

CHARACTERISATION OF TWO TOMATO LINES HIGHLY RESISTANT TO *TOMATO SPOTTED WILT VIRUS* FOLLOWING TRANSFORMATION WITH THE VIRAL NUCLEOPROTEIN GENE

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SUMMARY

Tomato spotted wilt virus (TSWV) is a serious threat to both horticultural and ornamental crops. Three fresh-market tomato (*Lycopersicon esculentum* Mill.) lines were transformed with the nucleoprotein gene of TSWV by *Agrobacterium tumefaciens*. Primary transformants for each line were analysed for transgene integration and expression. Results showed that inserted transgenic DNA was often rearranged. Progeny of selected primary transformants were obtained by self-pollination and tested for resistance to TSWV. One completely resistant line was identified having a single integration locus with multiple rearranged transgene copies and a true-to-type phenotype. It will be further tested for possible inclusion in breeding programs.

Key words: genetic transformation, TSWV, tomato, pathogen-derived resistance.

INTRODUCTION

Tomato spotted wilt virus (TSWV), genus *Tospovirus*, is an important pathogen of many plants worldwide. In tomatoes, resistance to TSWV can be introgressed by classical breeding through the *Sw-5* gene (Stevens *et al.*, 1992) from *Lycopersicon peruvianum*. Commercial hybrids are available, but virus isolates capable of overcoming the resistance induced by *Sw-5* have already been reported both in field and greenhouse trials (Cho *et al.*, 1996; Latham and Jones 1998). It is important therefore to explore other ways to obtain TSWV-resistant tomatoes. Among these, genetic engineering may furnish a means of adding a resistance character to the valuable genomic background already existing in specific parental lines. As a result, resistant hybrids satisfying local commercial demand could be produced.

The genetic engineering approach to TSWV resistance, based on pathogen-derived resistance (Sanford and Johnston 1985), began in 1991 (Gielen *et al.*, 1991)

with transformation of tobacco with the *N* gene of TSWV, providing the first example of genetically engineered resistance to a negative-strand virus. Since then, different plant species and constructs have been tested (reviewed in Accotto *et al.*, 2000). For tomato, limited protection to TSWV was obtained for R₁ seedlings of a transgenic tomato cultivar expressing the *N* gene (Kim *et al.*, 1994), while an inbred tomato line transformed with the *N* gene showed high levels of resistance that was maintained in derived hybrids (Ultzen *et al.*, 1995). In the current work we describe transformation of three different tomato parental lines of indeterminate growth red cluster type, and molecular characterisation and resistance assays of the transgenic tomato lines obtained.

MATERIALS AND METHODS

Agrobacterium tumefaciens strain EHA105 (Hood *et al.*, 1993) harbouring plasmid pSW9, a derivative of the binary vector pBin19, was used for transformation experiments. The plasmid pSW9 (Vaira *et al.*, 1995; Vaira *et al.*, 2000) contains an expression cassette (Fig. 1A) carrying the TSWV *N* gene derived from a virus-infected tomato collected in Liguria, Italy (GenBank acc. no. Z36882).

Three fresh-market tomato (*Lycopersicon esculentum* Mill.) breeding lines, L.276-76, L.149-88 and INB777, selected at the Istituto Sperimentale per l'Orticoltura were considered for transformation. L.276-76 is a vigorous line, well adapted to open-field culture, with good leaf coverage and smooth-round fruits. L.149-88 is adapted to greenhouse culture and characterised by sparse foliage, smooth-round fruits and early yield. INB777 is less vigorous than L.276-76, is adapted to both greenhouse and open-field conditions and has round, green fruits.

Preliminary regeneration and genetic transformation experiments were carried out in order to test pre-existing tomato protocols and improve transformation efficiency of the fresh-market tomato lines. The transformation procedure of tomato cotyledons was derived from McCormick *et al.* (1986), according to Cirillo *et al.* (1997). High-level organogenesis was obtained for each genotype with media containing glucose and zeatin supplemented with 0.1 mg/l IAA. Shoots were obtained

from kanamycin (Km)-resistant calli and cultured on selective medium. Several potential transformants were regenerated for each tomato genotype.

Independent transgenic lines were established by *in vitro* propagation of selected shoots on propagation medium (Murashige and Skoog 1962) containing 3% glucose and 0.6% agar (pH 5.8). Cultures were maintained at 21-25°C under a 16-8 h light/dark cycle with a quantum flux density of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$. *In vitro*-rooted plants were acclimatised in soil and used as a source for cuttings for R₀ molecular analysis. R₁ and R₂ progeny were obtained by self-pollination.

To confirm transformation, primary transformants were first analysed by PCR. Genomic DNA was isolated from 1 g of leaf tissue as described by Rogers *et al.* (1988). PCR reactions were performed to amplify a 679-bp fragment from the *nptII* gene using primers described by Dong and McHughen (1993). Sixteen primary transformants that were positive by PCR for presence of the marker gene, were considered for further experiments.

Integration of T-DNA was analysed by Southern blot hybridisation (Fig. 1A and 1B). 10 μg samples of plant DNA (from primary transformants and from R₂ progeny of selected lines) were digested with each of the following restriction enzymes *KpnI*, *BamHI*, *XbaI* or *PstI*. Digestions were always repeated at least twice.

Following electrophoresis through 1% agarose gel, the DNA was transferred to a positively charged nylon membrane (Roche, Basel, Switzerland) and hybridised with digoxigenin-11-dUTP-labelled TSWV *N*- or *nptII*-specific probes obtained by PCR (PCR DIG Probe Synthesis Kit; Roche, Basel, Switzerland); the templates used for probe synthesis were a pBluescript plasmid carrying the cloned TSWV *N* gene (Vaira *et al.*, 1995) and the pBin19 plasmid, respectively. The reactions were visualised by chemiluminescence using CDP-Star (Roche, Basel, Switzerland) as a substrate (Fig. 1B).

RESULTS AND DISCUSSION

The results were complex. Of the 16 lines tested (Table 1), five showed several *N* gene integrations: 30-4 and 113-12 (up to 4), 197-1 (up to 6), 127-1 (up to 7) and 20-2 (up to 8). Two clones (111-6, 114-1) consistently showed a single integration, while clone 118-2 apparently did not integrate the *N* gene. All the other lines showed at most 3 integrations. When integration of the *nptII* gene was checked, the results were similar. In clone 118-2, where the *N* gene could not be observed, integration of the *nptII* gene was confirmed.

Southern analysis was also used to check the stability of 30-4 and 110-1 R₂ progeny. R₂ seedlings from each line gave patterns identical to the corresponding parental R₀, indicating a single integration locus (not shown).

Southern analysis of transgenic lines proved useful but complex for evaluation of transgene integration. It showed that the 16 transgenic lines analysed derived

from independent transformation events, since each line showed a distinct pattern (Fig. 1B). However, when we used different enzymes and different probes to analyse the R₀ lines, results were not easy to interpret (Table 1). When experiments were repeated using the same template and restriction enzyme but different probes, or the same template and probe but different restriction enzymes, consistent estimation of the number of integrations could be obtained in some cases only.

With the 30-4 R₀ line, for example, the estimated number of integrations for the *N* gene was 1 (with *BamHI* and *KpnI*) or 4 (with *XbaI* and *PstI*). The same results were obtained when several individuals of the R₂ progeny were tested. This indicates that there is a single integration locus in line 30-4, containing more than one copy (probably 4) of the *N* gene. Some of these copies must not have been integrated *in toto*, as indicated by the loss of restriction sites *BamHI* and *KpnI*. When considering the *nptII* gene, the estimated number of integrations was 1 (with *BamHI*) or 3 (with *XbaI*). A different number of integrations of two genes present in the same T-DNA is again an indication of incomplete integrations.

As well as 30-4, several other lines did not show the same number of integrations for the two genes. An extreme case is line 118-2, which was selected according to its Km resistance and contains the *nptII* gene, but apparently has no integrated *N* gene moiety.

In conclusion, Southern analysis indicates that the majority of our primary transformants had undergone rearrangements of the T-DNA during transformation. It has been reported that vector backbone sequences, present when whole plasmids are used for transformation, can promote transgene rearrangements (Fu *et al.*, 2000).

To analyse *N* and *nptII* transcripts in transgenic clones, about 5 μg of total RNAs, extracted from young leaf tissue of primary transformants using the RNAwiz reagent (Ambion Inc., Austin, TX, USA) were loaded on 1% agarose gels after denaturing with glyoxal and formamide (McMaster and Carmichael, 1977). Following electrophoresis (Fig. 2C), the RNA was transferred to nylon membranes and hybridised with the *N* gene- or the *nptII* gene-specific probes already described (Fig. 2A and B). The reactions were visualised as for Southern blotting. *N* transgene expression was very variable among the 6 lines tested: lines 111-6 and 99-1 showed very high expression; lines 99-2 and 110-1 showed moderate and line 1-2c limited expression. *N* transgene expression in line 30-4 was complex; the expected band of about 1 kb, present for all the other clones, was absent and there was at least one strong band of lower mol. wt. and two bands of higher mol. wt.

The same lines, tested with the *nptII*-specific probe, showed very uniform expression except for line 30-4, which gave a complex multiple-band pattern.

This difference in the production of *N* and *nptII* transcripts is probably linked to the characteristics of the different promoters by which they are controlled, the enhanced 35S promoter (*N* gene) or the *nos* pro-

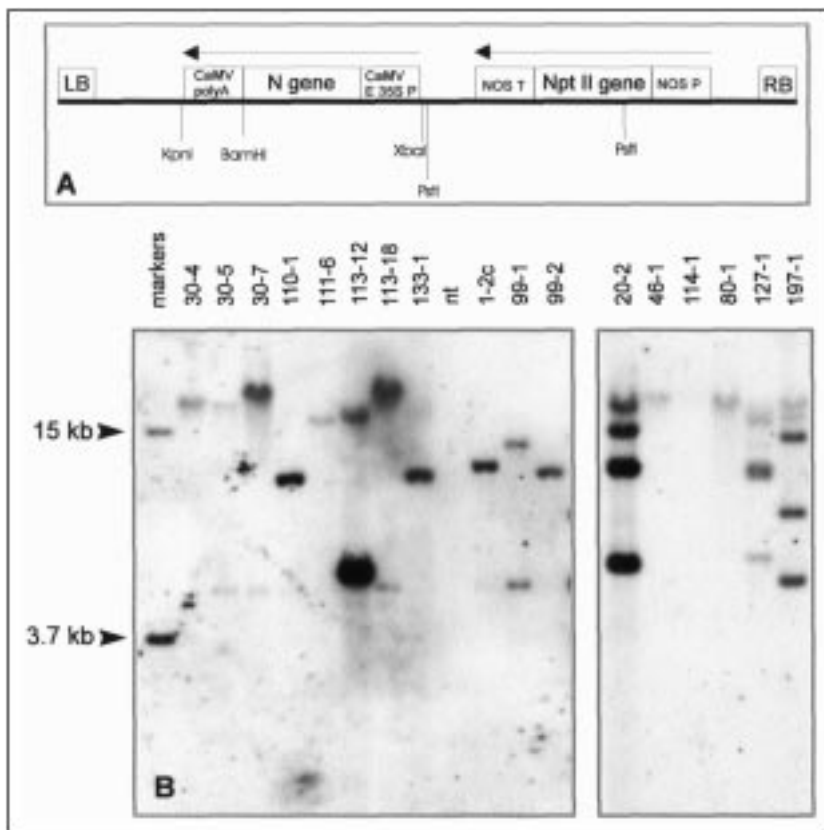


Fig. 1. A) Plasmid pSW9 T-DNA scheme, showing endonuclease cleavage sites. LB and RB, left and right borders; CaMV E35S P, *Cauliflower mosaic virus* enhanced 35S promoter; CaMV polyA, *Cauliflower mosaic virus* poly A termination sequence; NOS P, nopaline synthase promoter; NOS T, nopaline synthase termination sequence. Arrows indicate gene transcription directions. B) Southern blot analysis of primary transformant tomato lines. Genomic DNA was restricted with *Kpn*I and probed with an *N* gene DIG probe. Markers: linearized plasmids containing the TSWV *N* gene sequence. nt: non transgenic.

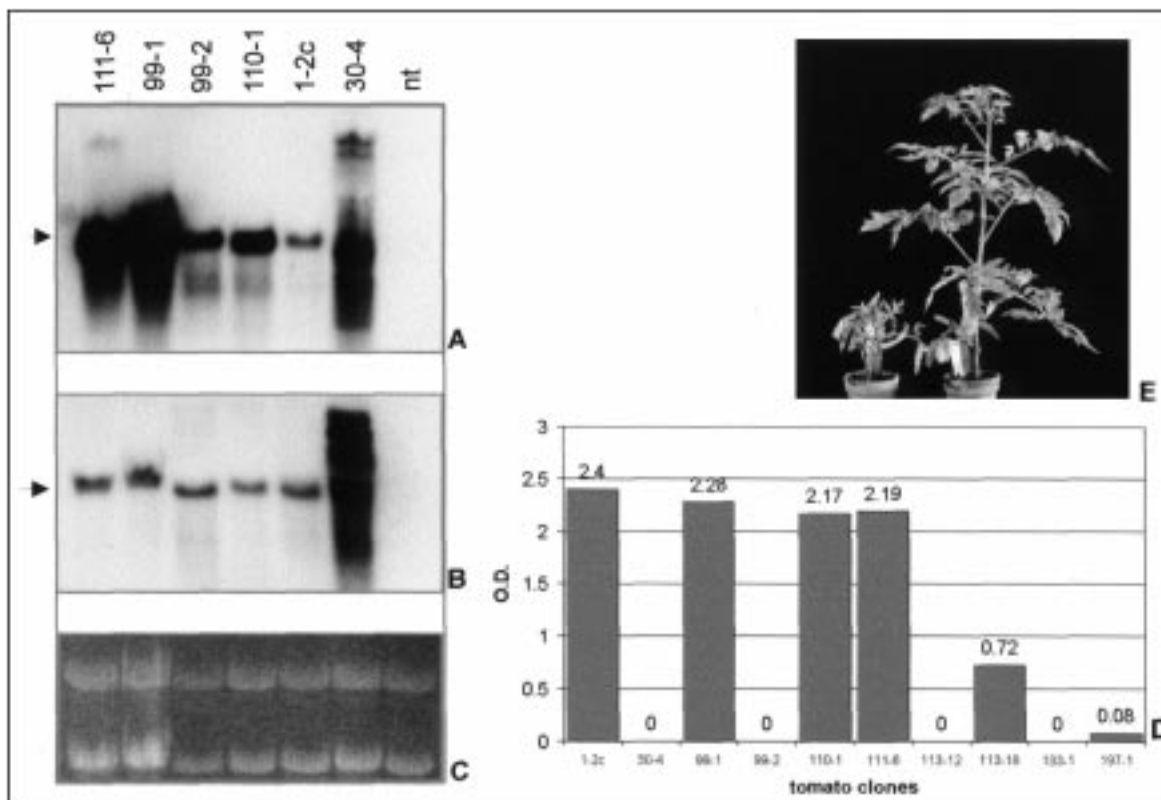


Fig. 2. A) Northern blot analysis of 5 µg total RNA from some primary transformants using *N* gene DIG probe; arrow indicates *N* gene transcript. nt: non transgenic. B) The same using *nptII* gene DIG probe; arrow indicates *nptII* gene transcript. C) Ethidium bromide-stained total RNAs after gel electrophoresis. D) ELISA evaluating transgenic *N* protein expression. O.D. values at 405 nm of 1/10 sample dilution; three times the healthy average value (always less than 0.03 O.D.) has been subtracted from the experimental values. At 1/10 sample dilution, lines classified in Table 2 as +++ and ++++ are not distinguishable owing to plateau absorbance values. E) Tomato plants six weeks after challenge inoculation with TSWV. Plant of transgenic line 30-4 R₂ on the right and control plant on the left.

moter (*nptII* gene). The peculiar patterns observed with line 30-4 are probably the result of the complex T-DNA integrations.

Production of the transgenic *N* protein was monitored in selected clones by double-antibody sandwich ELISA (Fig. 2D, Table 2), employing a kit for the detection of TSWV BR-01 nucleoprotein (Loewe Biochemica, Sauerlach, Germany). Crude plant sap extracts were used at dilutions of 1/10, 1/100, 1/1,000 and 1/10,000 (w/v). In some cases we found that integration of the construct did not correspond to detectable expression of transgene protein (clones 30-4, 113-12, 133-1, 99-2). In line 99-2, no protein was found even though apparently normal mRNA was detected. A high/very high transgene expression generally correlated with low numbers of integrations.

Four transgenic lines were selected to analyse virus resistance in their progeny (Table 2). Seeds obtained from R_0 or R_1 plants were sown under strictly controlled conditions and part of the R_1 and R_2 seedlings were sprayed with Km (300 mg l⁻¹) for 7-12 days to test uniformity and genetic stability. R_2 seedlings of lines 110-1 and 30-4 (150 plantlets per line) were all unaffected by the Km treatment, indicating that the transgene was homozygous; the same R_2 seedlings analysed by Southern blotting, showed complete pattern uniformity (Table 1).

Twenty-two R_1 seedlings from line 1-2c, twenty R_1 seedlings from line 114-1, and twenty R_2 seedlings each from lines 110-1 and 30-4, together with non-transgenic control plants were then mechanically inoculated with TSWV at four-leaf-stage, for the resistance tests (Vaira *et al.*, 1995). Young symptomatic leaves of *Nicotiana benthamiana* infected by a well-characterised TSWV isolate (IFA accession code P105) were used for preparation of virus inocula. The plants were maintained in an insect-proof glasshouse for 28 days. Virus infection, when not evident from symptoms, was checked by ELISA. As challenged plants might produce endogenous *N* protein, a triple-antibody sandwich (TAS) ELISA to detect TSWV glycoproteins was used (antibodies were from the collection of the Istituto di Virologia Vegetale, CNR, Torino, Italy; data not shown).

The six non-transgenic tomato controls showed local lesions at two weeks post-inoculation, with wilting and bronzing of young leaves at three weeks post-inoculation. The progeny of line 114-1 showed complete susceptibility: all the 20 R_1 seedlings behaved as non-transgenic controls. R_1 seedlings of line 1-2c were also readily infected by TSWV, except in one case out of 22, which was probably an escape.

When the two R_2 progenies of 30-4 and 110-1 were tested, none of the 20 seedlings of each line showed visible signs of infection. The transgenic plants showed complete resistance, that is, no local lesion or systemic symptoms throughout the resistance test (Table 2, Fig. 2E). Young leaves of all seedlings were tested by ELISA six weeks post-inoculation, and all were negative.

The phenotype of 30-4 progeny was normal, while 12

of 20 plants of the 110-1 progeny showed apical blindness.

Previous work done on *N. benthamiana* with the same pSW9 construct (Vaira *et al.*, 1995) demonstrated the induction of two kinds of TSWV resistance: protein-mediated, in high transgene-expressors that normally have a single or a limited number of integrations, and RNA-mediated, found in non- or very low-expressors with complex and multiple integrations. Line 110-1 might exhibit the first kind of resistance, but unfortunately linked to a genetic disorder probably due to the transformation event. Transgenic DNA integration may have occurred in a locus involved in the development of the apex, thus interfering with expression of important genes. The resistance observed in line 30-4, conversely, may be ascribed to the second, RNA-mediated type. In fact, although a relatively strong *N*-specific signal was present on Northern blots, full-length transgenic *N*-gene mRNAs were not observed. When *N*-specific transcripts were studied, in all cases deletions at the 3'-end were found (not shown), thus indicating the presence of aberrant mRNAs. This type of molecule has been found in association with homology-dependent post-transcriptional gene silencing (PTGS; Baulcombe 2000; Han and Grierson, 2002). Therefore the extremely high resistance observed in line 30-4 can be explained as a consequence of PTGS of TSWV-*N* sequences.

In conclusion, line 30-4, combining complexity of transgene rearrangements and high TSWV resistance, is worth considering for further resistance tests and worth being challenged with different TSWV isolates and with different tomato-infecting tospovirus species.

In this work we show that T-DNA rearrangement in plants transformed using *A. tumefaciens* is very frequent and may sometimes result in pathogen resistance. A single insertion site of the rearranged T-DNA should be present in order to easily obtain homozygous lines that can inherit the resistance character. Molecular characterisation is absolutely required, and has to be done on high numbers of R_0 lines, owing to the great variability of the transgenic patterns obtained. Testing transgene integration with Southern blotting is the most difficult to interpret and time-consuming step. New techniques, such as quantitative real time PCR (Ingham *et al.*, 2001; Mason *et al.*, 2002) may provide new and interesting perspectives for a different approach to the problem.

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Table 1. Number of transgene integrations for each R₀ line, as estimated from Southern blot analysis with different restriction enzymes and probes. Digestions with *KpnI* and *PstI* were repeated twice. When the replicated digestions produced different results, two figures separated by a comma are given. Figures in italics in brackets, for R₂ progeny, refer to the number of individual plants tested. Blank boxes: data not available.

Parental lines	Transgenic lines	<i>N</i> gene probe				<i>nptII</i> gene probe	
		<i>Bam</i> HI	<i>Kpn</i> I	<i>Pst</i> I	<i>Xba</i> I	<i>Bam</i> HI	<i>Xba</i> I
L.276-76	30-4	1	1	4	4	1	3
	113-18		2	3			
	113-12		2	2	4		
	111-6		1	1			
	110-1		1	2	1		
	133-1		2	3	3		
L.149-88	1-2c		1	2	2		
	99-2		1	1	2		
	99-1		2	1	1		
	197-1	3	5, 6	4, 5	4, 5	4	5
INB777	20-2	8	4		7	9	4
	46-1	2	1		1	1	
	80-1	1	1		2	1	1
	114-1	1	1			1	
	118-2	0	0		0	1	1
	127-1	4	4		7	4	9
R ₂ progeny	30-4	1(4)	1(19)		4(4)	1(4)	3(4)
	110-1	1(3)	1(5)		1(3)	1(3)	1(3)

Table 2. Summary of the results of molecular analysis and resistance assay. Blank boxes: data not available.

Parental line	Transgenic line	Expression of <i>N</i> protein (ELISA) ^a	Transgenic mRNA (Northern blotting) ^b		<i>N</i> transgene integration (Southern Blotting) ^c	TSWV resistance ^d Resistant/inoculated
			<i>N</i> ^e	<i>nptII</i> ^e		
L.276-76	30-4	-	+++	+++	1-4	20/20 (R ₂)
	113-18	++			2-3	
	113-12	-			2-4	
	111-6	++++	++++	+	1	
	110-1	++++	++	+	1-2	20/20 (R ₂)
	133-1	-			2-3	
L.149-88	1-2c	+++	+	+	1-2	1/22 (R ₁)
	99-2	-	++	+	1-2	
	99-1	+++	++++	+	1-2	
	197-1	+			3-6	
INB777	20-2				4-8	
	46-1				1-2	
	80-1				1-2	
	114-1				1	0/20 (R ₁)
	118-2				0	
	127-1				4-7	

^a - = no detectable expression; + = positive only at 1/10 dilution; ++ = positive at 1/10 and 1/100 dilutions; +++ = positive at 1/10, 100 and 1000 dilutions; ++++ = positive at all dilutions. A sample is considered positive when the O.D. value is more than three times the average value of the healthy control, calculated for each dilution.

^b Estimated by eye from Fig. 2A and B. Line 30-4 showed a complex pattern.

^c see Table 1 for details.

^d The progeny tested in brackets.

^e Probe-targeted gene.

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