Pathogen-derived Resistance to Tomato Spotted Wilt Virus in Transgenic Tomato and Tobacco Plants

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ABSTRACT. This study was undertaken to remedy significant yield losses in commercial tomato (Lycopersicon esculentum Mill.) and tobacco (Nicotiana tabacum L.) production caused by tomato spotted wilt virus (TSWV). One of the possible sources of resistance can be incorporation into the host plant of a viral nucleoprotein (N) gene by Agrobacterium-mediated transformation. Twelve primary transformants of tomato and 141 of tobacco were analyzed for the expression of the N gene and for resistance to the TSWV infection. The tests have demonstrated that transgenic plants were protected against virus infection irrespective of whether or not they contained detectable levels of the translational product.

Tomato spotted wilt virus (TSWV) causes one of the most severe diseases affecting tomato and brings about significant yield losses. At present, TSWV is spread all over the world and under diverse environmental conditions infects more than 850 plant species belonging to over 82 botanical families, including such important crops as tomato, tobacco, pepper (Capsicum annuum L.), potato (Solanum tuberosum L.), and lettuce (Lactuca sativa L.) (Goldbach and Peters, 1994; Prins and Goldbach, 1998). TSWV is a thrips-transmitted RNA virus, classified as genus Tospovirus in family Bunyaviridae (Francki and Hatta, 1981; Wijkamp et al., 1993). It was reported first in Australia in 1906 (Sakimura, 1962) in Europe (Smith, 1932), and in Poland (Jankowski et al., 1963), gradually becoming one of the major threats to fruit production under greenhouse conditions (Kaminska and Korbin, 1994).

The virus genome consists of three single-stranded linear RNA molecules: L, M, and S, which form pseudo-circular particles tightly complexed with the viral 28.8 kDa nucleoprotein (N). The L RNA [8.9 kilobases (kb)] is of negative polarity, while M (5.4 kb) and S (2.9 kb) RNAs are of ambisense polarity. The S RNA encodes the viral N protein in viral complementary sense (de Haan et al., 1990). The management of the TSWV disease requires chemical protection, culture practices and development of resistant varieties. The sources of genetic resistance in the genus Lycopersicon Mill. are not always effective, especially under strong infection pressure (Rosello et al., 1996). Since 1991, several reports have confirmed that engineered pathogen-derived resistance can be exploited successfully for TSWV by expressing its N gene in transgenic plants (Gielen et al., 1991; Kim et al., 1994; MacKenzie and Elis, 1992; Pang et al., 1992; Stoeva et al., 1998; Ulltzen et al., 1995). Further studies revealed that the transcript of the nucleoprotein gene mediates the resistance to the homologous and very closely related isolates of TSWV (de Haan et al., 1992; Pang et al., 1993; Vaira et al., 1995), while the nucleoprotein protection mechanism accounts for a broader-spectrum resistance that extends to distantly related tospoviruses although at a very low level (Pang et al., 1993; Vaira et al., 1995). Pang et al. (1996) reported on the protection against TSWV in transgenic lettuce expressing the nucleoprotein gene of the lettuce isolate of the virus via the N gene silencing mechanism or via a high level of accumulation of the transgenic N protein.

In this report we will show a high level of resistance to the TSWV infection obtained by the transformation of tomato and tobacco plants with the N gene of a Bulgarian TSWV isolate. The inheritance of the pathogen-derived resistance to TSWV was confirmed in two generations. Molecular analysis of the transgene expression pattern indicated that the engineered resistance to TSWV infection could be based on two different mechanisms: RNA-dependent (post-transcriptional silencing) or protein-dependent (protein accumulation). The paper gives some new information about the pathogen-derived resistance to TSWV showing that viral nucleoprotein expressed in plant tissue can cause high level of resistance.
Materials and Methods

PLANT MATERIAL AND PLANT EXPRESSION VECTORS. Three accessions of tomato: a breeding line non-ripening (nor) mutation (Serceżyńska et al., 1998), a breeding line KR, cultivar Potentat, and tobacco cultivar Petit Havana were used in the Agrobacterium-mediated [Agrobacterium tumefaciens (Smith and Town) Conn.] transformation experiments. Transgenic plants were produced by using the chimerical gene cassette constructed by Stoeva et al. (1998). It consisted of a 850-base-pair (bp) fragment comprising the full-length coding sequence of the nucleoprotein (N) gene from the Bulgarian L3 isolate of TSWV under two cauliflower mosaic virus (CaMV) 35S promoter sequences fused with untranslated leader sequence (UTR) from the plum pox virus (PPV), and the 35S CaMV terminator sequence (Stoeva et al., 1998). The cassette was cloned into the HindIII site in two plant expression vectors: pLX222 (Landsmann et al., 1988) and pCAMBIA-2300 (GenBank Accession No. AF234315; CAMBIA, Canberra, Australia). The resulting binary plasmids pXEL (Stoeva et al., 1998) and pCN were transferred into A. tumefaciens strain GV3101:MP90 (Koncz and Schell, 1986) by electroporation.

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION. Tomato transformation was performed as described by McCormick (1991) with modifications (Bartoszewski et al., 1996). Tobacco transformation was performed as described by Horsch et al. (1985). Tomato cotyledon explants and tobacco leaf disks were co-cultivated with A. tumefaciens strain GV3101:MP90 carrying either the pXEL or pCN plasmids. The shoots were regenerated on the MS medium (Murashige and Skoog, 1962) supplemented with adequate phytohormones (2 mg·dm–3 zeatin and 0.02 mg·dm–3 IAA for tomato and 1 mg·dm–2 BAP and 1.0 mg·dm–2 NAA for tobacco regeneration) and antibiotics (75 mg·dm–3 kanamycin sulfate and 400 mg·dm–3 carbenicillin). Regenerated shoots were rooted on the MS medium with the same antibiotics as above and supplemented with 0.2 mg·dm–1 IAA for the tomato and 0.1 mg·dm–1 NAA for the tobacco shoots. Primary transgenic plants were grown under greenhouse conditions.

ANALYSIS OF TRANSGENIC PLANTS. Southern-blot analyses were performed on R0 plants according to the Sambrook et al. (1989) procedure. DNA (12 μg) extracted from leaf material (Doyle and Doyle, 1990) was digested with HindIII or Dral and fractionated by electrophoresis in 0.8% agarose gels. HindIII restriction enzyme released an internal T-DNA fragment containing the TSWV chimeric N gene, while there was no Dral restriction site in the T-DNA insert. Fractionated DNA was blotted to Zeta-Probe GT membrane (Bio-Rad) and hybridized to a 32P-labeled 500-bp fragment of the N gene. Labeling was performed using Random Primed DNA Labeling Kit (Boehringer Mannheim). Hybridization, washings and autoradiography were performed as per membrane manufacturer’s protocol (Bio-Rad).

DAS-ELISA was applied for a serological detection of the viral nucleoprotein by using polyclonal rabbit antibodies (Agdia, Elkhart, Ind.).

ISOLATION OF NUCLEI AND NUCLEAR RUN-ON TRANSCRIPTION ASSAY. Plant material (5 g) was ground to a fine powder in liquid nitrogen, suspended in 40 mL of the extraction buffer (chemical constitution according to Dehio and Schell, 1994), supplemented with 1% Triton X-100 (Sigma, St. Louis), and briefly homogenized. After filtration through a 50-μm nylon mesh, the filtrate was centrifuged for 5 min at 1500 g. The pellet was resuspended in 2 mL of the same buffer and loaded on a gradient consisting of 6-mL layers of 80% and 35% (v/v) Percoll (Sigma) in extraction buffer, followed by a centrifugation for 15 min at 1000 g. Nuclei were collected from the interface of the 80% and 35% layers and diluted to 35 mL in the same buffer. Six millilitres of 35% Percoll was then layered under the pellet. After centrifugation, the nuclei-enriched pellet was resuspended in approximately three volumes of the nuclear storage buffer (Dehio and Schell, 1994). Aliquots of nuclei were counted in a Neubauer chamber under a fluorescence microscope after staining with DAPI solution (Partec, Münster, Germany). The run-on assay utilized 106 (in 50 μL) isolated nuclei and was conducted as described by Dehio and Schell (1994). The generated 32P-labeled transscripts were used for hybridization with identical filters containing 0.2 μg of DNA of a 500-bp fragment of nucleoprotein N gene, 0.2 μg of DNA of a 350-bp nptII gene, and 0.2 μg of DNA of the 400-bp 3′-end of 25S rRNA sequence. Hybridization and washing conditions were as described by Sambrook et al. (1989).

Results

ANALYSIS OF TRANSGENIC PLANTS. The A. tumefaciens-mediated transformation yielded a total of 12 tomato and 141 tobacco primary transformants. All these plants had proper ploidy levels and showed normal development, except five tomato plants that did not produce seeds. All transformants were studied for the transgene integration patterns, transgene expression, and were tested for resistance (Tables 1 and 2). Southern-blot analysis revealed different patterns of integration (one to three inserts) in the genomes of analyzed plants. Correct integration of the nucleoprotein gene was observed in eight tomato and 103 tobacco plants. Three tomato and 27 tobacco plants had correct insert and additional inserts of various lengths. One tomato and three tobacco plants showed integration of only shorter sequences, and eight tobacco plants were characterized by only longer sequences.

TESTING TRANSGENIC PLANTS FOR RESISTANCE. All plants that produced seeds for R2 generation (seven tomato and 141 tobacco lines) were tested for resistance to two strains of TSWV (Table 1). This revealed a wide range of responses, from susceptibility...
(S), through delayed reaction (D), recovery from infection (R), localized infection (LL), to a complete lack of symptoms (HR). The delayed reaction (D) is defined as the appearance of symptoms on transgenic plants at least 10 d later than on the control; the term recovery (R) applies to plants that originally showed the infection symptoms but which produced new shoots free of infection symptoms; localized infection (LL) refers to the appearance of mild local symptoms while resistance (HR) is a complete lack of virus invasion.

Resistance (HR) was found in 10 transformed tobacco plants (Fig. 1), local infection (LL) in one transformed tomato and 13 tobacco plants, and the delayed reaction (D) in one tomato and eight tobacco plants. A majority of tomato plants (five) showed the recovery (R) phenotype (Fig. 2).

Plants of the R2 generation showed the same reaction as the parents. The control plants that were back-inoculated with the inoculum prepared from the HR plant leaves showed no symptoms of virus infection. The absence of TSWV in the HR plant tissue was confirmed by the negative ELISA test (A405 below 0.1). The same result was obtained in the bio- and ELISA tests of R and LL plants. Control plants showed symptoms of virus infection following inoculation with the inoculum prepared from the D plants. High ELISA scores (mean A405>1.23) confirmed virus presence in the plant tissue.

EXPRESSION OF THE N GENE AND ITS LEVEL OF TRANSCRIPTION. The analyses were conducted on all the plants showing any of the resistant phenotype and additionally on four sensitive transgenic tobacco plants. Northern-blot of the total RNA detected a transcript of the \( \text{N} \) gene as a single band of the expected size (500 bp) with variable intensity (Fig. 3). The transcript of the \( \text{N} \) gene was present in five tomato and 27 tobacco plants. Plants that produced correct transcripts of the \( \text{N} \) gene accumulated virus nucleoprotein on different levels (Fig. 4). The OD 405 readings ranged from 0.04 to 2.76. Plants were classified as non-expressors when the readings were below 0.41, as low-expressors when the readings were between 0.41 and 1.20, as mid-expressors when OD405 were between 1.20 and 1.90, and as high-expressors when the readings were above 1.9 in accordance with Pang et al. (1996). Of the tobacco plants studied, 14 were classified as high-expressors, seven as mid-expressors, six as low-expressors, and two as non-expessors.

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>No. of plants</th>
<th>Transgene integration pattern</th>
<th>Expression of transcript</th>
<th>Expression of N protein</th>
<th>Hypothetical mechanism of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR</td>
<td>5</td>
<td>1–2 inserts</td>
<td>+</td>
<td>high or moderate</td>
<td>protein dependent</td>
</tr>
<tr>
<td>HR</td>
<td>5</td>
<td>more than 1 insert or insert of different lengths</td>
<td>–</td>
<td>absence or very low</td>
<td>RNA dependent</td>
</tr>
<tr>
<td>LL</td>
<td>10</td>
<td>1–2 inserts</td>
<td>+</td>
<td>moderate or high</td>
<td>protein dependent</td>
</tr>
<tr>
<td>LL</td>
<td>4</td>
<td>more than 1 insert or insert of different lengths</td>
<td>–</td>
<td>absence or very low</td>
<td>RNA dependent</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>1–2 inserts or insert of different lengths</td>
<td>+</td>
<td>moderate or high</td>
<td>?</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>1–2 inserts</td>
<td>–</td>
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<td>RNA dependent</td>
</tr>
<tr>
<td>R</td>
<td>5</td>
<td>1 insert</td>
<td>–</td>
<td>absence</td>
<td>RNA dependent</td>
</tr>
<tr>
<td>S</td>
<td>2</td>
<td>1–2 inserts or insert of different lengths</td>
<td>+</td>
<td>moderate or high</td>
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<tr>
<td>S</td>
<td>2</td>
<td>1–2 inserts</td>
<td>–</td>
<td>absence</td>
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</tr>
</tbody>
</table>

Table 1. Nucleoprotein gene expression pattern in transgenic tomato and tobacco plants with different resistance phenotype: highly resistant (HR), local infection (LL), delayed (D), recovered (R), and sensitive (S).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expression vector</th>
<th>No. of primary transforms</th>
<th>No. of resistant lines (HR, R, and LL)</th>
<th>Hypothetical mechanism of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato line nor</td>
<td>pCN</td>
<td>6</td>
<td>5/6</td>
<td>protein dependent</td>
</tr>
<tr>
<td>Tomato line KR</td>
<td>pCN</td>
<td>1</td>
<td>na</td>
<td>RNA dependent</td>
</tr>
<tr>
<td>Tomato ‘Potentat’</td>
<td>pCN</td>
<td>3</td>
<td>1/1</td>
<td>protein dependent</td>
</tr>
<tr>
<td>Tobacco ‘Petit Havana’</td>
<td>pCN</td>
<td>42</td>
<td>5/42</td>
<td>RNA dependent</td>
</tr>
<tr>
<td></td>
<td>pXEL</td>
<td>99</td>
<td>18/99</td>
<td>RNA dependent</td>
</tr>
</tbody>
</table>

Table 2. Number of primary transformants and lines resistant to the TSWV infection in the R1 and R2 generations of tomato and tobacco.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Expression vector</th>
<th>No. of primary transforms</th>
<th>No. of resistant lines (HR, R, and LL)</th>
<th>Hypothetical mechanism of protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato line nor</td>
<td>pCN</td>
<td>6</td>
<td>5/6</td>
<td>protein dependent</td>
</tr>
<tr>
<td>Tomato line KR</td>
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<tr>
<td></td>
<td>pXEL</td>
<td>99</td>
<td>18/99</td>
<td>RNA dependent</td>
</tr>
</tbody>
</table>

zHR = highly resistant, LL = locally infected, R = recovery.

yna = not analyzed.
and eight as non-expressors. Among the tomato plants, four were low-expressors while eight were non-expressors.

The run-on analysis revealed that the nucleoprotein gene was actively transcribed in plants resistant to the TSWV infection, in those showing high level of the $N$ gene transcript accumulation (expressors) as well as those without any detectable $N$ mRNA (non-expressors) (Fig. 5).

**Discussion**

The study showed that tomato and tobacco transformants expressing the sense form of the $N$ gene were resistant to the inoculation with Polish strains of TSWV and that resistance was present irrespective of the nucleoprotein accumulation. Plants with high accumulation as well as those not producing detectable $N$ gene transcripts and protein were resistant to the TSWV infection (Table 2). The lack of correlation between resistance and the presence of the nucleoprotein suggests that the engineered resistance can be based on one of two different mechanisms: RNA-dependent (post-transcriptional silencing) resistance or protein-dependent (protein accumulation) resistance.

A majority of the obtained tobacco plants (19) showed high expression of viral protein and exhibited the HR, LL, or D phenotypes, but the amount of the produced protein did not correlate with the level of resistance. The HR phenotype has never been reported before in plants accumulating the virus protein at high levels. Pang et al. (1992) reported that among the progeny of seven transgenic tobacco lines, resistance was mainly in plants accumulating very little or no N protein; plants accumulating high levels of the N protein were resistant at the local-lesions level. Vaira et al. (1995) indicated that plants with high levels of the N protein expression were protected against infection with unrelated virus strains as well as some other tospoviruses. In this study, two Polish TSWV strains, B and C, were used. Both are related to the BR-01 strain and belong to the I serotype, which is present in The Netherlands, Germany, and Italy (Korbin, 1995). The cassette used in this study included the DNA sequence of the $N$ gene from the Bulgarian L3 isolate that is related to Brazilian TSWV strains (Maiss et al., 1991). This may explain the presence of so many plants with high levels of the protein-dependent resistance.

It is surprising that resistance was not present in all the plants expressing high levels of protein. Two plants that were susceptible to infection showed high and moderate-level of protein expression. It is possible that these plants express partial resistance that depends on certain conditions, such as plant age, growing conditions, and the inoculum concentration. Vaira et al. (1995) observed such plants. Our results and some previous studies suggest that the current models of the virus replication blockage cannot explain virus-specific resistance. High accumulation of N protein or $N$ gene transcripts was expected to improve resistance, but this relationship was not confirmed. Variable levels of resistance-susceptibility were observed among plants that expressed no detectable N protein. Probably, the susceptible plants that
Fig. 3. Northern blot analysis of the N transgene in selected transgenic tobacco plants. Total RNA was isolated from transgenic plants (20 µg per lane), blotted and probed with the N gene. Lanes: 1, non-transgenic plant; 2–14, some of the transgenic plants, which were resistant to the TSWV inoculation. Transgenic plants that gave readings were rated as expressors (+). The density of the control and some transgenic plants was set at zero (−). Ethidium bromide stained rRNA was used as a control for equal loading of the RNA.

Fig. 4. The level of the nucleoprotein accumulation estimated by a double-antibody sandwich enzyme-linked immunoabsorbent assay (DAS-ELISA) in selected transgenic tobacco (A) and tomato (B) plants. The OD_{405} readings ranged from 0.04 to 2.76. Plants were classified as non-expressors when the readings were below 0.41, as low-expressors when the readings were between 0.41 and 1.20, as mid-expressors when OD_{405} were between 1.20 and 1.90, and as high-expressors when the readings were higher than 1.9. A. Lane 1, non-transgenic control tobacco plant, 2–36 transgenic tobacco plants. B. Lanes 1–3, non-transgenic control tomato plants; 4–15 transgenic tomato plants.
showed no detectable protein had an inactivated nucleoprotein gene (transcriptional silencing). RNA-dependent protection is based on the post-transcriptional silencing mechanism (PTGS) (Baulcombe, 1999). This mechanism does not increase the transcription level but rather, the degradation of transcripts produced by plant as well as by the genomic and subgenomic RNAs of the infecting virus. Run-on assay analysis conducted in this study confirmed that nucleoprotein gene was actively transcribed in the nuclei but the transcripts were not detectable. This suggests that resistance of plants expressing no detectable N protein is caused by a post-transcriptional silencing mechanism. Resistance is induced by an over-expression of identical transcripts or by aberrant transcripts (untranslatable, intron-less, self-complementary, aberrant length) (Waterhouse et al., 1998). This phenomenon was often observed in plants with multiple transgene copies. De Carvalho et al. (1992) described that the presence of single copy of the transgene in a haploid genome induced PTGS. Our Southern-blot analyses confirmed that plants with silenced transgene had more than one insert in the genome, or inserts of truncated sequences. It should be noted that the presence of multi-copies of a transgene is not sufficient per se to induce the silencing mechanism. Some plants with protein-dependent resistance also had more than one insert of the transgene. This suggests in turn that in plants that exhibit the PTGS, all integrated copies must be transcriptionally active. In plants expressing high levels of protein, additional inserts are either inactive or active at a low level, and the amount of produced transcripts do not reach the threshold level that induces silencing.

It is unclear why some plants with the induced silencing mechanism did not show high level of resistance. In theory, the PTGS is systemically spread and once induced it permanent and leads to a complete degradation of the virus RNAs (Waterhouse et al., 1999). Measurements of the virus protein accumulation in transgenic plants over time and in different leaves clearly showed that the N gene might be silenced to different degrees at different developmental stages (Pang et al., 1996). That may explain why some plants with the induced silencing mechanism showed the LL or D phenotypes.

All tomato plants with the R phenotype had single transgene insertions in their genomes and expressed no detectable N transcripts and proteins. After the viral infection and typical early stages of symptom development, the plants gradually outgrew the infection. Also the bio- and ELISA test proved the absence of the virus in R plants. This reaction may be indicative of the silencing mechanism induction. However previously described R plants (Lindbo et al., 1993) had 1–2 transgene copies and showed the moderate level of N gene expression. During the infection, transgene transcripts and the replicating virus genome act additively to trigger the switch for the transgene mRNA silencing, resulting in the recovery phenotype in the upper leaves at a later developmental stage (Waterhouse et al., 1999). The infection scenario of the R plants observed in this study was identical but the expression pattern differed from that described in the literature. The resistance is definitely RNA dependent but its mechanism remains unclear.

It should be noted that the position and orientation of the transgene integration, as well as plant factors that influence the transgene itself, play a significant role in the expression of resistance or susceptibility in transgenic plants.

**Literature Cited**


Gielen, J.J.L., P. de Haan, A.J. Kool, D. Peters, M.Q.J.M. van Grinsven,


Koncz, C. and J. Schell. 1986. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by novel type of 

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