Tomato Plants Transformed with the Inhibitor-of-Virus-Replication Gene Are Partially Resistant to *Botrytis cinerea*

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ABSTRACT

Loebenstein, G., Rav David, D., Leibman, D., Gal-On, A., Vunsh, R., Czosnek, H., and Elad, Y. 2010. Tomato plants transformed with the inhibitor-of-virus-replication (IVR) gene were partially resistant to *Botrytis cinerea*. This resistance was observed as a significant reduction in the size of lesions induced by the fungus in transgenic plants compared with the lesions on the nontransgenic control plants. This resistance was weakened when plants were kept at an elevated temperature, 32°C, before inoculation with *B. cinerea* compared with plants kept at 17 to 22°C prior to inoculation. Resistance correlated with the presence of IVR transcripts, as detected by reverse transcription-polymerase chain reaction. This is one of the few cases in which a gene associated with resistance to a virus also seems to be involved in resistance to a fungal disease.

The local lesion response in tobacco cultivars containing the N gene, one of the most striking resistance phenomena, has been associated with the presence of a protein with antiviral properties named inhibitor-of-virus-replication (IVR) (1,2,11–13,20,21,27). Transformation of susceptible *Nicotiana tabacum* cv. Samsun nn plants with the IVR-encoded NC330 clone driven by the Cauliflower mosaic virus 35S promoter resulted in the expression of variable resistance to both Tobacco mosaic virus (TMV) and *Botrytis cinerea* in a number of plants. However, even in the most resistant lines, resistance to TMV and *B. cinerea* was variable among self-crossing progenies, even after four generations, and not all of the plants were resistant (2).

Gray mold caused by *B. cinerea* is an important disease of numerous greenhouse and field crops throughout the world and infects many vegetable, ornamental, and horticultural species (5,6,9). It attacks flowers, fruits, leaves, and stems of tomato plants grown in greenhouses. The pathogen primarily infects leaves, but lesions are also found on the stems of infected plants (5,6,25). This disease is a major problem everywhere tomatoes are grown in high-wire cropping systems and it is often the reason why a crop is harvested earlier than planned. The disease also regularly causes losses in unheated and partially heated crops grown in Israel during the winter (24,26). Chemical control remains an important means of managing gray mold, although it is impeded by the development of pathogen resistance. Site-specific benzimidazole, dithiocarbamate, and N-phenylcarbamate fungicides have been used intensely since the early 1970s, and this use has led to the rapid selection of resistant strains of fungal pathogens in Israel and other countries (8,19). In recent years, some new botryticides with different modes of action have been introduced worldwide and in Israel. However, Korolev et al. (18) found resistance to the new botryticides, fenhexamid, fludioxonil, and pyrimethanil, in populations of *B. cinerea*. Studies in various crops have yielded a wide range of information regarding the conditions that promote *B. cinerea* infection in greenhouse crops (4). The temperature range for gray mold development in greenhouse crops is 12 to 30°C (16), with an optimal range of 15 to 20°C; although the pathogen is active even at temperatures as low as 0°C (8,9). Initial infection occurs on live or dead plant tissue (5). Programs for breeding resistance to *B. cinerea* are in progress and partial resistance has been identified in accessions of wild relatives of tomato, such as *Solanum habrobra* (10), as well as in transgenic tomatoes expressing a wheat oxalate oxidase (29). To date, none of these resistant lines have reached commercial fields.

IVR inhibited virus replication in protoplasts derived from both local lesion-resisting resistant Samsun NN and systemically responding susceptible *N. tabacum* cv. Samsun nn plants. IVR has also been obtained from the intracellular fluid of hypersensitive tobacco leaves infected with TMV and from induced-resistant tissue (1). A cDNA (NC330) was isolated from an expression library prepared from induced-resistant (uninfected) leaf tissue of Samsun NN. The NC330 expressed a 21.6 kDa IVR-like protein in *E. coli* that was recognized by an IVR antibody, which exhibited antiviral properties in biological tests (1). Here, we report that transformation of tomato plants with the IVR-encoding NC330 cDNA resulted in strong resistance to *B. cinerea*. This may be a first step toward the development of tomato plants that are resistant to *B. cinerea*.

MATERIALS AND METHODS

Plant transformation. The tomato (*Solanum lycopersicum*) cv. VF36 was used in this study. This tomato cultivar is not a commercial variety, but it was found to be very suitable for transformation (22). Tomato cv. VF36 seeds were germinated on Nitsch medium (24). Cotyledon explants were excised from 10-day-old seedlings, cut from both sides (proximal and distal, 2/3 of the cotyledon remaining), and cultured overnight on a 1-day-old feeder layer consisting of 3 ml of a 7-day-old tobacco suspension culture, plated on Murashige and Skoog (MS) agar medium and overlaid with sterile Whatman filter paper. *Agrobacterium tumefaciens* strain EHA 105, containing the binary vector pGA 492 with the IVR gene (the cDNA clone of tobacco origin), was

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grown overnight in 2× YT medium containing rifampicin (30 mg/liter), kanamycin (50 mg/liter), and tetracycline (10 mg/liter) and diluted to optical density at 600 nm (OD_{600nm}) = 0.3 with MS medium. Acetylsyringone (100 µM, final concentration), was added to the diluted bacterial suspension. Cotyledons were submersed in the bacterial suspension for 15 to 30 min, and then blotted on sterile Whatman No. 1 paper and placed back on the feeder plates for 45 h co-cultivation in the dark. The explants were then transferred to selective regeneration medium (solidified MS medium minerals (23) with Nitsch vitamin medium containing carbenicillin [400 mg/liter], kanamycin [70 mg/liter], and zeatin [1 mg/liter]) (23). Regenerated explants were transferred to the same fresh medium biweekly. Green shoots, 1 to 3 cm tall, were separated from calli and transferred to Nitsch medium containing carbenicillin (150 mg/liter), kanamycin (50 mg/liter), and indole butyric acid (1 mg/liter). Five rooted plants (T0) were transplanted to soil and transferred to a greenhouse. These five plants were allowed to flower and their offspring (T1) were screened for the presence of the IVR gene.

**Verification of the presence of the IVR gene.** Polymerase chain reaction (PCR) was used to evaluate the presence of the NPTII and NC300 genes. Specific primers were used. Among the offspring of NC, 4 to 5 plants out of 10 tested positive for both NPTII and NC300. Among the offspring of NC, 3 to 5 plants out of 10 contained both genes. IVR expression was evaluated using reverse transcription (RT)-PCR. A good correlation was observed between the lines that tested positive for the presence of the genes in the PCR analysis and those testing positive in the RT-PCR analysis (Fig. 1). The plants that tested positive for the gene in the PCR analysis were allowed to flower and daughter plants (T2) were raised from their seeds and tested for resistance to *B. cinerea*. Subsequently, we tested for resistance to *B. cinerea* among three additional generations of self-crossed, resistant plants derived from the initial transformants.

**Botrytis infection and disease evaluation.** *B. cinerea* (isolate Bc116) (14) was cultured on potato dextrose agar (Difco Laboratories, Detroit, MI) in petri dishes incubated at 20°C. Conidia were harvested from 14-day-old cultures by agitating small pieces of agar, bearing mycelia and conidia, in a glass tube. The suspension was then filtered through cheesecloth. The concentration of conidia was determined using a hemacytometer and adjusted to 5 × 10^7 cells ml⁻¹. Glucose (0.1%) was added to the final aqueous conidial suspension, together with 0.1% KH₂PO₄. Ten leaflets were removed from each tomato plant, placed on a tray and inoculated with 10-µl drops of the conidial suspension (2 drops per leaflet). Following inoculation, the trays were covered with plastic and inoculated at 20°C and 95% relative humidity, with 12 h of illumination each day. A similar procedure was followed with whole plants. Ten leaves on each whole tomato plant were inoculated as described for the detached leaflets. The severity of the resultant necrotic lesions was determined according to the scale described below. The diameter of a 10-µl drop on a tomato leaf was 5 mm, corresponding to an area of 19.6 mm². Disease developed gradually and was first visible 3 to 5 days after inoculation. The first symptoms were small necrotic lesions that covered only part of the area originally covered by the drop of the *B. cinerea* suspension. The diameter of a *B. cinerea* lesion on a typical tomato leaf at 9 to 10 days after inoculation was approximately 9 mm, corresponding to an area of 63.5 mm². A 12-mm-diameter lesion (corresponding to an area of 113 mm²) was used as a reference point and assigned a value of 100%. A pictorial scale of lesion sizes was used, including the following relative sizes: 0, 1, 2, 5, 10, 20, 40, 75, and 100%. Lesion size (a measure of disease severity) was determined for each lesion using this pictorial scale (12). Disease reduction was calculated according to the formula: 100 – 100 × A/B, in which A = disease level in the treatment and B = disease level in the untreated control.

**Statistical analyses.** Data in percentages were arcsine-transformed before further analysis. Disease severity and the calculated area under disease progress curve (AUDPC) data were analyzed using analysis of variance and Fisher’s protected least significant difference test. The statistical analyses were conducted using JMP software (SAS Institute, Cary, NC).

**RESULTS**

**Disease evaluation in IVR-transformed and control plants.** Marked resistance to *B. cinerea* was observed in several of the transgenic T2 lines. In a preliminary experiment with detached leaflets from transgenic NC5 tomato plants, the severity of the disease reached 3.0%. Meanwhile, in nontransgenic control plants, disease severity was 17.4% (82.7% disease reduction). In another experiment with detached leaflets, the AUDPC 6 days after inoculation was 62.9% × days in nontransgenic control plants; whereas in the transgenic plants, it was 29.0% × days (53% disease reduction). When whole plants were tested between 30 and 50% of the plants of two lines showed significant resistance to *B. cinerea*. In parallel experiments, a good correlation was found between disease severity at 6 days after inoculation (average 1.0% compared with 40.7% in the nontransgenic control) and the presence of the IVR sequence as detected by PCR and RT-PCR (Fig. 1).

**TABLE 1. Severity of Botrytis cinerea infection on leaves of inhibitor-of-virus-replication-transformed tomato plants**

<table>
<thead>
<tr>
<th>Tomato line</th>
<th>Evaluation time dpi</th>
<th>AUDPC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>6 May 2007 experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.0</td>
<td>20.0</td>
</tr>
<tr>
<td>NC3-3</td>
<td>0.4</td>
<td>5.6</td>
</tr>
<tr>
<td>NC3-6</td>
<td>2.1</td>
<td>6.1</td>
</tr>
<tr>
<td>NCS-3</td>
<td>5.2</td>
<td>10.5</td>
</tr>
<tr>
<td>NCS-5</td>
<td>1.1</td>
<td>15.0</td>
</tr>
<tr>
<td>NCS-7</td>
<td>2.5</td>
<td>4.0</td>
</tr>
<tr>
<td>NCS-9</td>
<td>4.5</td>
<td>15.4</td>
</tr>
<tr>
<td>20 July 2007 experiment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>24.1</td>
<td>63.7 a</td>
</tr>
<tr>
<td>NC3-5</td>
<td>23.6</td>
<td>36.0 b</td>
</tr>
<tr>
<td>NCS-7</td>
<td>20.0</td>
<td>49.6 b</td>
</tr>
<tr>
<td>NCS-7</td>
<td>23.7</td>
<td>39.4 b</td>
</tr>
<tr>
<td>NCS-5</td>
<td>1.0</td>
<td>25.1 c</td>
</tr>
<tr>
<td>NCS-6</td>
<td>20.3</td>
<td>35.6 b</td>
</tr>
</tbody>
</table>

Plants were inoculated with a 5 × 10⁵ cells ml⁻¹ suspension supplemented with 0.1% glucose and 0.1% KH₂PO₄. Disease severity is expressed as percentage of symptomatic leaf area, area under disease progress curve (AUDPC, days × % symptomatic leaf area). Numbers in columns followed by a common letter are not significantly different according to Fisher’s protected least significant difference test.

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**Fig. 1.** Detection of inhibitor-of-virus-replication transgene in transformed plants by polymerase chain reaction (PCR) and confirmation of the transcrip
tion of the transgene by reverse transcription (RT)-PCR. Total DNA and RNA from three transgenic plants (1t, 2t, and 3t) from line NC3 were used as templates for A, PCR and B, RT-PCR analyses. The NC330 plasmid (Pl) served as a positive control and genetic material from nontransgenic plants (non-t) was used as a negative control.
Representative results from several T2 lines are summarized in Table 1 and results from an additional experiment are presented in Figure 2. A marked decrease in disease severity was observed in almost all of the transgenic plants (Table 1; Fig. 2). By 2 weeks after inoculation, the control nontransgenic plants had lost all of their leaves and died, while many of the transgenic plants were still alive (Fig. 3). One of the transgenic plants survived for a full month after inoculation. Resistance to B. cinerea in the three subsequent generations was only partial and did not exceed 50% of the plants.

**Sensitivity of resistance to a higher temperature.** In additional experiments conducted during the summer, when greenhouse temperatures reached 30°C and more, no resistance to B. cinerea was observed. Following this observation, controlled experiments were performed to evaluate resistance in transgenic tomato at a higher temperature, in comparison to resistance in plants kept at lower temperatures.

Four to five weeks after transplanting, transgenic and nontransgenic control tomato plants were kept at 32°C for 10 to 12 days before being inoculated with B. cinerea. Control plants were kept at 17 to 22°C before inoculation. As shown in Table 2, the transgenic plants that were kept in a cooler environment were resistant to B. cinerea, while the level of resistance was markedly reduced in the plants kept at the higher temperature. Similarly, nontransgenic plants that were incubated at the higher temperature were significantly more susceptible to the disease later on. Disease reduction due to the presence of the IVR gene was 46.0 to 73.1% after incubation at 17 to 22°C and much lower (32.6 to 40.0%) in the plants that had been previously incubated at 32°C (Table 2).

In an additional experiment, uninfected transgenic and nontransgenic tomato plants were kept at 32 and 22°C to evaluate the effect of temperature on IVR expression. As shown in Figure 4,

<table>
<thead>
<tr>
<th>Temperature during incubation without pathogen</th>
<th>Disease severity at day 10 (%)</th>
<th>Disease reduction by IVR presence (%)</th>
<th>AUDPC during days 0 to 10 (% × days)</th>
<th>Disease reduction by IVR presence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17–22°C</td>
<td>41.4 ± 4.9 b</td>
<td>73.1</td>
<td>139.0 ± 7.0 b</td>
<td>76.0</td>
</tr>
<tr>
<td>30–35°C</td>
<td>65.2 ± 5.1 a</td>
<td>32.6</td>
<td>137.2 ± 14.6 b</td>
<td>40.0</td>
</tr>
</tbody>
</table>

*Plants were incubated at 20 ± 1°C and 95 ± 3% relative humidity. Treatments in each column followed by the same letter are not significantly different according to Fisher’s protected least significant difference test. + and – indicate the presence or absence of the IVR gene as determined using polymerase chain reaction.
RT-PCR clearly detected IVR transcript expression in the uninfected transgenic tomato plants kept at the different temperatures (22 and 32°C). Similar data were obtained using semi-quantitative RT-PCR (data not shown). Therefore, we can conclude that the exposure of the transgenic plants to high temperatures before their inoculation did not lead to depletion of the IVR mRNA.

**DISCUSSION**

We reported previously that tobacco plants transformed with a cDNA clone encoding an IVR-like protein were partially resistant to TMV and *B. cinerea* (2). Using an antibody raised against recombinant IVR, we were able to detect this protein in transgenic plants. However, we did not detect the protein in the control plants. TMV resistance was suppressed when the transgenic plants were kept at 32 to 34°C (2). Similarly, the production of IVR by infected protoplasts and by intact Samsun NN plants was almost completely suppressed at 35°C (11).

Transformation of tomato plants with the IVR gene also resulted in resistance to *B. cinerea*. In the future, this approach may become a useful tool for breeding resistant tomato cultivars. As with IVR-transformed tobacco, the observed resistance was only partial and did not exceed 50% of the plants. A similar variation in resistance levels has also been observed in transgenic Arabidopsis plants expressing a gene that increases tolerance to drought and salt stress (17). The lesions on the transgenic tomato plants were significantly smaller than those on the nontransgenic control plants. Similar to the IVR-transformed tobacco, the resistance in the transformed tomato plants was also sensitive to higher temperatures. When plants were kept at 32°C before they were inoculated, their resistance was markedly reduced, though not abolished. Although *B. cinerea* is sensitive to temperature and exposure of cucumber plants to temperatures as high as 30°C prior to their infection has been shown to result in more severe disease (7), the IVR-transgenic tomatoes exposed to temperatures of 30 to 36°C were still partially resistant (Table 2). When plants were kept at 20°C following inoculation with *B. cinerea*, the resistance mechanism was reactivated, though only to a limited extent. The sensitivity of the resistance to high temperatures resembles the behavior of TMV in hypersensitive tobacco. In that case, the virus moves systemically when plants are subjected to temperatures above 30°C; while at lower temperatures, the virus remains localized within the lesion area. Transferring uninfected transgenic tomatoes to the warmer environment did not inhibit the transcription of the gene, as observed by RT-PCR. Apparently, it is one or more of the later stages of gene expression, such as the production or degradation of the proteins involved in the resistance, that are affected by the higher temperatures.

The variation in resistance to *B. cinerea* among transgenic plants of the same line could be explained as follows. The transgene copy number was not completely characterized in IVR-tomato. Therefore, differential segregation of the transgene could be subject to various levels of RNA silencing; more copies are known to induce gene silencing in an epigenetic process based on a posttranscriptional gene-silencing mechanism (28).

The ability of the IVR gene to induce resistance to Botrytis is one of the very few reported instances in which R genes that confer resistance to a virus also confer resistance to a fungal pathogen, though genes from bacteria are known to induce resistance to fungi, including *B. cinerea* (15). In a similar situation, a viral R gene cloned from Arabidopsis that confers resistance to Turnip crinkle virus and the yellow mosaic strain of Cucumber mosaic virus also confers resistance to the fungus *Peronospora parasitica* (3). It will be interesting to see whether transformation of plants with the IVR gene will lead to resistance to other fungal pathogens or nematode pests.

**LITERATURE CITED**