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MOLECULAR AND MORPHOLOGICAL IDENTIFICATION OF TOMATO YELLOW LEAF CURL VIRUS (TYLCV) DISEASE IN TOMATO

P.K. SAMARAJEEWA, M.G.M.K. MEEGAHAKUMBURA
Plant Genetic Resources Center, Gannoruwa
and
R.M.S.L. RAJAPAKSE, C.G. GAMMULLA and V.A. SUMANASINGHE
Department of Agricultural Biology, Faculty of Agriculture, University of Peradeniya

ABSTRACT

Tomato Yellow Leaf Curl Virus (TYLCV) disease transmitted by whitefly *Bemisia tabaci* is a major constraint in tomato cultivation in Sri Lanka. The presently cultivated popular varieties are susceptible to the disease. Use of insecticides for its control is very expensive and partially successful due to the resistance of *B. tabaci* to insecticides. Development of resistant varieties requires identification of TYLCV resistant germplasm and the incorporation of resistant genes to commercial varieties. This study was carried out to screen some selected tomato germplasm accessions including wild and commercial types for resistance, to transfer the resistant genes to cultivated tomato and to identify a putative molecular marker for resistance. Twenty accessions were selected for the study. The plants were screened by using the virus vector, *B. tabaci* and by graft inoculation. Only *L. hirsutum* showed high resistance to the disease. Susceptible Marglobe was crossed with *L. hirsutum* in both directions to transfer the resistant genes. The crossing was successful only when Marglobe was used as the mother plant. F1 and F2 progenies were obtained and screened for resistance using whiteflies. The plants were morphologically characterized for trichome density and it was observe that the resistant plants carried more trichomes on leaves and stems. To identify a putative molecular marker linked with resistance, F2 generation was analyzed through Bulked Segregant Analysis using 10 selected RAPD polymorphic primers.

KEYWORDS: TYLCV, RAPDs, Bulk Segregation Analysis, Tomato.

INTRODUCTION

Tomato Yellow Leaf Curl Virus (TYLCV) disease transmitted by white fly *Bemisia tabaci* is the most severe viral disease of tomato in Sri Lanka (Ariyarathne *et al.*, 2004). It causes flower drop, impedes fruit set and reduces marketable fruit yield. Early infection always results in 100% yield loss (Agrios, 1997). Most of the commercial varieties, KWR, Roma, Marglobe, T146, T89, T245, Thilina, Vihara 2, Ravi, Tharindu and hybrids are susceptible to the disease (Peiris, 2002). Use of insecticides for its control is only partially successful as *B. tabaci* is resistant to insecticides and also it is very expensive and environmentally harmful. Breeding for resistance to TYLCV appears to be an ecological approach and sometimes the most effective way of reducing the level of infection.

According to a survey conducted recently (Anon. 2004) it has been found that incidence of TYLCV disease is high in Kandy, Matale and Nuwara-Eliya districts, and it affects over 25% of the tomato cultivation. In some areas about 75% infection was recorded.

If resistant varieties are to be developed through classical breeding, the germplasm resistant to virus should be identified. Although considerable amount of screening work for disease resistance has been done locally with local and introduced germplasm, no reports are available on the success rate of transfer of resistance to cultivated tomato. On the other hand, the local tomato germplasm collection in the gene bank of the Plant Genetic Resources Centre (PGRC), which is around 200 accessions, has not been systematically evaluated for TYLCV resistance. The collection comprises of several wild species, *L. hirsutum*, *L. pimpinellifolium* and *L. cheesmaneii* which may serve as a potential source of resistance (Hassan *et al.*, 1984b; Kasrawi *et al.*, 1994). Particularly, *L.hirsutum* has been reported to be resistant to TYLCV (Kasrawi *et al.*, 1994). However, its reaction to local viral isolates or the possibility of transferring the resistance factor to commercial cultivars is not known.

If resistance is found in a germplasm accession and molecular markers tightly linked to the responsible genes are found, it could help in the selection of breeding lines for the trait (Chague *et al.*, 1997). To determine a molecular marker, Bulked Segregant Analysis (BSA) method can be successfully used with fingerprinting techniques such as RAPD (Michelmore *et al.*, 1991).

The objective of the present work was to first screen the germplasm to identify the TYLCV resistant donors, and then to incorporate the resistant genes to commercial cultivars and finally to identify a RAPD marker linked to TYLCV resistance.

MATERIALS AND METHODS

Screening of Germplasm for TYLCV Resistance

Fourteen different cultivated tomato accessions and six wild tomato accessions belonging to four different species (Table 1) and F1 and F_2 populations derived from a cross between *L. esculentum* (Marglobe) × *L. hirsutum* were also screened for virus resistance. Some of the *L. esculentum* accessions were commercial tomato varieties. Selection of other tomato accessions was based on the analysis of characterization data to represent genetic variation. The seed samples of these accessions were obtained from the gene bank of PGRC and seed multiplication was done before the experiments. Insect transmission method and graft inoculation techniques were used for screening plants, under green house conditions.

Table 1. Tomato accessions used for screening against virus through insect transmission.

Number	*Lycopersicon species and accessions.*	*Common name/status*
1	*L. hirsutum*	Wild
2	*L. cheesmanii*	Wild
3	*L. peruvianum*	Wild
4	*L. pimpinellifolium*	Wild
5	*L. pimpinellifolium*	Wild
6	*L. pimpinellifolium*	Wild
7	*L. esculentum*	K.W.R.
8	*L. esculentum*	T_{245}
9	*L. esculentum*	Ravi
10	*L. esculentum*	Unknown
11	*L. esculentum*	Thilina
12	*L. esculentum*	Ratabatu
13	*L. esculentum*	L 390
14	*L. esculentum*	-
15	*L. esculentum*	Red setter
16	*L. esculentum*	Marmande
17	*L. esculentum*	Bianz
18	*L. esculentum*	Mas Thakkali
19	*L. esculentum*	Thakkali
20	*L. esculentum*	Goraka Thakkali

Screening for TYLCV resistance using *B. tabici*

Rearing white flies

The white flies that were naturally occurring on the eggplants (*Solanum melongena*) were collected from plants grown in vegetable fields at Horticultural Crop Research and Development Institute into test tubes in *maha* 2003 and immediately introduced to the seedlings of eggplant which were placed inside an insect proof cage in a green house. The white flies were reared until use for screening.

Production of TYLCV infected tomato plants

The TYLCV strain used for this study was collected from infected tomato plants collected from Marassana area in Kandy. However, Marglobe variety was used to maintain a source of inoculum for all the experiments. For this, three week old 50 seedlings were transplanted separately into black polythene bags of 8" width and 9"height. When the stems of seedlings were of pencil-thickness, they were grafted with the scions taken from infected Marglobe plants showing typical symptoms of viral infection. After three weeks, when the root stocks of successfully grafted plants started showing typical TYLCV symptoms, they were selected to be used in subsequent screening using white flies.

Screening via insect transmission

For screening of tomato accessions (Table 1), 10 plants of each accession were taken due to limited space in the green house. For analysis of F_1 generation, only 2 plants were used due to seed sterility. For characterization and evaluation of F_2 generation, 45 plants were included. The plants under screening and the TYLCV infected Marglobe plants were randomly arranged in soil beds. Two weeks after transplanting, the white flies were introduced to TYLCV infected Marglobe plants. Around 20 white flies were introduced to each plant. The screening was carried out in two different seasons. Those shown in Table 1 were screened in *maha* 2003. The progeny of Marglobe x *L .hirsutum*, F_1 and F_2 generations were screened in *maha* 2004. Three weeks after introduction of whiteflies, the test plants were observed for TYLCV symptoms using picture guides (Green and Kalloo, 1994).

In each accession, the total number of TYLCV infected plants was recorded and expressed as a percentage. The readings were taken twice, 31 days and 45 days after introduction of whiteflies. Based on the percentage data, the accessions were rated according to Ariyaratne and Weeraratne (2001). In the case of F_1 and F_2 populations, only the presence or absence of disease symptoms was recorded.

Screening by graft inoculation

Only 3 tomato accessions which exhibited resistance in the previous screening, were used for screening by graft inoculation. Seedlings used for this experiment were obtained from the nursery. Three-week old seedlings were transplanted into black polythene bags. Five of ten plants were used for graft inoculation and the rest were kept as controls. Graft inoculation was done when the plant stem was nearly of pencil-thickness (8-week old plants). The green house environment was maintained insect free by routine application of insecticides.

Hybridization between virus resistant and virus susceptible varieties

The most resistant *Lycopersicon* species *L. hirsutum* was reciprocally crossed with the highly susceptible varieties, Marglobe and Thilina. The F1 plants were raised and selfed to obtain the F_2 generation. The segregating population of the F_2 was then characterized phenotypically and evaluated for resistance.

Molecular Analysis: Plant DNA extraction

For molecular analysis, leaf samples were collected from the two parents (Marglobe and *L. hirsutum*), F1 and 45 F_2 plants which were planted for screening. DNA extraction was carried out according to the protocol in the Workshop Handbook, Genomics and Crop Improvement, ICGEB 2003. The DNA samples were quantified by Biomate 3 spectrophotometer. Optical density (OD) readings were obtained at 260nm and 280nm. DNA quantity was estimated based on 260nm reading. According to this value, the DNA samples were diluted to obtain a final concentration of 20ng/µl.

Primer selection for PCR amplification

PCR amplification was done for DNA of two parents (Marglobe and *L. hirsutum*) using 20 random primers (Operon primers) in order to select the most appropriate primers. The primers used were OPA 05, OPA 07, OPA 09, OPA13, OPA 16, OPA 18, OPA 20, OPC 03, OPC 04, OPC 7, OPD 20, OPF 15. OPK 15, OPK 16, OPJ 5, OPJ 06, OPJ 7, OPJ 20, OPR O6 and OPR 20. Based on the polymorphism obtained (Figure 2) between the two parents, 10 primers were selected.

Bulked Segregant Analysis of DNA

Bulk segregation method of molecular analysis described by Michelmore *et al.* (1991) was used for molecular analysis for identification of a putative marker. The DNA was diluted and then used to prepare resistant and susceptible bulks. PCR was carried-out for the resistant and susceptible parents, F_1, resistant bulk, each of the individual plants in the resistant bulk, and susceptible bulk as well as the individual plants used for the preparation of the susceptible bulk.

PCR Amplification and Gel Electrophoresis.

PCR amplification was performed using RAPDs, according to Williams *et al.* (1990). The PCR products were electrophoresed in 1.4% agarose gel. 1µl of loading buffer was mixed with 8µl of the sample and the mixture was loaded into wells in 1.4% agarose gel in 0.5 × TBE buffer. One kb DNA ladder was used as a size marker (Takara Schuzo Co. Ltd., Japan) and the samples were run in TBE (pH 8) buffer for 2 hours at 56V (5V / 1cm length). The gels were stained with ethidium bromide for 15-20 minutes and then destained for 10 minutes. The gels were then observed under the BIO-RAD gel documentation system with the "Quantity One" software package for analysis. Banding patterns were compared between the resistant bulk, susceptible bulk and the individuals showing resistance or susceptibility.

Plant characterization

The F_2 progeny was phenotypically characterized for leaf hair density. Trichome density was measured as described by Toscano *et al.* (2002). Two $1cm^2$ of leaf area near the main leaf vein and between the second and the third adjacent veins were selected and the counting was done under the light microscope with 10x amplification.

RESULTS AND DISCUSSION

Screening for TYLCV using white flies

The results of screening for TYLCV resistance ısing white flies are given in Table 2. Of 20 accessions studied, only the wild species *L. hirsutum* did not have symptoms of infection. *L. cheesmanii*, showed moderate resistance (symptoms). One of the *L. esculentum* accessions was moderately susceptible. The other 17 accessions were severely infected with TYLCV.

Table 2. Reaction of different tomato germplasm accessions to TYLCV when transmitted by whitefly.

Species name	*Common name*	*No. of TYLCV infected plants/Total No. of plants* Date 1	Date 2	*% of infection*	*Disease reaction*
L. cheesmanii	Wild	0	3/8	37.5	MR
L. hirsutum	Wild	0	0	0	HR
L. peruvianum	Wild	-	-	-	-
L. pimpinellifolium	Wild	7/8	8/8	100	S
L. pimpinellifolium	Wild	7/7	7/7	100	S
L. pimpinellifolium	Wild	8/10	10/10	100	S
L. esculentum	K.W.R.	8/9	8/9	100.0	S
L. esculentum	T 245	9/10	10/10	100.0	S
L. esculentum	Ravi	9/10	9/10	90.0	S
L. esculentum	Unknown	4/8	5/8	62.5	MS
L. esculentum	Thilina	7/7	7/7	100.0	S
L. esculentum	Rata Batu	4/9	8/9	88.8	S
L. esculentum	L 390	6/8	8/8	100	S
L. esculentum	-	8/9	9/9	100	S
L. esculentum	Red setter	7/10	10/10	100	S
L. esculentum	Marmande	6/8	8/8	100	S
L. esculentum	Bianz	9/10	10/10	100	S
L. esculentum	Mas thakkali	6/7	7/7	100	S
L. esculentum	Thakkali	5/9	8/9	88.8	S
L. esculentum	Goraka thakkali	5/10	8/10	80.0	S

Date 1 = 31 days after introduction of whiteflies
Date 2 = 45 days after introduction of whiteflies
MR- Moderately Resistant HR-Highly Resistant
S-Susceptible
MS-Moderately Susceptible

Screening by graft inoculation

In order to find out whether the plants which showed resistance had internal resistance to the virus, graft inoculation was carried out using *L. hisutum, L. cheesemani* and a *L. esculentum* accession. According to the results (Table 3) all the *L. cheesemani* plants and one *L. hirsutum* plant which could be successfully grafted showed TYLCV symptoms.

Table 3. Reaction of *Lycopersicon* species to TYLCV infection by graft inoculation.

Species name	*Common name*	*Total no. of plants grafted*	*No. of successful grafts*	*No. of TYLCV infected plants*
L. cheesmanii	Wild	5	4	4
L. hirsutum	Wild	5	1	1
L. esculentum	unknown	2	0	-

None of the plants maintained as control (plants that were not grafted) developed TYLCV symptoms.

The *L. hirsutum* species which showed total resistance to white fly screening was also found to be susceptible to graft inoculation. This indicates that the resistance of *L. hirsutum* to TYLCV is governed by an external mechanism. According to other workers, the four other wild species used here, *L. peruvianum, L. hirsutum, L. pimpinellifolium* and *L. cheesmaneii* are resistant to TYLCV (Hassan *et al.,* 1984; Kasrawi *et al.,* 1994). However, they used different accessions of the species, and genetic differences in the accessions would have contributed to the differential responses. It is also not clear whether the local isolate is genetically homologous to the strains in other regions.

Screening of progenies

In crosses of Marglobe with *L. hirsutum* fertile seeds could be obtained only when Marglobe was used as the female parent due to incompatibility between the two species as reported by Mueller (1996). Our phylogenetic analyses of tomato cultivars using molecular methods indicated that Marglobe was genetically quite distinct from many other cultivated types (SL – USA Report, 2004). Table 4 gives the evaluation results of the progenies of the cross between Marglobe and *L. hirsutum.*

Table 4 Screening of F_1 and F_2 progeny with white fly transmission.

Plant generation/species	*Total No. of plants*	*No. of plants infected with TYLCV*
F_1	2	0
F_2	45	23

F_1 plants showed complete resistance indicating that the trait is dominant. Due to the limited green house space, only a small population of F2 plants were assessed for resistance/susceptibility. However, our results indicate that the resistance factor has been transferred to the F2 generation (some were highly susceptible and some were moderately susceptible 45 days after whitefly introduction). Based on the above results, the five most susceptible and three most resistant F_2 plants were selected to identify a marker linked to resistance.

Molecular Analysis

Primer selection

It is prerequisite that one has to screen a large number of random primers in order to find a marker tightly linked to a trait. Markers also should display a fair amount of polymorphism between parents. Therefore, as a primary step, a number of primers were tested to select the ones putatively linked with the resistance character. The primers which gave high degree of polymorphism between the parents were OPA 5, OPA 16, OPA 20, OPC 04, OPD 20, OPF 15, OPJ 05, OPJ 06, OPJ 7 and OPR O6. The selected primers were used in the Bulked Segregant Analysis (BSA). Figure 1 shows the amplification products for nine primers.

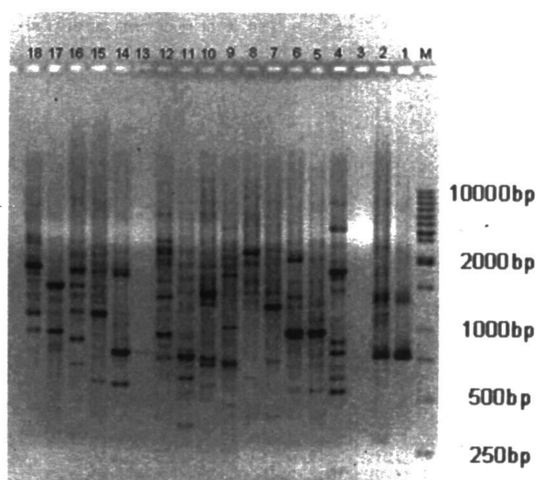

Figure 1. Gel photograph showing the emplified products of Marglobe and L.*hirsutum* with nine primers. (1,3,5,7,9,11,13,15,17-L.*hirsutum*; 2,4,6,8,10,12,14,16,1 - Marglobe; 1,2-OPR 20; 3,4-OPA 16; 5,6-OPJ 7; 7,8-OPA 20; 9,10-OPD 20; 11,12-OPK 15; 13,14-OPC 7; 15,16-OPJ 5; 17,18-OPF 15; M-Ladder).

Bulked Segregant Analysis (BSA)

As a primary step to find out a putative marker, Bulked Segregant Analysis (BSA) was used as it provides a rapid, technically simple alternative to identify markers linked to specific genes. The only prerequisite is the existence of a population resulting from a cross that segregates for the gene of interest (Michelmore *et al.*, 1991). The basis of BSA is that the loci of a particular bulk should represent the collection of all the loci of the individuals of the bulk. However, to confirm the linkage of the marker and the gene of interest, this should be followed by a linkage analysis.

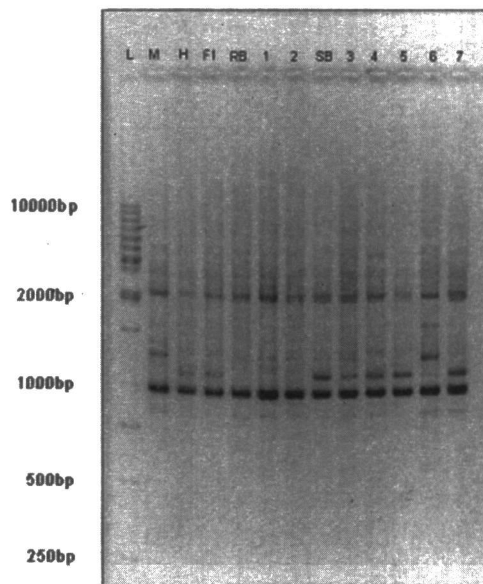

Figure 2. Gel photograph showing the emplification products of the primer OPJ 7 (L -1 kb ladder, M - Marglobe, H - L. *hirsutum*, F1 - resistant F_1 of the L. *esculentum* x L. *hirsutum*, RB - TYLCV resistant F_2 bulk, 1,2 - individuals of the resistant bulk, SB -susceptible F_2 bulk, 3,4,5,6,7 - individuals of the susceptible bulk.

The gel photograph in Figure 2 indicates the results of BSA with the OPJ 07 primer. In the present study, a specific band for virus resistance could not be identified. To identify a marker, analysis should be done with more primers. (Michelmore *et al.*, 1991).

Characterization of plants

Four resistant plants, three susceptible plants and both parents were studied for leaf hair density character. The results are given in Table 5.

Table 5. Leaf hair density of F_2 progeny and parents.

Plant generation/ Name	Plant number	Disease Reaction	No of hairs/cm² leaf area			
			Mature leaf	Middle aged leaf	Immature leaf	Average
F_2	28	Resistant	70,80	100,110	120, 150	135
F_2	39	Resistant	20,28	36, 38	44,43	44
F_2	44	Resistant	29,35	20,20	65,80	73
F_2	46	Resistant	25, 28	30, 34	40,46	44
F_2	1	Susceptible	5,7	4,8	14,17	16
F_2	2	Susceptible	6,9	12,17	25,33	29
F_2	32	Susceptible	4,6	10,13	14,16	15
L. esculentum (Marglobe)		Susceptible	6,7	9,12	5,22	14
L. hirsutum		Resistant	38,40	36,38	73,91	82

Data on number of hairs on leaves of the F_2 population (Table 5), suggest that there is an association between TYLCV resistance and leaf hair density. All four resistant individuals as well as the resistant parent had high leaf hair density. In contrast, all three susceptible plants and the susceptible parent had low leaf hair density. It has been reported that *L. hirsutum* carry four types of I, IV, VI and VII glandular trichomes (Channarayappa *et al.*, 1992). Therefore, resistance to whitefly could be related to the presence of glandular trichomes. These trichomes act as a barrier, preventing oviposition by the whitefly as observed by Williams *et. al.* (1980). Trichome based host plant resistance has also been observed in *L. hirsutum* for lacewings and aphids (Simmon and Gurr, 2004).

CONCLUSIONS

Total resistance to tomato yellow leaf curl virus with white fly screening was identified in wild species *L. hirsutum*. When screened with graft inoculation, all the wild species including *L. hirsutum* were found to be susceptible. Therefore, it can be confirmed that the mechanism of resistance in *L. hirsutum* is external. The resistance factor can be successfully introgressed into cultivated tomato yielding fertile progenies. Leaf hair density (trichome density) character could be associated with the TYLCV resistance in *L. hirsutum*. Random DNA primers selected could be used in bulk segregation analysis to search for molecular markers for resistance.

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