Marker-Assisted Selection of Tomato Genotypes with the *I-2* Gene for Resistance to *Fusarium oxysporum* f. sp. *lycopersici* Race 2

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ABSTRACT

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Fusarium vascular wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici*, affects tomatoes worldwide. Development of resistant varieties of tomato would constitute an economically and environmentally sound approach for the management of this disease. Resistance genes to *F. oxysporum* f. sp. *lycopersici* race 1 (*I-1* gene) and race 2 (*I-2* gene) were mapped to chromosome 11. The *I-2* gene cluster includes one functional copy and six nonfunctional homologs of the *I-2* gene. This report describes the design of primers based on the functional gene copy and the development of a multiplex polymerase chain reaction (PCR)-based method that has the ability to differentiate *I-2* genotypes from genotypes without the *I-2* gene. In these trials, 39 of the 40 genotypes tested with known reactions to race 2 gave the expected results. The only exception was the cultivar Plum Crimson carrying the *I-3* gene for resistance, which confers resistance to *F. oxysporum* f. sp. *lycopersici* races 1, 2, and 3. This method was validated in three countries and by bioassays.

Fusarium oxysporum f. sp. *lycopersici* (Sacc.) Snyder & Hansen is a soilborne fungus that causes a vascular wilt disease of tomatoes, and is now a worldwide problem. *Fusarium* species are known to produce chlamydospores that remain viable in infested soils for several years. Due to the inefficiency of fungicides and other conventional control methods, considerable breeding efforts have been directed toward the development of resistant tomato cultivars. Resistance genes to *F. oxysporum* f. sp. *lycopersici* races 1 and 2 have been introduced from *Solanum pimpinellifolium* L. (= *Lycopersicon pimpinellifolium* (L.) Mill.) and were mapped to the short arms (*I-1* locus) and long arms (*I-2* locus) of chromosome 11 (5,9). High-resolution mapping showed that *I-2* is located between markers *6-16* and *TG36*, and is 0.23 cM from *6-16* and 1.3 cM from *TG36* (6). Locus *I-3* was introgressed from *Solanum pennellii* Cornell (= *Lycopersicon pennellii* (Cornell) D'Arcy), and this gene provides resistance against *F. oxysporum* f. sp. *ly-*

copersici races 1, 2, and 3 (3), and was mapped to chromosome 7 (2,8). The *I-2* gene cluster was reported to include one functional copy of the resistance gene and six nonfunctional homologs (10).

In conventional breeding programs, marker-assisted selection (MAS) is considered a helpful tool that allows considerable savings of space and time. The DNA sequence of the *I-2* gene cluster has been published in GenBank, National Center for Biotechnology Information; however, to our knowledge, it has not been used for MAS. This paper describes the development of a multiplex polymerase chain reaction (PCR) that can be used for detection of the *I-2* gene for resistance to *F. oxysporum* f. sp. *lycopersici* race 2 in tomato genotypes.

MATERIALS AND METHODS

Plant material. In the initial PCR method trials, a total of 11 known tomato genotypes were used to evaluate the specificity of the PCR primers. Five of the genotypes (KH, BB [Peotec Seeds], Saint Pierre, Marmande Verte, and Mobaci [Peto Seeds]) do not contain genes for resistance to *F. oxysporum* f. sp. *lycopersici*. In addition, four other varieties (Chorouk [Peto Seeds], Karin [Royal Sluis], DRW 7216 [De Reuters], Motelle [INRA, France]) were resistant to *F. oxysporum* f. sp. *lycopersici* race 2, and thus supposedly had

the *I-2* dominant gene for resistance. Also, one breeding line, Ih902, with begomovirus resistance derived from *Solanum habrochaites* S. Knapp & D.M. Spooner (= *Lycopersicon hirsutum* Dunal) was tested. Ih902 is resistant to *Tomato yellow leaf curl virus* (TYLCV) (12) and contains the *I-2* gene. The variety Marporum (INRA, France) only has the *I-1* resistance gene. To further evaluate this PCR method, 24 additional genotypes with known resistance to *F. oxysporum* f. sp. *lycopersici* (Table 1) were evaluated at the University of Wisconsin-Madison.

Primer design. In order to detect the *I-2* gene for resistance to *F. oxysporum* f. sp. *lycopersici* race 2, the DNA sequences of the *I-2* active gene copy (accession no. AF118127) and its homologs *I-2C1* (accession no. AF004878), *I-2C2* (accession no. AF004879), and *I-2C3* (accession no. AF004880) were aligned using the CLUSTLAW version 1.83 (European Bioinformatics Institute, Cambridge, UK). This alignment indicated the presence of an insertion/deletion (indel, nt 3558–3631) in the 3' region of the active gene copy (9). Two reverse primers were designed in this region, TFusr1 (5'-CGA AGA GTG ATT GGA GAT-3') and TFusr2 (5'-CCT GGA TGA ACA GCT GAG-3'). The forward primer TFusF1 was designed in the 2996–3014 nt region of the *I-2* gene with the following sequence: TFusF1 (5'-CTG AAA CTC TCC GTA TTT C-3'). These two pairs of primers amplify a DNA fragment of approximately 600 bp. Another pair of primers for the *Ve* locus, Ve2F/Ve2R, which was reported to amplify a fragment of approximately 750 to 800 bp from all tomato genotypes tested (4), was used as the internal control in the multiplex PCR reaction. This Ve2 primer pair allowed for evaluation of the quality of extracted DNA and efficiency of the PCR reaction in each tube.

DNA extraction and PCR. DNA was extracted from fresh tomato leaves by using the lids of microfuge tubes to cut two leaf disks from expanding leaflets of the growing tips of tomato plants or seedlings. Leaf disks were ground in liquid nitrogen with a pestle attached to an electric drill. At the American University of Beirut (AUB), the ground samples were

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extracted according to the CTAB protocol with minor modifications (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 1% polyvinylpyrrolidone [PVP], 100 mM Tris-HCl, pH 8.0, and 0.2% mercaptoethanol [added just before use]) (13). The resulting pellet was washed in 75% ethanol, air-dried, and resuspended in 50 µl of double distilled water. The sample was diluted 20 times and then used as a template in the PCR reaction. At the University of Wisconsin-Madison, the ground samples were extracted with the Puregene DNA Isolation Kit, Cell and Tissue Kit (Gentra Systems, Minneapolis, MN) following the manufacturer's instructions. DNA concentration was adjusted to approximately 10 ng/µl.

For PCR, a 20- to 25-µl reaction mixture was used and comprised of 2 to 2.5 µl of 10× reaction buffer, 2 mM MgCl₂, 0.2 µM of each primer, 200 µM of each of the four dNTPs, 1 unit of *Taq* DNA polymerase, and 2 µl of DNA sample or approximately 20 to 30 ng of DNA. The PCR protocol consisted of an initial denaturation step at 95°C for 3 min followed by 35 cycles at: 94°C for 1 min, 57°C for 1 min, and 72°C for 2 min. The program ended with a final extension step of 72°C for 10 min. The same procedure was followed for multiplex PCR, but the concentration of the *I-2* gene specific primers was increased to 0.4 µM, while the internal control Ve2 primers were used at a concentration of 0.1 µM.

Bioassays. Biological tests were conducted on six tomato accessions from Guatemala (kindly provided by Luis Mejia) and on an F2 population ([Ih902 (*I-2/I-2*)

crossed with a local Lebanese land race, Baladi (*i-2/i-2*)], segregating for resistance to *F. oxysporum* f. sp. *lycopersici* race 2. Colonies of race 2 (kindly provided by Yael Rekah) were cultured on Czapek-Dox medium and incubated in the dark at 27°C for 7 days. The spores were harvested in a water suspension and the final concentration adjusted to 1 × 10⁶ spores/ml.

Tomato seeds, surface disinfested with sodium hypochlorite, were sown in 250-ml plastic containers filled with vermiculite. The roots of 13- to 14-day-old tomato seedlings were washed in tap water and trimmed. The seedlings (30 to 35 seedlings per accession and 101 F2 seedlings) were inoculated by dipping the roots of each tested line for 10 min in the spore suspension. Seedlings were then transplanted into pots filled with steam-sterilized soil (3.8% clay, 0.0% silt, 96.2% sand, and 0.4% organic matter) and maintained in the greenhouse at a temperature of 26 ± 2°C for 3 weeks, during which time visual disease symptoms (wilt and desiccation) were recorded. At 21-days postinoculation (DPI), seedlings were cut at 1 cm above soil level to determine vascular discoloration. A seedling was determined to be susceptible if wilt symptoms were apparent or it exhibited vascular discoloration. DNA was extracted from each seedling and tested by multiplex PCR for the presence of the *I-2* gene.

RESULTS

Marker-assisted selection for the *I-2* gene. In preliminary assays conducted at

AUB on the 11 genotypes, agarose gel analysis following PCR showed that both primer pair combinations TFusF1/TFusrr1 and TFusF1/TFusrr2 specifically amplified targets from plant tissue of genotypes with reported resistance to *F. oxysporum* f. sp. *lycopersici* race 2 (*I-2* gene) but not from other genotypes (Fig. 1).

To validate the usefulness of the Ve2 primers as internal control, assays were conducted in two different labs (AUB and University of Wisconsin-Madison) on DNA extracted from 54 tomato genotypes. The Ve2 primers consistently amplified a DNA fragment of approximately 800 bp in all genotypes tested (data not shown). Therefore, based on these results, these Ve2 primers were deemed reliable to use as the internal control primers in the multiplex PCR reactions.

Optimization of the multiplex PCR parameters (primer concentration and annealing temperature) allowed detection of two amplicons following gel electrophoresis: a specific amplicon of approximately 600 bp for the *I-2* genotypes, and another amplicon of approximately 800 bp for the internal control (Figs. 2 and 3). This allowed distinction between the *I-2* genotypes (both amplicons) and the genotypes without the *I-2* gene (800-bp amplicon). In some gels, the intensity of one of the bands was greater than that of the other; however, the addition of dimethyl sulfoxide to the reaction mix improved the results, giving bands of similar intensities.

This multiplex PCR method was evaluated at the University of Wisconsin-

Table 1. Tomato genotypes tested by multiplex polymerase chain reaction (PCR) for detection of *I-2* gene for resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2

Cultivar/line	Primer TFusF1/TFusrr2	Internal control primer Ve2F/2R	Resistance ^a	Source ^b
CLN2443A	–	+	Ty, TMV, F1	AVDRC, Peter Hanson
M82	–	+	Ve, F1	Favi Vidavski, HJ
F632	+	+	Ve, F1, F2, TMV, N, Ty	Favi Vidavski, HJ
Titrit	+	+	Ty, F1, F2, Ve, TMV, N, FCRR	Royal Sluis
Marina	+	+	F1, F2, N, ASC, GLS, BS1, Ve	Sakata Seed America
Moneymaker	–	+	None	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, F1, Cf5, N, Ve1	Vilmorin
Charanda	+	+	Ve, TMV, F1, F2, N	Vilmorin
Don Raul	+	+	TMV, V, F1	De Ruiter Seeds
Ih902	+	+	F1, F2, Ve, Ty	F. Vidavski, HJ
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; F1, F2, F3 = *Fusarium* wilt race 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 = tobacco or tomato mosaic disease; Ty = tomato yellow leaf curl disease; Ve = *Verticillium* wilt race 1.

^b AVRDC = Asian Vegetable Research and Development Center; UC-Davis = University of California-Davis; HJ = Hebrew University of Jerusalem.

Madison on an additional 24 genotypes, which were reported by the respective seed companies to either have or not have resistance to *F. oxysporum* f. sp. *lycopersici* race 2. The primers correctly detected the presence or absence of the *I-2* gene in 23 of the 24 genotypes. Only one cultivar, Plum Crimson, gave no band for the *I-2* gene, while it had been reported by the seed company to be resistant to *F. oxysporum* f. sp. *lycopersici* races 1, 2, and 3 (Table 1). Subsequent tests by Mark Lewis

(personal communication) with a PCR method and a biological assay verified that Plum Crimson had the *I-3* gene.

When genotypes such as Marporum and M82, containing only the *I-1* gene for resistance, were compared with other genotypes with both *I-1* and *I-2* genes of resistance using the developed multiplex PCR method, only those possessing the *I-2* gene gave the expected 600-bp amplicon.

Bioassays. With our experimental conditions, only inoculated seedlings from one

accession showed wilting symptoms at 21 DPI. Therefore, assessment for the other genotypes focused on development of stem vascular browning above the soil level. Some variability was experienced in the bioassay for resistance to race 2: some susceptible plants may escape infection; while a limited number of seedlings from resistant varieties or accessions may show vascular discoloration; and this may be influenced by the genetic background, whereby approximately 3 to over 10% of plants from resistant genotypes may show vascular discoloration (Table 2). However, the overall reaction of the six accessions from Guatemala correlated positively with the multiplex PCR results.

Out of the 101 F₂ seedlings tested, 72 were PCR positive for the *I-2* resistance gene (Fig. 3) and 29 were susceptible. Similar results were obtained in the biological test: 72 healthy seedlings and 29 with vascular discoloration. Chi square analysis indicated ($P > 0.05$) that this ratio of resistant to susceptible plants corresponds well to the expected 3:1 segregation ratio. Again, when the reactions of individual plants were compared, a high correlation was found between PCR results and biological assays, with 69/72 seedlings showing resistance in both tests and 26/29 seedlings showing susceptibility in both tests. With the multiplex PCR method, we obtained 3/72 (approximately 4%) PCR-resistant plants showing vascular browning symptoms, considerably closer to the results obtained by bioassays in Table 2.

DISCUSSION

Development of tomato varieties resistant to *F. oxysporum* f. sp. *lycopersici* represents the most environmentally sound and effective strategy for the management of Fusarium wilt. In breeding programs designed to introgress new traits into breeding lines, such as pathogen resistance, bioassays are needed to assess the inheritance of the introgressed trait in the breeding lines. In the case of Fusarium wilt, bioassays are time-consuming and complicated by variations in host genotype, inoculum concentration, stage of seedling development, soil temperature, and soil contamination by other pathogens or races (1,7). Moreover, breeding programs often aim at introducing genes for resistance to two or more diseases simultaneously (11). Subjecting plants in the breeding programs to infection by several pathogens may be cumbersome, and sometimes may lead to loss of important breeding material, which is resistant to a major pathogen. Therefore, the development of reliable molecular markers for resistance genes would be advantageous for rapid screening of progeny lines. In our breeding program, we are interested mainly in resistance to TYLCV. Therefore, only those lines that are resistant in field tests to TYLCV would be tested by multiplex PCR

Fig. 1. Gel electrophoresis of polymerase chain reaction (PCR)-amplified products in 1.5% agarose: Amplicons obtained with primer pair TFusF1/TFusrr1 (A) or of primer pair TFusF1/TFusrr2 (B). Lanes A to E are extracts of genotypes without the *I-2* gene for resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2: Baladi, Saint Pierre, Marmande Verte, and Marporum, respectively. Lanes F to I are samples of genotypes with reported resistance to race 2 (*I-2* gene): Chorouk, Karin, Motelle, and lh902, respectively. Lane Z is 100-bp ladder. The expected amplicon is approximately 600 bp for the *I-2* gene.

Fig. 2. Gel electrophoresis of multiplex polymerase chain reaction (PCR)-amplified products in 1.5% agarose. Amplicons obtained with primer pair TFusF1/TFusrr1. Lanes A to E and J to N are extracts from genotypes with no genes for resistance to *Fusarium oxysporum* f. sp. *lycopersici*. Lanes F to H and O to Q are extracts from genotypes with the *I-2* gene. Lanes A to H, have dimethyl sulfoxide (DMSO) added to the PCR mix. Lane Z is a 100-bp ladder.

Fig. 3. Gel electrophoresis in 1.5% agarose of multiplex polymerase chain reaction (PCR) from the F2 progeny of Ih902 (homozygous *I-2* gene of resistance) with a local Lebanese accession, Baladi (homozygous for *i-2*). Lanes B, C, F, I, J, K, M, N, O suggest seedlings with the *I-2* gene. Lanes A, D, E, G, H, L, P suggest seedlings without *I-2*. Lane Z is a 100-bp ladder.

Table 2. Comparison of results of biological tests with multiplex polymerase chain reaction (PCR) for determination of resistance to *Fusarium oxysporum* f. sp. *lycopersici* race 2^a

Accession	Biological test (no. of seedlings)			PCR results
	Healthy	Vascular discoloration	Wilted	
GTXA339	24	6	—	Resistant
GTXA258A	1	4	25	Susceptible
GTXB4C	23	7	—	Resistant
GTXC273B	20	10	—	Resistant
GTXC173B	27	3	—	Resistant
GTXA385	30	—	—	Resistant
Plum Crimson F1	30	—	—	Susceptible
Resistant control ^b (YA-603)	117	3	—	Total for 4 replicates
Susceptible control ^c (YA-604)	6	140	—	Total for 5 replicates

^a Data provided by Yael Rekah.

^b Homozygous resistant genotype.

^c Homozygous susceptible genotype.

for their resistance to *F. oxysporum* f. sp. *lycopersici*. The multiplex PCR method for the *I-2* gene successfully distinguished between the *I-2* genotypes in 39 out of 40 genotypes with known resistance reaction or reported responses to race 2. The only exception was Plum Crimson. Plum Crimson is reported to have resistance to races 1, 2, and 3. Therefore, the inability to detect the *I-2* gene specific amplicon in this variety was due to the presence of *I-3* resistance gene (Mark Lewis, *personal communication*), which confers resistance to *F. oxysporum* f. sp. *lycopersici* races 1, 2, and 3 (3). It is important to note that the 600-bp fragment for the *I-2* gene was never detected in a genotype that was reported to be susceptible to race 2. Additionally, since the *I-2* gene specific primers were designed to anneal to the sequences of the functional resistance gene, it is unlikely that the presence of this marker would not correspond to resistance to race 2. A multiplex PCR protocol was developed using an internal control with the Ve2-PCR primer set (4) to ensure that the quality of the DNA was satisfactory for PCR and to avoid false negative results.

The results obtained by the developed multiplex PCR method correlated well with those of the biological assays, when tested on accessions or on individual plants from an F2 population segregating for resistance to race 2. Overall, the PCR results correlated well with biological tests with race 2 except for the variety Plum Crimson, which contains the *I-3* gene for resistance as mentioned above.

In conclusion, this multiplex PCR method for the *I-2* gene could be effectively used in a breeding program to detect the presence of the *I-2* gene. It was validated in three laboratories in three different countries. Therefore, its use may greatly reduce the number of lines that would require further testing with a bioassay. Once parental breeding lines have been determined to contain the *I-2* gene by PCR and resistance was confirmed by bioassay, then segregating populations using this parental breeding line could be effectively evaluated with the multiplex PCR method for resistance to race 2. Therefore, the developed multiplex PCR method saves time and space, and avoids problems associated with environmental

conditions and inoculation uniformity. As this multiplex PCR method does not allow the differentiation of homozygous from heterozygous resistant genotypes, future efforts will be devoted to developing a PCR method to distinguish heterozygous genotypes.

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