Introduction

There are many molecular techniques that are now available to assist plant breeders in producing disease resistant crops. These resistant crops would negate the need for the use of chemicals and other expensive disease preventative methods. Many pathogens have become problems world wide due to the efficiency and speed of modern day travel, such as airplanes. Tomatoes are one such crop that has been ravaged world wide by a large variety of pathogens, such as *Fusarium oxysporum* f. sp. *lycopersici*. Among tomatoes there are wild species that have genes that confer resistance to many of the problematic diseases, but their fruits are not marketable. To assist plant breeders, techniques such as PCR (Polymerase Chain Reaction) are being used to identify and track specific disease resistance genes through generations of breeding lines. This allows for a more rapid production of disease resistant progeny at any locus of interest by allowing plant breeders to select only the resistant progeny for the next generation of breeding. This thesis discusses the uses of PCR and PCR primers that will enable researchers to follow disease resistance genes at a multiple loci, allowing for the rapid identification of a plants resistance or susceptibility to a pathogen.

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Chapter 1:
Analysis of *REX-1* Locus Sequences of *Solanum* (*Lycopersicum*) Species

Abstract

PCR fragments were obtained using the REXF1/R2 primers from *Solanum* species that had been chosen for their broad genetic diversity and/or used in breeding programs. Every plant tested gave a 720-bp fragment, which was sequenced and analyzed for SNPs, indels, heterozygosity, and homozygosity. The resulting sequences were used to create a phylogenetic tree, which showed four distinct clades. The phylgenetic tree was represented by a clade with *lycopersicum, pimpinelifolium,* and *cerasiforme,* and three others with accessions from *chilense, peruvianum,* and *habrochaites*. Each clade was characterized by unique SNPs and these were used to make inferences about evolutionary relationships among the *Solanum* species. These results also allowed the identification of the origin of sequence at the *REX-1* locus in breeding lines, which can be used by researchers to make predictions about phenotypic characteristics, such as resistance to root-knot nematodes or begomoviruses.

Introduction

Root-knot nematodes, aphids, and whiteflies are some of the most destructive pests that affect crops around the world; they can parasitize and act as vectors on wide-spread, commercially grown crops, such as tomatoes. The *Mi-1* gene is known to confer resistance to a variety of these pests (Smith, 1944). At the *Mi* locus there are two transcribed genes that have a 91% homology: *Mi-1.1* and *Mi-1.2*. The *Mi-1.2* gene confers resistance to root-knot nematodes (Martinez et al., 2004). The *Mi-1* gene is located on the short arm of chromosome six (6S) in *Solanum lycopersicum,* and was successfully introgressed from *S. peruvianum* (see Zhong et al., 1999). The *Mi-1* locus is known to confer resistance to nematodes which include: *Meloidogyne arenaria, M. incognita,* and *M. javanica.* In addition the *Mi-1* locus confers some resistance to the potato aphid (*Macrosiphum euphorbiae*) (Rossi et al., 1998) and the whitefly (*Bemisia tabaci*) (Nombela and Muniz, 2000 and 2003).

Williamson et al. (1994) reported the use of REXF1/REXR2 primers to amplify the *REX-1* product, which is located at the *REX-1* locus. The *REX-1* locus is closely linked to the *Mi-1* locus, and a *Taq*I restriction site is present in the REX-1 fragment only from resistant phenotypes. A comparison of the plant sequences at one locus is one way to find trends that may lead to inferences about their evolutionary history between plants (Nesbitt and Tanksley, 2002).

Besides the *Mi-1* locus being closely associated with the *REX-1* locus, there is evidence that the *Ty-1* gene, that is associated with begomovirus resistance from *S. chilense*, is also associated with the *REX-1* locus (Judith Milo, Hebrew University of Jerusalem). Thus, a better understanding of *REX-1* locus among the various *Solanum* species may allow for an improved determination of the origin of introgressions, such as the *Ty-1* gene, in tomato breeding lines and hybrids.

The purpose of this research was: i) to examine the sequence variation at the *REX-1* locus of *Solanum* species, ii) to develop a phylogenetic tree for this locus, iii) to use general patterns (SNPs or indels) found for each species to identify unique introgressions in various breeding lines, and iv) to use this information to make inferences about the relatedness of the various lines tested. To date, no research has been done to specifically explore the variation within the

sequences of the *REX-1* locus among the different *Solanum* species.

Material and Methods

Plant materials: The cultivars used as the standards for comparison were: Motelle (supplied by V. Williamson, UC-Davis) and Anahu (supplied by F. Vidavski, Hebrew University), which are both *S. lycopersicum* that have the *S. peruvianum* introgression for the *REX-1* locus (*Mi-1/Mi-1*), Moneymaker (supplied by V. Williamson, UC-Davis), which is an S. *lycopersicum* (*mi-1/mi-1, ty-1/ty-1*), TY52, which is a *S. lycopersicum* that has the *S. chilense* introgression at the *REX-1* locus (*Ty-1/Ty-1*, *mi-1/mi-1*) (supplied by H. Czosnek, Hebrew University), a *S. lycopersicum* var. *cerasiforme* (ty-1/ty-1 GT99, CERA, collected in Sanarate, Guatemala in 1999), and M82, which is a *S. lycopersicum* with no *Mi-1* gene (*ty-1/ty-1, mi-1/mi-1*) (supplied by F. Vidavski, Hebrew University). The remaining accessions are listed in Table 5. The REX-1 sequence (AY949616) from Heinz 1706 (supplied by Rich Ozminkowski, Heinz Seeds) was used as a standard to define the nucleotide numbers for all sequence comparisons. The *Solanum* species included: *S. lycopersicum*, *pimpinellifolium*, *habrochaites*, *chilense*, *peruvianum*, *habrochaites* f. sp. *glabratum*, and *lycopersicum* var. *cerasiforme*. These accessions were chosen for their potential genetic diversity (Nesbitt and Tanksley, 2002) and/or because many of them have been used in breeding programs for begomovirus resistance, eg. LA1969, in a breeding program at Hebrew University (Zamir et al., 1994)(Mejía et al., 2005) and LA1932 and LA2779 were used by J.W. Scott (Agrama and Scott 2006) to detect tomatoes with resistance to monopartite and bipartite begomoviruses. All of the genotypes labeled with an LA came from the Tomato Genetic Resource Center, University of California-Davis (UC-Davis).

DNA isolation, PCR amplification, sequencing, and sequence analysis: DNA was extracted from fresh leaves of plants grown in a plant growth chamber at the University of Wisconsin-Madison (UW-Madison). Thirty mg of young leaf tissue was frozen in liquid nitrogen in a 1.5-ml microfuge tube, and then ground with a sterilized Kontes™ micropestle (Kontes Glass, Vineland, NJ) and extracted with the PURGENE® DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. DNA concentrations were then measured and adjusted to 15 ng/µl and the extracted DNA was frozen at -20°C. The REXF1/REXR2 primers (Williamson et al., 1994) were purchased from Integrated DNA Technologies Inc., Coralville, IA. PCR parameters were for 50 µl reactions containing 5 µl 2.5 mM dNTPs, 5 µl of a 10x buffer, 5 µl 25 mM $MgCl_2$, 0.2 µl *Taq* DNA polymerase, 5 µl each of both forward and reverse primers at 10 µM, 5-7 µl of DNA extract, and H_2O. The PCR thermocycler parameters for fragment amplification were as follows: denaturation at 94°C for 3 min, then 35 cycles of 94°C for 30 sec, 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 done in the MJ DNA Engine PT200 Thermocycler™ (MJ Research Inc., Waltham, MA). All molecular biology chemicals for the PCR were purchased from Promega, Corp., Madison, WI. PCR fragments were then separated by gel electrophoresis using 1.5% Seakem LE™ agarose (BioWhittaker Molecular Applications Rockland, ME) in 0.5X TBE buffer, then stained with ethidium bromide and visualized with a Kodak Gel Logic 200 Imaging System. All PCR fragments were directly sequenced using the Big Dye Sequencing Kit™ (Biotechnology Center, Madison WI), both REXF1 and REXR2 primers were used to achieve a forward and a reverse sequence that could then be compared so that a reliable consensus sequence of 720 bp could be obtained. Analysis of the resulting sequences was accomplished by using the DNAMAN software (Lynnon Corp., Quebec, Canada). The phylogentic trees were created using a multiple sequence alignment with the Biology WorkBench 3.2 CLUSTALW

software (San Diego Supercomputer Center, University of California-San Diego).

Results and Discussion

The same size PCR fragment (720 bp) was obtained with the REX primers for all germplasm samples examined (Table 5). The REX-1 fragments were then sequenced and examined in order to detect any variation (indels and SNPs) and to determine heterozygosity or homozygosity for each accession, hybrid, or breeding line.

Analysis for homozygosity/heterozygosity at the *REX-1* locus: Heterozygosity in a sequence was identified when there were two peaks of similar intensity at one nucleotide location in the ABI sequence file (Fig. 1). The lines that were found to be homozygous at the *REX-1* locus were: *S. lycopersicum* [Heinz 1706, Rio Grande, Anahu, Motelle (LA2823), Motelle**,** LA3173, Moneymaker, and M82], *S. lycopersicum* var. *cerasiforme* [VFNT Cherry (LA1221) and CERA GT99], *S. pimpinellifolium* (LA1582), *S. chilense* (TY52, LA1932, LA2779, LA1968A, LA1969A and LA1969C-E), and *S. habrochaites* (LA1777, LA0386, LA1033, and LA3863). The heterozygous lines at the *REX-1* locus were *S. pimpinellifolium* (one heterozygous SNP, LA0121), *S. chilense* (12 heterozygous SNPs for LA1968B, D, and E, two heterozygous SNPs for LA1968C, six heterozygous SNPs for LA1969B, and one heterozygous SNP for LA1970), and *S. peruvianum* (one heterozygous SNP for LA0462).

The ability to accurately identify the heterozygous and homozygous plants in the progeny of breeding programs quickly and efficiently would be very advantageous, and would greatly help breeders and researchers. This knowledge would allow breeders to choose the next generation of parents from the homozygous plants, which would guarantee that the next generation of progeny would be homozygous for the locus of interest, ensuring that desired genes would in fact be passed on. The identification of homozygosity or heterozygosity is a technique that can be utilized for any locus that is of interest to breeders, this is only one specific example of its potential.

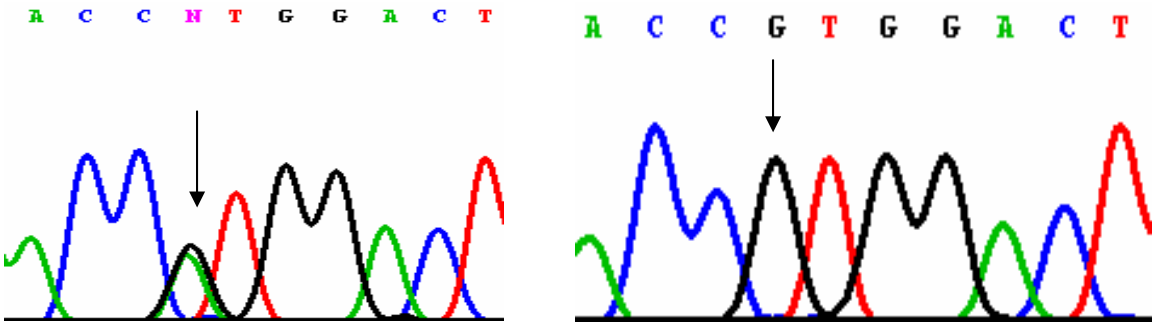


Figure 1: These ABI images of a heterozygous plant (image on the left) sequence compared to a homozygous sequence (image on the right) at the exact same nucleotide position. Observe how the heterozygous image has two peaks green and black (A and G, respectively) at one position, while the homozygous plant has only the black peak (G).

Diversity among single accessions: The *chilense* accession, LA1968 was most diverse among the accessions evaluated. Five different plants were tested, and from these five plants three different sequences were obtained. Sample LA1968A was the only sample out of the five that was homozygous, LA1968C had two heterozygous SNPs, and LA1968B, D, and E all had the same 12 heterozygous SNPs.

The *S. chilense* accession, LA1969, was also diverse; five plants of this accession were

evaluated, and plants LA1969A, C-E were all found to be homozygous at all positions, but plant LA1969B was heterozygous at six locations (6 SNPs). These two accessions show that there is no guarantee that the progeny of the same accessions will all produce the same result (i.e. all heterozygous or homozygous). The ability to distinguish these progeny and pick the homozygous plants to breed for the next generation would be advantageous, as this would exclude the necessity of checking the next generation for heterozygosity or homozygosity at this locus. *S. chilense* is a self-incompatible species so it is expected that there will be heterozygosity among plants from an accession.

Gene tree of the Solanum spp. REX-1 sequences: A phylogenetic tree was created from the *REX-1* consensus sequences for 22 plants (18 were homozygous and 4 were heterozygous). In the phylogenetic tree, only the sequences with less than three SNPs were included with the sequences from the homozygous plants. The samples with many heterozygous SNPs were not included because the computer program that was utilized to produce the phylogenetic tree was unable to recognize the heterozygous spots and analyze them. Instead this program would merely treat these areas as mistakes in the sequence, and these mistakes would cause erroneous results.

The phylogenetic tree showed four distinct clades (Fig. 2). The clade for *S. lycopersicum* also contains the two accessions of the *S. pimpinellifolium*. The other three clades represent the three species, *S. chilense*, *S. habrochaites*, and *S. peruvianum*. Within each clade there are conserved nucleotides that are completely unique to that species. When listing the SNPs, the first nucleotide represents the unique nucleotide found in all of the samples of the group and the nucleotide located after the slash is shared by all other plants not in the group. The SNPs identified here can be used to identify and then to classify the origin of the introgression at the *REX-1* locus.

***Unique SNPs found in the S. lycopersicum clade*:** The *lycopersicum* clade was composed of: Heinz 1706 (*lycopersicum*), M82 (*lycopersicum*), Rio Grande (*lycopersicum*), LA3173 (*lycopersicum*), LA0121 (*pimpinellifolium*), LA1582 (*pimpinellifolium*), and CERA–GT99 (*cerasiforme*). This clade contained the known *S. lycopersicum* var. *cerasiforme*, CERA-GT99, which is a wild relative of *lycopersicum* and known in Guatemala as Tomatillo, and another closely related species, *pimpinellifolium* (Spooner et al., 2005). The sequence comparisons showed that this group had five unique SNPs: G/T, A/G, A/G, A/C, and A/G at nt 39, 106, 144, 241, and 495, respectively. This information allows a positive identification of *lycopersicum* sequence at this locus.

Unique SNPs found in the S. chilense clade: The next group was a *chilense* clade which consisted of the *chilense* plant sequences: TY52 (*lycopersicum*, with *chilense* introgression at *REX-1* locus from LA1969), and the *chilense* accessions (LA1970, LA1969A, LA1968A, and LA1968C). This group shared one unique SNP C/T located at nt 151. This unique SNP would allow researchers to identify the *chilense* sequence.

LA1932 (*chilense*) was not placed directly within the clade, instead it was situated between the *lycopersicum* clade the *chilense* clade. This sample shared none of the unique SNPs of any other clade.

LA2779 (*chilense*) was not placed directly within the clade, instead it was situated between the *peruvianum* clade and the *habrochaites* clade. This sample does share the unique SNP that the *peruvianum* clade shares.

***Unique SNPs found in the S. peruvianum clade*:** The third group was characterized as a *peruvianum* clade, which contained lines that are either *peruvianum* (LA0462) or known *lycopersicum* with the *Mi-1* gene introgression from *peruvianum*. This clade had LA1221 (*lycopersicum* var. *cerasiforme*, VFNT Cherry with introgression from *peruvianum*), LA0462

(*peruvianum*), Motelle, and LA2823 (Motelle) (*lycopersicum* with *peruvianum* introgression), Anahu (*lycopersicum* with *peruvianum* introgression). This clade also shared only one unique SNP, G/A located at nt 70. As expected all of the *peruvianum* sequences are placed in this clade. Thus all that is needed to positively identify this clade is a single SNP that is easily located in a sequence alignment.

***Unique SNPs found in the S. habrochaites clade*:** The final group was a *habrochaites* clade which was composed of only accessions of *habrochaites.* The plants in this group were: LA1777, LA3863, LA0386, and LA1033. This group had five unique SNPs C/A, A/G, T/C, G/A, and G/A, located at nt 110, 133, 315, 632, and 633, respectively. This group has no outliers and is very distinct, which allows for quick and efficient identification of this locus for this species.

Sequence variation within the genus *Solanum* at the *REX-1* locus: In addition to samples sharing SNPs that were unique for the members of the clade, some plants had unique SNPs that distinguish them from other members within the clade itself. Some SNPs aren't restricted to a single clade and could be used to help infer evolutionary relationships between the clades themselves.

***Sequence variation within the S. lycopersicum clade*:** These samples showed the least variation of all of the groups. Between all seven samples compared to Heinz there are only four SNPs, and they are all found within the sequence of LA0121 (*pimpinelifolium*). All other samples within this group were identical to Heinz 1706. The *lycopersicum* clade shares none of its SNPs with any other clade, which supports the idea that this clade is the most evolutionarily distant of all of the clades studied in this research. If this clade shared more of the SNPs with plants of other clades, this would indicate a closer relationship, and would place this group in a closer proximity to the other clades in the phylogenetic tree.

Table 1: SNPs found within the *S. lycopersicum* clade. Heinz 1706 is used as the standard sequence. Two nucleotide letters in a box separated by a slash signifies a heterozygous plant at this nucleotide position. Heinz 1706 sequence was the same as sequence from M82, Moneymaker, LA1582, LA3173, Rio Grande, and CERA-GT99.

Identifier	Nucleotide variation within *lycopersicum* clade according to nucleotide position defined by Heinz 1706			
Nucleotide #	79	80	84	371
Heinz 1706	T	C	T	G
LA0121	A	T	A	G/T

***Sequence variation within the S. chilense clade*:** Four of the *chilense* accessions were placed in one clade, and two accessions were outside this clade. This group was by far the most diverse of any group tested, having 33 SNPs between only seven different germplasm sources.

Table 2: SNPs found within the *S. chilense* clade. Heinz 1706 is used as the standard sequence, so its sequence is listed at every position to compare to the sequence of the other samples. Two nucleotide letters in a box separated by a slash signifies a heterozygous plant at this nucleotide position. The X's in the table correspond to deletions (indels) that were found within the specific sequences in the sequence alignment.

Identifier	Nucleotide variation within *chilense* clade according to nucleotide position defined by Heinz 1706																																
Nucleotide #	24	39	59	70	91	94	106	138	139	140	144	151	164	233	241	267	285	306	325	369	374	385	421	444	461	469	495	528	555	565	592	624	663
Heinz 1706	A	G	G	A	G	T	A	A	C	G	A	T	C	T	A	C	T	A	A	T	T	T	G	A	C	G	A	C	C	C	T	G	T
TY52		T					G				G	C			C				C								G		A	G			
LA1970	A/C	T					G				G	C			C	T											G		A				
LA1968A		T					G				G	C			C	T							A				G		A				
LA1968B, D-E	A/C	T		A/G			G				G	C			C	C/T	A/T	A/T		C/T			A/G			G/T	G	C/T	A			A/G	G/T
LA1968C		T					G				G	C			C	C/T							A/G				G		A				
LA1932		T	T			C	G	X	X	X	G	C			C																		

Table 3: SNPs found within the *S. peruvianum* clade. Heinz 1706 is used as the standard sequence, so its sequence is listed at every position to compare to the sequence of the other samples. Two nucleotide letters in a box separated by a slash signifies a heterozygous plant at this nucleotide position.

Identifier	Nucleotide variation within *peruvianum* clade according to nucleotide position defined by Heinz 1706										
Nucleotide #	24	39	70	106	144	241	285	463	495	528	555
Heinz 1706	A	G	A	A	A	A	T	G	A	C	C
LA0462	C	T	G	G	G	C	A	A/G	G	T	A
Motelle	C	T	G	G	G	C	A		G	T	A
LA2823 (Motelle)	C	T	G	G	G	C	A		G	T	A
LA1221	C	T	G	G	G	C	A		G	T	A
Anahu	C	T	G	G	G	C	A		G	T	A

***Sequence variation within the S. habrochaites clade*:** This clade, like the *peruvianum* clade, showed conserved SNPs among the samples. Of the 12 total SNPs found among these accessions seven of them are conserved. Of the 12 total SNPs that the *habrochaites* clade has, eight are shared with the *chilense* clade. This means that the *habrochaites* clade shares 67% of its SNPs with the *chilense* clade, and the *chilense* clade shares 24% of its SNPs with the *habrochaites* clade. When the *habrochaites* clade is compared to the *peruvianum* clade there are eight SNPs that are shared, this means that the *habrochaites* clade is 72% related to the *peruvianum* clade and that the *peruvianum* clade is 80% related to the *habrochaites* clade. When the *habrochaites*, *chilense*, and *peruvianum* nucleotide variations are all compared with each other, we see that all three groups share eight SNPs among the three groups. This shows that these three clades are much more closely related to each other than to the *lycopersicum* clade, which shares no SNPs with any of these groups. This may mean that the common ancestor of all three of these closely knit groups most likely had all eight of these SNPs, and as the new clades diverged they become species.

All of these SNPs that the clades have in common give clues to the evolutionary relationships between them. This tells us that among the clades that have been detected, that the *habrochaites* is 66% related to the other two clades (*peruvianum* and *chilense*), and the *chilense* clade is 24% related to the other two (*peruvianum* and *habrochaites*), and finally that the *peruvianum* clade is 73% related to the other two clades (*habrochaites* and *chilense*).

From this study we suggest that the *habrochaites* and the *peruvianum* clades are the most closely related and then the *chilense* clade is the next closely related at the *REX-1* locus. Also we suggest that the lycopersicum is equally unrelated to all three of the other clades (*peruvianum*, *habrochaites*, and *chilense)* because it shares no additional SNPs with any of the other three clades.

Table 4: SNPs found within the *S. habrochaites* clade. Heinz 1706 is used as the standard sequence, so its sequence is listed at every position to compare to the sequence of the other samples. Two nucleotide letters in a box separated by a slash signifies a heterozygous plant at this nucleotide position.

Identifier	Nucleotide variation within *habrochaites* clade according to nucleotide position defined by Heinz 1706											
Nucleotide #	38	56	105	143	240	284	344	494	527	539	554	643
Heinz	G	G	A	A	A	T	T	A	C	T	C	T
LA1777	T		G	G	C	A		G			A	
LA3863	T	T	G	G	C	A		G		C	A	A
LA0386	T		G	G	C	A		G	T		A	
LA1033	T	C	G	G	C	A	G	G	T		A	

Practical application of REX sequences analysis at the *REX-1* locus: The results of this research can be used i) to identify the origin of the introgression from wild species at this locus, ii) to identify the parents used in a breeding line or hybrid, and iii) to follow the resistance genes (*Mi-1* and *Ty-1*) in a breeding program and iv) to determine homozygosity and heterozygosity of breeding lines at this locus.

***Application of REX sequences to determine information about breeding lines or hybrid parents*:** The use of the *REX-1* locus sequences to make inferences about tomato germplasm was evaluated by sequencing the locus for 12 breeding lines from the San Carlos University tomato breeding program and two accessions of wild species from Asian Vegetable Research Development Center (AVDRC). The plant samples were coded and the REX sequence determined. The single SNP G/A located at nt 69, which is associated with the *peruvianum* clade, was found in the REX locus sequences for breeding lines Gh2, Ih902, Gh1, GA3-a, and the two wild species TY22 and TY34. Gh2 and Gh1 were selected from a hybrid designated FAVI-12 and FAVI-9, respectively, and would be predicted to be resistant to root-knot nematode. Their resistance was confirmed by Dr. V. Williamson at UC-Davis. GA3-a would also be predicted to be resistant to root-knot nematode, but this line has not been tested in a bioassay. Line Ih902, which is an important source of resistance genes for begomoviruses (Vidavsky and Czosnek, 1998), was predicted to be resistance to root-knot nematode; however, bioassays at both Hebrew University of Jerusalem (F. Vidavski, pers. com.) and UC-Davis (V. Williamson, pers. com.) showed that this breeding line is susceptible. For the two wild species, TY22 and TY34, the REX sequence was in the *peruvianum* clade and these two accessions were listed as *S. peruvianum* by Dr. S. Green (AVDRC). These results demonstrate that in general, it is possible to infer some characteristics of lines tested. It is important to note that not all plants that have the REX locus sequence of *peruvianum* are going to be resistant to root-knot nematodes.

Four plants (Gh13, Gc172-1-2, Gc16, and H7996) had the SNPs unique to the *lycopersicum* clade. It would be predicted that all of these plants would be susceptible to root-knot nematode. Only Gh13 was evaluated and it was susceptible (V. Williamson, pers. com.). Two plants, Gc173 and Gc9 had the single unique SNP, C/T, that corresponds to the *chilense* clade. Both of these breeding lines were selected from begomovirus-resistant lines Fla024652-Y1 and Fla595-2, respectively, that had introgressions from *S. chilense* LA2779 (J.W. Scott, pers. com.). All plants are listed in Table 6.

This test showed that each sample clearly fit into a distinct group based on only the few unique SNPs that were previously identified. The simplicity evident in the rapid and clear

identification of the origin of the introgression at the given locus could be used in a breeding program to characterize a plant in question at this locus. This would allow a clear knowledge of the origin of the insertion and whether or not it was heterozygous or homozygous.

The applicability of this method of identification was also demonstrated by another example from the Guatemalan tomato breeding program. In this situation, breeding lines were miscoded as to the origin of resistance to begomoviruses. The breeding programs has lines derived from Ih902 (*habrochaites*) resistance or lines from J.W. Scott with *chilense* derived resistance. The Ih902 line has *peruvianum* sequence at the *REX-1* locus. Ih902 was then crossed with an unknown breeding line of *lycopersicum* origin and the F1 hybrids were named FAVI 9 and FAVI-12. From FAVI-12, line Gh2 was selected and has the *peruvianum* introgression. From FAVI-9, Gh1 (*peruvianum* introgression) and Gh13 (*lycopersicu*m introgression) were selected. So from these lines of tomatoes there could possibly be two different introgressions at the REX locus: *lycopersicum* or *peruvianum*.

The second group, the *chilense* derived lines, has three parents, Gc9 and Gc173 had *chilense* sequence at the REX locus, and Gc172 had *lycopersicum* sequence at this locus. From this, we know that we can track the *chilense*, or *peruvianum* sequences to either one breeding line or another. On the other hand since both breeding groups have lines with *lycopersicum* at the REX locus this introgression can't be traced to either one or another breeding group. There is also another complication and this relates to the REX *peruvianum* sequence being present in a susceptible parent to be used in the cross with the begomovirus-resistant parent. An example would be the commercial hybrid Marina, which has the REX *peruvianum* sequence linked to the *Mi-1* gene for root-knot nematode resistance. Thus, only if a REX *chilense* sequence was obtained could one be certain that one of the parental lines was either Gc9 or Gc173.

Samples labeled Gu143-1-1 and Gu143-1-2-2-1 (G = Guatemala and u = their unknown status) are two plants that were bred in Guatemala for the begomovirus resistance and due to incorrect labeling all parental information was lost. These plants showed great potential for begomovirus resistance, but because the lineage information was lost, the specific species resistance genes (*chilense* or *habrochaites*) were unknown. In order to determine the origin of these plants, samples were sequenced and analyzed in comparison to Heinz 1706. Line Gu143-1-1 was found to have the SNP that was unique to the *chilense* clade (C/T located at nt 150) and was identical to TY52 (contains *Ty-1* gene from *chilense*). So now breeders know that sample Gu143-1-1 was homozygous and had the *chilense* resistance genes, which had to come from either Gc9 or Gc173.

The second line labeled Gu143-1-2-2-1 was found to be heterozygous at this locus in nine positions. Six of these heterozygous positions: G/T, A/G, A/G, A/C, A/G were identified at nt 38, 105, 143, 240, and 494, respectively, and all of these positions corresponded to the unique SNPs found in the *lycopersicum* clade that was identified earlier. In addition one of the other SNPs was C/T located at nt 150, which corresponds to the unique SNP associated with the *chilense* clade. Due to the location of the various heterozygous SNPs, it is clear that this plant is a combination of *lycopersicum* and *chilense* sequences at the REX locus. Knowing the origin of this line's introgression again allows for identification of the lineage of this plant. Because this plant has *lycopersicum* and *chilense* in this region, we know that this plant was also from the *chilense* breeding group (either Gc9 or Gc173 as a parent), and in addition we know that this plant is heterozygous, and thus it may not be the best plant to select for future use because of its apparent heterozygosity.

The simplicity of these techniques for positive identification of the origin of the introgression at this locus is evident in these examples. Also, the efficiency of identifying if a

plant is heterozygous or homozygous at a certain locus can be important. If a plant is homozygous at this locus then plant breeders will be able to predict the presence of this locus and associated linked traits in a cross or subsequent self generations.

Acknowledgements

LA# accessions were provided by the Tomato Genetics Resource Center, UC-Davis. Heinz 1706, Motelle and Moneymaker, TY52 and M82, and Anahu were supplied by Rick Ozminkowski (Heinz Seed Co.), V. Williamson (UC-Davis), H. Czosnek (Hebrew University of Jerusalem, Rehovot, Israel), and F. Vidavski (Hebrew University of Jerusalem), respectively. This research was partially supported by CDR and MERC grants from USAID and by the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

Literature Cited:

Agrama, H.A., and Scott, J.W., 2006. Quantitative trait loci for *Tomato yellow leaf curl virus* and *Tomato mottle virus* resistance in tomato. J. Amer. Soc. Hort. Sci. 131:267-272.

Martinez de Llarduya, O., Nombela, G., Hwang, C., Williamson, V.M., Muñiz, M., and Kaloshian, I. 2004. Rme1 is necessary for Mi-1-mediated resistance and acts early in the resistance pathway. MPMI Vol. 17:55-61.

Mejía, L., R.E. Teni, F. Vidavski, H. Czosnek, M. Lapidot, M.K. Nakhla, and D.P. Maxwell. 2005. Evaluation of tomato germplasm and selection of breeding lines for resistance to begomoviruses in Guatemala. Acta Hort. 695:263-270.

Nesbitt, T.C., and Tanksley S.D. 2002. Comparative sequencing in the genus *Lycopersicon*: Implications for the evolution of fruit size in the domestication of cultivated tomatoes. Genetics 162:365-379.

Nombela, G., Beitia, F., and Muniz, M. 2000. Variation in tomato host response to *Bemisia tabaci* (Hemiptera: Aleyrodidae) in relation to acyl sugar content and presence of the nematode and potato aphid resistance gene Mi. Bull. Entomol. Res. 90:161-167.

Rossi, M., Goggin, F.L., Milligan, S.B., Kaloshian, I., Ullman, D.E., and Williamson, V.M., 1998. The nematode resistance gene Mi of tomato confers resistance against the potato aphid. Proc. Natl. Acad. Sci. U.S.A. 95:9750-9754.

Smith, P.G., 1944. Embryo culture of a tomato species hybrid. Proc. Amer. Soc. Hort. Sci. 44:413-416.

Spooner, D.P., Peralta, I.E., and Knapp, S. 2005, Comparisons of AFLPs with other markers for phylogenetic inference in wild tomatoes [*Solanum* L. section Lycopersicon (Mill.) Wettst.] Taxon 54:43-61.

Vidavsky, F., and Czosnek, H. 1998. Tomato breeding lines resistant and tolerant to tomato yellow leaf curl virus issued from *Lycopersicon hirustum*. Phytopathology 88:910-914.

Williamson, V.M., Ho, J.Y., Wu, F.F., Miller, N., and Kaloshian, I. 1994. A PCR-bases marker tightly linked to the nematode resistance gene, Mi, in tomato. Theor. Applied Genetics 87:757-763.

Zamir, D., Ekstein-Michelson, I., Zakay, Y., Navot, N., Zeidan, M., Sarfatti, M., Eshed, Y., Harel, E., Pleban, T., Van-Oss, H., Kedar, N., Rabinowitch, H., and Czosnek, H. 1994. Mapping and introgression of a tomato yellow leaf curl virus tolerance gene Ty-1. Theor. Appl. Genet. 88:141-146.

Zhong, X.B., Bodeau, J., Fransz, P.F., Williamson, V.M., van Kammen, A., de Jong, J.H., and Zabel, P. 1999. FISH of meiotic pachytene chromosomes of tomato locates the root-knot nematode resistance gene Mi-1 and the acid phosphatase gene Aps-1 near the junction of euchromatin and pericentromeric heterochromatin of chromosome arms 6S and 6L, respectively. Theor. Appl. Genet. 98:365-370.

Table 5: List of accessions, hybrids, and breeding lines used in sequence comparisons and in phylogenetic trees for the *REX-1* locus.

Identifier	Species Name [b]	GenBank Number	Disease Resistance[c]	HET/HOM (SNPs)[d]
LA 0462	*peruvianum*	AY796051		HET(1)
LA 1582	*pimpinellifolium*			HOM
LA 0121	*pimpinellifolium*			HET(1)
LA 1777	*habrochaites*		PI, GV	HOM
LA0386	*habrochaites*		GV	HOM
LA 1033	*habrochaites*	AY956441	PI	HOM
LA 3863	*habrochaites*		PI	HOM
LA 2779	*chilense*		GV, (Source of Ty-A)	HOM
LA1932	*chilense*		PBNV, GV, (Source of Ty-A)	HOM
LA 1968A	*chilense*	AY956442		HOM
LA 1968B	*chilense*			HET(12)
LA 1968C	*chilense*	AY956443		HET(2)
LA 1968D	*chilense*	AY956444		HET(12)
LA 1968E	*chilense*			HET(12)
LA 1969A	*chilense*	AY947717	GV (Source of Ty-1)	HOM
LA 1969B	*chilense*	AY947718	GV (Source of Ty-1)	HET(6)
LA 1969C	*chilense*		GV (Source of Ty-1)	HOM
LA 1969D	*chilense*		GV (Source of Ty-1)	HOM
LA 1969E	*chilense*		GV (Source of Ty-1)	HOM
LA 1970	*chilense*			HET(1)
TY52	*chilense*		Begomovirus-resistant germplasm, Introgression from *chilense,*LA1969	HOM
CERA-GT99	*cerasiforme*			HOM
LA 1221[a]	*cerasiforme*		F1, TMV, Ve, Introgression from *peruvianum* for Mi-1 gene	HOM
RIO GRANDE	*lycopersicum*		Ve, F1, F2	HOM
LA 3173	*lycopersicum*		I*cn*	HOM
LA 2823[a]	*lycopersicum*		Ve, St, F1, F2	HOM
M82	*lycopersicum*	AY596779	*Ve, F1*	HOM
HEINZ 1706	*lycopersicum*	AY949616	Ve, F1	HOM
MOTELLE	*lycopersicum*	AY589502	Mi-1, Introgression from *peruvianum,* Ve, F1, F2	HOM
Moneymaker	*lycopersicum*			HOM
Anahu	*lycopersicum*		Mi-1, Introgression from *peruvianum*	HOM

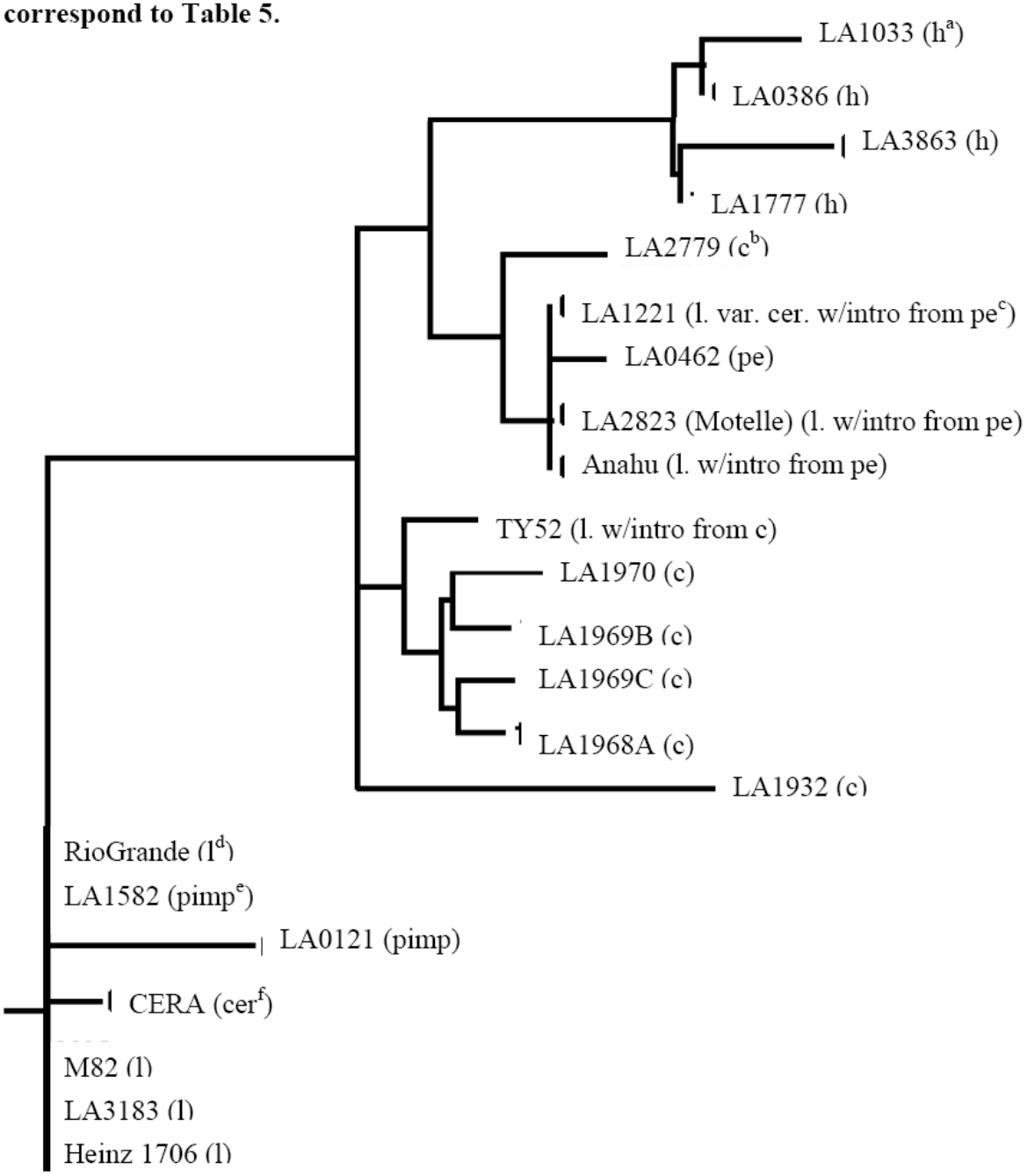
[a] LA1221=VFNT Cherry; LA2823=Motelle; CERA=collection made in Sanarate, GT in 1999 by L. Mejía, GT99.

[b] Spooner, D. P., Peralta, I. E., Knapp, S., Feb. 2005, Comparisons of AFLPs with other markers for phylogenetic inference in wild tomatoes [Solanum L. section Lycopersicon (Mill.) Wettst.]. Taxon 54:43-61

[c] Disease Resistance: PI= *Phytophthora infestans*; GV= geminivirus; Ve= *Verticillium*, F1, F2= Fusarium wilt race 1 and 2; PBNV= *Peanut bud necrosis virus*; TMV= *Tobacco mosaic virus*; St= *Stemphyllium* compared with Heinz 1706. Mi = *Mi-1* gene from *S. peruvianum*.

[d] HOM denotes an accessoin that was found to be homozygous for the *REX-1* locus. HET denotes an accession that was heterozygous for the *REX-1* locus. The number in parentheses following HET represents the number of SNPs for this locus in this plant.

Figure 2: One representative phylogeny of maximum likelihood, accession codes correspond to Table 5.



[a] **h represents *S. habrochaites***
[b] **c represents *S. chilense***
[c] **pe represents *S. peruvianum*.**
[d] **l represents *S. lycopersicum***
[e] **pi represents *S. pimpinellifollium***
[f] **cer represents *S. cerasiforme*, l. var. cer. represents *lycopersicum* var. *cerasiforme***

Table 6: List of all germplasm used to test the usefulness of the sequence analysis at the *REX-1* locus.

Identifier	**Disease Resistance**[a]	**Introgression at the REX-1 locus**
Gh2	GV, resistance from *S. habrochaites*, from FAVI 12 RKN resistant	*peruvianum*
Gh13	GV resistance from *S. habrochaites*, FAVI 9, RKN susceptible	*lycopersicum*
902-R2 from Ih902	GV resisitance from *S. habrochaites*, breeding line, RKN susceptible	*peruvianum*
Gc173	Selected from Fla024652-Y1, LA2779	*chilense*
Gc16	GV, from Fla658-2BK, LA1969/TY King	*lycopersicum*
Gc9	GV, from Fla 595-2, LA2779	*chilense*
Gc172-1-2	GV, from Fla024524-5 BK, LA2779/LA1932	*lycopersicum*
Gh1	GV resistance from *S. habrochaites*, FAVI 9, RKN resistant	*peruvianum*
H7996	BW, from Hawaii 7996	*lycopersicum*
GA3-a	GV resistance from either *habrochaites* or *chilense*	*peruvianum*
Gu143-1-2-2-1	GV resistance from either *habrochaites* or *chilense*	*chilense/lycopersicum*
Gu143-1-1	GV resistance from either *habrochaites* or *chilense*	*chilense*
TY22	from Dr. Green from AVRDC, *S. peruvianum*	*peruvianum*
TY34	from Dr. Green from AVRDC, *S. peruvianum*	*peruvianum*

[a]RKN= root-knot nematode, GV= geminivirus, BW= bacterial wilt.

Chapter II:
Degenerate PCR Primers as Internal Controls in Multiplex-PCR Reactions with Tomato DNAs

Abstract

Molecular techniques such as PCR have become a key tool for researchers and plant breeders, allowing for faster identification of resistant/susceptible or homozygous/heterozygous plants. This knowledge allows breeders to minimize the time for developing disease resistant hybrids. PCR fragments produced with the REXF1/R2 primers were sequenced from a variety of *Solanum* (*Lycopersicum*) species. The *REX-1* locus sequences of 13 different plants were aligned and from areas of conserved sequences, four primer pairs were designed that would amplify single fragments of different sizes (300, 400, 500, and 600 bp) at annealing temperatures of 53-57°C. These primers gave the expected fragment sizes with over 70 samples. Thus, it is anticipated that these four pairs of primers could be used in multiplex-PCR reactions as internal controls for evaluation of the quality of the sample DNA. This would allow researchers to rely on one set of internal controls that could be used in a wide range of reactions.

Introduction

In many third world countries, farmers rely on their crops to provide them with food and a small income. The problem that has become more evident is that these farmers must manage diseases caused by viruses, bacteria, fungi, and nematodes. In many situations, these growers have resorted to using high levels of pesticides in order to control disease (Larkin and Fravel, 1998), tomatoes are one such crop. For all of these potential pathogens, rapid and inexpensive techniques that offer reliable identification of resistance genes must be developed to assist tomato breeders in their choices of what plants should be used to produce disease resistant hybrids and lines. Although there are various wild species of tomatoes that are resistant to the different pathogens (Spooner et al., 2005), these wild varieties have fruits that are usually too small and unrealistic for commercial utilization (Nesbitt and Tanksley, 2002). So these wilds species are sources of resistance genes that can be introgressed into breeding lines that do produce fruits that are marketable.

For example, the *Mi-1* gene was introgressed from the wild species, *Solanum peruvianum* (see Seah et al., 2004), and this resistance gene confers resistance to three *Meloidogyne* species (*M. arenaria*, *M. incognita*, and *M. javanica*) (Martinez et al., 2003). The *Mi-1* gene has also been observed to confer resistance to potato aphids (*Macrosyphum eupharbiae*) and whiteflies (Seah et al., 2004). The *Mi-1* gene has been mapped to the short arm of chromosome 6 (6S). There are seven homologues of the *Mi* gene arranged in two groups of three and four (Seah et al., 2004). Williamson et al. (1994) designed a CAPS marker (Cleavage Amplified Polymorphic Sequence) that was linked to the *Mi-1* locus, this marker is the *REX-1* marker. This PCR-based method (REXF1/REXR2 primers) amplified an identical size fragment from both resistant and susceptible genotypes. These REX primers also amplified a fragment from germplasm with the *Ty-1* gene from *S. chilense* that is introgressed into the same region as the *Mi-1* gene (J. Milo, Hebrew University of Jerusalem). Thus, it seemed reasonable to expect that the REX primers would amplify a fragment from other wild tomato species, and therefore, this REX locus could be used to develop internal control PCR primers to use with multiplex-PCR.

The objectives of this research were i) to design degenerate PCR primers that would amplify different size fragments from conserved sequences of the *REX-1* locus from

tomato species, and ii) to evaluate these primers with DNA extracted from several tomato species. These degenerate PCR primers could then be used in multiplex-PCR reactions, providing researchers with a choice of fragment sizes and a range of annealing temperatures that would be useful with DNAs from any tomato germplasm.

Material and Methods

Plant material used for the REX-1 multiple sequence alignment: The cultivars used in the REX locus multiple sequence alignment to design the internal control (ICON) primers were: Motelle (supplied by V. Williamson, UC-Davis) (AY589502) which is *S. lycopersicum* that has the *S. peruvianum* introgression at the *REX-1* locus (*Mi-1/Mi-1*), TY52, which is a *S. lycopersicum* that has the *S. chilense* introgression at the *REX-1* locus (*Ty-1/Ty-1*, *mi-1/mi-1*), and Heinz 1706 (AY949616), which is *S. lycopersicum*. Ten additional germplasm accessions were also included in the alignment (Spooner et al., 2005): *S. pimpinelifolium* (LA0121 and LA1582) *habrochaites* (LA1777 and LA1033) *chilense* (LA1968A, LA1968B, LA1968C, LA1969A, LA1969B), *peruvianum* (LA1221) (Table 1). These accessions were chosen for their potential genetic diversity (Nesbitt et al., 2002) and/or because many of them have been use in breeding programs for begomovirus resistance, eg. LA1969 and LA1777. All of the genotypes labeled with an LA at the beginning of the identifiers came from the Tomato Genetic Resource Center, University of California-Davis (UC-Davis). See Table 1 for accession numbers for sequence at GenBank

DNA isolation, PCR amplification, sequencing, and sequence analysis: DNA was extracted from fresh leaves of plants grown in a plant growth chamber at the University of Wisconsin-Madison (UW-Madison). Thirty mg of young leaf tissue was frozen in liquid nitrogen in a 1.5-ml microfuge tube, and then ground with a sterilized Kontes™ micropestle (Kontes Glass, Vineland, NJ) and extracted with the PURGENE® DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. DNA concentrations were then measured and adjusted to 15 ng/µl and the extracted DNA frozen was at -20°C. The REXF1/REXR2 (Williamson et al., 1994) and ICONF1/ICONR1-4 primers were purchased from Integrated DNA Technologies Inc., Coralville, IA. PCR parameters were for 25 µl reactions containing 2.5 µl 2.5 mM dNTPs, 2.5 µl of a 10x buffer, 2.5 µl 25 mM $MgCl_2$, 0.1 µl *Taq* DNA polymerase, 2.5 µl each of both forward and reverse primers at 10 µM, 5-7 µl of DNA extract, and H_2O. The PCR thermocycler parameters for fragment amplification were as follows: denaturation at 94°C for 3 min, then 35 cycles of 94°C for 30 sec, annealing at 53-57°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 done in the MJ DNA Engine PT200 Thermocycler™ (MJ Research Inc., Waltham, MA). All molecular biology chemicals for the PCR were purchased from Promega, Corp., Madison, WI. PCR fragments were then separated by gel electrophoresis using 1.5% Seakem LE™ agarose (BioWhittaker Molecular Applications Rockland, ME) in 0.5X TBE buffer, then stained with ethidium bromide and visualized with a Kodak Gel Logic 200 Imaging System. All PCR fragments were directly sequenced using the Big Dye Sequencing Kit™ (Biotechnology Center, Madison WI), both forward and reverse REX primers were used to achieve a reliable consensus sequence of 720 bp. Analysis of the resulting sequences was accomplished by comparison with known *Solanum sp*. DNA sequences using the DNAMAN software (Lynnon Corp., Quebec, Canada).

Primer design: In order to design primers that would work on all *Solanum* species, the REX locus (Williamson et al., 1994) sequences from 13 different genotypes, which included a variety of species, were aligned, and areas of conserved sequences were used to design the new primers. From that consensus sequence alignment, one forward primer, ICONF1 (GGA GCC

TTG GTC TGA ATT TCC MGT C) was developed, and four reverse primers were designed: ICONR1 (GTT GCT CCC TCT TCA GCC TTW CCC AC), ICONR2 (GGA ACA GCA GGC TGG GCA AGA TCT GG), ICONR3 (GTG CAA GCA AAG GTA GTG CTG GAA YAG GTC), and ICONR4 (CCG ATG TGT CAA GCG ATA AGG GGA AGT G). The expected fragment sizes were 300, 400, 500, and 600 bp, respectively.

Results and Discussion

Results of the ICON Primers: As expected, the four sets of ICON primers each gave a single band of different lengths at annealing temperatures from 53-57°C (Fig. 1 and 2). The approximate length of the bands for each primer pair was as follows: ICONF1/ICONR1, 300 bp, ICONF1/ICONR2, 400 bp, ICONF1/ICONR3, 500 bp, and ICONF1/ICONR4, 600 bp (Fig. 1 and 2).

More than 70 samples were tested with the ICON primers, and these primers always gave the expected single band (Table 2). The samples included: *S. peruvianum*, *habrochaites*, *lycopersicum*, *lycopersicum* var. *cerasiforme*, *peruvianum*, and *chilense*. S. lycopersicum included: 25 commercial hybrids, eight experimental hybrids, 30 breeding lines from Guatemala, one land race, seven wild species, and four other breeding lines. These results show that these ICON primers can be reliable when used with germplasm of *Solanum* species and can be used at a temperature that can range from 53-57°C. It is expected that these ICON primers could be used in multiplex-PCR reactions as the internal control primers for assessing the quality of the DNA sample.

Literature Cited

Larkin, R.P., and Fravel, D.R., 1998. Biological Control of Fusarium wilt disease by nonpathogenic *Fusarium spp*.: Formulations and Field Efficacy, USDA, Biocontrol of Plant Diseases Laboratory, Beltsville MD 20705

Martinez de Llarduya, O., Nombela, G., Hwang, C., Williamson, V.M., Muñiz, M., and Kaloshian, I. 2004. Rme1 is necessary for Mi-1-mediated resistance and acts early in the resistance pathway. The American Phytopathological Society Vol. 17, No. 1, pp. 55-61.

Nesbitt, T.C., and Tanksley S.D. 2002. Comparative sequencing in the genus *Lycopersicon*: Implications for the evolution of fruit size in the domestication of cultivated tomatoes. Genetics 162:365-379.

Seah, S., Yaghoobi, J., Rossi, M., Gleason, C., and Williamson, V. 2004. The nematode-resistance gene, Mi-1 is associated with an inverted chromosomal segment in susceptible compared to resistance tomato. Theor. Appli. Genet. 108:1635-1642.

Spooner, D.P., Peralta, I.E., and Knapp, S., 2005. Comparisons of AFLPs with other markers for phylogenetic inference in wild tomatoes [Solanum L. section Lycopersicon (Mill.) Wettst.]. Taxon 54: 43-61.

Williamson, V.M., Ho, J.Y., Wu, F.F., Miller, N., and Kaloshian, I. 1994. A PCR-bases marker tightly linked to the nematode resistance gene, *Mi*, in tomato. Theor. Appli. Genet. 87:757-763.

Table 1: A list of accessions, hybrids, and breeding lines used in the multiple sequence alignment that was used to design the ICON primers.

Identifier	**Species Name** [b]	**GenBank Number**	**Disease Resistance**[c]	**HET/HOM (SNPs)**[d]
LA1582	*pimpinellifolium*			HOM
LA0121	*pimpinellifolium*			HET (1)
LA1777	*habrochaites*		PI, GV	HOM
LA1033	*habrochaites*	AY956441	PI	HOM
LA1968A	*chilense*	AY956442		HOM
LA1968B	*chilense*			HET (12)
LA1968C	*chilense*	AY956443		HET(2)
LA1969A	*chilense*	AY947717	GV (Source of Ty-1)	HOM
LA1969B	*chilense*	AY947718	GV (Source of Ty-1)	HET (6)
TY52	*lycopersicum*		Begomovirus-resistant germplasm,	HOM
LA1221[a]	*cerasiforme*		N, F1, TMV, Ve, Introgression from *peruvianum* for *Mi-1*	HOM
HEINZ 1706	*lycopersicum*	AY949616	Ve, F1	HOM
Motelle	*lycopersicum*	AY589502	N, Ve, F1, F2	HOM

[a] LA1221=VFNT Cherry.

[b] Spooner, D. P., Peralta, I. E., and Knapp, S., 2005, Comparisons of AFLPs with other markers for phylogenetic inference in wild tomatoes [Solanum L. section Lycopersicon (Mill.) Wettst.]. Taxon 54:43-61.

[c] Disease Resistance: PI= *Phytophthora infestans*; GV= geminivirus; Ve= *Verticillium*, F1, F2= Fusarium wilt race 1 and 2; TMV= *Tobacco mosaic virus*; N= *Meloidogyne incognita*; St= *Stemphyllium.*

[d] HOM denotes an accession that was found to be homozygous for the *REX-1* locus. HET denotes an accession that was heterozygous for the *REX-1* locus. The number in parentheses following HET represents the number of SNPs for this locus.

Figure 1: PCR with primers ICONF1/ICONR1-ICONR4, which were designed from sequence from the *REX-1* locus at 53°C annealing temperature. Lane 1 and 14, 100-bp DNA ladder (Invitrogen), lanes 2-4 are the ICONF1/ICONR1 combination with samples Crista (lane 2), Viva Italia (lane 3), and water (lane 4), lanes 5-7 are the ICONF1/ICONR2 combination with samples Crista (lane 5), Viva Italia (lane 6), and water (7), lanes 8-10 are the ICONF1/ICONR3 combination with samples Crista (lane 8), Viva Italia (lane 9), and water (lane 10), lanes 11-13 are the ICONF1/ ICONR1 combination with samples Crista (lane 11), Viva Italia (lane 12), and water (lane 13).

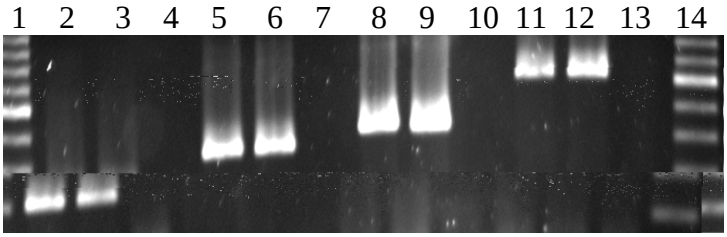


Figure 2: PCR with primers ICONF1/ICONR1-ICONR4, which were designed from sequences from the REX-1 locus, at 57°C annealing temperature. Lane 1 and 14, 100-bp DNA ladder (Invitrogen), lanes 2-4 are the ICONF1/ICONR1 combination with samples Crista (lane 2), Viva Italia (lane 3), and water (4), lanes 5-7 are the ICONF1/ICONR2 combination with samples Crista (lane 5), Viva Italia (lane 6), and water (lane 7), lanes 8-10 are the ICONF1/ICONR3 combination with samples Crista (lane 8), Viva Italia (lane 9), and water (lane 10), lanes 11-13 are the ICONF1/ICONR1 combination with samples Crista (lane 11), Viva Italia (lane 12), and water (lane 13).

Table 2: Germplasm used to evaluate the four sets of ICON primers.

Type	Identifier[a]
Commercial Hybrids	Antigua (H), Better Boy, M82, Heinz 1706 (H), Moneymaker, Titila, Titrit (S), Sheriff, Dominique, Tequila, Charanda, Don Raul, Nainemor, Celebrity (S), Anahu, Toro (HM), Crista (HM), Solar Fire (HM), Viva Italia (HM), Amelia VR (HM), El Cid (HM), Plum Crimson (HM), Rodeo (H), Antigua (H), Motelle
Experimental hybrids[b]	XA339, XC273B, XA258A, XA385, XA273A, XB4C, XC173B, XC273A
Breeding lines from San Carlos University, GT	Gc172-1-2, Gc173-2-1, Gh25-8-1, Gc43-4-3-1, Gh25-6-1-1-1, Ghpim44-1-3-a, Gh23-1-1-a-2, Gc43-4-1-1-a-1-SB, Gc43-4-1-1-a-1, Gh25-8-1-2-1-1, Gh25-2-1-2-1-1, LR171c-1-a, Gh137-1-1-1-1, Gh25-6-1-b-1, Ghc45-1-1-2, Gu143-1-1, Gc173-2-a, Gh105-1-a-2, Gu143-1-1-1-1, Ghp44-1-3-TF1-3-1-TF1, Ghp44-1-1-TF3-1-2-TF1, Gh105-1-a-b-1, Gc43-5-4-a, T44h-2-1-a, Gu143-1-2-2-1, Gh8632-1-2, Gc173A, Gh1, Gc16, Gh13
Land Race	Cerasiforme
Wild Species	LA1582, LA0121, LA1777, LA1033, LA1968, LA1969, LA1221
Breeding lines	CLN2443A, F632, Ih902, LA2711-2 (J.W. Scott)

[a] HM = Harris Moran; H = Heinz, S = Seminis Vegetable Seed

[b] Experimental hybrids from GenTropic Seeds, S.A., Guatemala

Chapter III:

Application of a PCR-based Technique for Detection of the *I-2* Gene for Resistance to Fusarium wilt in Tomato Breeding Lines for Guatemala

Abstract

For diseases that have become worldwide problems, the ability to detect resistance genes quickly and effectively has become vital. One such disease is Fusarium wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* race 2, which is one of three races of this pathogen that is known to devastate tomato plants around the globe. A great amount of effort has gone into rapid identification techniques, such as PCR, in hopes that this will help lead to varieties of resistant cultivars more quickly. This paper will evaluate a PCR method that may be used to quickly and effectively identify plants that are positive for the *Fusarium oxysporum* f. sp. *lycopersici* race 2 resistance gene, *I-2*.

Introduction

Fusarium oxysporum f. sp. *lycopersici* is a soil-borne fungus that infects plants and causes a wilt (Figure 1 and 2) that can drastically reduce yield and potentially kill the plant. Fusarium wilt has become a world-wide problem and this disease has been reported in many countries. Of these countries it is most severe where the climate is warm year round.

Pathogens that can spread world wide are becoming more and more common with the help of modern means of transport. While a certain level of disease control can be achieved with the use of chemicals, pathogens and insects have become resistant to them and chemicals have the potential to adversely affect the humans who apply them. Currently, chemicals such as methyl bromide are used to fumigate crops to reduce the presence of *Fusarium oxysporum* (Bao et al., 2000); also some nonpathogenic *Fusarium* species have been demonstrated to reduce Fusarium wilt on several crops in the greenhouse setting (Larkin and Fravel, 1998). Although both of these methods could be utilized, breeding Fusarium wilt resistant tomatoes is more economical in the long run because it negates or diminishes the need for chemicals or biocontrol agents altogether.

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resistance gene is present. A set of primers [Ve2F (ATT TGC TGC CCC TAC TAT TGA TCC) and Ve2R (TGA ATT GTA AGT TGT TGG AGG TCC)] were used as internal controls, and gave a fragment of approximately 800 bp (Kawchuk et al., 2001).

The ability to quickly and inexpensively identify resistant or susceptible genotypes of a plant at a specific locus of interest is very important to plant breeders. Biological assays are useful but they are also expensive and can be time consuming to perform. So a simple multiplex-PCR methods would prove to be advantageous for the positive identification of resistance or susceptibility at the *I-2* locus (El Mehrach et al., 2005).

The objectives of this research were to evaluate the reliability of this protocol with a diverse collection of germplasm to determine if it is able to distinguish between resistant and susceptible germplasm.

Material and Methods

Plant materials for Fusarium tests: The initial trials with this PCR method were performed in the American University of Beirut, Lebanon (Y. Abou Jawdah, pers. com.), and there were a total of eleven known tomato genotypes that were used to evaluate the specificity of the PCR primers. The PCR method was then further evaluated at the University of Wisconsin-Madison (UW-Madison) with 24 additional genotypes. These 24 genotypes were reported by the seed companies to either have or not to have resistance to *F. o.* f. sp. *lycopersici* race 2 (Table 2).

DNA isolation, PCR amplification, sequencing, and sequence analysis: DNA was extracted from fresh leaves of plants grown in a plant growth chamber at the UW-Madison. Thirty mg of young leaf tissue was frozen in liquid nitrogen in a 1.5-ml microfuge tube, and then ground with a sterilized Kontes™ micropestle (Kontes Glass, Vineland, NJ) and extracted with the PURGENE® DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's instructions. DNA concentrations were then measured and adjusted to 15 ng/µl and the extracted DNA frozen at -20°C. The FusF1/Fusrr2 and Ve2F/Ve2R primers were purchased from Integrated DNA Technologies Inc., Coralville, IA. PCR parameters were for 25 µl reactions containing 2.5 µl 2.5 mM dNTPs, 2.5 µl of a 10x buffer, 2.5 µl 25 mM $MgCl_2$, 0.1 µl *Taq* DNA polymerase, 4 µl each of FusF1/Fusrr2 primers at 10 µM, and 1 µl each of the Ve2F/Ve2R primers at 10 µM, 5-7 µl of DNA extract, and H_2O. The PCR thermocycler parameters for fragment amplification were as follows: denaturation at 94°C for 3 min, then 35 cycles of 94°C for 30 sec, annealing at 57°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 done in the MJ DNA Engine PT200 Thermocycler™ (MJ Research Inc., Waltham, MA). All molecular biology chemicals for the PCR were purchased from Promega, Corp., Madison, WI. PCR fragments were then separated by gel electrophoresis using 1.5% Seakem LE™ agarose (BioWhittaker Molecular Applications Rockland, ME) in 0.5X TBE buffer, then stained with ethidium bromide and visualized with a Kodak Gel Logic 200 Imaging System. All PCR fragments were directly sequenced using the Big Dye Sequencing Kit™ (Biotechnology Center, Madison, WI).

Biological Assay (Yael Rekah, Hebrew University of Jerusalem, pers. com.)**:** For the biological assay 30 seedlings of each of six different accessions were evaluated. The evaluation of the seedlings was as follows: seedlings were determined as diseased when they exhibited vascular discoloration accompanied with severe growth retardation and/or if wilt symptoms were apparent. Seedlings that were classified as healthy or resistant

remained without symptoms. Two different PCR markers (exact nature of markers is not available) were used in order to lend credence to the results of the biological test. One of the markers was located inside the gene. The PCR markers are able to detect the susceptible/resistant seedlings but they don't differentiate between the homozygous and the heterozygous resistant seedlings. Ten different samples of each accession were assessed by the PCR markers.

Results and Discussion

PCR: In every test the Ve2F/Ve2R primers resulted in a fragment that was always present at approximately 800 bp. In 23 of the 24 samples tested, the known disease resistance genes supported the results of the PCR test with the FusF1/Fusrr2 and Ve2F/Ve2R primers (Table 1). Of the 23 samples tested, all but one of the resistance genotypes resulted in a fragment of approximately 600 bp, and in all genotypes that lacked resistance, there was no band. One sample tested gave a conflicting result when compared with the reported disease resistance of the plant; this sample was Plum Crimson (Harris Moran). Plum Crimson was listed as resistant to *F. o.* f. sp. *lycopersici* race 2, but the FusF1/Fusrr2 primers gave no band with this sample. This hybrid, along with five others, was sent to Yael Rekah at the Hebrew University of Jerusalem for a biological assay with *F. o.* f. sp. *lycopersici* race 2. There were over 55 additional platns that this multiplex-PCR was tested on, see Table 4 for data.

Table 1: Bioassay done in Israel, Hebrew University of Jerusalem. I2-gene detected by multiplex-PCR, all parents are considered to be homozygous. See Table 3.

Hybrid			Parent 1		Parent 2	
Code	Genotype[a]	Bioassay[b]	Code	Genotype[c]	Code	Genotype
XB4C	I2/i2	R	Gh25-8-1-1	i2/i2	Gc43-5-4-1	I2/I2 (?)
XC173B	I2/I2	R	Ghp31-4-1-2-1-1	I2/I2	Gc43-4-1-1-a-1	I2/I2 (?)
XA258A	i2/i2	S	Ghp44-1-3-1	i2/i2 (?)	Gh105-1-a	i2/i2
XC273B	I2/i2	R	GhT44-2-1-a	i2/i2	Gc173-2-TF1-1	I2/I2
XA339	I2/-	R	Gh23-1-1-a-2		Gc173-2-TF1-1	I2/I2
XA385	I2/I2	R	Gc173-2-1-1-1	I2/I2	Ghp44-2-2-1-1	I2/I2

[a] I2 = Plant genotype has one copy of the dominant *Fusarium* race 2 resistant gene. i2 = Plant genotype has one copy of the recessive gene that doesn't confer resistance to *Fusarium* race 2.
[b] R = Resistant to *Fusarium* race 2. S = Susceptible to *Fusarium* race 2
[c] ? = genotype derived from closely related breeding line

Biological Assay: The results of the biological assay are shown in Table 3. For five of the six samples tested the PCR results supported the results of the biological assay. One of the samples gave conflicting results, again it was Plum Crimson. In this test the PCR primers gave a result that indicated that Plum Crimson lacked the I-2 gene. The biological test indicated that this hybrid was resistant to *F. o.* f. sp. *lycopersici* race 2 and of

the plants tested, no plants showed any form of vascular discoloration or wilting. So not only is this plant resistant but the level of its resistance is higher than at least four of the other samples, showing complete resistance to the pathogen.

These results indicate that in 96% of the cases the I-2 primers can be relied upon to give a positive identification of a plant that is either susceptible or resistant to *F. o.* f. sp. *lycopersici* race 2. There could be multiple reasons why Plum Crimson failed to give a band like all of the other resistant genotypes. One potential explanation could be the presence of *I-3* resistance gene located on chromosome 7, which has been reported to confer resistance to *F. o.* f. sp. *lycopersici* race 2 in addition to conferring resistance to race 3 (Bournival et al., 1990). Also a change in the nucleotide sequence of the target DNA may cause the primers to not anneal, but wouldn't necessarily change the gene so that it wouldn't confer resistance to *F. o.* f. sp. *lycopersici* race 2. Even if the FusF1/rr2 primers fail to recognize resistant phenotypes in a small percent of all total cases, it is very unlikely that these primers would ever anneal to a genotype that is susceptible, allowing researchers to be confident that positive results would indicate the presence if the *I-2* gene.

This technique would give researchers the ability to quickly and inexpensively identify resistant or susceptible genotypes of a plant at the *I-2* locus. Biological assays are very accurate, but they are also expensive and can be quite time consuming to perform. So this simple multiplex-PCR would prove to be very advantageous for choosing the next generation of parents from any available progeny in a very short period of time.

Acknowledgements

The Biological assay and PCR were performed by Yael Rekah at the Faculty of Agricultural, Food, and Environmental Quality Sciences, Department of Plant Pathology and Microbiology at the Hebrew University of Jerusalem. Heinz 1706, Motelle and Moneymaker, F632 and M82, and Anahu were supplied by Rick Ozminkowski (Heinz Seed Co.), V. Williamson (UC-Davis), H. Czosnek (Hebrew University of Jerusalem, Rehovot, Israel), and F. Vidavski (Hebrew University of Jerusalem), respectively. This research was partially supported by CDR and MERC grants from USAID and by the College of Agricultural and Life Sciences, University of Wisconsin-Madison.

Table 2: Genotypes tested by multiplex-PCR for detection of *I-2* gene for resistance to *F. Fusarium oxysporum* f. sp. *lycopersici* race 2.

Cultivar/Line	Primer TFusF1/TFusrr2	Primer Ve2F/Ve2R	Resistance[a]	Source[b]
CLN2443A	-	+	Ty-2,TMV,F1,Tm2a	AVDRC, Peter Hanson
M82	-	+	Ve,F1	Favi Vidavski, HUJ
F632	+	+	Ve,F1,F2,TMV,N,Ty	Favi Vidavski, HUJ
Titrit	+	+	Ty,F1,F2,Ve,TMV,N,FCRR	Royal Sluis
Marina	+	+	F1,F2,N,ASC,GLS,BS1,Ve	Sakata Seed America
Moneymaker	-	+		V. Williamson, UC-Davis
Motelle	+	+	Ve,F1,F2	V. Williamson, UC-Davis
Silverado	+	+	Ve,F1,F2	Ferry Morse
Sheriff	-	+	Ve,F1,N	Harris Moran
Dominique	+	+	V,F1,F2,TMV,N	Hazera Genetics
Tequila	-	+	TMV,F0,F1,Cf5,N,Ve1	Vilmorin
Charanda	+	+	Ve,TMV,F0,F1, F2, N	Vilmorin
Don Raul	+	+	TMV,V,F2	De Ruiter Seeds
Ih902	+	+	F1,F2,Ve,Ty	F. Vidavski, HUJ
Celebrity	+	+	Ve,F1,F2,N,TMV	Seminis Seeds
Toro	+	+	Ve,F1,F2,N	Harris Moran
Crista	+	+	Ve,F1,F2,F3,N,TSWV	Harris Moran
Solar Fire	+	+	F1,F2,F3,Ve	Harris Moran
Viva Italia	+	+	Ve,F1,F2,N	Harris Moran
Amelia VR	+	+	Ve,F1,F2,F3,N,St,TSWV	Harris Moran
El Cid	+	+	Ve,F1,F2,N,TMV	Harris Moran
Plum Crimson	-	+	Ve,F1,F2,F3	Harris Moran
Heinz 1706	-	+	Ve,F1	Heinz Seeds
Better Boy	-	+	Ve,F1,N	Northrup King

[a] Disease resistance: ASC = Alternaria stem canker; BS1 = Bacterial speck; Cf5 = Leaf mold; F0,F1,F2,F3 = Fusarium wilt, race 0, 1, 2, and 3, respectively; FCRR= Fusarium crown and root rot; GLS and St = Gray leaf spot; N = Root-knot nematode; TSWV = Tomato spotted wilt disease; TMV or Tm2a = Tobacco or Tomato mosaic disease; Ty = Tomato yellow leaf curl disease; Ty-2 = Tomato leaf curl disease; Ve or Ve-1 = Verticillium,wilt race 1.

[b] AVRDC = Asia Vegetable Research and Development Center; UC-Davis = University of California-Davis; HUJ = Hebrew University of Jerusalem.

Table 3: Results of the Biological assay and PCR performed by Yael Rekah at the Faculty of Agricultural, Food, and Environmental Quality Sciences, Department of Plant Pathology and Microbiology at the Hebrew University of Jerusalem.

ID # (Accession)	Biological test (No. of seedlings)			PCR results
	Healthy	Vascular discoloration	Wilted	
GT-06-01 (XA339)	24	6	-	Resistant
GT-06-02 (XA258A)	1	4	25	Susceptible
GT-06-03 (XB4C)	23	7	-	Resistant
GT-06-04 (XC273B)	20	10	-	Resistant
GT-06-05 (XC173B)	27	3	-	Resistant
GT-06-06 (XA385)	30	-	-	Resistant
Plum Crimson F1	30	-	-	Susceptible
Resistant Control	117	3		Summary of 4 replicates
Susceptible Control	6	140		Summary of 5 replicates

Figure 1: Dark brown vascular discoloration in tomato caused by Fusarium wilt.
(http://ohioline.osu.edu/hyg-fact/3000/3122.html)

Figure 2: Fusarium wilt of tomato causing the yellowing and death of leaves along the stem.
(http://ohioline.osu.edu/hyg-fact/3000/3122.html)

Table 4: List of additional lines, hybrids, and accessions tested with the Fus primers.

	Parents/Source		FusF1/rr2
Line	F	M	F2 Locus +/-
Gc16	Fla658-2BK		+
Gp10	PimperJ-13		-
Gh13	FAVI-9		+
TY22 (L.c.)	AVDRC		-
Nainemor	Vidavski	Vidavski	-
Gc172-1-2	Scott	Scott	+
Gc173-2-1	Scott	Scott	+
Gh25-8-1	Gh5	GC6	-
Gc43-4-3-1	Gc9	Marina	+
Gh25-6-1-1-1	Gh5	GC6	-
Ghp44-1-3-a	GP10 x Gh1	Marina	+
Gh8632-1-2	FAVI-9	Abigail	-
Gu143-1-1	Unknown	Unknown	+
Gc173-2-a	Scott	Scott	+
Gh105-1-a-2	Vidavski	Vidavski	-
Gu143-1-1-1-1	Unknown	Unknown	+
Gu143--1-2-2-1	Unknown	Unknown	+
GhT44h-2-1-a	FAVI9	Elios	-
Gc43-5-4-a	Gc9	Marina	+
Gh137-1-1-1-1	Gh2	Marina	+
Gh25-6-1-b-1	Gh5	GC6	-
Ghc45-1-1-2	Gc9	F131	+
LR171c-1-a	Scott	Scott	+
Gh25-2-1-2-1-1	Gh5	GC6	-
Gh25-6-1-1-1	Gh5	GC6	-
Bv/Bw0527-1			-
Gh25-8-1-2-1-1	Gh5	GC6	-
LA2711-2	Scott	Scott	+

	Parents		FusF1/rr2
Line	F	M	F2 Locus +/-
Bv/Bw514-sb			-
Bv/Bw519-sb			+
Gc43-4-1-1-a-1	Gc9	Marina	+
Gc43-4-1-a-1-1	Gc9	Marina	-
Bv0094-1			-
GhT44-2-1-a-TF1	FAVI9	Elios	-
LA2711-1	Scott	Scott	+
Gc173-2-1-1-1	Scott	Scott	+
Ghp31-4-1-2-1-1	Gh5	Gp11	+
Gc171-1-sb-1-2	Scott	Scott	+
Gc171-1-1-1-1	Scott	Scott	+
LA2711-a	Scott	Scott	+
Gc172-2-4-1-1	Scott	Scott	+
Gc171-2-2-a-1	Scott	Scott	+
Gc173-2-a-a-1	Scott	Scott	+
LA2711-b	Scott	Scott	+
Ghp44-1-2-a-1-2	Gp10 x Gh1	Marina	+
Gh25-4-1-1-a-1	Gh5	GC6	-
Ghc45-1-1-1-1-1	F131	Gc9	+
Gc43-5-4-1-1-1	Gc9	Marina	+
Gh124-1-a-1-1	Vidavski	Vidavski	+
Ghp44-1-2-1-1-1	Gp10 x Gh1	Marina	+
Gc143-1-1-3-a	Unknown	Unknown	+
Ghp44-1-2-2-1-1	Gp10 x Gh1	Marina	+
Gc180-1-b-1	Gc9	GC6	+
Ghp44-1-1-TF3-1-1	Gp10 x Gh1	Marina	-
Bv/Bw514-a			-
Gc9-a-1	Fla595-2		+

Literature Cited

Bao, J., Fravel, D., Lazarovits, G., Chellemi, D., van Berkum, P., and O'Neill, N. 2000. Population structure of *Fusarium oxysporum* in conventional and Organic tomato production in Florida. USDA, Biocontrol of Plant Diseases Laboratory, Beltsville MD 20705

Bournival, B. L., Vallejos, C.E., and Scott, J.W. 1990. Genetic analysis of resistances to races 1 and 2 of *Fusarium oxysporum* f. sp. *lycopersici* from the wild tomato *Lycopersicon pennellii.* Theor. Appl. Genet. 79:641-645.

El Mehrach, K., Hatimi, A., Mejía, L., Williamson, V.M., Vidavski, F., Gharsallah-Chouchane, S., Salus, M.S., Martin, C.T., and Maxwell D.P. 2005. PCR-based methods for tagging the *Mi-1* locus for resistance to root-knot nematode in begomovirus-resistant Tomato Germplasm. Acta Hort. 695:263-270.

Kawchuk, L., Hachey, J., Lynch, D., Kulcsar, F., van Rooijen, G., Waterer, D., Robertson, A., Kokko, E., Byers, R., Howard, R., Fischer, R., and Prufer, D. 2001. Tomato Ve disease resistance genes encode cell surface-like receptors. PNAS, USA 98:6511-6515.

Larkin, R.P., and Fravel, D. R., 1998. Biological Control of Fusarium wilt disease by nonpathogenic *Fusarium spp*.: Formulations and Field Efficacy, USDA, Biocontrol of Plant Diseases Laboratory, Beltsville MD 20705

Scott, J.W., and Jone, J.P. 1989. Monogenic resistance in tomato to *Fusarium oxysporum* f. sp. *lycopersici* race 3. Euphytica 40:49-53.

Sela-Buurlage, M., Budai-Hadrian, O., Pan, Q., Carmel-Goren, L., Vunsch, R., Zamir, D., and Fluhr, R. 2001. Genome-wide dissection of Fusarium resistance in tomato reveals multiple complex loci. Mol. Gen. Genom. 265:1104-1111.

Simons, G., Groenendijk, J., Wijbrandi, J., Reijans, M., Groenen, J., Diergarrde, P., Van der Lee, T., Bleeker, M., Onstek, J., de Both, M., Harring, M., Mes, J., Cornelissen, B., Zabeau, M., and Vos, P. 1998. Dissections of the Fusarium I2 gene cluster in tomato reveal six homologs and one active copy. Plant Cell 10:1055-1068.