

ABSTRACT

MOON, HYUNSOOK. Identification of AFLP markers linked to tomato spotted wilt virus resistance in tobacco. (Under the direction of Jennifer S. Nicholson.)

Tomato spotted wilt virus (TSWV) is a serious disease in several crops such as tobacco (*Nicotiana tabacum.*), peanut (*Arachis hypogaea.*), tomato (*Lycopersicon esculentum.*), and pepper (*Capsicum annuum.*). One source of resistance in tobacco is the breeding line Polalta, which contains a TSWV resistance gene introgressed from the wild relative *Nicotiana alata*. However, the resistance is associated with abnormal plant morphology and traditional backcrossing has been ineffective in producing normal plants with TSWV resistance. Molecular marker-assisted backcrossing allows for rapid identification of the plants that are most genetically similar to the recurrent parent and can be used to reduce the size of an introgressed chromosome segment. We applied AFLP (amplified fragment length polymorphism) technology and bulk segregant analysis to identify markers linked to TSWV resistance. One TSWV resistant bulk and two susceptible bulks were constructed by combining DNA from 5 doubled haploid lines from the cross K326 x Polalta for each bulk. A total of 128 primer combinations were used to screen one resistant and two susceptible bulks, and I found 48 potential markers linked to the TSWV resistance. Thirty two markers were selected for further study based on their reproducibility. A population of 88 F₂ plants and 23 doubled haploid lines were screened with 32 markers and a 2.5 cM map with 24 markers was constructed. Eleven AFLP fragments between 100 to 400 bp in size linked in coupling phase to TSWV resistance were isolated and sequenced to develop PCR based markers. Four AFLP

fragments were successfully converted to sequence characterized amplified region (SCAR) markers.

An F_2BC_2 population of 160 plants was screened with 5 AFLP coupling phase markers to select resistant plants that have fewer Polalta-derived markers and more K326-derived markers. No resistant line was identified with a reduced introgression from *N. alata*. In an F_2BC_3 population of about 200 plants, 8 plants were selected based on an improved phenotype and screened with the 17 AFLP coupling phase markers. Four plants with a reduced introgression were identified and will form the basis for future backcrossing. This approach is expected to facilitate development of a line with TSWV resistance and a normal phenotype.

**IDENTIFICATION OF AFLP MARKERS LINKED TO TOMATO SPOTTED
WILT VIRUS RESISTANCE IN TOBACCO**

by

HYUNSOOK MOON

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APPROVED BY:

Dr. Jennifer S. Nicholson
Co-chair of Advisory Committee

Dr. Arthur K. Weissinger
Co-chair of Advisory Committee

Dr. Jim B. Holland

Dr. J. Paul Murphy

BIOGRAPHY

Hyunsook Moon was born on December 14, 1975 in Daegu, Republic of Korea.

Hyunsook received her Bachelor of Science and Master of Science Degree from the Department of Agronomy, Kyungpook National University in 1998 and 2000, respectively.

The title of her master's thesis is “ Growth and Optimum Harvesting Time of Pod-edible Peas (*Pisum sativum* L.)” under the direction of Dr. Young-Hyun Hwang. In the fall of 2002, she began her studies at North Carolina State University to pursue a Ph.D. degree in Crop Science under the direction of Dr. Jennifer S. Nicholson.

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CHAPTER I. LITERATURE REVIEW

Tomato spotted wilt virus

The disease reported as “spotted wilt” was first described in Australia in 1915 (Brittlebank, 1919). In 1930, Samuel and co-workers were able to show that the disease was caused by a virus, which they named tomato spotted wilt virus (TSWV) (Samuel et al., 1930). TSWV infects over 900 plant species, including both monocots and dicots, and is a serious disease in many crops such as tobacco (*Nicotiana tabacum.*), peanut (*Arachis hypogaea.*), tomato (*Lycopersicon esculentum.*), and pepper (*Capsicum annuum.*) (Moyer, 1999).

TSWV is a relatively new disease on tobacco in the USA. It was first identified on tobacco in Louisiana in 1973 (Lucas, 1975). Since 1973 TSWV has become widespread in the southeastern U.S. (Melton et al., 2002). This disease is particularly severe in Georgia, and in recent years there is typically a 30 % incidence and 15% yield loss due to TSWV, which is jeopardizing the viability of future production in this state (Bickers, 2006). TSWV is gradually affecting more areas of North Carolina tobacco production. North Carolina flue-cured tobacco losses due to TSWV were particularly

high in 2002 and 2005, resulting in estimated losses of \$45 million and \$17 million, respectively (Broadwell and Gutierrez, 2005).

Symptoms of TSWV

The symptoms caused by the tospoviruses are diverse. On non-systemic hosts, symptoms are generally restricted to local lesions, with chlorosis and necrosis. With systemic hosts, symptoms include ring spots, line patterns, wilting, stunting, mottling, chlorosis, and necrosis. The symptom expression is also variable depending on the age of the plant, number of virus strains infecting the host plant, level of infection, and environmental factors, particularly temperature (German et al., 1992).

Infection usually occurs in epidermal cells through wounds made by feeding thrips. Symptoms may appear within 2-4 days after infection. The virus causes concentric necrotic rings and zonate necrotic spots of tissue on the younger leaves. Frequently the spots coalesce. At first, the spots are yellow, but later the dead areas turn red-brown. Infested plants are stunted, and the apical bud droops or bends over. Young leaves infected on one side are frequently distorted or puckered as a result of unequal growth. Plants severely attacked do not grow for several weeks, and the leaves droop and finally die (Lucas 1965; Shew and Lucas, 1991) (Figure 1.2).

Biology of TSWV

TSWV is the type member of the plant infecting genus *Tospovirus* in the arthropod-borne family *Bunyaviridae* (Elliott, 1990). The TSWV virion is bound by a lipid envelop containing two types of glycoproteins (G1 and G2) which encompasses a tripartite RNA genome containing S (2.9 kb), M (4.9 kb), and L (8.9 kb) RNAs. These RNA molecules are packaged with numerous copies of nucleocapsid protein (N) subunits (De Haan et al., 1990 and 1991; German et al., 1992; Law et al., 1992). The L RNA has one negative sense open reading frame that encodes the viral RNA-dependant RNA polymerase (330-kDa) (Adkin et al., 1995; De Haan et al., 1991; Van Poelkijk et al., 1997). The M RNA and S RNA (de Haan et al., 1990) each have two ORFs in ambisense coding mode. The putative movement protein (NSm) and the precursor of the glycoproteins (G1 and G2) are encoded by the M RNA (Kormelink et al., 1992; 1994). The S RNA encodes the nucleocapsid protein (N protein) which has been used widely as the source of pathogen-derived resistance (Kim et al., 1994; Pang et al., 1992; 1996; Sherman et al., 1998; Ultzen et al., 1995; Vaira et al., 1995) and the nonstructural protein (NSs) which is reported to suppress gene silencing (Bucher et al., 2003; De Haan et al., 1990).

TSWV is transmitted by several thrips species (Ullman et al., 1993; Wijkamp et al., 1993). The most common thrips vectors are tobacco thrips (*Frankliniella fusca* Hinds) and western flower thrips (*Frankliniella occidentalis* Pergande) in the southeastern United States (Groves et al., 2001). Thrips acquire the virus in the first instar stage when the larvae feed on the virus infected leaves. The second instar larvae and adults can become transmitters of *Tospoviruses* after the virus is acquired. (Sakimura, 1962; Wijkamp et al., 1993). Winged adult thrips can survive for 30-45 days, during which they can transmit TSWV if they have acquired it by feeding on infected plants as first instar larvae.

After acquisition by thrips, the virus moves into midgut epithelial cells which are the initial site of TSWV entry and infection (Ullman et al., 1993; Nagata et al., 1999). After the virus replicates in the midgut epithelial cells, the virus moves to the salivary glands; thus the insect vectors not only transmit the virus, but also serve as a host (Ullman et al., 1993; Wijkamp et al., 1993; Negata et al., 1999). The glycoproteins of TSWV are reported to be involved in TSWV entry to the midgut and circulation throughout the thrips body (Bandla et al., 1998; Kikkert et al., 1998; Medeiros et al., 2000). After transmission to plant cells, the virus is released of its membrane, and

nucleocapsid enters into the cytoplasm. At this stage, the viral RNA will be either transcribed or replicated by the viral RNA dependent RNA polymerase (Matthews, 1992).

Genetic variation of TSWV

Plant viruses evolve by mutation, recombination, and reassortment (Roossinck, 1997). Much of the genetic variations in virus populations are the result of errors that occur during viral genome replication because RNA viruses lack proofreading ability (Domingo and Holland, 1997). Reassortant isolates can be synthesized by combining two genetically different isolates during the mixed infection of viruses with segmented genomes (Pringle, 1996). It is believed that for RNA viruses in the family *Bunyaviridae*, mutation and genome segment reassortment are the major sources of variability (Elliott, 1995). Qiu et al. (1998) reported that TSWV isolates easily exchanged genome segments in a nonrandom fashion and the intergenic region of the S RNA was correlated with competitiveness of the individual segments in reassortant isolates.

The high genetic variability in TSWV contributes to the ability of the virus to overcome various sources of resistance. TSWV is able to overcome transgene-mediated resistance through natural selection for resistance-mediated isolates in the field (Herrero et al., 2000; Jankulova et al., 1999), and through genomic reassortment under

experimental conditions (Qiu et al., 1998). Hoffman et al. (2001) has shown that the M RNA of TSWV might be responsible for overcoming host- and transgene-mediated resistance. Qiu et al. (1998) also found that the intergenic region (IGR) of the S RNA was correlated with competitiveness of the individual segments in reassortant isolates. In addition, the ability to overcome host resistance genes *Sw-5* in tomato and *TSW* in pepper mapped to the M and S RNA of TSWV, respectively (Jahn et al., 2000).

Control measures

Tomato spotted wilt has been difficult and expensive to control. Damage from TSWV can be reduced with the application of the plant defense activator acibenzolar-S-methyl (Actigard) and the insecticide imidacloprid (Admire). Pappu et al. (2000) evaluated the effect of these chemicals on control of TSWV in flue-cured tobacco in multiple locations in Georgia. The results showed Actigard alone significantly reduced final disease incidence to 5.8% compared to 17.3% the nontreated control. Admire reduced disease incidence to 30% in comparison to 60 % disease incidence in the nontreated control. When both were applied together the disease incidence was near zero for three of the four locations (Pappu et al., 2000). However, Actigard treatments are

expensive and may not be cost effective in all situations. In addition, these treatments do not offer complete control.

Sources of resistance

In flue-cured tobacco, sources of naturally available host plant resistance to TSWV are very limited. No resistance has been reported in the cultivated species, *N. tabacum* (Gajos, 1981). Opoka (1969) reported that three wild species showing complete resistance to TSWV; *N. glauca* Graham, *N. alata* Link & Otto, and *N. noctiflora* Hooker. Gajos (1978) noted a hypersensitive response in *N. alata*, *N. affinis* Hort. ex Moore, and *N. langsdorffii* Weinmann. Jankowski (1980) found six species showing a hypersensitive response to TSWV, *N. sanderae* Hort, *N. fragans* Hooker, *N. longiflora* Cavanilles, *N. trigonophylla* Dunal, *N. alata* and *N. affinis*.

In 1987, Gajos released the first resistance tobacco variety 'Polalta' by introgressing the TSWV resistance from *N. alata* into the cultivated species *N. tabacum*. Introgression is the movement of a gene from one species into the gene pool of another by backcrossing an interspecific hybrid with one of its parents. He produced an F₁ hybrid of *N. tabacum* x *N. alata*, and one male fertile TSWV resistant plant was selected and chromosome doubled by colchicine treatment. However, this hybrid could not be crossed

directly to tobacco. Another interspecific hybrid, *N. tabacum* x *N. otophora* Grisebach, was used as a bridging parent. Progeny of this interspecific hybrid was used as a female in a cross with the *N. tabacum* x *N. alata* hybrid. The resulting progeny was then backcrossed to tobacco. This source of resistance has never been used in commercial cultivars due to a linkage of the resistance with an abnormal plant type, including an altered leaf shape, thickened midveins, and a tendency to produce tumors. The tendency to produce tumors has been observed in crosses of *N. tabacum* with members of the *Alatae* section of the genus, and is thought to be a symptom of altered auxin regulation (Durbin, 1979)

Genetically engineered TSWV resistance

Due to the serious problems caused by TSWV and the lack of natural resistance, genetically engineered resistance has been developed in lettuce, tomato, tobacco, peanut, and chrysanthemum (Pang et al., 1996; Gonsalves et al., 1996; Pang et al., 1992; Kim et al., 1994; Magbanua et al., 2000; Sherman et al., 1998). In 1985, Sanford and Johnston first proposed pathogen-derived resistance. This is the way to generate a virus resistance gene by using a gene from the pathogen to impart resistance in the host.

In most cases the expression of the sense or antisense RNA of the TSWV N gene has been used to achieve genetically engineered resistance (Kim et al., 1994; Sherman et al., 1998). The TSWV NSm gene, which encodes the putative viral movement protein, has also been used to develop resistance in transgenic tobacco plants (Prins et al., 1997).

Several research groups have transformed tobacco with the TSWV nucleocapsid gene to achieve resistance. Two different mechanisms of resistance have been observed in transformed tobacco with the nucleocapsid gene of TSWV (Pang et al., 1992; 1994; Vaira et al., 1995). The resistances differed in the levels of nucleocapsid protein (NP) accumulations. Transgenic *N. benthamiana* plants producing small amounts of the N protein were highly resistant to the homologous isolate and closely related isolates, whereas plants producing large amounts of the N protein possessed moderate levels of protection against the homologous isolate and two distantly related isolates (Pang et al., 1992; 1994). Vaira et al. (1995) supported the previous studies by reporting that *N. benthamiana* transformed with the N gene of TSWV expressing at a low level of protein conferred TSWV-specific resistance, whereas high expression of N protein retarded disease development of TSWV.

In plants expressing a high level of N protein that show moderate TSWV

resistance, it has been proposed that the protein accumulation is interfering with disease development. However, in plants expressing a low level of N protein and RNA, the resistance has been shown to be an RNA-mediated virus resistance that works through post-transcriptional gene silencing (PTGS) (Pang et al., 1996; 1997; Prins, et al., 1996). PTGS is involved in the suppression of foreign genetic elements such as viruses and transposons through a specific RNA breakdown mechanism. PTGS is induced by dsRNA which can be produced most commonly by viruses during replication, or by transgenes that contain inverted repeat structures. In addition, a dsRNA may be produced from transgene without an inverted repeat through the action of a plant RNA –dependent RNA-polymerase. The dsRNAs are degraded into short interfering RNAs (21-25 nucleotides) which are recruited into an RNA-induced silencing complex (RISC) and act as sequence-specific templates for further degradation of homologous RNA. In RNA-mediated virus resistance, the transgene RNA is broken down in a sequence-specific manner by PTGS, as well as homologous RNA from an infecting virus (reviewed by Goldbach et al., 2003).

Transgenic tobacco lines with RNA-mediated resistance have been shown to have high levels of resistance to TSWV in the field. Herrero et al. (2000) demonstrated that N-gene transformed tobacco plants had significantly reduced incidence of infection when

exposed to natural inoculum in a field with high levels of TSWV. Levin et al. (2005) also evaluated several transgenic lines under field conditions with very high TSWV disease pressure. Most transgenic lines had 0 % infection, while the susceptible non-transgenic control had an infection incidence of 61 %.

TSWV resistance genes in other crops

Genes conferring a high level of resistance to TSWV have been characterized in tomato and pepper. The *Sw-5* gene confers resistance to tomato spotted wilt virus (TSWV) in tomato and acts as a dominant gene. The gene has been introgressed into cultivated tomato from the wild species *Lycopersicon peruvianum* and provides resistance against TSWV isolates from different geographical locations (Boiteux and Giordano 1993; Stevens et al. 1992). In addition, it also provides resistance against two other tospovirus species, tomato chlorotic spot virus (TCSV) and groundnut ring spot virus (GRSV) (Boiteux and Giordano 1993). Stevens et al. (1995) mapped *Sw-5* between two RFLP markers (CT71 and CT220) near a telomeric area of chromosome 9, and also identified one RAPD marker that is localized within 0.5 cM of *Sw-5*. Chagué et al. (1996) identified six RAPD markers (4 coupling phase and 2 repulsion phase) linked to the *Sw-5* gene using bulked segregant analysis. One of the coupling markers close to the gene was

used to develop a SCAR marker. The 4 RAPD markers identified were integrated into chromosome 9 on the RFLP tomato map developed by Tanksley et al. (1992) (Chagué et al., 1996).

The *Sw-5* gene has been isolated by a genetic map-based approach called chromosome landing. Using one previously identified RFLP marker, CT220, which is very tightly linked to the *Sw-5* gene on chromosome 9, it was shown that the *Sw-5* locus is located on a chromosomal segment of 100 kb (Brommonschenkel and Tanksley, 1997). To determine whether the *Sw-5* locus consisted of a cluster of functional resistance genes, Folkertsma et al. (1999) developed a physical map of the *Sw-5* locus by screening a bacterial artificial chromosome (BAC) library of tomato cultivar ‘Stevens’ with two tightly linked markers, CT220 and SCAR 421.

Spasova et al. (2001) identified two highly homologous (95%) resistance gene candidates within 40 kb of CT220. The genes, *Sw5-a* and *Sw5-b* encode proteins of 1245 and 1246 amino acids, respectively and are members of the coiled-coil, nucleotide-binding-ARC, leucine-rich repeat group of resistance gene candidates. Promoter and terminator regions of the genes are also highly homologous. Both genes significantly resemble the tomato nematode and aphid resistance gene *Mi* and, to a lesser extent,

Pseudomonas syringae resistance gene *Prf*. Transformation of the *Sw5-a* and *Sw5-b* into tobacco plants revealed that only the *Sw5-b* gene is necessary and sufficient for conferring resistance against tomato spotted wilt virus.

In pepper, *Capsicum annuum*, the only locus known to confer resistance to TSWV has been designated *Tsw* (Black et al., 1991; Boiteux et al., 1993). This locus originated from *Capsium chinense* and was introgressed into pepper cultivars (Costa et al., 1995). *Tsw* has been identified in 5 different *C. chinense* lines that exhibit a hypersensitive response upon infection with TSWV (Boiteux 1995; Moury et al., 1997).

The *Tsw* gene has been tagged with a RAPD marker that is located 3.45 cM from the *Tsw* locus, and has been mapped to the distal portion of chromosome 10 (Jahn et al., 2000). Moury et al. (2000) found 4 RAPD markers linked to the *Tsw* locus spanning 20 cM. A close RAPD marker was converted into CAPS marker linked 0.9 cM away from the gene to facilitate marker - assisted selection.

Molecular marker technologies

Molecular markers can be very useful in improving the efficiency of plant breeding. Several molecular marker technologies are available and are described below.

Restriction Fragment Length Polymorphisms (RFLP)

Using RFLP markers, genetic maps have been developed in many plant species. RFLPs detect differences in restriction enzyme sites in genomic DNA. In order to perform RFLP reactions, genomic DNA is digested with a restriction enzyme and the DNA fragments separated by electrophoresis are transferred to nitrocellulose or nylon filter. A labeled probe is hybridized to the DNA fragments on the blot to detect differences in restriction digestion between different genotypes. The bands are inherited in a co-dominant manner (reviewed by Staub et al., 1996). Rice is the species that has been analyzed most extensively by RFLPs (Causse et al., 1994; Kurata et al., 1994). The major disadvantages of RFLP are that it is labor intensive and time consuming due to technical complexity (reviewed by Mohan et al., 1997).

Random Amplified Polymorphic DNA (RAPD)

RAPD analysis, which is a PCR-based molecular marker technique, was developed independently by Welsh and McClelland (1990) and Williams et al. (1990). Short single random primer sequences (10-12 base pairs) are used to amplify DNA in a thermocycler, and amplification products can be separated by electrophoresis on an agarose gel. RAPDs are dominant markers with polymorphisms between individuals

defined as the presence or absence of a particular RAPD band. Although RAPD methodology is relatively easy and inexpensive, its lack of reproducibility and sensitivity to experimental conditions are problematic (reviewed by Staub et al., 1996).

Amplified Fragment Length Polymorphism (AFLP)

AFLP method combines the use of restriction enzymes with PCR selective amplification of fragments, and detects fragment length polymorphisms (Vos et al., 1995). The first step is to double-digest genomic DNA with two restriction enzymes (rare cutter and frequent cutter). Vos et al. (1995) explained that the rationale for using two different restriction enzymes is the following. “1) The frequent cutter will generate small DNA fragments, which will amplify well and are in the optimal size range for separation on sequence gel. 2) The number of fragments to be amplified is reduced by using the rare cutter, since only the rare cutter/frequent cutter fragments are amplified. This limits the number of selective nucleotides needed for selective amplification. 3) The use of two restriction enzymes makes it possible to label one strand of the ds PCR products, which prevents the occurrence of ‘doublets’ on the gels due to unequal mobility of the two strands of the amplified fragments. 4) Using two different restriction enzymes gives the greatest flexibility in tuning the number of fragments to be amplified. 5) Large numbers

of different fingerprints can be generated by the various combinations of a low number of primers.”

Next, two specific short DNA sequences (adapters) are ligated to the ends of the fragment to serve as primer binding sites. The primers are designed to match to the two different adapters, but also carry short extensions of 1-3 nucleotides for selective amplification of DNA fragments. This method can generate a large number of bands for detection of polymorphisms. The amplified DNA fragments can be altered by the composition of nucleotides in the primers.

High reproducibility, rapid generation and high frequency of identifiable AFLP polymorphisms make AFLP DNA analysis an attractive technique for identifying polymorphisms, determining linkages by analyzing individuals from a segregating population, high resolution mapping and marker assisted cloning. However, AFLP analysis is technically difficult and requires expensive fluorescently labeled primers and sequencing gel equipment or radioactivity (reviewed by Neil et al., 1997).

Simple sequence repeats (SSRs)

Simple sequence repeats (SSR) or microsatellites are an advanced PCR-based molecular marker. Plant genomes contain a large number of tandemly repeated short

sequences which are widely scattered at many hundreds of loci throughout the chromosome. SSR polymorphism reflects polymorphisms in the number of repeat units in a short motif that is repeated multiple times or insertions/deletions in the regions flanking microsatellites repeats. Nucleotide sequences flanking the repeat motif are used to design primers to amplify the repeated sequence. This type of the marker system has the advantage of being codominant and highly reproducible. These primers are very useful for rapid and accurate detection of polymorphic loci. However, start-up costs for developments of SSR primers are large, and primer sequences for detecting SSRs are not available in all species of plants (Neil et al., 1997; Mohan et al., 1997).

Conversion of RAPD or AFLP into simple PCR markers

Converting specific AFLP or RAPD markers into single locus PCR markers such as cleaved amplified polymorphic sequences (CAPS) or sequence characterized amplified region (SCAR) markers is desirable because PCR markers are easy to use, less laborious, and inexpensive for simple locus assays. SCAR markers are detected with two primers designed from sequenced ends of a RAPD or AFLP marker. DNA sequence differences are indicated by the presence or absence of a single unique band. They are more reproducible than RAPDs. The newly designed primers for detecting SCAR markers

often generate monomorphic amplifications and loss of the initial polymorphism (Paran and Michelmore, 1993). To retrieve this polymorphism, some fragments can be converted to codominant CAPS markers by digestion with endonucleases. CAPS markers are a form of genetic variation in the length of DNA fragments generated by the restriction digestion of PCR products. The source of the sequence information for the primers can come from genomic or cDNA sequences, or cloned RAPD or AFLP bands. Utility of a desired RAPD or AFLP marker can be increased by sequencing a specific fragment and designing longer primers (e.g. 24 nt) for specific amplification of the markers. These primers can then be used directly on genomic DNA in a PCR reaction to amplify the polymorphic region. These allele-specific markers are useful for marker-assisted selection and can enhance the mapping precision by the detection of heterozygous plants (reviewed by Mohan et al., 1997).

Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are polymorphisms where the alleles differ by the replacement of a single nucleotide in the DNA sequence. The discovery of single nucleotide polymorphisms (SNPs) has been simplified by recent developments in sequencing technology. There are several different ways to discover SNPs, including

direct sequencing of amplified DNA fragments from several individuals, and electronic SNP discovery in shotgun genomic libraries or EST libraries.

SNP discovery in many crop species, such as corn and soybean became relatively straightforward because of their high level of intraspecific nucleotide diversity and the availability of many gene and expressed sequence tag (EST) sequences.

Technological improvements make the use of SNP markers attractive for high-throughput use in marker-assisted breeding, EST mapping, and the integration of genetic and physical maps (reviewed by Rafalski 2002). There are numerous methods available for genotyping SNPs. SNPs can be identified using PCR and gel electrophoresis, by developing CAPS or dCAPS markers, or by allele-specific PCR. Bundock et al. (2006) showed that the allele-specific PCR method was highly successful for SNP genotyping in expressed barley sequences. An allele-specific PCR method comprised of two flanking-primers (forward and reverse primers) combined with an internal allele-specific primer produced for the SNP site, and run as nested three primer PCR. Yeam et al. (2005) reported the development and use of CAPS markers based on the SNPs in recessive viral resistance alleles in *Capsicum*, and demonstrated that the application of the marker system is a promising tool to improve for pepper breeding.

Newer methods for SNP detection are designed for high throughput detection and use luminescent or fluorescent labeled primers or nucleotides to detect the polymorphism, or mass spectrometry to detect mass differences in the products of different alleles. There are three aspects to these types of genotyping assays: allelic discrimination, assay format, and detection methodology. For allelic discrimination, sequence-specific detection relies on four general mechanisms: allele-specific hybridization, allele-specific nucleotide incorporation, allele-specific oligonucleotide ligation, and allele-specific invasive cleavage. Kwok (2001) reviewed in great detail. “With allele-specific hybridization approach, two allele-specific probes are designed to hybridize to the target sequence when they match perfectly. Allele-specific nucleotide incorporation mechanism is based on primer extension approach that performs reactions with amplified target DNA and analyze the products to determine the identity of the base incorporated at the polymorphic site. Allele-specific oligonucleotide ligation mechanism is using the nature of DNA ligase. When two adjacent oligonucleotides are annealed to a DNA template, they are ligated only if the oligonucleotides perfectly match the template at the junction. Therefore, they can infer the alleles present in the target DNA by determining whether ligation has occurred. For allele-specific invasive cleavage method, structure-specific enzymes cut a

complex formed by the hybridization of overlapping oligonucleotide probes. When probes are designed such that the polymorphic site is at the point of overlap, the correct overlapping structure is formed only with the allele-specific probe but not with the probe with a one-base mismatch“.

The assay format can be a solution or a solid support such as a latex bead or a glass slide. Detection mechanisms include monitoring light emission, measuring the mass of the products, or detecting a change in the electrical property. Monitoring light emission is the most widely used detection and methods include luminescence, fluorescence, time-resolved fluorescence, fluorescence resonance energy transfer, and fluorescence polarization. These three aspects (the allelic discrimination mechanisms, reaction formats, and detection modalities) can be combined in various ways to produce the many SNP genotyping approaches in use today (reviewed by Kwok, 2001).

As one example in a plant system, Torjek et al. (2003) established two procedures, multiplexed SNaPshot assays and matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) assays, for rapid mapping with SNP based markers for *Arabidopsis*. The multiplexed SNaPshot assay relies on the 5' extension of a specific primer adjacent to the SNP, using four different fluorescently labeled dideoxynucleotides. The reaction

products are separated and visualized by the sequencer. The matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) assay is a method that applies mass spectrometry in combination with the primer extension reaction to detect primer extension products in multiplex reactions, and the intrinsic mass differences between the primers extension products are assayed.

Marker assisted selection.

Genetic markers that are associated with economically important traits can be used as selection tools in plant cultivar development programs by plant breeders. Marker-assisted selection (MAS) provides new solutions for increasing selection efficiency by allowing for earlier selection and reducing the numbers of lines which should be tested in field trials (reviewed by Staub et al., 1996).

MAS can facilitate several aspects of plant breeding (reviewed by Collard et al., 2005, Mohan et al., 1997). First, MAS can reduce time required for backcrossing. Compared with conventional backcrossing, the use of DNA markers thus offers faster recovery of the recurrent genotype and more efficient selection of genomes that have recombination events close to the target gene. Frisch et al. (1999) demonstrated with a computer simulation that a genotype with 98 or 99 % genetic similarity to the recurrent

parent can be accomplished in one or two generations less with MAS than conventional backcrossing. As one example, MAS was used to improve the resistance to bacterial blight in a popular rice line (Chen et al., 2000). The authors used two flanking markers tightly linked to the *Xa21* gene for selection of the presence of the *Xa21* gene, which confers high levels of resistance to a broad spectrum of the pathogen races, and subsequently used 128 RFLP markers to guide selection of progeny with genetic backgrounds essentially equivalent to that of the recurrent parent. The resulting improved version of the rice cultivar was agronomically similar to the recurrent parent in most respects, but showed the same spectrum of bacterial blight resistances as the donor parent.

MAS also facilitates breeding when phenotypic evaluation is difficult. Disease resistance phenotypes are often simple and oligogenic in nature, yet the difficulties in establishing reliable inoculation and scoring methods can be challenging. In cases like these, MAS can provide an extremely powerful tool with which to guide selection. An example is soybean cyst nematode (SCN; *Heterodera glycines*). A widely used phenotypic assay takes five weeks to complete, is difficult to score with conventional techniques and requires extensive greenhouse space and labor. After years of efforts to

develop DNA markers, an SSR marker designated Satt309 that tagged *rhg1*, the gene responsible for SCN resistance became available (Cregan et al., 1999). Currently, the SSR marker can be effective in a breeding program, and is more than 90% accurate in genotypic selection (reviewed by Young 1999).

MAS is also useful for a pyramiding gene, i.e., combining multiple genes in a single recipient genotype. Initially, donor material may consist of several lines, each possessing one of the positively acting genes, or a single line in which all major genes were previously accumulated. The resistance genes then can be stacked into an elite cultivar by backcrossing and MAS (reviewed by Masojć, 2002). A successful use of marker-assisted selection for pyramiding three major genes (*xa5*, *xa13*, and *xa21*) for bacterial blight resistance in a single rice (*Oryza sativa* L.) cultivar was described by Huang et al. (1997) and Singh et al. (2001).

Lastly, MAS can assist in avoiding the transfer of undesirable or deleterious genes. For some traits, the only source of favorable genes is exotic germplasm. Many important traits have been introgressed into cultivated plants by backcrossing to the recurrent parent after interspecific hybridization. The gene of interest introduced into cultivated plants is flanked by segments of DNA derived from the donor parent. Backcrossing results in the

transfer not only of the genes of interest, but also of additional linked genes, a phenomenon known as linkage drag (Tanksley et al., 1989). Markers can be used to select for rare progeny in which there have been recombinations near the target gene. Thereby breaking the linkage between the gene of interest and the chromosomal regions with which they were associated in the donor plant. Young and Tanksley (1989) demonstrated that plants containing desirable recombination near a resistance gene were rarely selected during backcrossing. Therefore, the conventional backcrossing breeding method was not successful in reducing the size of linked DNA around the resistance gene.

Molecular markers in tobacco

Ren and Timko (2001) used AFLP analysis to determine the degree of intra- and inter-specific genetic variation in the genus *Nicotiana*. Forty six diverse accessions of tobacco (*N. tabacum* L.) and seven wild *Nicotiana* species, including *N. sylvestris*, *N. tomentosiformis*, *N. otophora*, *N. glutinosa*, *N. suaveolens*, *N. rustica*, and *N. longiflora* were analyzed. The amount of genetic polymorphism among cultivated tobacco was limited as evidenced by the high degree of similarity in the AFLP profiles, while a greater amount of genetic polymorphism did exist among wild species of *Nicotiana*. This observation is consistent with the observation of Bogani et al. (1997) following their

analysis of interspecific variation using RAPD analysis.

Because of the low abundance of polymorphic markers within *N. tabacum*, it has been difficult to find markers linked to disease resistance genes originating from within the species. Using RAPD markers with *N. tabacum*, the general experience is that less than one polymorphism is revealed for every ten decamer primers tested (Wernsman, 1999). This apparent lack of molecular diversity could be related to the fact that tobacco is a natural allotetraploid formed by the hybridization of *N. sylvestris* as a maternal contributor and either *N. tomentosiformis* or *N. otophora* as a paternal contributor (Goodspeed, 1954; Gerstel, 1960). However, molecular markers have been easily identified for disease resistance genes introgressed from other *Nicotiana* species into tobacco.

The root-knot nematode resistance gene (*Rk*) which confers resistance to races 1 and 3 of the root-knot nematode (*Meloidogyne incognita*) has been mapped in tobacco with RAPD markers (Yi et al., 1998). NC 528 is a burley breeding line containing high resistance to race 1 and 3 of the root-knot nematode due to the presence of the *Rk* gene. This line was developed by crossing the flue-cured cultivar NC 95 to the cultivar Burley

21 and backcrossing the F₁ to Burley 21 to transfer the *Rk* gene. Resistant burley tobacco NC528, containing the *Rk* gene, and the susceptible cultivar Ky14 were screened with 1,500 random decamers. A low rate of genetic polymorphism (<10%) was detected between the parental lines. Two populations (F₁ and F₃) of maternal doubled haploid (MDH) lines from the cross NC528 x Ky 14, were used to construct a map of the *Rk* gene. NC528, Ky14, three *Rk*-resistant DNA bulks, and three *Rk*-susceptible bulks generated from F₁-derived MDH individuals were screened with the primers for bulked segregant analysis. Sixteen RAPD markers were positioned at six loci in a map 24.1 cM long. Six RAPD markers were mapped at the same position with *Rk* gene locus.

A single gene conferring complete resistance to a wide spectrum of black root rot isolates was first transferred to burley tobacco from *N. debneyi* Domin by interspecific hybridization and backcrossing (Hoffbeck et al., 1965; Clayton 1969). A flue-cured tobacco breeding line 'PB19' containing a resistant gene introgressed from *N. debneyi* Domin, and a flue-cured tobacco cultivar grown in Canada 'Delgold' which is moderately susceptible to black root rot were used as parental lines to identify RAPD markers (Bai et al., 1995). A total of 2594 reproducible polymorphic RAPD fragments were generated by 441 primers. Only seven of these primers produced eight polymorphic

bands between parents. These eight markers were used to test for the association with black root rot resistance gene in the F₂ population developed from the cross of PB19 and Delgold. One repulsion and one coupling phase marker were found to be linked with the resistance gene. Later, the coupling phase RAPD marker (UBC418₁₀₅₀) was cloned and sequenced. This marker designated Tnd-1 was found to be part of a retrotransposon that came from the *N. debneyi* genome (Kenward et al., 1999).

Johnson et al. (2002) reported that RAPD analyses were used to identify markers linked to the 'Ph' locus conferring resistance to the black shank disease in flue-cured tobacco (*N. tabacum*). The flue-cured cultivar Coker 371-Gold (C 371-G) has high levels of resistance to *P. parasitica* var. *nicotianae* race 0, and is controlled by the single dominant gene 'Ph' originating from *N. plumbaginifolia*. DH lines produced from the F₁ of C 371-G x K326 were used for this study. Sixty RAPD markers were found linked to the *Ph* locus by screening with 1237 decamer primers in one resistant and one susceptible bulk. Thirty RAPD markers, 26 in coupling and 4 in repulsion phase, were randomly selected for estimating linkage distance between the markers and *Ph* locus. Five marker recombination events were observed in K326-BC₂F₁ DH lines. Four events occurred in a single DH line, and two recombination events involved one RAPD marker. No

recombinants were observed for RAPD markers linked in repulsion phase to *Ph* locus.

Thus, determination of linkage distance was not feasible.

Blue mold is caused by the fungal pathogen *Peronospora tabacina* D.B. Adam, and resistance has been introgressed into cultivated tobacco from *N. debneyi*, *N. goodspeedii* Wheeler, and *N. velutina* Wheeler. Milla et al. (2005) identified RAPD and SCAR markers linked to the resistance. Bulk segregant analysis was used to screen for polymorphisms between DNA bulks from susceptible doubled haploid and resistant doubled haploid lines possessing resistance derived from cultivar Ovens 62 using 1216 RAPD primers. Fifteen RAPD markers (12 in coupling phase linkage with resistance and three in repulsion phase) were identified as being linked to the major resistance locus to blue mold and mapped to a single linkage group of 36.6 cM. Two RAPD markers flanking the most likely QTL position were converted to SCAR markers, so these markers should be useful for development of blue mold resistant tobacco cultivars.

Although development of molecular markers for disease resistance genes of tobacco origin is difficult, such markers have successfully been developed for bacterial wilt and potyvirus resistance. Bacterial wilt caused by *Ralstonia solanacearum* is a soil-borne disease. Breeding for resistant commercial varieties of tobacco is complicated by

the oligogenic nature of the resistance. Nishi et al. (2003) found one QTL linked to bacterial wilt resistance in tobacco. The susceptible Michinoku1 and the resistant W6 were screened for AFLP polymorphisms with 3,072 primer combinations. One hundred and seventeen polymorphic markers were identified. These markers were analyzed in 125 doubled haploid lines, derived from F1 hybrids of W6 x Michinoku 1, for analyzing the association between the markers and bacterial wilt resistance, and a linkage map consisting of ten linkage groups was drawn. One QTL for bacterial wilt resistance was identified on a 32 cM linkage group consisting of 15 markers. This QTL explained more than 30% of the variation in resistance.

Noguchi et al. (1999) identified RAPD markers linked to the tobacco *Va* locus for potato virus Y (PVY) susceptibility using two nearly isogenic lines (NILs) that differed in their susceptibility to PVY. Over 100 RAPD fragments that differed between the NILs were identified using each of 500 primers and 800 pairs of primers. An F₂ population obtained from a cross between the susceptible line BY4 and the resistant *va*²-bearing line F55 was screened with these RAPD primers. Ten RAPD markers were linked to the *Va* locus, and Southern analysis revealed that the resistance conferred by *Va* is due to deletions at the *Va* locus governing susceptibility to PVY.

SCAR markers linked to three disease resistances; black root rot, blue-mold, and PVY susceptibility, were reported by Julio et al. (2006). AFLP analysis was conducted on a set of 92 *N. tabacum* L. accessions of diverse market types and breeding origins to identify markers associated with disease resistances. The eleven most informative primer pairs out of sixty four primer combinations identified in preliminary AFLP assays were used to screen these accessions, and 33 reproducible polymorphic fragments were identified. A neighbor joining tree analysis revealed that seven AFLP fragments were associated with three different resistances: two for the blue mold resistance derived from *N. debneyi* Domini, two for the *Va* gene (potato virus Y susceptibility) and three for the black root rot resistance of *N. debneyi* origin. Each of these markers was converted into SCAR markers, and they were tested in recombinant inbred lines or doubled haploid lines to reveal the genetic distance between the SCAR marker and the resistance factor. The genetic distances between the SCAR marker and the black root rot resistance factor and the SCAR marker and PVY susceptibility were estimated to be 1 cM and 5.1 cM, respectively.

Flue-cure tobacco losses due to TSWV in NC

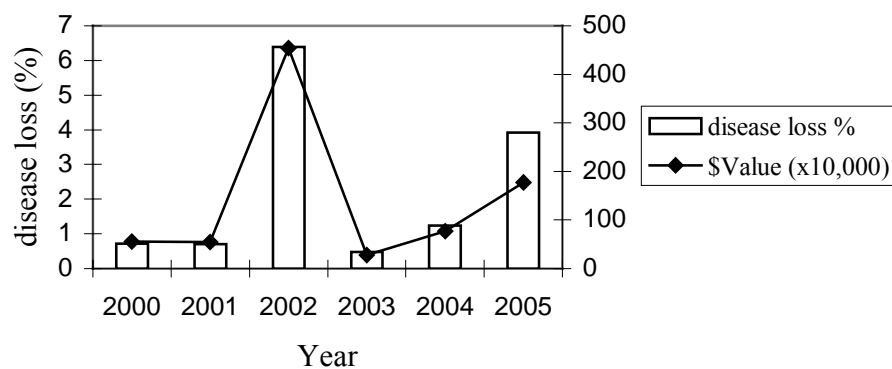


Figure1.1 Flue-cured tobacco disease loss estimates-North Carolina



Figure 1.2 K326 (left) and Polalta (right) symptoms after TSWV inoculation

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CHAPTER II. IDENTIFICATION OF AFLP MARKERS LINKED TO TOMATO SPOTTED WILT VIRUS RESISTANCE IN TOBACCO

ABSTRACT

Tomato spotted wilt virus (TSWV) is a serious disease in several crops such as tobacco (*Nicotiana tabacum.*), peanut (*Arachis hypogaea.*), tomato (*Lycopersicon esculentum.*), and pepper (*Capsicum annuum.*). One source of resistance in tobacco is the breeding line Polalta, which contains a TSWV resistance gene introgressed from the wild relative *Nicotiana alata*. Link & Otto. However, the resistance is associated with abnormal plant morphology and traditional backcrossing has been ineffective in producing normal plants with TSWV resistance. Molecular marker-assisted backcrossing allows for rapid identification of the plants that are most genetically similar to the recurrent parent and can be used to reduce the size of an introgressed chromosome segment. We applied AFLP (amplified fragment length polymorphism) technology and bulk segregant analysis to identify markers linked to TSWV resistance. One TSWV resistant bulk and two susceptible bulks were constructed by combining DNA from 5 doubled haploid lines from the cross K326 x Polalta for each bulk. A total of 128 primer

combinations were used to screen one resistant and two susceptible bulks, and I found 48 potential markers linked to the TSWV resistance. Thirty two markers were selected for further study based on their reproducibility. A population of 88 F₂ plants and 23 doubled haploid lines were screened with 32 markers and a 2.5 cM map with 24 markers was constructed. Eleven AFLP fragments between 100 to 400 bp in size linked in coupling phase to TSWV resistance were isolated and sequenced to develop PCR based markers. Four AFLP fragments were successfully converted to sequence characterized amplified region (SCAR) markers.

An F₂BC₂ population of 160 plants was screened with 5 AFLP coupling phase markers to select resistant plants that have fewer Polalta-derived markers and more K326-derived markers. No resistant line was identified with a reduced introgression from *N. alata*. In an F₂BC₃ population of about 200 plants, 8 plants were selected based on an improved phenotype and screened with the 17 AFLP coupling phase markers. Four plants with a reduced introgression were identified and will form the basis for future backcrossing. This approach is expected to facilitate development of a line with TSWV resistance and a normal phenotype.

INTRODUCTION

TSWV is the type member of the plant infecting genus *Tospovirus* in the arthropod-borne family *Bunyaviridae* (Elliott, 1990). The TSWV virion is bound by a lipid envelop containing two types of glycoproteins (G1 and G2) which encompasses a tripartite RNA genome containing S (2.9 kb), M (4.9 kb), and L (8.9 kb) RNAs. These RNA molecules are packaged with numerous copies of nucleocapsid protein (N) subunits (de Haan et al., 1990; 1991; German et al., 1992; Law et al., 1992).

TSWV was first identified on tobacco in Louisiana in 1973 (Lucas, 1975). Since 1973 TSWV has become increasingly widespread in the southeastern U.S. (Melton et al., 2002). This disease is particularly severe in Georgia, and in recent years there is typically a 30 % incidence and 15% yield loss due to TSWV, which is jeopardizing the viability of future production in this state (Bickers, 2006). TSWV is gradually affecting more areas of North Carolina tobacco production. North Carolina flue-cured tobacco losses due to TSWV were particularly high in 2002 and 2005 resulting in estimated losses of \$45 million and \$17 million, respectively (Broadwell and Gutierrez, 2005). The symptoms of TSWV in tobacco include chlorosis, ring patterns, mottling, stunting, and plant death (German et al., 1992). Infected tobacco plants exhibit all of these symptoms and all

growth stages are susceptible.

Damage from TSWV can be significantly reduced by application of the insecticide imidacloprid (Admire), or by the plant defense elicitor acibenzolar-S-methyl (Actigard). Pappu et al. (2000) evaluated the effect of these chemicals. The results showed Actigard alone significantly reduced final disease incidence to 5.8% compared to 17.3% the nontreated control. Admire reduced disease incidence to 30% in comparison to 60 % disease incidence in the nontreated control. When both were applied together the disease incidence was near zero for three of the four locations. However, the most effective way to minimize the damage of TSWV would be to grow resistant cultivars. In the case of flue-cured tobacco, sources of naturally available host plant resistance to TSWV are very limited. No resistance has been reported in the cultivated species, *N. tabacum* (Gajos, 1981). Opoka (1969) reported three wild species showing complete resistance to TSWV; *N. glauca* Graham, *N. alata*, and *N. noctiflora* Hooker. Gajos (1987) introgressed the *N. alata* resistance into the tobacco breeding line 'Polalta'. Yancheva (1990) reported that resistance derived from *N. alata* was inherited as a single dominant gene. However, the resistance is associated with abnormal plant morphology, which is particularly severe when Polalta is crossed with other tobacco lines. Traditional

backcrossing has not been effective in separating the resistance from the abnormal plant type. However, marker assisted backcrossing allows rapid identification of alleles conferring desirable traits and can be used to select for introgressions of reduced size. Lewis et al. (2005) showed increased potential for obtaining recombinants with minimal amounts of deleterious alien chromatin by screening with a set of AFLP markers linked to the N gene which is a gene for resistance to tobacco mosaic virus derived from *N. glutinosa* L.

The objectives of this study were (1) to identify AFLP markers linked to tomato spotted wilt virus resistance, (2) convert closely linked coupling phase AFLP markers into SCAR markers and (3) use these markers in a marker-assisted backcrossing program to incorporate tomato spotted wilt virus resistance into an acceptable cultivar.

MATERIALS AND METHODS

Population development

Seeds of the TSWV resistant cultivar Polalta and the susceptible flue-cured cultivar K326 were obtained from the USDA Tobacco Germplasm collection. To generate maternal haploid plants, K326 was crossed to Polalta, and the F_1 hybrid was crossed to *N. africana* (Burk et al., 1979). To generate paternal haploid plants, anther culture was performed on the F_1 hybrid. After haploid plants were generated, they were chromosome doubled in tissue culture using a midvein culture technique. The doubled haploid plants were used to form the resistant and susceptible bulks for bulked segregant analysis.

An F_2 population from K326 x Polalta was used to construct a map of the TSWV resistance locus. The genotype of each F_2 plant was determined by inoculating selfed ($F_{2:3}$) and backcrossed to K326 progeny (F_2BC_1). We inoculated 9 plants each from 88 F_2BC_1 plants. If the genotype of F_2 plant is homozygous resistance, all 9 plants of backcrossed progeny is resistant, and the genotype of F_2 plant is heterozygous resistance, backcrossed progeny is segregating as 1:1 (resistance:susceptible). Resistant F_2 plants

were backcrossed to K326 to produce F₂BC₂ and F₂BC₃ plants as breeding materials to be used in marker-assisted selection (Figure 2.1).

Production of F₁ DH and F₂ population

In order to develop a population for identifying AFLP markers linked to TSWV resistance, the TSWV susceptible flue-cured cultivar K326 was crossed to the TSWV resistant breeding line Polalta. F₁ plants of this cross were pollinated with *N. africana* to produce 36 maternal haploid plants, and seven paternal haploids were generated from anther culture of K326 x Polalta. Over 200 *N. africana* crosses were performed, which was expected to easily produce over 100 haploid plants. However, the crosses with Polalta produce plants with an abnormal leaf shape, which made it very difficult to visually select haploid plants. Twenty-three of the haploid lines were successfully chromosome doubled to produce doubled haploid lines.

Haploid production

To produce maternally derived haploids, K326 (a popular cultivar susceptible to TSWV) was crossed to Polalta (a line containing an introgression from *Nicotiana alata*). The F₁ hybrid was crossed to *N. africana*. To produce paternally derived haploids, floral buds of the F₁ hybrid were obtained when the calyx and corolla were of equal length.

Buds were surface sterilized, and anthers were aseptically cultured according to produce described by Kasperbauer and Collins (1972). Murashige and Skoog Basal Medium without growth regulators (Murashige and Skoog, 1962) with 3 % sucrose and 0.8 % agar was used for anther culture. Haploid plantlets started to emerge in 4-6 weeks, and they were transferred to MS medium containing indole acetic acid (IAA) (0.5 mg/ml) for rooting.

Doubled haploid production

Midribs from haploid plants were collected when they were at least 18 inches tall and before flowering. A 1 inch section of the midrib from the base of the leaf was sterilized and aseptically cultured on the same medium for anther culture, but containing IAA (0.5 mg/ml) and benzyladenine (BA) (1 mg/ml). After 3-4 weeks, shoots were transferred to rooting medium, and rooted plantlets were transplanted to soil.

TSWV inoculation

The Hawaiian L isolate of tomato spotted wilt virus used in this study was obtained from Tom German of the University of Wisconsin. The virus was maintained in *Emilia sonchifolia* L. The inoculation procedure was similar to that reported by Best (1964), with modifications. Plants were inoculated about 30 days after germination at the

4-6 leaf stage. The inoculation buffer (0.01 M Tris-HCl pH 7.8-8.0, 0.01 M Na₂SO₃, 0.1 % cysteine) was kept on ice before and during inoculation. The virus stock was collected from *E. sonchifolia*, and ground thoroughly in inoculation buffer (1:10 W/V) in a prechilled mortar and pestle. Carborundum (600 mesh) was dusted on the leaves to be inoculated, and the inoculum buffer was painted onto the leaves with a brush. The inoculated leaves were rinsed with water within 15 minutes after inoculation. The plants were kept at room temperature for 2-3 days and then placed in a 25 °C growth room. Plants were scored for disease symptoms 14 days after inoculation. Resistant plants showed a hypersensitive type response at the invasion sites, and susceptible plants developed spreading necrotic lesions on the inoculated leaf and a systemic infection that usually resulted in plant death.

AFLP analysis

The AFLP technique was performed using the method described by Vos et al. (1995) with modifications by Myburg et al. (2000). All primers and adaptors were obtained from Sigma Genosys (The Woodlands, TX) except for the labeled primers. Labeled primers were obtained from Licor Inc (Lincoln, NE).

Digestion of DNA DNA was extracted from leaf tissue using Plant DNAzol from Invitrogen (Carlsbad, CA) according to the manufacture's instructions. A total of 500 ng DNA was digested with *EcoRI* and *MseI* for 1.5 hr at 37 °C in a final volume of 30 µl containing 6 µl of 5 X reaction buffer (50 mM Tris-acetate, 50 mM magnesium acetate, 250 mM potassium acetate, 25 mM DTT, and 250 ng/ul BSA). After incubation, 10 µl of the reaction was run on a 0.8 % agarose gel to test for success of the complete digest.

Adaptor ligation Five µl of the adaptor ligation mix (1 µl of 5X R/L buffer (50 mM Tris-acetate, 50 mM magnesium acetate, 250 mM potassium acetate, 25 mM DTT, and 250 ng/ul BSA), 5 pMol *EcoR* I adaptor, 50 pMol *Mse* I adaptor, 10 mM ATP, and 0.5 U of T4 DNA ligase) was added to 20 µl of digested DNA samples, incubated overnight at 37 °C, and diluted 1:10 with dH₂O.

Pre-amplification Preamplifications were performed with *EcoR* I+A, *Mse* I+C primers. PCR reactions consisted of 5 µl diluted restriction-ligation product, 10 X PCR buffer (100 mM Tris HCl pH 8.3, 15 mM MgCl₂, 500 mM KCl), 2.5 mM dNTP mixture, and 1.2 U Taq polymerase (New England Biolabs, Ipswich, MA). PCR conditions for preamplifications were 94 °C 15 s, 60 °C 30 s, 72 °C 60 s +1 sec/cycle extension (28 cycles), with a final extension at 72 °C for 2 min. Ten µl of preamplification product was

separated on a 0.8 % agarose gel. Amplified products were visible as a smear in the 100 to 1200 bp range. The remaining 10 µl was diluted 1:20 by adding low TE.

Selective amplification and polyacrylamide gel electrophoresis Selective amplification was done with *Mse*I and *Eco*RI primers containing three selective nucleotides (Table 2.1). The *Eco*RI primers were labeled with fluorescent tags (IRD-700 or IRD-800) for display in polyacrylamide gels. The reaction mixture contained 10 X PCR buffer (100 mM Tris HCl pH 8.3, 15 mM MgCl₂, 500 mM KCl), 2.5 mM of each dNTP, 30 ng *Mse* I primer, 5 ng labeled *Eco*R I primer, 1.2 U Taq DNA polymerase, and 5 µl of diluted preamplification product. Selective amplification was carried out in a Eppendorf mastercycler equipped with a hot bonnet using the following cycle profile: 13 cycles of 10 s at 94 °C, 30 s at 65 °C –0.7 °C/cycle after the first cycle and 60 s at 72 °C: followed by 25 cycles of 10 s at 94 °C, 30 s at 56 °C, and 60 s +1 s/cycle extension at 72 °C with final extension of one cycle of 2 min at 72 °C. Upon completion of amplification, reaction products were mixed with 10 µl of formamide loading dye (95 % deionized formamide, 20 mM EDTA and 0.8 mg/ml bromophenol blue). The mixture was denatured for 3 min at 94 °C and then immediately chilled on ice for at least 10 min. About 0.1-0.2 µl of each sample was loaded on a 8 % polyacrylamide gel.

Electrophoresis was performed on a Licor DNA analyzer 4300 with the following conditions: 1500V, 40mA, 40W, 45°C for 3 hr. A IRDye 700 or 800 labeled molecular weight standard (50-700 bp) was loaded on each gel for sizing of the AFLP fragments. The AFLP Quanta 1.0 software (KeyGene Products B.V., The Netherlands) was used to score polymorphic bands.

Bulked segregant analysis

Bulked segregant analysis (BSA) (Michelmore et al., 1991) was used to identify markers linked to TSWV resistance. Bulks were prepared by combining 100 ng of DNA (total DNA per reaction = 500 ng) from doubled haploid plants identified by inoculation. One resistant and two susceptible bulks were constructed and screened along with K326 and Polalta to identify potential markers. We named the markers as *Mse* I and *EcoR* I primer combination and its size in bp.

Linkage analysis

Chi-square analyses were conducted to test 3(+) : 1(-) segregation of markers in the F₂ segregating population. Linkage between markers and the TSWV resistant gene was performed with Joinmap 3.0 (Ooijen and Voorrips, 2001). The marker and phenotypic data were coded as follows: an F₂ individual homozygous susceptible='A',

heterozygous='H', and homozygous resistant='B' and missing data='-', Not genotype A (dominant b-allele)='C' and Not genotype B (dominant a-allele)='D'. A genetic map was constructed based on a LOD score of 3 and a maximum recombination fraction of 0.4. Map distances were calculated using the Kosambi mapping function (Kosambi, 1944) which assumes a moderate amount of interference between crossovers.

Development of single locus PCR markers

Isolation and sequencing of AFLP markers For conversion of coupling phase AFLP markers into locus specific markers, 11 AFLP fragments between 100 and 400 bp were selected. The banding pattern for each of these reactions was first simplified by amplifying with an *EcoR* I +3 primer in combination with an *Mse* I+6 primer that was identified by AFLP mini-sequencing as described by Brugmans et al. (2003). AFLP-mediated mini-sequencing was performed to determine the fourth, fifth, and sixth selective nucleotide following the AFLP-restriction site using a generalized set of 12 degenerate primers (Table 2.2). The reaction and temperature cycling conditions were the same as described for the selective amplification except for that the +3/+3 selectively amplified product was diluted by 100 times and used as the template, and the *EcoRI* and *MseI* primer concentrations were 0.5 pmol and 3 pmol, respectively.

AFLP fragments in the polyacrylamide gel were visualized in the gel by scanning on a Licor Biosciences Odyssey Infrared Imaging system set at the following conditions: Resolution 169, Quality medium, Focus Offset 0.3 mm, Intensity 9.0. The desired bands were cut out of the gel and successful fragment extraction was verified by re-scanning the gel. The extracted gel plug were placed in 50 μ l of 1 x TE, and frozen at -20°C for ~ 30 min. After thawing, samples were mashed and centrifuged for 20 min at 15,000 g. Five μ l of supernatant was taken for re-amplification. Two procedures, pre-amplification, and selective amplification, were performed using the same primers that were originally used to generate the band with the same PCR cycling conditions. Reamplified PCR products were purified with the Promega Wizard Gel and PCR CleanUp Kit (Promega, Madison, WI). The concentration of the purified DNA was checked on a 0.8% agarose gel by comparison with a Low DNA mass ladder (Invitrogen, Carlsbad, CA). Sequencing reactions were carried out by Northwoods DNA Inc (www.nwdna.com).

Conversion of AFLP markers to SCAR markers Specific forward and reverse primers were designed from the sequence information of each AFLP fragment using the Primer 3 program (Rozen and Skaletsky, 2000). PCR with the newly designed internal primers was performed in a 15 μ l total volume consisting of 200 ng genomic DNA, 10 μ M each

primer and Promega PCR master mix. The PCR cycle was one cycle of 2 min at 94 °C , 34 cycles of 30 s at 94 °C, 30 s at annealing temperature and 40 s at 72 °C with a final extension at 72 °C for 5 min. The primer sequences and annealing conditions are listed in Table 2.4. For several markers, the internal primers amplified a similar fragment in both K326 and Polalta. To attempt to develop cleaved amplified polymorphic sequences (CAPS) markers, these fragments were screened for internal polymorphisms with the restriction enzymes *Alu* I, *Hinf* I, *Hind* III, *Hpa* II, *Hpy*CH4V, *Mse* I, *Hae* III, *Sca* I, *Taq* I. Ten µl of the PCR product was digested for 2 hr in total volume of 20µl with 10 units of the restriction enzyme. After digestion, 10µl of the restriction digest was analyzed for DNA fragment length on a 2 % agarose gel.

RESULTS

Characteristics of TSWV resistance

K326 is a popular flue-cured tobacco cultivar that is susceptible to TSWV, and Polalta is a breeding line which contains a TSWV resistance gene introgressed from *N. alata*. When Polalta plants are mechanically inoculated with TSWV, they exhibit a hypersensitive response on the inoculated leaf. Susceptible K326 plants develop expanding TSWV lesions on the inoculated leaf and a systemic infection that usually results in the death of the plant. The F₁ hybrid plants of K326 x Polalta are fully resistant to TSWV. In 144 plants in an F₂ population inoculated with TSWV, 108 were resistant and 36 were susceptible. The segregation of resistant:susceptible plants was not significantly different from the 3:1 ratio expected for the segregation of a single dominant gene ($\chi^2=0$, P=1). This result agreed with the previously reported inheritance of this resistance as a single dominant gene (Yancheva 1990).

The resistance in two additional European breeding lines was also examined. Virginia wiktoria and Virginia ZG8 have resistance to TSWV derived from Polalta. These two lines also show the hypersensitive-response type of resistance when they are inoculated with TSWV. The F₁ plants from a cross of Virginia wiktoria to K326 and

Virginia ZG8 to K326 showed the same resistance response, indicating that the resistance was dominant and acts in the same way as the Polalta resistance.

Bulked segregant analysis

Out of twenty three doubled haploid lines, seven of these doubled haploid lines were TSWV resistant and 16 lines were susceptible. DNA of five lines for each bulk was combined to form one resistant and two susceptible bulks for bulked segregant analysis. A total of 128 AFLP primers were used to identify polymorphisms between the susceptible bulks and K326 and the resistant bulk and Polalta. These primers generated 48 polymorphic markers potentially linked to TSWV resistance. Thirty one markers were in coupling phase with TSWV resistance and 17 markers were in repulsion phase. Thirty-two markers (20 coupling and 12 repulsion) were selected based on their reproducibility and were screened against an F₂ population of 88 plants from K326 x Polalta for construction of a linkage map. The F₂ plants were selfed and backcrossed to K326 for progeny testing to determine the TSWV resistance genotype.

Construction of linkage map

Of 32 markers screened against the F₂ population, 17 coupling and 7 repulsion markers (Figure 2.2 and Figure 2.3) were found to be linked to the TSWV resistance gene

and a 2.3 cM map of the locus was constructed (Figure 2.4). When this map was combined with the marker data identified in 23 doubled haploid lines to improve the precision, a 2.5 cM map of the locus was constructed (Figure 2.5). Very little recombination was observed in this population. In the combined map, fifteen coupling phase markers and four repulsion markers were mapped at the same location with the TSWV resistance.

TSWV resistance originated from *N. alata*. The AFLP markers linked to TSWV resistance were tested for their presence in *N. alata*. All the coupling phase markers were present, confirming that this species was the origin of TSWV resistance.

There were two additional breeding lines, Virginia wiktoria and Virginia ZG8, with TSWV resistance derived from Polalta. These two lines were screened with the AFLP markers linked to TSWV resistance. These lines showed an almost identical banding pattern to Polalta and contained all of the coupling phase markers, indicating that they contained a similar introgression (Table 2.5).

Marker assisted backcrossing for TSWV resistance

An F₂BC₂ population of 160 TSWV resistant plants was screened with 5 AFLP coupling markers (CCCACT169, CCGAAC248, ACAACG279, CGAAAG228,

CCCACT54) to select resistant individual plants that have fewer Polalta-derived markers.

Among 160 F₂BC₂ plants, there was no resistant line identified with a reduced introgression from *N. alata*.

In the F₂BC₃ generation, approximately 200 TSWV resistant plants were selected after inoculating 1080 plants. From these, 8 plants were selected based on an improved phenotype although not completely normal, and screened with 17 AFLP coupling phase markers. Four lines were identified that have between 2 –13 fewer Polalta derived markers (Table 2.6). In this generation, it was also observed that there were several plants with the abnormal plant type associated with Polalta that were TSWV susceptible, suggesting that the resistance and abnormal plant type could be separated.

Conversion of AFLP markers into SCAR markers

For allele specific markers, AFLP markers are less suitable for marker-assisted selection or map-based cloning because AFLP markers are expensive and labor-intensive for large-scale single locus screenings. To overcome this problem, there is a need to convert specific AFLP markers into simple PCR markers such as cleaved amplified polymorphic site (CAPS) or sequence characterized amplified region (SCAR) markers. SCAR markers provide many advantages in comparison with AFLP, and they are useful

for marker-assisted selection in many segregating populations. As potentially codominant markers, they may allow the detection of heterozygous plants and enhance the mapping precision.

For conversion of the AFLP markers into SCAR markers, eleven coupling phase AFLP fragments between 100 and 400 bp were chosen. The original primers contained three selective nucleotides in the genomic DNA following the AFLP restriction site. The fourth through sixth selective nucleotides following the *Mse* I site were determined by AFLP mini-sequencing using a generalized set of 12 primers. The main purpose of AFLP-mini sequencing is to reduce the number of amplified fragments, which increases the probability of successful excision of the desired fragments from the polyacrylamide gels. Information on the fourth, fifth, and sixth selective nucleotides could be determined unambiguously and was used to generate *Mse* I + 6 primers (Table 2.3, and Figure 2.6). The newly amplified AFLP fingerprint using *Eco*R I + 3 / *Mse* I + 6 primers produced reactions with much lower fragment complexity than *Eco*R I + 3 / *Mse* I + 3 primers (data not shown).

Eleven AFLP fragments were isolated from polyacrylamide gels and sequenced in order to develop PCR-based markers. Four AFLP markers (CCCAAC172, CCGACG169,

CGAAAG228, CTAAC268) were successfully converted into SCAR markers. Out of four, three SCAR markers (CCCAAC172, CCGACG169, CGAAAG228) produced a single amplification product that is present in Polalta and four resistant doubled haploid lines, but absent in K326 and four susceptible doubled haploid lines using genomic DNA as template, and one SCAR marker from the CTAAC268 AFLP fragment generated a fragment in both the susceptible and resistant lines but there is a clearly visible difference in the size of these fragments (Figure 2.7, Figure 2.8, and Figure 2.9). Therefore, this marker still can be useful as SCAR marker. However, the PCR primers developed from six AFLP markers (CCCAAG292, CACACG279, CGGAAC138, CCCACT169, CCGAAC248, CTGAAC507) amplified a similar fragment in both K326 and Polalta. In the case of AFLP marker CGAAGC160, the AFLP fragment was excised from the gel, but it failed to re-amplify for sequencing. An attempt to develop CAPS markers for AFLP fragments from CCGAAC248 and CTGAAC507 was made. The fragments amplified from K326 and Polalta were digested with several restriction enzymes, but no polymorphisms in restriction digests were observed.

DISCUSSION

This study reports identification of AFLP and SCAR markers linked to tomato spotted wilt virus resistance in a tobacco breeding line carrying a resistance gene derived from *N. alata*. In this study, the AFLP technique was combined with bulked segregant analysis to identify DNA markers linked to the TSWV resistance. The identification of 24 markers within a single linkage group associated with the TSWV resistance confirmed that a single genetic locus controls resistance in Polalta. The tight linkage with all 24 markers is indicative of low recombination of this locus with the tobacco genome. Although tight linkage is a prerequisite for successful MAS, the presence of 17 very tightly linked coupling markers indicated reduced recombination between the introgressed chromosomal block and tobacco genome. Recombination between the markers and the TSWV resistance gene was highly suppressed in the introgressed block, as very few recombinants were observed in the F₂ population used in this study. A similar situation exists for resistance to black root rot, root knot nematode and black shank (Bai et al., 1995; Yi et al., 1998; Johnson et al., 2002). Low recombination in introgressed regions around interspecific resistance genes has also been reported in tomato (Chungwongse et al., 1994; Young and Tanksley, 1989). Suppressed recombination in

regions around an introgressed interspecific resistance gene makes reducing the effect of linkage drag difficult. Therefore, traditional backcrossing can be ineffective in separating the alien segment from the resistance allele (Tanksley et al., 1989). The molecular marker- assisted backcrossing approach has the potential to increase the chance to develop a plant with TSWV resistance and a normal phenotype by rapid identification of the plants that are most genetically similar to the recurrent parent.

Due to limitations of the AFLP technique, AFLP markers cannot be conveniently used for large-scale selection. In contrast, the conversion of these markers into simple, PCR-based markers will expand the usefulness of the technique. The conversion procedure of AFLP markers into single locus marker has been presented by Brugmans et al. (2003). The most likely difficulty encountered in the conversion of AFLP markers was the extraction of AFLP fragments from polyacrylamide gels. Contamination by undesirable AFLP fragments which are co-segregating with the AFLP fragment of interest may cause the extraction to contain multiple fragments. In such a case, background products from the standard AFLP fingerprint (*Mse* I+3/*Eco*R I+3) appear as very clear bands in the AFLP-mediated mini sequencing (*Mse* I+6/*Eco*R I+3). Therefore, AFLP-mediated mini sequencing increases the probability of successful isolation of the desired

fragments (Brugmans et al., 2003). For all 11 AFLP fragments, AFLP-mediated mini sequencing could be useful to reduce the complexity adjacent to the AFLP fragments of interest. Consequently, four SCAR markers obtained provided a single amplification product using the internal primers using genomic DNA. Several of the AFLP markers could not be converted to SCAR markers because the primers selected amplified similar bands in both parents. In this case, the AFLP polymorphism is likely to be a small difference in the *EcoR* I or *Mse* I restriction site or in the selective nucleotides.

The two purposes of molecular markers used in recurrent backcrossing are to trace the presence of a target allele and to identify individuals with a low proportion of the undesired genome segment from the donor parent. Frisch et al. (1999) studied the minimum number of individuals to be genotyped to obtain desired individuals which carry the target allele and have a maximum proportion of the recurrent parent genome on the carrier chromosome. Their analysis indicated that for varying chromosome length between one end of the chromosome and the target locus (25 –200 cM) with tightly linked flanking markers (2 cM), 300-500 individuals are required in order to obtain at least one recombinant with 99 % probability. Consequently, several hundreds or thousands of individuals may need to be genotyped to achieve ultimate success by a

molecular assisted backcrossing approach to eliminate linkage drag effects for further study. However, in our study, four plants with an improved phenotype and a partially reduced introgression have been identified in the F₂BC₃ generation. These plants are being backcrossed to K326 and the next cycle of selection will begin using the SCAR markers and phenotype selection. By initially using phenotypic selection, several thousand plants could be examined for resistance and improved plant type. The best plants could then be examined with the SCAR or AFLP markers to select those plants with the smallest introgressed block.

If this approach is successful in producing a TSWV resistant cultivar with acceptable yield and quality, it would likely be popular in areas with high TSWV pressure. Some resistance genes of interspecific origin have a negative effect on yield and quality, such as the *Ph* gene for black shank resistance or the *N* gene for tobacco mosaic virus resistance (Lewis et al., 2005). However, these resistances can be successfully deployed in hybrid form and this may also be necessary for TSWV resistance. One possible concern with the use of this gene is the ability of TSWV to eventually overcome resistance genes such as the *Sw-5* gene in tomato. However, the Polalta resistance is the only TSWV resistance available in tobacco at the present time.

So far, RAPD, AFLP, and SCAR molecular markers have been identified that are linked to root knot nematode (*Rk*), black shank (*Ph*), potato virus Y (*Va*), black root rot, blue mold and bacterial wilt resistance (Yi et al., 1998; Johnson et al., 2002; Noguchi et al., 1999; Bai et al., 1995; Milla et al., 2005; Nishi et al., 2003; Julio et al., 2006). These markers are already being used in marker-assisted selection in tobacco breeding to select for breeding lines with multiple disease resistance traits. In the future, these markers and the TSWV resistance markers identified here will provide a starting point for the cloning of resistance genes to determine the structure of these genes and how they interact with pathogens.

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Table 2.1 *EcoR* I /*Mse* I primer combinations used to screen for polymorphisms among K326, Polalta, one resistant bulk and two susceptible bulks

<i>EcoR</i> I primer	<i>Mse</i> I primer
5'-GACTGCGTACC AATTC NNN-3'	5'-GATGAGTCCTGAG TAA NNN-3'
Selective nucleotides	Selective nucleotides
AAC	CAA
AAG	CAC
ACA	CAG
ACC	CAT
ACT	CTA
ACG	CTC
AGC	CTG
AGG	CTT
	CCA
	CCC
	CCG
	CCT
	CGA
	CGC
	CGG
	CGT

Table 2.2 The generalized set of 12 primers for AFLP-mediated mini-sequencing

Primer name	Sequence	
3N+A	GATGAGTCCTGAGTAA NNNA	For determination of the fourth selective nucleotide
3N+C	GATGAGTCCTGAGTAA NNNC	
3N+G	GATGAGTCCTGAGTAA NNNG	
3N+T	GATGAGTCCTGAGTAA NNNT	
4N+A	GATGAGTCCTGAGTAA NNNNA	For determination of the fifth selective nucleotide
4N+C	GATGAGTCCTGAGTAA NNNNC	
4N+G	GATGAGTCCTGAGTAA NNNNG	
4N+T	GATGAGTCCTGAGTAA NNNNT	
5N+A	GATGAGTCCTGAGTAA NNNNNA	For determination of the sixth selective nucleotide
5N+C	GATGAGTCCTGAGTAA NNNNNC	
5N+G	GATGAGTCCTGAGTAA NNNNNG	
5N+T	GATGAGTCCTGAGTAA NNNNNT	

(Brugmans et al., 2003)

Table 2.3 Eleven selected AFLP marker conversion with their extra selective nucleotide for the *Mse* I primer

Fragment and fragment length (bp)	Fourth, fifth and sixth selective nucleotide for the <i>Mse</i> I primer
M-CCC/E-ACT-169	ATA
M-CCG/E-AAC-248	AGA
M-CAC/E-ACG-279	TTG
M-CCG/E-ACG-169	AGT
M-CCC/E-AAG-292	AAA
M-CCC/E-AAC-172	CAA
M-CGA/E-AAG-228	AAT
M-CGG/E-AAC-138	TAG
M-CGA/E-AGC-160	GTT
M-CTA/E-ACT-268	GGA
M-CTG/E-AAC-507	TCC

Table 2.4 Forward and reverse primer sequences for SCAR makers

AFLP Fragment	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)	Products of Polalta (bp)	Products of K326 (bp)
CCCAAC172	AGCTTCTTTTCTCTTTCCATTTT	CAGAAGAAAACTGCTGGAGCTAT	57	117	-
CTAACT268	CTGATCGTTCCAGCAGGTTCTTAT	GGAGCTATTTCCAGACACGAA	57	161and200	200
CCGACG169	ACTTTTCACACCAAAAACTCACG	GATGATGATAAAGATTGAAGAAAACAA	50	105	-
CGAAAG228	TAGATGTCATGAATGGAACACGG	TTTGATCGAAAAACCCAACC	55	117	-

Table 2.5 Evaluation of a population of doubled haploid lines, and two breeding lines with 24 AFLP markers linked to tomato spotted wilt virus resistance

Classif.	Sus	Res	Res	Res	Res	Res	Res	Res	Res	Res	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Sus	Res	Res	Res
Markers	K326	Pol	DH2	DH3	DH8	DH31	DH49	DH52	DH50	DH6	DH9	DH24	DH29	DH30	DH44	DH46	DH53	DH59	DH61	DH58	DH48	DH25	DH54	DH56	DH23	N.ala	V.W	V.Z
CTGAAC340	+	-	+	+	+	+	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	
CCCACT169	-	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CGAAAG94	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CCGAAC248	-	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	
CTGAAC514	-	+	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CACACG279	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CCGACG169	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CCCAAG292	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CAGACG432	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CTGAAC507	-	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CCCAAC172	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CGAAAG228	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CCCACT54	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CGGAAC138	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CGAAGC160	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CTAACT268	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CGAAAG84	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CGTAGG75	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
CCGACC300	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
CAAACG320	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
CCGAGG113	+	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CCCAAC317	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CGTAAG207	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
CCGAGC424	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-

Res= resistant, Sus=susceptible, N.ala= *N. alata*, V.W=Virginia wiktoria, V.Z= Virginia ZG8. White color: coupling marker, Yellow color:Repulsion marker.

Table 2.6 Screening with 17 coupling AFLP markers for 8 most promising F₂BC₃ plants selected by phenotype

AFLPmarkers	K326	Polalta	GH05-664	GH05-766	GH05-797	GH05-811	GH06-1	GH06-7	GH06-30	GH06-33
CGAAAG84	-	+	+	-	+	-	+	+	+	+
CTAACT268	-	+	+	+	+	+	+	+	+	+
CGTAGG75	-	+	+	+	+	-	+	+	+	+
CAGACG432	-	+	+	-	+	-	+	+	+	+
CACACG279	-	+	+	-	+	-	+	+	+	+
CCCAAC172	-	+	+	+	+	-	+	+	+	+
CGAAAG94	-	+	+	+	+	-	-	+	+	+
CCCACT54	-	+	+	+	+	+	-	+	+	+
CCCAAG292	-	+	-	+	+	-	+	+	+	+
CGAAGC160	-	+	+	-	+	-	+	+	+	+
CCGACG169	-	+	+	+	+	+	+	+	+	+
CGAAAG228	-	+	+	+	+	+	+	+	+	+
CGGAAC138	-	+	+	+	+	-	+	+	+	+
CTGAAC514	-	+	+	+	+	-	+	+	+	+
CTGAAC507	-	+	+	+	+	-	+	+	+	+
CCCACT169	-	+	+	+	+	-	+	+	+	+
CCGAAC248	-	+	-	-	+	-	+	+	+	+

Red color : four F₂BC₃ plants that have fewer Polalta derived markers

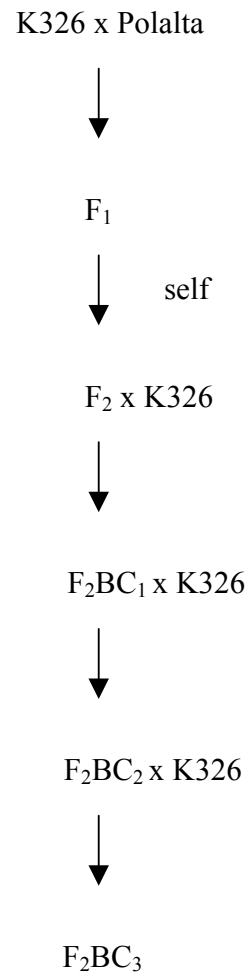


Figure 2.1 Development of populations used for marker-assisted backcrossing

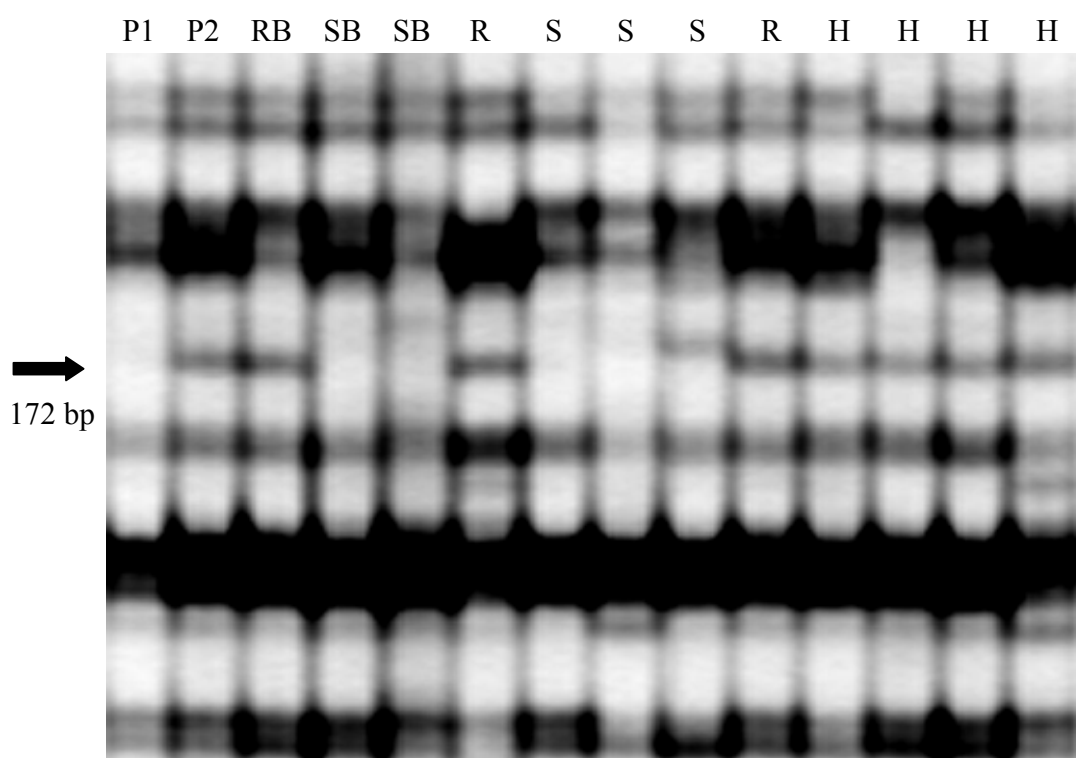


Figure 2.2 CCCAAC172 coupling phase AFLP marker Screening of F_2 population with AAC *EcoR* I and CCC *Mse* I primer combination. P1: Susceptible parent (K326), P2: resistant parent (Polalta), RB: resistant bulk, SB: susceptible bulk, R: homozygous resistant, S: susceptible, H: heterozygous resistant.

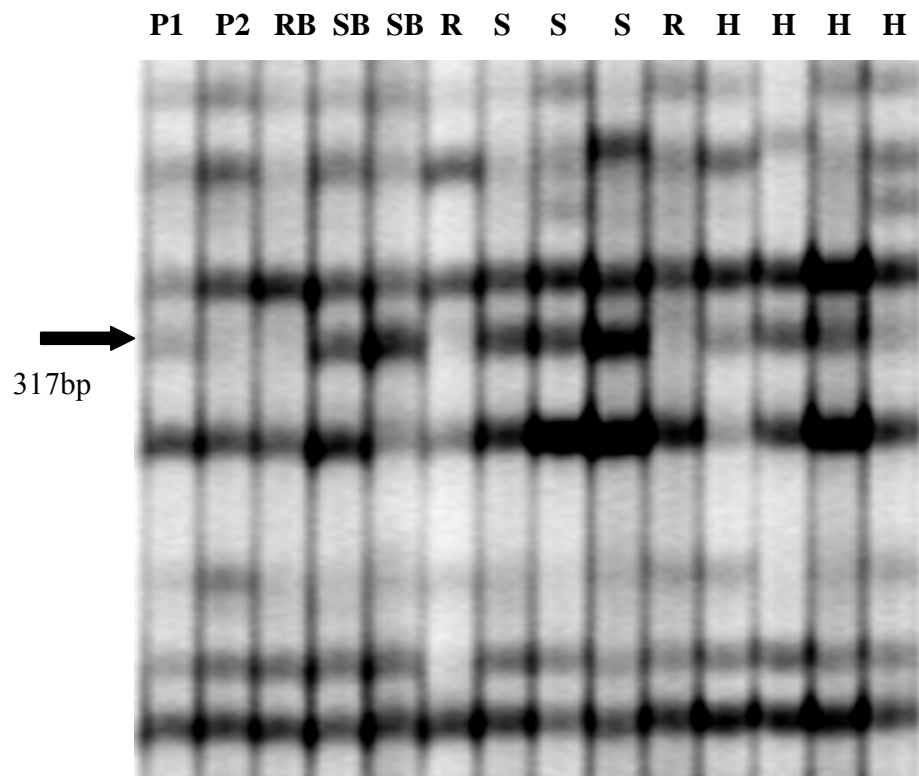
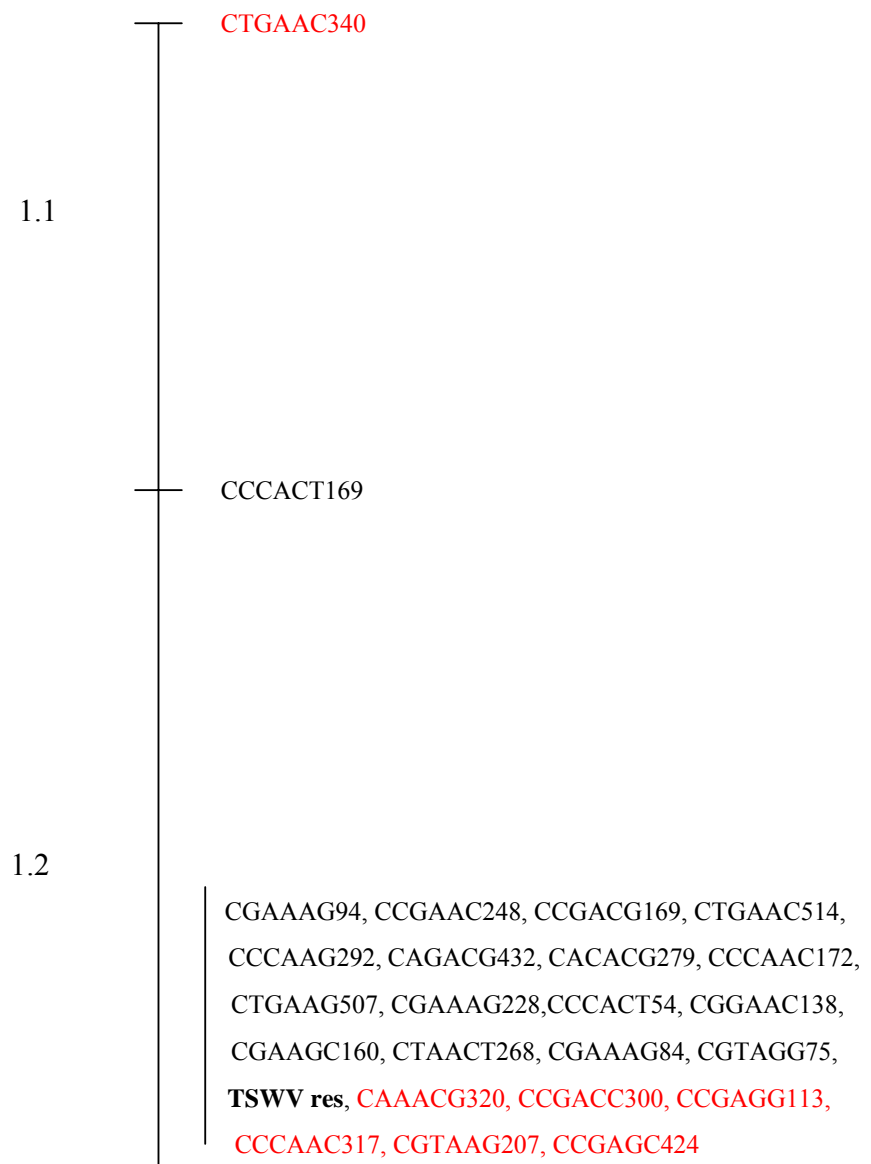


Figure 2.3 CCCAAC317 repulsion phase AFLP marker Screening of F_2 population with AAC *EcoR* I and CCC *Mse* I primer combination. P1: Susceptible parent (K326), P2: resistant parent (Polalta), RB: resistant bulk, SB: susceptible bulk, R: homozygous resistant, S: susceptible, H: heterozygous resistant.

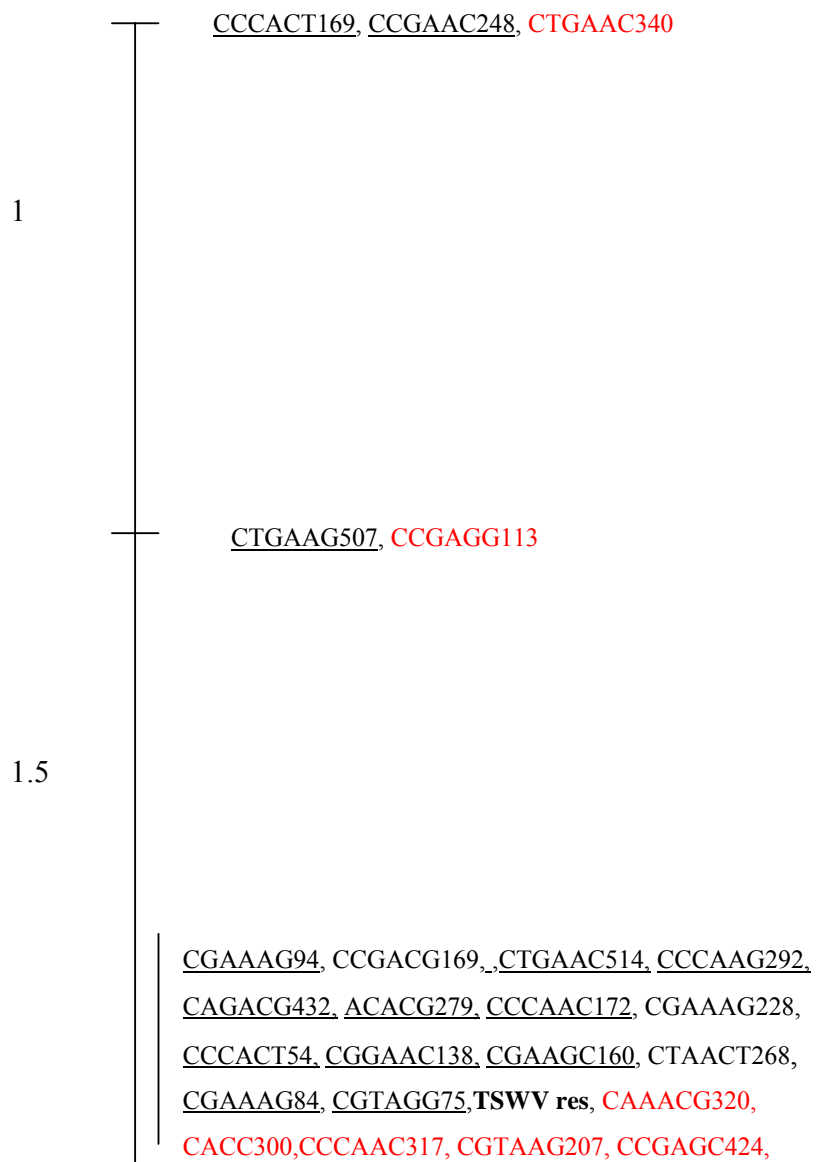


Red color : repulsion phase markers

Black color : coupling phase markers

Figure 2.4 Genetic mapping of the TSWV resistance gene based on 88 F₂ plants

The map includes 24 loci and covers a genetic distance of 2.3 cM. Joinmap was used to construct the map using a LOD score of 3 and a maximum recombination fraction of 0.4. Map distance in cM shown on left.



Red color : repulsion phase markers

Black color : coupling phase markers

Underline : coupling markers that are missing in F_2BC_3 plants selected by phenotype

Figure 2.5 Combined genetic mapping of the TSWV resistance gene based on 88 F_2 plants and 23 doubled haploid lines The map includes 24 loci and covers a genetic distance of 2.5 cM. Joinmap was used to construct the map using a LOD score of 3 and a maximum recombination fraction of 0.4. Map distance in cM shown on left.

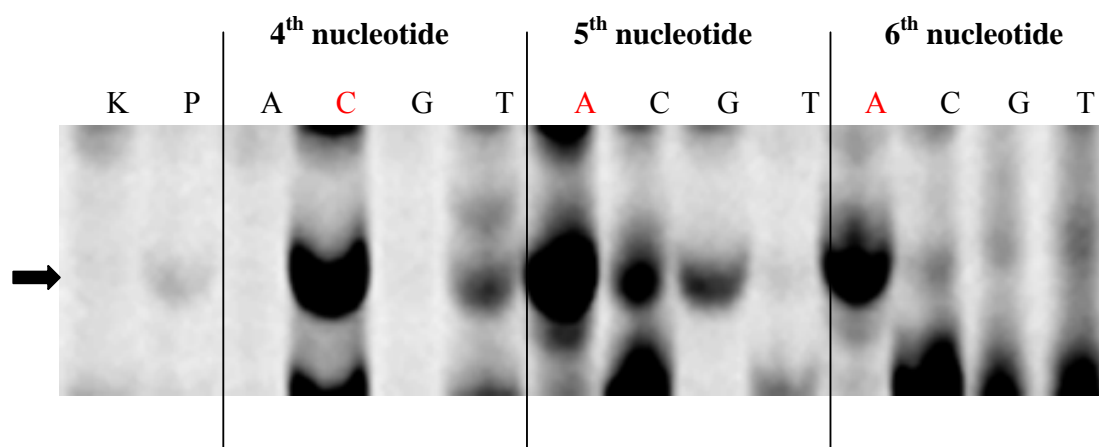


Figure 2.6 AFLP-mediated mini-sequencing For determination of the fourth, fifth, and sixth selective nucleotide following AFLP-restriction site. K: K326, P: Polalta
e.g. fourth, fifth, and sixth selective nucleotides for the fragment are C-A-A.

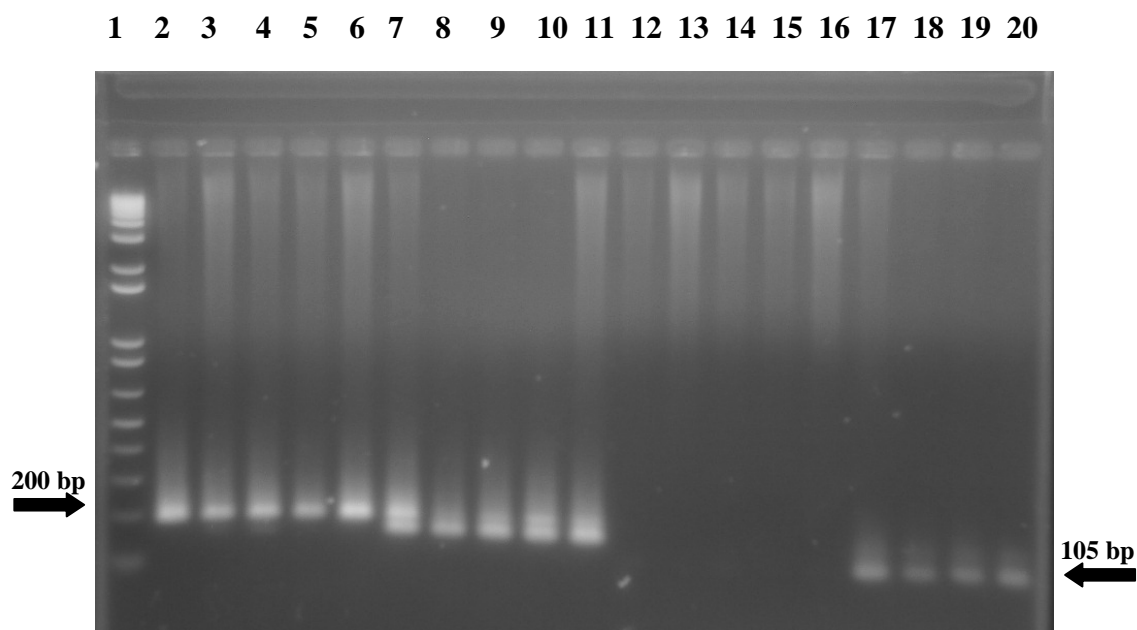


Figure 2.7 SCAR marker S-CTAACT268 and S-CCGACG169 Lane 2-11 : S-CTAACT268, Lane 12-20: S- CCGACG169, Lane 1: 1 kb ladder, Lane 2: K326, Lane3-6 : susceptible doubled haploid lines, Land 7: Polalta, Lane 8-11 : resistant doubled haploid lines, Lane 12: K326, Lane13-16: susceptible doubled haploid lines, Land 17: Polalta, Lane 18-20: resistant doubled haploid lines.

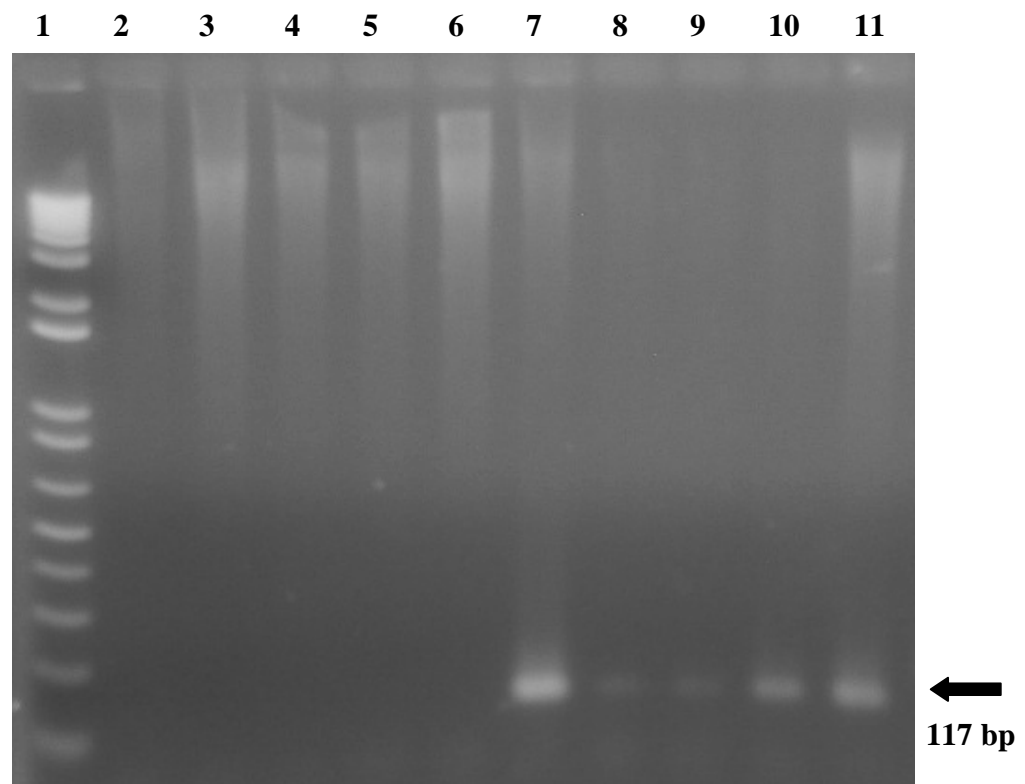


Figure 2.8 SCAR marker S-CCCAAC172 Lane 2-11: S-CCCAAC172, Lane 1: 1 kb ladder, Lane 2: K326, Lane3-6 : susceptible doubled haploid lines, Land 7: Polalta, Lane 8-11 : resistant doubled haploid lines.

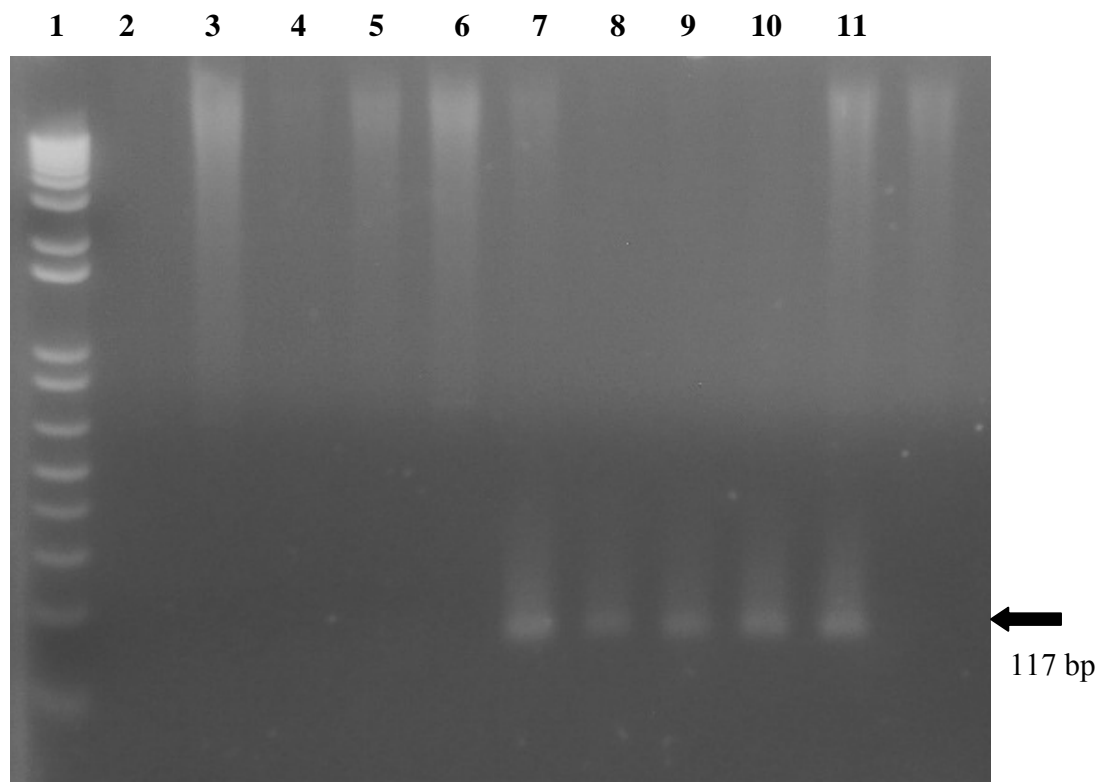


Figure 2.9 SCAR markers S-CGAAAG228 Lane 2-11: S-CGAAAG228 Lane 1: 1 kb ladder, Lane 2: K326, Lane3-6: susceptible doubled haploid lines, Land 7: Polalta, Lane 8-11 : resistant doubled haploid lines