

Broad Resistance to Tospoviruses in Transgenic Tobacco Plants Expressing Three Tospoviral Nucleoprotein Gene Sequences

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Transgenic tobacco plants have been obtained expressing nucleoprotein (N) gene sequences of three different tospoviruses known to affect vegetable crops: tomato spotted wilt virus (TSWV), tomato chlorotic spot virus (TCSV), and groundnut ringspot virus (GRSV). The chimeric plant transformation vector used comprised the three viral N gene sequences, each with a copy of the CaMV 35S promoter and the nos terminator. Despite the high levels of homology between the different N gene sequences (74–82%) and the presence of repeated promoter and terminator sequences in this construct, unrearranged copies of this triple N gene construct were stably maintained in both *Escherichia coli* and *Agrobacterium tumefaciens* plasmids used during the cloning process, as well as in several generations of transgenic tobacco plants. A transgenic tobacco line was obtained that exhibited high levels of resistance to all three tospoviruses, showing the possibility of producing transgenic plants with a broad resistance to tospoviruses by introducing tandemly cloned viral N gene sequences. DNA analysis of this transgenic plant line shows that the multiviral resistance trait is confined to a single genetic locus, which is very convenient for further breeding purposes.

Additional keywords: *Nicotiana tabacum*, nucleoprotein gene, virus resistance.

The tospoviruses are a group of plant-infecting, negative-strand RNA viruses, which form a separate genus within the arthropod-borne family of Bunyaviridae (Francki *et al.* 1991). Based on serological differences (Avila *et al.* 1992) and sequence divergence of the nucleoprotein gene (Avila *et al.* 1993), four different tospoviruses have so far been identified: tomato spotted wilt virus (TSWV), tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV), and impatiens necrotic spot virus (INSV).

The type species of the genus *Tospovirus*, TSWV, has a very broad host range, encompassing more than 400 plant species within 50 families (Peters *et al.* 1990), including many important crops and ornamentals. Also the host ranges

of both TCSV and GRSV are very broad (Avila *et al.* 1992), while the host range of INSV is relatively narrow and mainly restricted to ornamental plants (Law and Moyer 1990).

Tospoviruses are the only plant viruses that are transmitted by thrips species (*Thysanoptera*) in a propagative manner (Sakimura 1962; Wijkamp *et al.* 1993). The complete nucleotide sequence of the three genomic RNAs of TSWV has been elucidated (De Haan *et al.* 1990, 1991; Kormelink *et al.* 1992) and revealed the presence of five open reading frames that specify six mature viral proteins. The L RNA is of negative polarity and encodes the putative viral polymerase of 331.5 kDa, present in virus particles (Van Poelwijk *et al.* 1993). The M and S RNAs both have an ambisense coding arrangement. The M RNA codes for the precursor of the membrane glycoproteins G1 and G2 of 78 and 58 kDa, respectively, and a nonstructural protein (NS_M) of 33.6 kDa, which represents the putative viral cell-to-cell movement protein (Kormelink *et al.* 1994). The S RNA codes for the nucleoprotein (28.8 kDa) and another nonstructural protein (NS_S) of 52.4 kDa.

Engineered resistance to tomato spotted wilt tospovirus (TSWV) has been accomplished by expressing the viral nucleoprotein (N) gene in transgenic tobacco (Gielen *et al.* 1991; MacEnzie and Ellis 1992; Pang *et al.* 1993) and tomato plants (Ultzen *et al.* 1994). Similar levels of protection have been obtained when an untranslatable N gene was expressed, indicating that the resistance is, at least for a major part, RNA mediated (De Haan *et al.* 1992).

Tobacco and tomato plants expressing TSWV N gene sequences are only resistant to isolates and strains of TSWV but not to other tospoviruses, such as TCSV and GRSV (De Haan *et al.* 1992; Ultzen *et al.* 1994). Although heterologous tospovirus protection in plants expressing high levels of TSWV N protein has been reported (Pang *et al.* 1993, 1994), this protection concerned limited delay in symptom development rather than immunity as observed for RNA-mediated resistance. Furthermore, delay of symptom development was only observed for INSV, but not for TCSV, a virus more closely related to TSWV than INSV.

Although the economic impact of novel tospoviruses remains to be further established, it is clear that TSWV resistance should be extended to resistance against the other

tospoviruses TCSV and GRSV in vegetable crop plants, and to INSV in ornamental plants.

As a first step towards such broad spectrum virus resistance in vegetable crops, a DNA construct has been made comprising the N genes of the three different vegetable crop-infecting tospoviruses.

We here demonstrate that this construct is genetically stable and capable of conferring high levels of resistance to all three tospoviruses.

RESULTS

Transformation of tobacco with three different tospoviral N genes.

A chimeric DNA construct, pTOSPO 3N-A, was made, comprising three different tospoviral nucleoprotein gene sequences, derived from TSWV, TCSV, and GRSV (Fig. 1). Each tospoviral N gene was supplied with a copy of the

CaMV 35S promoter. The original tospovirus-specific leader sequence of 123–124 nucleotides in length was maintained in front of all three N genes. At the 3' end of the N cistrons the transcription-termination signal of the nopaline synthase (nos) gene was inserted. The N gene cassettes were subsequently cloned into the binary vector pBIN19 in the order "Left Border-TSWV-TCSV-GRSV-NPTII-Right Border." Finally, the combined pTOSPO 3N-A cassette was introduced in *Nicotiana tabacum* 'SR1' plants, via *A. tumefaciens*-mediated leaf disk transformation.

To determine the amount of transgenically produced tospoviral N proteins, leaf extracts of transgenic plants were used in a Western blot analysis. Remarkably, in all cases the total amount of N proteins accumulating in the pTOSPO 3N-A transformed tobacco plants was low (data not shown) when compared to the amounts in the previously analyzed TSWV N protein expressing plants (Gielen *et al.* 1991). In addition, low levels of transgenic transcripts were produced, albeit at

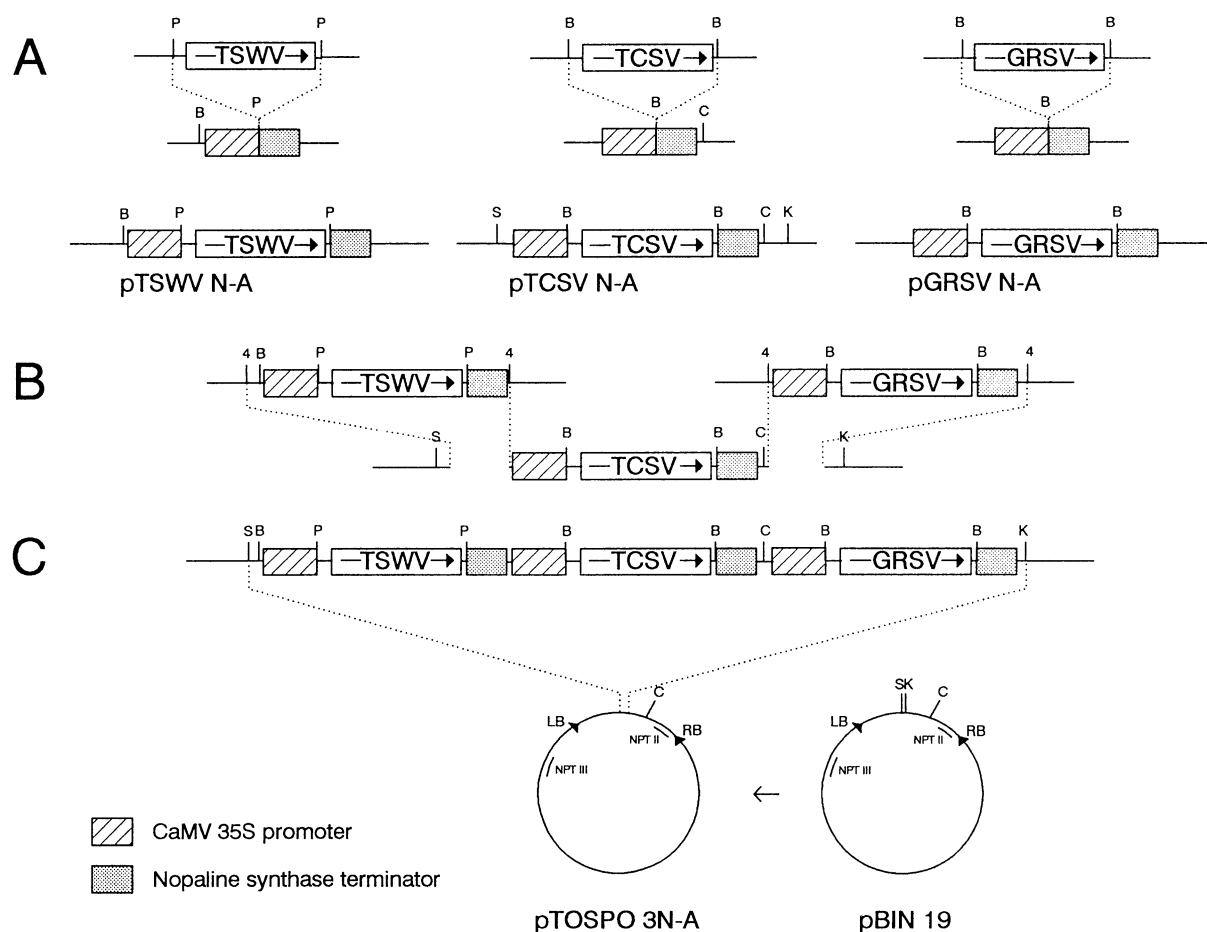


Fig. 1. Construction of the triple N gene containing plant transformation vector pTOSPO 3N-A. A and B indicate the construction of progenitor plasmids, while C shows the construction of the final transformation construct pTOSPO 3N-A. A, Sequences containing the complete open reading frames of the TSWV, TCSV, and GRSV nucleoproteins were independently cloned in pZU-A vectors. The TCSV and GRSV cDNA clones were inserted in the *Bam*HI site of pZU-A(Bam), creating pTCSV N-A and pGRSV N-A, respectively. The TSWV N gene had been previously cloned in the *Pst*I site of pZU-A(Pst) by Gielen *et al.* (1991) and designated pTSWV N-A. B, The expression cassettes of the TSWV and GRSV N genes were subsequently cloned into pTCSV N-A by blunt-end ligation of T4 DNA polymerase-treated DNA fragments, since no useful compatible sticky restriction sites were available. C, Finally the triple N gene expression cassette was cloned between the left and right border sequences of the pBIN19 transformation vector, resulting in construct pTOSPO 3N-A. Sites used in the cloning process as well as in the Southern blot analysis (Fig. 3A and B) are indicated. B = *Bam*HI; C = *Cla*I; K = *Kpn*I; S = *Sst*I; P = *Pst*I; 4 = blunt after treatment with T4 DNA polymerase. Positions of the selection markers NPTII and NPTIII as well as left-(LB) and right border sequences (RB) in the pBIN19 and pTOSPO 3N-A vectors are also shown.

an equal level as some TSWV resistant lines described by De Haan *et al.* (1992).

Resistance levels in transgenic tobacco plants.

S1 progeny plants of 22 original transformants were first assayed for resistance to inoculation with tomato chlorotic spot virus (TCSV, strain BR-03). Resistance levels of up to 65% were observed in these segregating populations. Four of the 22 lines showed a considerable level of resistance (30–65% resistance), from each of these lines up to eight plants were maintained for seed production after self-pollination. The S2 progeny of the resistant lines 3, 6, 10, and 14 was subsequently inoculated with TCSV, TSWV, or GRSV, and monitored for the development of systemic disease symptoms. Asymptomatic plants were tested in ELISA for the presence of NS_s protein. In none of these plants detectable amounts of NS_s protein could be found after virus inoculation, indicating that these plants remained free of virus. The levels of resistance in the different transgenic tobacco lines to the different tospoviruses are listed in Table 1. In all of these experiments, nonexpressing segregants (SR1-c) as well as previously described TSWV N gene expressing plants (SR1-12), which are resistant to TSWV only (Gielen *et al.* 1991), were used as negative or positive controls, respectively.

Two lines (lines 10 and 14) showed a clear delay of 3–8

days in the development of systemic symptoms, but appeared to be only moderately protected to the tospoviruses tested. Some S2 progeny lines derived from line 6 displayed immunity to inoculation with TCSV, but were more susceptible to the other tospoviruses, which might be explained by the initial selection for TCSV resistance in the S1 generation. One of the lines (line 3), however, showed— independent of the amount of virus used in the inoculations— high levels of resistance to all three viruses separately. Plants of three of these S2 lines were also simultaneously inoculated with all three viruses and displayed high levels of resistance as listed in Table 2 and exemplified in Figure 2. Immunological analysis (ELISA) confirmed that the symptomless plants remained virus-free and that the broad protection was based on true immunity.

Stability of the construct in bacteria and plants.

Three identical copies of the promoter (CaMV 35S) and terminator (nos) sequences are present in the pTOSPO 3N-A DNA construct, whereas the different cloned N gene sequences are highly homologous (72–84% sequence identity). It was considered that the repetition of highly homologous sequences could have caused genetic rearrangements in the pTOSPO 3N-A construct in bacteria or in transgenic plants after introduction in the genomic DNA. Therefore, the integrity of this construct was carefully monitored during passages in *Escherichia coli* strain DH5 α F' and *A. tumefaciens* strain LB4404, respectively. Although recombination in the *Rec A*[–] *E. coli* strain DH5 α F' could not be expected, the occurrence of recombination in *A. tumefaciens* still seemed possible. Southern blot analyses of total DNA of *A. tumefaciens* used in the transformation experiments, however, always showed the presence of the unaltered pTOSPO 3N-A DNA construct (data not shown).

Finally, Southern blot analyses of S2 progeny plants of original tobacco transformants (Fig. 3A and B), demonstrated integration of unrearranged copies of the triple N gene cassette in the genomes of those plants. Hence, even after several generations, this construct was stably maintained in the genome of transgenic plants and did not undergo major genetic rearrangements, thereby showing that this method is useful for introducing resistance to several different viruses by a single transformation.

Number of transgenic insertions.

Southern blot analysis of several transgenic S2 plants revealed a single large DNA fragment when genomic plant DNA was digested with *Cla*I and probed with TSWV N gene sequences (Fig 3A, lane C). In addition, slight cross-hy-

Table 2. Resistance levels to simultaneous inoculation with tospoviruses TSWV, TCSV and GRSV in S2 progeny of transgenic tobacco plants transformed with the pTOSPO 3N-A construct

S2 line	Number of resistant plants upon simultaneous inoculation with TSWV, TCSV, and GRSV	
3-4	20/20	
3-5	15/20	
3-6	20/20	
SR1-c	0/20	
SR1-12	0/20	

Table 1. Resistance levels to TSWV, TCSV, and GRSV inoculation in S2 progeny of transgenic tobacco plants transformed with the pTOSPO 3N-A construct

S2 line	Number of resistant plants upon inoculation with		
	TSWV	TCSV	GRSV
3-1	10/10	8/10	9/10
3-2	10/10	7/10	5/10
3-3	10/10	5/10	8/10
3-4	10/10	9/10	8/10
3-5	7/10	5/10	7/10
3-6	10/10	9/10	9/10
6-1	0/10	3/10	0/10
6-2	6/10	10/10	0/10
6-3	6/10	7/10	5/10
6-4	3/10	10/10	1/10
6-5	7/10	4/10	3/10
6-6	7/10	10/10	6/10
6-7	1/10	8/10	1/10
10-1	1/10	1/10	0/10
10-2	1/10	1/10	0/10
10-3	1/10	3/10	0/10
10-4	1/10	1/10	0/10
10-5	4/10	1/10	0/10
10-6	5/10	1/10	0/10
10-7	0/10	0/10	2/10
10-8	1/10	3/10	0/10
14-1	3/10	0/10	0/10
14-2	1/10	0/10	0/10
14-3	5/10	0/10	0/10
14-4	0/10	0/10	0/10
14-5	1/10	2/10	0/10
14-6	3/10	4/10	0/10
SR1-c	0/10	0/10	0/10
SR1-12	10/10	0/10	0/10

bridization to a 2.4-kb pTOSPO 3N-A specific *Cla*I fragment containing sequences of the GRSV N gene was observed. In view of the positions of the two *Cla*I sites in the pTOSPO 3N-A transgene insert (Fig. 1), the observation of only a single genomic DNA fragment containing TSWV N gene sequences, demonstrates that the original transformants 3 and 6 contained a single transgenic insertion in their genome. The insertion sites, however, were different for both original transformants, considering the different sizes of the hybridizing genomic *Cla*I fragments. The presence of the TSWV N gene used as a probe, plus bordering promoter and terminator sequences in the DNA of all transgenic plants tested, is shown by *Bam*HI fragments of the expected size of 2.0 kb (Fig. 3A, lanes B).

DISCUSSION

Previous studies in our laboratory (Gielen *et al.* 1991; De Haan *et al.* 1992) have shown that transgenic plants expressing TSWV viral N gene are only resistant to strains and isolates of the homologous virus but not to other tospoviruses, even though there is a considerable sequence homology between these viruses with respect to the transgenically expressed sequences. This is also true for most other reported cases of virus resistance based on transgenically expressed pathogen-derived sequences (reviewed in Wilson 1993). In only a few cases low but significant levels of resistance have been observed to more than one virus (Stark and Beachy 1989; Lawson *et al.* 1990; Donson *et al.* 1993).

As a first step towards a broad resistance to tospoviruses, a DNA construct has been made to investigate the feasibility of simultaneous introduction of three tospoviral N gene sequences. Following this approach, resistance against three tospoviruses was aimed, tomato spotted wilt virus (TSWV), tomato chlorotic spot virus (TCSV), and groundnut ringspot virus (GRSV). These three tospoviruses have overlapping host ranges and are known to infect crop plants such as tomato, (sweet) pepper, lettuce, melon, and peanut. Impatiens necrotic spot

virus (INSV), was not included in these experiments, since INSV is unable to induce systemic symptoms in *N. tabacum* and is, moreover, not a vegetable crop-infecting tospovirus.

Despite high homologies in the sequences used and identity of the promoter and terminator regions, the TOSPO 3N-A construct was stable in *E. coli* and *A. tumefaciens* and inherited unaltered over several generations of plants. Apparently, the incidence of homologous recombination within the triple N gene cassette in the used plant and bacterial systems is very low, and only unrearranged forms of multiple N gene expressing constructs are present in transgenic plants. The stability of highly homologous repeats introduced in plant genomes has not been studied in great depth, although several other multiple transgene products with several copies of identical promoter/terminator cassettes have been reported and used (e.g., Lawson *et al.* 1990; Yie *et al.* 1992), apparently without construct stability problems.

In this paper it is shown that broad tospovirus resistance can be accomplished by transforming tobacco plants with a multiple N gene-expressing DNA construct. Broad spectrum resistance to the three different tospoviruses TSWV, TCSV, and GRSV has been obtained in transgenic *N. tabacum* 'SR1' plants. Our previous research (De Haan *et al.* 1992) has shown that N gene-based resistance to TSWV is mainly, if not completely, caused by the presence of the transgene RNA transcript rather than the translation product, a phenomenon also reported for several other plant-virus combinations (Van der Vlugt *et al.* 1992; Lindbo and Dougherty 1992). This suggests a resistance mechanism based either on antisense inhibition of virus multiplication by direct RNA-RNA interaction between transgenic and viral RNAs, or sense inhibition of the N gene messenger or the viral complementary-strand RNAs, by a mechanism that involves cytoplasmic breakdown of specific RNA sequences induced by the expression of transgenic RNA (Lindbo *et al.* 1993). The induced breakdown of specific RNA sequences may also explain the small amounts of transgenic RNA observed in Northern blot analysis, while, on the contrary, the observed levels of resistance are very high. However, it cannot be

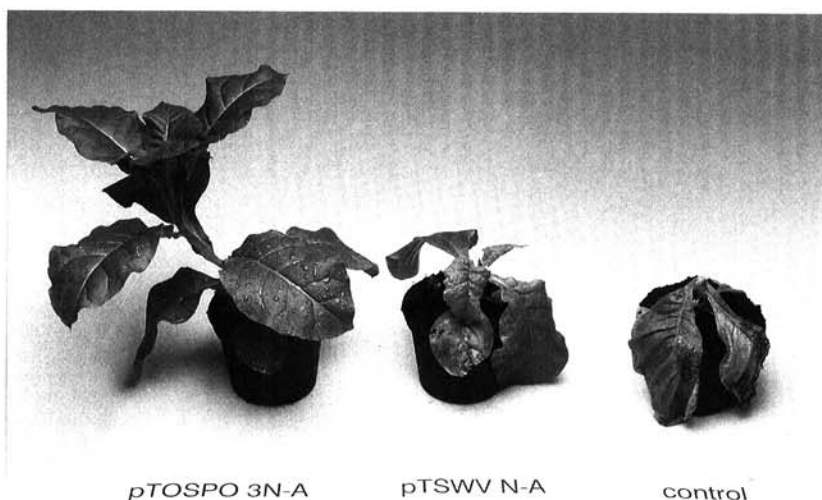


Fig. 2. Phenotype of tobacco plants mechanically inoculated with TSWV, TCSV, and GRSV simultaneously. pTOSPO 3N-A: Plant of multiple tospovirus resistant line 3-6 transformed with the pTOSPO 3N-A construct. pTSWV N-A: Plant of line 12 transformed with the pTSWV N-A construct, only resistant to TSWV, but susceptible to TCSV and GRSV. control: Nontransgenic *Nicotiana tabacum* 'SR-1' plant.

excluded that the observed low level of expression of the transgene product (RNA or protein) in leaf samples, is the result of variations in activity of the CaMV 35S promoter in various tissue and cell types (Benfey *et al.* 1989a, 1989b).

Although the introduced genes are integrated at the same locus in the plant genome, they phenotypically behave as three independent genes in terms of resistance (see Table 1). Transgenic line 6 for instance displayed a clear difference in the levels of protection to the different tospoviruses, while, on the contrary, line 3 exhibited similar high resistance levels for all three viruses. Apparently, resistance to one of the tospoviruses does not automatically imply that the plant is also resistant to the other viruses. Screening for resistance to all three viruses over several generations of transgenic plants is necessary, to select the proper transgenic lines. This seemingly independent behavior of the three genes may be the result of the site of insertion in the plant genome. Moreover, different levels of resistance to viruses of which the active transgenes are incorporated at the same locus in the plant genome, can be caused by co-suppression due to the presence of three identical promoter sequences, which may favor the expression of one of the three genes (Matzke and Matzke 1993).

In the approach described in this paper, sequences from different viruses are transgenically introduced to one locus in the plant genome. The expression of these sequences results in high levels of resistance to three different vegetable-infecting tospoviruses. This approach may be further extended to other viruses, thereby providing a flexible strategy for creating broad spectrum virus resistance in transgenic plants. If desired, resistance traits can be stacked by crossing several of these transgenic plant lines.

MATERIALS AND METHODS

All methods involving DNA or RNA were according to standard procedures (Sambrook *et al.* 1989).

Viruses and plants.

The different tospovirus strains, i.e., TSWV strain BR-01, TCSV strain BR-03, and GRSV strain SA-05, have been described by Avila *et al.* (1990, 1992, 1993) and were maintained on systemic hosts *Nicotiana rustica* 'America' or *N. tabacum* 'SR1.'

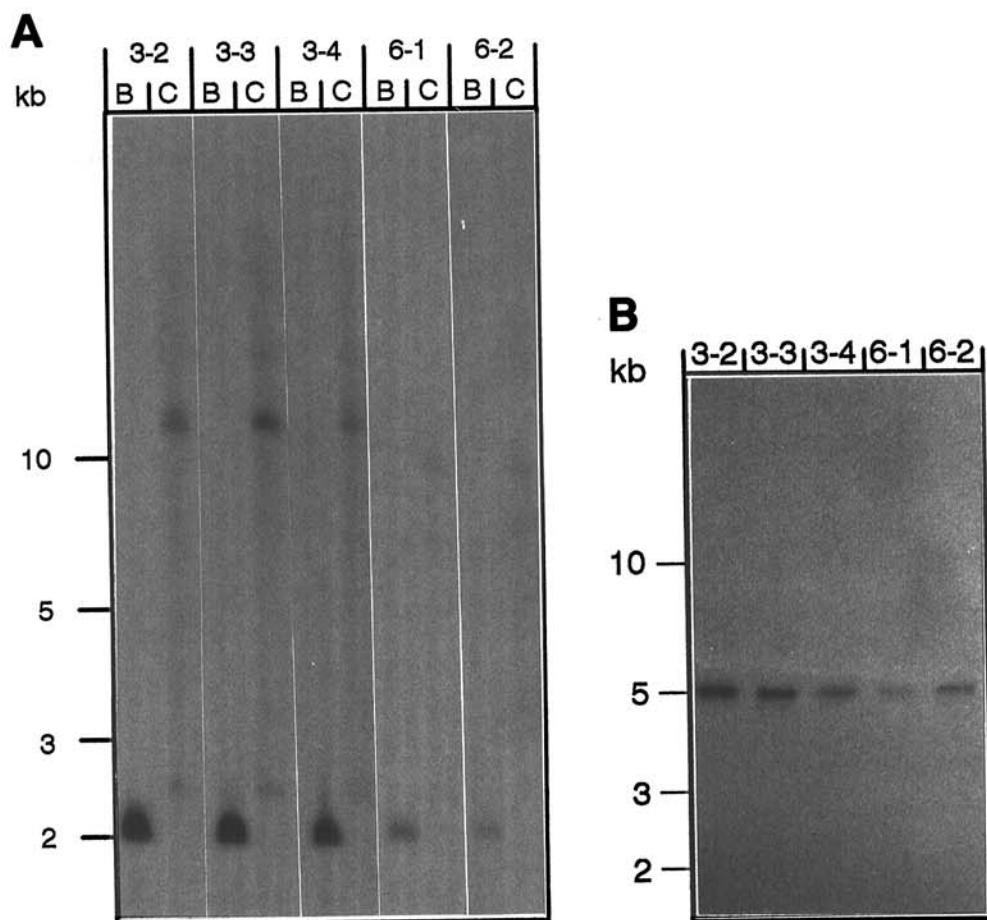


Fig. 3. A, Southern blot analysis of genomic DNA of pTOSPO 3N-A transformed S2 tobacco lines derived from two different original transformants (3 and 6). Total genomic DNA was isolated from 4-wk-old plants, digested with the appropriate restriction enzymes and fractionated on a 1% agarose gel, transferred to a Hybond membrane and hybridized to a 32 P-dATP-labeled DNA fragment containing the complete TSWV N gene. The numbers above the lanes correspond to the transgenic tobacco lines. Two different restriction enzymes were used in this experiment. B = *Bam*HI; C = *Cla*I. Sites in the pTOSPO 3N-A construct recognized by these two enzymes, are indicated in Figure 1. **B,** Southern blot analyses of plants from S2 lines 3-2, 3-3, 3-4, 6-1, and 6-2 after digestion with both *Sst*I and *Kpn*I, releasing the complete 5.0-kb insert containing the three N genes with their respective promoter and terminator sequences. This shows these lines contain the intact 5.0-kb triple N gene cassette.

Recipient plants used in the transformation experiments were *N. tabacum* 'SR1' plants. All manipulations with transgenic plant material were carried out under conditions (PKII) imposed by the Dutch authorities (VROM/VCOGEM).

Construction of the multiple nucleoprotein expression vector.

Nucleoprotein gene sequences of TCSV and GRSV were obtained from cDNA clones described by Avila *et al.* (1993), and cloned in the plant transformation vector pZU-A (Gielen *et al.* 1991) between a copy of the CaMV 35S promoter and a copy of the nopaline synthase (nos) terminator. The TSWV N gene construct used has previously been described as TSWV N-A (Gielen *et al.* 1991). The TSWV and GRSV expression cassettes were subsequently cloned in the TCSV N gene containing vector and finally, the triple N gene construct was inserted in the binary vector pBIN19 (Bevan 1984). Details of the cloning schedule are presented in Figure 1.

Transformation of tobacco.

The pBIN19-derived vector pTOSPO 3N-A was introduced in *A. tumefaciens* strain LB4404 (Ditta *et al.* 1990) by triparental mating, using pRK2013 (Horsch *et al.* 1985) as a helper plasmid.

N. tabacum 'SR1' plants were transformed and regenerated as described by Horsch *et al.* (1985).

Analysis of protection of transgenic plants against TSWV, TCSV, and GRSV.

Twenty S1 progeny plants from 22 original pTOSPO 3N-A transformed *N. tabacum* 'SR1' plants, were inoculated with TCSV, approximately 5 wk after sowing. Resistant plants were left to set seeds. From a selection of S2 progeny lines, 10 plants were inoculated with each of the three different tospoviruses TSWV, TCSV, and GRSV independently, and 20 plants with the three viruses simultaneously. Inoculation was done according to standard procedures (Gielen *et al.* 1991). The appearance of systemic symptoms was monitored on a daily basis. Plants were scored susceptible when leaves younger than the inoculated leaf showed severe stunting and chlorosis, usually followed by death of the plant within a week. Approximately 5 wk after the first inoculation, leaf samples from visually healthy plants were collected to check for the presence of the NS_s gene product using ELISA, using a polyclonal antisera directed against TSWV NS_s protein expressed in a baculovirus expression system (Kormelink *et al.* 1991). This antiserum also strongly cross reacts with NS_s proteins present in TCSV- and GRSV-infected plant cells.

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