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Resistance to Tospoviruses in *Nicotiana benthamiana* Transformed with the N Gene of Tomato Spotted Wilt Virus: Correlation Between Transgene Expression and Protection In Primary Transformants

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***Nicotiana benthamiana* was transformed with the nucleoprotein (N) gene of an Italian isolate of tomato spotted wilt virus (TSWV). Forty-five T_1 primary transformant lines were analyzed for the expression of N protein and for resistance to TSWV and three other tospoviruses: impatiens necrotic spot virus (INSV), groundnut bud necrosis virus (GBNV), and groundnut ringspot virus (GRSV). Thirteen of these lines were further characterized. Resistance to all TSWV isolates tested was found in two lines. The expression of the transgene (N mRNA) was lower in these resistant lines than in any of the susceptible lines, and the transgene N protein was either absent or present below detectable levels. These lines were susceptible to the other tospoviruses tested, but they developed symptoms milder than controls when inoculated with GRSV. Some of the lines producing high levels of N protein showed delays (of 2–3 weeks) in symptom expression with at least one of the TSWV isolates tested and symptom delay or attenuation with INSV or GRSV (or both). From our results it appears that high expression of TSWV N protein retards, in some cases, disease development by TSWV and INSV. In contrast, the lack of detectable expression of the transgenic N protein, accompanied by limited production of N transcripts, conferred TSWV-specific resistance.**

Additional keywords: Bunyaviridae, coat protein–mediated protection, gene expression, transgenic plants.

Tomato spotted wilt virus (TSWV) is the best characterized member of the genus *Tospovirus,* family *Bunyaviridae.* The TSWV genome is composed of three single-stranded RNAs (L, M, and S): L RNA is of negative polarity, and M and S RNAs are of ambisense polarity. The virus particle is composed of the genomic RNAs tightly associated with the viral N protein, surrounded by a glycolipid membrane. TSWV infects hundreds of plant species in many families, and its most important vector, the thrips *Frankliniella occidentalis,* is rapidly colonizing new regions and is very difficult to control (Peters *et al.* 1991). The tospoviruses TSWV and impatiens necrotic spot virus (INSV) (Law and Moyer 1990) have caused severe losses in both horticultural and ornamental crops in Italy during recent years (Vaira *et al.* 1993).

Expression of the N gene of TSWV induced resistance in transgenic *Nicotiana tabacum* (Gielen *et al.* 1991; Pang *et al.* 1992; MacKenzie and Ellis 1992). This protection (de Haan *et al.* 1992) was primarily correlated with the presence of N gene transcripts rather than protein in transgenic plants. However, the resistance, where tested, was limited to strains of TSWV and was not effective against other tospoviruses (Pang *et al.* 1992; de Haan *et al.* 1992).

In this work we describe the cloning and sequencing of the N gene from an Italian isolate of TSWV into a binary vector under the control of the 35S promoter, transformation of *Nicotiana benthamiana* (systemic host of all tospoviruses) by *Agrobacterium tumefaciens,* analysis of 45 primary transformants for expression of the N protein, and resistance to TSWV and three other tospoviruses: INSV, groundnut bud necrosis virus (GBNV) (Yeh *et al.* 1992; Adam *et al.* 1993), and groundnut ringspot virus (GRSV) (de Avila *et al.* 1993). The use of primary transformants allowed us to test several viruses on the same vegetatively propagated clonal line. Thirteen of these lines were further tested for resistance to three other TSWV isolates and were analyzed for the presence of the N gene and N mRNA.

RESULTS

Cloning and sequencing the N gene.

The sequence of the N gene in pSW3 (TSWV-IT) (EMBL accession number Z36882) consists of 777 nucleotides. When compared with the sequences of three other published TSWV isolates, its homology was 97.4% with BR-01 (EMBL accession number P25999), originally derived from tomato in Brazil; 98.2% with BL (EMBL accession number P26000) (Pang *et al.* 1992), originally derived from lettuce in Hawaii; and 97.3% with L3 (Maiss *et al.* 1991), originally derived from tobacco in Bulgaria. When translated into protein, the TSWV-IT N gene coded for a polypeptide of 258 amino acids, which is the same size as the N protein of the other isolates. When identity and similarity were considered, the homology to the other TSWV N proteins described was 97–98% and above 99%, respectively. TSWV-IT differed from BR01 by four amino acids, differed from BL by two, and differed from L3 by two.

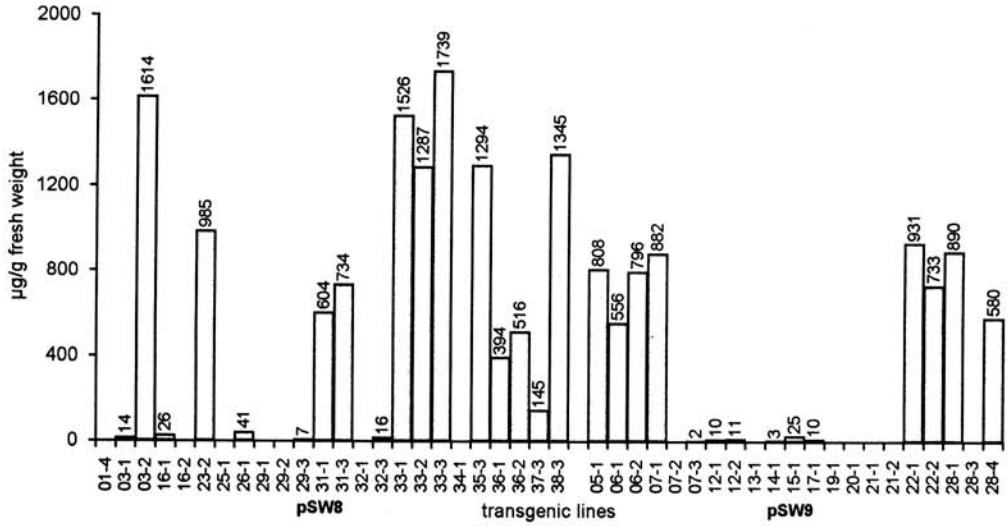

Fig. 1. Quantification of N protein production by double antibody sandwich enzyme-linked immunosorbent assay in *Nicotiana benthamiana* transgenic lines obtained with pSW8 and pSW9. The numbers above the columns represent micrograms of N protein per gram of leaf (fresh weight) and are the average of two measurements.

Complete sequencing of two other independent clones showed base variations at three positions. One had G instead of A at coordinate 673 (asparagine to aspartic acid), the other had T instead of C at coordinate 764 (alanine to valine), and both had C instead of A at coordinate 762 (silent mutation). These changes could reflect variability in the original virus population or may be due to amplification errors during the polymerase chain reaction (PCR).

Plant transformation.

Putative transgenic calli and buds appeared on selective medium, from almost all *A. tumefaciens*–infected leaf strips, 15–20 days after co-cultivation. There was no difference between the two *A. tumefaciens* constructs in the efficiency of transformation. At the end of 1 month, shoots were long enough to be cut and transferred to selective propagation medium. At least 80% of the selected shoots were able to grow further, root, and establish plant clones.

The transformations were carried out with *A. tumefaciens* isolates carrying the N gene coupled to the 35S promoter of cauliflower mosaic virus (CaMV) in plasmids pSW8 and pSW9. These plasmids differed with respect to the orientation of the transgene in the T-DNA (see Materials and Methods). A total of 24 lines transformed with pSW8 and 21 transformed with pSW9 were propagated and used for resistance tests.

Expression of N protein in transgenic plants.

The level of expression of the N protein was quantified in all lines by enzyme-linked immunosorbent assay (ELISA) (Fig. 1). Both pSW8 and pSW9 constructs produced transgenic lines with different levels of N protein, although pSW9 lines did not reach the very high level of some pSW8 lines, which yielded up to 1,700 µg of protein per gram of fresh leaf.

Seven pSW8 and seven pSW9 lines did not produce detectable N protein, while five pSW8 and six pSW9 lines produced a limited amount (up to 50 µg per gram of fresh leaf). An N protein level between 51 and 500 µg per gram of fresh leaf was detected in two pSW8 lines only. Eighteen lines (10 pSW8 and eight pSW9) produced high amounts of protein, above 500 µg per gram of fresh leaf. When tested in Western blot experiments using total protein extracts, the N protein produced by transgenic lines was indistinguishable from that found in TSWV-infected plants (Fig. 2).

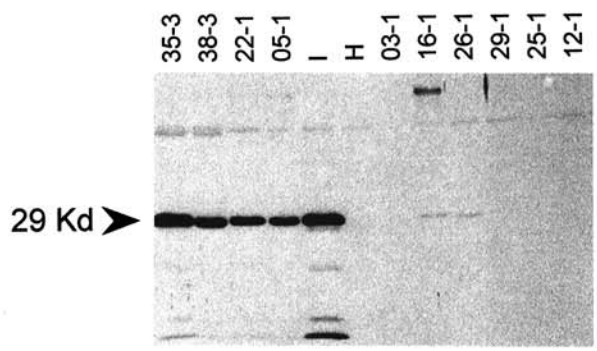

Fig. 2. Western blot analysis of transgenic N protein expressed by some lines, in comparison with N protein expressed by TSWV during infection of *Nicotiana benthamiana*. Numbers of the transgenic lines are given above the lanes. I and H are total protein extracts from infected and healthy *N. benthamiana*, respectively. The molecular mass of the TSWV N protein is indicated on the left.

Testing transgenic plants for resistance.

All lines were challenged with TSWV T365, INSV, GRSV, and GBNV (Table 1). This initial screening produced a wide pattern of response, from susceptibility to delay, attenuation, and complete lack of systemic symptoms. We define *delay* as the appearance of systemic symptoms on transgenic plants at least 2 weeks later than on controls; *attenuation* as the appearance of mild symptoms but continued plant growth; and *resistance* as the complete lack of systemic invasion, even after a second inoculation.

In the first screening, resistance to T365 was found in one pSW8 line (01-4) and one pSW9 line (21-2). Some lines did not react consistently against T365 when several individuals

were challenged. No resistance was found against the other tospoviruses tested. The results with GBNV should be interpreted with caution, because of the great instability of this virus and the difficulties in obtaining consistently reproducible results. Delay in symptom expression was found in three pSW8 and nine pSW9 lines inoculated with INSV. Delay in symptom development was not detected in any line following inoculation with GBNV or GRSV. However, GRSV produced attenuated symptoms on four pSW8 and seven pSW9 lines.

Thirteen lines were selected for further biological and molecular characterization. They included the two T365-resistant lines, together with lines that showed some level of protection against one or more tospoviruses and lines susceptible to all of them. New plantlets from all the selected lines were challenged with three other TSWV isolates: L3, GB-01, and VE214.

The two T365-resistant lines (01-4 and 21-2) also proved to be resistant to the three other TSWV isolates, as tested by ELISA and by back-inoculation to *N. benthamiana,* while three others (25-1, 29-1, and 14-1), whose reaction to T365 was not consistent, were susceptible to one or more TSWV isolates. Interestingly, both resistant lines produced no detectable transgenic N protein; they were susceptible to INSV and GBNV, and they produced attenuated symptoms following inoculation with GRSV.

Line 35-3, when inoculated with INSV, became infected af-

Table 1. Infectivity of 45 primary transformants of *Nicotiana benthamiana* inoculated with tomato spotted wilt virus (TSWV), impatiens necrotic spot virus (INSV), groundnut bud necrosis virus (GBNV), and groundnut ringspot virus (GRSV)[a,b]

	Transgenic	TSWV isolate						
Plasmid	line	T365	L3	GB-01	VE214	INSV	GBNV	GRSV
pSW8	01-4	R, R	R	R	R	S, S	S	A
	03-1	S, S	S	S	S	S, S	D, S	S
	03-2	S	…[c]	…	…	S	S	S
	16-1	S, S	S	S	S	S	S	S
	16-2	S	…	…	…	S	S	S
	23-2	S	…	…	…	D	S	S
	25-1	R, R, S	R	S, S	R	S	S	S
	26-1	S, S	S	S	S	S	S	S
	29-1	R, A	S_2	S	…	S, S	S	S
	29-2	S	…	…	…	S	S	S
	29-3	S	…	…	…	S	S, S	S
	31-1	S	…	…	…	S	S	S
	31-3	Da, D	…	…	…	S	S	S
	32-1	D	…	…	…	S	S	S
	32-3	S	…	…	…	S	S	S
	33-1	S	…	…	…	S	S	A
	33-2	D	…	…	…	S	S	S
	33-3	D	…	…	…	S	S	S
	34-1	R, S, S	…	…	…	S, S	S	A
	35-3	Da, S_2, S	S_2	S	D	Da, Da, Da	S	S
	36-1	S_2, S, S	…	…	…	D	S	S
	36-2	D	…	…	…	S	S	S
	37-3	S	…	…	…	S	S	A
	38-3	D, D, D, S	S	S	Da	A	S	S
pSW9	05-1	D, S	S	S	D	D	S	S
	06-1	S	…	…	…	S	S	A
	06-2	D	…	…	…	D	S	A
	07-1	D, D	…	…	…	D	S	S
	07-2	S	…	…	…	S	S	A
	07-3	S	…	…	…	S	…	S
	12-1	S, S, S	S	S	S	D	S	S
	12-2	S	…	…	…	D	S	S
	13-1	S	…	…	…	D	S	S
	14-1	R, R, S_2	S	S	…	S	S	A
	15-1	S	…	…	…	S	S	S
	17-1	S_2	…	…	…	S	…	S
	19-1	S	…	…	…	S	S, S	S
	20-1	S	…	…	…	S	…	S
	21-1	S_2, S	…	…	…	S	S	A
	21-2	R, R	R, R	R	R	S	S	A
	22-1	S, S	R, Da	S	S	S	S, S	S
	22-2	D	…	…	…	D	S, S	S
	28-1	S	…	…	…	D	S	S
	28-3	S, S, S	…	…	…	S	S	S
	28-4	D	…	…	…	D	S	A

[a] One or more plants were inoculated with each virus; results are given for each plant.
[b] A = symptom attenuation. D = symptoms delayed at least 2 weeks. Da = symptoms delayed and attenuated. R = resistant. S = susceptible. S_2 = susceptible after reinoculation.
[c] Not tested.

ter a delay of 2 weeks, and with systemic symptoms that were milder than those of controls. Another line, 38-3, did not show delay in INSV symptom development, but the plants were much less affected by the virus. Plants of both these two lines survived for a long time, and ELISA with antibodies against INSV showed detectable but limited virus accumulation (results not shown). Lines 35-3 and 38-3, susceptible to GBNV and GRSV, showed partial resistance to TSWV and were among those expressing very high amounts of N protein (Fig. 1).

Molecular analysis of transgenic plants.

The 13 lines selected were analyzed for the presence of the transgene, the expression of specific mRNA, and the absence of contaminating *A. tumefaciens*. PCR amplification of a 273-bp fragment of the N gene and a 326-bp fragment of the virC1 gene showed that all lines contained the transgene and were not contaminated by *A. tumefaciens* (not shown).

When N-specific mRNAs were analyzed in Northern blots, a transcript of about 1 kb was detected in most but not all lines (Fig. 3). The level of mRNA expression usually, but not always, correlated with that of N protein measured previously. No mRNA was detected in three lines; two of these (01-4 and 21-2) produced no detectable protein either, while the third (12-1) expressed low levels of protein (Fig. 1 and Table 2).

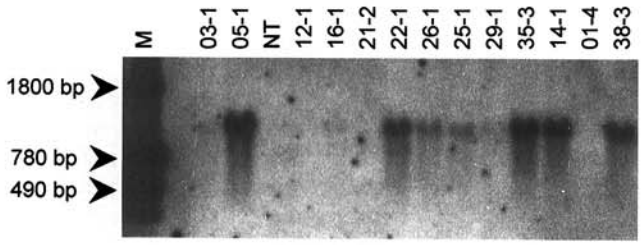

Fig. 3. Analysis of N-specific mRNA in transgenic lines by Northern blotting. NT = Nontransgenic *Nicotiana benthamiana.* The molecular masses of size markers (M) are given on the left.

Table 2. Molecular analysis and infectivity of 13 selected primary transformants of *Nicotiana benthamiana* inoculated with tomato spotted wilt virus (TSWV), impatiens necrotic spot virus (INSV), and groundnut ringspot virus (GRSV)

Plasmid	Transgenic line	Expression[a] of: N protein	Expression[a] of: N transcript	Reaction[b] to: TSWV	Reaction[b] to: INSV	Reaction[b] to: GRSV
pSW8	01-4	–	+	R	S	PP
	03-1	+	++	S	S	S
	16-1	+	++	S	S	S
	25-1	–	++	PP	S	S
	26-1	+	++	S	S	S
	29-1	–	++	PP	S	S
	35-3	+++	+++	PP	PP	S
	38-3	+++	+++	PP	PP	S
pSW9	05-1	+++	+++	PP	PP	S
	12-1[c]	+	+	S	PP	S
	14-1	+	+++	PP	S	PP
	21-2	–	+	R	S	PP
	22-1	+++	+++	PP	S	S

[a] Expression of N protein as determined by double-antibody sandwich enzyme-linked immunosorbent assay: – = none detected; + = less than 50 µg/g of leaf; +++ = more than 500 µg/g of leaf. Level of N transcript as determined by Northern blots and rapid amplification of cDNA ends (RACE) polymerase chain reaction (PCR): + = very low level, detectable by RACE PCR only; ++ = low level; +++ = high level.

[b] PP = partially protected. R = resistant. S = susceptible.

[c] This line had transcripts of unusual size, as a result of rearrangement in the expression cassette (see text).

In order to check the integrity of the whole transformation cassette in those three lines apparently expressing no mRNA, the complete cassette of about 1.8 kb, containing the double promoter, the N gene, and the terminator sequences, was amplified by PCR (not shown). Lines 01-4 and 21-2 contained the entire cassette, while line 12-1 appeared to have a cassette reduced by a deletion of approximately 500 bp.

Rapid amplification of cDNA ends (RACE) PCR, performed on the two lines having the complete cassette, amplified a 600-bp fragment corresponding to 470 3′-terminal nucleotides of N gene plus a poly(A) tail. The specificity of this fragment was confirmed by Southern blot using an N-specific probe (Fig. 4). From line 12-1, containing a modified cassette, DNA fragments of different sizes were amplified.

DISCUSSION

The sequences of the TSWV-IT N gene and protein, compared with other TSWVs described, showed an extremely high level of conservation, for virus isolates coming from distant parts of the world; this appears to reflect strong constraints on both RNA and amino acid variation. High conservation of the N gene has previously been observed in each tospovirus species (de Avila *et al.* 1993).

We used primary transformants expressing the TSWV-IT N gene, in order to test resistance to four tospoviruses on the same clonal line. However, in some lines, results obtained from different individuals of the same line were not completely consistent (see T365 in Table 1). We consider these lines to be partially protected, as they may be resistant in some circumstances (age of the plant, growth conditions, inoculum concentration) but partially or even completely susceptible in others.

To identify lines displaying broad-spectrum resistance to TSWV we inoculated the plants with four isolates, none of which was used to obtain the transgene. Resistance to all

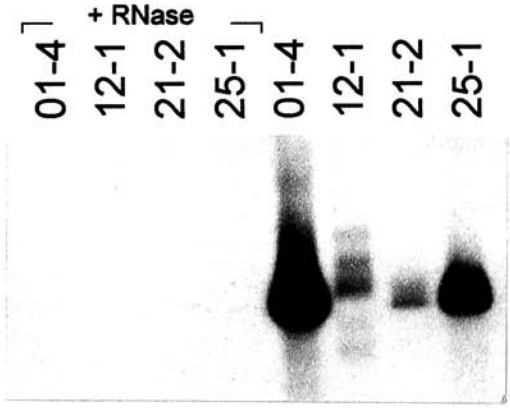

Fig. 4. Amplification of a 600-bp DNA fragment by rapid amplification of cDNA ends (RACE) polymerase chain reaction of mRNAs from the lines in which N-specific mRNA was not detectable by Northern blotting and hybridization to labeled N gene. Line 25-1 was introduced as a control. The lanes marked "+ RNase" were treated with ribonuclease prior to analysis.

TSWV tested isolates was found in only two lines (01-4 and 21-2) out of 45. It has been shown that higher resistance is achieved when transgenic plants are inoculated with the isolate originally used to obtain the N gene, rather than with related isolates (Pang *et al.* 1992). In the end, virus-specific, not only isolate-specific, protection will be needed if resistance is to hold up in different geographical locations and under different biological conditions.

When other tospovirus species were tested, no line showed resistance, although symptom attenuation was observed after inoculation with GRSV and significant symptom delay was found after inoculation with INSV. All lines were susceptible to GBNV. GRSV, also known as SA-05, was previously classified as a TSWV serotype (de Avila *et al.* 1990), and its nucleoprotein shows an amino acid sequence identity of about 78% with TSWV N (de Avila *et al.* 1993). It is therefore not surprising that the TSWV N gene influenced the reaction to GRSV infection. In similar tests, de Haan *et al.* (1992) used GRSV to challenge the progeny of *N. tabacum* plants transformed with a TSWV N gene construct similar to ours. In those tests the transformed lines were as susceptible as the controls.

INSV is genetically distinct in the N protein from TSWV (about 55% of amino acid sequence identity), and the finding that 13 lines out of 45 showed some level of protection was unexpected. In another study recently published on resistance to tospoviruses in *N. benthamiana* transgenic for the TSWV N gene, Pang *et al.* (1994), analyzing the progeny of primary transformants, came to conclusions similar to ours on TSWV and INSV and demonstrated that resistance to TSWV in low expressors is RNA-mediated, while partial protection against both viruses in high expressors is protein-mediated. They found symptom delay with GRSV in high expressors, while we found attenuation in several lines, without clear correlation with gene expression levels. They did not test GBNV.

GBNV has been proposed as a new species (Adam *et al.* 1993), since it is serologically distinct from all other members of the genus. Although no sequence data are available to us, the complete susceptibility of our transgenic lines to GBNV suggests that it will prove genetically more distant from TSWV than INSV itself.

Our aim was not only to look for resistant lines following introduction of the TSWV N gene but also to seek any correlation between resistance and transgene expression at either the mRNA or the protein level. The complete resistance to TSWV of lines 01-4 and 21-2 corresponded to no detectable N protein and limited amounts of N transcripts, while partial protection was shown mainly, but not exclusively, by lines expressing high levels of protein. All seven lines showing partial protection against both TSWV and INSV (35-3, 38-3, 05-1, 06-2, 07-1, 22-2, and 28-4) produced high levels of protein. These results are in accordance with observations by Pang *et al.* (1992) on the progeny of seven *N. tabacum* lines: resistance to TSWV isolates was mainly found in plants accumulating very little or no N protein, while plants accumulating high levels were resistant to INSV at the local-lesion level. Gielen *et al.* (1991), although their transgenic tobaccos had a range of N protein levels as varied as ours, detected no correlation between amounts of N protein and the level of resistance. MacKenzie and Ellis (1992) measured levels of TSWV N protein in transgenic tobacco and then tested for resistance, selecting only the progeny of lines producing detectable amounts.

It has to be noted (see Tables 1 and 2 and Figs. 1, 3, and 4) that high expression is not sufficient per se to give partial protection, and that very low expression does not determine per se virus-specific resistance. We conclude that very limited expression of the N gene seems a prerequisite for virus-specific resistance, and that a high content of transgenic N protein seems required for partial protection against both TSWV and INSV.

Regarding the relation between resistance and mRNA expression, it has been shown that the protection conferred by the TSWV N gene appears to be RNA-mediated (de Haan *et al.* 1992; Pang *et al.* 1993, 1994). In our experiments the two lines showing resistance to TSWV and containing no detectable N protein produced limited amounts of N-specific transcripts. On the other hand, lines showing partial protection to both TSWV and INSV and high levels of N protein expression had high steady-state transcript levels (see lines 35-3, 38-3, and 05-1).

Although we cannot attribute the protection to either RNA or protein, our results confirm those of Pang *et al.* (1992, 1993, 1994) and point to the conclusion that different mechanisms may be responsible for partial protection and for resistance. Virus-specific resistance could be RNA-mediated, in the presence of low levels of transcript, while partial protection to both TSWV and INSV could be protein-mediated, in the presence of high amounts of N protein.

Virus-specific resistance cannot be explained by current models of blockage of virus replication by accumulation of N protein or its transcript, which assume that high levels of the protecting component should give better resistance. In addition, any explanation has to clarify why resistance is not found in all lines that express no detectable N protein and symptom delay or attenuation is not shown by all lines expressing high levels of protein. A new and unusual case has recently been reported with tobacco etch potyvirus (TEV)

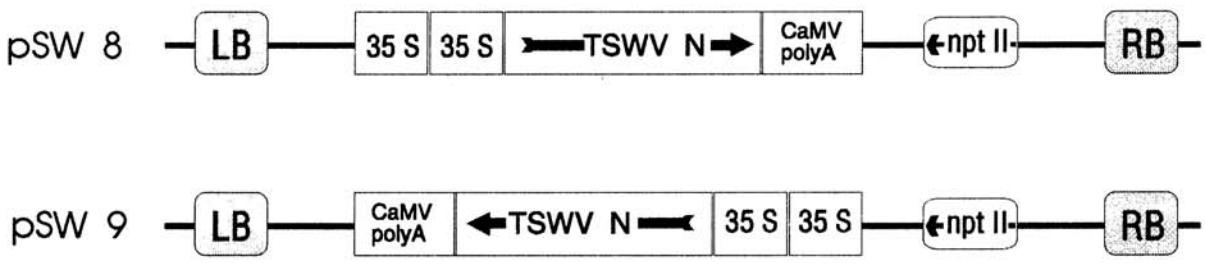

Fig. 5. Expression cassettes of the two plasmids used for transformation. LB = left border; RB = right border.

(Lindbo *et al.* 1993): plants transformed with untranslatable versions of the coat protein gene are initially infected by TEV but then recover, and the newly grown part of the plant is free of virus and immune to new infection. However, this is not our case, since our resistant plants are resistant from the beginning, and we never observed any recovery in any of the infected plants. The site of integration or host factors interacting with the transgene probably play an important role in producing a resistant or susceptible phenotype.

MATERIALS AND METHODS

Viruses.

The TSWV isolates used in this work were IT and T365, from tomato in Liguria, Italy (V. Lisa, unpublished); L3 (Maiss *et al.* 1991), from lettuce in Bulgaria (supplied by G. Adam, Braunschweig, Germany); GB-01 (Vaira *et al.* 1993), isolated in England (supplied by D. Wright, Harpenden, U.K.); and VE214, from tomato in Spain (supplied by E. Moriones, Barcelona, Spain).

INSV isolate Sta 9 (V. Lisa, unpublished), GBNV, and GRSV (both provided by G. Adam) were also used.

All viruses were maintained in *N. benthamiana* by mechanical inoculation.

Cloning and sequencing the N gene.

Based on the sequence of TSWV BR-01 (de Haan *et al.* 1990), two synthetic 30-mer primers were designed. The first primer (P414, 5′-TACCCGGGAAGCTTCATGTCTAAGGTTAAG) contained 15 bases of adaptor arm with *Sma*I and *Hin*dIII restriction sites and 15 bases complementary to the region near the 3′ terminus of the S RNA that corresponds to the 5′ terminus of the N gene. The second primer (P413, 5′-CTAGTCGACGGATCCTCAAGCAAGTTCTGC) contained 15 bases homologous to the S RNA region where the N gene 3′ end resides and 15 bases of adaptor arm with *Bam*HI and *Sal*I sites.

Total RNAs were extracted from 2 g of tomato leaves infected with TSWV-IT, by the guanidinium thiocyanate method (Chirgwin *et al.* 1979). cDNA was synthesized by the use of a commercial kit (cDNA Synthesis System, Amersham), with 20 µg of total RNAs, 100 ng of primer P414, and avian myeloblastosis virus reverse transcriptase in a 20-µl reaction for 90 min at 42° C. After dilution to 250 µl, 1 µl was used for the amplification reaction, with both primers at final concentrations of 0.5 µM and AmpliTaq polymerase (Perkin-Elmer) in a 100-µl reaction, according to the manufacturer's instructions. Forty cycles of denaturation at 95° C, annealing at 53° C, and extension at 72° C were performed, followed by a final extension of 20 min at 72° C. The presence of a DNA fragment of expected size (about 0.8 kb) was checked on 1% agarose gels.

Using the sites contained in the adaptor arms, the amplified DNA was digested with *Hin*dIII and *Bam*HI and cloned into pBluescript KS+ (Stratagene) cut with the same enzymes. One of the several clones obtained (pSW3) was sequenced on both strands by the dideoxyribonucleotide method with Sequenase 2.0 (United States Biochemicals) and synthetic primers. Two other independent clones were also fully sequenced.

Data were assembled and analyzed with the PC/GENE software package (Intelligenetics).

Construction of plant expression vectors and plant transformation.

To obtain an expression cassette, the sequence encoding the N gene was excised from pSW3 with *Hin*dIII and *Bam*HI, recovered from gels, and cloned into pJIT163BGL (kindly provided by P. M. Mullineaux, Norwich, U.K.) digested with the same two enzymes. Plasmid pSW7 was obtained, which contained the expression cassette with a double 35S promoter, the N gene, and the CaMV poly(A) terminator sequence, flanked by *Bgl*II restriction sites. The 1.8-kb *Bgl*II fragment was cloned into *Bam*HI-cut pBin19 (Bevan 1984), yielding pSW8 and pSW9, in which the N cassette had the opposite orientation and the same orientation, respectively, as the NPT II expression cassette (Fig. 5). These two binary transformants were mobilized into *A. tumefaciens* LBA 4404 by direct transformation (Hoefgen and Willmitzer 1988), giving LBA4404 pSW8 and pSW9.

In vitro plantlets of *N. benthamiana* were established from surface-sterilized seeds and maintained on MS basal medium (Murashige and Skoog 1962) containing salts and vitamins, sucrose (30 g/l), and 1% agar. Leaf strips 3–4 mm wide were excised from these plantlets and co-cultivated with the two recombinant *A. tumefaciens* strains and then treated essentially as described by Horsh *et al.* (1985). Individual shoots were propagated and rooted on MS basal medium containing kanamycin sulfate and cefotaxime, each at 100 µg/ml.

Testing transgenic plants for resistance.

In order to have enough single plants from each line to be tested against each virus, transgenic lines were first multiplied *in vitro* and then adapted to the glasshouse in pots with a mixture of sterilized soil and Agriperlite for 1–2 weeks. This procedure yielded plants of heterogeneous size. As viral symptoms take longer to appear on older plants, and we wished to measure the delay of symptom expression in transgenic lines, we minimized the effects of size diversity by inoculating a new batch whenever a group of adapted plants was grown to the eight- to 12-leaf stage.

For preparation of virus inocula, young symptomatic leaves of *N. benthamiana* were ground in the presence of extraction buffer (20 mM phosphate buffer, pH 7, containing 5 mM sodium diethyldithiocarbamate, 1 mM EDTA, and 5 mM thioglycolic acid Na salt, plus 5 mg of activated charcoal per milliliter). The extracts, brought to a final dilution of 1/100 (w/v) with the same buffer, were used to mechanically inoculate Carborundum-dusted transgenic *N. benthamiana* plants, followed by nontransgenic control plants. With GBNV, a 1/10 dilution was used, as the 1/100 dilution proved unreliable.

The plants were maintained for observation at about 25° C. Those not showing symptoms after 1 month were reinoculated. When individual plants appeared resistant to T365, other individuals of the same line were challenged. New individuals of the 13 lines selected for further characterization were then inoculated with the three other TSWV isolates.

Plants showing no symptoms even after reinoculation were checked for the presence of TSWV by back-inoculation and triple-antibody sandwich ELISA (see below).

All transgenic plants and imported viruses were manipulated under stringent containment conditions.

Expression of N protein in transgenic plants.

For Western blotting, total proteins were extracted from leaves by trichloroacetic acid (Wu and Wang 1984). The extracts were loaded on 12.5% discontinuous polyacrylamide gels (Laemmli 1970) and transferred to nitrocellulose membranes (Towbin *et al.* 1979). The membranes were incubated with the polyclonal antiserum A308 prepared against recombinant TSWV N protein expressed in *Escherichia coli* (A. M. Vaira *et al.*, manuscript in preparation), followed by an anti-rabbit horseradish peroxidase conjugate. Reactions were visualized by chemiluminescence (Renaissance Kit, DuPont) according to the manufacturer's instructions. Double-antibody sandwich ELISA (Clark and Adams 1977) was used to quantitate the amount of transgenic N protein; a commercial kit for the detection of TSWV BR-01 nucleocapsid was employed (Loewe, Otterfing, Germany). Crude sap extracts of *N. benthamiana* were diluted 1/10, 1/100, 1/1,000, and 1/10,000 in extraction buffer containing (w/v) 2% polyvinylpyrrolidone and 0.2% bovine serum albumin. As standard for quantitation of N protein in transgenic lines, a purified TSWV nucleocapsid preparation, obtained by the protocol of Adam *et al.* (in press), was used. Standard curves, corrected for the effect of crude leaf sap, were used to calculate the amount of transgenic protein per gram of leaf (fresh weight).

In order to detect TSWV infection in plants producing endogenous N protein, ELISA plates were incubated with polyclonal antibodies against TSWV glycoproteins (IFA-AS292) (Adam *et al.*, in press), crude sap extracts, monoclonal antibodies Mab 2B6 (Adam *et al.* 1991) specific for TSWV G1 glycoprotein, and anti-mouse alkaline phosphatase–conjugated immunoglobulin Gs (triple-antibody sandwich ELISA).

Molecular analysis of transgenic plants.

Standard molecular biology techniques (Sambrook *et al.* 1989) were used, unless otherwise specified.

PCR was used to search for the N transgene in plants and to check the possible residual presence of *A. tumefaciens*. Mainly to avoid cross-contamination among samples, a small piece of alkali-treated leaf was subjected to PCR without any extraction (Klimyuk *et al.* 1993). Since the efficiency of this procedure is increased when small DNA fragments are amplified, synthetic oligonucleotides were designed to obtain a 273-bp fragment from the TSWV N gene and a 326-bp fragment from the virC1 gene of *A. tumefaciens* (accession number Y00535) (Close *et al.* 1987). The two N-gene specific primers were PTSW302 (5′-GGGTCAGGCTTGTTGAGGAAAC) and PTSW575 (5′-TTCCCTAAGGCTTCCCTGGTG). The two virC-specific primers were PVIRC2775 (5′-CTCGCTCAGCAGCAGTTCAATG) and PVIRC3101 (5′-GACGGCAAACGATTGGCTCTC). PCR conditions were as described above, except that an annealing temperature of 60° C was maintained, and the reaction volume was reduced to 50 µl. Products were analyzed by gel electrophoresis on 4% NuSieve agarose (FMC BioProducts). In the case of virC1, PCR products were blotted on nylon membranes and probed with a virC1-specific digoxigenin-labeled DNA fragment. This probe was obtained by amplification of *A. tumefaciens* LBA 4404 with the two virC primers described above, according to the protocol of Emanuel (1991).

To analyze N transcripts in transgenic lines, 20 µg of total RNAs, extracted as described above, was loaded on 1% agarose gels following denaturation with glyoxal and formamide (McMaster and Carmichael 1977) and transferred to nylon membranes. Membranes were hybridized with ^{32}P-labeled random primed cloned DNA of the N gene.

In cases where amplification of the entire 1.8-kb transformation cassette was needed, the M13/pUC universal (-20) and the M13/pUC reverse (-24) sequencing primers were used.

In order to detect very small amounts of N transcript that could escape Northern analysis, rapid amplification of cDNA ends (RACE) PCR was performed as described by Frohman *et al.* (1988), using 50 µg of total RNA and the (dT)17-adaptor primer for the cDNA synthesis and the adaptor primer together with PTSW302 for the amplification, with an annealing temperature of 60° C. After 30 cycles, products were analyzed on agarose gels, transferred to nylon membranes, and hybridized as described for Northern blots.

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