

# ***Progress Towards Development of a Molecular Marker for Begomovirus Resistance in Tomato***

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## ***Abstract***

Each year begomoviruses cause millions of dollars in damage to tomato crops in Central America. Attempts to create a resistant plant with a suitable fruit through breeding programs have been unsuccessful. This study hoped to develop a molecular marker for the gene that controls resistance to begomoviruses. A successful molecular marker would enable breeders to quickly determine a tomato's susceptibility or resistance to begomoviruses and greatly aid in the creation of a commercially viable resistant hybrid. We hypothesized that a molecular marker for begomovirus resistance could be found within either the resistance gene hotspots on chromosomes one or seven, or the resistance gene homologue hotspots on chromosomes two, four, or eleven. To accomplish our goal we used a PCR-based tagging method to identify differences in sequences between resistant and susceptible tomato breeding lines. The results of the study indicate that the begomovirus resistance gene is not located at any of the tested hotspots.

## ***Introduction***

### ***Background on Geminiviruses***

Tomatoes in Central America are plagued by a series of geminiviruses that are transmitted by the whitefly, *Bemisia tabaci* (Jones, 2003). Geminiviruses transmitted by the white fly in this manner are called begomoviruses. The effect of the disease is near total loss of crops and annual damages range in the millions of dollars (Morales and Anderson, 2001; Nakhla *et al.*, 2004). In some areas of Nicaragua and Guatemala losses have been so extensive that the crop is no longer grown. Suitable resistant cultivars are currently unavailable, and methods of control are mostly restricted to insecticides that must be applied every third day (Nakhla *et al.*, 2004). Unfortunately, these drastic measures have been ineffective in controlling the virus. The insecticides are only partially effective even as a preventative measure, yet the farmers in the area continue to spray infected crops. This costs the farmers money that they will not recoup due to the loss of the infected crop (Maxwell, D., pers. com.).

Preliminary genetics studies indicated that tolerance to begomoviruses is controlled by between one and five genes, some dominant some recessive. Subsequent studies indicated that complete resistance to begomoviruses is controlled by a single dominant gene (Vidavsky and Czosnek, 1998). This study focused its efforts on locating the single dominant gene that controls resistance.

### ***Previous Attempts to introduce resistance through breeding programs***

*Lycopersicon hirsutum* and *Lycopersicon chilense* are wild species of tomato that have shown resistance to *Tomato yellow leaf curl virus*, which is a monopartite begomovirus (Vidavsky and Czosnek, 1998). However, the shape and size of the plant's fruits make them unsuitable for commercial use. Breeding programs have been underway for some time with the goal of creating a resistant hybrid plant that produces a healthy fruit (Chen *et al.*, 2003; Mejia *et al.*, 2004; Narasegowda *et al.*, 2003; Scott *et al.*, 1995). After crossing parent plants, the F1 and F2 generations must be tested in order to determine if the gene for resistance has been inherited. Current methods of doing this testing involve growing the plants to maturity in a field that has been shown to produce 100% infection of susceptible plants. Each cycle takes five months and there can be an incorrect diagnosis of plant resistance due to escapes. Thus far, breeding programs have been ineffective in producing a successful resistant hybrid for Central America.

Therefore, in order to more quickly produce a resistant hybrid, a molecular marker for the resistance gene is needed. The molecular marker could be used to track the resistance gene through successive generations with Polymerase Chain Reaction (PCR), which is a method of amplifying DNA. An accurate molecular indicator would eliminate the need to grow the plants to maturity and eliminate false positives.

### ***Background on hotspots***

Restriction fragment length polymorphism (RFLP)-based probes have been used to help develop a map of the tomato genome (Solanaceae Genomics Network, 2004). The results of this work have produced evidence for two

types of hotspots; resistance gene hotspots (Rgh) and resistance gene homologue hotspots (RGHH) (Pan *et al.*, 1999). Many resistance genes have already been located on the tomato genome, and Rgh are defined as places on the genome where two or more disease resistance genes are located in close proximity. The tendency for resistance genes to appear in these areas indicates a high probability of finding a molecular marker within these regions.

Resistance gene homologues are spots on the tomato chromosome that contain a genomic sequence with significant homology to genes that confer resistance to disease (Pan *et al.*, 1999). RGHH are defined as places where two or more of these homologous sequences are located in close proximity. The strong correlation between the resistance gene sequence and the RGHH sequence indicates a high probability of finding a molecular marker within that region.

Overall, there are 14 known Rgh and 30 known RGHH. Previously the Maxwell lab group tested Rgh on chromosomes six and eleven and concluded that a molecular marker for the begomovirus resistance gene could not be found at those locations (Mejia *et al.*, 2004). For this study, hotspots were chosen to be tested based on the number of resistance genes or resistance homologues at the hotspot.

In addition, previous research has shown that introgressions from a wild species of tomato tend to be quite large, on the order of 5-50 million base pairs (Monforte and Tanksley, 2000). Thus, sequence from only a single RFLP-based probe is sufficient to determine whether or not an introgression is located at a certain hotspot.

### ***General approach***

Therefore, we hypothesized that a molecular marker for begomovirus resistance could be found within either the Rgh on chromosomes one or seven, or the RGHH on chromosomes two, four, or eleven. Specifically, the hotspots on the genomes of three plants resistant to begomoviruses were tested to determine if there was a DNA introgression of *L. hirsutum* or *L. chilense*, respectively. We tested these hotspots using a PCR-based tagging method that identifies resistance genes (Czosnek *et al.*, 2004; Nesbitt and Tanksley, 2002). The sequences of the tested hotspot were compared against a control, susceptible tomato. Differences in the sequences as small as 3-4% were indicative of an introgression from a wild species. When sequence differences were found, additional sequencing was done with other susceptible and resistant lines in the hopes of finding a significant correlation.

## ***Materials and Methods***

### ***Plant Lines being used***

We used the tomato breeding lines, Gh13, Gc9, and Gc173, that are resistant to the bipartite begomoviruses in Guatemala (Mejía *et al.*, 2004; Nakhla *et al.*, 2004). Gh13 is the F7 generation and is a homogeneous breeding line with resistance derived from *L. hirsutum*. Gc173 and Gc9 are at least F8 breeding lines with resistance genes introgressed from *L. chilense* by J. W. Scott (Scott *et al.*, 1995).

As a control, we used the breeding line Heinz 1706. Heinz 1706 is the tomato cultivar being sequenced in an international sequencing project (Budiman *et al.*, 2000; Ozminowski, 2004), and is susceptible to geminiviruses (Hapidat, M., pers. com.). The susceptibility of Heinz 1706 to geminiviruses was confirmed through testing with *Tomato Yellow Leaf Curl Virus*, which is a begomovirus (Maxwell, D., pers. com.).

The begomovirus resistant lines, Gh13, Gc9, and Gc173, were supplied by Dr. L. Mejía, Universidad de San Carlos, Guatemala City. The susceptible line, Heinz 1706, was supplied by Dr. R. Ozminowski, Heinz Seed Co., Stockton, CA. In cases where significant sequence differences were identified in the initial plant lines, additional susceptible and resistant plant lines were put through the same procedure in the hopes of finding a strong correlation.

### ***DNA Extraction***

DNA was extracted from the fresh leaves of plants grown in a plant growth chamber at the University of Wisconsin-Madison. Thirty mg of tissue were frozen in liquid nitrogen in a microfuge tube, then ground with a sterilized Kontes™ micropestle (Kontes Glass, Vineland, NJ). The DNA was extracted with the PUREGENE® DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. DNA concentrations were adjusted to 10ng/μl and the extracts were frozen at -20°C.

### ***Primer Development***

#### ***General approach***

The partial sequences of the RFLP probes are located on the Cornell website (Solanaceae Genomics Network, 2004). Appropriate DNA data bases (National Center for Biotechnology Information, 2004; Schoof *et al.*,

2003) were accessed to determine if these sequences were associated with known plant genes. Where possible, primers were designed to anneal to the exon regions and amplify at least one intron.

#### *Rgh primer development*

Primers were developed for two hotspots, one on chromosome 7 and one on chromosome 1. The hotspot on chromosome 7 is located between RFLP probes TG128 and TG662 on the long arm of the chromosome. The hotspot on chromosome 1 is located on the long arm of the chromosome between RFLP probes TG125 and CT2 (Pan *et al.*, 1999).

#### *RGHH primer development*

Primers were developed for three hotspots, one on chromosome two, one on chromosome four, and one on chromosome eleven. The hotspot on chromosome two is located between RFLP probes R45S and CT205 on the short arm of the chromosome (Pan *et al.*, 1999). The hotspot on chromosome four is located between RFLP probes TG182 and TG75 on the long arm of the chromosome. The hotspot on chromosome eleven is located between RFLP probes TG651 and TG36 and spans both arms of the chromosome (Pan *et al.*, 1999).

#### **PCR Reactions**

PCR fragments from each set of primers, for each of the four genotypes, were obtained using methods developed in the Maxwell lab (Czosnek *et al.*, 2004). PCR parameters were for 50- $\mu$ l reactions containing: 5- $\mu$ l 2.5mM deoxynucleotide triphosphates (dNTPs), 5- $\mu$ l 10X buffer, 5- $\mu$ l 25 mM MgCl<sub>2</sub>, 0.2- $\mu$ l *Taq* DNA polymerase, 5- $\mu$ l each forward and reverse sense primer at 10 $\mu$ M, 5-7  $\mu$ l of DNA extract, and H<sub>2</sub>O. Some PCR reactions were run with 25- $\mu$ l reactions. When this was the case, the concentrations of all chemicals were exactly half of what appeared in the 50- $\mu$ l reactions. PCR cycle parameters for fragment amplification were as follows: denaturation at 94°C for 3 min, then 35 cycles at 94°C for 30 sec each, annealing at 50°C for 1 min, and extension at 72°C for 1 min. These cycles were followed by a reaction at 72°C for 10 min, and then the reaction was held at 4°C. PCR reactions were performed in the MJ DNA Engine PT200 Thermocycler™ (MJ Research Inc., Waltham, MA).

#### **PCR Fragment Analysis**

The PCR-amplified DNA was run on an electrophoresis gel of 1.5% Seakem LE™ agarose (BioWhittaker Molecular Applications Rockland, ME) in 0.5X TBE buffer, stained with ethidium bromide, and visualized with a Kodak Gel Logic 200 Imaging System. This allowed us to determine the quality of the amplified DNA. If the primer pair produced multiple bands, we redesigned the primer and did PCR again. If the primer pair produced only one band, this PCR fragment was directly sequenced.

#### **Sequencing and Comparison**

After successful amplification of the tomato genomic DNA, PCR fragments were directly sequenced using Big Dye Sequencing Kit™ (Biotechnology Center, Madison, WI). Analysis of the sample sequences was accomplished by comparison with the DNAMAN software (Lynnon Corp., Quebec, Canada). In comparing the DNA sequences of Gh13, Gc9, and Gc173 with Heinz 1706, we looked for an introgression of *L. hirsutum* or *L. chilense* DNA. Sequence differences as small as 3-4%, such as SNPs or indels, between Gh13, Gc9 or Gc173 and Heinz 1706 were evidence of an introgression and had the potential to be used as molecular markers for begomovirus resistance. When sequence differences were found, additional sequencing was done with other susceptible and resistant lines in the hopes of finding a significant correlation.

#### **Presentation of Agarose Gel Pictures**

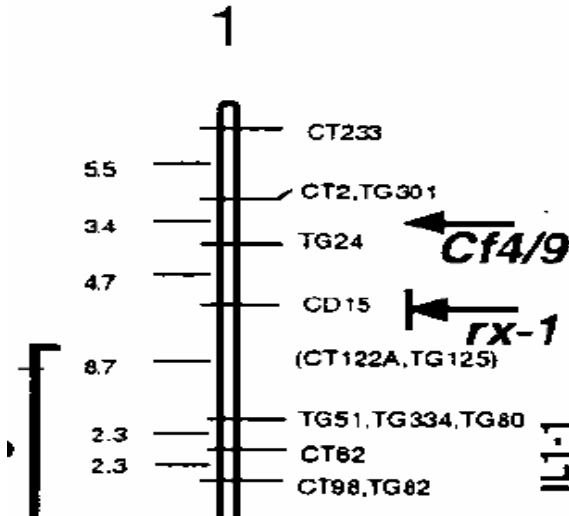
Often times multiple primers from different RFLP probes were run on the same Agarose gel. In order to clearly present the results, the pictures of the gels were often cut or cropped so that primers from only one RFLP probe were left in the picture. This allowed us to present the RFLP probes one at a time. The appearance of the bands was never altered and no significant data has been omitted.

## Results

### Chromosome 1

PCR primer pairs were designed for one RFLP probe: TG301 (Fig. 1).

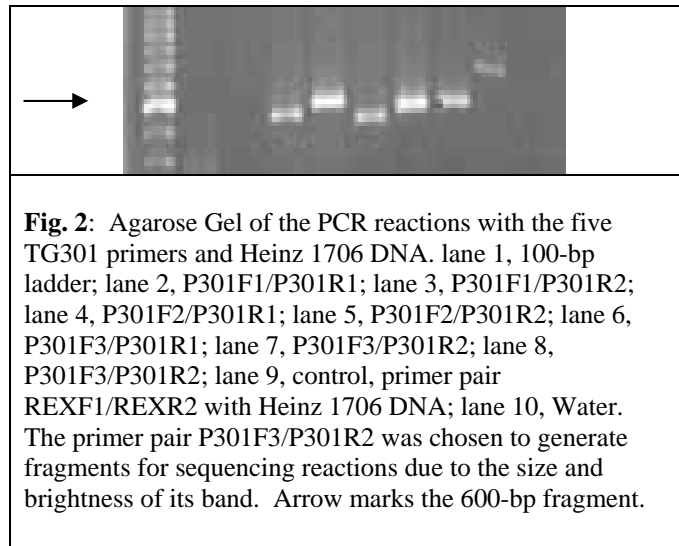
**Fig. 1:** RFLP map of the top of Chr. 1 (Adapted from Pan et. al., 1999).



**Table 1:** Primers from the TG301 probe on Chr 1.

TG301	Primer Sequence (5' to 3')
P301F1	GTGGGAGTTCTTGTCTGAATAAGC
P301F2	GATGACAAGACATGTGAAGAGCG
P301F3	GGAAATTGAAGCACAGTGG
P301R1	CCACAGTGACAATCTTGATCTGCACC
P301R2	GTTTAGGTCTGATTCCCAGC

**TG301 RFLP Probe:** Five primers were designed from the TG301 RFLP probe: P301F1, P301F2, P301F3, P301R1, and P301R2 (Table 1). All primer combinations were tested with Heinz 1706 DNA (Fig. 2). Primer pairs P301F2/P301R1, P301F2/P301R2, P301F3/P301R1, P301F3/P301R2, and P301F3/P301R2 gave a single band. Their sizes varied from 550 bp to 650 bp. The primer pair P301F3/P301R2 gave the most intense band of the largest size, so this primer pair was used with the additional genotypes. Heinz 1706, Gc173, Gh13, and Gc9 produced strong bands of 580 bp. These PCR fragments were sequenced with both the forward and reverse primers. The sequence from Gh13 (acc. no. DQ066448), Gc9 (acc. no. DQ066449), and Heinz 1706 (acc. no. DQ66450) was submitted to GenBank. When the sequences were aligned one INDEL was found at nt number 416 (Table 2). Heinz 1706 had a single thiamine nucleotide that none of the other tested sequences had. As a result, 11 additional samples (Gh11, Gc16, Gc173-2, Gc16, Gc173-2-1, H7996, Dominique, Don Raul, Gc173-2-a, M82, M82-2, Silverado) were sequenced with these primers. All of the other samples had the same sequence as Gc9, Gc173, and Gh13. Thus, it was concluded that the Heinz 1706 sequence was unique, and that the SNP was not associated with begomovirus resistance. Subsequently, *Solanum habrochaites* accessions LA1777 (acc. no. DQ222939) and LA0386 (acc. no. DQ437767) were tested with the P301F3/P301R2 primer pair. These species differed from each other at five SNP and one INDEL and from the breeding lines at seven SNP and two INDEL (Table 2). It is interesting to note that the LA0386 and LA1777 sequences had been identical when tested at other locations. The source of these differences may warrant additional investigation. However, there was no correlation between the species sequences and any of the breeding lines. Therefore, there is no evidence that supports an introgression of a begomovirus-resistance gene from *S. habrochaites* at the TG301 locus.



**Fig. 2:** Agarose Gel of the PCR reactions with the five TG301 primers and Heinz 1706 DNA. lane 1, 100-bp ladder; lane 2, P301F1/P301R1; lane 3, P301F1/P301R2; lane 4, P301F2/P301R1; lane 5, P301F2/P301R2; lane 6, P301F3/P301R1; lane 7, P301F3/P301R2; lane 8, P301F3/P301R2; lane 9, control, primer pair REXF1/REXR2 with Heinz 1706 DNA; lane 10, Water. The primer pair P301F3/P301R2 was chosen to generate fragments for sequencing reactions due to the size and brightness of its band. Arrow marks the 600-bp fragment.

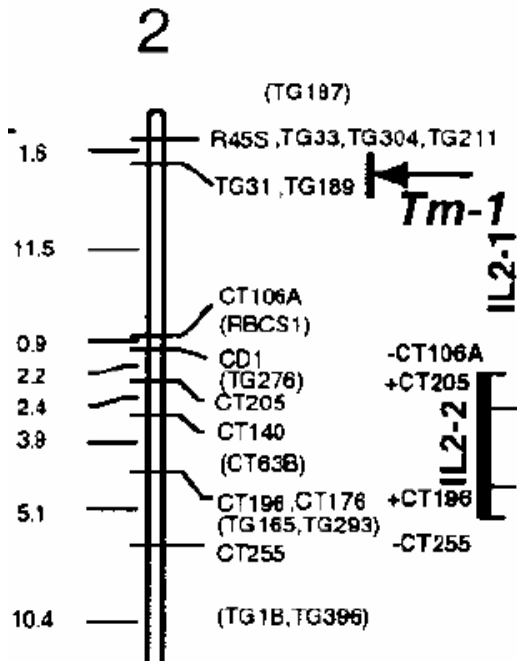
**Table 2:** Sequence differences found at the TG301 locus. All tested samples not listed matched LA3918 exactly. The sequences of the lines listed were identical in between the listed SNP and INDEL. The nt position is relative to Gh13.

Line	SNP 1	SNP 2	SNP 3	INDEL	SNP 4	SNP 5	INDEL	SNP 6	INDEL	SNP 7
LA1777	A	G	A	.	T	A	.	G	.	A
LA0386	C	G	A	.	C	G	.	C	C	G
Heinz 1706	C	A	T	T	T	G	T	C	.	A
Gh13	C	A	T	T	T	G	.	C	.	A
nt Position	186 bp	291 bp	349 bp	353 bp	366 bp	387 bp	416 bp	417 bp	418 bp	435 bp

### Chromosome 2

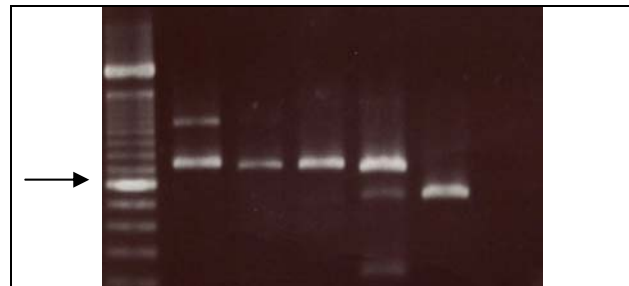
PCR primer pairs were designed for four RFLP probes: CT140, TG293, CT205, and TG480 (Fig. 3).

**Fig. 3:** RFLP map of the top of Chr. 2 (Adapted from Pan et. al., 1999).



**Table 3:** Primers from the CT140 probe on Chr. 2.

CT140	Primer Sequence (5' to 3')
PCT140F1	CACAAGGCTAGAGTTGTTCGGG
PCT140F2	CGGGAAAGGAAGGAACGTCCG
PCT140R1	GTTTTTTTGGTTTACCCATCAGTGTCC
PCT140R2	CAGTGTCCAAAACCAGCTCGCC



**Fig. 4:** Agarose gel of the PCR reactions with the four CT140 primer pairs and Heinz 1706 DNA. lane 1, 100-bp marker; lane 2, PCT140F1/PCT140R1; lane 3, PCT140F1/PCT140R2; lane 4, PCT140F2/PCT140R1; lane 5, PCT140F2/PCT140R2; lane 6, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA. The strongest bands in the lanes 2-5 are at 750 bp. The primer pair PCT140F2-CT140R1 was chosen to generate fragments for the sequencing reactions due to the intensity of its band. Arrow marks the 600-bp fragment.

**CT140 RFLP Probe:** Four primers were designed from the CT140 RFLP probe: PCT140F1, PCT140F2, PCT140R1 and PCT140R2 (Table 3). All four primer combinations gave the strongest bands of 750 bp with Heinz 1706 DNA (Fig. 4). Primer pairs PCT140F1/PCT140R1 and PCT140F2/PCT140R2 gave two bands. Primer pairs PCT140F1/PCT140 and PCT140F2/PCT140R1 gave a single band. The primer pair PCT140F2/PCT140R1 gave the most intense single band, so this primer pair was used with the additional genotypes.

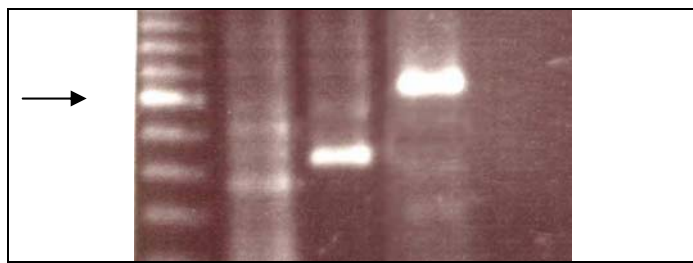
Heinz 1706, Gc173, and Sheriff gave a strong band at 750 bp; and Gc9 and Gh13 produced a weak 750-bp band. These PCR fragments were sequenced with both the forward and reserve primers. Sequence for Heinz 1706 (acc. no. DQ222941), Gc173, and Gh13 were obtained with both primers, and sequence was only obtained with the forward primer for Sheriff and Gc9. When these sequences were aligned, there were no SNPs or INDELS that distinguished the begomovirus-resistant genotypes, Gc9, Gh13, and Gc173, from the susceptible genotype, Heinz 1706. Thus, there was no evidence that supports an introgression of a begomovirus-resistance gene from *S. habrochaites* at the *CT140* locus.

**TG293 RFLP probe:** Two primers were designed from the TG293 RFLP probe: PTG293F1 and PTG293R1 (Table 4). The PTG293F1/PTG293R1 primer combination gave an intense single band at around 450 bp with Heinz 1706 DNA (Fig. 5). Subsequently, the primer pair was run with additional genotypes. Heinz 1706 again gave a very strong band at 450 bp. However, no other tested germplasm line (Gc9, Gc173, Gh13) gave a band with these primers. The reaction was tried three times, but each time only Heinz 1706 produced the band. The Heinz 1706 PCR fragment was sequenced with both the forward and reverse primers. The Heinz 1706 sequence (acc. no. DQ097528) was aligned with the RFLP probe from which it was designed in order to determine the reason that the primers did not work with any other germplasm lines. The sequence matched the RFLP probe exactly. No further analysis was conducted on this RFLP probe. It is likely that there are genetic differences between the lines that prevented the primers from binding. It is possible that additional primers could be designed to amplify this region and explore those differences. However, with regard to begomovirus resistance, it was unlikely that an introgression would be found in this region. The CT140 RFLP probe is less than 2cM away, and evidence from this probe indicates that an introgression was not present.

**CT205 RFLP Probe:** Two primers were designed from the CT205 RFLP probe: PCT205F1 and PCT205R1 (Table 5). The PCT205F1/PCT205R1 primer pair produced a streaky band that was not deemed suitable for sequencing (Fig. 5).

**Table 4:** Primers from the TG293 probe on Chr. 2.

TG293	Primer Sequence (5' to 3')
PTG293F1	GTACCAGCACCAACGCCAAATTTTCGC
PTG293R1	GGTGAGTGACTAAGTTACATCATTAAATG



**Fig. 5:** Agarose gel of the PCR reactions with the one CT 205 primer pair, and the one TG293 primer pair with Heinz 1706 DNA. lane 1, 100-bp ladder; lane 2, PCT205F1/PCT205R1; lane 3, PTG293F1/PTG293R1; lane 4, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA; lane 5, Water. The PTG293F1/PTG293R1 primer pair produced a strong single band and was chosen for use with additional genotypes. Arrow marks the 600-bp fragment.

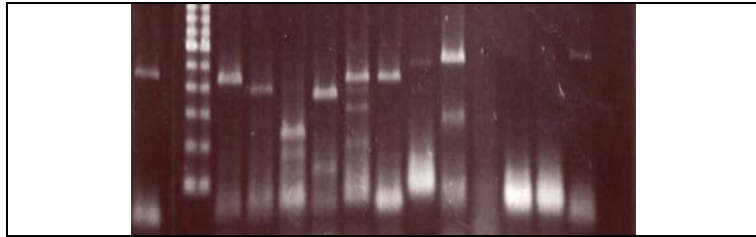
**Table 5:** Primers from the CT205 probe on Chr. 2.

CT205	Primer Sequence (5' to 3')
PCT205F1	GCAATGGAGAAGGTGAGGAGAACGG
PCT205R1	AATTGAAGGGAGAATCTTGTGTTTGCTC

**Table 6:** Primers from the TG480 probe on Chr. 2. Primers from different groups cannot be used together.

TG480 RFLP Probe (located within 1 cM of R45S): Three groups of primers were designed from the TG480 RFLP probe with four primers in each group for a total of twelve primers, Group 1: PTG480F1, PTG480R1, PTG480F2, PTG480R2, GROUP 2: PTG480F3, PTG480F4, PTG480R3, PTG480R4, GROUP 3: PTG480R5, PTG480R6, PTG480F5, PTG480F6 (Table 6). Primers from the different groups cannot be used with each other due to the place on the RFLP probe from which they were designed. The TG480F1/TG480R1, TG480F1/TG480R2, TG480F2/TG480R1, TG480F4/TG480R3, and TG480F4/TG480R4 primer pairs gave a single band. Their sizes varied from 400 bp to 600 bp (Fig. 6). The TG480F2/TG480R2, TG480F3/TG480R3, TG480F3/TG480R4, and TG480F5/TG480R6 primer pairs gave multiple bands. The TG480F5/TG480R6, TG480F6/TG480R5, and TG480F6/TG480R5 primer pairs gave no bands. The PTG480F1/PTG480R2 and PTG480F4/PTG480R3 primer pairs gave the largest and most intense bands and were thus chosen for use with additional genotypes. The PTG480F1/PTG480R2 primer pair produced a strong band with Heinz 1706 DNA and a weak band with Gc173 DNA at 450 bp. The PTG480F4/PTG480R3 primer pair produced bands with Heinz 1706, Gh13, Gc173, and Sheriff at 450 bp. These PCR fragments were sequenced with both the forward and reverse primers. None of the reactions produced usable sequence data. Subsequently, the PCR reactions were tried again, but similar results were obtained each time. It is possible that by choosing one of the other TG480 primer combinations we would achieve better results.

TG480	Primer Sequence (5' to 3')
GROUP 1	
PTG480F1	GGAAGTGAGAGATTTTCATTGGCGG
PTG480R1	CTTACAAGCATCCCTCAAGCC
PTG480F2	GATAGAGAACGGCGACGGCAGAC
PTG480R2	GGAGAGATGGATCTGGTACTGTTGG
GROUP 2	
PTG480F3	GGCTTGAGGGATGCTTGTGAAG
PTG480F4	CCAACAGTACCAGATCCATCTCTCC
PTG480R3	CTTGTTGAATAGCTCGTGTGGG
PTG480R4	GCAGGTTTAAGTATAGGAAGAGGCAGAGTG
GROUP 3	
PTG480R5	GGGAGACAGCTTGCATGCCTGC
PTG480R6	GGCAGGTGAGTGCACAGTGGTTTC
PTG480F5	CCCACACGAGCTATTCAACAAG
PTG480F6	CACTCTGCCTCGGCCTATACTTAAACCTGC



**Fig. 6:** Agarose gel of the PCR reactions with the 12 TG480 primer pairs and Heinz 1706 DNA. lane 1, TG480F1/TG480R1; lane 2, 100-bp Ladder; lane 3, TG480F1/TG480R2; lane 4, TG480F2/TG480R1; lane 5, TG480F2/TG480R2; lane 6, TG480F3/TG480R3; lane 7, TG480F3/TG480R4; lane 8, TG480F4/TG480R3; lane 9, TG480F4/TG480R4; lane 10, TG480F5/TG480R6; lane 11, TG480F5/TG480R6; lane 12, TG480F6/TG480R5; lane 13, TG480F6/TG480R5; lane 14, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA; lane 15, Water. The PTG480F1/PTG480R2 and PTG480F4/PTG480R3 primer pairs were chosen for sequencing reactions based on their size and the intensity of their bands. Arrow marks the 600-bp fragment.

## Chromosome 4

PCR primer pairs were designed for three RFLP probes: TG208, TG287, and TG633 (Fig. 7).

Fig. 7: RFLP map of the middle of Chr. 4 (Adapted from Pan et. al., 1999).

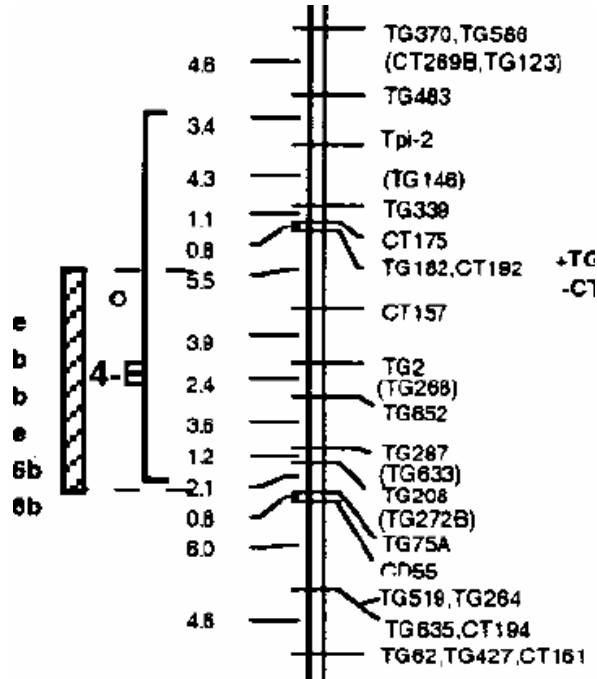


Table 7: Primers from the TG208 probe on Chr.4.

TG208	Primer Sequence (5' to 3')
PTG208F1	CCCTCTCCAGATCTACTTTTATTGGGTACG
PTG208F2	GCATTCACAAAGTACGGACAAGGTTAGG
PTG208R1	CATATCCAACAGCATGGCTATCAG
PTG208R2	GAAGCAGAGAATAACCGGTGAAGACTC

**TG208 RFLP Probe:** Four primers were designed from the TG208 RFLP probe: PTG208F1, PTG208F2, PTG208R1, and PTG208R2 (Table 7). All four primer combinations gave intense single bands with Heinz 1706 DNA that were from 400 bp to 500 bp (Fig. 8). The PTG208F2/PTG208R2 gave the largest band and was chosen for use with additional genotypes. Heinz1706, Gc9, Gh13, and Sheriff each gave intense single bands at 500 bp. Gc173 gave a weak band at 500 bp. These PCR fragments were sequenced with both the forward and reverse primers. When these sequences were aligned three distinct patterns were found. The sequences were different due to SNPs in three different places. Gc9 (acc. no. DQ120939), Gh13, and Gc173 each had one pattern while Heinz 1706 (acc. no. DQ120940) had a distinctly different pattern (3 SNPs). Sheriff (acc. no. DQ223930) was heterozygous for both patterns (Table8). In order to determine the significance of this pattern, sequence was obtained for 20 additional plant lines with the PTG208F2/PTG208R2 primer pair (Dominique, Gpim10, Marina, Celebrity, Gc16, Gh902a, H7996, Moneymaker, Naenemor, Silverado, LA1968a, LA0386, Gp11, Ih902a, LA1777, Hc7880, Motelle, LA0462b, LA3900, and Toro). Gpim10, Dominique, and Marina each had the same pattern as Gc9, Gh13, and Gc173. Celebrity, Gc16, Gh902a, H7996, Moneymaker, Naenemor, Silverado, and LA 1968a each had the same pattern as Heinz 1706. LA0386, Gp11, Ih902a, and LA1777 each had the same sequence as Sheriff. Hc7880, Motelle, LA0462b, LA3900, and Toro failed to sequence, despite being run in the same reaction as all of the other DNAs (Table 8). The groups of plant lines do not correlate with any known morphological difference or any known resistance gene, geminivirus related or otherwise. Thus, there is no evidence of a molecular marker for the begomovirus resistance gene at the TG208 locus. It is possible that the groups of

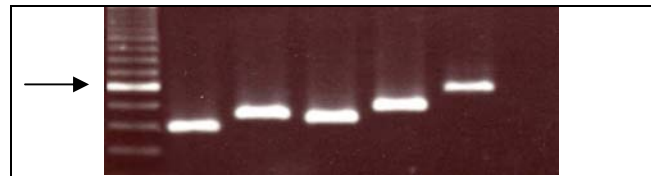


Fig. 8: Agarose gel of the PCR reactions with the four TG208 primers and Heinz 1706 DNA. lane 1, 100-bp ladder; lane 2, PTG208F1/PTG208R1; lane 3, PTG208F1/PTG208R2; lane 4, PTG208F2/PTG208R1; lane 5, PTG208F2/PTG208R2; lane 6, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA; lane 7, water. The PTG208F2/PTG208R2 gave the largest band and was chosen for use with additional genotypes. Arrow marks the 600-bp fragment.

patterns that we found correlate with an as yet to be found morphological difference or resistance gene, and these correlations may warrant additional research.

**Table 8:** Sequence patterns associated with the TG208 RFLP probe. The first SNP is at 37 bp, the second is at 230 bp, and the third is at 290 bp in reference to Heinz 1706.

Pattern (TGT)	Pattern (CAC)	Pattern (Heterozygous)	Failed to Sequence
Gc9	Heinz	Sheriff	Hc7880
Gh13	Celebrity	LA0386	Motelle
Gc173	Gc16	Gp11	LA0462b
Gpim10	Gh902a	Ih902a	LA3900
Dominique	H7996	LA1777	Toro
Marina	Moneymaker		
	Naenemor		
	Silverado		
	LA1968a		

**TG287 RFLP Probe:** Recent Cornell data (Solanaceae Genomics Network, 2004) shows that his RFLP probe is located roughly 4 cM above the TG208 RFLP probe on Chr. 4. Primers were designed for this RFLP probe in order to determine how far the introgression extended above the TG208 RFLP probe on chromosome four. Four primers were designed from the TG293 RFLP probe: PTG287F1, PTG287F2, PTG287R1, and PTG287R2 (Table 9). All four primer combinations gave single bands between 480 bp and 900 bp with Heinz 1706 DNA (Fig. 9). PTG287F2/PTG287R2 primer pair gave the most intense single band and was thus used with additional genotypes. Heinz 1706, Gc9, Gc173, and Sheriff each gave strong bands at 480 bp. These PCR fragments were sequenced with both the forward and reverse primers. Heinz 1706 (acc. no. DQ222943), Gc173 (acc. no. DQ222942), Gh13, Gc9, and Sheriff each gave sequence with both primers. Just as with the TG208 primers, two patterns were found. Gc173, Gc9, and Gh13 each had the same pattern, while Heinz had a second unique pattern. Sheriff was heterozygous for both patterns (Table 10). Thus, because the TG287 probe has the same correlation between genotypes as the TG208 probe, this would indicate that the introgression extends at least 4 cM above the TG208 locus. Just as with the TG208RFLP probe, the sequence differences do not correlate with begomovirus resistance. Therefore, a molecular marker for begomovirus resistance could not be found at the TG287 RFLP probe. However, the groups of patterns may warrant additional research.

**Table 9:** Primers from the TG287 probe on Chr. 4.

TG287	Primer Sequence (5' to 3')
PTG287F1	CTCAGAGGTGGGGGCGGAATGG
PTG287F2	CGAACTCAAAGAAGCAACAGAGGGTG
PTG287R1	GGTGTGTTCTCTCTGCTTTATATGC
PTG287R2	CCCCATATATTAACAAAGTGCAAACG



**Fig. 9:** Agarose gel of the PCR reactions with the four TG287 primer pairs and Heinz 1706 DNA. The PCR reactions were run in two separate gels. In gel one: lane 1, 100-bp ladder; lane 2, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA; lane 3, water; lane 4, PTG287F1/PTG287R1; lane 5, PTG287F1/PTG287R2, lane 6, PTG287F2/PTG287R1. In gel two: lane 1, 100-bp ladder; lane 2, PTG287F2/PTG287R2. The PTG287F2/PTG287R2 primer pair was chosen for sequencing reactions based on the intensity of its band. Arrow marks the 600-bp fragment.

**Table 10:** Sequence patterns associated with the TG287 RFLP probe. The INDEL is at 51 bp and the SNP is at 122 bp into the sequence.

Pattern (TA)	Pattern (_G)	Pattern (Heterozygous)
Gc173	Heinz 1706	Sheriff
Gc9		
Gh13		

**TG633 RFLP Probe:** Recent Cornell data (Solanaceae Genomics Network, 2004) shows that this RFLP probe is located less than 1 cM below the TG208 RFLP probe on Chr. 4. These primers were designed in order to determine the how far the introgression extended below the TG208 RFLP probe on Chr. 4. Four primers were designed from the TG633 RFLP probe: PTG633F1, PTG633F2, PTG633R1, and PTG633R2 (Table 11). Three of the four primer combinations gave bands with Heinz 1706 DNA. The PTG633F1/PTG633R1 primer pair produced a strong band at 250 bp. The PTG633F1/PTG633R2 and PTG633F2/PTG633R1 primer pairs produced a single weak band at 150 bp and 500 bp, respectively (Fig. 10). Though it had a weak band, the PTG633F2/PTG633R1 primer pair was chosen for sequencing reactions because it was the only primer pair to produce a band over 300 bp. In the subsequent PCR the primer pair produced a very strong single band with Heinz 1706, Gc9, Gc173, Gh13, and Sheriff at 400 bp. Upon sequencing, Heinz 1706 produced good sequence with both the forward and reverse primers, but no other samples gave readable sequence data. In many of the cases it appeared as if the thiamine peaks were missing. It is likely that if this reaction were run again that it would produce good results. However, evidence from the TG208 and TG287 RFLP probes in this region would indicate that an introgression that correlated with begomoviruses is not likely to be found in this area. Thus, further investigation of this RFLP probe is not warranted with regards to begomovirus resistance, but may be warranted with regards to the overall sequence differences.

**Table 11:** Primers from the TG633 probe on Chr. 4.

TG633	Primer Sequence (5' to 3')
PTG633F1	CAATATGAGCACTTAACTGTTCTTTTCGG
PTG633F2	CAGGTGTGGGGACAGAGCGGAAAC
PTG633R1	GTGAAATCTTCCTTAGTCTCCTCCTC
PTG633R2	GGTGTGGGGACAGAGCGGAAACTTG

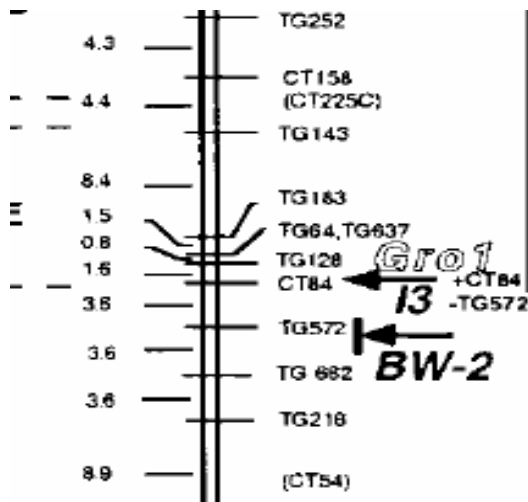


**Fig. 10.** Agarose gel of the PCR reactions with the four TG633 primers. lane 1, 100-bp ladder; lane 2, PTG633F1/PTG633R1; lane 3, PTG633F1/PTG633R2; lane 4, PTG633F2/PTG633R1; lane 5, PTG633F2/PTG633R2; lane 6, water; lane 7, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA. Though it had a weak band, the PTG633F2/PTG633R1 primer pair was used to generate PCR fragments for sequencing reactions because it was the only primer pair to produce a band over 300 bp in size. Arrow marks the 600-bp fragment.

## Chromosome 7

PCR primer pairs were designed for four RFLP probes: TG149, TG143, TG662, and TG572 (Fig. 11).

**Fig. 11:** RFLP map of the middle of Chr. 7 (Adapted from Pan et. al., 1999).



**Table 12:** Primers from the TG149 probe on Chr. 7.

TG149	Primer Sequence (5' to 3')
P149F1	GCCAGTATCAGAAGGTAAC
P149F2	GGGTAACCTAAGCTTTCTCTCCTCC
P149R1	GCAGAACCCTCCATATGATAGTC
P149R2	GCCAGTATCAGAAGGGTAACTAAGC

**TG149 RFLP Probe (2.5 cM below the TG143 RFLP probe):** Four primers were designed for the TG149 RFLP probe: P149F1, P149F2, and P149R1 (Table 12). None of the primer combinations gave a single band. It is possible that new primers could be designed around this probe that would give a single band.

**Table 13:** Primers from the TG143 probe on Chr. 7.

TG143	Primer Sequence (5' to 3')
P143F1	GCCTGCAGATTCTCACTCTC
P143 F2	CCCTACTGATCCCAATACGAC
P143R1	GGGAGATTACTTAGTTTGGCG
P143R2	CTGAAGAATGGGTACTGAG

**TG143 RFLP Probe:** Four primers were designed for the TG143 RFLP probe: P143F1, P143 F2, P143R1, and P143R2 (Table 13). The P143F1/P143R1 and P143F1/P143R2 primer pairs did not produce bands (Fig. 12). The P143F2/P143R1 and P143F2/P143R2 primer pairs produced single bands with Heinz 1706 DNA at 300 bp. Both were used with additional genotypes. The P143F2/P143R1 primer pair produced a band only with the Heinz 1706 DNA. The P143F2/P143R1 primer pair gave strong a single band with Heinz 1706, Gc173, Gc9, and Gh13 at 300 bp. As a result, only P143F2/P143R1 was used to generate a fragment for sequencing reactions. The PCR products were sequenced with both the forward and reverse primers. The resulting sequence produced strong peaks in the ABI file, but the sequence was unreadable in many places. It is possible that there are two fragments of roughly equal size, but of different sequence. This would explain the erratic sequence results. It is possible that new primers could be designed around this probe that would give single bands and produce readable sequence.



**Fig. 12:** Agarose gel of the PCR reactions with the four TG143 primers and the four TG662 primers with Heinz 1706 DNA. lane 1, 100-bp ladder; lane 2, P143F1/P143R1; lane 3, P143F1/P143R2; lane 4, P143F2/P143R1; lane 5, P143F2/P143R2; lane 6, P662F1/P662R1, lane 7, P662F1/P662R2; lane 8, P662F2/P662R1; lane 9, P662F2/P662R2; lane 10, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA; lane 11, water. The P143F2/P143R1 and P143F2/P143R2 primer pairs produced single bands and were used with additional genotypes. Arrow marks the 600-bp fragment.

**TG662 RFLP Probe:** Four primers were designed around the TG662 RFLP probe: P662F1, P662F2, P662R1, and P662R2 (Table 14). None of the primer combinations produced usable bands (Fig. 12). It is possible that new primers could be designed around this probe that would give single bands.

**Table 14:** Primers Designed for RFLP Probe TG662 on Chromosome 7.

TG662	Primer Sequence (5' to 3')
P662F1	GAATTGGGCCCTCTAGATGC
P662F2	CCGCCAGTGTGATGGATATC
P662R1	CCCTCCAACACTTTAAGG
P662R2	CCCAATGAGAGCAATATCCCC

## Chromosome 11

PCR primer pairs were designed for three RFLP probes: TG384, TG400, and TG523.

Fig 14: RFLP map of the bottom 2/3 of Chr. 11 (Adapted from Pan et. al., 1999).

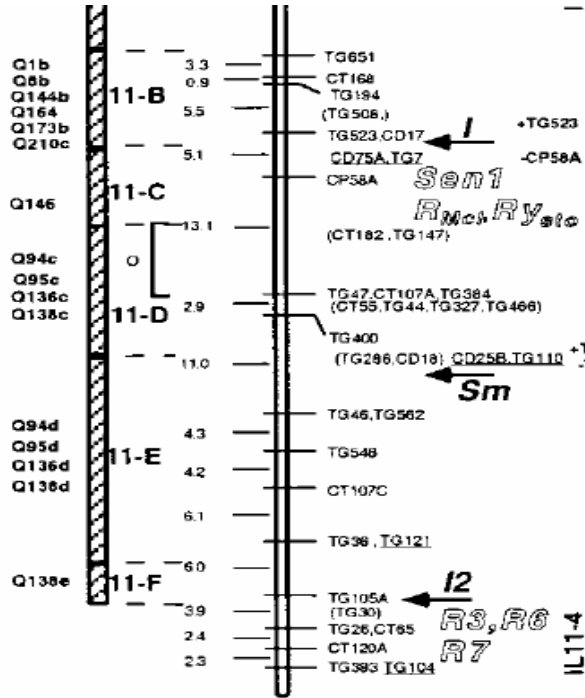


Table 17: Primers from the TG384 probe on Chr. 11.

TG384	Primer Sequence (5' to 3')
PTG384F1	GCAACTTTGGCACCATAGCTG
PTG384F2	GAACTTGTATGTTAGGCTGTGCTGGG
PTG384R1	GATGGCATCAAGGGCAGAAACC
PTG384R2	GATACAGTATCTACTTGAACCAGTTG

TG384 RFLP Probe: Four primers were designed around the TG384 RFLP probe: PTG384F1, PTG384F2, PTG384R1, and PTG384R2 (Table 17). The PTG384F1/PTG384R1 primer pair gave a strong band at 1000 bp and several smaller bands with Heinz 1706 DNA. The PTG384F2/PTG384R1 and PTG384F2/PTG384R2 primer pairs did not give bands with Heinz 1706 DNA. The PTG384F1/PTG384R2 primer pair gave a strong single band at 900 bp and was used with additional genotypes (Fig. 15). The primer pair produced a strong band at 900 bp with Heinz 1706, Gc9, Gh13, Sheriff, and Gc173. The PCR products were sequenced with both the forward and reverse primers; however, none of the reactions produced usable sequence. The experiment was tried again, but never again gave useable PCR products.

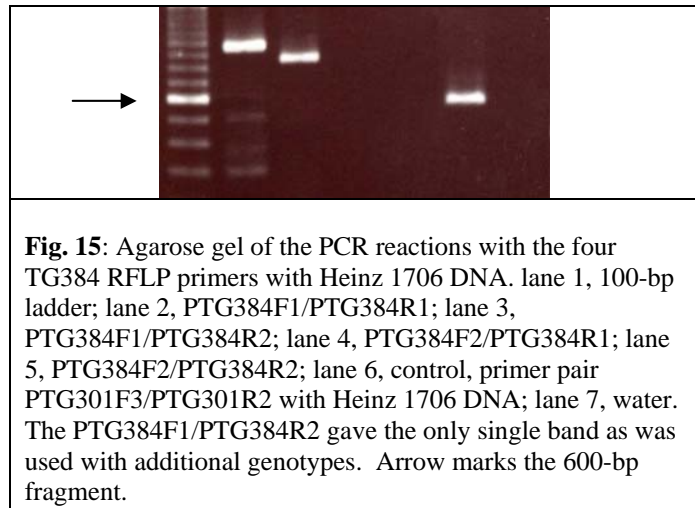
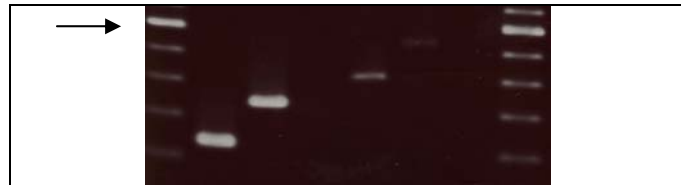


Fig. 15: Agarose gel of the PCR reactions with the four TG384 RFLP primers with Heinz 1706 DNA. lane 1, 100-bp ladder; lane 2, PTG384F1/PTG384R1; lane 3, PTG384F1/PTG384R2; lane 4, PTG384F2/PTG384R1; lane 5, PTG384F2/PTG384R2; lane 6, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA; lane 7, water. The PTG384F1/PTG384R2 gave the only single band as was used with additional genotypes. Arrow marks the 600-bp fragment.

**Table 18:** Primers from the TG400 probe on Chr. 11.

TG400	Primer Sequence (5' to 3')
PTG400F1	CCATTGGCCATTGAATTGACAGC
PTG400F2	CCTGTTGCTTGCTTGGATATATG
PTG400R1	GTAAGAGTAGCAGTACAGAATAAGC
PTG400R2	CATGTGAGTGTGAGAAATCCAGAGC

**TG400 RFLP Probe:** Four primers were designed around the TG400 RFLP probe: PTG400F1, PTG400F2, PTG400R1, and PTG400R2 (Table 18). The PTG400F1/PTG400R1, PTG400F1/PTG400R2, and PTG400F2/PTG400R2 primer pairs produced single bands with Heinz 1706 DNA at 220 bp, 320 bp, and 400 bp, respectively (Fig. 16). The PTG400F2/PTG400R1 primer pair did not produce a band. Though the band was not as intense as the others, the PTG400F2/PTG400R2 primer pair was chosen for use with additional genotypes because it produced the largest band. This primer pair produced a single band at 400 bp with Heinz 1706, Gc9, Gh13, and Sheriff. These PCR products were sequenced with both the forward and reverse primers. None of the sequencing reactions produced readable sequence. The PCR reactions were tried again three times. Each time the PCR reaction produced an erratic banding pattern. This would seem to indicate that this primer combination is not suitable for sequencing reactions. It is possible that additional primers could be designed to amplify this region.



**Fig. 16:** Agarose gel of the PCR reactions with the four TG400 primers and Heinz 1706 DNA. lane 1, 100-bp ladder; lane 2, PTG400F1/PTG400R1; lane 3, PTG400F1/PTG400R2; lane 4, PTG400F2/PTG400R1; lane 5, PTG400F2/PTG400R2; lane 6, control, primer pair PTG301F3/PTG301R2 with Heinz 1706 DNA (this band is very faint); lane 7, water; lane 8, 100-bp ladder. Though the band was not as intense as the others, the PTG400F2/PTG400R2 primer pair was chosen for use with additional genotypes because it produced the largest band. Arrow marks the 600-bp fragment.

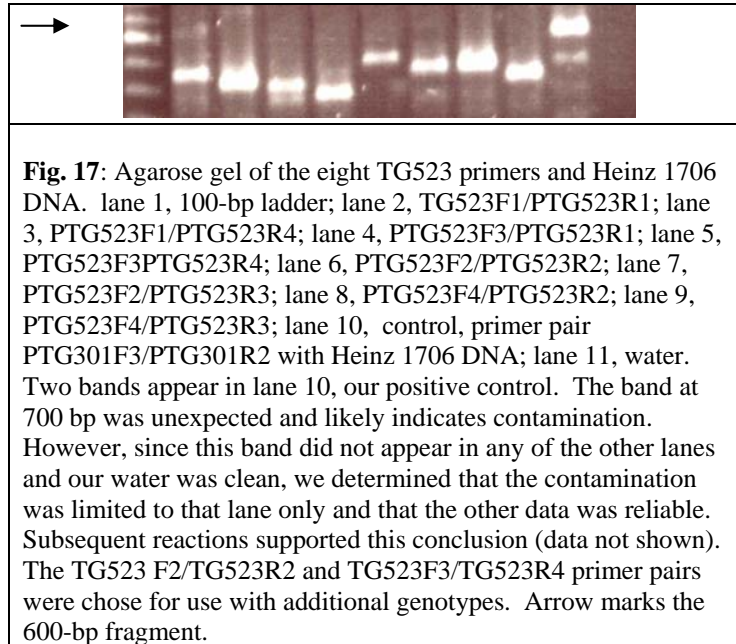
**TG523 RFLP Probe:** Two groups of primers, with four primers in each group were designed from the TG523 probe: GROUP 1- PTG523F1, PTG523F3, PTG523R1, PTG523R4, GROUP 2- PTG523R2, PTG523R3, PTG523F2, and PTG523F4 (Table 19). Primers from the different groups cannot be used with each other due to the place on the RFLP probe from which they were designed. The PTG523F3/PTG523R4, PTG523F2/PTG523R2, PTG523F2/PTG523R3, and PTG523F4/PTG523R2 primer pairs each gave single bands with Heinz 1706 DNA with sizes ranging from 400 bp to 600 bp (Fig. 17). The other primer combinations produced multiple bands or streaky bands. One primer pair was chosen from each of the two groups; TG523 F2/TG523R2 and TG523F3/TG523R4

**Table 19:** Primers from the TG523 probe on Chr. 11.

TG523	Primer Sequence (5' to 3')
GROUP 1	
PTG523F1	GGAAGAGGAGGATTTTCAGTCCTGTAG
PTG523F3	CCTGGATTCGTTTTCTTCTCAAGATGG
PTG523R1	GATGTTCTAAGTCAAAAAGTCACAACC
PTG523R4	CCTACTACTTCACTTCTGTCATG
GROUP 2	
PTG523R2	GACCACATTCACAAAACACTCTTTTAACC
PTG523R3	CATGAAGGAAATACTAACCAGGAACC
PTG523F2	CCAGTAAGGAGCTTCATTCAATCTATG
PTG523F4	GTTGTTTTGTTCTCTCTCTTTCTATTTCG

produced the largest and most intense single bands and were thus used with additional genotypes. Heinz1706, Gc9, Gc173, Gh13, Sheriff, LA1777, and LA1968 each produced a strong band at 500 bp with the TG523F2/TG523R2 primer pair. LA1777 and LA1968 were not run with the TG523F3/TG523R4 primer pair, but the remaining samples were and each produced a strong band at 450 bp. These PCR fragments were directly sequenced with both the forward and reverse primers. Sequence was obtained for both the forward and reverse TG523F2/TG523R2 primer pair with Heinz 1706 (acc. no. DQ097530), Gh13 (acc. no. DQ097531), Gc9 (acc. no. DQ097529), Sheriff (acc. no. DQ097532), and Gc173 (acc. no. DQ097533), and LA1968. Sequence was obtained for LA1777 with only the forward TG523F2 primer. Sequence was also obtained for both the forward and reverse TG523F3/TG523R4 primer pair with Heinz 1706 (acc. no. DQ097537), Gc9 (acc. no. DQ097534), Gc173 (acc. no. DQ097535), Gh13 (acc. no.

DQ097536), and Sheriff (acc. no. DQ097538). When these sequences were aligned, there were no SNPs or INDELS detected that distinguished the begomovirus-resistant genotypes, Gc9, Gh13, and Gc173, from the susceptible genotype, Heinz 1706. When Heinz 1706 was compared to the LA1777 and LA1968 species there were 6 SNPs between Heinz and LA1777, and 7 SNPs and 2 INDEL between Heinz and LA1968 (Table 20). Thus, there is no evidence that supports an introgression of a begomovirus resistance gene at the *TG523* locus.



**Table 20:** Sequence differences found at the *TG523F2/TG523R2* locus. In instances where an SNP or INDEL was found but not all of the lines gave sequence in that area, the absence of sequence for that line is indicated by N/A. All tested lines not listed matched exactly with Heinz 1706. The nt position is relative to Heinz 1706.

Line	INDEL	INDEL	SNP 2	SNP 4	SNP 5	SNP 6
Heinz 1706	GTT	T	T	T	C	G
LA1777	N/A	N/A	N/A	C	T	G
LA1968	...	.	G	C	C	A
nt Position	76-78 bp	93 bp	104 bp	152 bp	163 bp	202 bp
Line	SNP 7	SNP 8	SNP 9	SNP 10	SNP 11	
Heinz 1706	C	A	G	A	A	
LA1777	C	T	T	T	G	
LA1968	T	T	T	A	G	
nt Position	277 bp	304 bp	316 bp	344 bp	408 bp	

## Overall Discussion

Altogether we designed 70 primers for use with 15 different RFLP probes on Chromosomes 1, 2, 4, 7, and 11. Reliable sequence data was obtained at eight of the RFLP probes, and lead to the submission of 22 sequences to Genbank. This sequence data produced significant evidence against an introgression of the begomovirus resistance gene at five of the RFLP probes (*TG301* on Chr. 1, *CT140* on Chr. 2, *TG208* and *TG287* on Chr. 4, and *TG523* on Chr. 11). Data at the other RFLP probes was inconclusive, but was not deemed worthy of additional study. Previous studies have shown that introgressions from *L. hirsutum* tend to be on the order of 5-50 cM (Monforte and Tanksley, 2000). Therefore, the resistance gene is not likely to be found in a 10-20 cM interval around the RFLP probes which produced significant results. Overall, no evidence of an introgression from *L. hirsutum* or *L. chilense* was found at any of the tested Rgh or RGHH.

### Benefits of This Study

This study narrows the required scope of future investigations of the source of begomovirus resistance. There are 14 known Rgh and 30 known RGHH on the tomato chromosome, and thus this study has eliminated 4 of the 44 likely locations for begomovirus resistance. In addition, molecular markers for LA1777 were found at all of the RFLP probes that produced significant results. These markers could be used by breeders who are trying to introduce an introgression from LA1777 into one of these areas. Finally, the primers designed in this study have been used by colleagues who needed primers specific to the relevant RFLP probe. Specifically, our lab has used the primers to test the validity of LA1777 introgressions at specific areas of the tomato genome.

### Future Studies

Future studies should attempt to analyze the remaining hotspots. Subsequently, a method that detected variability over the entire tomato genome, such as AFLP analysis, could be used to locate likely sources of the introgression. In addition, future studies should investigate the interesting sequence data obtained at TG293, TG208, TG287, and TG633. In the case of TG293 and TG633, Heinz 1706 produced good sequence data, but no other tested lines were interpretable. In the case of TG208 and TG287 significant patterns were found that did not correlate to any known resistance gene or morphological difference. These anomalies may be related to important phenotypic or genotypic differences; however that investigation was beyond the scope of the current study.

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