Development of a Molecular Marker for Geminivirus Resistance Genes in Tomatoes through Analysis of Tomato Resistance Gene Homologues

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Abstract

Each year geminiviruses 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 hopes to develop a molecular marker for the gene that controls resistance to geminiviruses. We hypothesize that a molecular marker for geminivirus resistance can be found within the resistance gene homologues on chromosomes two and eleven. To accomplish our goal we will use a PCR-based tagging method to identify differences in sequences between resistant and susceptible tomato breeding lines. Sequence differences would be indicative of a DNA introgression from a resistant species and could be used as a molecular marker. A successful molecular marker would enable breeders to quickly determine a tomato's susceptibility or resistance to geminiviruses and greatly aid in the creation of a commercially viable resistant hybrid.

Significance and Originality

Tomatoes in Central America are plagued by a number of geminiviruses that are transmitted by the whitefly, *Bemisia tabaci* (Jones, 2003). The effect of these diseases is near total loss of crops (Morales and Anderson, 2001; Nakhla *et al*., 2004). *Lycopersicon hirsutum* and *Lycopersicon chilense* are wild species of tomato that have shown resistance to geminiviruses (Scott *et al*., 1995; 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 an acceptable fruit (Chen *et al*., 2003; Mejia *et al*., 2004; Narasegowda *et al*., 2003; Scott *et al*., 1995; Vidavsky and Czosnek, 1998). 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. Therefore, in order to facilitate the breeding efforts, 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 incorrect characterizations of a plant's resistance.

This study hopes to develop a molecular marker for resistance. We will look for a marker within tomato resistance gene homologues. 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 shown that there 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). These homologous sequences are defined as resistance gene homologues (RGH). The strong correlation between the resistance gene sequence and the RGH sequence indicates a high probability of finding a molecular marker within that region. Therefore, we hypothesize that a molecular marker for geminivirus resistance can be found within the tomato RGH. No previous researchers have ever looked for a molecular marker for geminivirus disease resistance within an RGH. This study will be the first to do so.

Project Summary

We will be using the tomato breeding lines, Gh13, Gc9, and Gc173, that are resistant to geminiviruses in Guatemala (Mejía *et al*., 2004; Nakhla *et al*., 2004). As a control, we will be using the breeding line Heinz 1706. Heinz 1706 is the tomato cultivar being sequenced in an international sequencing project (Budiman *et al*., 2000; Ozminkowski, 2004), and is susceptible to geminiviruses (Maxwell, D., pers. com.). 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).

Places on the tomato chromosome that contain several RGH are referred to as hotspots. For this research, hotspots are defined as a place on the genome where two or more RGH are located in close proximity. Hotspots on chromosomes two and eleven within the genome of Gh13, Gc9, and Gc173 will be tested to determine if there is a DNA introgression of *L. hirsutum* or *L. chilense*, respectively. Hotspots were chosen based on their concentration of RGH. We will test these hotspots by obtaining PCR fragments for the experimental lines and comparing them to the control, susceptible tomato, Heinz 1706. Differences in the sequences as small as 3-4% would be indicative of an introgression from a wild species. As a final step, the differences would then be compared against sequence from *L. hirsutum* and *L. chilense* in order to determine the origin of the introgression. If an introgression is found and these results can be verified in other lines of tomato, then we will use that hotspot as a marker for the resistance gene.

DNA Extraction

The geminivirus resistant lines, Gh13, Gc9, and Gc173, will be supplied by Dr. L. Mejía, Universidad de San Carlos, Guatemala City. The susceptible line Heinz 1706 will be supplied by Dr. R. Ozminikowski, Heinz Seed Co., Stockton, CA. The wild species *L. hirsutum* and *L. chilense* will be obtained from the Tomato Genetics Resource Center,

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

Primer Development

Primers will be developed for two hotspots, one on chromosome two and one on chromosome eleven. The hotspot on chromosome two is located between RFLP probes R45S and CT205 (Pan *et al.*, 1999). The hotspot on chromosome eleven is located between RFLP probes TG651 and TG36 (Pan *et al*., 1999). 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) will be accessed to determine if these sequences are associated with known plant genes. Where possible, primers will be designed to anneal to the exon regions and amplify at least one intron. Maxwell's lab group has completed this primer development process many times before, and the proposed experiment should pose no special problems (Czosnek, *et al*., 2004).

PCR Reactions and Fragment Analysis

PCR fragments from each set of primers, for each of the genotypes, will be obtained using methods developed in the Maxwell lab (Czosnek *et al*., 2004). The PCR-amplified DNA will be 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. If the primer pair produces multiple bands, we will redesign the primer and do PCR again. If the primer pair produced one band, this PCR fragment will be directly sequenced using Big Dye Sequencing Kit™ (Biotechnology Center, Madison, WI).

Sequencing and Comparison

Analysis of the sample sequences will be 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 will be looking for an introgression of *L. hirsutum* or *L. chilense* DNA. Sequence differences as small as 3-4%, such as single nucleotide polymorphisms (SNPs) or insertions/deletions of sequence (indels), between Gh13, Gc9 or Gc173 and Heinz 1706 would be evidence of an introgression. Once differences are found, those regions will be compared to sequence from *L. hirsutum* and *L. chilense* in order to determine the source of the introgression.

Expected Results

We expect to find an introgression of *L. hirsutum* or *L. chilense* DNA in at least one of the hotspots. In order to determine the validity of this introgression, several known geminivirus-resistant breeding lines would be tested with our PCR-based marker. If the introgression of *L. hirsutum* or *L. chilense* was present in all of the resistant lines and not the susceptible lines then this sequenced hotspot could be used as a molecular marker for resistance.

It is possible that no difference will be found between Gh13, Gc9, or Gc173 and Heinz 1706 at the hotspots we will be testing. This type of result would indicate that the two hotspots we had evaluated were not the location of the geminivirus-resistance gene. Future studies on other RGH hotspots would then be necessary in order to locate the gene responsible for resistance. There are only eighteen known hotspots for RGH on the tomato genome. Therefore, this type of result would still prove valuable as it would narrow down possible locations for a molecular marker for geminivirus resistance. If no evidence is found for the location of a marker at a known hotspot, then a method that detects genetic variability over the whole genome, such as amplified fragment length polymorphism (AFLP)-based tagging (Parella *et al.*, 2004), would be used to develop new starting points for the experiment.

With either a positive or negative outcome, the results of this experiment will bring us closer to developing a molecular marker for resistance gene to geminiviruses. In the end this research will result in saving millions of dollars and countless tomato crops throughout Central America.

Timeline (Summer 2005)

Task	June	July	August
DNA Extraction	X		
Primer Development	X		
PCR Reactions	X	X	
PCR Fragment Analysis		X	
Sequencing and Comparison		X	X
Presentation of Results			X

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