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Original Article

Resistance to *tomato yellow leaf curl virus* -Thailand isolate (TYLCTHV-[2]) and markers loci association in BC_2F_1 population from a cross between Seedathip 3 and a wild tomato, *Solanum habrochaites* 'L06112' clone no.1

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Abstract

The BC_2F_1 population from a cross between wild tomato, *Solanum habrochaites* 'L06112' and recurrent susceptible variety, Seedathip 3 was investigated for a resistance to *Tomato yellow leaf curl Thailand virus* (TYLCTHV-[2]). Five stem cuttings from each of the 196 lines were inoculated with TYLCTHV-[2] using viruliferous whiteflies as the inoculation vector. Disease response was recorded weekly intervals and scored for three weeks according to the severity of the symptoms. The presence of TYLCTHV-[2] was confirmed by enzyme-linked immunosorbent assay (ELISA) at three weeks after inoculation. ELISA readings showed a normal distribution for the BC_2F_1 population ranging from 0.055 (resistant) to 0.930 (susceptible). DNA samples from BC_2F_1 population were analyzed for genes and markers association; *Ty-2* on chromosome 11 using AVRDC primer number 11-090.0 (TG105A), and *Ty-3* on chromosome 6 using marker C2_At3g11210. Results showed that *Ty-2* was lost during stepwise selection at BC_1F_1 generation, while *Ty-3* showed no relationship to marker C2_At3g11210 and the amount of virus detected from ELISA reading. This indicated that the TYLCTHV-[2] resistant phenotype response in the BC_2F_1 population neither came from *Ty-2* nor linked to the *Ty-3* gene.

Keywords: breeding, disease resistance, TYLCV, TYLCTHV-[2], wild tomato introgression, *Solanum habrochaites*

1. Introduction

Tomato yellow leaf curl disease is transmitted by whiteflies (*Bemisia tabaci*), and it is caused by the *tomato yellow leaf curl virus* (TYLCV). TYLCV is a circular single-stranded DNA with quasi-isomeric particles. Most isolates found are monopartite, while the Thailand isolate (TYLCTHV-[2]) is comprised of two genomes; DNA-A and DNA-B and hence named bipartite. The obvious symptoms are systemic in expression, starting with chlorotic curling of the youngest leaves. Plants infected at an early growth stages show stunting soon after infection and are incapable of producing any kind of quality crop.

The disease can be managed by cultural practices, selecting and planting resistant species to the virus or by managing the whitefly population through insecticides. Since

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frequent insecticide applications are resource costly, both in time and money, breeding for resistance is a desirable approach in order to rectify this serious problem. Introgression of TYLCV resistant genes from wild to commercial species has been done for decades with several species of *Solanum; S. peruvianum*, *S. pimpinellifolium*, *S. habrochaites* and *S. cheesmaniae* (Scott *et al.*, 1995), and in most cases, multiple responsible genes (1 to 5 genes, either recessive or dominant resistance) were found in each species depending upon its geographic origin (Vidavsky and Czosnek, 1998).

Several accessions from *S. habrochaites* were used as donor parents for a source of TYLCV resistance. Hassan *et al.* (1984) found *S. habrochaites* accession 'LA386' was TYLCV-resistant in Jordan, while Ioannou (1985) found that 'LA1777' resistant to TYLCV in Cyprus. In 1998 Vidavsky and Czosnek developed a resistant line by combining 'LA386' and 'LA1777' and found that their new variety resisted TYLCV in Israel.

Previous reports on H24, the cross between *S. habrochaites* f. *glabrarum* accession 'B6013' and *S. lycopersicum* 'Hisar Arun' followed by 4 backcrossing and 2 generations for self-crossing, showed high levels of resistance to viral isolates from Taiwan (TYLCV-TW), and tolerance to TYLCV-SL from Srilanka and TYLCV-Ban3 from India (Zamir *et al.*, 1994). A genetic study for molecular markers revealed the gene responsible for TYLCV resistance was located on chromosome 11 between RFLP markers TG36 and TG393 and named *Ty-2* (Hanson *et al.*, 2000).

In 2007, H24 and several other tomato germplasm were crossed with the Thailand commercial susceptible cultivar, Seedathip3. Parents with their F_1 hybrid were infected with Thailand's viral isolate (TYLCTHV-[2]) and they showed the highest level of resistance with no symptoms to TYLCTHV-[2] in H24, while F_1 progenies showed a moderate resistance (Chomdej *et al.*, 2007).

Because most of Thai commercial cultivars are susceptible to TYLCV, this study has been based mainly on exploiting traits from the wild tomato, *Solanum habrochaites* accession no.L06112 clone no.1 (L06112-C1) into a domestic cultivar, Seedathip3. Screening for resistance and studying the distribution in BC_2F_1 of tomato population was validated using enzyme-linked ommunosorbent assay (ELISA). In addition, phenotypes for disease response were analyzed for a relationship to Ty-2 and Ty-3 markers for further breeding programs.

2. Materials and Methods

2.1 Plant materials

One hundred and ninety six lines from advanced breeding no. B-7 at BC_2F_1 generation from the cross between *S. habrochaites* accession 'L06112' clone no.1 and *S. lycopersicum* 'Seedathip3' were planted and kept for multiplication in an insect-proof greenhouse at Kasetsart University, Kamphaeng Sean Campus, Nakhon Pathom, Thailand for three months. Replications were done by five stem cuttings of each tomato line, including donor and recurrent parents, selected resistant lines at F_1 and BC_1F_1 generation from the previous research (Chomdej *et al.*, 2008).

2.2 Plant inoculums and whitefly cultures

A susceptible tomato cultivar, Seedathip3, was used as a source of TYLCTHV-[2] culture and inoculums were propagated by cleft grafting from the infected plants to one month old seedlings in a glasshouse. Infected plants developed full symptoms in two weeks after being grafted. Whiteflies were reared on eggplants in a separate greenhouse to avoid contamination.

2.3 Whitefly-mediated inoculation

Whiteflies were subjected to inoculated plants and allowed to feed for a week, and then five cuttings of tested plants (BC_2F_1, BC_1F_1, F_1, L06112, and Seedathip3) were caged together with viruliferous whiteflies. Shoots of infected plants were scored at weekly intervals for three weeks starting at the first week after inoculation according to disease incidence and disease severity (Figure 1).

2.4 Detection of TYLCTHV-[2]

Viral accumulation was quantified from infected plants three weeks after inoculation using ELISA technique (Gajanandana *et al.*, 2002). New shoots from each tested plant were ground in extraction buffer in the ratio of 1:250 g/ml and coated to the 96-well ELISA plate. The plate was incubated

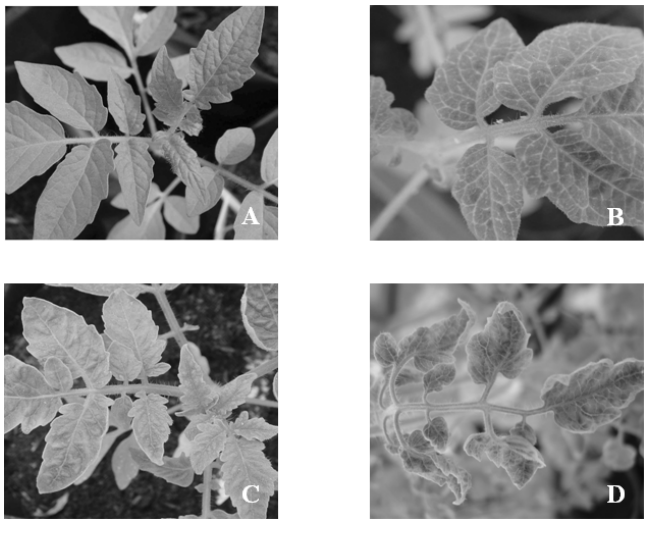

Figure 1. TYLCV symptom rating was determined using scale of 0 to 3 where 0 = No symptoms (A), 1 = Mild, light yellowing along the leaf margins but no curling (B), 2 = Moderate, foliar yellowing and curling (C) and 3 = Severe, leaf curling, puckering and plant stunning (D)

at 37°C for two hours and washed three times at 5 min intervals with phosphate buffer, containing 0.05% tween-20 (PBST). The plate was blocked with 2%BSA in PBST and then incubated for one hour at 37°C. After washed three times with PBST, a specific monoclonal antibody was added to each well and the plate was incubated at 37 °C for 90 min. The plate was washed again for three times at 5 min. intervals and a goat anti-mouse antibody conjugated with alkaline phosphate was added to each well and then incubated for another 90 min at 37°C. A substrate solution containing *p*-nitrophenyl phosphate in diethanolamine buffer was added to each well and incubated at 37°C around 30 min for color reaction. The reaction was stopped by adding 3N NaOH in each well and was measured as absorbance (optical density = OD at 405 nm on an ELISA plate reader, Multiskan EX, Themolabsystems OY, Finland). Tested plants were evaluated for infection by comparing to a positive threshold value (PTV). Plants that had an ELISA reading three times higher than a negative control was considered as infected (Sutara *et al.*, 1986).

2.5 Markers linkage analysis

The DNA of 196 lines from BC_2F_1 population was extracted according to the methods described by Fulton *et al.* (1995). Samples were amplified by polymerase chain reaction (PCR) technique with forward and reverse specific primers; (TG105A for *Ty-2* and C2_At3g11210 for *Ty-3*) linked to resistant allele at loci on chromosome 11 and 6, respectively (Hanson, personal communication; Garcia and Maxwell 2007; Ji *et al.*, 2007; Sol Genomic Network). Amplification conditions started at 35 cycles of 2 min at 94°C DNA denaturation, 30 sec at 50°C annealing for marker TG105A and 60°C annealing for marker C2_At3g11210. Then the extension period was 1 min at 72°C for both markers. After that amplified DNA products were cut with restriction enzymes, *taq*I for marker TG105A at 65°C for 3-4 hours and *Hinf* I for marker C2_At3g11210 at 37°C for 3-4 hours incubation period. Then the digested fragments were separated in 2% agarose gels. Linkage between markers and phenotypes for TYLCTHV-[2] response were analyzed on BC_2F_1 population by MapQTL® version 4.0 with the Kruskal-Wallis one–way analysis of variance (van Ooijen *et al.*, 2002).

3. Results and Discussion

Even though wild species contain numerous sources of resistances for both insects and diseases (Rick and Chetelat, 1995), gene complexity and linkage drag could be a major obstacle factor for plant breeders. Because the accession no. L06112 from *Solanum habrochaites* and Seedathip3 from *S. lycopersicum* are not closely related, interspecific hybridization only occurred when using Seedathip3 as a female parent (Whankaew *et al.*, 2005). Moreover, the F_1 hybrid still showed paternal self-incompatibility and did not bear fruit at the F_2 generation. Therefore plants at the F_1 generation were backcrossed to the recurrent parent (Chomdej and Chunwongse, 2006; Chomdej *et al.*, 2007).

In previous research, the BC_1F_1 generation was screened for TYLCTHV-[2] resistance and the results showed the top five performance lines were A-1, B-1, B-7, B-10, and O-4, which contained low viral concentrations asymptomatic to mild symptoms in infected tested plants. Fruits from selfing in BC_1F_1 generation were varied in size, shape and color; therefore breeding line no. B-7, which acquired closer characteristics to their recurrent parent, Seedathip3, has been selected for advancements into a further breeding program. Unfortunately, seeds derived from selfing BC_1F_1 generation exhibited very low germination rates, which was less than 50% of the best rate of germination (unpublished data). Consequently another attempt at backcrossing has been proceeded (Chomdej *et al.*, 2008) (Figure 2).

Five stem cuttings in each of the 196 lines from the offspring of B-7 (BC_2F_1 generation) and Seedathip3 were subjected to whitefly inoculation and screened for TYLCTHV-[2] resistance. Symptomatic phenotypes were scored and analyzed. Results have shown segregation in viral responses at BC_2F_1 population, ranging from susceptibility, over tolerance, to resistance. Lines which susceptible to the disease (20% of the tested population) were visually observed and had started expressed moderate to severe symptoms at the second week after inoculation. Infected lines showed extremely accelerated levels of infection at the third week after inoculation with almost ninety percent of the population showing TYLCV symptoms (score $\geq$ 2); yellowing and curling symptoms at the leaf margins with venal chlorosis and puckering as shown in Figure 1.

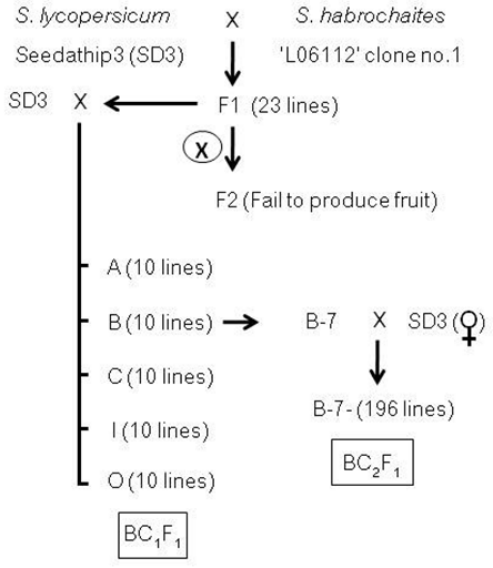

Figure 2. Diagram of a breeding plan; *Solanum habrochaites* accession no. L06112 was crossed to *S. lycopersicum* var. Seedathip3 to produced F_1 generation. The resistant hybrids were backcrossed to a susceptible Seedathip3 to generate BC_1F_1 and BC_2F_1 generation.

The disease severity and viral titer in susceptible lines showed a positive correlation by ELISA detection. Plants containing high values of TYLCTHV-[2] accumulation, also showed moderate to severe disease symptoms (score $\geq$ 2). Only ten out of 196 plants had ELISA reading values lower than the PTV value (0.260) and the average symptom scoring were ranging from 1.4 to 2.4.

ELISA readings showed a normal distribution for the BC_2F_1 population ranging from 0.055 (resistant) to 0.930 (susceptible) (Figure 3). This indicated that the resistance response to TYLCTHV-[2] from 'L06112' is quantitatively inherited and controlled by multiple genes. Several reports have shown similar results that wild tomatoes, which were crossed to introgress genes for resistance, appeared to be controlled by more than one gene depending on plant source and background (Vidavsky and Czosnek, 1998; Hanson *et al.*, 2000; Ilana *et al.*, 2009; Ji *et al.*, 2009).

Tomato commercial line, H24 was analyzed for a resistant gene *Ty-2* from a cross to a wild species, *S. habrochaites* accession B6013. This resistance was mapped to the long arm region of chromosome 11 between marker TG393 and TG36 (Kaloo and Banerjee, 1990; Hanson *et al.*, 2000). AVRDC primer number 11-090.0 (Hanson, personal communication), later published as TG105A in 2007 by Garcia and Maxwell, was used to identify the *Ty-2* gene in our population. The results showed allele was lost over selection at BC_1F_1 generation that was initially heterozygous (Figure 4). However resistance to the disease is still remaining at the BC_2F_1 generation. This indicated that the TYLCTHV-[2] resistance found in our BC_2F_1 generation derived from B-7 was not accountable for the *Ty-2* gene.

Ji *et al.* (2007) reported that *Ty-3* allele is located on chromosome 6 between markers cLEG-31-p16 and TG1079. This resistant gene was mapped using wild tomato; *Solanum chilense* accessions LA1932, LA2779 and LA1938, with crossed to cultivated tomato *S. lycopersicum*. In our study, a CAPS marker, C2_At3g11210 adjacent to the *Ty-3* loci at 24.5 cM distance was probed to identify association to phenotype at BC_2F_1 generation. The phenotypic and genotypic data were analyzed with single marker analysis to identify marker linked to TYLCTHV-[2] resistance gene. Results from a Chi-square test showed a segregating population in the marker C2_At3g11210 detection in the 196 tested lines from BC_2F_1 with a segregation ratio of 1:1 (102:94 lines). In addition, the ELISA mean values of 102 plants that were detected by the marker C2_At3g11210 and 94 plants that did not link to the marker were 0.531088 and 0.502606, respectively. These numbers showed no correlation between *Ty-3* marker and tomato lines that expressed resistance at a statistical significance ($p<0.05$) based on the Kruskal-Wallis one–way analysis of variance in MapQTL® 4.0.

Further confirming in detection of Ty-3 marker showed no association to resistance by comparing two groups of DNA samples (extremely resistant and susceptible) in the BC_2F_1 population. Nucleotide polymorphism was found between parents' designated L06112 carrying *Ty-3* gene, which resisted to monopartite TYLCV and *Tomato mottle virus* (ToMoV) in genus begomovirus. Tested tomatoes that showed low levels of viral concentration at BC_2F_1 generation (BC_2F_1–resistant) were detected for Ty-3 marker at the ratio of 7/10 plants, while high levels of viral titer (BC_2F_1–susceptible) showed 6/10 plants (Figure 5). It indicated that *Ty-3* was not responsible for the TYLCTHV-[2] resistance. However, this gene appears to be useful practically for broad spectrum improvement purposes and would be incorporated into our breeding program. In the ongoing research genome scan using more markers is underway to localize QTLs controlling TYLCTHV-[2] resistance.

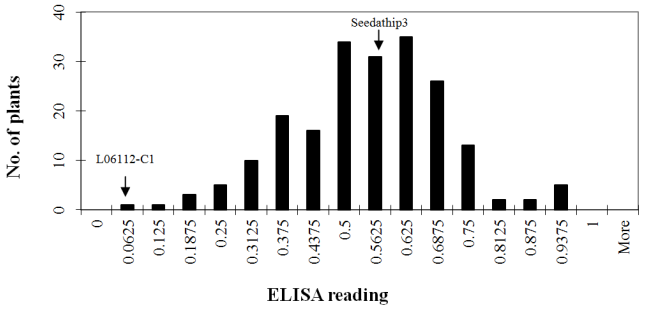

Figure 3. ELISA readings showed a normal distribution of response to TYLCTHV-[2] resistance in BC_2F_1 population from a cross between Seedathip3 and a wild tomato, *Solanum habrochaites* 'L06112' clone no.1 at three weeks post inoculation.

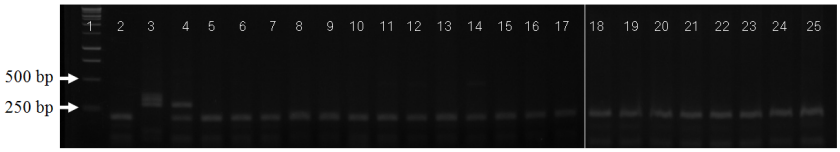

Figure 4. PCR fragments with the *Ty-2* locus primers (TG105A) Lane 1: 100-bp marker (Promega Corp.); Lane 2: Seedathip3 (susceptible), Lane 3: L06112-C1 (resistant to TYLCTHV-[2]); Lane 4: line B (F_1, resistant to TYLCTHV-[2]); Lane 5: line B-7 (BC_1F_1 resistant to TYLCTHV-[2]); Lane 6-15: BC_2F_1 population (extremely resistant to TYLCTHV-[2]); Lane 16-25: BC_2F_1 population (extremely susceptible to TYLCTHV-[2]).

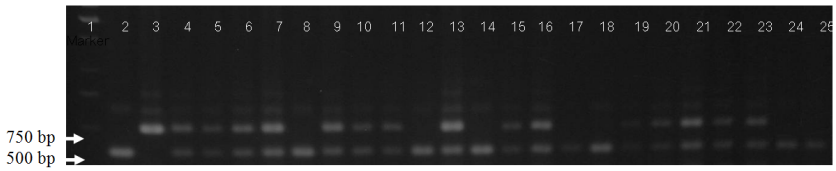

Figure 5. PCR fragments with the *Ty-3* locus primers (C2_At3g11210) Lane 1: 100-bp marker (Promega Corp.); Lane 2: Seedathip3 (susceptible), Lane 3: L06112-C1 (resistant to TYLCTHV-[2]); Lane 4: B (F_1, resistant to TYLCTHV-[2]); Lane 5: B-7 (BC_1F_1 resistant to TYLCTHV-[2]); Lane 6-15: BC_2F_1 population (extremely resistant to TYLCTHV-[2]); Lane 16-25: BC_2F_1 population (extremely susceptible to TYLCTHV-[2]).

4. Conclusion

In conclusion, progress in breeding traits from wild species is a slow process, primarily because of the complex genetics of the plants. Multiple genes are involved in the resistance, but TYLCTHV-[2] resistance found in our BC_2F_1 population derived from a cross between *Solanum habrochaites* 'L06112' clone no.1 and Seedathip3 was not affected by the *Ty-2* and *Ty-3* gene. However, both genes will be a great benefit for further breeding programs attempting to broaden the base of resistance for TYLCV around the world. From this study we believe that TYLCTHV-[2] resistance was quantitatively inherited from donor parents and controlled by a concert of multiple genes, either major or minor.

Acknowledgements

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