1.5 µl sterile distilled water. Samples were incubated at the appropriate temperature for the enzyme for at least 3 hours. After incubation the samples were loaded on 2-3% agarose gels in 1X TAE buffer (0,25 M Tris base, 12,75 M EDTA adjusted to 1 L with distilled water and pH: 8.3 with acetic

acid). Samples were run at 110 V for at least 2 hours. Staining the gels with ethidium bromide allowed the identification of marker bands under UV light. Polymorphic markers were selected and then applied to the whole population.

2.2.3.3. SRAP Analysis

For the SRAP markers 14 forward (Me) and 17 reverse primers (Em) were used in this study (Table 2.1).

Table 2.1. Forward and reverse SRAP primers

Forward Primers	**Reverse Primers**
Me1	Em1
Me2	Em2
Me3	Em3
Me4	Em4
Me5	Em5
Me6	Em6
Me7	Em7
Me8	Em8
Me9	Em9
Me10	Em10
Me11	Em11
Me12	Em12
Me13	Em13
Me14	Em14
	Em15
	Em16
	Em17

At first, SRAP markers were combined with 5 COSII markers. In this way, 310 combinations were done. DNA amplification was carried out in 20 µL reaction mixture containing 2 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 2 µl $MgCl_2$, 0.7 µl dNTP (0.2 mM), 2 µl SRAP and 1 µl COSII primers (10 pmol) 0.3 µl Taq polymerase (0.25 U), 10.5 µl sterile distilled water, and 1.5 µl DNA (~55 ng/µl). Furthermore, SRAP markers were combined with each other resulting in 238 combinations. DNA amplification was carried out in 20 µL reaction mixture containing 2 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 2 µl

$MgCl_2$, 0.7 µl dNTP (0.2 mM), 2 µl forward and 2 µl reverse primers (10 pmol), 0.3 µl Taq polymerase (0.25 U), 9.5 µl sterile distilled water, and 1.5 µl DNA (~55 ng/µl).

Samples were amplified in a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems; Authorized Thermal Cycler, Mastercyler epgradientS, Eppendorf; C1000 Thermal Cycler™, BIO-RAD) using the PCR program: one step of 5 min at 94°C, 5 cycles were performed with 1 min at 94°C, 1 min at 35 °C as annealing temperature, 1 min at 72°C, 35 cycles with 1 min at 94°C, 1 min at 55 °C as annealing temperature, 1 min at 72°C and a final extension step of 10 min at 72°C. PCR products were separated on 3% agarose gels in 1X TAE buffer. They were stained with ethidium bromide and visualized under UV light. Polymorphic markers were selected and then applied to the whole population.

2.2.3.4. SSR Analysis

Thirteen SSR primers (listed in Table 2.2) were used. DNA amplification was carried out in 25 µL reaction mixture containing 2.5 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 2 µl $MgCl_2$, 0.5 µl dNTP (0.2 mM), 1 µl forward and 1 µl reverse primers (10 pmol), 0.25 µl Taq polymerase (0.25 U), 15.75 µl sterile distilled water, and 2 µl DNA (~55 ng/µl).

Table 2.2. SSR primers and melting temperatures (Tm).

SSR Primers	**Tm**	**SSR Primers**	**Tm**
HPMS 1-3	65°C	HPMS E117	63°C
HPMS 2-41	65°C	HPMS E025	63°C
HPMS 1-117	50°C	GPMS 171	50°C
HPMS E102	63°C	GPMS 163	59°C
HPMS E098	63°C	SSCP_B322	53°C
HPMS E082	64°C	SSCP_B54	45°C
HPMS E007	63°C		

Samples were amplified in a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems; Authorized Thermal Cycler, Mastercyler epgradientS, Eppendorf;

C1000 Thermal Cycler™, BIO-RAD) using the PCR program: one step of 5 min (3 min for SSCP primers) at 94°C, 35 cycles with 30 sec at 94°C, 30 seconds at the appropriate annealing temperature as given in Table 2.2, 1 min at 72°C and a final extension step of 5 min at 72°C. PCR products were separated on 3% agarose gels in 1X TAE buffer. They were stained with ethidium bromide and visualized under UV light. Polymorphic markers were selected and then applied to the whole population.

2.2.3.5. SCAR Analysis

The ten SCAR markers listed in Table 2.3 were used (Tai et al. 1999; Dijan-Caporalino et al. 2007). DNA amplification was carried out in 25 µL reaction mixture containing 2.5 µl 10X PCR buffer (50 mM KCl, 10 mM Tris-HCl, 1.5 mM MgCl2, pH: 8.3), 2 µl $MgCl_2$, 0.5 µl dNTP (0.2 mM), 1 µl forward and 1 µl reverse primers (10 pmol), 0.25 µl Taq polymerase (0.25 U), 15.75 µl sterile distilled water, and 2 µl DNA (~55 ng/µl).

Table 2.3. SCAR primers and melting temperatures (Tm).

SCAR Primers	**Tm**
SCAR A2	60°C
SCAR S2	55.5°C (F), 53°C (R)
SCAR E1	53°C (F), 55°C (R)
SCAR F1	58°C (F), 63°C (R)
SCAR G1	65°C (F), 52°C (R)
SCAR B3	52°C (F), 51°C (R)
SCAR S19	58°C
SCAR S45	59°C (F), 57°C (R)
SCAR_CD	59.1°C
SCAR_B94	53°C

Samples were amplified in a thermocycler (GeneAmp® PCR System 9700, Applied Biosystems; Authorized Thermal Cycler, Mastercyler epgradientS, Eppendorf; C1000 Thermal Cycler™, BIO-RAD) using the PCR program: one step of 5 min at 94°C, 35 cycles with 30 sec at 94°C, 30 seconds depending on each primers melting temperature, 1 min at 72°C and a final extension step of 5 min at 72°C. PCR products

were separated on 3% agarose gels in 1X TAE buffer. Gels were stained and visualized under UV light. Polymorphic markers were selected and then applied to whole population.

2.2.3.6. HRM Analysis

HRM analysis was conducted with primers HPMS E007, HPMS E082, HPMS E098, HPMS E102, HPMS E117, HPMS 2-41, HPMS 1-3, HPMS 1-117, SSCP_B322, SSCP_B54, SCARB94 and GPMS 163. DNA amplification was carried out in 25 µL reaction mixture including Qiagen Type-it HRM PCR Kit, 12.5 µl 1 x 1.3 ml of 2x HRM PCR Master Mix (contains HotStarTaq Plus DNA Polymerase, EvaGreen dye, optimized concentration of Q-solution, dNTPs, and $MgCl_2$), 8 µl RNase-free water, 1.75 µl forward and 1.75 µl reverse primers and 1 µl (~ 10-50 ng/µl) DNA.

Samples were amplified in Qiagen Rotor-Gene Q using the programme: one step of 5 min at 95°C, 40 cycles with 10 sec at 95°C, 30 seconds depending on each primers melting temperature and final extension step of 10 seconds at 72°C. For HRM analysis, annealing temperature was ramped from 65°C to 95°C, rising by 0.1°C each step.

2.2.3.7. Data Analysis

Segregation data were analyzed with a Chi-square goodness of fit test. The MAPMAKER V3 computer program (Lander et al. 1987) was used for linkage analysis of molecular markers. A minimum LOD score of 3.0 was used. Maximum likelihood method was used to estimate the recombination frequencies and their standard errors. The Kosambi's mapping function (Kosambi 1944) was used to estimate distances between markers in centiMorgans (cM).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Phenotypic Characterization of Nematode Resistance

Phenotypic characterization of nematode resistance was performed on the F2 population using two different isolates of *M. incognita*. For isolate D5, 78% of individuals were evaluated as resistant according to their gall formation (that is having only 0 to 2 galls). Thus, 22% of individuals were evaluated as susceptible according to their gall formation (that is having more than 2 galls) (Figure 3.1).

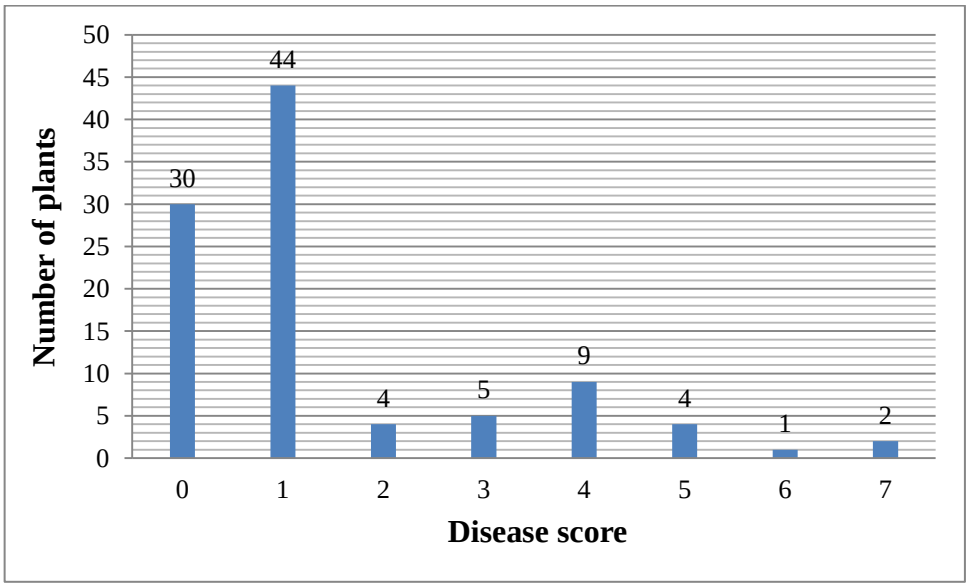

Figure 3.1. Distribution of disease (resistance) score in F2 plants treated with D5 isolate of *M. incognita*.

For G3 isolate application, 73% of individuals were evaluated as resistant while 27% of individuals were evaluated as susceptible according to their gall formation (Figure 3.2). The results with both isolates indicated that the resistance gene is inherited dominantly with the classical Mendelian segregation ratio of 3:1. This segregation was confirmed by a Chi-square goodness of fit test (p= 0.49 and 0.65 for D5 and G3, respectively).

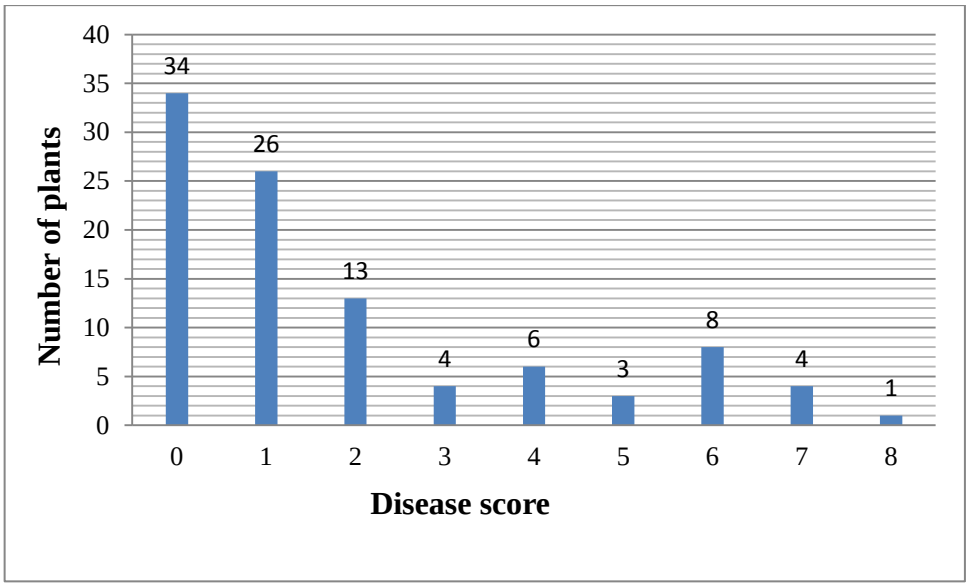

Figure 3.2. Distribution of disease (resistance) score in F2 plants treated with G3 isolate of *M. incognita*.

3.2. COSII Analysis

COSII markers C2-At5g06130, C2-At3g09925, C2-At5g58410, C2-At2g37240, C2-At2g29210, C2-At3g09920 were used to amplify parental DNA. Amplification products were then digested with 66 enzymes (all except for C2-At5g58410 as only Alu and Taq1 enzymes were used for that marker) but polymorphism was not detected. Enzymes which were used in the survey are listed in Table 3.1. Tru1l digestion of CAPS_F4 and R4 primers showed polymorphism and this marker-enzyme combination was applied to the F2 population. Susceptible and resistant alleles of individuals were evaluated according to the parents' banding patterns. As can be seen in Figure 3.3, individuals which showed the same pattern as the susceptible parent were labeled as S while individuals that showed the same banding pattern as the resistant parent were labeled as R. This primer pair was used for the construction of the linkage map.

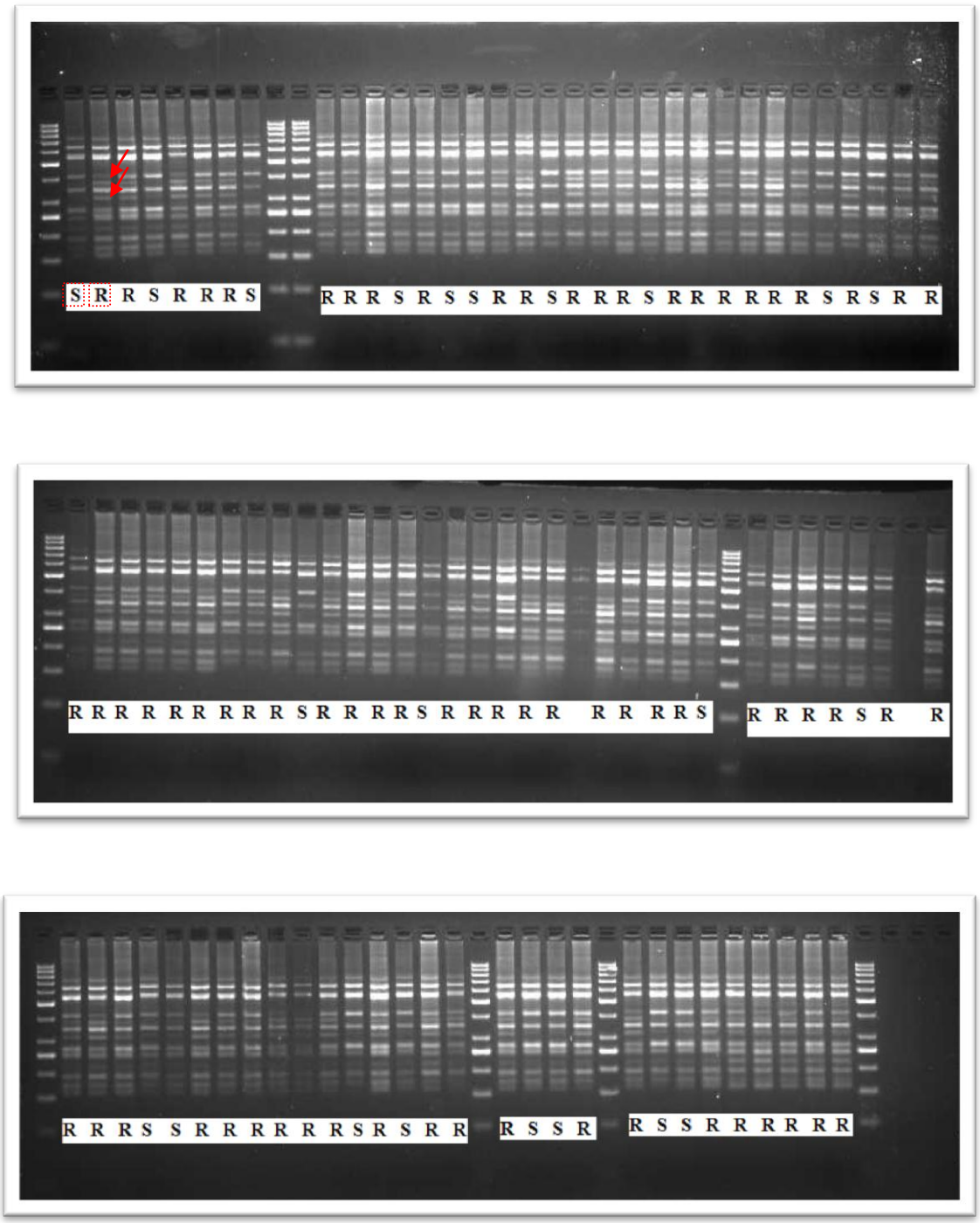

Figure 3.3. Tru1l digestion of CAPS_F4 and R4 on F2 population. Susceptible paren(S) and resistant parent (R) are indicated by a red rectangle, polymorphic bands are shown with arrows and F2 individuals were labeled according to parents' banding patterns.

Table 3.1. List of restriction enzymes which were used in survey.

ENZYMES	ENZYMES	ENZYMES
AluI	DpnI	MvaI
Alw21I	DpnII	NcoI
ApaI	DraI	NdeI
ApoI	Eco130I(StyI)	NmuCI(Tsp45I)
BamHI	Eco47III(AfeI)	NsiI
BanI FD	Eco47I(AvaII)	PdmI(XmnI)
Bcl I	Eco32I(EcoRV)	PstI
BcuI	Eco24I(BanII)	PvuII
BgI II	Eco24I(HgIJ II)	RsaI
Bme1390 I (ScrFI)	EcoRI	SacI
BseG I	HhaI	ScaI
BseDI(BsaJI)	Hin6I	SspI
Bsh1236I(BstUI)	Hin1II(NIaIII)	TaaI(HpyCH4III)
BseLI (BsiYI)	HincII(HindII)	TaiI
BsuRI(HaeIII)	HindIII	TaqI
Bsp119I(BstBI)	HinfI	TasI(TspEI)
BspTI(AfIII)	HpyF3I(DdeI)	TscAI(TspRI)
Bsp143I(Sau3AI)	KpnI	Tru1I(MseI)
BoxI (PshAI)	MboI	VspI(AseI)
Csp6I(CviQI)	MspI(HpaII)	XbaI
CfoI	Mph1103I(NsiI)	XapI(ApoI)
Cfr13I(Sau96I)	Mph1103I(AvaIII)	XmiI(AccI)

3.3. SRAP Analysis

Combinations of 14 forward and 17 reverse SRAP primers comprising a total of 238 combinations, were applied to parents and bulks of susceptible and resistance individuals. However polymorphism was not detected. Also SRAP primers were combined with 5 COSII markers on parents and bulks, in this way 64 primer new combinations (21%) were found to be polymorphic. These polymorphic primer combinations are listed in Table 3.2. These polymorphic primers were applied to the F2 population and primers which showed clear segregation on the F2 population were selected for construction of the linkage map (Table 3.3). The combination of C2-At2g29210 (R) and Em14 is a good example of a marker which showed clear segregation in the F2 population (Figure 3.4). Markers which gave unclear banding patterns were not used for mapping as the results would be unreliable.

Table 3.2. List of polymorphic COSII-SRAP primers combinations.

COS primers	**SRAP primers**	**COS primers**	**SRAP primers**
C2-At5g06130 (F)	Em1	C2-At2g29210 (F)	Em4
C2-At5g06130 (F)	Em3	C2-At2g29210 (F)	Em7
C2-At5g06130 (F)	Em5	C2-At2g29210 (F)	Me11
C2-At5g06130 (F)	Me8	C2-At2g29210 (F)	Me14
C2-At5g06130 (F)	Me10	C2-At2g29210 (R)	Em3
C2-At5g06130 (F)	Me3	C2-At2g29210 (R)	Em12
C2-At5g06130 (F)	Me6	C2-At2g29210 (R)	Em14
C2-At5g06130 (F)	Me12	C2-At2g29210 (R)	Me12
C2-At5g06130 (R)	Me5	C2-At3g09920 (F)	Me2
C2-At5g06130 (R)	Me9	C2-At3g09920 (F)	Me4
C2-At5g06130 (R)	Me10	C2-At3g09920 (F)	Me9
C2-At5g06130 (R)	Em10	C2-At3g09920 (F)	Me11
C2-At3g09925 (F)	Me2	C2-At3g09920 (F)	Me13
C2-At3g09925 (F)	Me5	C2-At3g09920 (F)	Em2
C2-At3g09925 (F)	Me4	C2-At3g09920 (F)	Em3
C2-At3g09925 (F)	Me7	C2-At3g09920 (F)	Em5
C2-At3g09925 (F)	Me10	C2-At3g09920 (F)	Em11
C2-At3g09925 (F)	Em2	C2-At3g09920 (F)	Em12
C2-At3g09925 (F)	Em5	C2-At3g09920 (F)	Em13
C2-At3g09925 (F)	Em8	C2-At3g09920 (R)	Me1
C2-At3g09925 (F)	Em10	C2-At3g09920 (R)	Me4
C2-At3g09925 (F)	Em11	C2-At3g09920 (R)	Me10
C2-At3g09925 (F)	Em14	C2-At3g09920 (R)	Me11
C2-At3g09925 (R)	Em3	C2-At3g09920 (R)	Me13
C2-At2g37240 (F)	Me3	C2-At3g09920 (R)	Em1
C2-At2g37240 (F)	Me4	C2-At3g09920 (R)	Em2
C2-At2g37240 (F)	Me13	C2-At3g09920 (R)	Em4
C2-At2g37240 (F)	Em1	C2-At3g09920 (R)	Em5
C2-At2g37240 (F)	Em3	C2-At3g09920 (R)	Em10
C2-At2g37240 (F)	Em11	C2-At3g09920 (R)	Em14
C2-At2g37240 (R)	Me4	C2-At3g09920 (R)	Em15
C2-At2g37240 (R)	Em17	C2-At3g09920 (R)	Em17

Table 3.3. COSII-SRAP primers which were used for construction of linkage map.

COSII primers	SRAP primers	COSII Primers	SRAP primers
C2-At5g06130 (F)	Em3	C2-At3g09925 (F)	Me10
C2-At5g06130 (F)	Em1	C2-At3g09925 (F)	Me5
C2-At5g06130 (F)	Me3	C2-At3g09925 (F)	Me4
C2-At5g06130 (F)	Me12	C2-At2g37240 (F)	Me4
C2-At5g06130 (R)	Me5	C2-At2g37240 (R)	Me4
C2-At5g06130 (R)	Me10	C2-At2g29210 (F)	Em7
C2-At3g09925 (F)	Em10	C2-At2g29210 (R)	Em7
C2-At3g09925 (F)	Em8	C2-At2g29210 (R)	Em14
C2-At3g09925 (F)	Em11	C2-At2g29210 (R)	Me12

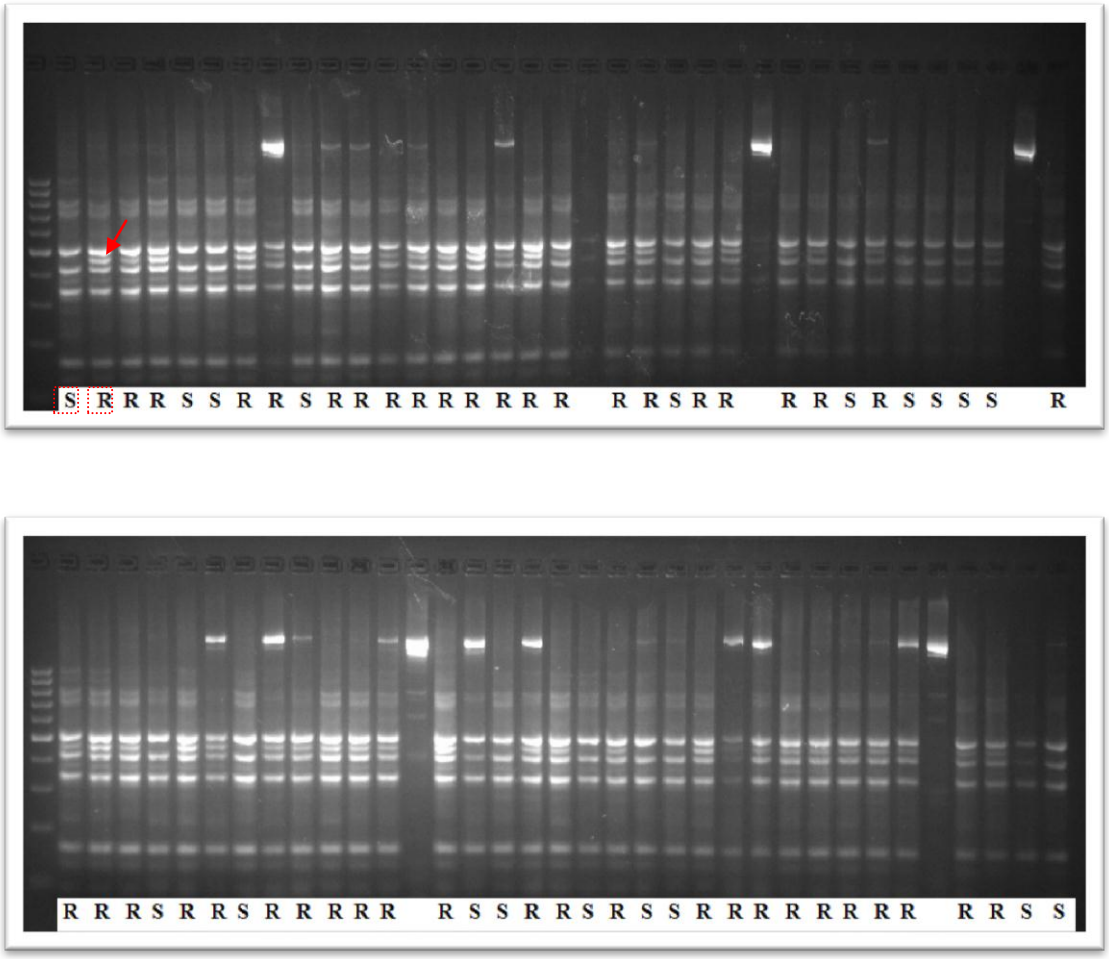

Figure 3.4. Combination of C2-At2g29210 (R) and Em14 on F2 population. Susceptible parent (S) and resistant parent (R) are indicated by a red rectangle, polymorphic bands are shown with arrows and F2 individuals were labeled according to parents' banding patterns.

(cont. on next page)

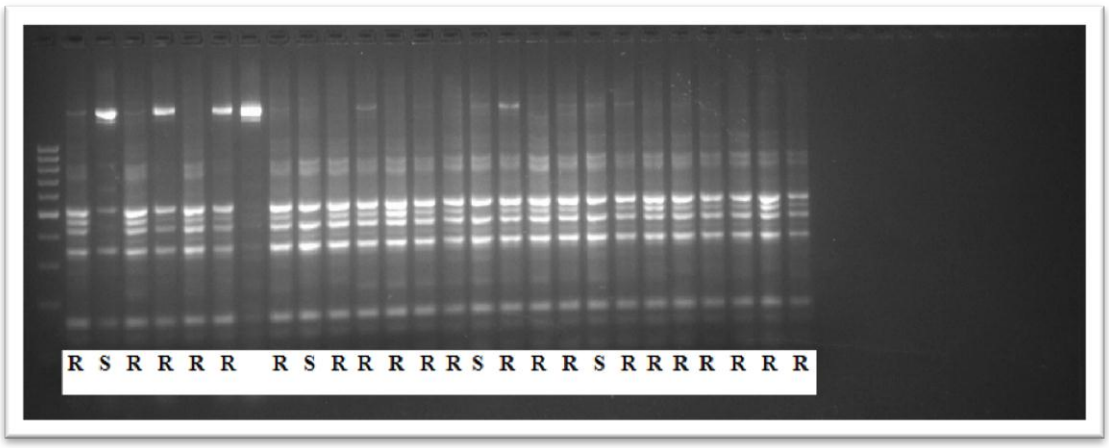

Figure 3.4. (cont.)

3.4. SSR Analysis

Thirteen SSR primers: HPMS 1-3, HPMS 2-41, HPMS 1-117, HPMS E102, HPMS E098, HPMS E082, HPMS E007, HPMS E117, HPMS E025, GPMS 171, GPMS 163, SSCP_B322, and SSCP_B54 were applied to parents and bulks to detect polymorphism. Among these primers only one (8%), GPMS 171, showed clear segregation and it was applied to the F2 population. Differences between homozygous individuals were clearly observed and some individuals showed heterozygosity. Individuals that showed both resistant and susceptible parents' banding patterns were labeled as heterozygous (H). This primer, GPMS 171, was used for the construction of the linkage map (Figure 3.5). Other primers also showed segregation on gels but were not as clear as GPMS 171. For this reason, HRM analysis was carried out with those primers (see section 3.6).

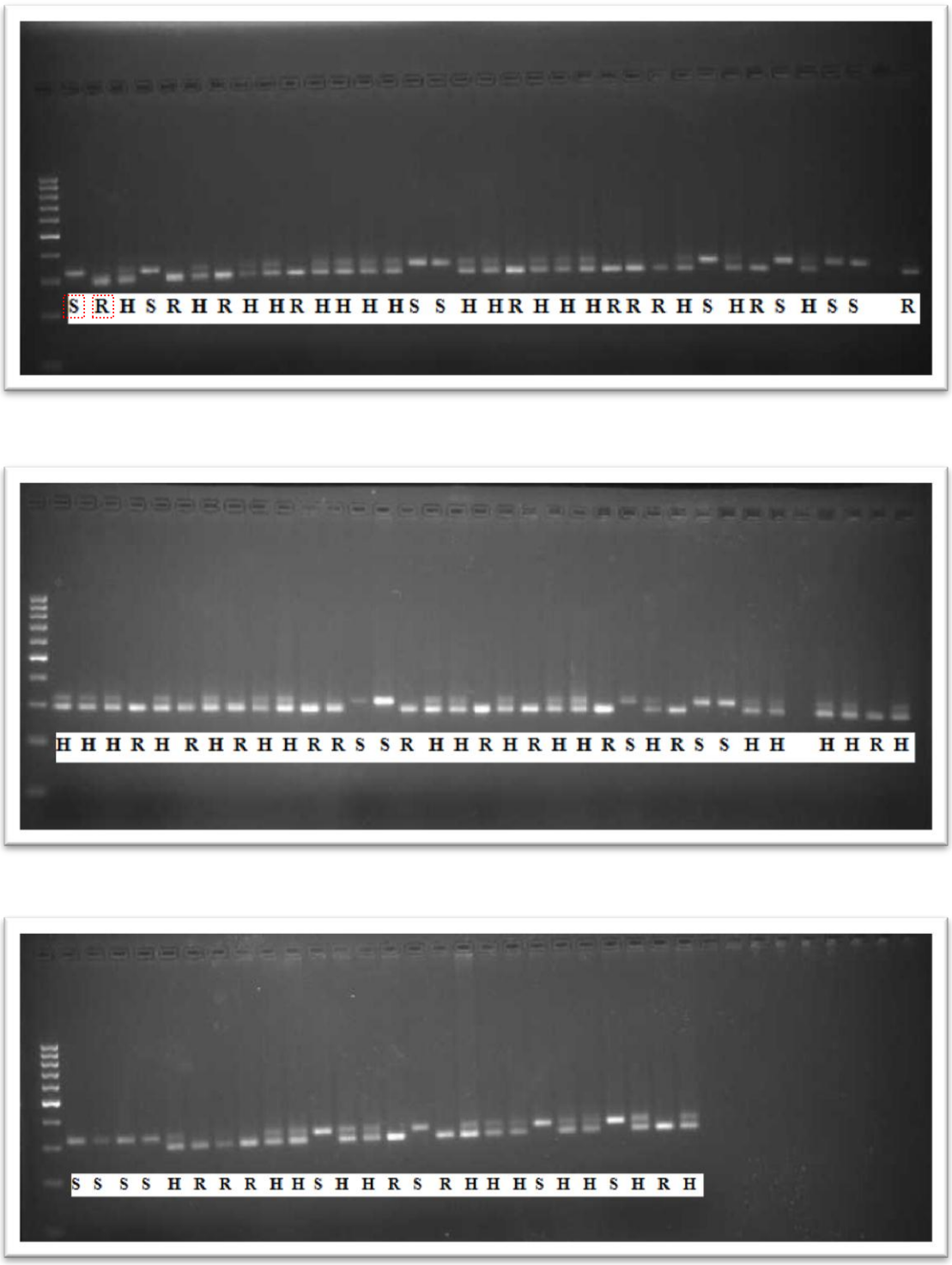

Figure 3.5. GPMS 171 application on F2 population. Susceptible parent (S) and resistant parent (R) are indicated by a red rectangle, F2 individuals were labeled according to parents' banding patterns.

3.5. SCAR Analysis

Ten SCAR markers were applied to parents and bulks to detect polymorphism, however just two (20%) of them, SCAR_CD and SCAR_B94, showed polymorphism. Clear segregation was detected with SCAR_CD and it was applied to the F2 population. Homozygous individuals were clearly distinguished and some individuals showed heterozygosity (H). Therefore, this marker was used for construction of the linkage map (Figure 3.6). HRM analysis was carried out with SCAR_B94 to see the polymorphism clearly before application to the F2 population.

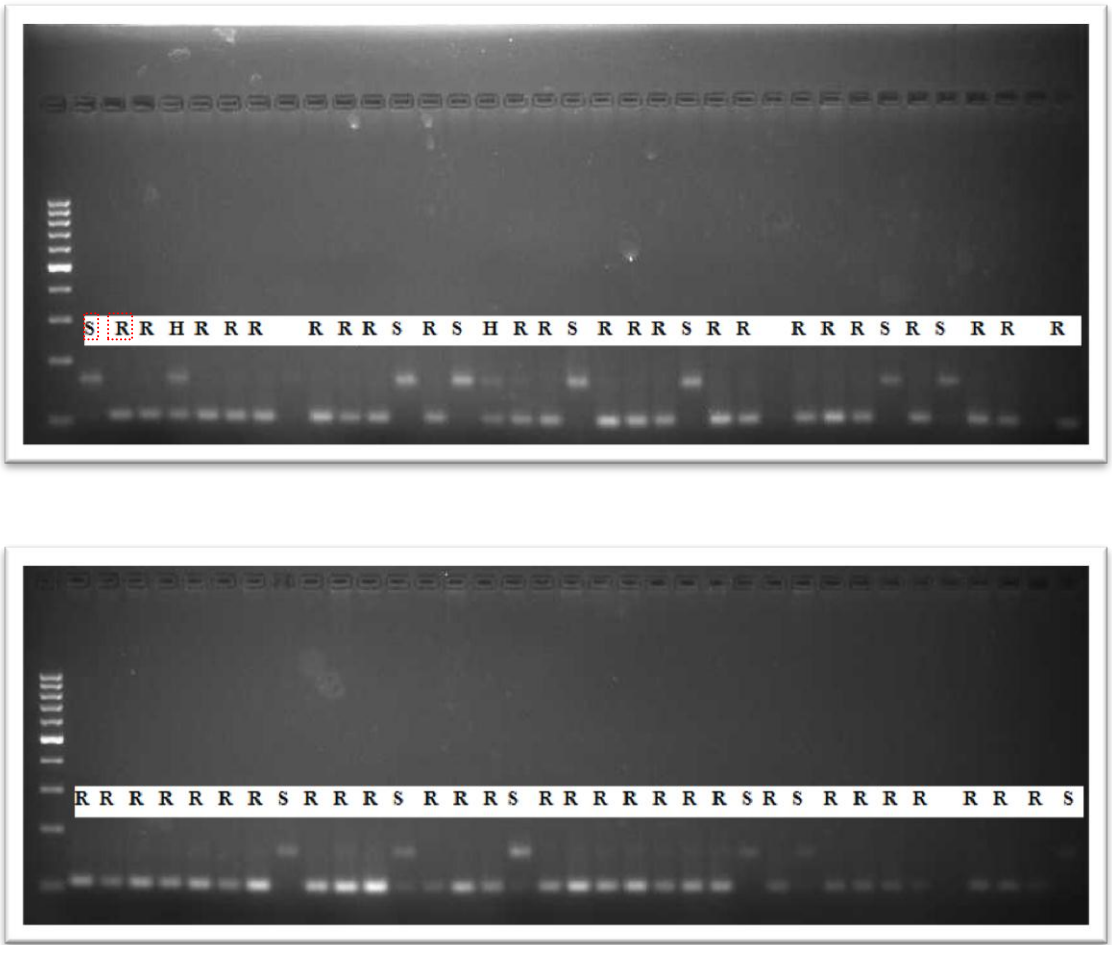

Figure 3.6. SCAR_CD application on F2 population. Susceptible parent (S) and resistant parent (R) are indicated by a red rectangle, F2 individuals were according to parents' banding patterns.

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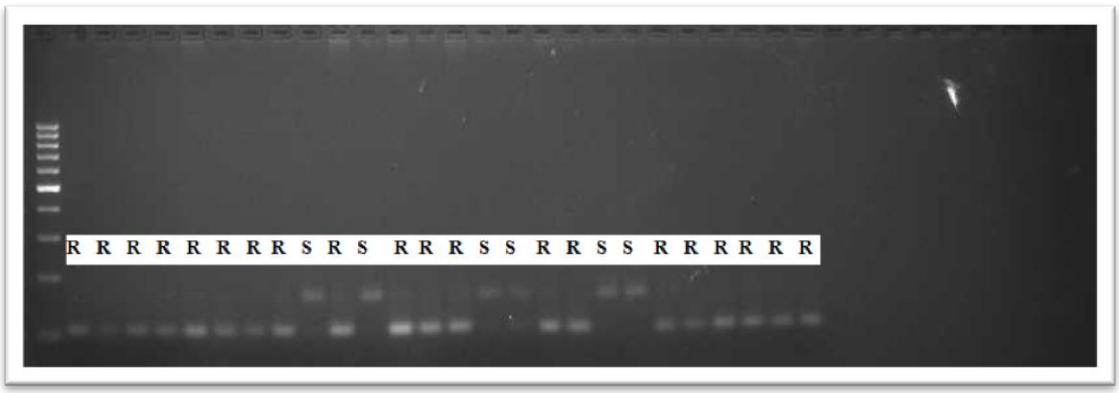

Figure 3.6. (cont.)

3.6. HRM Analysis

Primers HPMS E098, HPMS E102, HPMS E117, HPMS 2-41, HPMS 1-3, HPMS 1-117, HPMS E007, HPMS E082, SSCP_B322, SSCP_B54, SCAR_B94 and GPMS 163 were used in HRM analysis. Five of them, HPMS E007-HPMS E082-HPMS E117-HPMS E098-GPMS 163, did not show segregation between susceptible and resistant parents. The other primers (58%) showed segregation and were applied to the F2 population. Two examples of HRM analysis results are shown in Figure 3.7 and 3.8. As can be seen in these figures, some individuals showed heterozygosity.

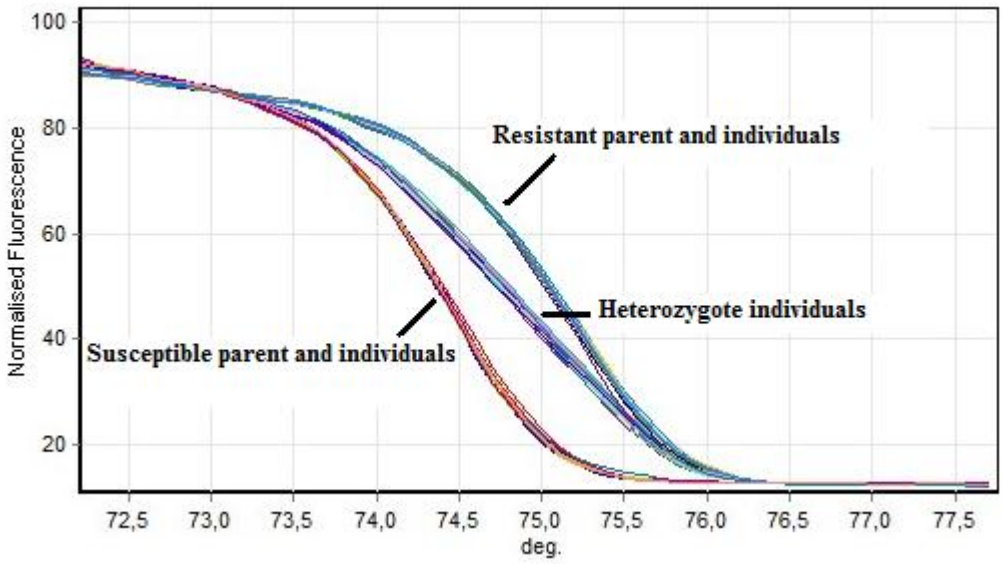

Figure 3.7. HPMS 1-117 analysis on F2 population containing 70 individuals.

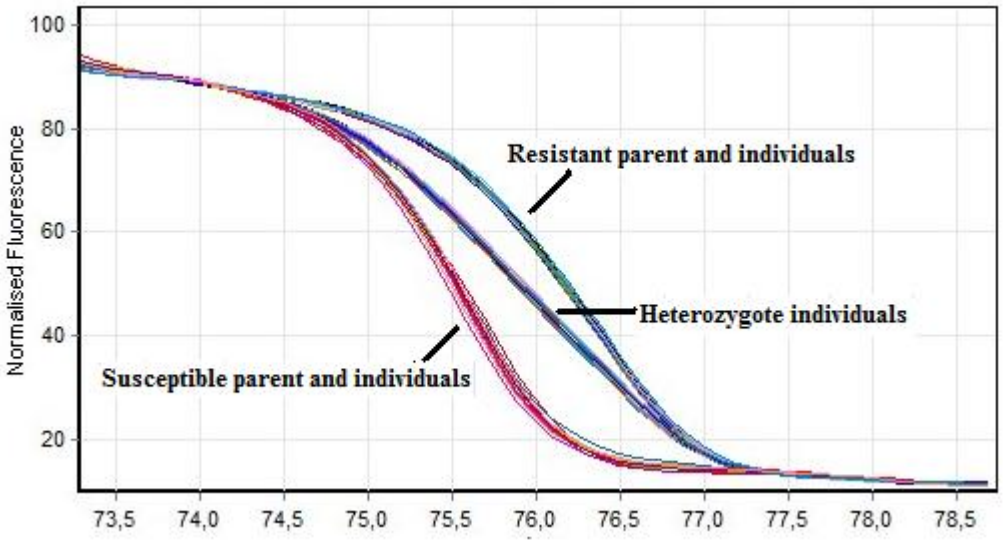

Figure 3.8. HPMS 1-3 analysis on F2 population containing 70 individuals.

3.7. Marker Polymorphism

In the Solanaceae family, marker polymorphism is usually abundant in interspecific populations but less abundant in intraspecific populations (Foolad et al. 2012). A total of 30 standard markers, consisting of 13 SSRs, 10 SCARs, 6 COSII, and 1 CAPS, and 548 new marker combinations, consisting of 238 SRAP combinations, and 310 COSII-SRAP combinations, were tested for polymorphism. Of these 578 markers, 75 of them (13%) were polymorphic. Among the 30 standard markers, 11 of them (37%) were polymorphic. For the 548 new marker combinations, 64 (12%) were found to be polymorphic. Thus, although polymorphism was very limited in the intraspecific pepper population, the use of new marker combinations yielded a significant number of new polymorphism and increased the polymorphic markers by nearly seven-fold. Previous studies also showed that new marker combinations increased reproducibility and the number of polymorphisms found in plants (Mutlu et al. 2008; Castonguay et al. 2010; Li et al. 2011).

3.8. Construction of Linkage Map

Skewed segregation is very common in interspecific populations (Frary et al. 2004). Skewed segregation can be also seen in intraspecific populations (Lefebvre et al. 2002). Eighteen of the markers (64%) segregated dominantly and were expected to fit a

3:1 Mendelian ratio. Of these 18 markers, 10 markers (56%) did not fit the 3:1 ratio as determined by a Chi-square goodness of fit test at $p \leq 0.05$. The remaining markers were codominant and a 1:2:1 ratio was tested for these 10 markers (36%) . Of these, five of the 10 markers (50%) did not fit the expected ratio (Table 3.4). Overall, 54% of the markers showed skewed segregation.

A total of 28 primers showed clear segregation on the F2 population. Linkage map analysis of these markers conducted with the MAPMAKER programme revealed that just 3 of them (11%) were near the Me1 gene on chromosome 9. Therefore these markers; SCAR_CD, CAPS_F4R4 and SSCP_B54 were used for the construction of a linkage map. The distance between SCAR_CD and Me1 gene was 1.1 cM. CAPS_F4R4 and Me1 gene were 14.2 cM apart while SSCP_B54 and Me1 gene were 25.1 cM apart. In a previous study, with the use of different pepper parental lines and nematode strains, CAPS_F4R4 was found to be the closest marker to Me1 at a distance of 1.5 cM while SCAR_CD was the second closest marker with 2.7 cM (Dijan-Caporalino et al. 2007). In this sudy we observed that SCAR_CD is the nearest marker to Me1 gene (Figure 3.9). Markers distances were different because of the use of different populations by the two studies. Because of its proximity to the Me1 gene and its co-dominant nature, SCAR_CD is appropriate for MAS (marker-assisted selection).

Table 3.4. Chi-square goodness of fit test of markers with clear segregation.

Marker Name	**Expected ratio[a]**	**X^2 value**	**p-value**
C2-At5g06130 (F)-Em3	3:1	2.88	0,09
C2-At5g06130 (F)-Em1	3:1*	187.65	<0.0001
C2-At5g06130 (F)-Me3	3:1*	213.26	<0.0001
C2-At5g06130 (F)-Me12	3:1	3.62	0,06
C2-At5g06130 (R)-Me5	3:1*	211.85	<0.0001
C2-At5g06130 (R)-Me10	3:1*	7.85	0,005
C2-At3g09925 (F)-Em10	3:1	4.01	0,05
C2-At3g09925 (F)-Em8	3:1*	85.48	<0.0001
C2-At3g09925 (F)-Em11	1:2:1*	231.97	<0.0001
C2-At3g09925 (F)-Me10	3:1*	105.81	<0.0001
C2-At3g09925 (F)-Me5	3:1	2.25	0,13
C2-At3g09925 (F)-Me4	3:1	1.56	0,21
C2-At2g37240 (F)-Me4	3:1*	21.23	<0.0001
C2-At2g37240 (R)-Me4	3:1*	13.14	0,0003
C2-At2g29210 (F)-Em7	3:1*	4.57	0,033
C2-At2g29210 (R)-Em7	3:1	1.72	0,19

(cont. on next page)

Table 3.4. (cont.)

C2-At2g29210 (R)-Em14	3:1	3.93	0,05
C2-At2g29210 (R)-Me12	3:1*	158.59	<0.0001
GPMS171	1:2:1	2.7	0,26
HPMS 2-41	1:2:1*	10.32	0,006
HPMS 1-3	1:2:1	1.5	0,47
HPMS 1-117	1:2:1	1.76	0,41
HPMS E102	1:2:1	0.82	0,66
SSCP_B54	1:2:1	2.94	0,23
SSCP_B322	1:2:1*	95.55	<0.0001
Caps F4-R4	3:1	1.49	0,22
Scar_CD	1:2:1*	50.11	<0.0001
Scar_B94	1:2:1*	12.52	0.0019

a Marker data that do not fit the expected ratio are marked with *, p≤0.05

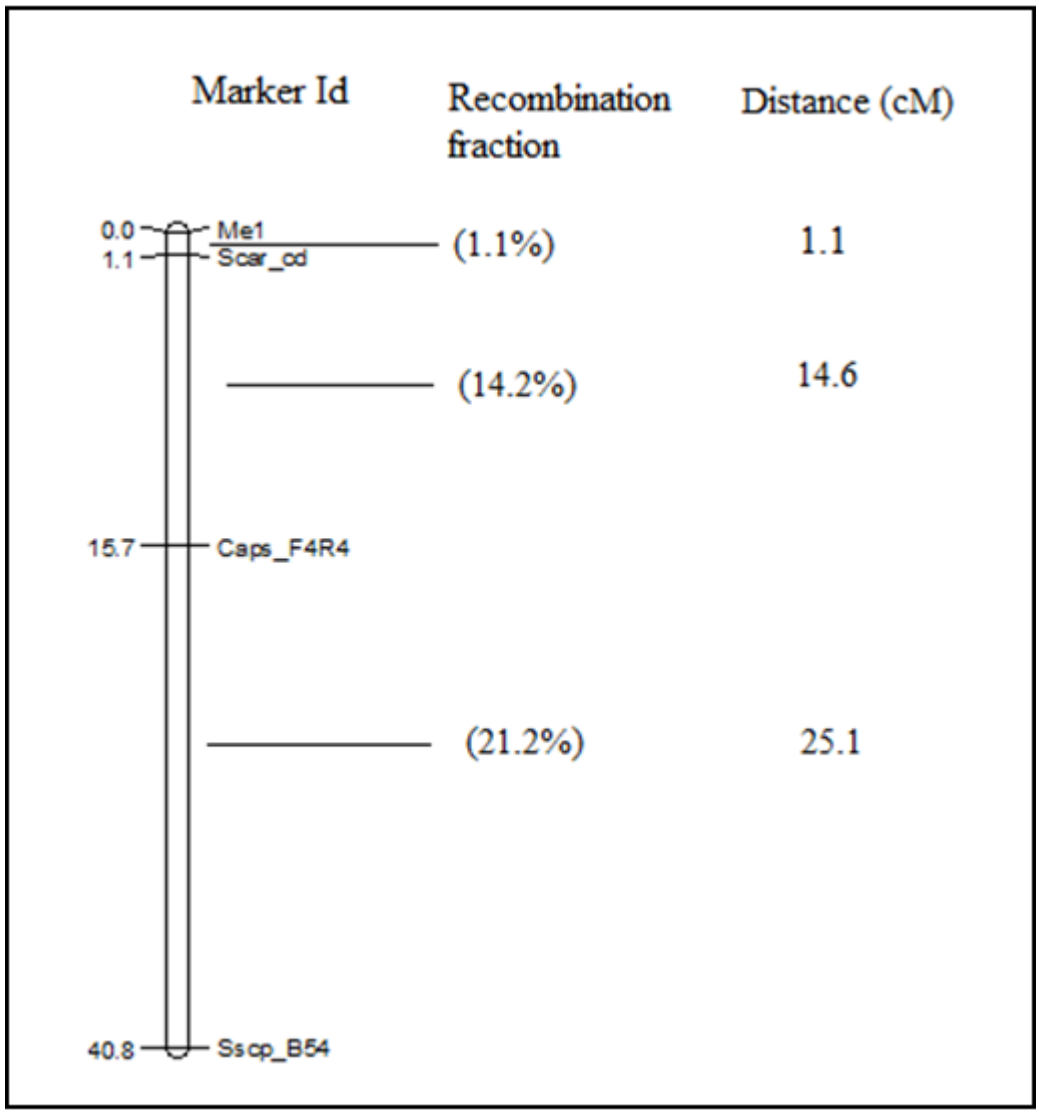

Figure 3.9. Map of link markers that linked to Me1 gene.

CHAPTER 4

CONCLUSION

Pepper (*Capsicum annuum* L.) is an agronomically important plant which has a significant role in the economy, human diet and pharmaceutical industry. Plant nematode parasitism restricts pepper growth, quality and yield. In Turkey, *Meloidogyne incognita* is the most damaging root-knot nematode that affects production of pepper. The Me1 gene which confers resistance to *M. incognita* was mapped in pepper to a 28 cM region on chromosome 9. The aim of this study was to saturate this interval by developing molecular markers linked to the resistance gene.

A total of 238 SRAP markers combinations, 6 COSII markers, 310 combinations of SRAP-COSII markers, 13 SSR markers, 10 SCAR markers and one CAPS marker were tested. Among these markers, the polymorphic ones were tested on and F2 population and 28 markers showed clear segregation on the F2 population. In all, 18 of the markers (64%) segregated dominantly, and 10 of the markers (36%) segregated codominantly. With linkage map analysis it was found that three of the markers: SCAR_CD, CAPS_F4R4 and SSCP_B54 were located near the Me1 gene so these markers were used for the construction of a linkage map. The nearest marker to Me1 gene was SCAR_CD which was 1.1 cM away from the gene. This marker is codominant and can be applied for marker assisted selection of nematode resistance in pepper.

Marker assisted selection (MAS) is useful for transferring new genes and their alleles. Use of a marker identified to be linked to a trait of interest, makes it easier to select an individual that has the trait. Therefore, these markers will provide selection at the genotypic level by marker-assisted selection and this will increase the efficiency and effectiveness of pepper breeding for nematode resistance.

REFERENCES

Castonguay, Y., Cloutier J., Bertrand A., Michaud R. and Laberge S. 2010. SRAP polymorphisms associated with superior freezing tolerance in alfalfa (Medicago sativa spp. sativa). *Theoretical and Applied Genetics,* 120:1611–1619.

Cetintas, R. and Yarba M.M. 2010. Nematicidal effect of five plant essential oils on the southern root-knot nematode, *M.incognita* race 2. *Journal of animal and veterinary advance,* 9 (2): 222-225.

Delaney, T.P. 2009. Plant defence against pathogens. *AccessScience.* Retrieved from http://www.accessscience.com/content/Plant-defense-against pathogens/YB090125.

Djian-Caporalino C., Fazari A., Arguel M.J., Vernie T., Vandecasteele C., Faure I., Brunoud G., Pijarowski L., Palloix A., Lefebvre V. and Abad P. 2007. Root-knot nematode (*Meloidogyne spp*.) Me resistance genes in pepper (*Capsicum annuum* L.) are clustered on the P9 chromosome. *Theoretical and Applied Genetics,* 114: 473-486.

Eisenbach, J.D. and Triantaphyllou H.H. 1991. Root-knot nematodes: *Meloidogyne* species and races. *Manual of agricultural nematology*, 281-286.

Foolad, M.R. and Panthee D.R. 2012. Marker-Assisted Selection in Tomato Breeding. *Plant Sciences* 31(2): 93-123.

Frary, A., Fulton T.M., Zamir D. and Tanksley S.D. 2004. Advanced backcross QTL analysis of a Lycopersicon esculentum L. pennellii cross and identification of possible orthologs in the Solanaceae. *Theoretical and Applied Genetics,* 108: 485-496.

Fuller V.L., Lilley C.J. and Urwin P.E. 2008. Nematode Resistance. *New Phytologist,* 180: 27-44.

Fulton, T.M., Hoeven R.V.D., Eannetta N.T. and Tanksley S.D. 2002. Identification , Analysis , and Utilization of Conserved Ortholog Set Markers for Comparative Genomics in Higher Plants. *The Plant Cell*, 14: 1457-1467.

Jones, N., Ougham H., Thomas H. and Pasakinskiene I. 2009. Markers and mapping revisited: finding your gene. *New phytologist,* 183: 935-966.

Kosambi, D.D. 1944. The estimation of map distances from recombination values. *Ann Eugen,* 12:172-175.

Kumar, L.S. 1999. DNA markers in plant improvement: An overview. *Biotechnology Advances,* 17: 143-182.

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

Lefebvre, V., Pflieger S., Thabuis A., Caranta C., Blattes A., Chauvet J.C., Daubèze A.M. and Palloix A. 2002. Towards the saturation of the pepper linkage map by alignment of three intraspecific maps including known-function genes. *Genome,* 45: 839–854.

Li, G., Quiros C.F. 2001. Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction : its application to mapping and gene tagging in Brassica. *Theoretical and Applied Genetics,* 103: 455-461.

Li, W., Zhang J., Mou Y., Geng J., McVetty P.B., Hu S. and Li G. 2011. Integration of Solexa sequences on an ultradense genetic map in Brassica rapa L. *BioMed Central Genomics,* 12:249.

Ma, Z. Q., Roder M. and Sorrells M.E. 1996. Frequencies and sequence characteristics of di-, tri-, and tetra-nucleotide microsatellites in wheat. *Genome,* 39: 123-130.

Masi, L.D., Siviero P., Castaldo D., Cautela D., Esposito C. and Laratta B. 2007. Agronomic, chemical and genetic profiles of hot peppers (Capsicum annuum ssp .). *Molecular nutrition and food research,* 51: 1053-1062.

Mitkowski, N.A. and Abawi G.S. 2003. Root-knot nematode. *The plant health instructor.*

Mohan, M., Nair S., Bhagwat A., Krishna T.G., Yano M., Bhatia C.R. and Sasaki T. 1997. Genome mapping , molecular markers and marker-assisted selection in crop plants. *Molecular Breeding*, 3: 87-103.

Morgante, M. and Olivieri A.M. 1993. PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal*, 3(1): 175-182.

Mutlu, N., Boyacı F.H., Göçmen M. and Abak K. 2008. Development of SRAP, SRAP-RGA, RAPD and SCAR markers linked with a Fusarium wilt resistance gene in eggplant. *Theoretical and Applied Genetics,* 117: 1303-1312.

Niebel, A., Gheysen G., Montagu M. V. 1994. Plant-cyst nematode and plant-root-knot nematode interactions. 10: 424-430.

Paran, I., Ben-chaim A., Kang B. and Jahn M. 2007. Capsicums. *Genome Mapping and Molecular Breeding in Plants*, 5: 209-226

Pickersgill, B. 1997. Genetic resources and breeding of Capsicum spp. *Euphytica*, 96: 129-133.

Rohami, M. and Mohammadi A., Khosroshahlı., Ahmadi H., Darandeh N. 2010. Karyotype Analysis of several Ecotypes of Capsicum annuum L . in Iran. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca,* 38 (3): 177-180.

Sanchez-Puerta, M. V. and Masuelli R.W. 2011. Evolution of nematode-resistant Mi-1 gene homologs in three species of Solanum. *Molecular genetics and genomics,* 285: 207-218.

Tai, T., Dahlbeck D., Stall R.E., Peleman J. and Staskawicz B.J. 1999. High-resolution genetic and physical mapping of the region containing the Bs2 resistance gene of pepper. *Theoretical and Applied Genetics*, 99: 1201-1206

Varshney, R.K., Graner A. and Sorrells M.E. 2005. Genic microsatellite markers in plants: features and applications. *Trends in biotechnology*, 23 (1): 48-55.

Varshney, R.K. and Tuberosa R. 2007. Genomics-Assisted Crop Improvement. *Genomics Approaches and Platforms*, 1: 13-29.

Vossen, R.H.A.M., Aten E., Roos A. and Dunnen J.T.D. 2009. High-resolution melting analysis (HRMA): more than just sequence variant screening. *Human mutation,* 30 (6): 860-866.

Wang, L.H., Gu X.H., Hua M.Y., Mao S.L., Zhang Z.H., Peng D.L., Yun X.F. and Zhang B.X. 2009. A SCAR marker linked to the N gene for resistance to root knot nematodes (*Meloidogyne spp*.) in pepper (*Capsicum annuum* L.). *Scientia Horticulturae,* 122: 318-322.

Wenzl, P., Raman H., Wang J., Zhou M., Huttner E. and Kilian A. 2007. A DArT platform for quantitative bulked segregant analysis. *BioMedCentral Genomics,* 8: 196.

Williamson, V.M. and Gleason C.A. 2003. Plant – nematode interactions. *Current Opinion in Plant Biology*, 6: 327-333.

Williamson, V.M. and Kumar A. 2006. Nematode resistance in plants : the battle underground. *Trends in genetics*, 22 (7): 396-403.

Wojdacz, T.K. and Dobrovic A. 2007. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Research*, 35 (6).

Wu, F., Eannetta N.T., Xu Y., Durrett R., Mazourek M., Jahn M.M. and Tanksley S.D. 2009. A COSII genetic map of the pepper genome provides a detailed picture of synteny with tomato and new insights into recent chromosome evolution in the genus Capsicum. *Theoretical and applied genetics*, 118: 1279-1293.

Wu,W. and Huang B. 2006. Strategy for the mapping of interactive genes using bulked segregant analysis method and Mapmaker/Exp software. *Chinese Science Bulletin*, 51 (21): 2619-2623.