Resistance to *Cucurbit yellow stunting disorder virus* in Cucumber

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ABSTRACT

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Three hundred accessions of *Cucumis sativus*, including wild cucumbers, land races, traditional cultivars, and breeding lines, were evaluated under natural-infection conditions in order to identify potential sources of resistance to *Cucurbit yellow stunting disorder virus* (CYSDV). Although 100% of the susceptible control plants showed typical yellowing symptoms induced by CYSDV, another 24 *C. sativus* accessions showed partial or total absence of yellowing symptoms. In contrast, when CYSDV inoculation was carried out under controlled conditions, only two (A1 and A2) of these 24 accessions showed resistance to the virus. The nature of the resistance found in A1 and A2 plants was characterized by studying the pattern of virus accumulation and symptom development under controlled infection conditions, and by analyzing the possible nonpreference of *Bemisia tabaci* for these accessions under free-choice conditions. There was a delay in the establishment of the CYSDV infection in A1 and A2 plants which was evident shortly after inoculation and in apical leaves of the plants at long times after inoculation. Symptom severity was also less for A1 and A2 than for a susceptible control at 8 and 12 weeks postinoculation. Thus, delayed viral infection appeared to be associated with decreased symptom severity in A1 and A2 plants. Our results also showed nonpreference for plants of the A2 accession by *B. tabaci*, the CYSDV vector.

Additional keywords: antixenosis, closterovirus, melon, systemic movement

Whitefly-transmitted viruses cause severe economic losses in many crops in warm and temperate areas. In particular, the whitefly-transmitted crinivirus *Cucurbit yellow stunting disorder virus* (CYSDV; family *Closteroviridae*) (13,14) has been described in many areas of the world (1,3,4,5,7,10,21). In Spain, CYSDV was first detected in 1992 (3), and it has since become the prevalent virus in cucurbit crops of southeastern regions, where frequently up to 100% of plants are infected. CYSDV induces interveinal chlorotic spots in mature leaves that may enlarge and eventually coalesce, resulting in yellowing of the entire leaf except for the veins, which remain green (3,21). Yellowing symptoms are accompanied by

substantial reductions in fruit yield and quality, and therefore, this virus is of significant economic importance. CYSDV virus particles are flexuous rods 750 to 800 nm in length (8). The virus encapsidates two molecules of single-stranded (ss) positive-sense RNA designated RNA 1 and RNA 2 (2). The genetic variability of CYSDV has been studied locally (12) and globally (15,16). These studies showed that CYSDV variability is unusually low compared with other members of the family *Closteroviridae*, although it is still possible to differentiate two genetic groups, the so-called Eastern and Western CYSDV subpopulations (15,16).

Control of CYSDV is currently based on preventive cultural practices and chemical treatments against its vector, both with limited success. The use of cultivars that carry genetic resistance is a potential option for control of the disease induced by CYSDV, although information concerning any sources of resistance to this virus is scarce (9,11). In particular, no resistance to CYSDV has been reported in cucumber. In this work, we have evaluated under natural-infection conditions 300 accessions of cucumber in order to identify potential sources of resistance to CYSDV. The nature of the apparent resistance found in two accessions was characterized by

studying the pattern of virus accumulation and symptom development over time after controlled inoculation, and also by analyzing whitefly preference for these accessions in a free-choice experiment.

MATERIALS AND METHODS

Germplasm. Three hundred cucumber (*Cucumis sativus* L.) accessions were provided by Dr. Carravedo (Banco de Germoplasma Hortícola de Zaragoza, Zaragoza, Spain) and Seminis Vegetable Seeds (Almería, Spain) and included wild cucumbers, land races, traditional cultivars, and 12 breeding lines of the seed company. Geographical distribution of accessions (except breeding lines) was as follows: 112 accessions from Europe (7 from Central Europe and 105 from Spain), 55 accessions from Japan, 41 accessions from Central Asia, 38 accessions from the Far East and India, 22 accessions from the Middle East and Egypt, 13 accessions from America, and 7 accessions from Indonesia.

Resistance screening under natural inoculation conditions. The 300 accessions were grown in a polyethylene greenhouse at the Seminis Vegetable Seeds Ibérica at El Ejido (Almería, Spain) in an artificial substrate (coco peat) commonly used for cucumber production in southern Spain, using drip irrigation and fertilization. The sidewall windows of the greenhouse remained open during the test in order to permit free entry of whiteflies to the plants. The planting plot size was 0.66 m² per plant. Two replications with a randomized layout of the 300 accessions were used; 8 plants of each accession were evaluated in each replication. The susceptible control was cucumber cv. Toril (Semini Iberica, Spain), a commercial hybrid cultivar known to be susceptible to CYSDV.

Resistance screening under controlled inoculation conditions. Twenty-four accessions were also inoculated under controlled conditions and analyzed 15 days postinoculation (PI) to detect CYSDV. Ten plants of each accession were evaluated. The CYSDV isolate used (CYSDV-AILM) was obtained from a naturally infected melon plant collected in Almería (Spain) in 1997 and maintained in La Mayora on melon cv. Amarillo through serial transmission by *Bemisia tabaci* (2). Non-viruliferous *B. tabaci* colonies were main-

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tained on healthy melon plants (cv. Amarillo) in an insect-proof glasshouse (20 to 32°C, 45 to 85% relative humidity) with artificial light supplemented as needed. Whitefly inoculation of test plants with CYSDV was performed essentially as described by López-Sesé and Gómez-Guillamón (9). Briefly, groups of adult whiteflies were given a 24-h acquisition access period by feeding on virus source melon plants (cv. Amarillo). Following the acquisition access period, whiteflies were given a 48-h inoculation access period on test plants using 60 whiteflies per plant. Plants used for inoculation were 2 to 3 weeks old, with the first true leaf fully expanded. Virus acquisition and inoculation were performed using clip-on cages placed abaxially onto plant leaves (9). Cages were polyethylene cylinders 5 cm long and 3 cm in diameter. After the inoculation access period, whiteflies were carefully removed and the plants were treated with insecticide. Fifteen days after inoculation, the leaves on which the whiteflies had fed and laid eggs were removed. The same protocol was used for controls except that nonviruliferous whiteflies were used.

CYSDV was detected by RNA:RNA hybridization in tissue prints of petiole cross-sections. The petioles of the first leaf above the inoculated leaf were cut with a razor blade, and cross-sections were immediately blotted onto positively charged nylon membranes (Roche Diagnostics GmbH, Mannheim, Germany). CYSDV

RNA on membranes was detected by molecular hybridization using an RNA digoxigenin-labeled probe (11,17).

Pattern of CYSDV accumulation and symptom development in putative sources of resistance. Accessions used for the virus accumulation studies were: A1 (Seminis Vegetable Seeds, Almería, Spain), A2 (Banco de Germoplasma Hortícola de Zaragoza, Zaragoza, Spain), and cucumber cv. Toril (Seminis Vegetable Seeds), the susceptible control. Twenty-four plants of each genotype were inoculated with CYSDV-AILM and maintained in an insect-proof glasshouse for 3 weeks after inoculation. Twelve plants at each of two locations were subsequently transplanted to a commercial greenhouse in El Ejido (Almería, Spain) or in Estación Experimental “La Mayora” (Málaga, Spain). Four plants of each genotype at Almería and Málaga were chosen at random and sampled at 5, 8, and 12 weeks PI. The leaves sampled for each plant were as follows: at 5 weeks PI, leaves 3, 7, 11, and 15; at 8 weeks PI, leaves 11, 15, 18 to 20, and 23 to 25; at 12 weeks PI, leaves 11, 15, 18 to 20, 23 to 25, 26 to 28, and 30 to 32. The inoculated leaf was numbered 0. Quantitative dot-blot hybridization was used to measure the amount of CYSDV RNA in leaf samples (11,17). In this same experiment, average symptom severity was estimated at 8 and 12 weeks PI for each genotype. Symptom severity in individual plants was evaluated based on a 0 to 5

severity index scale specifically designed for each observation time point (Tables 1 and 2).

Free-choice test. The five genotypes used were as follows: A1, A2, and Toril, as described above; cucumber cv. Panda (Seminis Iberica, Spain), a commercial hybrid cultivar derived from A1; and melon (*Cucumis melo* L.) TGR-1551, an accession known to be resistant to *B. tabaci* (19). Twelve plants from each genotype with two to three true leaves were distributed at random in a structure covered with an insect-proof net (mesh 50 threads per cm) inside an insect-free greenhouse. Whiteflies for the test were collected randomly from the colonies reared on melon and introduced into clip-on cages in groups of 60 whiteflies per cage. To guarantee that an initial distribution of whiteflies was sufficiently uniform, a cage with whiteflies was placed near each plant in the experiment, and cages were simultaneously opened and whiteflies released. After 1 week, the number of whiteflies present on the third true leaf of each plant was recorded (20).

Data analysis. The results of the virus accumulation and free-choice experiments were subjected to analysis of variance (one-way ANOVA), and for cases in which the ANOVA results indicated significance, the Fisher protected least significant difference (LSD) test (18) was calculated for means comparisons. Data from the virus accumulation experiment were previously transformed ($\log[x + 1]$). Analyses were carried out using the package SPSS v11 for Windows (SPSS Inc., Chicago).

Table 1. Symptom severity index scale at 8 weeks postinoculation

Value	Description
5	Complete yellowing of leaves 1 to 4 ^a . Interveinal chlorosis of leaves 5 to 12-13. Chlorotic mottling of leaves 13-14 to 18-19. Three or 4 apical leaves with no symptoms ^b .
4	Complete yellowing of leaves 1 and 2. Interveinal chlorosis of leaves 3 to 8-9. Chlorotic mottling of leaves 9-10 to 15-16. Six or 7 apical leaves with no symptoms.
3	Yellowing of leaves 1 and 2 except for the veins, which remain green. Interveinal chlorosis of leaves 3 to 6-7. Chlorotic mottling of leaves 7-8 to 11-12. Ten or 11 leaves with no symptoms.
2	Interveinal chlorosis of leaves 1 to 5-6. Chlorotic mottling of leaves 6-7 to 9-10. Twelve or 13 leaves with no symptoms.
1	Interveinal chlorosis of leaves 1 and 2. Chlorotic mottling of leaves 3 to 5-6. Sixteen or 17 leaves with no symptoms.
0	No symptoms.

^a Inoculated leaf was numbered 0.

^b Average number of leaves was 22.

Table 2. Symptom severity index scale at 12 weeks postinoculation

Value	Description
5	Complete yellowing of leaves 1 to 18-19 ^a . Interveinal chlorosis of leaves 19-20 to 25-26. Chlorotic mottling of leaves 26-27 to 30-31. Four or 5 apical leaves with no symptoms ^b .
4	Complete yellowing of leaves 1 to 13-14. Interveinal chlorosis of leaves 14-15 to 21-22. Chlorotic mottling of leaves 22-23 to 28-29. Six or 7 apical leaves with no symptoms.
3	Complete yellowing of leaves 1 to 4-5. Interveinal chlorosis of leaves 5-6 to 18-19. Chlorotic mottling of leaves 19-20 to 24-25. Ten or 11 leaves with no symptoms.
2	Complete yellowing of leaves 1 and 2. Interveinal chlorosis of leaves 3 to 11-12. Chlorotic mottling of leaves 12-13 to 16-17. Eighteen or 19 leaves with no symptoms.
1	Interveinal chlorosis of leaves 1 to 5-6. Chlorotic mottling of leaves 6-7 to 9-10. Twenty-five or 26 leaves with no symptoms.
0	No symptoms.

^a The inoculated leaf was numbered 0.

^b The average number of leaves was 35.

RESULTS AND DISCUSSION

Resistance screenings. Of the 300 cucumber accessions screened for resistance to CYSDV under natural infection conditions, 24 accessions were selected because of partial or total absence of yellowing symptoms. One hundred percent of the susceptible control plants showed typical yellowing symptoms induced by CYSDV, indicating that inoculum pressure was very high. These 24 accessions were subsequently tested under controlled-inoculation conditions. Fifteen days after inoculation, CYSDV was detected in all plants, but hybridization signal was low for plants of the A1 and A2 accessions (data not shown), suggesting that virus accumulation in A1 and A2 plants was reduced compared with controls. All plants of these two accessions were symptomless at 15 days PI, whereas approximately 90% of the plants of the other accessions and 100% of the plants of the susceptible control showed yellowing symptoms typically induced by CYSDV. These results suggested that accessions A1 and A2 were putative sources of partial resistance to this virus. Data also suggested that complete resistance to CYSDV is uncommon in cucumber, as it is in melon (9,11). The

finding that only two accessions showed virus resistance after controlled inoculation of the 24 candidates may be the consequence of a number of factors. Among these, nonpreference of the whiteflies for accessions that showed no symptoms under natural infection conditions is possible. Because of the high genetic stability of CYSDV in this geographical area (12), differences among virus isolates are unlikely.

Pattern of CYSDV accumulation and symptom development in putative sources of resistance. The distribution of CYSDV in accessions A1, A2, and a susceptible control was studied over a period

of 12 weeks after inoculation. At 5 weeks PI, the viral RNA content was highest for the basal leaves of susceptible control plants, although differences with middle and apical control leaves were not significant (Fig. 1A). The viral RNA content in leaves of the A1 and A2 plants was lower than in the control plants, especially in the apical leaves, where the virus was almost undetectable (Fig. 1A, leaf 11). However, at 8 weeks PI, the amount of viral RNA in leaves of A1 and A2 plants did not differ significantly from that in the susceptible controls (Fig. 1B). At 12 weeks PI, there were significant differences in the amounts of viral RNA between plants of the A1 and

A2 accessions and the susceptible controls, but only in apical leaves (Fig. 1C, leaves 23 to 31). In basal leaves, there were no significant differences between the three accessions (Fig. 1C, leaves 11 to 19). Therefore, CYSDV infection in A1 and A2 whole plants may be delayed in comparison to the susceptible controls. This partial resistance to CYSDV could be due to restriction of the virus movement in the vascular system of the plants because of the presence of preexisting barriers and/or to an induced response of the plants that prevents high levels of virus accumulation, as has been proposed for the resistance to CYSDV found in a melon accession (11).

The A1 and A2 plants were asymptomatic during the entire cultivation period under natural inoculation conditions. However, in the experiment to determine the pattern of CYSDV accumulation in these accessions, systemic yellowing symptoms were observed in A1 and A2 plants 3 weeks after controlled inoculation. To measure the severity of the symptoms that CYSDV can induce in A1 and A2 plants after controlled inoculation, mean symptom severity was calculated for each accession based on a 0 to 5 severity index scale (Tables 1 and 2). Symptom severity was lower for A1 and A2 plants than for plants of the susceptible control at both 8 and 12 weeks PI (Table 3). Thus, the observed delay in the establishment of a systemic infection in A1 and A2 plants may be associated with decreased symptom severity compared with susceptible controls. Probably, reduced symptom severity is a direct consequence of reduced viral content, although it has been shown in a few other instances that both traits may be independently controlled (6). This is an aspect that remains to be elucidated.

Free-choice test. Data from experiments under natural and controlled inoculation conditions initially appeared contradictory. Under natural inoculation, CYSDV-induced symptoms were never observed in A1 and A2 plants during the

Fig. 1. Accumulation of *Cucurbit yellow stunting disorder virus* (CYSDV) RNA in cucumber accessions A1, A2, and Toril at **A**, 5 weeks, **B**, 8 weeks, and **C**, 12 weeks postinoculation. Numbers on the horizontal axis represent the relative position of the leaf sampled with respect to the inoculated leaf (inoculated leaf was considered leaf no. 0). Numbers on the vertical axis correspond to the logarithmic transformation of the average amounts of viral RNA expressed in ng/ μ g of total plant RNA. Each bar corresponds to the average viral RNA content of leaf samples from four independent plants. Amount of viral RNA was estimated by quantification of hybridization signals in dot-blot of total RNA extracted from leaf tissues.

Table 3. Average symptom severity index of plants of three cucumber accessions inoculated under controlled conditions with *Cucurbit yellow stunting disorder virus* (CYSDV)^a

Accessions ^b	Time postinoculation	
	8 weeks	12 weeks
A1	2.33 $\pm$ 0.62	3.50 $\pm$ 0.85
A2	2.25 $\pm$ 0.75	2.60 $\pm$ 0.46
Toril	4.08 $\pm$ 0.29	4.50 $\pm$ 0.37

^aUsing a symptom severity index scale of 0 to 5, a value was assigned to each plant at each observation time and average symptom severity index, and deviations, were calculated for each accession; 24 plants per accession, grown in two commercial greenhouses, were evaluated in this experiment.

^bAccessions A1 and A2 were putative sources of resistance to CYSDV; Toril (*Cucumis sativus* cv. Toril) was included as a control.

Table 4. Average number of whiteflies on the third true leaf of plants of four cucumber and one melon accession 7 days after insect release^a

Accession ^b	Whitefly average number
A1	17.92 ab ± 11.91
A2	7.08 a ± 4.31
Toril	25.75 b ± 7.74
Panda	30.25 b ± 17.45
TGR-1551	7.00 a ± 3.38

^aTwelve plants (2 to 3 true leaves) of each genotype were distributed at random in a structure covered with an insect-proof net. Sixty insects per plant were released, and 1 week later the number of whiteflies present on the third true leaf of each plant was counted.

^bAccessions A1 and A2 were putative sources of resistance to *Cucurbit yellow stunting disorder virus* (CYSDV); Toril (*Cucumis sativus* cv. Toril) was included as a control; Panda (*C. sativus* cv. Panda) is a commercial hybrid cultivar derived from A1; and *Cucumis melo* TGR-1551 was included due to its known resistance to *Bemisia tabaci* (19).

entire cultivation period, whereas under controlled inoculation conditions, symptoms were evident 3 weeks PI. This could be due to a number of reasons, including nonpreference of the whiteflies for these accessions under free-choice conditions. We estimated the number of whiteflies present on each plant 1 week after insect release in plants of accessions A1, A2, and cv. Toril. In the same experiment, we included plants of a commercial hybrid cultivar (cv. Panda) derived from A1 and one melon accession (TGR-1551) resistant to *B. tabaci* (19). The lowest numbers of whiteflies were observed on A2 plants together with the resistant melon, which differed significantly ($P \leq 0.01$) from the values obtained for cvs. Toril and Panda (Table 4). The average number of whiteflies for accession A1 was smaller but not significantly different from that of the susceptible control (Table 4). Therefore, it appears that accession A2 has an antixenotic effect on the insects.

In conclusion, we have identified and characterized two cucumber accessions that appear to carry traits of partial resistance to CYSDV and, in one case, nonpreference to the CYSDV vector. How these traits are inherited remains to be determined, but our initial analyses suggest that the resistance to CYSDV in these

accessions is controlled by more than one recessive gene (J. Abad, *unpublished*). Given the importance of CYSDV and the scarcity of sources of resistance to CYSDV in cucumber, the virus resistance found in accessions A1 and A2 and the nonpreference to whiteflies identified in accession A2 should be introgressed into commercial cultivars.

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