CAPS marker for detection of Ty3a-locus associated with tomato inbred line, Gc171, which is resistant to whitefly-transmitted begomoviruses

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Introduction

Geminiviruses threaten many crops in Central America (Jones, 2003). There are three subgroups of geminiviruses in plants. The *Begomovirus* genus, also known as subgroup III, infects dicotyledonous plants and is transmitted by the whitefly *Bemisia tabaci* (Fauquet et al., 2003). These whitefly-transmitted viruses pose significant threats to crop production in southern regions of the United States as well as tropical and subtropical locations worldwide (Jones, 2003). Most viruses in subgroup III have a bipartite genome. The genome contains DNAs A and B, which are single-stranded circular DNAs (Fauquet et al., 2003), about 2,600 nucleotides. Component A contains a gene that is responsible for DNA replication and the coat protein, while the B component has two genes that code for proteins which allow for movement through the plant (Hanley-Bowdoin et al., 1999). The *Tomato yellow leaf curl virus* (TYLCV), which belongs to the *Begomovirus* genus, has an ssDNA monopartite genome, however (Navot et al., 1991). This monopartite genome is similar to DNA A of the bipartite genome. The loss of crops due to geminiviruses has been so overwhelming for the Central American region that production of some crops has vanished in select areas (Jones, 2003). The standard management strategy against this epidemic has been a massive overuse of insecticides (Morales and Jones, 2004). Insecticides as a form of control are not only expensive, but they tend to have limited success and cause environmental contamination and health risks for those working with them (Morales and Jones, 2004).

Resistance to TYLCV has been observed in several wild-type species, *Solanum chilense, S. pimpinellifolium, S. peruvianum, S. habrochaites, and S. cheesmaniae* (Ji and Scott, 2006). However, still no resistance has been observed in the *Solanum lycopersicum* (Ji and Scott, 2006). Three *Solanum chilense* accessions, LA1932, LA2779, and LA1938, each have three different regions on chromosome six of their genome that are associated with resistance to TYLCV and another begomovirus, *Tomato*

mottle virus (ToMoV) (Agrama and Scott, 2006). This implies that resistance to the TYLCV involves at least three different loci. Studies have been done that suggest that two of the regions, Ty1 and Ty3 may be linked, and the presence of both resistance genes in a single genome may provide greater resistance (Ji and Scott, 2006).

Located at 25 centimorgans (cM) of chromosome six is the region of interest for this research project, the Ty3 region (Maxwell, pers. com.). This region explains roughly 65% of variance in resistance to TYLCV in F2 progeny from an initial cross of a susceptible *S. lycopersicum* with a resistant advanced breeding line having an introgression (Ji and Scott, 2006). These findings regarding Ty3 indicate that it is a significant locus in resistance to TYLCV (Ji and Scott, 2006). Three different alleles have been observed at the Ty3 locus by sequence analysis (Maxwell, pers. com.). The Ty3 and Ty3a alleles are associated with begomovirus resistant breeding lines in Guatemala (Maxwell, pers. com.). The ty-3 allele, however, is associated with susceptible plants (Maxwell, pers. com.). The purpose of this study is to develop an assay for detection of the Ty-3a allele associated with tomato breeding line, Gc171, which has the Ty3a allele (Maxwell, pers. com.).

Materials and Methods

Tomato Germplasm

Gc171 and GIh902b are tomato breeding lines that are resistant to begomovirus in Guatemala (Mejía and Maxwell, pers. com.). Gc171 originated from an introgression from *S. chilense* LA1932 by J. W. Scott (Maxwell and Mejia, pers. com.) and has a different sequence at 25 cM region of chromosome six than the sequence for M82-1-8 (susceptible to begomoviruses) and GIh902b (resistant to begomoviruses, C.T. Martin and D.P. Maxwell, pers. com.). M82-1-8 (*S. lycopersicum*) has ty-3 allele and GIh902b (introgression from *S. chilense* LA 2779 and/or S. *habrochaites*) has the Ty3 allele. Gc171 (introgression from *S. chilense* LA 1932), which is resistant to begomoviruses in Guatemala, was used as the source of the Ty-3a allele.

DNA Extraction

DNA was extracted from young, fresh tomato leaves from tomato plants grown using a plant growth cart in the Maxwell Laboratory at Russell Laboratories. Plants were ready for extraction approximately two-three weeks after planting. Roughly, 30 mg of

young growth from each plant was frozen in liquid nitrogen and crushed into small segments with a Kontes Pestle in a 1.7-mL microfuge tube. The PUREGENE DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) was used for DNA extraction following the procedure outlined by the manufacturer. The DNA was adjusted to approximately 10ng/µl.

Primer Development and Primer Analysis

Primers were developed from the FER BAC clone (AY678298) of *Solanum lycopersicum*, on chromosome six in the 25 cM area of interest. The sequence was selected from gene eight of the BAC clone, which is located at 170,770-173,873 nucleotides. Primers were developed to anneal at exons while amplifying one complete intron, and yielding a PCR fragment of approximately 300-800 nucleotides. Two introns from gene eight were used to develop two sets of primers. From each intron, two forward and two reverse primers were designed (Fig. 1). All primers were evaluated using polymerase chain reaction (PCR) and electrophoresis analysis (Fig. 2 and 3), and those that consistently produced a single visible band were selected for use in this project. The forward primer, (FER-G8F1, 5'-CATCCCGTGCATCATCCAAAGTGAC-3'), and the reverse primer, (FER-G8R1, 5'-CTAAGGGTGTACCCCAAGGGAAC-3') annealed at nucleotide 171,587 and 172,113, respectively, and produced a 530-bp fragment.

PCR and Fragment Analysis

PCR was used to exponentially amplify the desired fragments of DNA. The PCR reaction master mix was prepared using methods established in the Maxwell Lab. The 25 µl PCR mixture contained 5 µl water, 2.5 µl 2.5 mM dNTPs, 5 µl 5X buffer, 2.5 µl 2.5 mM MgCl2, 0.1 µl (0.5 units) Go Taq DNA polymerase (Promega Corp., Madison, WI), 2.5 µl forward primer at 10 µM, 2.5 µl reverse primer at 10 µM, and 5 µl DNA extract. PCR cycles include 94 C for 3 min., then 35 cycles of 94 C for 30 sec., 55 C for 1 min., and 72 C for 1.5 min. Once the cycles were finished, they were then followed by 10 min. at 72 C. The reactions were held at 4 C following completion of PCR. After completing the cycles in the MJ DNA Engine PT200 Thermocycler (MJ Research Inc., Waltham, MA), the PCR-amplified DNA was separated by agarose gel electrophoresis. A 1.5% agarose gel was used in 0.5X TBE buffer bath, which ran at an electric current of 135 volts. After movement of the negatively charged DNA through the matrix of agarose, the

gel was soaked and stained with ethidium bromide for approximately 10 min. to allow for visualization of the bands of DNA using UV light. The Kodak Gel Logic 200 Imaging System was used to capture an image.

Sequencing and Comparison

Sequencing of DNA involved a three reaction procedure. The ssDNA was digested in PCR reactions with 1 µl shrimp alkaline phosphatase (Progmega Corp.), 1 µl exonuclease I (Epicentre, Madison, WI), and 5 µl of PCR reaction mixture in a "cut and kill" PCR protocol, which consisted of 37 C for 15 min. followed by 80 C for 15 min. These PCR reaction mixtures were directly sequenced with Big Dye Sequencing Kit reagents. The Big Dye reaction mixture contained 3 µl buffer, 2 µl Big Dye, 1 µl primer, 7 µl water and 7 µl PCR fragments from the cut and kill PCR procedure. The sequencing Big Dye PCR cycles were: 36 cycles of 96 C for 10 sec., 55 C for 15 sec. and 60 C for 3 min., which were followed by 72 C for 7 min. and the reaction sat at 4 C indefinitely. Magnetic beads (Beckman Coulter Company, Beverly, MA) were applied following the Big Dye reaction. The mixture was then analyzed by the Biotechnology Center, University of Wisconsin-Madison. The sequences were aligned and evaluated using computer programs, Chromas and DNAMAN. Figure 4 illustrates the comparison of the three DNA segments, one from each line M82-1-8, GIh902b, and Gc171, amplified using selected primer pair, FER-G8F1 and FER-G8R1.

Restriction Digestion and Analysis

The restriction enzyme digestion reaction was a 25 µl reaction mixture containing 11 µl water, 3 µl buffer, 0.25 µl BSA, 1 µl *Taq*I restriction enzyme (Promega Corp.), and 10 µl PCR reaction mixture. The reaction mixture was placed in a 65 C water bath for about 3 hrs. Analysis of digestion was completed with agarose gel electrophoresis, using 2% agarose gel in a 0.5X TBE buffer. Electrophoresis ran for approximately 40 min. at 105 volts. For visualization of bands, the gel was stained with ethidium bromide for approximately 10 min., and then viewed with UV light. The Kodak Gel Logic 200 Imaging System was used to capture the gel image.

Results and Discussion

The primers, FER-G8F1/FER-G8R1, gave approximately 500-bp fragments using DNAs Gc171, M82-1-8, and GIh902b (Fig. 5). The DNA sequences were aligned and

compared for insertions and deletions (indels) and single nucleotide polymorphisms (SNPs, Fig. 4). The aligned sequences were visibly scanned for restriction sites by observing palindromic sequences (Fig. 4). *Taq*I site, 5'-TCGA-3', was not observed in the M82-1-8 sequence. However, Gc171 had one *Taq*I site (*Ty-3a* locus), and two *Taq*I sites were found in GIh902b sequence (*Ty-3* locus), (Fig. 4). PCR fragments were digested with *Taq*I restriction enzyme and the expected fragment sizes were obtained for each genotype (Fig. 6). The FER-G8 PCR fragment of M82-1-8 had the same size band as the fragment without being digested, this is due to the lack of a *Taq*I site in this fragment. The PCR fragment from Gc171, however, gave two bands. This observation is the result of Gc171 having one *Taq*I site. As a result of two *Taq*I sites, GIh902b gave three bands after the digest.

Conclusion

The FER BAC sequence is located in a region of chromosome 6 that has been associated with gene(s) for resistance to begomoviruses (Ji and Scott, 2006). The PCR molecular markers (FLUW25F/R) for the *Ty3* allele in this region did not yield a PCR fragment from begomovirus-resistant line Gc171. The PCR primer set FER-G8 F1/R1 amplified a 500-bp fragment from genotype *ty3/ty3* (*S. lycopersicum*), *Ty3/Ty3* (introgression from *S. chilense* LA2779), and *Ty3a/Ty3a* (introgression from *S. chilense* LA1932). The sequences of the FER-G8 F/R fragment from these three genotypes were different. Single nucleotide polymorphisms and indels were observed in the consensus sequence among these three DNAs. For SNPs, there were 13 single nucleotide differences among the three DNA sequences. One indel, an additional adenine nucleotide was present in the Gc171 PCR DNA fragment only. From the sequence comparison, it was determined that *Taq*I restriction enzyme would distinguish among these genotypes. Thus, the FER-G8 fragment could be used as a Cleavage Amplified Polymorphic Sequence (CAPS) marker for the three alleles.

This CAPS marker allows the selection of the *Ty-3a* allele in segregating populations, such as F2 populations. When homozygous *Ty-3a* plants are crossed with homozygous *ty3* plants, the F1 progeny are all *Ty-3a/ty3* heterozygotes. When this plant is selfed, it will produce three different genotypes in the F2 generation, *Ty-3a* homozygous, *ty3* homozygous, and *Ty-3a/ty3* heterozygous. Before now, one could not

distinguish between the *Ty-3a/ty3* heterozygous plant and the *Ty-3a* homozygous plant, because they both carry the resistant gene in their genome. This research allows one to distinguish between the resistant plants and select for the *Ty-3a* homozygous plants. This is advantageous because easy selection for *Ty-3a* homozygous plants ensures that all plants in the next selfed generation will be homozygous for this allele.

In conclusion this CAPS marker, FER-G8, will be useful for detection of the *ty3*, *Ty3*, and *Ty3a* alleles in plants, which are part of a tomato breeding program for resistance to begomoviruses.

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Region of Gene 8 from BAC Clone (AY678298) used for primers F/R 1 and 2

```
        caaagaaatt gaaatcgcat gagaagtcta acagatttca tcttatgacc
        cttcttatca tacagaggga ctgatcggat ccctgttctc aactctgaga caggcaaaca
170881  tgtttgtcca ccaaaatcat ccttctccga catgtcatat tctcgtactt caattcgaag
170941  caaagccagc tctggtactg tcaatggaaa attaaactct tcatcccata cgggacacca
171001  gtcatcctcc agtatccttg tctttttctt acgactatct gcaggaactc caactaaata
171061  tagctgcgac ataaggatca atagagttat aataagttcc tgcagcctct tggagtataa
171121  agcagaacat caatgaccat acaaatatgt aaagtaaaaa ggcaaggaca caccaaacaa
171181  gaaacataac accttaatta tgagctcagg acagggtcaa agctacgaat gtgttaattc
171241  aggtaagcaa atgagtcgtg gaagagggaa cagtcactgt cactaacctt tgtgtagaag
171301  tctggtggtg aataggcatc aaaatgtgtg tggctgaaat ccaagcgcca cccatctccc
171361  atatatactc taacctgcag
```

Primer Names:

KJG8F1 catacagagggactgatcgg
KJG8F2 gtccaccaaaatcatccttctcc

KJG8R1 gagatgggtggcgttgg
KJG8R2 ccctcttccacgactcatttgc

Region of gene 8 from BAC Clone (AY678298) used for primers F/R 3 and 4

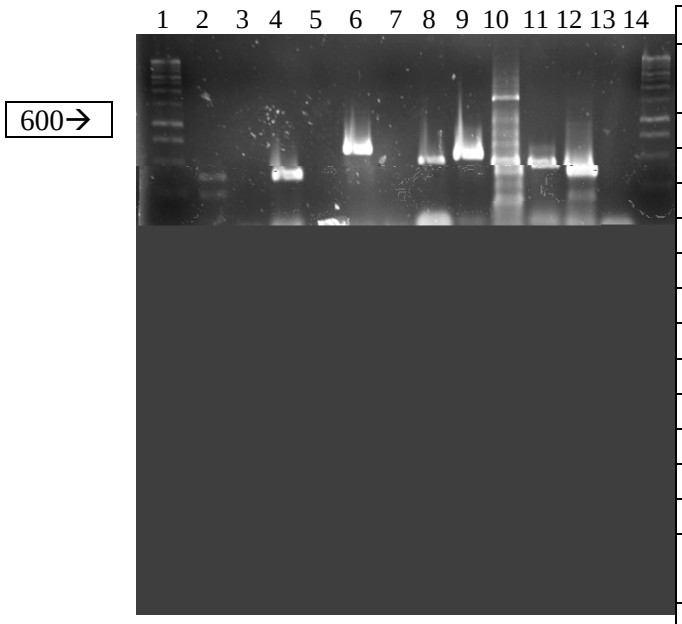
```
171481  gcttacctgt aaagtttgct tcactggcaa ttttacttta ggatcaaata cctcattgtt
171541  tgggccttta tccatgagaa attgaggttt tttcacatag ccacagcttc cattagacct
171601  aaacatcccg tgcatcatcc aaagtgactt cccatatccc tatttataca gttagcaatt
171661  agacagcagg tatggaacca aattgattag aacgataaaa tcgggagaca aaatagttga
171721  attatctatt aaatcatact cattgccaac acatttaaga agaggagttt cttagggaca
171781  taaagaactg cagaagcaat cggcaccaat gagatgtaag gcatggatgc ccttgaatgt
171841  taagttagtc aagcaagctc aagctgcaga gctgatacag tatcttacat gctagtttgc
171901  attttgattg atatatatca gcctgtacaa gaccttccaa caaacactac atactaatca
171961  tgatgttaat gcacataact acggatgcac cagcattcag aagttcaaac taacctgcat
172021  gttgaatgct accatctgag ctccatgcat ccagccagtc ataggcttga aatttgagga
172081  ggtcactctt gttcccttgg ggtacaccct tagaatattt ttctgtgtga acctagcaaa
```

Primer Names:

KJG8F3 cctcattgtttgggcctttatcc
KJG8F4 catcccgtgcatcatccaaagtgac (FER-G8F1)

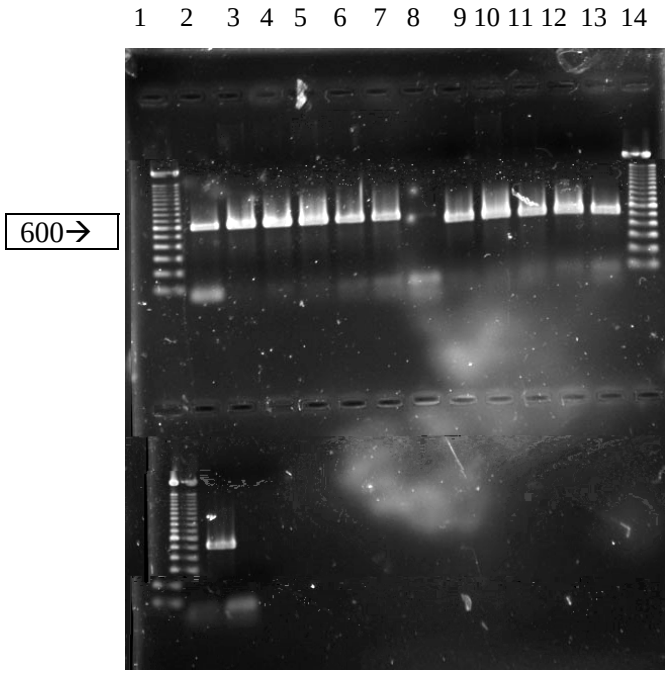
KJG8R3 ctaagggtgtaccccaagggaac (FER-G8R1)
KJG8R4 ctatgactggctggatgcatggag

Fig. 1. Primers designed from gene 8 of the FER BAC clone (AY678298) chromosome 6, 25 cM.



Lane	DNA	Primers
1	100-bp Invitrogen Marker	
2	W100	F1/R1
3	W100	F2/R1
4	W100	F2/R2
5	W100	F1/R2
6	W100	F3/R3
7	W100	F3/R4
8	W100	F4/R4
9	W100	F4/R3
10	W101	F1/R1
11	W101	F1/R2
12	W101	F2/R2
13	W101	F2/R1
14	100-bp Invitrogen Marker	
15	100-bp Invitrogen Marker	
16	W101	F3/R3
17	W101	F3/R4
18	W101	F4/R4
19	W101	F4/R3

Fig. 2. PCR with various primer pair combinations using Ty3a DNA, Gc171 (W100 and W101), and annealing at 55 C.



Lane	DNA	Primer
1	Ladder	
2	902b	F3/R3
3	Gh13	F3/R3
4	Heinz	F3/R3
5	M82-1-8	F3/R3
6	Gc171	F3/R3
7	Gc171	F3/R3
8	Water	F3/R3
9	902b	F4/R3
10	Gh13	F4/R3
11	Heinz	F4/R3
12	M82-1-8	F4/R3
13	Gc171	F4/R3
14	Ladder	
15	Ladder	
16	Gc171	F4/R3
17	Water	F4/R3

Fig. 3. PCR, annealing at 55 C with primer sets that worked most favorably (F3, F4, R3, R4) in above gel (Fig. 2) using various DNA to further examine their ability.

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M82-G8-F4R3 CTTCCATTAGACCTAAACATCCCGTGCATCATCCAAAGTGACTTCCCATATCCCTATTTA     60
100-G8-F4R3 CTTCCATTAGACCTAAACATCCCGTGCATCATCCAAAGTGACTTCCCATATCCCTATTTA     60
902b-G8F4R3 .........GACCTAAACATCCCGTGCATCATCCAAAGTGACTTCCCATATCCCTATTCA     51
Consensus            gacctaaacatcccgtgcatcatccaaagtgacttcccatatccctatt a

M82-G8-F4R3 TACAGTTAGCAATTAGACAGCAGGTATGGAACCAAATTGATTAGAACGATAAAATCGGGA    120
100-G8-F4R3 TACAGTTAGCAATTAGACAGCAGaTATGGAACCAAATTGATTAGAACGATAAAATtGGGA    120
902b-G8F4R3 TACAGTTAGCAATTAGACAGCAGaTATGGAACCAAATTGATTAGAACGATAAAATtGGGA    111
Consensus   tacagttagcaattagacagcag tatggaaccaaattgattagaacgataaaat ggga

M82-G8-F4R3 GACAAAATAGTTGAATTATCTATTAAATCATACTCATTGCCAACACATTTAAGAA.GAGG    179
100-G8-F4R3 GACAAAATAGTTGAATTATCTATTAAATCATACTCATTGCCAACACATTaAAGAAaGAGG    180
902b-G8F4R3 GgCAAAATAGTTGAATTATCTATTAAATCATACTCATTGCCAACACATTaAgaAA.GAGG    170
Consensus   g caaaatagttgaattatctattaaatcatactcattgccaacacatt a  aa gagg

M82-G8-F4R3 AGTTTCTTAGGGACATAAAGAACTGCAGAAGCAATCGGCAACAATGAGATGTAAGGCATG    239
100-G8-F4R3 AGTTTCTTAGGGACAaAAAGAAaTGCAGAAGCAATCGaCAACAATGAGATGTAAGGCATG    240
902b-G8F4R3 AGTTTCTTAGGGACATAAAGAAaTGCAGAAGCAATCGaCAAaAATGAGATGTAAGGCATG    230
Consensus   agtttcttagggaca aaagaa tgcagaagcaatcg caa aatgagatgtaaggcatg

M82-G8-F4R3 GATGCCCTTGAATGTTAAGTTAGTCAAGCAAGCTCAAGCTGCAGAGCTGATACAGTATCT    299
100-G8-F4R3 GATGCCCTTGAATGTTAAGTTAGTCAAGCAAGCTCAAGCTGCAGAGCTGATACAGTATCT    300
902b-G8F4R3 GATGCCCTTGAATGTTAAGTTAGTCgAGCAAGCTCAAGCTGCAGAGCTGATACAGTATCT    290
Consensus   gatgcccttgaatgttaagttagtc agcaagctcaagctgcagagctgatacagtatct

M82-G8-F4R3 TACATGCTAGTTTGCATTTTGATTGATATATATCAGCCTGTACAAGACCTTCCAACAAAC    359
100-G8-F4R3 TACATGCTAGTTTGCATTTTGATTGATATATATCAGCCTGTACAAGACCTTCCAACAAAC    360
902b-G8F4R3 TACATGCTAGcTTGCATTTTGATTGATATATATCAGCCTGTACAAGACCTTCCAACAAAC    350
Consensus   tacatgctag ttgcattttgattgatatatatcagcctgtacaagaccttccaacaaac

M82-G8-F4R3 ACTACATACTAATCATGATGTTAATGCACATAACTACGGATGCACCAGCATTCAGAAGTT    419
100-G8-F4R3 ACTACATACTAATCATGATGTTAATGCACATAACTACGGATGCACCAGCATTCAGAAGTT    420
902b-G8F4R3 ACTACATACTAATCATGATGTTAATGCACATAACTACGGATGCACCAGCATTCAGAAGTT    410
Consensus   actacatactaatcatgatgttaatgcacataactacggatgcaccagcattcagaagtt

M82-G8-F4R3 CAAACTAACCTGCATGTTGAATGCTACCATCTGAGCTCCATGCATCCAGCCAGTCATAGG    479
100-G8-F4R3 CAAACTAACCTGCATGTTGAATGCTACCATCTGAGCTCCATGCATCCAGCCAGTCATAGG    480
902b-G8F4R3 CAAACTAACCTGCATGTTGAATGCTACCATCTGAGCTCCATGCATCCAGCCAGTCATAGG    470
Consensus   caaactaacctgcatgttgaatgctaccatctgagctccatgcatccagccagtcatagg

M82-G8-F4R3 CTTGAAATTTGAGGAGGTCACTCTTGTTCCCTTGGGGTACACCCTTAG               527
100-G8-F4R3 CTTGAAATTTGAGGAGGTCACTCTTGTTCCCTTGGGGTACACCCTTAG               528
902b-G8F4R3 CTTGAAATTTGAGGAGGTCACTCTTGTTCCCTTGGGGTACACCCTTAG               518
Consensus   cttgaaatttgaggaggtcactcttgttcccttggggtacacccttag
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TCGA = Sequence cut by restriction enzyme *TaqI*
Blue = Signifies a place of sequence differences among the three different DNAs.

Fig. 4. Multiple sequence alignment to observe for differences among the three DNAs of interest, M82= M82-1-8 (susceptible), Gc171 (100) (resistance from LA1932), GIh902b (resistance from introgression similar to LA2779), in the segment annealed by primers FER-G8F1/FER-G8R1.

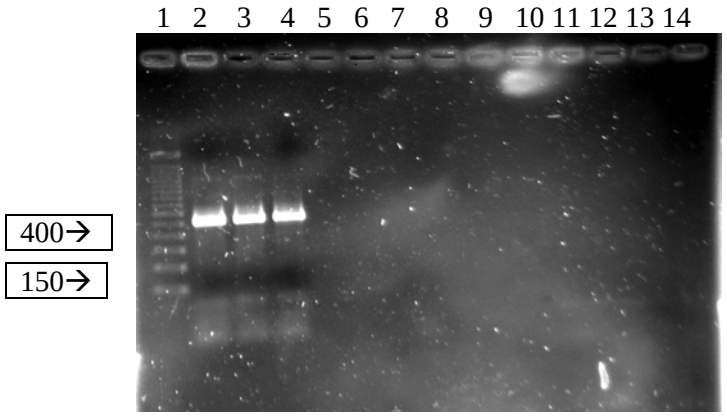

Fig. 5. PCR with primers FER-G8F1/FER-G8R1 at annealing temperature 55 C. Lane 1, 100-bp marker (Invitrogen); lane 2, Gc171 (Ty3a/Ty3a); lane 3, M82-1-8 (ty3/ty3); lane 4, GIh902b (Ty3/Ty3).

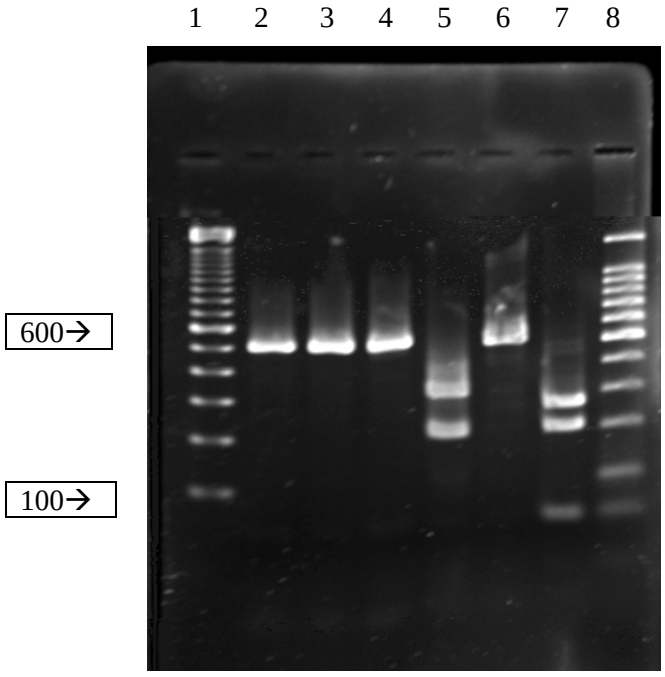

Fig. 6. PCR with primers FER-G8F1/FER-G8R1 at annealing temperature of 55 C and digest with restriction enzyme *Taq*I at 65 C. Lane 1, 100-bp marker (Invitrogen); lane 2, undigested Gc171; lane 3, undigested M82-1-8; lane 4, undigested GIh902b; lane 5, digested Gc171; lane 6, digested M82-1-82; lane 7, digested GIh902b; lane 8, 100-bp marker (Invitrogen).