



## Molecular determinants influencing the inheritance of transgenic virus resistance in segregating tobacco families transformed with the nucleocapsid gene of tomato spotted wilt virus

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### Abstract

The successful introgression of transgenic virus resistance to control tomato spotted wilt virus (TSWV) epidemics relies on the stable inheritance of this type of resistance. We developed five segregating tobacco families transformed with sense or antisense copies of the TSWV nucleocapsid gene to determine the usefulness of TSWV-resistant transgenic parents in a breeding program, and to define the molecular determinants (transgene dosage, transcript and protein accumulation) controlling the expression of resistance. Three generations per family were evaluated: (1) R<sub>3</sub> or R<sub>4</sub> progeny derived from resistant transgenic parents used in crosses, (2) F<sub>1</sub> hybrids from crosses between the resistant transgenic parents and untransformed tobacco, and (3) F<sub>2</sub> progeny derived from resistant F<sub>1</sub> plants. Analysis of two of these families revealed that resistance was correlated with either two insertions in hemizygous plants or a single homozygous insertion. In contrast, in two other families, there was no correlation between the number of insertions and the resistance response, but rather resistance was correlated with a single, actively transcribed insertion. Transgene protein accumulation was not detected in any of the plants analyzed, indicating that resistance is probably RNA-mediated. As inheritance of the transgene does not always result in the expression of resistance, special care may be necessary when using transgenic resistant parents in a breeding population.

### Introduction

Tomato spotted wilt virus (TSWV) belongs to the genus *Tospovirus*, a group of plant viruses with a tripartite RNA genome enclosed in a glycoprotein envelope (German et al. 1992; Mumford et al. 1996; Ullman et al. 1997; Prins and Goldbach 1998). The *Tospovirus* genus constitutes one of the most economically important groups of plant viruses worldwide. TSWV itself infects over 800 plant species and ranks among the ten most detrimental plant viruses. The rapid emergence of new species of *Tospovirus* and the spread of existing ones can be attributed to expansion of their most important thrips vector, *Frankliniella occidentalis*. The method of choice for control of TSWV epidemics is the use of genetic resistance, however, the

development of resistant cultivars has been hampered by the scarcity of resistance genes available to crop breeders.

The advent of plant genetic engineering has revolutionized crop improvement methods, and developing cultivars resistant to TSWV is no longer limited by the availability of resistance genes in closely related plant species. Since Powell-Abel et al. (1986) first obtained resistance to tobacco mosaic virus (TMV) by transforming tobacco with the TMV coat protein gene, a large body of work has been published on the utility of viral genes for developing resistance in a variety of economically important crops (Malpica et al. 1998). Although the precise mechanisms underlying transgenic virus resistance are poorly understood, it is clear that resistance can be mediated by either the trans-

gene protein or accumulation of the RNA transcript in the absence of protein expression (Prins and Goldbach 1996; Miller and Hemenway 1998; Baulcombe 1999).

TSWV-resistant plants of tobacco, tomato, lettuce and chrysanthemum have been obtained after transforming them with genes encoding for the TSWV nucleocapsid (N) or putative movement (NSM) proteins (Gielen et al. 1991; MacKenzie and Ellis 1992; Kim et al. 1994; Pang et al. 1996; Prins et al. 1997; Sherman et al. 1998). The expression of resistance is highly variable from plant to plant and under different environmental conditions. In our studies with tobacco and chrysanthemum, only a small proportion of the transformed plants (less than 10% ) have agronomically acceptable levels of resistance (Sherman et al. 1998; Herrero et al. 2000). Others also report large variations in the expression of resistance of plants transformed with viral transgenes, from completely susceptible to various degrees of resistance, to plants that become diseased, but later recover. By comparing transformed plants that differ in their expression of resistance, factors such as transgene dosage, transgene length, and type and structure of transgene viral sequences have all been correlated with expression of resistance (Vaira et al. 1995; Goodwin et al. 1996; Pang et al. 1996, 1997; Prins et al. 1996; Sijen et al. 1996; Tenllado and Diaz-Ruiz 1999).

Ideally, once stable resistant transgenic lines have been developed, commercialization of virus-resistant transgenic cultivars would be facilitated by introgressing transgenes into elite breeding material. This in turn would avoid time-consuming and costly processes such as transformation, regeneration and selection of resistant transgenic plants. However, the successful deployment of such stable transgenic resistance relies on our understanding of how transgene expression and the resulting phenotype vary within a segregating population. While the large body of work published in this area has contributed significantly to such an understanding, it has centered mostly on the analysis of regenerants obtained from different transformation events and not on populations that resemble breeding material, that is, segregating families derived from the progeny of a single transgenic parent. The work we present here is aimed at investigating the expression of resistance in a breeding population obtained by developing segregating transgenic families derived from a single resistant transgenic plant. In turn, this study will allow us to correlate the phenotypic expression of susceptibility or resistance with the segregation of molecular events in such a population. Similar to what

other authors have reported, our observations demonstrate that inheritance of the transgene does not always impart resistance in a breeding population. In addition, we show that transgenic resistance to TSWV in the transgenic tobacco families studied depends on either transgene dosage or the presence of an actively transcribed insertion.

## Materials and methods

### *Selection of TSWV-resistant transgenic lines*

Greenhouse and field evaluation of advanced tobacco (*Nicotiana tabacum*) lines independently transformed with sense and antisense versions of the nucleocapsid gene (N gene) of a dahlia isolate of TSWV (TSWV-D) identified transgenic lines of cv. Burley 21 highly resistant to TSWV-D and partially resistant to field strains (Herrero et al. 2000). For use in this study, lines transformed with constructs pTSWVN- and pTSWVNt were selected. These constructs have been described previously (Sherman et al. 1998) and contain, respectively, an antisense version of the N gene and a sense copy of the N gene encoding a 27 kDa truncated protein which is smaller than the 29 kDa wild-type protein. Parents for the crosses included a resistant plant from one R<sub>2</sub> line (12-9/N-) transformed with construct pTSWVN-, and one resistant plant from each of two R<sub>3</sub> lines (47-2-1/Nt and 47-2-2/Nt) derived from sib plants of the same 47-2/Nt R<sub>2</sub> line transformed with construct pTSWVNt.

### *Crosses and generation of segregating families*

To generate five different segregating families, TSWV-resistant plants from the lines described above were either selfed or used as the female parent in crosses to untransformed burley tobacco cvs. KY-14, TN-90 or TN-86 (Figure 1). Families 1 and 2 were developed by crossing the same resistant R<sub>3</sub> plant, 47-2-1(5)/Nt, to cv. KY-14 or cv. TN-90, respectively. Families 3 and 4 were derived by separately crossing two sib R<sub>3</sub> plants, 47-2-2(3)/Nt and 47-2-2(5)/Nt, to cv. TN-86. Family 5 was derived by crossing an antisense R<sub>2</sub> resistant plant, 12-9(6)/N-, to cv. KY-14. Three generations per family were evaluated: (1) R<sub>3</sub> or R<sub>4</sub> lines derived from selfing the resistant transgenic parents, (2) the F<sub>1</sub> hybrids, and (3) F<sub>2</sub> lines derived from resistant F<sub>1</sub> plants (Figure 1).

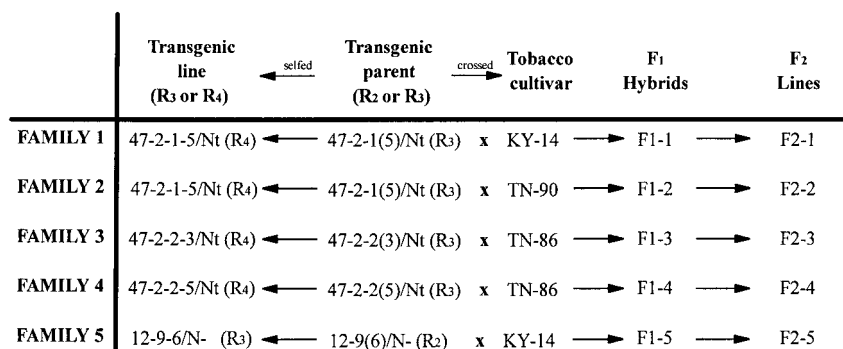


Figure 1. Development of transgenic tobacco families transformed with either a sense (Nt) or an antisense (N-) version of the nucleocapsid gene of TSWV. A resistant transgenic parent was either selfed to generate advanced transgenic lines (arrows pointing left) or use as a female parent in crosses with different tobacco cultivars.

### Inoculation experiments

Seeds were surface-sterilized and germinated on hormone-free Murashige and Skoog (MS) medium containing 50 µg/ml kanamycin as previously described (Daub et al. 1994). After 2 weeks, germinated seeds were transferred to fresh MS medium + kanamycin and grown for an additional 2–4 weeks. Surviving 4–6 week old seedlings were transplanted to soil and grown in the greenhouse for 2–3 additional weeks. Plants that had 3–4 fully expanded leaves were mechanically inoculated with inoculum of the homologous isolate TSWV- D (1 g of TSWV infected *Nicotiana benthamiana* tissue per 10 ml of buffer consisting of 0.01 M Tris pH 7.8, 0.01 M sodium sulfite and 0.1% cysteine HCl). Transgenic families were evaluated in two different greenhouse experiments, and at least 12 plants per generation were inoculated. Disease progress was monitored for ten and seven weeks, respectively, in the two experiments. In all experiments, plants rated as resistant lacked systemic infection or had very few small and scattered systemic lesions that never expanded. The susceptible controls included cv. Burley 21 transformed with the plasmid vector pBI121 (Clontech, Palo Alto, CA) and untransformed cvs. Burley 21, KY-14, TN-90 and TN-86.

### Southern and northern analysis

For Southern blot analysis, tissue was collected from a subset of susceptible and resistant plants for each generation tested within a given family. A total of 15 µg of plant DNA was extracted (Dellaporta et al. 1983), digested with *Hind*III and electrophoresed in a 0.8% agarose gel. Plants from families 3, 4 and 5

which had been analyzed by Southern blotting were also assayed by northern analysis. RNA from tissue that had been collected prior to inoculations was extracted using an RNA Isolator kit (Genosys, The Woodlands, TX). A total of 20 µg of total RNA was electrophoresed in a 1.2% formaldehyde denaturing gel. Transfer of DNA or RNA and hybridization with a double-radiolabeled [<sup>32</sup>P] N gene probe were carried out according to the protocol of the membrane's manufacturer (MSI, Westborough, MA), except pre-hybridization and hybridization steps were done at 60°C.

### ELISA and western analysis

Tissue from all plants evaluated in the greenhouse from families 3, 4 and 5 was collected prior to inoculations to determine if individual plants were expressing the transgene N protein. DAS-ELISA tests were carried out according to the manufacturer with a diagnostic TSWV kit from Agdia (Elkhart, Indiana). TSWV N protein accumulation was estimated by reading microtiter plates at OD<sub>490</sub>. Reactions with either an OD<sub>490</sub> of 0.1 or three standard deviations greater than the mean of the healthy control were scored as positive. Detection of the N protein by western analysis was carried out as previously described (Sherman et al. 1998).

### Kanamycin assay for homozygosity

Resistant and susceptible F<sub>2</sub> plants from families 1 and 2 that harbored a single insertion were selected to determine if the insertion was homozygous or hemizygous (Tenllado and Diaz-Ruiz 1999). F<sub>3</sub> seeds from each F<sub>2</sub> parent plant were sown on plates of MS

Table 1. Percentage of TSWV-resistant transgenic tobacco plants from three generations of segregating families<sup>1</sup>.

Families	Generations <sup>2</sup>		
	Selfed transgenic parental generation <sup>3</sup>	F <sub>1</sub>	F <sub>2</sub>
Family 1 47-2-1(5)/Nt × 'KY14' Sense	50%	92%	33%
Family 2 47-2-1(5)/Nt × 'TN90' Sense	50%	25%	58%
Family 3 47-2-2(3)/Nt × 'TN86' Sense	83%	83%	64%
Family 4 47-2-2(5)/Nt × 'TN86' Sense	75%	33%	67%
Family 5 12-9-6/N- × 'KY14' Antisense	77%	10%	29%

<sup>1</sup>Plants were mechanically inoculated with TSWV-D, and final disease incidence was recorded at 7–10 weeks after inoculation. In all experiments, 100% of control plants (cv. Burley 21, KY14, TN86, TN90, and Burley 21 transformed with the vector construct pBI121) developed systemic symptoms.

<sup>2</sup>The generations evaluated included (1) selfed progeny from transgenic parent used in crosses, (2) F<sub>1</sub> hybrid progeny and (3) F<sub>2</sub> progeny.

<sup>3</sup>For families 1–4, selfed progeny was evaluated at the R<sub>4</sub> generation, while for family 5, selfed progeny was evaluated at the R<sub>3</sub> generation.

medium amended with 50 µg/ml of kanamycin, and segregation ratios for kanamycin resistance were calculated. Significant differences at the probability level of 0.05 were estimated by  $\chi^2$  tests.

## Results

### *Inheritance of TSWV resistance in transgenic tobacco families*

For each of the five families, the F<sub>1</sub>, F<sub>2</sub>, and selfed R<sub>3</sub> or R<sub>4</sub> generations were produced, and then plants of all generations were tested together in greenhouse inoculation tests (Table 1). For both transgenic plants and untransformed controls, local lesions developed on inoculated leaves. Plants were scored as susceptible if the virus spread systemically and produced

symptoms on uninoculated leaves. In all cases, control plants became severely diseased over the course of the experiments, with 100% of susceptible controls becoming systemically infected within 2–3 weeks. The response of the transgenic families varied, depending both on the family and on the generation (Table 1). Parents used to generate families 1, 2, 3, and 4 were resistant R<sub>3</sub> plants derived from sib plants of the same R<sub>2</sub> line and are highly related. Families 1 and 2 were derived from an R<sub>3</sub> plant from R<sub>2</sub> plant 47-2(1)/Nt, and families 3 and 4 were derived from two R<sub>3</sub> plants from R<sub>2</sub> plant 47-2(2)/Nt. The response of selfed and hybrid progeny from these parents varied. Only 50% of the plants of the selfed R<sub>4</sub> line derived from the transgenic parent plant of families 1 and 2 were resistant to TSWV infection. The proportion of resistant plants was lower than expected since the parental R<sub>3</sub> line from which its resistant parent was obtained was strongly resistant (89%) (data not shown).

The F<sub>1</sub> progeny of families 1–4 segregated for resistance, and major differences were seen in the expression of resistance between families (Table 1). Families 1 and 2 have the same common R<sub>3</sub> transgenic parent, 47-2-1(5)/Nt, and were developed by crossing this plant to either 'KY-14' (family 1) or 'TN-90' (family 2) (Figure 1). However, the response of the F<sub>1</sub> progeny differed dramatically, with 92% of the F<sub>1</sub> plants of family 1 showing resistance as compared to only 25% for family 2. Families 3 and 4 were generated by crossing two sib R<sub>3</sub> plants, 47-2-2(3)/Nt and 47-2-2(5)/Nt, both to 'TN-86'. Significant differences were seen between the two families with 83% and 33% of the F<sub>1</sub> plants of families 3 and 4, respectively, showing resistance to infection. Segregation was expected and was seen in the F<sub>2</sub> generation, but segregation ratios did not fit an obvious Mendelian pattern. Family 5, containing the antisense construct (pTSWVN-), also showed segregation for resistance in all generations tested. Of all families, family 5 showed the least resistance in the F<sub>1</sub> and F<sub>2</sub> generations.

### *Southern analysis*

To understand the basis of the unusual segregation patterns seen in expression of resistance, plants were analyzed by Southern analysis. Plants from families 1 and 2 had two N gene insertions (6.0 and 4.5 kb) (Figure 2). The 6.0 kb insertion segregated in selfed progeny and in the F<sub>1</sub> generation, indicating that the transgenic parent plant was hemizygous for this insertion. With one exception (a susceptible F<sub>1</sub> plant

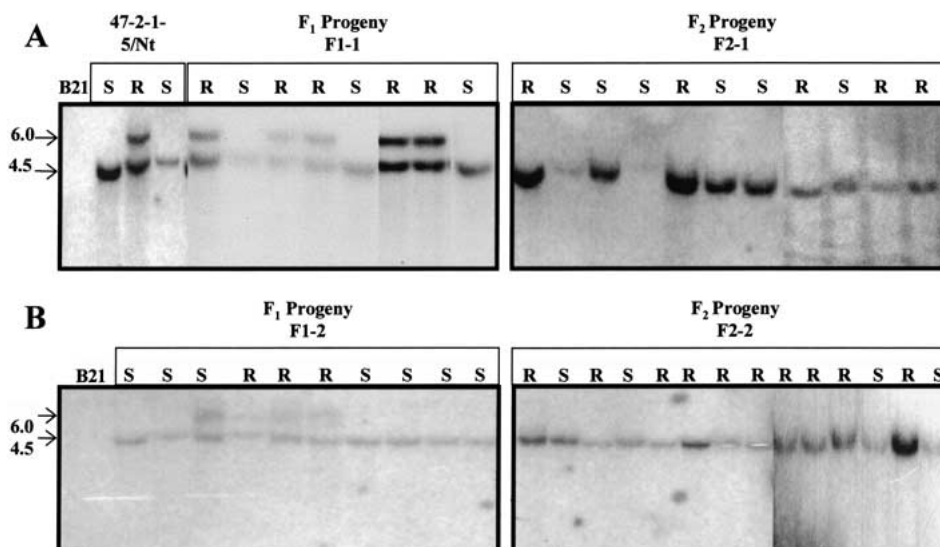


Figure 2. Southern analysis of selected susceptible (S) and resistant (R) plants from (A) family 1 and (B) family 2. B21 is the untransformed tobacco cv. Burley 21. Plants from three generations per family were analyzed: (1) parental R<sub>4</sub> transgenic line 47-2-1-5/Nt which is common to both families, (2) F<sub>1</sub> hybrids F1-1 and F1-2, and (3) F<sub>2</sub> lines F2-1 and F2-2. The size (kb) of detected insertions is indicated on the left-hand side of both panels.

from family 2 with two insertions; Figure 2B, lane 4), the presence of both insertions was correlated with resistance in the R<sub>4</sub> selfed progeny and in the F<sub>1</sub> generation. However, this correlation could not be established in the F<sub>2</sub> generation of either family, where all plants analyzed had only one insertion (4.5 kb) independent of the disease rating (Figure 2).

In order to determine if resistance or susceptibility was correlated with the homozygous or hemizygous state of this insertion, F<sub>3</sub> seed was collected from 2 resistant or 3 susceptible F<sub>2</sub> plants and screened for segregation for kanamycin resistance (Table 2). In all cases, F<sub>3</sub> progeny derived from susceptible F<sub>2</sub> plants segregated 3:1 for kanamycin resistance, indicating that the susceptible F<sub>2</sub> plants were hemizygous for the transgene. F<sub>3</sub> progeny derived from F<sub>2</sub> plants that were TSWV resistant did not segregate for kanamycin resistance, indicating homozygosity of the transgene insertion. These data indicate that the single 4.5 kb insertion is sufficient to impart resistance to TSWV infection if present in the plant in a homozygous state. If heterozygous, a second transgene insertion is required for resistance. Thus, for families 1 and 2, there was a strong correlation between the transgene dosage and the resistance phenotype.

Plants from families 3 and 4 were derived from two different parental sib plants from the same transgenic line, and all plants in all generations of both families

segregated for 2 insertions (6.0 kb and 8.0 kb) (Figures 3A and 4A). In addition, some plants of family 3 segregated for a 10 kb insertion (Figure 3A). Unlike families 1 and 2, there was no direct correlation between the number of insertions and TSWV resistance. Nonetheless, 82% of the resistant plants inherited the 8.0 kb insertion alone (12 plants) or in combination with the 6.0 kb insertion (11 plants), while 77% of the susceptible plants had only the 6.0 kb insertion (10 plants), with only 3 plants having both insertions. Consequently, in most instances the presence of the 8.0 kb insertion was correlated with the resistance phenotype. As some of the F<sub>1</sub> plants (hemizygous) in both families 3 and 4 had a single insertion and were also resistant, there was no correlation seen in these families between transgene dosage and TSWV resistance.

Members of family 5 harbored an antisense version of the N gene. We detected three segregating insertions (>13.0, 10.2 and 8.6 kb), and no correlation was found between the insertions present and the resistance response for any of the generations (Figure 5A).

#### Northern analysis

All plants from families 3, 4, and 5 that were analyzed by Southern blotting were also subjected to northern analysis (Figures 3B, 4B, and 5B). Two (1.0

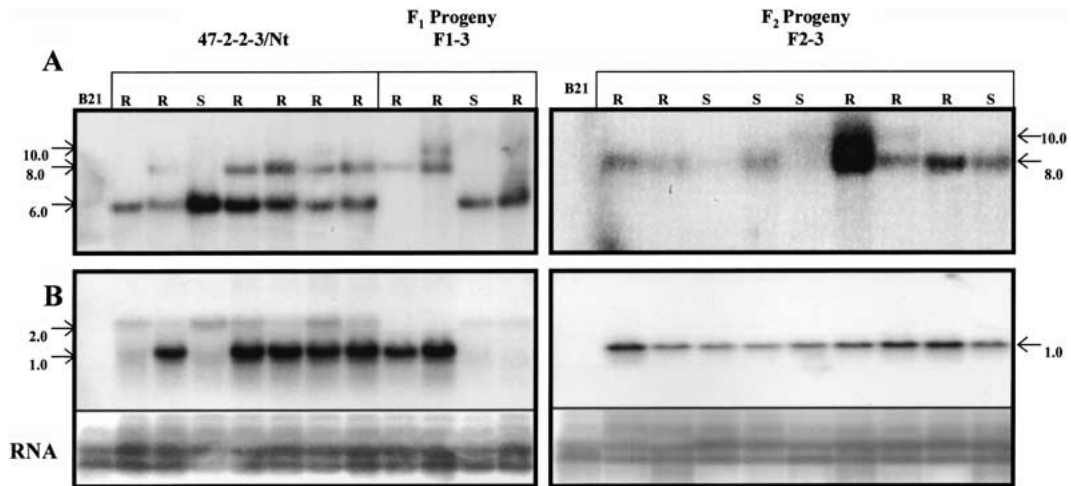


Figure 3. Southern (A) and northern (B) analysis of selected susceptible (S) and resistant (R) plants from family 3. B21 is the untransformed tobacco cv. Burley 21. Plants from three generations were tested: (1) the parental R<sub>4</sub> line 47-2-2-3/Nt, (2) F<sub>1</sub> hybrid F1-3, and (3) F<sub>2</sub> line F2-3. The size (kb) of detected insertions or transcripts is indicated on the left- and right-hand sides of both panels. The rRNA was detected after hybridization of the northern blot membrane with a ribosomal DNA probe to ascertain equal loading.

and 2.0 kb) and three different size transcripts (1.0, 1.6 and 2.0 kb) were observed for plants in families 3 and 4, respectively (Figures 3B and 4B). In both families, the 1.0 kb transcript was the most highly expressed. Accumulation of the 1.0 kb and 2.0 kb transcripts was strongly correlated with Southern profiles, suggesting that they may be separately transcribed by the 8.0 kb or 6.0 kb insertions, respectively. The 1.6 kb transcript in family 4 occurred in association with the 1.0 kb transcript, and it might be distinct transcript transcribed from the same 8.0 kb insertion. Such an observation suggests that the transgene may have undergone rearrangements during insertion or during subsequent generations. Alternatively, the possibility exists that premature termination of transcription or degradation intermediates of full-size RNA give rise to the formation of RNAs of different sizes (Goodwin et al. 1996; Morino et al. 1999).

From a total of 28 resistant plants analyzed from families 3 and 4, 14 plants showed significant accumulation of the strongly expressed 1.0 kb transcript, 9 plants were expressing both the 1.0 kb and 2.0 kb transcripts, and 5 had only the 2.0 kb transcript (Figures 3B and 4B). Of the susceptible plants, 4 plants expressed the 1.0 kb transcript, 10 plants expressed the 2.0 kb transcript, and 3 plants expressed both. Thus, although resistance is most often associated with the presence of the strongly expressed 1.0 kb transcript, the correlation is not absolute, indicating additional factors impact resistance.

A single transcript (1.3 kb) was detected for all members analyzed in family 5 (Figure 5B). Few differences were observed in transcript accumulation from plant to plant, and there was no direct correlation between transgene expression and the resistance phenotype.

#### ELISA and western analysis

All plants from families 3, 4 and 5 that were evaluated for resistance to TSWV were also tested for transgene protein expression using a DAS-ELISA kit. Transgene protein expression was not detected in any of the 119 plants analyzed from families 3 and 4. Additionally, 42 plants from family 3 were analyzed by western analysis with a polyclonal antiserum, and in no instance was accumulation of the N protein detected.

## Discussion

Numerous studies have used plants transformed with viral genes obtained from different transformation events to correlate the expression of transgenic resistance to various molecular events (Vaira et al. 1995; Goodwin et al. 1996; Pang et al. 1996, 1997; Prins et al. 1996; Sijen et al. 1996; Tenllado et al. 1999). In contrast with this approach, we developed and analyzed tobacco families segregating for the TSWV N gene that were derived from a single transformed resistant plant. The use of such families resembles the

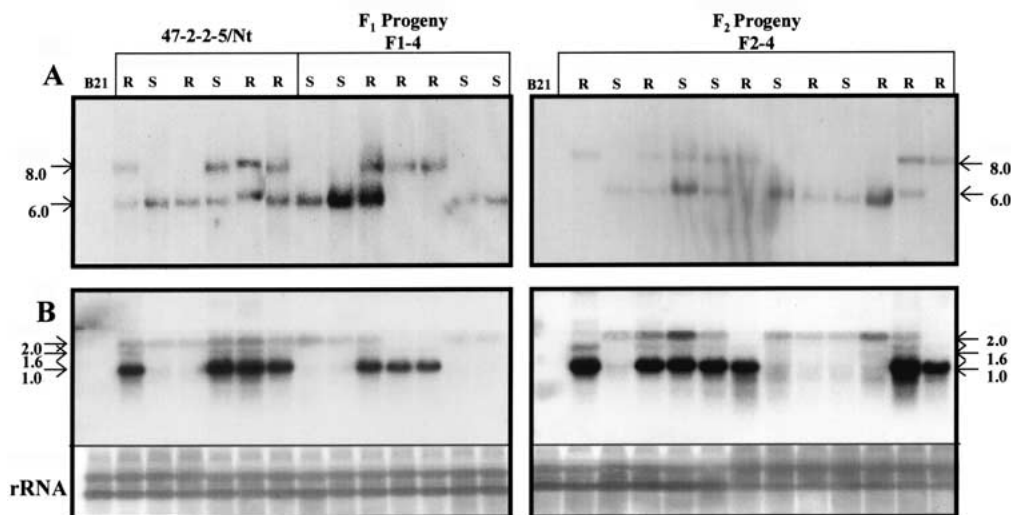


Figure 4. Southern (A) and northern (B) analysis of selected susceptible (S) and resistant (R) plants from family 4. B21 is the un-transformed tobacco cv. Burley 21. Plants from three generations were tested: (1) the parental R<sub>4</sub> line 47-2-2-5/Nt, (2) F<sub>1</sub> hybrid F1-4, and (3) F<sub>2</sub> line F2-4. The size (kb) of detected insertions or transcripts is indicated on the left- and right-hand sides of both panels. The rRNA was detected after hybridization of the northern blot membrane with a ribosomal DNA probe to ascertain equal loading.

populations used in breeding programs and thus allowed us both to investigate the usefulness of resistant transgenic parents in establishing a breeding population, and to elucidate the molecular determinants of viral protection that co-segregate with the resistance phenotype.

Our results support many of the conclusions of studies based on analyzing different transgenic plants. For example, molecular analysis of families 1 and 2 indicated that in these families, transgene dosage correlates with transgenic virus resistance in an additive manner. These results support previous findings with tobacco etch virus (Goodwin et al. 1996), pepper mild mottle tobamovirus (Tenllado et al. 1999), and TSWV (Pang et al. 1996). In these studies the authors observed that hemizygous progeny had reduced levels of resistance as compared to homozygous resistant plants. In addition, our observations support previous results where sense constructs (families 1–4) are able to confer resistance more efficiently than antisense ones (family 5) (Pang et al. 1993; Smith et al. 1994).

It is well known that resistance can be mediated by either the transgene protein or accumulation of the RNA transcript in the absence of protein expression (Miller and Hemenway 1998; Baulcombe 1999). Because we were not able to detect nucleocapsid protein accumulation in any of the plants analyzed by either ELISA or western blotting, we conclude that protec-

tion in resistant plants is more likely RNA-mediated. This type of resistance is brought about by a process known as homology-dependent post-transcriptional gene silencing that is characterized by the accumulation in the nucleus of transgene-derived RNA that is post-transcriptionally degraded in the cytoplasm (van den Boogaart et al. 1998). In our study, analyzed resistant plants generally accumulated high levels of steady-state RNA, contrasting with previous reports where low steady-state mRNA accumulation was observed in resistant plants (Goodwin et al. 1996; Pang et al. 1996; Sijen et al. 1996). Sampling for northern analysis was done prior to inoculation, therefore it is possible that the proposed post-transcriptional gene silencing mechanism is triggered only upon inoculation and viral replication, after which threshold RNA levels would be high enough for a degradation mechanism to be turned on (Baulcombe 1996).

As pointed out in Results, different size transcripts were detected. A possible explanation is that the transgene may have undergone rearrangements and/or that transcripts are defective. Our data are most consistent with the hypothesis that the single 6.0 kb insertion is transcribed into a 2.0 kb transcript, and the 8.0 kb insertion into a 1.6 kb transcript. Premature termination of this 8.0 kb transgene or degradation of full-size RNA transcripts may give rise to a distinct 1.0 kb fragment that accumulates to very high levels (Goodwin et al. 1996; Morino et al. 1999).

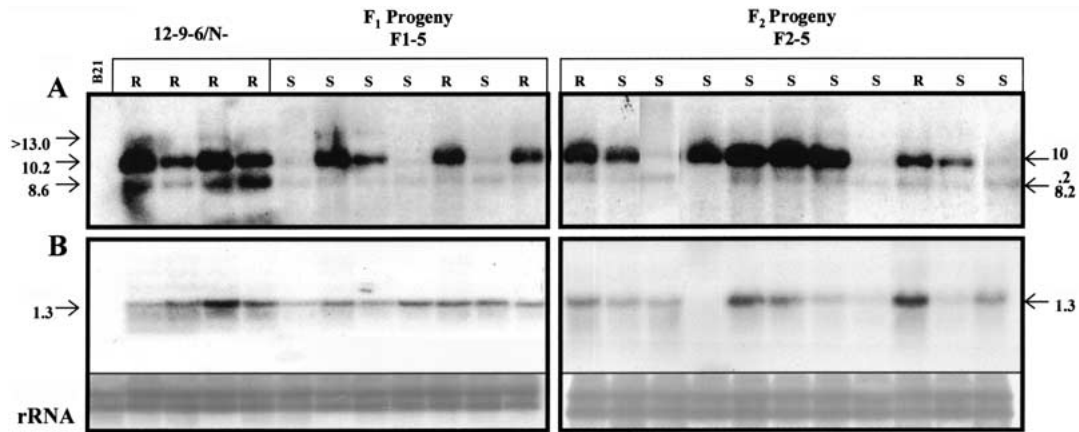


Figure 5. Southern (A) and northern (B) analysis of selected susceptible (S) and resistant (R) plants from family 5. B21 is the untransformed tobacco cv. Burley 21. Plants from three generations were tested: (1) the parental R<sub>3</sub> line 12-9-6/N, (2) F<sub>1</sub> hybrid F1-5, and (3) F<sub>2</sub> line F2-5. The size (kb) of detected insertions or transcripts is indicated on the left- and right-hand sides of both panels. The rRNA was detected after hybridization of the northern blot membrane with a ribosomal DNA probe to ascertain equal loading.

Table 2. Segregation ratios for kanamycin resistance in F<sub>3</sub> progeny derived from TSWV-susceptible or resistant F<sub>2</sub> plants of families 1 and 2.

Family	F <sub>2</sub> plant phenotype <sup>1</sup>	Number of transgene insertions in F <sub>2</sub> plant <sup>2</sup>	Number of kanamycin-resistant F <sub>3</sub> progeny/ number tested		$\chi^2$ <sup>3</sup>	
			observed	expected	(3:1)	(1:0)
1	Susceptible	1	33/45	34/45	0.02	n.a. <sup>4</sup>
1	Susceptible	1	86/117	88/117	0.04	n.a.
1	Resistant	1	92/93	93/93	n.a.	0.01
1	Resistant	1	98/98	98/98	n.a.	0.00
2	Susceptible	1	67/93	70/93	0.11	n.a.

<sup>1</sup>Phenotype of F<sub>2</sub> plant of families 1 and 2.

<sup>2</sup>Number of insertions detected by Southern hybridization. All plants contained only a single 4.5 kb insertion.

<sup>3</sup> $\chi^2$  of 3.84 is significant at  $p \leq 0.05$ . The expected ratios from a F<sub>2</sub> hemizygous or homozygous F<sub>2</sub> plant are 3:1 and 1:0 (R:S) respectively.

<sup>4</sup>Not applicable.

In addition to analyzing molecular determinants of resistance, our study also points out potential problems that may be encountered when using transgenic materials as parents in a breeding program. All transgenic parents used in this study were derived from selfing resistant plants through multiple generations. In spite of using plants from advanced (R<sub>3</sub>) generations, analysis of the families demonstrated that plants were still segregating for the transgene, even in the R<sub>4</sub> generation. We selected and advanced our lines based on disease resistance, and the material used for this study came from the most resistant lines available (Herrero et al. 2000). The maintenance of heterozygosity in these lines suggests that a certain level of heterozygosity,

which is greater in lines containing multiple transgene insertions, is advantageous for the expression and stability of resistance. If so, we would hypothesize that the presence of multiple homozygous transgene copies might favor the loss of resistance. This hypothesis is supported by findings where transgene methylation, and thus gene inactivation, correlated directly with the number of integrated copies (Meyer 1995). Thus advancing of lines based on screening for resistance alone may not be sufficient to establish stable, homozygous lines for use as breeding stock.

In summary, we used transgenic tobacco families transformed with the TSWV N gene to investigate their usefulness as breeding material and to elucidate

the molecular determinants of viral protection by analyzing segregating populations. We conclude that the use of transgenic resistant parents introduces additional variables to the breeding process, as inheritance of the transgene does not guarantee expression of resistance. Although our data suggest that resistance correlated either with gene dosage or with the presence of a highly transcribed insertion, the correlation was not absolute. In every case, plants were recovered that contained the appropriate gene dosage or a highly transcribed insertion, and remained susceptible to infection. These results demonstrate that factors other than transgene inheritance and expression impact the expression of transgenic resistance. Thus the ability to generate populations of uniformly resistant plants through breeding may be more problematic than with conventional resistance genes.

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