Accumulation of Tomato Yellow Leaf Curl Virus DNA in Tolerant and Susceptible Tomato Lines

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ABSTRACT

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Four tomato lines (M-60, M-54, 1970, and 2950), an F_1 hybrid (TY-20) tolerant to tomato yellow leaf curl virus (TYLCV), and the susceptible cultivars 1651, 1630, 614-1, and 199-2 were compared for accumulation of viral DNA in TYLCV-infected plants. DNA was analyzed by either alkaline transfer or dot spot hybridization using a cloned viral DNA as a probe. Results obtained with either procedure indicated that in all tested lines viral DNA accumulation peaked at 12–15 days after inoculation. At this time, accumulation of viral DNA in the tolerant genotypes was 10–50% lower than in the susceptible ones. Viral DNA then decreased up to 50% in both the tolerant and the susceptible lines, and it remained at that level. Differences in viral DNA among the tested genotypes were found throughout the experiment, but differences were most pronounced when DNA accumulation was at a peak. Viral DNA accumulation was positively correlated to symptom severity. Thus, this approach may provide a useful means of selecting TYLCV-tolerant genotypes.

Tomato yellow leaf curl virus (TYLCV) is a whitefly-transmitted geminivirus that causes devastating damage to tomato (*Lycopersicon esculentum* Mill.) crops in Israel and in several Mediterranean countries (3,12,13). All commercially grown tomato cultivars worldwide are susceptible to TYLCV. Chemical control of the virus vector, *Bemisia tabaci* Gennadius, is inefficient because of resistance of the insect to insecticides (5). Breeding for resistance to TYLCV seems to be a promising approach for control. Resistance to TYLCV has been reported in wild relatives of *L. esculentum*. This has led to initiation of several breeding programs (8–10,15,22). Tolerance to TYLCV was introduced from *L. peruvianum* (L.) Mill. to a cultivated tomato by Pilowsky and Cohen, yielding a determinant commercial F_1 hybrid (TY-20) adapted for growth in Israel (16,17). The present study was initiated to compare the accumulation of viral DNA and the development of symptoms in tomato lines tolerant and susceptible to TYLCV and to evaluate this procedure for selection of tolerant genotypes.

MATERIALS AND METHODS

Virus source. The TYLCV used in this study is from the original culture described by Cohen and Harpaz (3). The virus was maintained in *Datura stramonium* L., and inoculation was carried out by whiteflies.

Tomato lines. The TYLCV-tolerant material used was F_1 hybrid TY-20 (16) and the breeding lines M-54, M-60 (16), 1970, and 2950. These lines are characterized by milder symptoms and better vegetative growth compared to the susceptible lines, 1651, 1630, 614-1, and 199-2, which are of different genetic backgrounds. Line 1651 was chosen as a representative susceptible line on the basis of former results showing high similarity in viral DNA accumulation and in symptom severity among these four genotypes. The tolerant material was selected on the basis of differences in symptom severity. In the field, TYLCV-infected plants of TY-20 yielded about 60 tha compared to 3 tha in the susceptible cultivars (17).

Whitefly maintenance and plant inoculation. *B. tabaci* was raised on cotton plants (*Gossypium hirsutum* L.) grown in muslin-covered cages held in an insectary greenhouse at 26–32 C. Female adult whiteflies were used for inoculation. Insects were given a 48-hr acquisition access on TYLCV-infected *D. stramonium* L. source plants, after which they were allowed a 48-hr inoculation access on tomato test plants. Plants were inoculated at the two-leaf stage, using 40 whiteflies per plant as described elsewhere (15,16). Following inoculation, plants were sprayed with senprotatherine (Smash) and held in an insect-proof greenhouse for further analysis.

TYLCV symptom severity rating. Symptom severity was evaluated on the following scale: 1 = slight yellowing of the leaflets margins of the apical leaf; 2 = yellowing of the leaflet ends, reduction in leaf size, and slight stunting of plants; 3 = leaf yellowing, decrease in leaf size, plant stunting, and upward twisting of the leaf; 4 = yellowing of leaf, a substantial reduction in leaflet size, severe leaf curl, and plant stunting; and 5 = very severe plant stunting, erect branches, leaves with very small leaflets exhibiting severe interveinal chlorosis, and upward cupping to assume an erect position.

Plant sampling and extraction of total DNA from TYLCV-infected tomato plants. Tissue samples for viral DNA analysis were collected at midday from the two top leaves (2). The amount of viral single-stranded DNA (ssDNA) obtained by the procedure of Dellaporta et al (4) was approximately 10 times greater than that obtained by the CTAB (hexadecyltrimethylammonium bromide) extraction method (6). Both procedures yielded similar amounts of viral double-stranded DNA (data not shown). Thus, the procedure of Dellaporta et al was used for DNA extraction. Following isopropanol precipitation, pellets were resuspended in 500 μl of 50 mM Tris and 10 mM EDTA and brought to a final concentration of 0.5 μg/μl as determined by spectrophotometry.

Alkaline transfer blotting of total DNA from TYLCV-infected tomato plants. The DNA extracted from plant samples was separated in 1% agarose gels by electrophoresis in Tris-acetate buffer (pH 7.8) and then blotted onto nylon membranes by alkaline transfer (20).

Preparation of sap extracts for dot spot hybridization. Leaf disks (6 mm in diameter) were removed from the top leaf of the tested plants and crushed individually in microfuge tubes in 15 μl of 0.4 N NaOH. Tubes were centrifuged at 10,000 *g* for 2 min, and 3 μl of the supernatant of each sample was spotted on nylon membranes for dot spot hybridization assays.

Probe preparation. Specific TYLCV probes representing a full length virus genomic unit (1) were produced in a riboprobe system. The viral clone in KS^+ bluescript plasmid (Stratagene, La Jolla, CA) served as a template for the production of in vitro ^{32}P-labelled viral transcipts complementary to the virion DNA. The transcription reaction was carried out by T3 RNA polymerase according to the manufacturer's instruc-

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tions (Stratagene). A fragment (1,150 bp) of tomato ribosomal RNA gene cloned in pUC8 was provided by E. Lifschitz (Haifa University, Haifa, Israel). This cloned DNA was ^{32}P-radiolabelled by a random priming kit (Boehringer GmbH, Mannheim, Germany) and served to probe for total plant ribosomal DNA as an internal standard in hybridization experiments.

Hybridization tests. Following alkaline transfer of DNA, membrane blots were washed in 1 × SSC and hybridized in the presence of 50% formamide according to the membrane manufacturer's instructions (Gelman, Rehovot, Israel). At the end of the hybridization procedure, membranes were washed once in 1 × SSC and 0.1% SDS at room temperature and twice in 0.1 × SSC and 0.1% SDS at 60 C. Membranes were exposed to an X-ray film in the presence of an intensifying screen for a period of 4–16 hr in order to obtain sufficient viral and plant ribosomal DNA signals.

Quantification of TYLCV DNA accumulation in infected tomatoes. Samples of total DNA from the compared tomato lines were blotted on nylon membranes as described above. Following hybridization with the viral-specific probe, membranes were accurately aligned with their autoradiograms over a light table, and the relevant pieces of membrane, each carrying a DNA band or spot, were excised. Each piece of membrane was counted (in cpm) individually in Insta-Gel II (Hewlett-Packard, Palo Alto, CA) by a scintillation counter (model LS1701, Beckman Instruments, Fullerton, CA).

Quantification of plant genomic DNA in the tested samples. The probe derived from the cloned ribosomal gene fragment was used to detect plant ribosomal DNA, which served as an internal standard for total genomic DNA. In this case the upper part of each blot carrying the plant genomic DNA bands was rehybridized with the ribosomal probe under the conditions described above. Radioactivity of plant genomic DNA in each sample was measured (in cpm) by scintillation counting as described above.

Assessment of viral DNA content in the tested samples. In experiments in which the alkaline transfer blotting procedure was used, standardization of the amount of viral DNA in each tested sample was obtained by calculating the ratio of viral DNA cpm to plant ribosomal DNA cpm. In some of these experiments the amount of DNA was calculated in absolute units according to Lorens et al (11). The minimum amount of detected vi: ıl DNA was 3 pg/μg plant DNA. In cases in which dot spot hybridization was used, comparisons among lines were based on determination of viral DNA content in standard multiple leaf disk samples. The viral DNA content in the compared samples was expressed in cpm.

Statistics. Data analysis was performed with the Statistical Analysis System (SAS Institute, 1982), using general linear models and repeated measure analysis of variance. Treatments were compared by Duncan's multiple range test at the 0.05 protection level.

RESULTS

Quantitative assessment of TYLCV-DNA. As indicated in Figure 1, the viral riboprobe hybridized specifically with the viral DNA but did not react with the plant genomic DNA. Viral DNA in infected tissues was detectable by hybridization of alkaline transfer blots as soon as 3 days after inoculation. At this time, viral DNA reached about 15 pg/μg plant DNA in the susceptible line 1651 and about 3 pg/μg plant DNA in the tolerant line M-60.

The results of spectrophotometric determination of the total DNA content in the samples were inconsistent. Therefore, a tomato ribosomal DNA probe was used as an internal standard for the detection and quantification of plant DNA (Fig. 1). Dot spot hybridization was found to be an alternative and simpler method to detect viral DNA. The results (Fig. 2) were consistent with those obtained by the alkaline transfer blotting analysis (Fig. 1).

Time course of TYLCV DNA accumulation in tolerant and susceptible tomato lines. To compare viral DNA

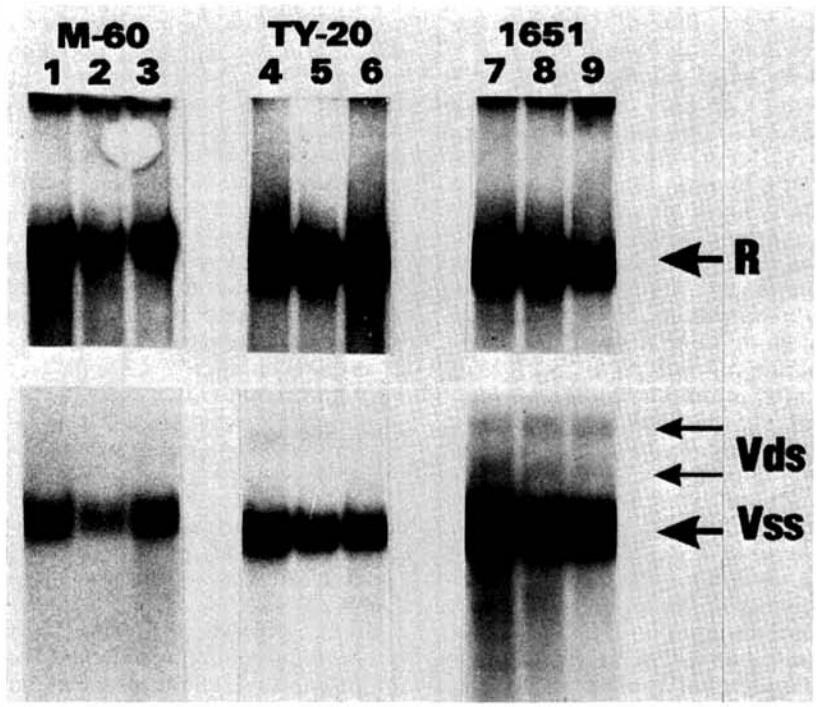

Fig. 1. Accumulation of TYLCV DNA in tolerant and susceptible tomato plants, with alkaline transfer blot analysis at 13 days after inoculation. Total DNA from the two top leaves of three plants of the tolerant lines M-60 (lanes 1-3) and TY-20 (lanes 4-6) and of the susceptible line 1651 (lanes 7-9) was fractionated on agarose gel and blotted to a nylon membrane. Hybridization with a TYLCV-specific probe (bottom) and a tomato ribosomal DNA-specific probe (top) was carried out sequentially on the same blot. The viral replicative double-stranded (Vds, thin arrows) and single stranded (Vss) DNA forms, as well as the plant ribosomal DNA (R), are indicated.

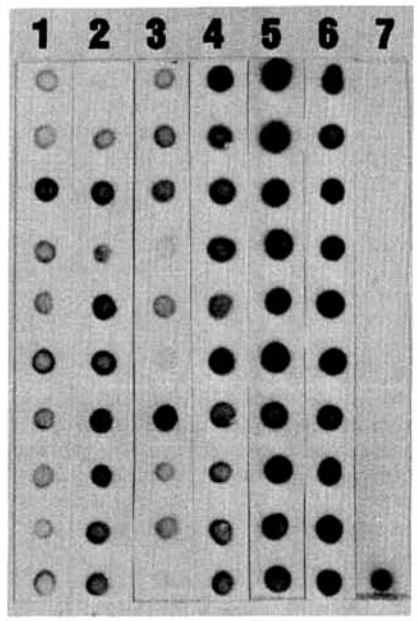

Fig. 2. Accumulation of TYLCV DNA in tolerant and susceptible tomato plants, with dot spot analysis at 13 days after inoculation. Total DNA from leaf disks from the top leaves of 10 plants of the tomato lines M-54 (lane 1), M-60 (lane 2), 2950 (lane 3), TY-20 (lane 4), 1970 (lane 5), and 1651 (lane 6) was spotted on a nylon membrane for dot spot hybridization with a TYLCV-specific probe; dot spot hybridization with DNA from nine uninfected plants (lane 7) and a positive viral DNA control (lane 7, bottom).

accumulation in tolerant and susceptible lines, six plants each of M-60, TY-20, and 1651 were inoculated with TYLCV. DNA was isolated from the two upper leaves of each plant at six time intervals after inoculation and analyzed using alkaline transfer blots (Fig. 3) . In the three lines, DNA accumulation peaked 13 days postinoculation (DPI), just prior to symptom appearance. At this time, the accumulation of viral DNA in the tolerant M-60 and TY-20 was only 23 and 49%, respectively, of that in the susceptible line 1651. Subsequently, viral DNA amount decreased, and differences among lines were less distinct. To simplify DNA analysis and enable mass testing of the six lines, we checked the possibility of employing dot spot hybridization instead of the more time-consuming DNA extraction and alkaline transfer blotting procedures. A representative experiment (of five) is shown in Figure 2. To analyze the kinetics of viral DNA accumulation, 10 plants of each line were sampled at six time points during 38 DPI. Each sample was extracted separately and analyzed for viral DNA by the dot spot hybridization procedure. Uninfected tomato plants of line 1651 served as a control. As indicated in Figure 4, and in agreement with the alkaline transfer blot hybridization analysis, viral DNA accumulation peaked at 13 DPI in all lines. Subsequently, the viral DNA decreased, and the lower amount remained stable until the end of the experiment (38 DPI).

The accumulation of viral DNA in the five tolerant lines during the experiment was significantly lower than in the susceptible line 1651. The DNA accumulation in the tolerant lines M-54, M-60, and TY-20 at 13 DPI reached 28, 45 and 67%, respectively, compared with the susceptible line 1651. Using Duncan's multiple range test at the 0.05 protection level, statistically significant differences among the five tolerant lines were found only between 13 and 17 DPI. The 5% LSD for the compared lines at any DPI was 2713.

Timing of symptom appearance and correlation between symptom severity and accumulation of TYLCV DNA. As indicated in Table 1, the time required for symptom appearance was different for each line and consistent with its tolerance to TYLCV, as shown in Figures 3 and 4. Our previous observations indicated that whitefly-mediated inoculation with 20 insects or less per plant resulted in variable symptom appearance within a given line. This information was used to produce variability within the tested lines and enable us to analyze correlation between DNA level and symptom severity within, as well as between, lines.

Symptom severity was indexed at 17 DPI and reconfirmed 5 days later. At that time, differences in symptom severity were most pronounced. Viral DNA was quantified at 12, 15, and 19 DPI. The highest correlation between symptom severity and viral DNA content was found within the individual lines at 15 DPI. At that time, the correlation coefficient ranged between 0.70 and 0.91, at $P < 0.05$ (Fig. 5). The mean values of symptom scores against the mean DNA accumulation for each line at 12, 15, and 19 DPI were found to have correlation coefficients of 0.77, 0.72, and 0.68, respectively. These data were highly significant at $P < 0.0001$ (Fig. 5). A decreased correlation coefficient value of 0.46 was observed for the means at 38 DPI.

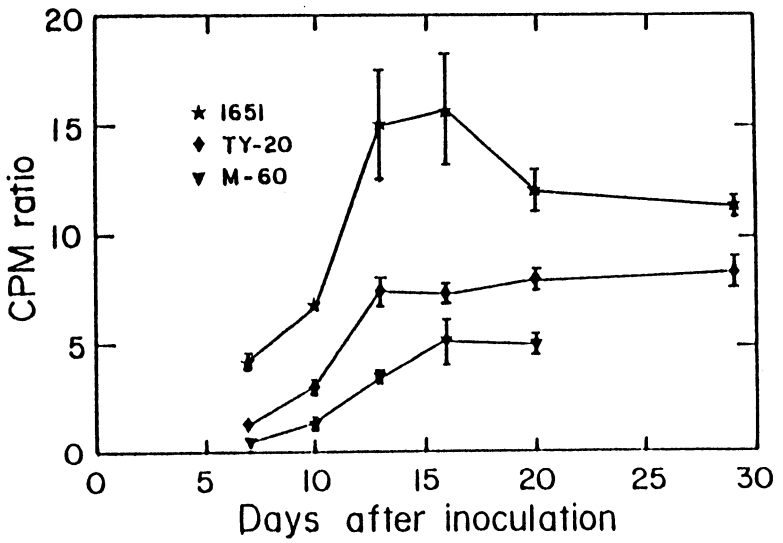

Fig. 3. Time course of TYLCV ssDNA accumulation in tolerant and susceptible tomato plants. Total DNA from six plants of each of the three lines in Figure 1 was extracted at the indicated time points after inoculation and analyzed by alkaline transfer blot hybridization as described (Fig. 1). The hybridization bands corresponding to the viral ssDNA (Vss) and the plant rDNA (R) of each plant were excised from the membrane, and their radioactivity was monitored. The mean Vss/R cpm ratio for each line was plotted against its corresponding time of DNA extraction, with SE bars on both of its sides.

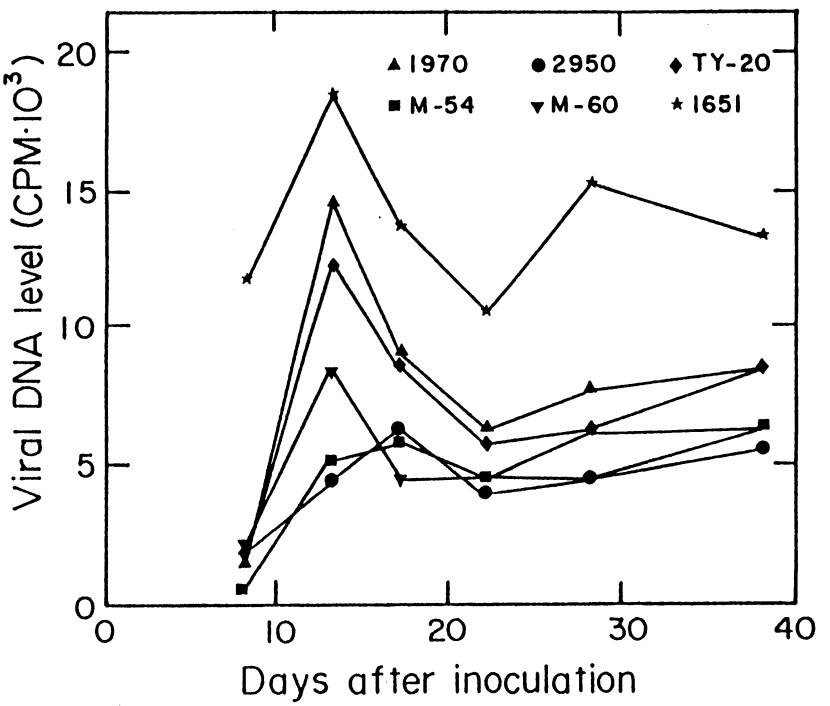

Fig. 4. Time course of TYLCV DNA accumulation in tolerant and susceptible tomato plants. Total DNA from 10 individual plants of each line was extracted at the indicated time points after inoculation and analyzed by dot spot hybridization as described in Figure 2. The 10 resultant hybridization spots of each line were excised from the membrane, and the mean radioactivity (in cpm) was plotted against its corresponding time of DNA extraction. Statistically significant differences among the five tolerant lines were found only between 13 and 17 days after inoculation. Statistical analysis was done by Duncan's multiple range test at the 0.05 protection level. 5% LSD for the compared lines at any day after inoculation is 2,713.

Table 1. Percentage of tomato plants showing symptoms at different days after inoculation

Tomato line	Days after inoculation		
	17	22	38
1651	100	100	100
2950	81	100	100
1970	81	100	100
M-54	81	100	100
M-60	50	75	100

DISCUSSION

Breeding for disease resistance is often restricted by a lack of reliable and efficient screening procedures. Resistant or tolerant individuals may be identified by symptomatology or serology, but in some situations specific antisera are not available, and quantitative assessment of visual symptoms is not always precise and reproducible. This situation may lead to contradictory results attributing different resistance levels to the same genetic source, as is the case for TYLCV (10,11,22). In such cases, molecular procedures may be used for more accurate detection and quantification of plant viruses. The use of nucleic acid probes to estimate virus titers in plant tissues as a tool for selection of resistant genotypes has been described elsewhere (7,11,19).

In this report we describe a procedure for quantitative determination of TYLCV DNA in tomato plants. A cloned TYLCV DNA served as a specific probe for detection of viral DNA by the dot spot hybridization procedure, using crude extracts of plant leaf disks. This procedure offers a rapid and reliable method for comparison of relative viral DNA accumulation in TYLCV-infected tomato plants. The results obtained by this procedure were as reliable as those obtained by alkaline transfer blot hybridization, with the advantage of this method being less tedious and costly. This procedure is also superior to the squash-blot technique (14,22), which gave us nonspecific signals and was found unsuitable for quantitative comparisons of DNA content. The time course of TYLCV DNA accumulation shown here is consistent with a previous study in which a TYLCV-susceptible line was tested (2), and it resembles that shown for another geminivirus, bean golden mosaic virus (21).

In this study the maximum differences in viral DNA content among tolerant and susceptible lines occurred about 2 wk after inoculation (2–4 days before symptom appearance), when the viral DNA was at the highest accumulation. The decrease in viral DNA content that followed the peak accumulation was accompanied by smaller differences among the tested lines (Figs. 3 and 4). A positive correlation was established between TYLCV symptom severity and viral DNA level. This fits the general concept that higher titer of virus means more damage to the plants (18). Our results and observations indicate that tolerance against TYLCV is characterized by lower accumulation of viral DNA, milder symptoms, and delayed symptom appearance. The mechanism of tolerance in the tested tomato lines is still unknown, but the lower accumulation levels of viral DNA may reflect either a reduction in the capacity of the virus to replicate and/or spread in the infected tissue. This study shows that relative accumulation of TYLCV DNA in tomato plants under standard conditions may serve as a reliable tool to complement visual scoring of symptoms severity in the selection of tolerant tomato genotypes.

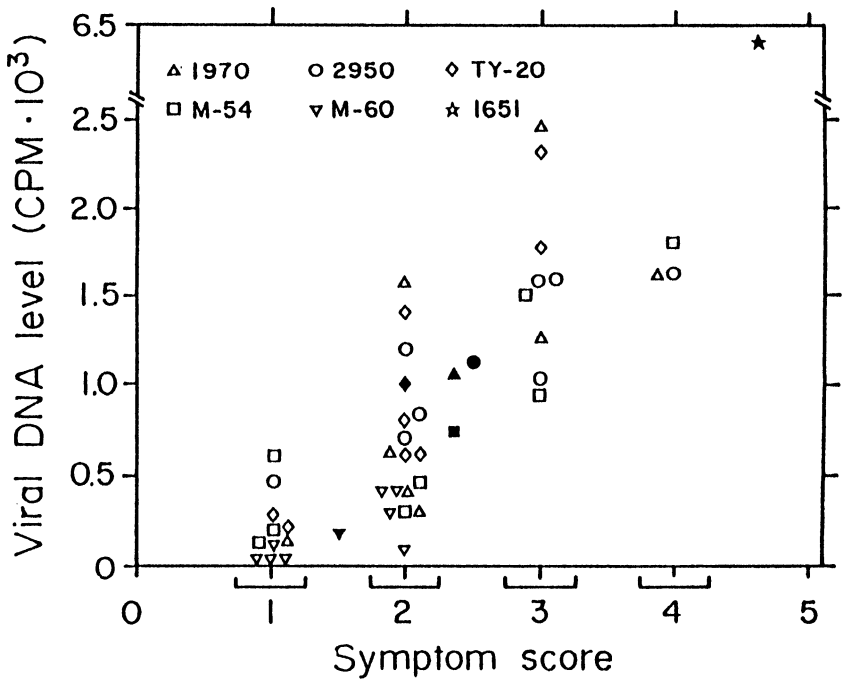

Fig. 5. Correlation between viral DNA level and TYLCV disease symptoms. Symptoms were recorded on the 17th day after inoculation according to a severity rating of 1-5 (see Materials and Methods). The symptom score of each of eight individual plants in each line was plotted against the corresponding viral DNA content that was determined 15 days after inoculation (open symbols). Correlation coefficients within the six individual lines ranged between 0.70 and 0.91, $P < 0.05$. A correlation between symptom severity and viral DNA level among the lines was established by plotting the mean value of symptom score against the mean DNA content for each line (solid symbols). The susceptible line 1651 is represented by its mean value only (solid asterisk) . The correlation coefficient of line mean values was 0.72, $P < 0.0001$.

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