CHARACTERISATION OF TOSPOVIRUS RESISTANCE IN TRANSGENIC PLANTS

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STELLINGEN

1. Het is mogelijk planten te (pre)immuniseren tegen virussen door ze te transformeren met virale sequenties en daarmee een al aanwezig mechanisme vroegtijdig te activeren.

   Dit proefschrift; Lindbo *et al.* (1993) *Plant Cell* **5**: 1749-1759; Mueller *et al.* (1995) *Plant Journal* **7**: 1001-1013; Sijen *et al.* (1996) *Plant Cell* **8**: 2277-2294.

2. Waardplantresistentie tegen het geminivirus ACMV, op basis van transgene expressie van het BC1 gen, is waarschijnlijk "RNA-mediated".

   Hong and Stanley (1996) *Molecular Plant-Microbe Interactions* **9**: 219-225

3. De conclusie dat TSWV glycoproteïnen ophopen aan de plasmalemma van met recombinant baculovirus geïnfecteerde *Spodoptera frugiperda* cellen op basis van perifere labeling met glycoproteïne-antilichamen, gaat voorbij aan de pathologie van het gebruikte vectorsysteem en is derhalve prematuur.

   Adkins *et al.* (1996) *Phytopathology* **86**: 849-855.

4. De titel van een onlangs verschenen proefschrift: "Quantitative Resistance to Peanut Bud Necrosis Virus in Groundnut", geeft aan dat met de naamgeving van dit tospovirus onzorgvuldig is omgesprongen.

   Buiel (1996) Proefschrift, LU Wageningen.

5. De opvallend snelle ontwikkeling van een complexe prebiotische "RNA wereld", tesamen met indicaties voor fossiele biogene activiteit op Mars, suggereren dat de "RNA wereld" niet op aarde is ontstaan.

   Gersteland and Atkins (eds.) The RNA world. (1993) Cold Spring Harbour Press, USA; McKay *et al.* (1996) *Science* **273:** 924-930.

6. Het promoveren binnen vier jaar wordt extra bemoeilijkt door het beperkte aantal geschikte data dat de universiteit daarvoor beschikbaar stelt.

7. Alle commotie rond de invoering van de nieuwe spelling van de Nederlandse taal is nogal overdreven, aangezien door de huidige opmars van de Engelse taal als voertaal in Nederland verdere veranderingen in de toekomst waarschijnlijk niet meer nodig zullen zijn.

8. Het architectenbureau Mecano heeft bij het ontwerpen van de nieuwbouw van de vakgroep Virologie de brede blik die wetenschappers op de wereld behoren te hebben, wel wat erg letterlijk genomen.

9. Het verdient aanbeveling een nieuw type promotie-onderzoek te introduceren als de toekomstige promovendus, na het invoeren van een "studiebeurs", is verworden tot de kandidaatsstudent van weleer.

10. Het gebruik van moleculair-biologische technieken bij de productie van virusresistente (transgene) gewassen laat zien dat ook hedendaagse plantenvirologie nog steeds "groen" kan zijn.

Stellingen behorende bij het proefschrift:

Characterisation of tospovirus resistance in transgenic plants

Wageningen, 21 februari 1997 Marcel Prins

"... to boldly go where no one has gone before..."

Voorwoord

Zo'n voorwoord is een mooie plaats voor mij om ook eens bescheiden te doen en anderen te bedanken die mij hebben geholpen deze promotie tot een goed einde te brengen:

Rob, jou wil ik bedanken voor het initiëren van mijn project. Je constante (prettige) betrokkenheid bij mijn werk en zeker ook de snelheid waarmee je gecorrigeerde manuscripten doorgaans retourneerde hebben zeker bijgedragen aan een prettige AIO-tijd bij viro. Het was voor mij dan ook een eenvoudige keuze om er nog een paar jaar aan vast te knopen toen de kans zich voordeed.

Peter, je bent natuurlijk niet voor niets mijn co-promoter geworden. Met jouw voorwerk is het allemaal begonnen en staande op de schouders van reuzen is het makkelijk om ver te kijken. Ook denk ik met plezier terug aan onze vele telefonische werkbesprekingen en ik hoop dat we nog lang op deze voet kunnen blijven samenwerken.

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Naast mijn sfeerbepalende kamergenoten Richard en Frank, en later Erwin wil ik alle tospovirogolen bedanken voor hun niet te onderschatten bijdrage aan de bijzonder prettige werksfeer op het lab. Dit geldt zeker ook voor de (oud)baculows en alle andere los en vast medewerkende virologen.

Titia, dankjewel voor de ontelbare nuttige discussies over modellen van transgene resistentie/silencing en het helpen in de beeldvorming over wat er nu eigenlijk allemaal gaande is in onze resistente planten. Want ook al zijn CPMV en TSWV heel verschillende virussen, het resistentiemechanisme in transgene planten vertoont grote overeenkomsten, dat moge nu wel duidelijk zijn.

De medewerkers van de kassen en met name Bert ben ik erg erkentelijk voor het met grote zorg behandelen van de (vele) planten en de flexibele werkhouding daarbij.

Heel belangrijk voor de in dit proefschrift vermelde resultaten waren ook "mijn" studenten: René, Cahya, Corine, Ronald, Wendy, Marco, William, Harry, Carolien en Hans, allemaal bedankt voor jullie bijdrage.

De samenwerking met de (bio)technologie afdeling van S&G Seeds werd naast de hele belangrijke "zak met geld" vooral gekenmerkt door een altijd bijzonder prettige sfeer. Mart, Jan, Tony, Alie, Tineke en anderen, bedankt daarvoor en zeker ook voor vele belangrijke "favours" als het maken van transgene planten en primers.
Het stelletje meelopers van "Waarheen, waarvoor?" wil ik danken voor de broodnodige afleiding van belangrijker zaken, al kan het organiseren van een volledige ploeg ook behoorlijk in de stress lopen.
Hans en Wijnand, bedankt voor jullie vriendschap en de bereidheid mij op het podium bij te staan.
Gonneke, jou wil ik als laatste bedanken, niet voor je wetenschappelijke bijdrage aan dit boekje, maar wel voor je heel belangrijke bijdrage in het relativeren van zaken en het gladstrijken van motivationele hobbeltjes.

CONTENTS

Chapter 1

GENERAL INTRODUCTION

Although tomato spotted wilt virus (TSWV) was already identified in 1930 as the causal agent of a major viral disease in many crops (Samuel *et al.*, 1930), it was not until the 1980's that studies on the ecology and molecular biology of this pathogen were intensified. This was concomitant with a marked emergence of the virus, due to increased world trade and to the rapid expansion of one of its major vectors, the western flower thrips (*Frankliniella occidentalis*). With an estimated annual crop loss of over $1,000,000,000 for several crops, TSWV ranks among the ten most detrimental plant viruses worldwide (Goldbach and Peters, 1994).

The increasing importance of TSWV as a plant pathogen, i.e. the rising problems concerning the control of its spread, is most likely based on its very broad host range on one hand, and on the effective vectoring by thrips, a group of minute insects on the other. Since thrips have become resistant against most of the applied insecticides over the recent years, they are notoriously hard to control (Brødsgaard, 1994; Zhao *et al.*, 1995).

Therefore, the major future challenge is to develop strategies to restrain TSWV, and other tospoviruses, by non-chemical means. A number of possibilities may contribute to a durable control of tospovirus diseases, of which a prominent one should be on breeding for resistance. Hitherto, only a limited number of natural resistance genes suitable for introduction into plant breeding programs have been identified (Boiteux *et al.*, 1993; Boiteux and Giordano, 1993; Stevens *et al.*, 1994).

Molecular biology of Tospoviruses

Based on structural and physical evidence, TSWV was proposed to be a possible member of the family *Bunyaviridae* (Milne and Francki, 1984). This was further substantiated by sequencing the genome of TSWV (De Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992c), which revealed that the genomic organisation of TSWV indeed exhibited features similar to those of the animal infecting Bunyaviridae. As a result,

TSWV was classified as the type species of a newly designated genus *Tospovirus* within the family *Bunyaviridae* by the International Committee on Taxonomy of Viruses (Francki *et al.*, 1991). Over the past six years, several viruses have been reported that are related to TSWV, but distinct in host range, geographic distribution, serology and nucleotide sequence. Based on their nucleoprotein gene sequence information, six species have now been established within the genus *Tospovirus*, while at least seven tentative species await further identification (Table 1.1)

Table 1.1: Members of the genus tospovirus

Established	
Tomato Spotted Wilt Virus	TSWV[1]
Tomato Chlorotic Spot Virus	TCSV[2]
Groundnut Ringspot Virus	GRSV[2]
Impatiens Necrotic Spot Virus	INSV[3]
Watermelon Silverleaf Mottle Virus	WSMV[4]
Groundnut Bud Necrosis Virus	GBNV[5*]
Tentative	
Groundnut Yellow Spot Virus	GYSV[6*]
Iris Yellow Spot Virus	IYSV[7]
Melon Spotted Wilt Virus	MSWV[8]
Chrysanthemum Stem Necrosis Virus	CSNV[9]
Onion tospovirus	....[9]
Zucchini Lethal Chlorosis Virus	ZLCV[9]
Groundnut Chlorotic Fanspot Virus	GCFV[10*]

[1]De Haan *et al.*, 1991; [2]Àvila *et al.*, 1993; [3]Law and Moyer, 1991; [4]Yeh *et al.*, 1995; [5]Satyanarayana *et al.*, 1996; [6]Reddy *et al.*, 1990; [7]Peters *et al.*, *unpublished*; [8]Kato, 1995; [9]Resende *et al.*, 1995; [10]Chen and Chiu, 1995. *Some papers mention Peanut instead of Groundnut.

The tospoviral particle consists of a core of nucleocapsids in which three genomic RNA molecules are tightly associated with nucleoprotein (Figure 1.1). Typical for Bunyaviridae, these nucleocapsids are surrounded by a lipid membrane carrying two types of glycoproteins (G1 and G2). Both the nucleoprotein (N) and the precursor to the

glycoproteins are encoded on the viral complementary strand of the ambisense S and M RNAs, respectively. The L RNA is of complete negative polarity, encoding the viral RNA dependent RNA polymerase (L).

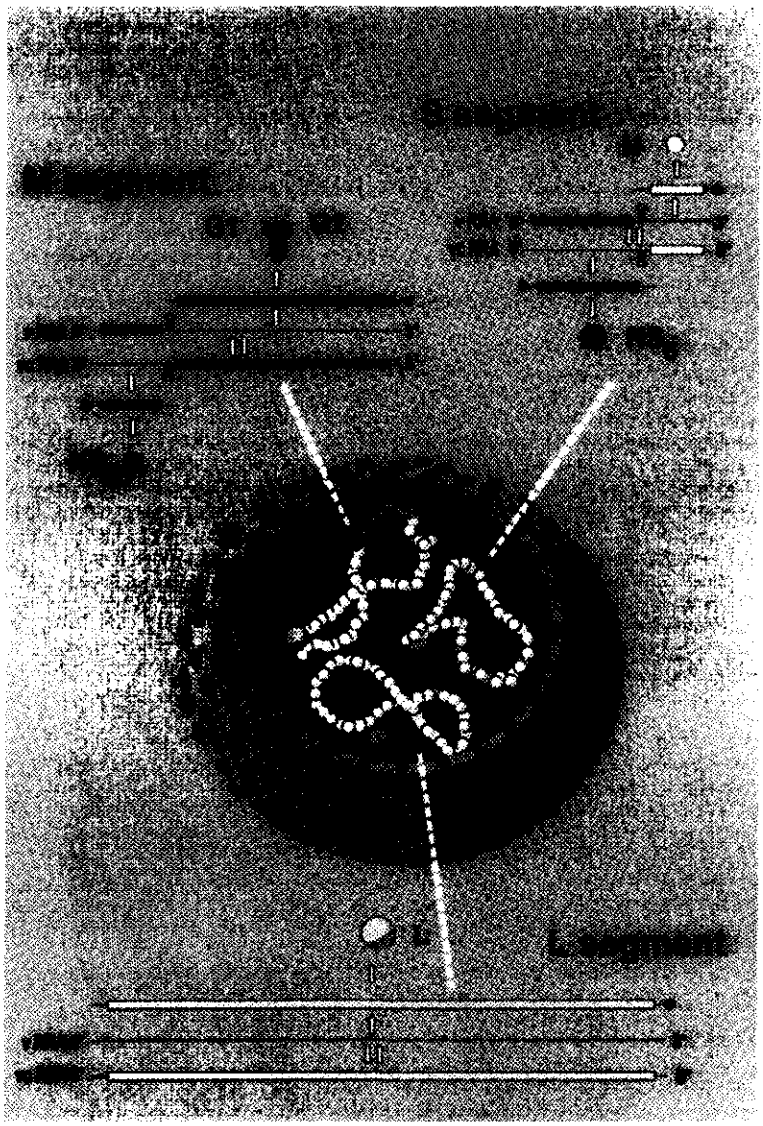

Figure 1.1: Morphology and genome expression of TSWV

Since tospoviruses are principally negative strand viruses, they require the presence of the viral polymerase (L) in the particle. The presence of the L protein, albeit in low abundancy, could indeed be confirmed for TSWV (Van Poelwijk *et al.*, 1993). Apart from replicating the viral genomic RNAs the L protein is also responsible for the transcription of the viral messenger RNAs. For the latter purpose, cap structures plus 12-20 additional nucleotides are snatched from host messenger RNAs and are subsequently used to prime the transcription reaction (Kormelink *et al.*, 1992b; Van Poelwijk *et al.*, 1996). This cap-snatching mechanism has also been shown to occur in other Bunyaviridae and members of the Arenaviridae and Orthomyxoviridae negative-strand virus families (Bishop, 1996).

On the viral strands of both M and S RNAs, tospoviruses encode the respective non-structural proteins, NS_M and NS_S. Production of these proteins requires replication prior to transcription, which may suggest a late function in the infection process. Whereas the function of the NS_S protein has remained largely unsolved, evidence is accumulating that NS_M represents the viral movement protein, necessary for tubule guided cell-to-cell transport of tospoviruses (Kormelink *et al.*, 1994; Storms *et al.*, 1995; Chapter 8).

Infection cycle of tospoviruses in plant tissue

In natural infections, tospoviruses enter the plant cell during probing or feeding of viruliferous thrips, in which these viruses have been shown to replicate (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993). Under laboratory conditions, virus infection can be mimicked by mechanical inoculation using an abrasive (e.g. carborundum powder) to slightly damage the leaf tissue. Upon entry of the virus in the cell the virus is relieved of its membrane and infectious nucleocapsids are released into the cytoplasm. At this stage the viral RNA will be either transcribed or replicated. Based on observations of similar processes in the infection cycle of other negative strand viruses (e.g. Banjeree and Barik, 1992; Baudin *et al.*, 1994), the transcription to replication switch is thought to be controlled by the cytoplasmic free nucleoprotein concentrations. At low N concentrations, i.e. at the onset of the infection process, the replicase will produce messenger RNAs, resulting, after translation, in accumulation of the various viral proteins. Upon subsequent elevation of the N protein concentration, the polymerase switches to "replicase-mode"

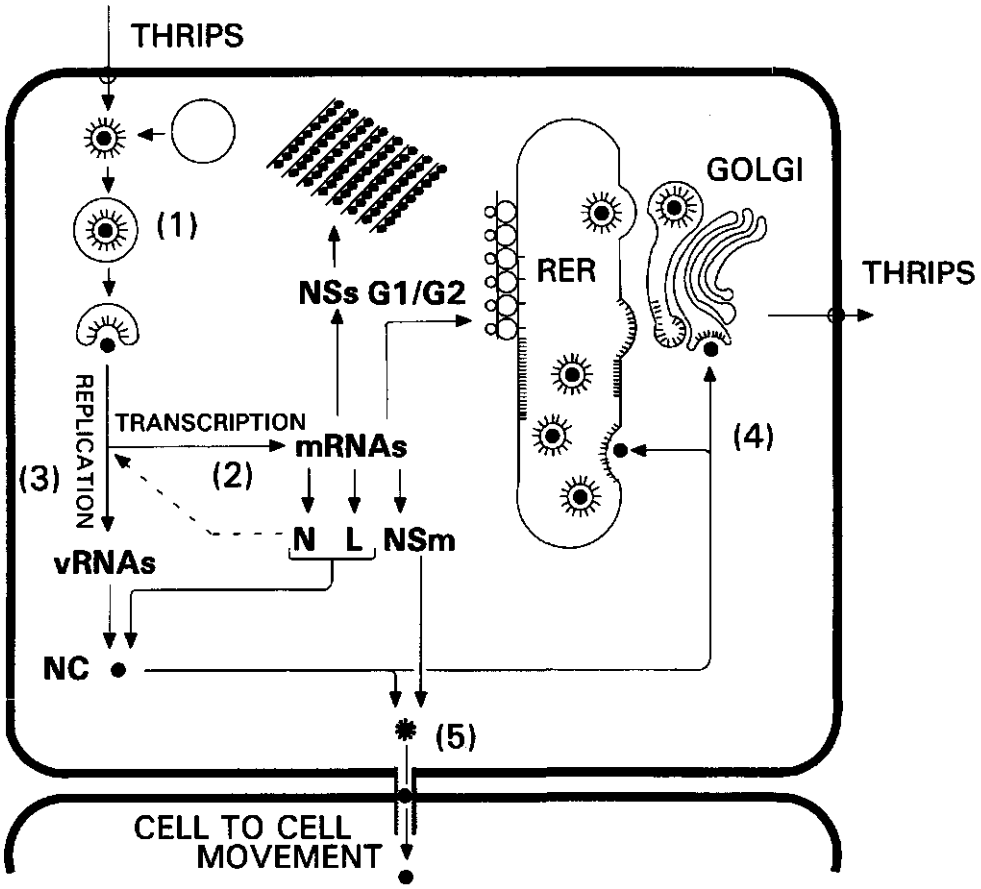

Figure 1.2: Infection cycle of tospoviruses in plant cells. After initial (thrips mediated) entry of the virus into the cell, the viral nucleocapsids are released (1). At low N protein concentrations, the polymerase produces viral messenger RNAs that can be translated into viral proteins (2). Upon elevation of the N protein levels the polymerase switches its mode to replication and viral progeny is produced (3). These can either move to an adjacent cell via NS_M induced tubules (4) or bud into golgi or ER to form mature virus particles (5). These particles can then be taken up by thrips and transmitted to other plants.

upon which viral genomic RNAs are multiplied. For some isolates the NS_S protein accumulates to large amounts in paracristalline arrays in the cytoplasm (Kormelink *et al.*, 1991). The purpose of these structures as well as the function of the protein have remained unknown. The precursor to the glycoproteins contains a signal sequence that allows translation on the RER. After glycosylation and proteolytic cleavage, the glycoproteins are transported to the site of budding.

Replicated viral genomic RNAs associate with N protein to form progeny nucleocapsids to which L protein attaches (Van Poelwijk *et al.*, 1993). Subsequent association of nucleocapsids with the NS_M protein allows transport to adjacent cells through tubular structures (Kormelink *et al.*, 1994; Storms *et al.*, 1995). Alternatively, nucleocapsid structures can form new virus particles by associating with the glycoproteins and budding into the ER or Golgi lumen. Eventually, newly formed particles can be taken up by thrips upon feeding. A schematical representation of the tospovirus infection cycle in plant cells is depicted in Figure 1.2.

Scope of the investigations

Since sources of natural resistance to TSWV are very limited, there is an urgent need for novel forms of tospovirus resistance involving plant transformation strategies. The aim of the research as described in this thesis was to develop, extend and understand at the molecular level, novel forms of host plant resistance against tospoviruses, based on strategies involving genetic modifications. Previous research revealed that transgenic resistance using the viral nucleoprotein (N) gene can be effective against TSWV (Gielen *et al.*, 1991; De Haan, 1991).

At the onset of this research, the mechanism of N gene-mediated resistance was not understood. In contrast to the theory of pathogen-derived resistance that was used to devise the nucleoprotein-mediated resistance strategy, the observed resistance in the transgenic plants did not reflect the amount of protein expressed. Therefore the requirement for the translational expression of the N gene was investigated. In Chapter 3 it is shown that the transcript of the viral transgene rather than the expressed nucleoprotein is responsible for the observed resistance. During the course of this project similar features were observed for other viruses, and a general overview of current

knowledge on "RNA-mediated" resistance to viruses is presented in Chapter 2.

Because the phenotype of this RNA-mediated resistance against TSWV is accompanied by a rather narrow resistance spectrum, it was aspired to extend the resistance of transgenic plants to related tospoviruses. This was attempted by expressing three different tospoviral N gene sequences at the same time from a single locus on the plant genome (Chapter 4). To further expand the possible use of tospoviral sequences for transgenic resistance, a large array of other parts of the viral genome were expressed in transgenic plants (Chapters 5 and 6). More detailed studies were needed to unravel the mechanism of the observed resistance. Nuclear transcription rates of resistant and non-resistant plants, were compared to their steady state cytoplasmic RNA levels, which revealed a co-suppression type of resistance. Additional experiments were carried out to investigate whether the resistance mechanism targets the viral genomic RNA or viral mRNAs (Chapter 7). In Chapter 8 it is shown that expression of viral proteins can have undesired side-effects, that are nonetheless informative for the characteristics of the expressed protein.

Chapter 2

RNA-MEDIATED VIRUS RESISTANCE IN TRANSGENIC PLANTS

In recent years the concept of pathogen-derived resistance (PDR) has been successfully exploited for conferring resistance against viruses in many crop plants. Starting with coat protein-mediated resistance, the scala has broadened to the use of other viral genes as a source of PDR. However, in the course of the efforts, often no clear correlation could be made between expression levels of the transgenes and observed virus resistance levels. Several reports mentioned high resistance levels using transgenes incapable of producing protein, but in these cases even plants accumulating high amounts of transgene RNA were not most resistant. To accommodate these unexplained observations, a resistance mechanism involving specific breakdown of viral RNAs has been proposed. Recent progress towards understanding the RNA-mediated resistance mechanism and similarities with the co-suppression phenomenon will be discussed.

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Concept of pathogen-derived resistance

As RNA-mediated resistance approaches arose as unforeseen spin off from the concept of pathogen-derived resistance, this notion will first be discussed in more detail.

In their original description of the concept of pathogen-derived resistance (PDR), Sanford and Johnston (1985) proposed the possible broad application of pathogen originated genes in generating specific host resistance, as had been observed for bacteriophage Qß genes expressed in its host. Deliberate expression of such genes at e.g. modified expression levels or at untimely stages in the pathogen's life cycle was suggested to be applicable in most host-parasite systems. In addition, greater knowledge of the "resistance gene" is available, while the isolation of these genes is relatively easy, taken into account that the size of the pathogen's genome is generally considerably smaller than that of its host. For the described case of the Qß phage, the authors report several types of approaches: deriving resistance from the Qß coat protein, using a modified replicase, expressing a replicase binding site, using antisense RNA and interfering with the maturation of the particle. The blocking of the attachment of the phage to the pili of the bacterium, thereby preventing the spread of the pathogen through the host population was also mentioned. A prerequisite for the use of PDR, of course, is that none of the approaches should interfere with essential host functions. Since all parasites require replication of their genome and spread from the original infection site through the environment, and encode genes that are essential for these functions, PDR was suggested to be widely applicable in host systems that can be modified in such a way that they express genes derived from the parasite. Principally there is no restraint to the hosts that can be exploited for this purpose, provided its genetic modification can be achieved both technically and ethically.

Besides bacterium-phage combinations, the PDR concept provides excellent possibilities for all systems involving viruses, considering the relative simplicity of their genomes and the detailed knowledge of many viral gene functions. It is therefore not surprising that most applications of PDR have been reported in viral systems, and especially those involving plant viruses. The latter seems mainly due to the availability of efficient transformation protocols for model plants such as tobacco, which is susceptible to most plant viruses.

Use of PDR against plant viruses

Considering the PDR theory there are several targets in the plant viral infection process at which resistance can be pursued without interfering with essential host functions (Beachy, 1993). Three types of viral genes have been most widely used for PDR: coat protein genes, replicase genes and movement protein genes.

Based on observations involving classical cross protection (Hamilton, 1980; Fulton, 1982), it was envisaged that the use of viral coat protein (CP) untimely expressed in transgenic plants might interfere with the uncoating of the virus in such a way that it would be less available for initiation of viral replication (Osbourn *et al.*, 1989). Because of the genetic structure of most plant (RNA) viruses, encoding their most abundant structural protein (CP) at the 3' terminal part of the genome, clones of these genes were first available for genetic studies. Forthcoming information, combined with the fact that the CP gene was available in cloned format, resulted in the use of viral CP genes for initial strategies of PDR for plant viruses. Indeed, coat protein-mediated resistance has been reported to be successful for several viruses as described in numerous previous reviews on this subject (Beachy *et al.*, 1990; Wilson, 1993; Hackland *et al.*, 1994; Hull, 1994; Kavanagh and Spillane, 1995; Lomonossoff, 1995). In the current review it is, therefore, not aimed to describe the outcome of these studies once more, unless the RNA of the expressed CP gene played a major role in the observed resistance.

Replicase-mediated resistance as a PDR concept (Carr and Zaitlin, 1993) was first applied by Golemboski and co-workers (1990). By expressing the 54 kilodalton (kD) readthrough part from the tobacco mosaic virus (TMV) replicase protein, transgenic plants proved highly resistant to the virus. Also other initial reports were focused on the role of the (mutated) replicase protein and some evidence was presented for its role in resistance, however, in most cases no direct correlation could be made between protein expression levels and resistance (Anderson *et al.*, 1992; MacFarlane and Davies, 1992; Longstaff *et al.*, 1993; Donson *et al.*, 1993; Audy *et al.*, 1994; Carr *et al.*, 1994; see also review by Baulcombe, 1994).

A number of more recent publications strongly suggest the involvement of replicase gene RNA sequences, rather than the protein. Resistance to pepper mild mottle tobamovirus (PMMV), using its 54 kD protein gene occurred in two phenotypes (Tenllado

et al., 1995), one pre-established phenotype resembling immunity and another, induced type of resistance, resulting in highly resistant plants after initial infection. This resistance was effective against high inoculum doses of PMMV isolates, not related to transgene expression levels, and broken by related tobamoviruses like TMV. Also, transgenic expression of a truncated 54 kD protein resulted in PMMV resistance (Tenllado *et al.*, 1996) indicating that the (full-length) protein is not necessary for resistance. Replicase-mediated resistance against cymbidium ringspot tombusvirus (CyRSV) appeared to correlate with low rather than high expression levels and was not functional against related viruses such as artichoke mottled crinkle virus (AMCV) and carnation italian ringspot virus (CIRV) (Rubino and Russo, 1995) suggesting the involvement of transgene RNA rather than protein. Replicase-mediated resistance experiments with potato virus X (PVX) revealed similar resistance phenotypes (Braun and Hemenway, 1992; Longstaff *et al.*, 1993) and expression of the RNA alone was proven sufficient for resistance (Mueller *et al.*, 1995). Similarly, untranslatable cucumber mosaic virus (CMV) RNA2 resulted in high levels of resistance (De Haan, *pers.comm.*), implying RNA-mediated resistance.

The third important target for PDR is viral movement. Most cases described so far discuss the use of mutated movement protein genes and result in an attenuated or delayed virus infection. A reduced TMV accumulation at non-permissive temperatures was observed in tobacco plants transformed with movement protein sequences derived from a temperature sensitive movement mutant (Malyshenko *et al.*, 1993). Inhibition of disease symptom development of TMV as well as two other tobamoviruses: tobacco mild green mosaic (TMGMV) and sunnhemp mosaic virus (SHMV), in plants expressing a defective movement protein lacking three amino acids at its N-terminus, has been reported by Lapidot and co-workers (1993). Furthermore, these plants show a delay of several days in the appearance of viral symptoms when inoculated with other non-related viruses (Cooper *et al.*, 1995). The expression of a mutated form of the white clover mottle potexvirus (WCIMV) "triple gene block" movement proteins (Beck *et al.*, 1994), showed broad resistance to WCIMV and related viruses, even against potato virus S (PVS), a Carlavirus, but not against TMV. In conclusion, it seems that the use of defective movement proteins results in relatively broad resistance. A type of resistance,

however, that is broken with increased inoculum doses. The use of the TSWV movement protein gene proved successful for obtaining resistance (Chapter 5), but untranslatable transcripts appeared equally effective, suggesting RNA-mediated resistance.

Discovery of RNA-mediated resistance

With increasing number of reports on the use of viral genes for PDR as described in the previous paragraph, deviations from the original PDR concept became more frequently observed. A consistent lack of correlation between expression level of the transgenic protein and levels of resistance was reported, which seemed to be in conflict with the PDR theorem (e.g. Stark and Beachy, 1989; Golemboski *et al.*, 1990; Lawson *et al.*, 1990; Kawchuk *et al.*, 1991; Gielen *et al.*, 1991; Van der Wilk *et al.*, 1991). Some were even unable to show any protein product, suggesting that the expression of the protein was not essential for resistance. In addition, three reports were published rather contemporarily (De Haan *et al.*, 1992; Van der Vlugt *et al.*, 1992; Lindbo *et al.*, 1992a) in which untranslatable sequences were used to confer resistance to tomato spotted wilt virus (TSWV), potato virus Y (PVY) and tobacco etch virus (TEV). The observed phenotype of the resistance was indistinguishable from plants expressing a translatable transgene, yet markedly different from reported cases of strictly protein mediated resistance (Powell *et al.*, 1990). The phenotype of the resistance in all three cases is independent of the inoculum dose and as such resembles immunity, whereas typical coat protein-mediated resistance levels decreased with increasing virus titers. Another difference was the spectrum of the resistance. RNA-mediated resistance proved to be specific for the virus from which the transgene was derived, while protein-mediated resistance also has an effect on related other viruses. As for protein expression levels, no direct correlation could be made between RNA expression levels of the transgene and the levels of resistance against virus infection. For RNA-mediated resistance against TSWV a strict, negative correlation was reported (Pang *et al.*, 1993). Previous work of De Haan and co-workers (1992) using the same transgene (N), did not show such a strict correlation between RNA expression levels and resistance. However, also here a tendency was observed that resistant plants generally had lower expression levels. This was also found in resistant plants expressing TSWV NS_M sequences (Chapter 5). These

observations, together with others that will be discussed in more detail below, suggested that RNA-mediated resistance is not a form of antisense resistance, directed against the replicative strand of the virus, but must operate in a different manner. To date, many more cases of RNA-mediated resistance have been reported, whereas for many (established) cases of pathogen-derived resistance, the contribution of the expressed RNA to the resistance has not been examined.

Molecular basis of RNA-mediated resistance

The first clue on the molecular background of RNA-mediated resistance was revealed by Lindbo and co-workers (1993). They observed recovery from TEV infection in transgenic plants expressing TEV CP sequences. Diseased plants developed new shoots that remained virus free and were resistant to subsequent inoculations. Occurrence of recovery coincided with a substantial drop in cytoplasmic transgenic RNA levels. The latter phenomenon prompted the investigation of nuclear expression levels of the transgenes in these plants. Surprisingly, the run-on assays that were used to differentiate nuclear expression of the transgene showed no notable change between unchallenged and recovered tissue. It was therefore concluded that an induced, post-transcriptional, cytoplasmic activity was responsible for the reduction of transgenic RNA levels and that consequently the same activity may be responsible for virus resistance. It was suggested that resistant plants that do not show the recovery phenotype utilize the same cytoplasmic activity, but in these plants the so called "resistant state" is permanently induced. This proposed mechanism also explains the lack of correlation between resistance and steady state RNA expression levels, since resistant plants actively degrade their transgenically expressed RNA, resulting in low steady state RNA levels.

In follow up experiments (Dougherty *et al.*, 1994) it was shown that root stocks of these resistant plants were unable to transfer the resistance phenotype to grafts of susceptible plants. In contrast to systemically acquired resistance (Farmer and Ryan, 1992; Ryals *et al.*, 1994; Ryals *et al.*, 1995) this type of resistance does not involve diffusible factors and must therefore be effective at the cellular level. Indeed, protoplasts derived from "recovered" tissue proved to block TEV replication. An inverse correlation between transgene-derived RNA steady state levels and resistance was observed also

in plants expressing untranslatable PVY CP RNAs (Smith *et al.*, 1994). A clear correlation was found between methylation of the transgenic DNA, nuclear expression levels, steady state expression levels and resistance. Resistant plants showed markedly lower steady state transgenic RNA levels, higher nuclear expression levels and transgenic DNA sequences were more extensively methylated. This suggested that methylation of transgene DNA sequences may be responsible for the induction of specific cytoplasmic RNA degradation.

Some other interesting features of transgenic sequences involved in RNA-mediated type of resistance were put forward by Mueller and co-workers (1995). Their work involved tobacco plants transformed with various forms of the PVX replicase. Previous studies (Longstaff *et al.*, 1993) had shown that this part of the virus could be exploited to induce resistance. Although originally designed to induce PDR based on a mutated form of the viral replicase, resistance turned out to be equally effective in plants transformed with the wild-type replicase gene sequences. In addition, plants with the highest levels of resistance appeared to have the lowest levels of protein accumulation. Not only did these plants produce little protein, also RNA expression levels were low when compared to sensitive plants. Susceptible transgenic plants, expressing high levels of RNA, were crossed with resistant plants displaying low RNA steady state levels. This resulted in a reversible resistant phenotype with low RNA expression, showing that the resistance conferring locus was capable of *trans*-silencing its homologue. Whether *trans*-silencing of the potentially high expressor locus was regulated at the genomic level by blocking transcription or at a post-transcriptional stage by increased turnover rates of transgenic transcripts was investigated by nuclear run-on experiments. These proved the latter option to be true, confirming earlier observations by Lindbo *et al.* (1993). Since the resistance-conferring locus is capable of post-transcriptionally silencing other genes *in trans*, it was considered feasible that the same mechanism can also degrade incoming viral RNAs *in trans* in the cytoplasm, resulting in a resistance phenotype. Indeed this was shown for a recombinant PVX virus carrying bacterial ß-glucuronidase (GUS) sequences. Plants transformed with the same bacterial sequences in which the transgene was silenced (Hobbs *et al.*, 1993) (see also next paragraph) appeared to be resistant against this novel virus (English *et al.*, 1996). Moreover, it turned out that

primarily the 3' region of this GUS gene in the recombinant PVX virus was targeted, coinciding with methylations in this region of the transgene DNA insert in the nucleus.

RNA-mediated resistance against PVY was also obtained in potato plants expressing viral *CP* gene sequences (Smith *et al.*, 1995). Also here, resistant plants generally had lower transgene RNA expression levels and a higher number of transgenic inserts. This feature was studied further in TEV resistant tobacco plants (Goodwin *et al.*, 1996). A series of plants was made by crossings and doubling of haploid genomes in such a way that plants contained zero, one, two or three transgenic inserts in their DNA. Plants harbouring one or two transgenes generally displayed a recovery phenotype, while three transgenic insertions resulted in plants with a highly resistant phenotype. In all resistant plants, a sequence specific post-transcriptional RNA degradation mechanism coincided with the resistance. This RNA decline appeared to be initiated by cleaving the target RNA molecule at specific sites, for which no consensus sequence or structure was observed. An interesting observation was discussed mentioning the complete lack of (nuclear) transcription in transgenic plants harbouring more than eight transgenes, suggesting that large numbers of homologous transgenes are silenced already at the nuclear transcription level.

Typical RNA-mediated resistance features were observed by Swaney *et al.* (1995), by expressing another part of the TEV genome, the 6K/21K central region of the viral RNA. The authors suggested that principally all (TEV) sequences are amenable for inducing resistance, but that the size of the transgene may be important for eliciting the response and that, in addition, specific nucleotide sequences or secondary structures could play a role. For the bipartite cowpea mosaic virus (CPMV), engineered resistance was shown to be directed against the viral RNA from which the transgene was derived (Sijen *et al.*, 1995) and could also be induced by untranslatable RNA sequences (Sijen, *pers. comm.*). Also for TSWV, post-transcriptional silencing of transgenes has been demonstrated in resistant transgenic tobacco (Chapter 7) and lettuce plants (Pang *et al.*, 1996). Surprisingly, only transgenic sequences derived from the nucleoprotein (N) and movement protein (NS_M), were capable of inducing resistance. Transgenes expressing antisense and sense RNA, as well as untranslatable versions, were equally effective, while RNA sequences derived from other TSWV genes or parts thereof were not

(Chapter 6). Two possible explanations for this sequence specificity were proposed. First, the relevance of the targeted genes for the infection of plants. Since TSWV also replicates in its thrips vector (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993) it is envisaged that some TSWV genes are not essential for replication and spread in plants. Suppressing the expression of these genes (even when successful) would not result in resistant transgenic plants, provided that the naked viral mRNAs are target of the induced resistance and permanently encapsidated viral genomic RNAs are not. Second, the actual primary or secondary structure of the transgenes may be involved in inducing the resistance. This might also explain why RNA-mediated resistance has not been found for several viruses or some specific viral genes.

RNA-mediated virus resistance and co-suppression share similar features

Some unexpected results were obtained when petunia plants where transformed with additional copies of endogenous genes involved in the flower pigmentation pathway. Instead of an increase, a dramatic decrease in expression levels was observed, resulting in completely white flowers (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). Steady state RNA levels even decreased fifty fold when compared to natural expression levels. Similar phenomena have been found e.g. in tomato (Smith *et al.*, 1990) and tobacco (De Carvalho *et al.*, 1992). The process appeared to be meiotically reversible, was correlated to homozygous transgenic insertions (De Carvalho *et al.*, 1992; Hart *et al.*, 1992; Dehio and Schell, 1994; Dorlhac de Borne *et al.*, 1994) and was originally labelled "co-suppression". An essential functional role of the expressed protein was ruled out by expressing non-functional genes that were also capable of inducing co-suppression, suggesting involvement of the RNA (Smith *et al.*, 1990; Goring *et al.*, 1991; Van Blokland *et al.*, 1994). Nuclear run-on experiments showed that expression levels in the nucleus were relatively high in co-suppressed plants and did not correlate to RNA steady state levels in the cytoplasm. Therefore, the suppression was suggested to operate on a post-transcriptional level (De Carvalho *et al.*, 1992; Niebel *et al.*, 1995a,b; Van Blokland *et al.*, 1994). A possible involvement of methylation of transgenes has been suggested by Wassenegger and co-workers (1994). In their experiments transgenically expressed viroid cDNA copies became extensively methylated only after RNA replication of viroids

in these plants. It was suggested that high expression levels of (replicated) RNA molecules can be a signal for methylation of corresponding transgenes. The involvement of a general mechanism in plants regulating highly expressed genes was discussed with respect to frequently observed methylation of transgenes with multiple insertions and co-suppression of endogenous genes for which this mechanism may also (partly) account. A correlation between silencing and *de novo* methylation of the transgene was also shown by Ingelbrecht *et al.* (1994). RNA-directed methylation of transgenic DNA sequences was suggested, involving RNA transcribed from the transgene itself. As a result, transgenes were not silenced at the transcriptional level as shown by run-on experiments, but steady state RNA levels decreased, confirming a post-transcriptional inactivation of these sequences.

Various potential mechanisms of co-suppression (or post-transcriptional gene silencing) have been discussed in a number of reviews on the subject (Kooter and Mol, 1993; Matzke and Matzke, 1993; Flavell, 1994; Jorgensen, 1995; Matzke and Matzke, 1995a,b). A biochemical switch model was proposed (Meins and Kunz, 1995) involving a threshold RNA expression level at which silencing is induced. This supports most observed cases of post-translational silencing, but seems to be contradictory to obser ved co-suppression induced by a promoterless transgene or other genes with low (nuclear) expression levels (Van Blokland *et al.*, 1994). Beside the quantity, also the influence of the quality (aberrancy) of expressed transgene RNAs was drawn into the discussion (Metzlaff *et al.*, 1996). When probing the structure of the transcripts of silenced genes it was noted that the 3' terminus of the target sequences was much less abundantly transcribed than its 5' end, also deletions were observed in this end of the transcript. This prompted the authors to suggest that accumulation of aberrant RNAs might be involved in the induction of co-suppression. In addition, small antisense RNAs were reported in relatively high abundancy suggesting involvement of these molecules in recognition and/or degradation of sense RNA.

Often, co-suppression has only been observed in plants that were homozygous for a transgene and/or carried multiple copies, while heterozygous plants expressing the same transgene were not silenced (De Carvalho *et al.*, 1992; Hart *et al.*, 1992; Dehio and Schell, 1994; Dorlhac de Borne *et al.*, 1994; Jorgensen, 1995). This suggested a gene

dosage effect lifting total expression levels past threshold levels. Not only the number of genes present in transgenic plants appears to be important, but also the nature of the transgene locus (Hobbs *et al.*, 1993) and even intrinsic properties, like primary or secondary structure, of the transgene transcript itself (Elomaa *et al.*, 1995). Plants expressing a single gene can give high expression levels, whereas plants harbouring complex transgenic insertions can have low expression. By crossing these lines it could be shown that silenced genes are capable of *trans*-silencing genes that are transcriptionally active when expressed alone (English *et al.*, 1996). Genes silenced in this way also became methylated, like the low expressor transgenes. Homologous genes provided *in trans* by particle bombardment, could thus be silenced. Even when sequences homologous to the transgene were provided *in trans* by a (PVX) viral vector, that operates in the cytoplasm, these were silenced. This suggests a cytoplasmic (post-transcriptional) component of the silencing mechanism, capable of silencing virus expressed sequences (English *et al.*, 1996). Co-suppression of endogenous genes involved in the carotenoid pathway was even suggested to occur *in trans* by homologous sequences (sense or antisense) cytoplasmically expressed from a TMV based viral vector (Kumagai *et al.*, 1995).

An alternative type of transgenic silencing involves extensive methylations of the promoter region of the transgene, thereby heritably inactivating the gene at the transcriptional level (e.g Meyer and Heidmann, 1994; Park *et al.*, 1996). Even though, without doubt, promoter methylation occurs in transgenic plants expressing viral sequences, it has not been reported in relation to RNA-mediated resistance, nor can this be envisaged mechanistically. Therefore, promoter methylations will not be discussed further here. Excellent reading on the subject is provided in several reviews (Matzke and Matzke, 1993; Flavell, 1994; Matzke and Matzke, 1995a,b; Meyer, 1995a,b,c).

Current model for RNA-mediated virus resistance and co-suppression

Since transgenic RNA-mediated resistance against viruses and co-suppression of endogenous genes share so many similarities it is conceivable that they are both (induced) manifestations of a basic mechanism residing in plants (and perhaps other organisms) that is involved in the regulation of gene (over)expression. Based on current

knowledge we will attempt to construct a model explaining (most of the) observed phenomena (Figure 2.1). The core of the model is formed by a cellular mechanism that can be induced by the expression of transgenes and subsequently leads to sequence specific RNA degradation. For both RNA-mediated resistance and co-suppression, passing a threshold level of transgenic (nuclear) expression seems an adequate explanation for the induction of the silencing mechanism. Some reports, however, present strong evidence against this assumption (Van Blokland *et al.*, 1994). Therefore, quantitative expression of a transgene may not be the (only) requirement for silencing, but also the quality of the transcript (Baulcombe *et al.*, 1996; Metzlaff *et al.*, 1996). Methylations of transgenic loci related to co-suppression, may be induced initially by high expression levels of the transgene RNA sequences, either already in the nucleus (Wassenegger *et al.*, 1994) or after redirectioning of cytoplasmic RNAs to the nucleus (Lindbo *et al.*, 1993). Extensive methylations of the transgene may cause aberrations in the transcribed messenger RNAs, that subsequently trigger a resident RNA-dependent RNA polymerase present in the cytoplasm (Dorssers *et al.*, 1982; Dorssers *et al.*, 1983; Van der Meer *et al.*, 1984) to synthesize (short) antisense RNA molecules. These could than form the core of the highly specific RNA-degrading complex that can target specific cellular or viral RNA molecules in the cytoplasm. Thereby explaining why the mechanism is so versatile in targeting different sequences, yet operates in a very sequence specific manner. Ribonucleic acids have been described as essential part of enzymes involved in RNA cleavage and sequence specific recognition e.g for RNase P and snRNPs (Baserga and Steitz, 1993; Kirsebom and Svard, 1994; Altman, 1995; Kirsebom, 1995).

Antisense transgenes have been shown capable of downregulating expression of endogenous genes as efficient as sense genes (e.g. Van der Krol *et al.*, 1988; Van der Krol *et al.*, 1990). The original rationale behind the use of antisense transgenes aims at the expression of stoichiometric amounts of antisense transcripts that can anneal to sense transcripts, thereby making them instable. Even though successful as a concept, expression levels of antisense RNA often appeared to be low, displaying features very much resembling sense suppression, like reduced steady state levels (Van Blokland, 1994). This suggests that suppression of sense as well as antisense sequences can lead to the post-transcriptional degradation. How silencing of an endogenous gene can be

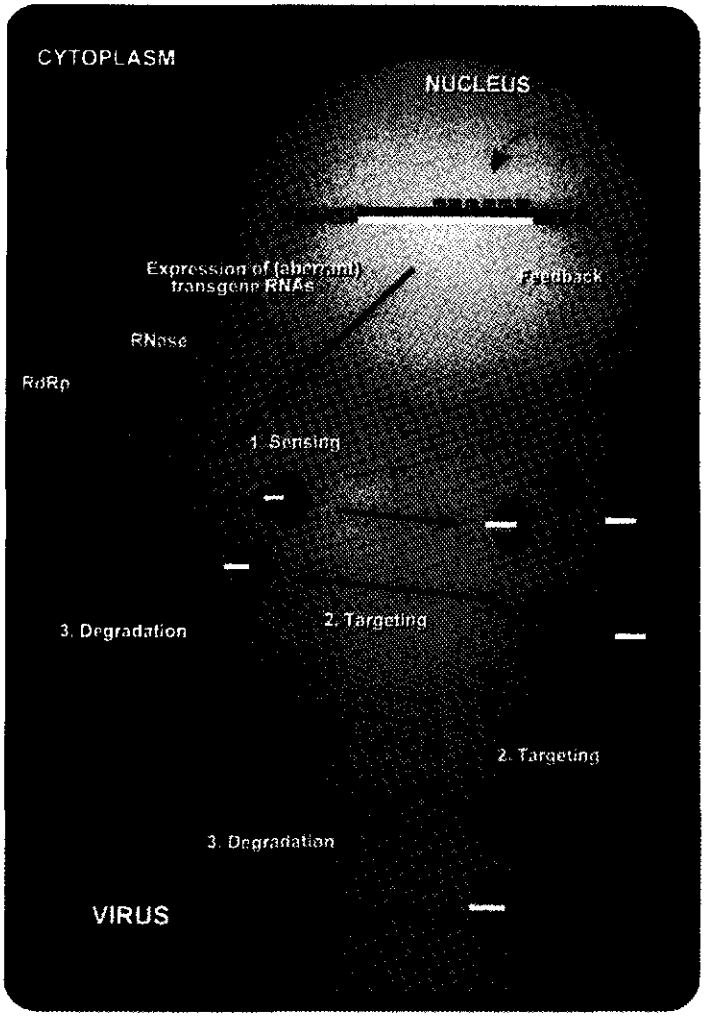

Figure 2.1: Current model for RNA-mediated resistance. Expression of transgenes in the nucleus leads to an unacceptable level of (aberrant) transcripts. This is sensed (1) by a cytoplasmic factor that includes an RNA-dependent RNA polymerase activity which transcribes short antisense RNAs. These RNAs form the core of a sequence specific ribonuclease that targets (2) and degrades (3) sequences identical or complementary to the transgene, resulting in low steady state transgene RNA levels. Feedback to the nuclear transgene resulting in transgene methylation (m) may cause increased aberration frequencies, thereby reinforcing the silencing. Upon entry of the virus, the viral RNAs, which have the same sequence as the transgene, are also targeted and degraded, resulting in virus resistance.

achieved by post-transcriptionally silencing of its antisense transgene requires the ability of the silencing mechanism to operate on both strands. To accomplish this, part of the targeted RNA molecule may not be degraded but retained in the RNA degradation complex, making it capable of targeting both sense and antisense RNAs.

Relevance of RNA-mediated resistance: implications for applications

Even though silencing of transgenes can impose great drawbacks in the transgenic expression of proteins in plants (Finnegan and McElroy, 1994), for pursuing virus resistance it has turned out to be a very powerful strategy. Endeavouring co-suppression-like RNA-mediated resistance against plant viruses is a general straight-forward strategy, which remains to be explored for many viruses, even for those in which protein expression was initially designed as a means of PDR, but where protein expression and resistance levels did not directly correlate. Several distinctive properties characterize RNA-mediated virus resistance. First, the observed phenotype resembles immunity in that it is not broken by increased doses of virus or application of viral RNA instead of virus. Second, it has a narrow scope of operation and can be broken by heterologous related viruses. This limitation can be overcome by simultaneous expression of multiple RNA sequences (Chapter 4). A third property of this resistance based on RNA is that it is expectedly more durable than protein mediated resistance. RNA-mediated resistance can be broken by related viruses displaying up to 90% sequence homology in the target gene, this still requires substantial modification of the viral genome of the homologous virus. In contrast, even a single point mutation in a protein has been shown to have major consequences on virus resistance both in transgenic and natural resistance gene sources (Turner *et al.*, 1991; Santa Cruz and Baulcombe, 1994). A fourth, beneficial, aspect of RNA-mediated virus resistance is the advantage of deliberate use of untranslatable RNAs, possibly with extra precautions like introduced stopcodons. This further decreases the chance of possible unwanted recombinations between transgenic transcripts and RNAs of invading viruses, resulting in novel viruses (Greene and Allison, 1994). Furthermore transcapsidation (Bourdin and Lecoq, 1991; Candelier-Harvey and Hull, 1993; Lecoq *et al.*, 1993) cannot occur in plants expressing untranslatable RNA of viral coat protein genes. Expression of a viral

sequence (not encoding any protein), that induces its own breakdown, resulting in (undetectably) low steady state RNA expression levels due to the induction of a resident plant response capable of very specifically destroying viral sequences, is biosafe and therefore very suitable with respect to public acceptance of genetically modified crops.

Concluding remarks

Post-transcriptional silencing of transgenes homologous to viral sequences represents a newly discovered phenomenon which can be successfully applied for developing novel forms of resistance against plant viruses. Accessibility of the viral RNA to be targeted by the silencing mechanism obviously plays an important role. The genomes of plus strand RNA viruses are relatively easy accessible. The genomes of negative strand RNA viruses are associated with nucleoprotein throughout their replication cycle and may therefore be less susceptible to RNA degradation. However viral mRNAs are not encapsidated and thus accessible for sequence specific degradation as shown for TSWV. Potentially this form of resistance could also operate against DNA viruses (see experiments by Hong and Stanley, 1996). Even though replication of these viruses takes place in the nucleus, messenger RNAs still end up in the cytoplasm. Targeting of mRNAs by the silencing mechanism, thereby preventing the formation of essential proteins (e.g. involved in replication and movement), could inhibit virus multiplication or spread.

For the resistance mediated by some viral transgenes only an effect of the protein was reported, while plants expressing untranslatable RNAs were all sensitive. Although often biased by preselecting for plants expressing high levels of transgenic protein or transcripts, these incidents may indicate that not all viral sequences are capable of conferring RNA-mediated resistance. In analogy, some transgenes are apparently unable to confer co-suppression to endogenous genes (Elomaa *et al.*, 1995). Possibly, a requirement for specific primary or secondary structure elements in the expressed sequence needs to be met, for which the chance can be elevated by increasing the size of the transgene.

It is not known whether post-transcriptional gene silencing does at all occur in other organisms than higher plants, although phenomena very similar to co-suppression have

been observed in the filamentous fungi *Neurospora crassa* (Cogoni *et al.*, 1994) and *Ascobolus immersus* (Barry *et al.*, 1993). In higher vertebrates, the use of short antisense oligomers is being used in anti-cancer therapy and treatment of some virus infections, but pharmaceutical addition of chemicals is used rather than actual transformations of cells. Beside these experiments, successful use of ribozymes flanked by antisense sequences *in vitro* and in cell lines has been reported (Rossi, 1993; Yamada *et al.*, 1996). Deliberate transformation of animal cell(-line)s with e.g. (sense) viral gene sequences in order to investigate the possibility of co-suppression has not been reported so far.

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Chapter 3

N GENE-MEDIATED RESISTANCE AGAINST TOMATO SPOTTED WILT VIRUS IS BASED ON TRANSCRIPTIONAL EXPRESSION OF THE TRANSGENE

High levels of resistance against tomato spotted wilt virus have been obtained by expressing its nucleoprotein (N) gene in transgenic tobacco plants. Protein expression levels and degree of resistance did not correlate and prompted the investigation of the role of the transgenically expressed viral RNA sequence. It was demonstrated that untranslatable tomato spotted wilt virus (TSWV) N gene RNA sequences were equally effective in conferring resistance to the virus. Additionally, plants proved resistant when viruliferous thrips *Frankliniella occidentalis* (Perg.) were used for inoculation, but appeared sensitive to other tospoviruses.

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INTRODUCTION

In the past few years numerous outbreaks of tospovirus infections have been reported in many crops and ornamental plant species in the Northern hemisphere (Peters *et al.*, 1991; De Haan, 1992). In most cases tomato spotted virus (TSWV) isolates were identified as the causal agent. TSWV is the type species of the newly designated genus *Tospovirus* within the arthropod-born family of Bunyaviridae (De Haan *et al.*, 1990; Elliot, 1990; Francki *et al.*, 1991). Several distinct species have been reported within this genus (Àvila *et al.*, 1990; Law and Moyer, 1990; Sreenivasulu, *et al.*, 1991; Àvila *et al.*, 1992; De Haan, 1992). Due to limited data, economical and ecological impact of these novel tospoviruses has remained unclear. Transmission of tospoviruses is mediated by several thrips species of which *Frankliniella occidentalis* is the most important (Sakimura, 1962; Paliwal, 1974). Typical for Bunyaviridae the tospoviral particle comprises a lipid envelope with glycoprotein protrusions (Ie, 1964; Kitajima, 1965; Milne, 1970). The tripartite RNA genome is wrapped with nucleoprotein (N) to form stable nucleocapsids (De Haan *et al.*, 1989). The S RNA is 2.9 kilobases (kb) long and codes for the N protein (28.8 kilodalton; kD) and a non-structural protein (NS_S, 52.4 kD) in an ambisense arrangement (De Haan *et al.*, 1990). Also the 4.9 kb M RNA is ambisense, encoding the precursor to the membrane glycoproteins G1 and G2 (127.4 kD) and another non-structural protein (NS_M) of 33.6 kD (Kormelink *et al.*, 1992c). The L RNA (8.9 kb) contains only one ORF in the viral complementary strand (331.5 kD), which most likely represents the viral RNA polymerase (De Haan *et al.*, 1991). Availability of the TSWV N gene in a cloned format created the possibility to investigate its potential for inducing transgenic resistance. Indeed, transgenic resistance to this negative strand virus has been reported after transformation of tobacco plants (Gielen *et al.*, 1991; MacKenzie and Ellis, 1992). Two hypotheses were proposed to explain the molecular mechanism underlying the resistance. First, untimely expression of high levels of viral N protein may interfere with transcription or replication of the incoming viral genome, by altering the mode of the viral polymerase (Beaton and Krug, 1986; Franze-Fernandez *et al.*, 1987; Vidal and Kolakofski, 1989). Second, high levels of N gene transcripts in transgenic plants may result in antisense inhibition of viral replication (Hemenway *et al.*, 1990; Cuozzo *et al.*,

1988; Kawchuk *et al.*, 1991).

Here we have extended studies on N gene-mediated resistance and focussed on the possible role of transgene transcripts in the resistance mechanism.

RESULTS

Construction of an N gene-based transformation vector expressing untranslatable RNAs

Since resistance observed in plants expressing N protein was not correlated to expression levels (Gielen *et al.*, 1991), the potential role of the transgene transcript was investigated. Two different TSWV nucleoprotein gene transformation constructs have been described previously (Gielen *et al.*, 1991), comprising the complete N gene sequence cloned behind the CaMV 35S promoter. Construct pTSWV N-A contained the viral leader sequence, while in construct pTSWV N-B this leader had been replaced by the 5' untranslated leader of TMV in order to increase translation.

To investigate the contribution of the N gene transcript to the observed resistance, an untranslatable construct was devised by site-directed mutagenesis of pTSWV N-B. The translational startcodon was removed, and a frameshift was introduced at position +8, resulting in pTSWV N-C (Figure 3.1).

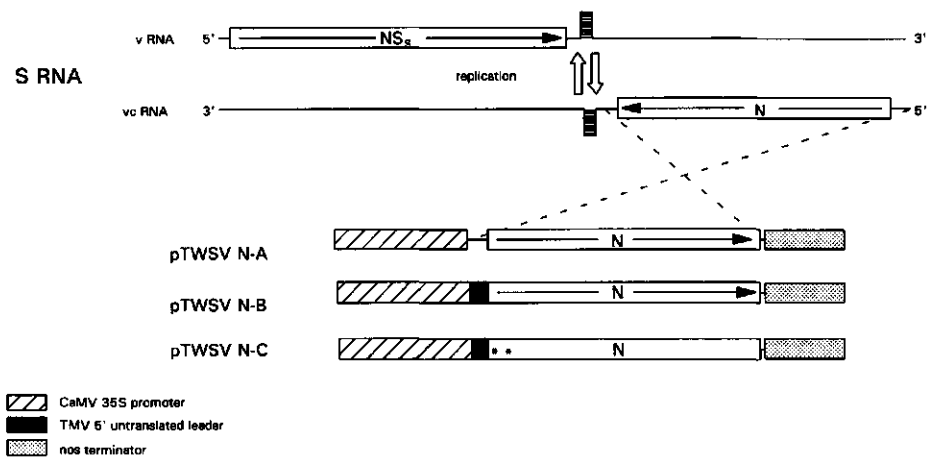

Figure 3.1: Construction of plant transformation vectors pTSWV N-A, N-B and N-C and the origin of the used RNA sequences on the TSWV S RNA segment. Point mutations in the N-C construct resulting in an untranslatable gene are indicated by asterisks.

Analysis of pTSWV N-C transformed plants

Twenty-three independent transformants were obtained containing the pTSWV N-C construct. These were examined for the presence of TSWV specific sequences using Northern blot analysis (Figure 3.2). When compared to transgenic plants expressing translatable transcripts, both RNA levels and number of plants were comparable. Protein expression from alternative startcodons was checked using a polyclonal antiserum in DAS-ELISA (Resende *et al.*, 1991a) and proved to be negative for all 23 lines.

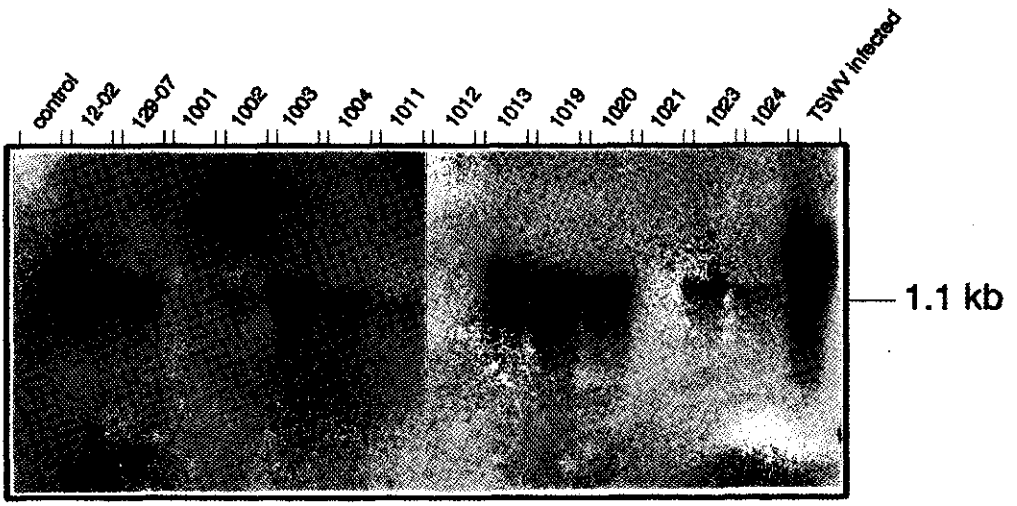

Figure 3.2: Northern blot analysis of the N gene transcripts in pTSWV N-C transformed plants (lines 1001 through 1024). For reference RNA of non-transformed plants (control) was added as well as RNA isolated from pTSWV N-A and N-B transformed resistant lines (12-02 and 129-07 respectively). TSWV infected tobacco was added to indicate the size of the S RNA and the N mRNA. The expected length of the transcripts is indicated on the right.

Virus resistance of pTSWV N-C transformed lines

Transgenic plants were allowed to set seed, and resulting S1 plants were assayed for virus resistance. Four pTSWV N-C transformed lines: 1004, 1011, 1019 and 1023 showed resistance to TSWV. Thirty to eighty percent of the plants escaped infection with TSWV (Figure 3.3), and were completely virus-free when tested with DAS-ELISA.

Symptoms of infected plants were indistinguishable from those in untransformed plants. Also the other 19 pTSWV N-C lines showed identical susceptibility levels as untransformed control plants. Both percentage of transgenic lines and levels of resistance were comparable to N protein expressor plants. This indicates that previously reported N gene-mediated resistance is due to transcription of the transgene rather than translation of the viral transgene.

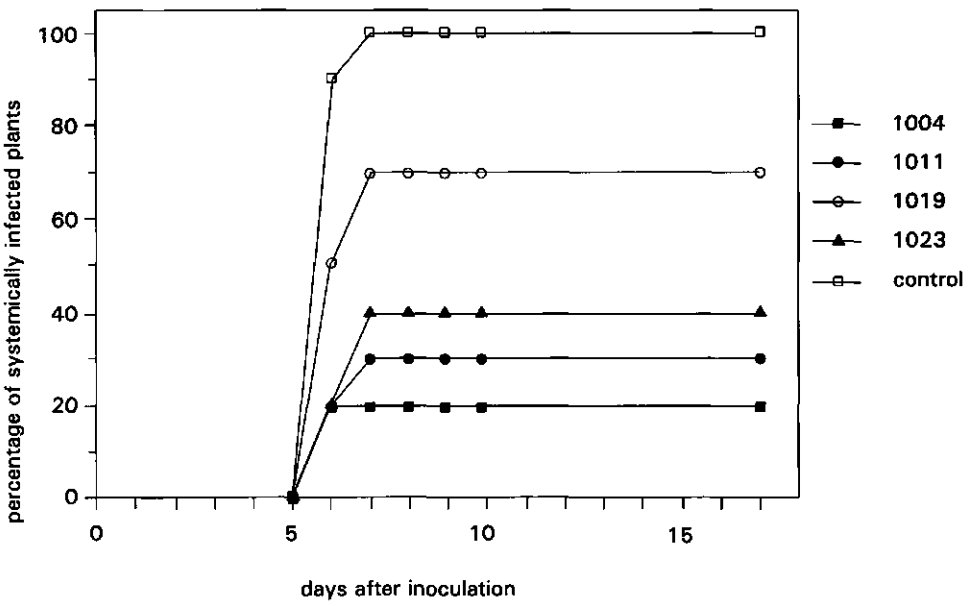

Figure 3.3: Development of systemic disease symptoms on the S1 progeny of 23 independent regenerants of pTSWV N-C transformed tobacco plants. Four resistant lines are indicated, 19 other lines proved completely susceptible and developed symptoms at a severity and timing identical to non-transgenic control plants.

Increased resistance in homozygous transgenic plant lines

As for pTSWV N-A and N-B lines 12 and 129 respectively (Gielen *et al.*, 1991), resistance increased to 100% in homozygous S2 lines derived from line 1004. Three homozygous S2 lines: 12-02, 129-07 and 1004-02 were used for further experiments concerning inoculation with viruliferous thrips and with other tospoviruses.

Resistance assays using viruliferous thrips

Mechanical inoculation experiments revealed that only tobacco plants younger than six weeks could be infected efficiently (results not shown). When three viruliferous adult thrips were fed on four-week old tobacco plants for an inoculation access period (IAP) of three days, typical feeding scars caused by mechanical damage of leaf tissue were observed on all plants. Non-transgenic control plants became systemically infected after four to five days post inoculation. In contrast, both N protein-expressing and RNA-expressing lines remained completely healthy (Figure 3.4). Thus transgenic plants are not only resistant to mechanical inoculation, but also when the natural vector for TSWV transmission is used for inoculating virus.

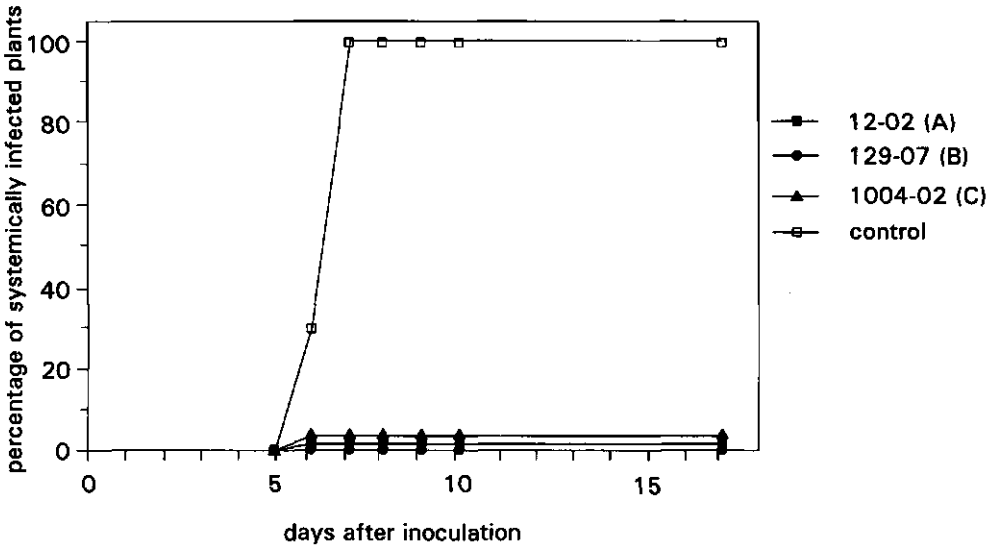

Figure 3.4: Resistance of homozygous pTSWV N-A, N-B and N-C transformed tobacco lines to inoculation using viruliferous thrips. Three thrips were allowed to feed for three days on the plants to be tested, and the development of the disease symptoms was followed on a daily basis. Non-transformed tobacco plants served as a susceptible control.

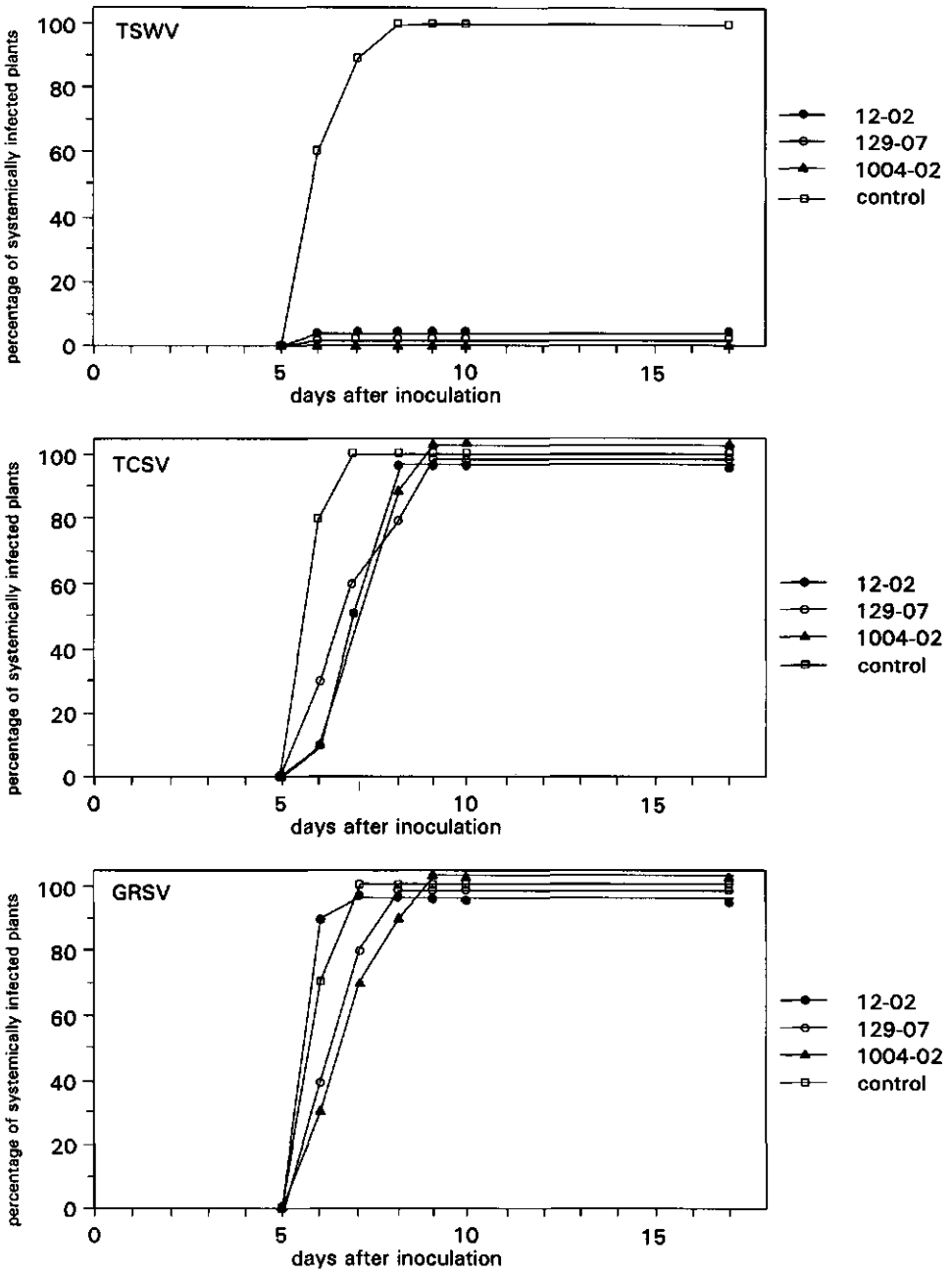

Figure 3.5: Inoculation of TSWV resistant S2 plant lines (12-02, 129-07 and 1004-02) with related tospoviruses GRSV and TCSV. Untransformed *N.tabacum* var. SR1 plants serve as susceptible control.

Resistance to other tospoviruses

Beside TSWV, a number of other viruses have been established within the tospovirus genus (Law and Moyer, 1990; Àvila *et al.*, 1992). Of these viruses *Impatiens* necrotic spot virus (INSV) seems mainly restricted to ornamentals, while tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV) have a broader host range, similar to TSWV. INSV does not spread systemically in *N. tabacum*, therefore this virus was not included in the challenge experiments. TCSV and GRSV share approximately 80% sequence homology with TSWV at the N gene RNA level (Àvila *et al.*, 1993). Upon inoculation of plants resistant to TSWV with these two other tospoviruses, all plants proved fully susceptible and developed symptoms characteristic of these viruses (Figure 3.5).

DISCUSSION

The results shown in this chapter indicate that the previously reported N gene-mediated resistance to TSWV (Gielen *et al.*, 1991) is to a major degree the consequence of the expression of N gene transcripts, since phenotypes of resistant plants incapable of expressing N protein are indistinguishable from those expressing detectable amounts of protein. Four out of 23 tobacco lines containing the translationally defective pTSWV N-C transgenic insert, showed high levels of resistance to TSWV. This ratio closely resembles the one previously found for N protein expressing transgenic plants (Gielen *et al.*, 1991). Furthermore, the levels of resistance within these lines are also similar, both in S1 and S2 generation plants. In addition it was shown that plants resistant to mechanical inoculation were equally well protected against virus inoculation by viruliferous thrips.

Increased resistance in homozygous S2 lines suggests a gene dosage effect, implying the level of resistance may be determined by the amount of transgene DNA copies and resulting increase in transcript (or protein) production. However, the expression level of transgenic N gene transcripts in individual plants does not correlate with the level of resistance. This may be influenced by varying degrees of promoter activity depending on tissue and cell type (Benfey *et al.*, 1989a,b).

TSWV specific transcripts expressed in all transgenic plant lines (pTSWV N-A, N-B and N-C) are of antigenomic polarity with respect to the viral genome. It is therefore conceivable that virus replication is blocked by antisense inhibition of the viral S RNA. Alternatively or additionally, transcripts may compete for viral and/or host encoded (replication) factors. Since the resistance was shown to be primarily RNA-mediated, a minimal sequence homology between the transgene and challenging RNA should be required. In agreement with this, the N gene sequence of TSWV, which shares approximately 80% homology at the nucleotide level with TCSV and GRSV (Àvila *et al.*, 1993), was unable to confer protection against challenge by these viruses. On the other hand, the N gene of TSWV was effective in protecting against a variety of strains and isolates of TSWV with less heterogeneity in their N gene sequences. This requirement for a high level of sequence homology has practical consequences if one aims at engineering broad resistance against tospoviruses.

Similar to experiments described here, engineered resistance based on transgenic expression of viral RNA sequences was found for potyviruses PVY (Van der Vlugt *et al.*, 1992) and TEV (Lindbo and Dougherty, 1992b), indicating RNA-mediated resistance is not limited to negative strand viruses. For practical applications it may be favourable to use RNA-mediated resistance over protein-mediated resistance, since no foreign protein accumulates in the transgenic plants.

Finally, it is important that transgenic plants also show high levels of resistance to TSWV infection upon inoculation by its main natural vector *F. occidentalis*, one of the major causes for the rapid emergence and global spread of TSWV infections.

MATERIALS AND METHODS

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 Àvila *et al.*, (1990, 1992 and 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America.

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

Construction of the mutant N gene sequence expression vector

Based on construct pTSWV N-B as described by Gielen *et al.* (1990), plants transformation vector pTSWV N-C was made by site directed mutagenesis. The sequence was modified in such a way that the authentic ATG startcodon of the N gene open reading frame was replaced by CTG, additionally a frameshift was introduced by adding a G residue at position +8 (Figure 3.1).

Transformation of tobacco

The pBIN19-derived pTSWV vectors were introduced in *Agrobacterium tumefaciens* strain LB4404 (Ditta *et al.*, 1990) by triparental mating, using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid.

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

Analysis of protection of transgenic plants against TSWV and other related tospoviruses

Twenty S1 progeny plants from 24 original pTSWV N-C transformed *N. tabacum* var. SR1 plants, were inoculated with TSWV, approximately 5 weeks after sowing. Inoculation was done according to standard procedures (Gielen *et al.*, 1991). The appearance of systemic symptoms was monitored on a daily basis. Resistant plants were left to set seeds.

A second experiment involved inoculation of several S2 lines that were completely resistant to TSWV (lines 12-02 and 129-07, described by Gielen *et al.* (1990) and 1004-02, this chapter) with related tospoviruses TCSV and GRSV.

Inoculation of transgenic plants using viruliferous thrips

Cultures of *Frankliniella occidentalis* (Perg.) were maintained on bean pods (*Phaseolus vulgaris*). In all experiments thrips were kept in modified Tashiro cages (Tashiro, 1967) at 27°C at a photoperiod of 16 hours. Prior to each experiment, first instar (L1) larvae (0-12 h after hatching) were allowed to acquire the virus by feeding on TSWV-infected *Datura stramonium* plants for three days. Larvae were subsequently maintained on healthy *D.stramonium* leaves until the adult stage was reached. After emergence, adult

thrips were individually tested for infectivity on *Petunia hybrida* (Allen and Matteoni, 1991). Three viruliferous adult thrips were concomitantly transferred to 20 transgenic and 10 non-transgenic tobacco seedlings of four weeks old. After an inoculation access period (IAP) of three days, thrips were killed by spraying with dichloorvos and plants were monitored on a daily basis for the development of local and systemic symptoms.

Acknowledgements

The authors wish to thank Antonio de Àvila for kindly providing tospovirus isolates and for helpful discussions and Paul Grooteman for skilled assistance in the inoculation experiments.

Chapter 4

BROAD RESISTANCE TO TOSPOVIRUSES BY SIMULTANEOUS EXPRESSION OF THREE TOSPOVIRAL NUCLEOPROTEIN GENES

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 chimaeric 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 *E. coli* and *A. 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 multivirus resistance trait is confined to a single genetic locus, which is very convenient for further breeding purposes.

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INTRODUCTION

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 (Àvila *et al.*, 1992) and sequence divergence of the nucleoprotein gene (Àvila *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.*, 1991), including many important crops and ornamentals. Also the host ranges of both TCSV and GRSV are very broad (Àvila *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.*, 1992c) 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 kD, 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 kD and 58 kD, respectively, and a non-structural protein (NS_M) of 33.6 kD, which represents the putative viral cell-to-cell movement protein (Kormelink *et al.*, 1994). The S RNA codes for the nucleoprotein (28.8 kD) and another non-structural protein (NS_S) of 52.4 kD.

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; MacKenzie and Ellis, 1992; Pang *et al.*, 1992) and tomato plants (Ultzen *et al.*, 1995). 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 (Chapter 3).

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 (Chapter 3; Ultzen *et al.*, 1995). 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 economical 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 (Figure 4.1). 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 *N. tabacum* var. SR1 plants, via *A. tumefaciens*-mediated leaf disk transformation.

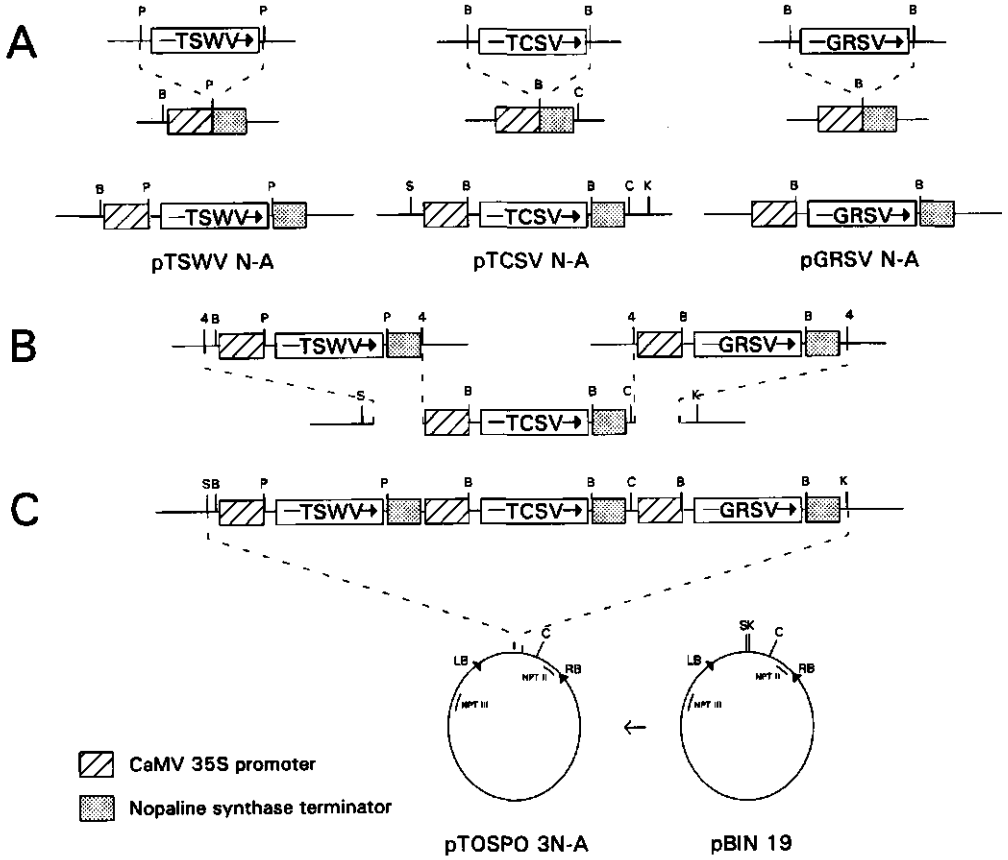

Figure 4.1: Construction of the triple N gene containing plant transformation vector pTOSPO 3N-A. A and B indicate the construction of progenitor plasmids, while C shows the construction of the final transformation construct pTOSPO 3N-A.

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 BamHI site of pZU-A(Bam), creating pTCSV N-A and pGRSV N-A, respectively. The TSWV N gene had been previously cloned in the PstI 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 (Figure 4.3A and Figure 4.3B) are indicated.

B=BamHI; C=ClaI; K=KpnI; S=SstI; P=PstI; 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.

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 analysed TSWV N protein expressing plants (Gielen *et al.*, 1991). In addition, low amounts of transgenic transcripts were produced, albeit at an equal level as some TSWV resistant lines described in Chapter 3.

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 4.1. In all these experiments, non-expressing 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 three to eight 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

Table 4.1: Resistance levels to TSWV, TCSV and GRSV inoculation in S2 progeny of transgenic tobacco plants transformed with the pTOSPO 3N-A construct.

	Number of resistant plants upon inoculation with		
S2 line	**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

high levels of resistance as listed in Table 4.2 and exemplified in Figure 4.2. Immunological analysis (ELISA) confirmed that the symptomless plants remained virus-free and that the broad protection was based on true immunity.

Table 4.2: 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

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 *E. coli* strain DH5αF', and *A. tumefaciens* strain LB4404, respectively. Although recombination in the *Rec A⁻ E.coli* strain DH5αF' 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 (Figure 4.3A and Figure 4.3B), 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.

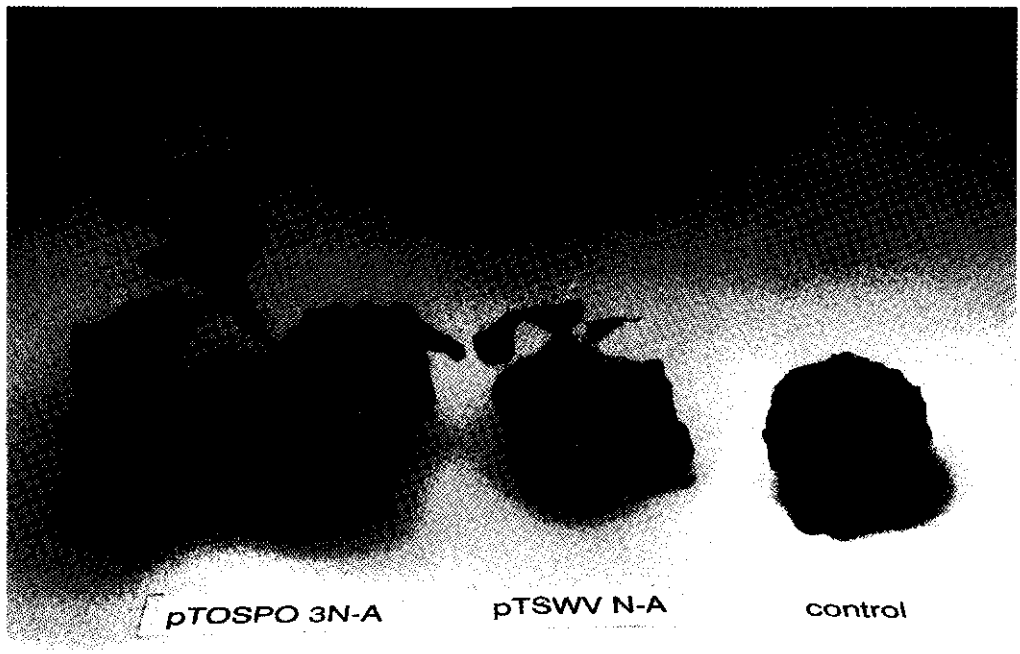

Figure 4.2: Phenotype of tobacco plants mechanically inoculated with TSWV, TCSV and GRSV simultaneously. **pTOSPO 3N-A**: plant of multiple tospovirus resistant line 3-6 transformed with the pTOSPO 3N-A construct. **pTSWV N-A**: plant of line 12 transformed with the pTSWV N-A construct, only resistant to TSWV, but susceptible to TCSV and GRSV. **control**: non-transgenic *N.tabacum* var. SR-1 plant.

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 ClaI and probed with TSWV N gene sequences (Figure 4.3A, lanes C). In addition, slight cross-hybridisation to a 2.4 kb pTOSPO 3N-A specific ClaI fragment containing sequences of the GRSV N gene was observed. In view of the positions of the two ClaI sites in the pTOSPO 3N-A transgene insert (Figure 4.1), 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

hybridising genomic ClaI 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 BamHI fragments of the expected size of 2.0 kb (Figure 4.3A, lanes B).

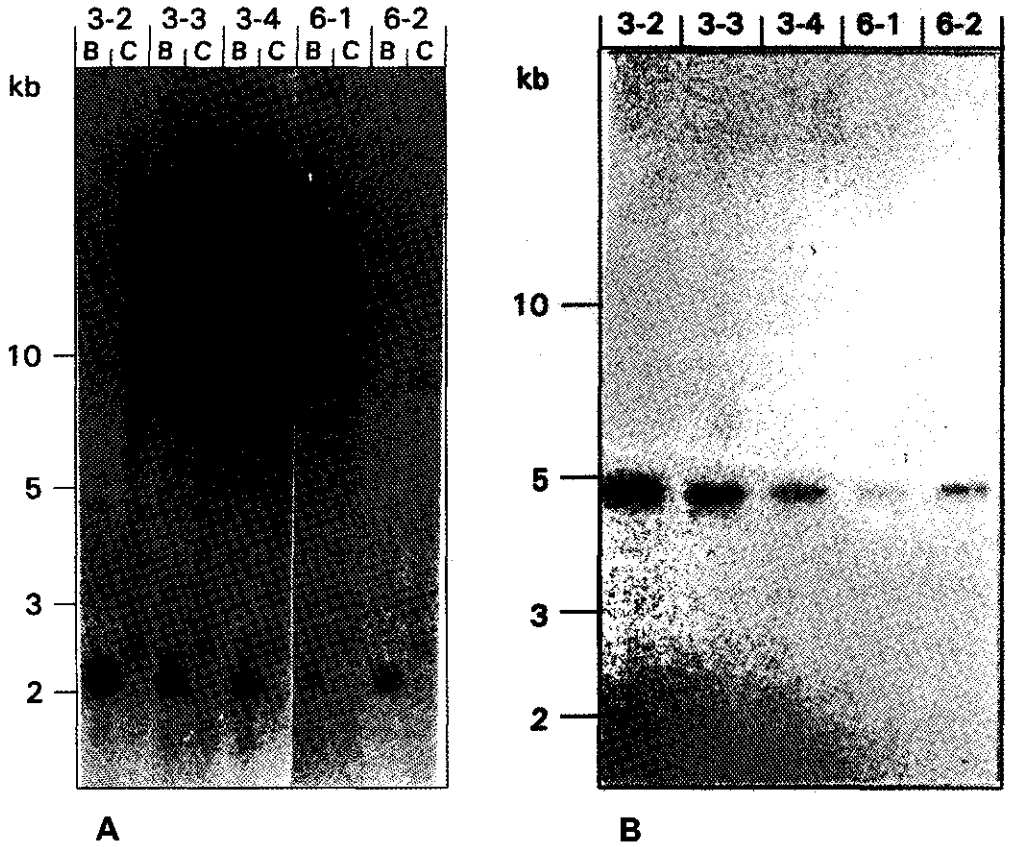

Figure 4.3: A) Southern blot analysis of genomic DNA of pTOSPO 3N-A transformed S2 tobacco lines derived from two different original transformants (3 and 6). Total genomic DNA was isolated from 4 week-old plants, digested with the appropriate restriction enzymes and fractionated on a 1% agarose gel, transferred to a Hybond membrane and hybridised 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. Three different restriction-enzymes were used in this experiment.
B=BamHI; C=ClaI. Sites in the pTOSPO 3N-A construct recognised by these two enzymes, are indicated in Figure 4.1.
B) Southern blot analysis of plants from S2 lines 3-2, 3-3, 3-4, 6-1 and 6-2 after digestion with both SstI and KpnI, 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.

DISCUSSION

Previous studies in our laboratory (Gielen *et al.*, 1991; Chapter 3) 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* var. SR1 plants. Our previous research (Chapter 3) 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, 1992a). 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 low amounts of transgenic RNA observed in Northern blot analysis, while, on the contrary, the observed levels of resistance are very high. However, it can not be 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,b).

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 4.1). 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 behaviour 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 favour 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 Àvila *et al.* (1990, 1992 and 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America or *N. tabacum* var. SR1.

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

Construction of the multiple nucleoprotein expression vector

Nucleoprotein gene sequences of TCSV and GRSV were obtained from cDNA clones described by Àvila *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 4.1.

Transformation of tobacco

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

N. tabacum var. 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* var. SR1 plants, were inoculated with TCSV, approximately 5 weeks after sowing. Resistant plants were left to set seeds. From a selection of S2 progeny lines, ten plants were inoculated with each of the three different tospoviruses TSWV, TCSV and GRSV independently, and twenty 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 weeks 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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Chapter 5

RNA-MEDIATED RESISTANCE TO TOMATO SPOTTED WILT VIRUS IN TRANSGENIC TOBACCO PLANTS EXPRESSING NS_M GENE SEQUENCES

Transgenic *Nicotiana tabacum* plants expressing RNA sequences of the tomato spotted wilt virus NS_M gene, which encodes the putative viral movement protein, were found to be highly resistant to infection with the virus. Expression of untranslatable as well as antisense RNA of the NS_M gene resulted in resistance levels as high as those in plants expressing translatable RNA sequences. For all three types of transgenic plants resistance levels of up to 100% were reached in the S2 progeny. These results indicate that the resistance mediated by the NS_M gene is accomplished by expression of transcripts rather than protein in transgenic plants, similar to previously observed N gene-mediated resistance. It is concluded that transgenic expression of NS_M RNA sequences is a successful strategy to protect host plants against tomato spotted wilt virus infection. Possible mechanisms underlying the resistance observed will be discussed.

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INTRODUCTION

The tospoviruses form a distinct genus of phytopathogenic viruses within the arthropod-borne family of Bunyaviridae (Murphy *et al.*, 1995) which is otherwise restricted to animals. Based on serological differences (Àvila *et al.*, 1992) and sequence divergence of the nucleoprotein gene (Àvila *et al.*, 1993; Law and Moyer, 1990), four different tospovirus species have so far been established: tomato spotted wilt virus (TSWV), tomato chlorotic spot virus (TCSV), groundnut ringspot virus (GRSV), and impatiens necrotic spot virus (INSV). Based on serological data, groundnut bud necrosis virus Reddy *et al.*, 1992), watermelon silverleaf mottle virus (Yeh *et al.*, 1992) and groundnut yellow spot virus (Reddy *et al.*, 1990) have been proposed as additional members of the *Tospovirus* genus. Tospoviruses are the only plant viruses that are transmitted by thrips species (*Thysanoptera*; Sakimura, 1962) in a propagative manner (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993).

The type species of the genus *Tospovirus*, TSWV, has a very broad host range, encompassing more than 650 plant species belonging to 70 different families (Goldbach and Peters, 1994), including many important crops and ornamentals.

The TSWV particle consists of a nucleocapsid core, in which the three genomic RNAs are tightly associated with the nucleoprotein (N), surrounded by a lipid membrane containing two types of glycoprotein-protrusions, G1 and G2 (Tas *et al.*, 1977). In addition, several copies of the putative viral RNA-dependent RNA polymerase are present in the virus particle (Van Poelwijk *et al.*, 1993). Nucleotide sequence determination revealed five open reading frames in the TSWV genome, specifying six mature viral proteins (De Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992c). The L RNA is of complete negative polarity and encodes the putative viral polymerase of 331.5 kilodalton (kD). The M and S RNAs both have an ambisense coding arrangement and are translationally expressed via subgenomic messenger RNAs (Kormelink *et al.*, 1992a). The M RNA codes for the common precursor to the membrane glycoproteins G1 and G2 (of 78 kD and 58 kD, respectively) and a non-structural protein (NS_M) of 33.6 kD, which represents the putative viral movement protein (Kormelink *et al.*, 1994; Storms *et al.*, 1995). The S RNA codes for the N protein of 28.8 kD and another non-structural protein

(NS_S) of 52.4 kD.

When compared to coding arrangements of the genomic RNAs of other members of the family Bunyaviridae, tospoviruses are unique in having an ambisense M RNA segment. The additional presence of the NS_M gene on the viral strand seems to be an evolutionary adaptation of Bunyaviridae to the plant kingdom. Results presented by Kormelink and co-workers (1994) and Storms and co-workers (1995) indicate that the NS_M gene product is involved in viral cell-to-cell movement.

Engineered resistance to TSWV has been accomplished previously by expressing the viral nucleoprotein (N) gene in transgenic tobacco plants (Gielen *et al.*, 1991) and was confirmed by others (MacKenzie *et al.*, 1992, Pang *et al.*, 1992). Recently, engineered TSWV resistance has been introduced in tomato plants (Kim *et al.*, 1994) and tomato hybrids (Ultzen *et al.*, 1995). Similar levels of protection, i.e. complete protection to the virus in homozygous S2 plants, have been observed when an untranslatable N gene was expressed, indicating that the N gene-based resistance is, at least for a major part, RNA-mediated (Chapter 3). This N gene-mediated resistance appeared to be virus-specific and is broken by other tospoviruses. Simultaneous introduction of several tospoviral N gene sequences to one genetic locus in the plant genome, led to broad-spectrum immunity to several tospoviruses (Chapter 4).

Besides the use of the nucleoprotein of tospoviruses and the widespread use of coat protein sequences for positive strand RNA viruses (for references see Wilson, 1993), other, non-structural gene sequences have been used to confer engineered virus resistance, including replicase, protease and movement protein genes. (e.g. Golemboski *et al.*, 1990; MacFarlane *et al.*, 1992; Carr *et al.*, 1992; Maiti *et al.*, 1993; Malyshenko *et al.*, 1993; Vardi *et al.*, 1993; Lapidot *et al.*, 1993; Longstaff *et al.*, 1993; Carr *et al.*, 1994; Audy *et al.*, 1994; Brederode *et al.*, 1995; Cooper *et al.*, 1995).

To date, limited results have been obtained in resistance to viral pathogens using transgenic expression of (defective) viral movement protein sequences. Transgenically produced movement protein has been proposed as a source for resistance to systemic spread of geminiviruses by Von Arnhim and Stanley (1992), based on the observation that systemic movement of African cassava mosaic geminivirus in *N.benthamiana* plants is inhibited by co-inoculation with constructs expressing movement protein sequences

of the related geminivirus tomato golden mosaic virus (TGMV). A reduction of tobacco mosaic virus (TMV) accumulation at non-permissive temperatures in tobacco plants transformed with movement protein sequences derived from temperature sensitive cell-to-cell movement mutant Ni2519 has been reported (Malyshenko *et al.*, 1993). Inhibition of disease symptom development of TMV as well as two other tobamoviruses (tobacco mild green mosaic, TMGMV and sunnhemp mosaic virus, SHMV) in plants expressing a defective movement protein lacking three amino acids at its N-terminus, has been reported by Lapidot and co-workers (1993). Furthermore, these plants show a delay of several days in the appearance of viral symptoms when inoculated with other non-related viruses (Cooper *et al.*, 1995). In general, interference with cell-to-cell movement or systemic spread of the target virus might be a powerful means to protect plants from viral pathogens.

Here we report that high levels of resistance to the negative-stranded tomato spotted wilt virus can be obtained in transgenic tobacco plants expressing sequences derived from its putative viral movement protein gene, NS_M.

Table 5.1: Resistance levels in NS_M sequence expressing transgenic tobacco S1 plants and S2 progenies

progenies	S1 line	#resistant/#tested	100% resistant S2
pTSWV NS_M-A	A2	6/20	4/6
	A13	2/20	0/2
	A23	5/20	0/5
	A30	2/20	2/2
	29 others	0/20	-
pTSWV NS_M-B	B3	10/20	4/10
	25 others	0/20	-
pTSWV NS_M-C	C14	10/20	3/10
	C22	3/20	3/3
	22 others	0/20	-

RESULTS

Resistance levels in transgenic tobacco plants

In total, 85 transformed plants were obtained that expressed NS_M-derived sequences. Of these plants, 35 expressed NS_M-A sequences, 26 the NS_M-B insert and 24 transformants were obtained containing NS_M-C. All original transformants were maintained for seed production. Twenty plants of the resulting S1 progenies were subsequently assayed for resistance to TSWV. Resistant plants were maintained for S2 seed production and resulting S2 plants were tested for their ability to resist infection by TSWV.

Four of the pTSWV NS_M-A transformed plants showed virus disease-like symptoms during their development. Whether these malformations are the result of the expression of the NS_M protein remains to be investigated. From the 35 original transformants two plants were unable to set seed and therefore no progeny could be tested. Thirty-three S1 lines, derived from the remaining transformants were tested and four displayed resistance to TSWV (Table 5.1). From the progenies of those plants, six lines displayed complete resistance to TSWV, while the other S2 lines displayed partial resistance, probably due to segregation of the transgene. Progeny lines derived from fully resistant S2 lines also displayed complete resistance, suggesting the resistant S2 lines are homozygous for the transgene.

Of the 26 S1 lines transformed with the NS_M-B construct, only one showed resistance to the virus, albeit at a considerable level of 50% in the S1 generation. In four of the resulting ten S2 lines complete resistance was observed in all plants. In two of the segregating NS_M-C S1 lines resistance levels of up to 50% were observed, and resistance levels of up to 100% were reached in six of the resulting S2 lines. No viral NS_S protein, as a proof for viral replication (Wijkamp *et al.*, 1993), was detected by ELISA in neither inoculated nor systemic leaves of any of the resistant plants. Figure 5.1 illustrates the temporal development of symptoms in S1 lines that were most resistant to TSWV (A2, B3 and C14) and some of their S2 progeny lines.

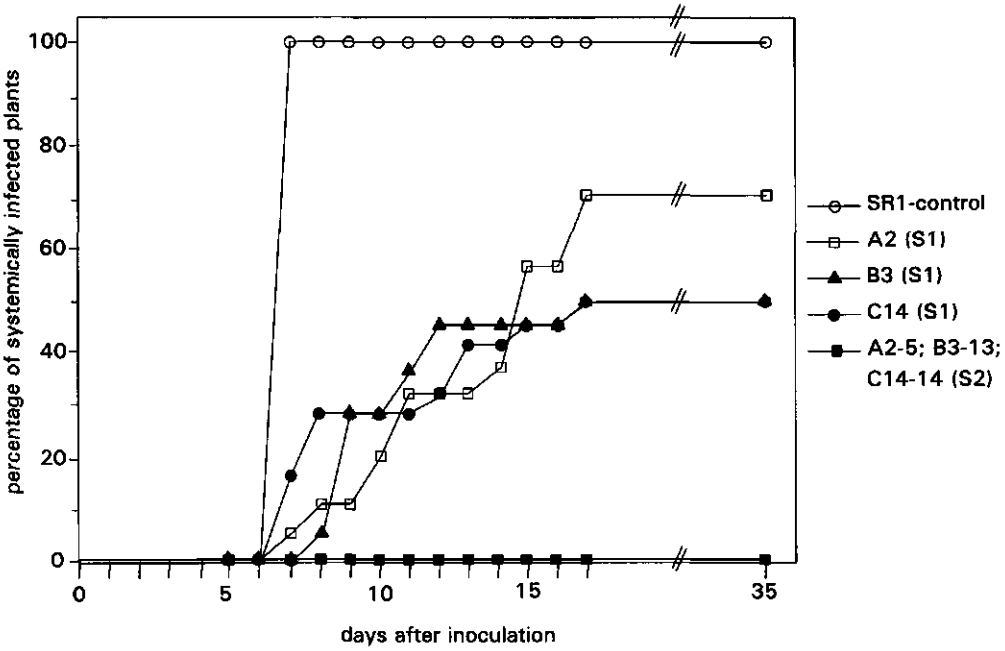

Figure 5.1: Development of systemic symptoms in transgenic tobacco plants expressing TSWV movement protein (NS_M) sequences. The best performing S1 lines of the three different groups of transformants are indicated (A2, B3 and C14), as well as some S2 lines derived plants from these S1 lines that show complete immunity to tomato spotted wilt virus (lines A2-5, B3-13 and C14-14). *N. tabacum* var. SR1 plants from a segregant line lacking transgenic sequences were used as a TSWV susceptible control.

Resistance to other tospoviruses

Plants from all 16 S2 lines that displayed immunity to TSWV (derived from S1 lines A2, A30, B3, C14 and C22) were inoculated with TSWV, TCSV and GRSV, both simultaneously and separately. All plants of these lines were susceptible to TCSV and GRSV, and developed disease symptoms typical for these viruses, indicating that the NS_M gene-mediated resistance, like N gene-mediated resistance, is highly virus-specific and does not hold upon inoculation with other tospoviruses.

Expression of NS_M specific RNA and protein in transgenic plants

The transcriptional expression of NS_M sequences was checked by Northern blot analysis, using a ^{32}P-dATP labeled double stranded NS_M cDNA probe. In the resistant lines, derived from all three different constructs, transgenically produced RNA was detected, but in much lower levels than those observed in virus-infected plants. Remarkably, no correlation between steady-state expression levels of the transgene RNA and resistance levels was observed in any of the NS_M expressing lines, as was the case with N gene RNA-mediated resistance (Chapter 3). Figure 5.2 shows the steady state RNA expression levels for 13 NS_M-C lines, and is representative for all NS_M transformed plant lines.

In leaf extracts from NS_M-A transformed plants no NS_M protein could be detected neither by Western blot analysis, nor by ELISA techniques. NS_M protein, however, may have been present in subdetectably low levels.

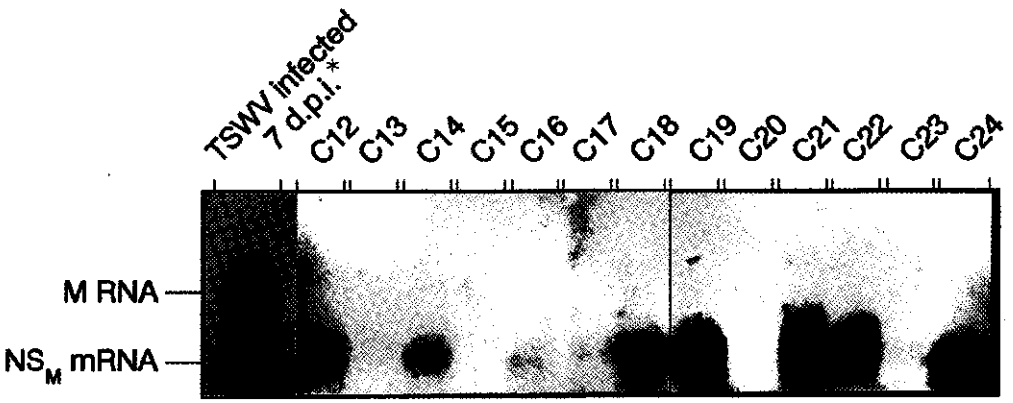

Figure 5.2: Northern blot analysis of 13 lines transformed with the pNS_M-C construct. RNA isolated from TSWV-infected plant material revealing genomic M RNA and NS_M mRNA was added as a control. The lane containing RNA of infected plants contained 5 times less material and was exposed 10 times shorter than the lanes containing transgenic RNAs, indicating the relatively low abundance of transgenic NS_M RNA in plants.

DISCUSSION

Viral coat protein and nucleoprotein sequences have been successfully applied to confer resistance to the corresponding viruses in transgenic plants. More recently, expression of sequences corresponding to other viral genes have become a successful tool for inducing pathogen-derived resistance, such as replicase sequences (Golemboski *et al.*, 1990; Carr *et al.*, 1992; MacFarlane *et al.*, 1992; Longstaff *et al.*, 1993; Carr *et al.*, 1994; Audy *et al.*, 1994; Brederode *et al.*, 1995) protease sequences (Maiti *et al.*, 1993; Vardi *et al.*, 1993) and (defective) movement protein sequences (Malyshenko *et al.*, 1993; Lapidot *et al.*, 1993; Cooper *et al.*, 1995).

In the research described in this paper we show that, besides expression of N gene sequences of tospoviruses (Gielen *et al.*, 1991; Chapter 3 and Chapter 4), also expression of sequences derived from the NS_M gene, the putative tospoviral movement protein gene, confers resistance in transgenic tobacco plants. Observed resistance in NS_M gene sequence expressing plants reaches similar high levels as were observed in plants expressing nucleoprotein gene sequences. NS_M-A transformed plants did not accumulate detectable amounts of NS_M protein, but detectable levels of transgene transcripts were present in most of the transformed NS_M-A lines. The high levels of resistance in plants expressing untranslatable (NS_M-B) or antisense (NS_M-C) RNA support the view that in all types of transformants, including the NS_M-A lines, the transgenically expressed RNA, confers the observed resistance. The manifestations of the RNA-mediated resistance phenomenon induced by NS_M sequences is similar to that of the previously described N gene RNA-mediated resistance. Resistance induced by NS_M and N sequences both result in high levels of resistance to TSWV in homozygous S2 progeny lines. Moreover, they both share the same spectrum of resistance, and only hold against the virus (TSWV) of which the transgene sequence was derived. Similar characteristics have also been found for RNA-mediated resistance to other viruses, e.g. potyviruses TEV (Lindbo *et al.*, 1992a) and PVY (Van der Vlugt *et al.*, 1992).

A number of theories have been proposed for the mechanism of such an RNA-mediated resistance. The transgenically produced RNA may interact with the incoming viral RNA or with replicative forms of the virus by an antisense mechanism, involving

direct RNA-RNA interactions. Another model implies competition between transgenic RNAs and viral RNAs for essential plant or viral factors involved in virus replication. Lindbo *et al.* (1993) proposed a mechanism that implies the induction of an anti-viral state in the cytoplasm of transgenic plants, similar to the sense- or co-suppression phenomenon observed in transgene expression studies of endogenous genes in plants (for review see e.g Matzke and Matzke, 1995a). This co-suppression causes dramatically decreased endogenous RNA steady-state levels upon expression of homologous transgene sequences as a result of a post-transcriptional RNA-degrading mechanism. RNA-mediated resistance in plant lines expressing the TEV coat protein RNA sequences that were immune to the virus was suggested to be a result of increased RNA turnover of (transgene) viral sequences, since nuclear *de novo* synthesis in run-on assays remained high in these plants, while cytoplasmic steady-state RNA levels were low (Dougherty *et al.*, 1994). Our results with run-on studies (Chapter 7) suggest that also TSWV resistance operates via a co-suppression-like mechanism similar to that described for potyviruses Lindbo *et al.*, 1993; Dougherty *et al.*, 1994).

As it has now been demonstrated that, besides N gene sequences (Chapter 3), also NS_M gene sequences can induce virus resistance, the question arises whether any randomly chosen sequence from all genomic regions would also induce virus resistance upon transgenic expression. This will be discussed further in Chapter 6.

MATERIALS AND METHODS

All manipulations with DNA or RNA were accomplished by using 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 Àvila (1990; 1992; 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America or *N. tabacum* var. SR1.

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

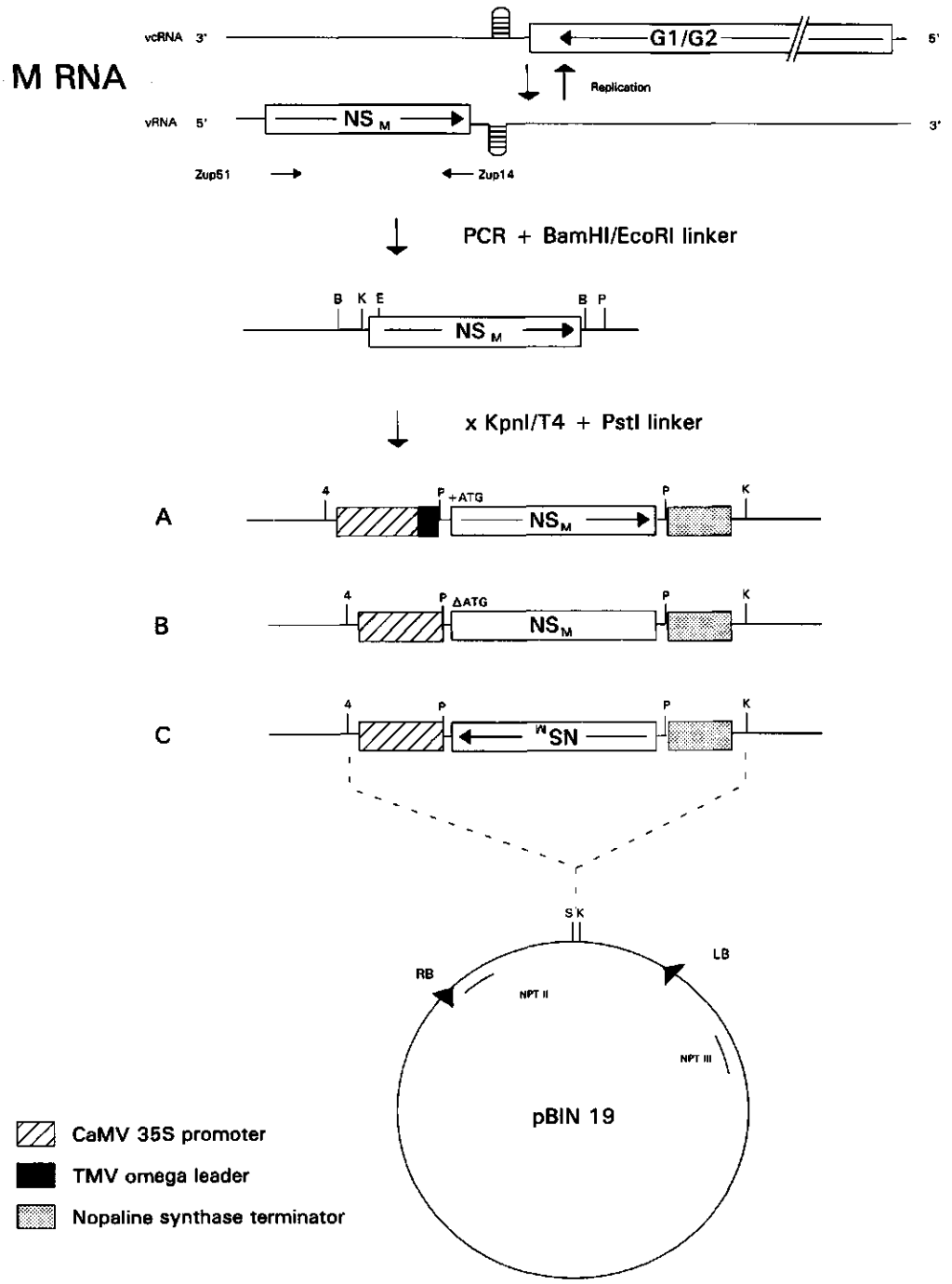
M RNA
vcRNA 3'
G1/G2
5'
Replication
vRNA 5'
NS M
3'
Zup51
Zup14
PCR + BamHI/EcoRI linker
B K E
B P
NS M
x KpnI/T4 + PstI linker
A
4
P
+ATG
NS M
P
K
B
ΔATG
C
SN
S K
RB
LB
NPT II
NPT III
pBIN 19
CaMV 35S promoter
TMV omega leader
Nopaline synthase terminator

Figure 5.3: Construction of plant transformation vectors pTSWV NS_M-A, NS_M-B and NS_M-C. NS_M sequences were PCR amplified from a genomic cDNA clone of TSWV, using primers that added a BamHI restriction site to the 3' end of the gene (Zup014) and a EcoRI site immediately downstream of the original startcodon (Zup051). An oligonucleotide linker sequence containing an in frame startcodon was ligated to the 5' end of the PCR fragment, thereby restoring the NS_M ORF. Directly upstream of the ATG a unique KpnI site is present. This fragment was inserted in the BamHI site of a pUC18 vector, resulting in a PstI site 3' of the NS_M sequence. By treating KpnI linearised DNA with T4 DNA polymerase, blunt ends were created to which PstI linkers were ligated prior to cloning the NS_M gene as a PstI fragment in the PstI site of the pZU-A plasmid in both sense (A) and antisense orientation (C). In the pTSWV NS_M-A construct, the pZU-A vector had been previously supplied with TMV translation enhancing sequences upstream of the cloning site. Incubation with T4 DNA polymerase for a longer time at an elevated temperature resulted in the removal of extra nucleotides by the exonuclease activity of T4 DNA polymerase, these extra nucleotides included the A residue of the NS_M startcodon. Ligation of a PstI linker yielded an ATG-deficient NS_M sequence, which is hence not translatable (B). After cloning in the PstI site of the pZU-A vector, the NS_M sequences are supplied with a CaMV 35S promoter at the 5' end and flanked at their 3' ends by a nopaline synthase terminator. Finally, all three NS_M constructs were cloned in the KpnI and SmaI sites of binary vector pBIN19. B=BamHI; E=EcoRI; K=KpnI; P=PstI; S=SmaI; 4=blunt after treatment with T4 DNA polymerase. RB and LB are right and left border sequences, respectively. The ΔATG indicates removal of the ATG startcodon.

Construction of NS_M gene sequence expression vectors

NS_M gene sequences of TSWV (Kormelink *et al.*, 1992c), were modified using PCR in such a way that an EcoRI site was generated immediately downstream of the original startcodon, using primer Zup051 (dGGGAATTCTTTTCGGTAACAAGAGGCC) located at position 108 to 129 of the viral M RNA and primer Zup014 (dCCCTGCAGGATCCGA-AATTAAAGCTTAAATAAGTG) located at position 1043 to 1023 of the viral complementary M RNA. The resulting PCR fragment was digested with EcoRI and a EcoRI/BamHI linker including an internal KpnI site and an in frame start codon

```
5' GATCCGGCAACGAAGGTACCATGGG      3'
3'     GCCGTTGCTTCCATGGTACCCTTAA 5'
   BamHI          KpnI NcoI EcoRI
```

was ligated. This slightly modified NS_M gene (starting with aminoacid sequence Met.Leu.Ile... in stead of Met.Thr.Val...) was cloned in a pUC18 vector as a BamHI restriction fragment. The resulting plasmid was linearised using KpnI, and PstI linkers were ligated after creating blunt ends using T4 DNA polymerase. The 5' to 3' exonuclease activity of T4 DNA polymerase was used to create an untranslatable NS_M sequence devoid of its start codon. Different reaction temperatures and incubation times

were used to vary the extent of 5' to 3' exonuclease degradation. The resulting clones were checked by sequence analysis and a clone was selected in which the original startcodon was mutagenised to CTG. The PstI restriction fragments, i.e. one with an in frame ATG start codon and the mutant, were ligated in plant transformation vector pZU-A (Gielen *et al.*, 1991) between the CaMV 35S promoter and the nopaline synthase (nos) terminator. In case of the pTSWV NS_M-A construct, the untranslated leader sequence of TMV (Gallie *et al.*, 1987) was inserted immediately upstream of the NS_M gene. In addition, an antisense construct was selected. Finally, three NS_M constructs were inserted in binary vector pBIN19 (Bevan, 1984), yielding pTSWV NS_M-A (sense polarity), pTSWV NS_M-B (sense/untranslatable) and pTSWV NS_M-C (antisense polarity). Details of this cloning schedule are presented in Figure 5.3.

Transformation of tobacco

The pBIN19-derived vectors pTSWV NS_M-A, NS_M-B and NS_M-C were introduced in *Agrobacterium tumefaciens* strain LB4404 (Ditta *et al.*, 1980) by triparental mating, using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid. *N. tabacum* var. SR1 plants were transformed and regenerated as described by Horsch and co-workers (1985).

Analysis of protection of transgenic plants against TSWV

Inoculations were done according to standard procedures (Gielen *et al.*, 1991) and repeated after two weeks to exclude plants escaping inoculation being scored resistant. The appearance of systemic symptoms was monitored on a daily basis until day 35 after the first inoculation. Plants were scored susceptible when leaves younger than the inoculated leaf showed characteristic tospovirus induced symptoms i.e. severe stunting and chlorosis, usually followed by death of the plant within a week. Samples of both inoculated and systemic leaves from visually healthy plants were collected to check for the presence of the NS_S gene product by ELISA, using a polyclonal antiserum directed against TSWV NS_S protein (Kormelink *et al.*, 1991). This antiserum also recognizes the NS_S proteins of established tospoviruses TCSV and GRSV.

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Chapter 6

ENGINEERED RNA-MEDIATED RESISTANCE TO TOMATO SPOTTED WILT VIRUS IS SEQUENCE SPECIFIC

To test whether the previously reported RNA-mediated resistance to tomato spotted wilt virus in tobacco plants expressing viral nucleoprotein (N) or movement protein (NS_M) gene sequences, can be induced by any randomly chosen viral genome sequence, transgenic plants were produced that expressed a wide range of parts of the TSWV RNA genome or its complement. Testing the progenies of these plants revealed that only those plants that expressed N or NS_M gene sequences showed resistance to TSWV. All plants expressing other parts of the L, M or S segment were still susceptible to TSWV infection irrespective whether these viral sequences contained terminal or internal sequences, and translatable or untranslatable regions. The suggested important role for N and NS_M gene products in early stages of tospovirus infection of plants will be discussed.

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INTRODUCTION

Since the first report on pathogen-derived resistance (PDR) against a virus (Powell Abel *et al.*, 1986) many reports of coat protein-mediated resistance to plus strand RNA viruses have been published (for references see Wilson, 1993). Also expression of the nucleoprotein of the negative strand RNA virus TSWV resulted in resistance (Gielen *et al.*, 1991; MacKenzie and Ellis, 1992; Pang *et al.*, 1992). More recently, PDR has been reported by expressing other (modified) virus genes, e.g. genes encoding replicases, movement proteins and other non-structural proteins of various viral sources (for references see e.g. Beachy, 1993).

Many of these reports indicate the involvement of the transgenically produced protein. However, a number of reports has shown that transgenic plants also exhibit resistance when using untranslatable forms of coat protein sequences (Lindbo *et al.*, 1992; Van der Vlugt *et al.*, 1992), and the RdRp gene of PVX (Mueller *et al.*, 1995). The same holds true for sequences derived from the nucleoprotein gene of the negative strand tospoviruses (Chapter 3). Resistance in plants expressing untranslatable forms of coat protein and nucleoprotein genes indicate an RNA-mediated type of resistance. Most of the proposed mechanisms for this RNA-mediated resistance involve inhibition of viral replication by an antisense effect, or by binding factors involved in the replication process. Lindbo and co-workers (1993) proposed a process similar to co-suppression (Matzke and Matzke, 1993) as a possible mechanism for this RNA-mediated resistance, based on their observation of suppression of transgenic RNA levels upon infection with the corresponding virus in resistant transgenic plants.

Tomato spotted wilt virus is the type species of the genus *Tospovirus*, which encompasses the thrips-transmitted plant-infecting members of the *Bunyaviridae* (Francki *et al.*, 1991).

The genome of tomato spotted wilt virus consists of three RNA species, of which the L RNA (8.9 kb) is of complete negative polarity while both the M RNA (4.8 kb) and S RNA (2.9 kb) have an ambisense coding strategy. The five viral open reading frames are translated from viral messenger RNAs (Kormelink *et al.*, 1992a) and specify a total of six mature viral proteins, two non-structural proteins (NS_S and NS_M of 52.4 kD and 33.6 kD

resp), the two envelope glycoproteins (G1 and G2 of 78 kD and 58 kD), the putative viral polymerase (L of 331.5 kD) and the nucleoprotein (N) of 28.8 kD (De Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1992c).

In some cases the appearance of defective interfering RNAs (DIs) derived from the L RNA have been observed in tospovirus-infected plants. The presence of these DIs generally causes an attenuation of viral symptoms (Resende *et al.*, 1991b and 1992).

In previous studies, translatable N gene sequences were introduced in transgenic *N.tabacum* var. SR1 plants. Several lines of these N protein-expressing plants, produced completely immune S2 progeny plants (Gielen *et al.*, 1991). Further studies revealed that the presence of transgenic RNA rather than protein is responsible for the observed immunity (Chapter 3). Our recent work revealed similar resistance to the virus when using sequences derived from the TSWV NS_M gene (Chapter 5), which encodes the putative viral movement protein (Kormelink *et al.*, 1994; Storms *et al.*, 1995). Expression of translatable and untranslatable forms of the NS_M gene resulted in immune transgenic S2 lines, as did the NS_M antisense RNA (Chapter 5).

The results with the N and NS_M gene sequences raises the question whether the resulting RNA mediated-resistance is gene specific or whether any, randomly selected sequence of the TSWV genome would confer such resistance upon transgenic expression.

RESULTS

Transformation of tobacco with genomic sequences of TSWV

To investigate whether RNA-mediated resistance can be obtained by transgenic expression of any randomly chosen genomic RNA sequence of TSWV, 14 different viral cDNA constructs were made. Together with the previously analysed N and NS_M gene constructs (Gielen *et al.*, 1991; Chapter 3; Chapter 5), this set of constructs spans over 70% of the TSWV genome, covering virtually the entire M and S segments and a large part of the L segment. The positions of the tospoviral RNA sequences expressed from these constructs in the RNA genome of TSWV are indicated in Figure 6.1.

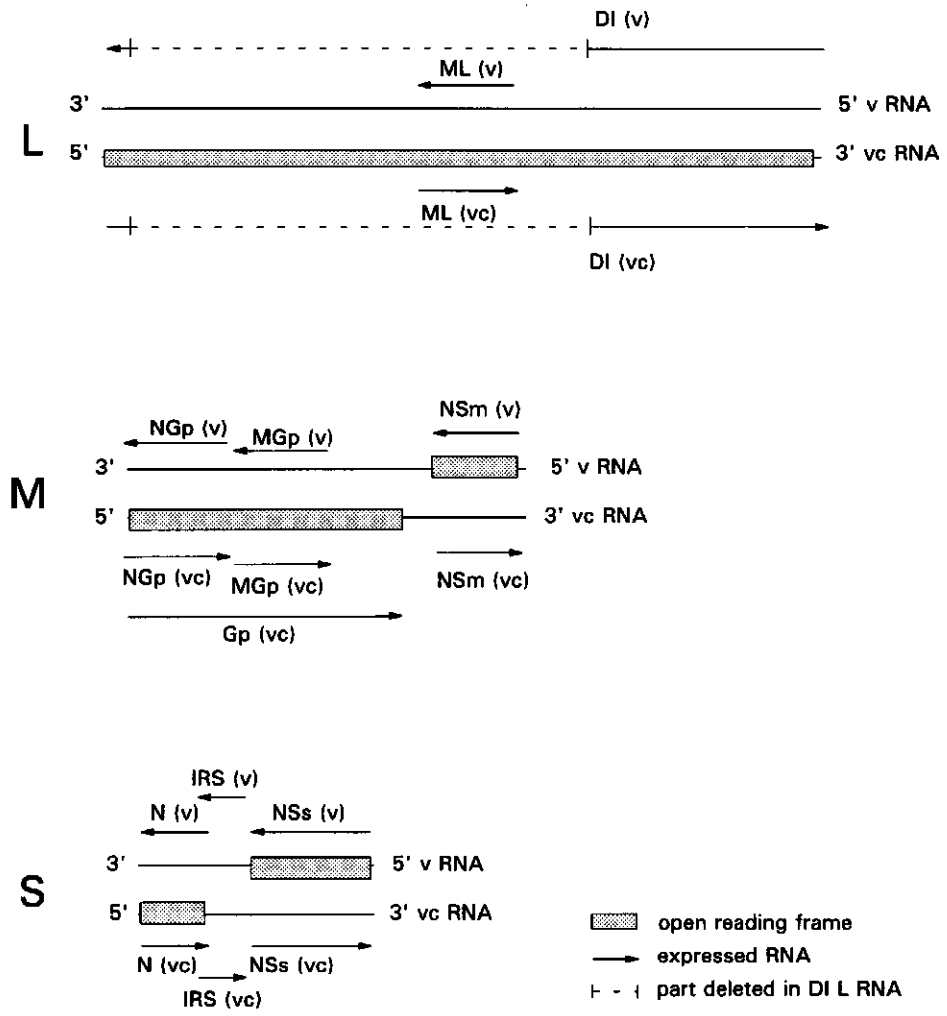

Figure 6.1: Position on the TSWV genome of the viral cDNA constructs used for transformation of tobacco plants. DI encompasses L RNA sequences found in a naturally occurring defective interfering RNA, ML spans the middle of the L ORF including the majority of the conserved polymerase motifs. Clone NSm encodes the entire NS_M ORF. NGp comprises the N terminal third of the precursor to the glycoproteins, MGp the central part of this precursor, while Gp transcribes the complete glycoprotein ORF. N and NSs clones include the respective genes and with their viral 5' untranslated leader sequences. IRS contains the cDNA of the highly internally basepaired intergenic region of the S RNA.

All genomic fragments, supplied with a CaMV 35S promoter and a nos terminator sequence, were cloned in binary vector pBIN19 (Bevan, 1984) and subsequently used for *Agrobacterium tumefaciens*-mediated transformation of *Nicotiana tabacum* var. SR1 leafdiscs.

Table 6.1. Resistance levels inoculation in S1 and S2 progenies of transgenic tobacco plants expressing parts of the TSWV genomic RNAs.

		Percentage of resistant plants	
line	**(# resistant lines/# lines)**	**S1**	**S2**
N[1] (vc/ORF)	4/25	25-90%	100%
N[2] (vc/ATG-)	4/23	30-80%	100%
N (v)	4/24	10-50%	100%
IRS (v)	0/17	0%	-
IRS (vc)	0/17	0%	-
NS_S (v/ORF)	0/10	0%	-
NS_S (vc)	0/22	0%	-
NG (v)	0/22	0%	-
NG (vc)	0/18	0%	-
MG (v)	0/15	0%	-
MG (vc)	0/20	0%	-
G1/G2(vc/ORF)	0/21	0%	-
NS_M[3] (v/ORF)	4/33	10-30%	100%
NS_M[3] (v/ATG-)	1/26	50%	100%
NS_M[3] (vc)	2/24	15-50%	100%
ML (v)	0/11	0%	-
ML (vc)	0/25	0%	-
DI (v)	0/13	0%	-
DI (vc)	0/ 7	0%	-

[1] Gielen *et al.*, 1990; [2] Chapter 3; [3] Chapter 5

Testing transgenic plants for resistance to TSWV

Between 7 and 25 original transformants were obtained for each construct (see Table 6.1), adding up to a total of 242 transformed plants in addition to the 131 lines tested in previously described experiments (Gielen *et al.*, 1991; Chapter 3; Chapter 5). The S1 progenies of these plants were assayed for resistance to TSWV. Subsequently, the S2

progenies of surviving plants were also inoculated with the virus. To obtain highly resistant progeny, i.e. RNA-mediated resistance phenotype, all plants were inoculated twice within a two week interval, using a high titer of virus (TSWV strain BR-01).

Despite the high inoculum concentrations and repeated inoculations, the amount of originally surviving antisense N expressor plants, as well as the number of these lines showing resistant plants were comparable to experiments involving the messenger sense N and both the sense and antisense NS_M sequences. Of plants expressing anti-messenger sense N sequences, the S2 progenies were completely immune to high doses of virus, similar to S2 generation plants expressing messenger sense N gene sequences and plants expressing sequences of the NS_M gene. These resistant S2 plants remained virus free, as verified by ELISA.

In contrast to the antisense N expressor plants and the previously described N and NS_M sequence expressor lines (Gielen *et al.*, 1991; Chapter 3; Chapter 5), all lines expressing other parts of the TSWV genome were completely susceptible to the virus, indicating that for TSWV, the RNA-mediated pathogen-derived resistance is sequence specific and restricted to plants expressing N or NS_M gene sequences (Table 6.1). Resistance inducing ability of different TSWV sequences in transgenic plants is visualized in Figure 6.2.

Expression of RNA and protein

Expression of transgenic RNA in S1 lines was checked by Northern blot analysis and always showed low levels of RNA accumulation when compared to RNA levels reached during virus infection. Possible expression of the NS_S and glycoproteins (G1 and G2) when translatable RNAs were expressed in transgenic lines was assayed by ACP and TAS-ELISA, respectively, using NS_S polyclonal antiserum (Kormelink *et al.*, 1991) and monoclonal antibodies against glycoproteins (Huguenot *et al.*, 1990). In none of the S1 lines expressing translatable NS_S or glycoprotein RNAs detectable amounts of protein could be shown, despite the presence of transgenic RNAs.

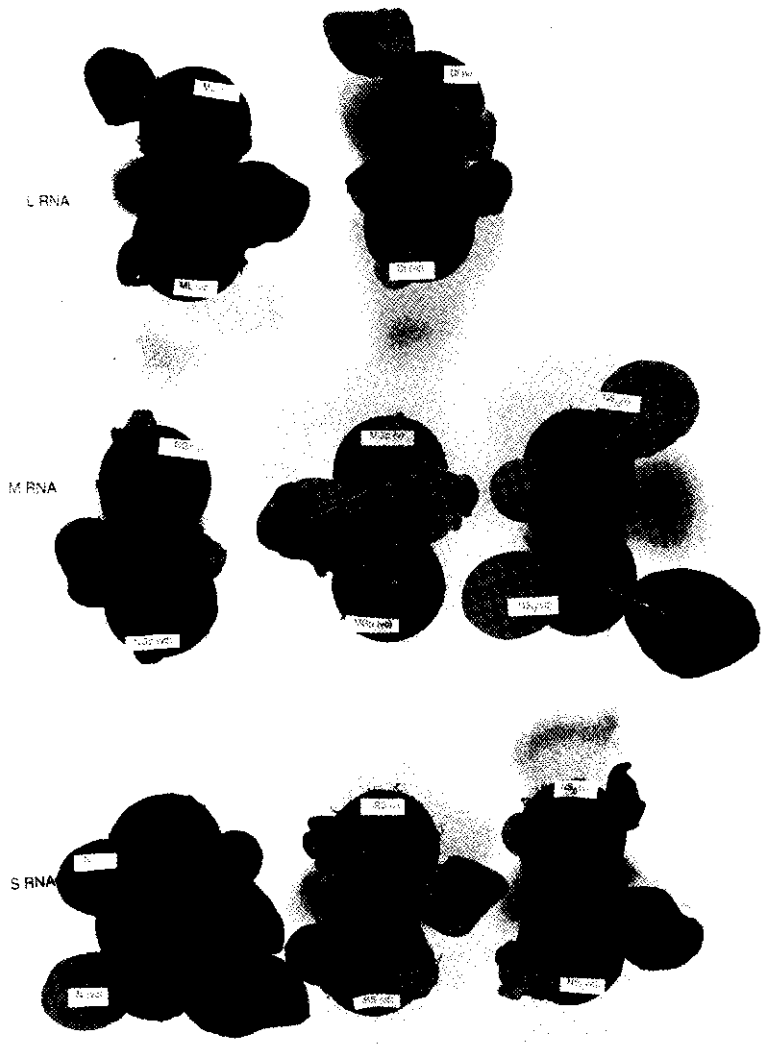

Figure 6.2: Susceptibility to virus infection of plants expressing a variety of TSWV sequences. Plants were photographed 7 days after inoculation with TSWV. Only plants expressing N or NS_M sequences (both viral and viral complementary sense) are resistant to the virus.

Resistance to other tospoviruses

The antisense N (i.e. viral RNA strand) expressing S2 progeny plants that were completely immune to infection with TSWV, were also challenged with two other closely related tospoviruses: tomato chlorotic spot virus and groundnut ringspot virus (TCSV strain BR-03 and GRSV strain SA-05; Àvila *et al.*, 1993). All inoculated plants appeared to be completely susceptible to both viruses, similar to previous observations with the TSWV resistant sense N (i.e. vc strand) expressing plants (Chapter 3) and plants expressing NS_M sense and antisense sequences (Chapter 5). The other established, more distantly related tospovirus, impatiens necrotic spot virus (INSV), was not tested, since this virus is not capable of inducing systemic symptoms in *N. tabacum*.

DISCUSSION

Only sequences derived from the N and NS_M gene regions of the TSWV genome appear to be able to confer resistance to transgenic plants. All other RNA sequences used in this extensive experiment, covering most of the TSWV genome, are not able to confer resistance to transgenic plants. This sequence specificity indicates that inhibition of virus replication by direct interfering with the viral genomic RNAs, based on antisense interference as proposed previously (e.g. Chapter 3), seems a less likely model for the observed transgenically induced RNA-mediated resistance to TSWV. The explanation for the sequence specificity of the resistance should reside in the mode of its action. Possibly, the mechanism that causes RNA-mediated resistance can not interfere with the viral genomic RNA, as it remains encapsidated in N protein throughout the infection cycle, but only with the (non-encapsidated) viral mRNAs. The relevance of the viral proteins, encoded by these RNAs, may be crucial for the effectiveness of several of the constructs used. A thorough knowledge of the tospoviral infection cycle in plants is therefore required. The infection process of tospoviruses in plants is thought to involve five major steps (Figure 6.3). First the virus enters the cell through feeding of viruliferous thrips and viral nucleocapsids are released. Second, viral mRNAs are transcribed at low cellular concentrations of the N protein (i.e. the initial phase of viral infection). From these unencapsidated mRNAs, viral proteins are translated (Kormelink *et al.*, 1992a).

Upon elevation of the N protein levels the viral RdRp (L) switches its mode to replication of vRNAs (step 3). These newly produced RNAs are encapsidated with N protein and associate with copies of the L protein. At this stage the nucleocapsid structures are either budded in the RER or golgi-membranes (step 4), thereby forming new viral particles that can be taken up by feeding thrips. Cell-to-cell movement within plants is mediated by association of nucleocapsids with the NS_M protein that mediates tubule guided movement through modified plasmodesmata (step 5) (Kormelink *et al.*, 1994; Storms *et al.*, 1995).

The observation that RNA-mediated resistance against TSWV is not effective with most parts of the viral genome is quite unexpected and seems to contradict some of the results obtained with positive strand RNA viruses. For potato virus X (PVX) for instance, it has been found that even nonviral (GUS) sequences can induce RNA-mediated virus resistance provided only that this sequence has been introduced in the viral genome (English *et al.*, 1996). A possible explanation why only the N and NS_M sequences of TSWV are capable of introducing transgenic resistance may be that its genomic RNA segments, like those of all negative stranded RNA viruses, remain packaged with nucleocapsid protein throughout the infection cycle. The RNA-mediated resistance might therefore not operate on (encapsidated) genomic level but rather on (non-encapsidated) viral mRNA level. Along this line the relative importance of the viral protein encoded by the different tospoviral mRNAs may be crucial for the effectiveness of several of the constructs used. Indeed both the N protein (regulator of transcription-to-replication switch) and NS_M protein (movement protein, Kormelink *et al.*, 1994; Storms *et al.*, 1995; Chapter 8) are essential for systemic host plant infection, while inhibition of some other functions (e.g. G1/G2 and NS_S) may only interfere with replication in (Wijkamp *et al.*, 1993) or spread by (Resende *et al.*, 1991b) the insect vector. Sequences derived from the putative viral RNA dependent RNA polymerase gene (L) seem to be unable to confer resistance, although this protein is obviously indispensable for replication. Perhaps it is necessary that the complete open reading frame, or at least most of the RNA sequence is expressed in plants in order to give resistance and not just partial gene sequences (constructs ML and DI, Figure 6.1) as used in this experiment. Another possibility may be that the limited number of polymerase molecules already present in the virus particle

(Van Poelwijk *et al.*, 1993) are sufficient to carry out the initial rounds of replication, which may be essential in overcoming the inhibition of virus replication in transgenic plants. Alternatively, a small number of catalytic L protein molecules, sufficient for viral replication, may be produced despite the suppression of the mRNA.

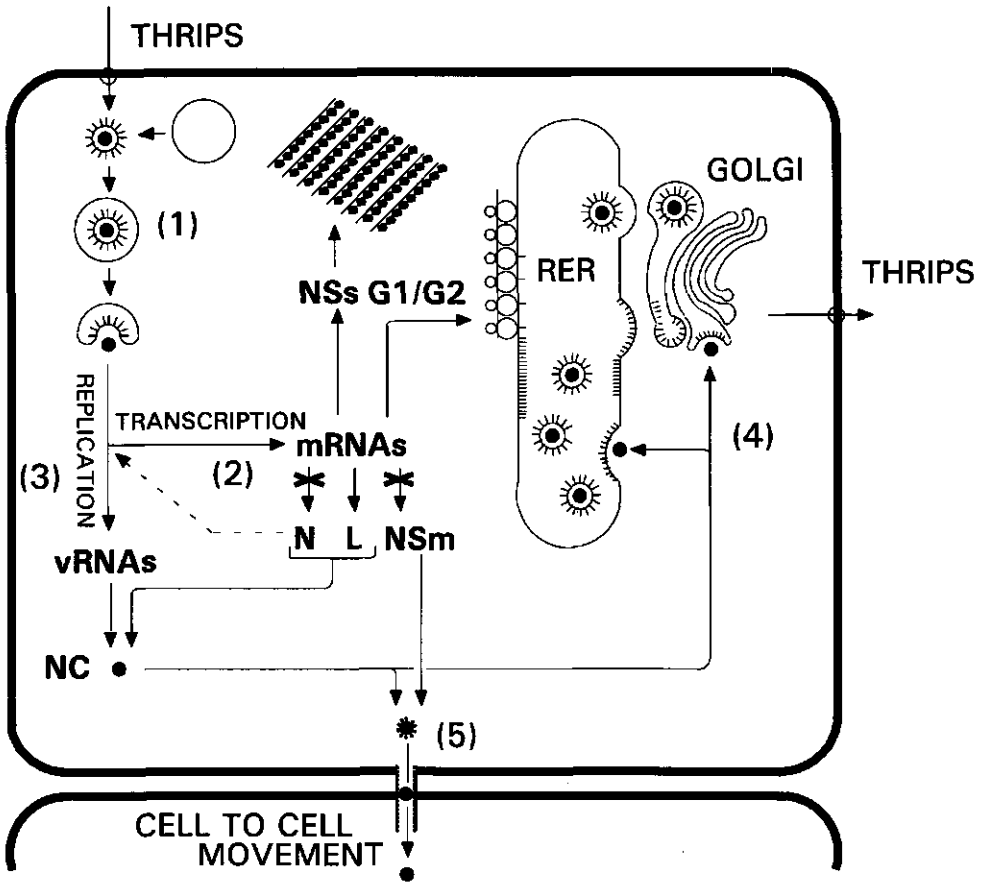

Figure 6.3: Infection cycle of TSWV in plant cells. The following steps can be distinguished: cell entry and nucleocapsid release (1), transcription (2), replication (3), budding and virus particle formation (4) and cell-to-cell movement (5). Processes potentially targeted by successful RNA-mediated resistance strategies (involving N and NS_M sequences) are marked with a cross.

The DI construct used in this experiment contained the cDNA of a naturally occurring defective interfering L RNA molecule, which retained 2966 nt of the viral 5' end and 138 nt of the 3' end. Presence of this DI L RNA sequence in TSWV isolates attenuates virus symptoms. Beside the inability to support RNA-mediated resistance, expression of these sequences does not attenuate virus symptoms as wild type DI molecules do *in planta*. This may be explained by the inability of the transgenic DI transcript to co-replicate with the helper virus, due to the presence of extra nucleotides at both the 5' and 3' ends.

It seems likely that the transgenically expressed RNAs can interfere with the production or translation of the mRNAs of the challenger virus. Since only N and NS_M sequences can confer resistance, this may indicate that both N and NS_M proteins play an essential role in tospoviral infection of plants. Interfering with viral messenger RNAs and hence production of new viral proteins can occur in two ways, either by antisense inhibition and subsequent degradation by a dsRNase, or by sense inhibition, e.g. by a mechanism proposed by Lindbo *et al.* (1993), which involves the induction by the expressed transgenic RNA of a cytoplasmic factor that specifically degrades viral RNA, and may act similar to the co-suppression phenomenon observed in various other transgene expression systems (for references see Flavell, 1994; Matzke and Matzke, 1995a).

A second explanation for the presented data might be that for TSWV the majority of the genome does not suit yet unknown criteria needed for induction of or accessibility to post-transcriptional transgene silencing, like e.g. short stretches of primary or secondary structure in the expressed RNA. Perhaps only the N or NS_M gene sequences or parts thereof have these capabilities, resulting in resistance of host plants to TSWV. This view is supported by the observation that, despite a large number of lines tested, no (co-suppression-like) RNA-mediated resistance to potyviruses was observed in plants expressing potyviral CI and NIb sequences (Maiti *et al.*, 1993; Audy *et al.*, 1994). Furthermore, in numerous transgenic plant lines expressing chalcone isomerase sequences no plants were observed that displayed co-suppression (Kooter, personal communication). Obviously, further experiments need to be carried out to support or exclude the alternative interpretations for sequence specific RNA-mediated resistance against TSWV.

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 Àvila *et al.*, (1990, 1992 and 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America or *N. tabacum* var. SR1.

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

Construction of expression vectors

TSWV cDNA fragments situated in the genome at positions indicated in Figure 6.1 were cloned as PstI fragments, either direct or after addition of PstI linkers in the single PstI site in expression vector pZU-A (Gielen *et al.*, 1991) immediately downstream of a CaMV 35S promoter and flanked a their 3' ends by a nopaline synthase terminator. For the DI construct an alternative approach was followed. First, it was PCR amplified using primer pDH001 (5' CCCCGGATCCTCGAGAGCAATCAGGTAACA 3') which is complementary to both ends of the TSWV genomic L RNA and contains a BamHI restriction site. Subsequently, this PCR fragment was inserted in a modified pZU-A plasmid. Modifications consisted of the addition of TMV translation enhancing sequences (Gallie *et al.*, 1987) fused to the CaMV 35S promoter, these sequences were included to enhance the expression of the protein that is potentially encoded by the DI RNA (Resende *et al.*, 1992). In this modified pZU-A vector, a BamHI cloning site is present between the translational enhancer sequences and the nos terminator. All 14 different pZU-A derived expression cassettes containing TSWV cDNA inserts were finally cloned in pBIN19 transformation vector (Bevan, 1984).

Transformation of tobacco

All TSWV sequence containing pBIN19-derived vectors were introduced in *Agrobacterium tumefaciens* strain LB4404 (Ditta *et al.*, 1990) by triparental mating, using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid. *N. tabacum* var. SR1 plants were transformed and regenerated as described by Horsch *et al.* (1985).

Analysis of protection of transgenic plants against TSWV

All original regenerants were allowed to set seed and twenty S1 progeny plants of each individual transformed plant were grown and inoculated with tomato spotted wilt virus. Inoculations were in essence done according to Gielen *et al.* (1991), but plants were inoculated at a younger stage using higher doses of virus. 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 weeks after the first inoculation, leaf samples from visually healthy plants were collected to check by ELISA for the presence of the NS_S gene product, a reporter for viral replication (Wijkamp *et al.*, 1993), using a polyclonal antiserum 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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Chapter 7

FURTHER STUDIES ON THE MECHANISM OF RNA-MEDIATED RESISTANCE AGAINST TOMATO SPOTTED WILT VIRUS

Transgenic host plant resistance against TSWV has previously been demonstrated to be mediated by the transgenic RNA and could only be observed when N or NS_M gene derived sequences were used as transgenes. No correlation was observed between steady state RNA expression levels and resistance. Here it is demonstrated that resistant plants specifically express high levels of transgenic transcripts at the nuclear level but have steady state RNA levels comparable to those of susceptible transgenic plants. This indicates the induction of an active sequence-specific RNA breakdown mechanism similar to co-suppression, that confers resistance by degrading viral RNA sequences identical to the transgene.

While the phenotypes of the resistance in N and NS_M transformed plants are indistinguishable, inoculation studies using protoplasts revealed a clear difference at the cellular level: protoplasts derived from transgenic plants expressing N gene sequences appeared resistant to TSWV infection, while protoplasts from NS_M transgenic plants were susceptible. Implications for the mechanism of the sequence specific resistance are discussed.

Parts of this chapter concerning the nuclear run-on experiments, have been published in: Prins, M., Resende, R. de O., Anker, C., Van Schepen, A., De Haan, P. and Goldbach, R. (1996) Engineered RNA-mediated resistance to tomato spotted wilt virus is sequence specific. *Molecular Plant-Microbe Interactions* **9** (5): 416-418. Protoplast experiments were included in: Prins, M., Kikkert, M., Ismayadi, C., De Graauw, W., De Haan, P. and Goldbach. R. Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NS_M gene sequences. *Plant Molecular Biology* **33**: 235-243.

INTRODUCTION

The concept of pathogen-derived resistance (Sanford and Johnston, 1985) has been applied for many virus-plant combinations (see reviews by Beachy *et al.*, 1990; Wilson, 1993; Scholthof *et al.*, 1993; Lomonossoff, 1995). Following the original design of the PDR concept, transgenic expression of large amounts of protein was pursued, and plants were pre-selected for their protein production abilities prior to screening for resistance. This resulted in operational resistance against a considerable number of viruses, but manifestations of the resistance differed greatly, while underlying mechanisms appeared to be diverse and not universally applicable.

As has now been shown for several other virus-plant combinations, transgenic resistance against tomato spotted wilt virus was shown to be largely mediated by the transgenic expression of viral RNA sequences, and not protein, since untranslatable versions of the transgenes resulted in plants with indistinguishable phenotypes when compared to their translatable counterparts, as did the antisense versions of these genes (Chapters 3 and 5). In all cases, observed resistance correlated neither to protein nor to RNA expression levels, suggesting no direct (stoichiometric) interaction between expressed RNAs and invading viral RNAs based on antisense interaction with viral sequences complementary to the transgene. Work by Lindbo *et al.* (1993) suggested that by transgenic expression of sequences derived from the tobacco etch virus coat protein gene, a plant response could be induced, resulting in the specific degradation of the expressed sequences. Ultimately this may have resulted in resistance, since viral RNA sequences were identical to the transgene and may therefore also be targeted and degraded. Similar observations have been made earlier in transgenic plants expressing additional copies of resident endogenous genes (Van der Krol *et al.*, 1990; Napoli *et al.*, 1990; Smith *et al.*, 1990; De Carvalho *et al.*, 1992). In some of these cases, expression of the endogene was completely silenced by addition of the transgene and tentatively called "co-suppression" (Napoli *et al.*, 1990). From nuclear run-on experiments it became clear that this silencing occurred post-transcriptionally, since both the transgene as well as the endogene displayed high levels of nuclear transcription, while steady state RNA levels were low (De Carvalho *et al.*, 1992; Van Blokland *et al.*, 1994; Niebel *et al.*,

1995a,b). This suggested the induction of a mechanism capable of sequence-specific targeting and degradation of transgene transcripts.

Currently, RNA-mediated resistance has been shown to operate via a mechanism similar to co-suppression or post-transcriptional gene silencing for two positive strand viruses: TEV and PVX (Lindbo *et al.*, 1993; Mueller *et al.*, 1995). Here we investigated whether the observed RNA-mediated resistance against the negative strand virus TSWV also operates in a fashion similar to co-suppression.

Since resistance to TSWV using the N gene was shown to be mediated by the RNA, this led to the expectation that also other parts of the viral genome would potentially be able to confer resistance. Therefore, the observation that RNA-mediated resistance against TSWV was not effective with most parts of the viral genome (Chapter 6) was quite unexpected. Two possible mechanisms that may explain this observation have been discussed. First it may be possible that the transgenically expressed RNA has to meet certain unknown criteria in order to be able to induce the silencing mechanism. These may include elements of the transgene like e.g. primary or secondary structures. Second, it may be possible that all sequences are principally capable of inducing co-suppression, but that the targeting and degradation of specific sequences is not effective in conferring resistance to the virus. Consequently, since transgenic expression of different parts of the genome of TSWV is not equally effective in conferring resistance, this implies that the viral messenger RNAs must be targeted by the suppression mechanism and not the viral genomic RNAs. Along this line the relevance of the suppressed viral genes for plant cell infection could then be critical for acquiring resistance or not (Chapter 6). The observation that viral messenger RNAs do not associate with nucleoprotein in contrast to their genomic counterparts (Kormelink *et al.*, 1992a), therefore making them more accessible for RNA degradation, supports this view.

In this chapter additional evidence is presented that RNA-mediated resistance of the transgenic lines operates by targeting viral messenger RNAs, thereby inhibiting specific gene functions.

RESULTS

Nuclear run-on assays of transgenic lines

For studying the nuclear transcription rate of several lines of transgenic plants expressing the TSWV N gene RNA in a non-translatable form, nuclei from leaf tissue of several plant lines were isolated. Two sensitive and two resistant lines were chosen based on similarity in steady state expression levels. Line 1004-01 was chosen as a control, since it has an identical history as the other lines, but has lost its transgenic copy by segregation. Isolated nuclei of these plants were used in run-on experiments and hybridized to single stranded probes, that had been slot blotted on a nylon membrane. Single stranded sequences were derived from M13 phages, therefore M13 sequences were included as control for aspecific hybridisations to these sequences. Single stranded DNA complementary to the messenger RNA of the rubisco small subunit (SSU) was included to compensate for the varying amount of nuclei per assay. Beside the transcription of the transgene driven by the strong CaMV 35S promoter, possible transcription of antisense transcripts from plant promoters in the vicinity of the transgenic insert was also monitored. As is shown in Figure 7.1, varying degree of SSU was compensated for by different exposure times. It became evident that only in resistant plant lines, nuclear transcription of the sense N gene greatly exceeded that of the internal standard and there was a clear relation between high nuclear transcription levels and resistance, in contrast to steady state RNA levels and resistance. Obviously, plants that display high nuclear transgene expression, yet have low steady state RNA levels, must have an active mechanism for specific breakdown of these (transgene) viral RNA sequences, consequently resulting in resistance to the virus. Hence, the observed RNA-mediated resistance against TSWV resembles the homology dependent gene silencing observed in other transgenic plants (De Carvalho *et al.*, 1992; Lindbo *et al.*, 1993; Van Blokland *et al.*, 1994).

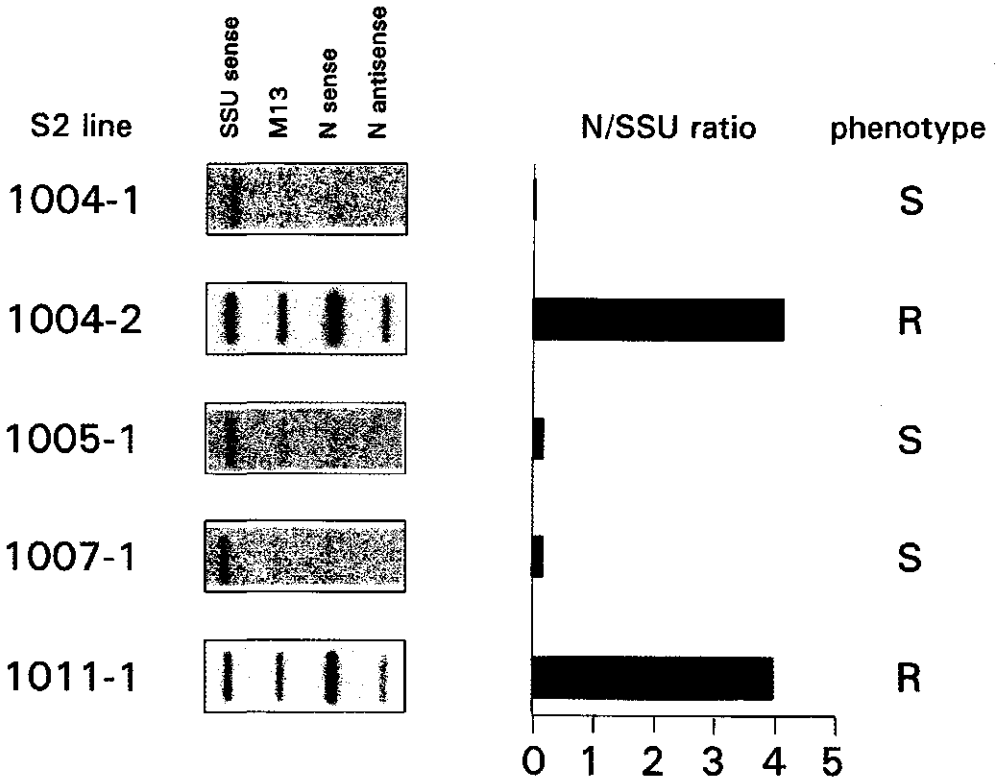

Figure 7.1: Transgene transcription levels in isolated nuclei of TSWV N gene-transformed plants. Steady state RNA levels in all lines except segregant line 1004-1, lacking the transgene, are similar. SSU indicates the nuclear expression of the small subunit of rubisco, used as an internal standard. M13 accounts for aspecific hybridisation to single stranded M13 sequences. N sense and N antisense show hybridisation of respective run-on transcript to complementary sequences. N/SSU ratio indicates the relative expression level of the transgene compared to the rubisco internal expression, which is assumed constant. No hybridisation of antisense N sequences (induced e.g. by a plant promoter) was observed. R and S are resistant and susceptible phenotypes of the transgenic plants, respectively.

In none of the plant lines tested, nuclear expression of antisense N gene transcripts was observed, indicating that production of antisense in the nucleus is not essential for the observed silencing. This in disagreement with one of the proposed models for co-suppression based on the possible expression of antisense from a plant promoter in the vicinity of the transgene (Grierson *et al.*, 1991).

Resistance mediated by the N gene operates at the cellular level while NS_M-mediated resistance does not

Four transgenic S2 lines that are completely resistant to TSWV at the plant level were selected. In two of these lines (12-02 and 1004-02), resistance was mediated by a translatable and an untranslatable version of the N gene, respectively (Gielen *et al.*, 1991; Chapter 3), while in lines A2-05 and B3-13 (Chapter 5) resistance was brought about by NS_M transgenic sequences. Following the inoculation of the protoplasts, virus accumulation was monitored on Western blot using anti N polyclonal antisera (Figure 7.2) and the percentage of infected protoplasts determined by immunofluorescence (Table 7.1). In all cases a *N. tabacum* var. SR1 segregant lacking a transgenic insert was used for reference.

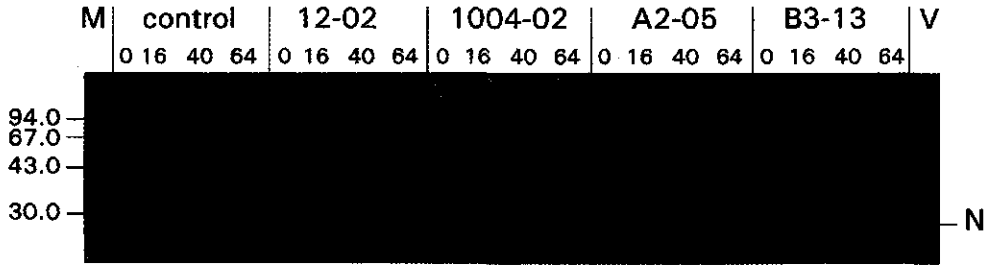

Figure 7.2: Western blot analysis of TSWV infected protoplasts generated from untransformed control tobacco plants and transgenic lines 12-02(N), 1004-02(N), A2-05 (NS_M) and B3-13 (NS_M). Virus infection was monitored using antibodies against the N protein. V is purified virus, numbers indicate the number of hours post inoculation. Sizes of low molecular weight markers (kD) are indicated on the left.

In the protoplasts expressing NS_M sequences, infection percentages and viral antigen accumulation levels comparable to those in susceptible control plants were observed. In contrast, N gene expressing resistant plants proved immune at the cellular level allowing no accumulation of viral nucleocapsids. N protein observed at t=0 in lines 12-02 and 1004-02 resulted from the inoculation and is slowly degraded, the low expression of N protein at later times in line 12-02 (Figure 7.2) results from expression of the transgene and is also observed in uninoculated protoplasts (results not shown).

Table 7.1. Percentage of TSWV infected protoplasts derived from N and NS_M transgenic tobacco lines.

S2 tobacco line	% infected protoplasts (64 h.p.i.)*
non-transgenic control	80%
12-02 (N)	<5%
1004-02 (N)	<5%
A2-05 (NS_M)	70%
B3-13 (NS_M)	50%

* determined by immunofluorescence using FITC-coupled antibodies against the viral N protein.

DISCUSSION

For the negative strand virus TSWV, transgenic expression of both N and NS_M gene sequences of TSWV have been shown to confer resistance to plants (Chapters 3 and 5). The observed resistance reaches similar high levels for sense and antisense expressing plants in both cases. Resistance was also obtained when untranslatable sense genes were introduced, indicating an RNA-mediated type of resistance. At the whole plant level, the manifestations of the RNA-mediated resistance phenomenon induced by NS_M sequences are indistinguishable from those caused by the N gene RNA-mediated resistance and both result in complete resistance to TSWV in homozygous S2 progeny lines. Moreover, they both share the same spectrum of resistance, and only hold against the virus (TSWV) of which the transgene sequence was derived. Similar characteristics have also been found for RNA-mediated resistance to other viruses, e.g. tobacco etch virus (TEV) (Lindbo *et al.*, 1992a,b), potato virus Y (PVY) (Van der Vlugt *et al.*, 1992), potato virus X (PVX) (Mueller *et al.*, 1995) and cowpea mosaic virus (CPMV) (Sijen *et al.*, 1995).

A mechanism for RNA-mediated resistance that implies the induction of an anti-viral state in the cytoplasm of transgenic plants was proposed by Lindbo and co-workers

(Lindbo *et al.*, 1993), based on similarities to the sense- or co-suppression phenomenon observed in transgene expression studies of endogenous genes in plants (for review see e.g Matzke and Matzke, 1995a). This co-suppression causes dramatically decreased endogenous RNA steady-state levels upon expression of homologous transgene sequences as a result of a post-transcriptional RNA-degrading mechanism. In immune plant lines expressing TEV coat protein RNA sequences, nuclear *de novo* synthesis in run-on assays remained high, while cytoplasmic steady-state RNA levels were low (Lindbo *et al.*, 1993; Dougherty *et al.*, 1994). This suggested that RNA-mediated resistance was a result of increased RNA turnover of (transgene) viral sequences. Here we have shown that also for TSWV resistance operates via a co-suppression-like mechanism. Of several transgenic plant lines tested that are expressing the same construct to similar steady state cytoplasmic RNA levels, only the plants with a resistant phenotype exhibit high nuclear transcription rates, indicating the presence of an active RNA-degrading mechanism. This suggests that the co-suppression is caused by high expression levels, which are not tolerated by the plant. The mechanism that is thought to be responsible for this phenomenon may involve a resident regulatory function of plants cells necessary for downregulating overexpressed genes. In this fashion it could be involved in developmental regulation of specific genes. Targeting by the resistance mechanism is very sequence specific and even sequences up to 80% homology at the nucleotide level are not recognized, yet it is very versatile and can be induced by many (if not all) transgenic sequences. This suggests antisense RNA may be present within the targeting/degrading complex. The source of this antisense RNA does not seem to be nuclear, since no antisense production was observed in run-on experiments (Figure 7.1). Because co-suppression operates post-transcriptionally, it can be conceived that silencing operates in the cytoplasm. This is supported by the effect it has against viruses that replicate in the cytoplasm. Possibly, a resident, non-viral RNA-dependent RNA polymerase activity as observed in plant cells (Dorssers *et al.*, 1982 and 1983; Van der Meer *et al.*, 1984), is involved in the production of short antisense molecules forming the core of the specific RNase complex. A current model for the co-suppression-like resistance mechanism against TSWV is presented in Figure 7.3 (page 88).

Surprisingly, the transgenic expression of the majority of the genome of TSWV does not result in virus resistance, indicating gene specific effects (Chapter 6). Unlike positive-strand RNA viruses, the genomic and anti-genomic RNAs of negative strand viruses remain protected by N protein throughout the entire replication cycle (Kormelink *et al.*, 1992a) and may therefore be unaccessible as targets for the resistance mechanism. Hence, TSWV resistance in transgenic plants may be conferred by interference with (non-encapsidated) messenger RNAs. Inhibiting the formation of sufficient amounts of proteins essential for e.g. viral replication (N) or movement (NS_M) of the virus, may be the basis of the observed resistance. Synchronous infection of single plant cells derived from N gene transgenic plants revealed a block in the accumulation of virus. Preliminary results of Western blot analysis and immunofluorescence studies indicate accumulation of NS_S protein in inoculated protoplasts derived from N gene transformed plant lines. This indicates that the genomic S RNA segments are not targeted, but that the lack of replication and accumulation of N protein must have been caused by degradation of the N messenger. RNA-mediated resistance does not operates at the cellular level for the NS_M transgenic plants, because an accumulation of N protein to wild type levels was observed, demonstrating that these cells still can support virus replication. Considering the phenotype of the NS_M gene-mediated transgenic resistance is identical to N gene-mediated resistance at the whole plant level, this would require an inhibition of cell-to-cell movement in these plants due to the inability of the virus to produce sufficient amounts of NS_M protein required for transport-tubule formation.

As stated previously (Chapter 6), silencing of other viral genes may not be effective for blocking virus replication and/or spread in plants. Additional research can substantiate if these sequences indeed are targeted in some transgenic lines, for example by introducing the target sequence in viral vectors like e.g. PVX (English *et al.*, 1996) or TMV (Kumagai *et al.*, 1995). Transgenic progeny plants incapable of conferring resistance to TSWV can be screened for effective silencing of transgenic sequences, consequently resulting in resistance against this recombinant (positive strand) virus, due to inserted TSWV sequences. In such a way, large amounts of plant lines can be screened for the presence of a TSWV specific co-suppression-like RNA degradation mechanism, without the necessity to perform labourious run-on experiments for all these (242) lines.

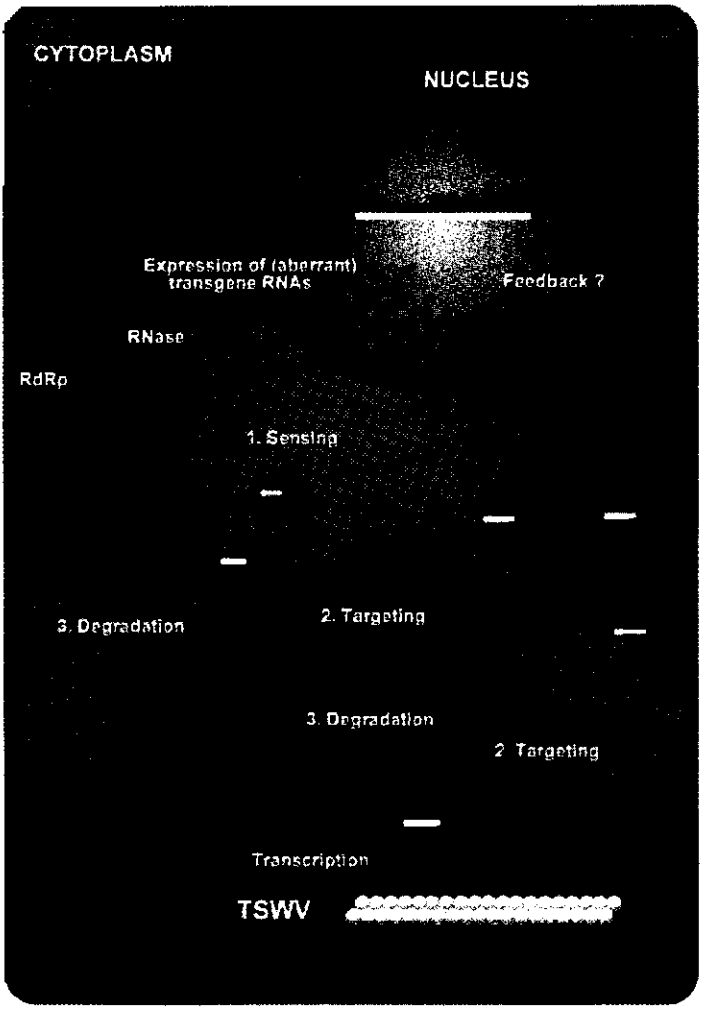

Figure 7.3: Current model for co-suppression-like RNA-mediated resistance directed against TSWV N and NS_M mRNAs. In silenced plants, transgenes are expressed to an "unacceptable" level and may contain aberrancies, possibly due to DNA methylations. This is sensed (1) by a cytoplasmic factor, that is able to specifically target the transgenic RNA sequences (2) and subsequently degrade it (3). Upon entry of the virus, the unencapsidated viral mRNAs, which have the same sequence as the transgene (i.e. N or NS_M), are also targeted by the cytoplasmic factor and degraded, rendering the virus unable to carry out essential functions in replication and movement, respectively. Viral genomic RNAs are protected from degradation by encapsidation with N protein.

MATERIALS AND METHODS

Isolation of nuclei

Nuclei were isolated according to Van Blokland *et al.* (1994), with slight modifications to adapt to larger amounts of leaf material.

In general, twenty to forty grams of expanded *N. tabacum* leaves of approximately eight week old plants were ground in liquid nitrogen. The powder was slowly resuspended in 50 ml ice-cold buffer A (10 mM NaCl, 10 mM MES pH 6.0, 5 mM EDTA, 0.15 mM spermidine, 20 mM ß-mercaptoethanol, 0.250 M sucrose and 0.6% triton X-100) while thawing. The suspension was passed twice through Miracloth filter (Calbiochem) to remove debris and pelleted at 2,000 *g*. After washing once with buffer A, the pellet was suspended in 88% Percoll (w/w) in buffer A. Centrifugation at 4000 *g* for 10 minutes yielded nuclei floating on top. Collected nuclei were washed with run-on transcription buffer (10 mM Tris-HCl pH 7.7, 100 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 5 mM ß-mercaptoethanol) resuspended in the same buffer and stored at -80^{0}C after the addition of an equal volume of glycerol in 1 ml portions.

Nuclear run-on assays

By adding and equal volume of transcription buffer cells were washed and after precipitation resuspended in 200 μl transcription buffer. Nucleotides (except UTP) were added to a final concentration of 0.5 mM each. The actual run-on was performed at 27^{0}C for 30 min. after the addition of 100 μCi (0.125 nmol) ^{32}P-UTP. The reaction was stopped by adding SDS up to 2.5% and incubating at 50^{0}C for 10 min. After phenol/chloroform extraction and ethanol precipitation, DNA was digested with 3 μg of RNase-free DNase. Another phenol/chloroform extraction and ethanol precipitation were carried out prior to passing the water-dissolved transcripts through a Sephadex G-50 column. Ethanol precipitated transcripts were resuspended in 100 μl water and incorporation was estimated using scintillation counting.

Hybridisation of run-on transcripts

Single stranded M13 DNA was isolated according to Sambrook *et al.* (1989). Per slot, one μg of DNA (in 10*SSC) was spotted on Hybond N membrane and UV cross-linked. Prehybridisation (in 50% formamide, 5*SSPE, 1% PVP, 0.1% Ficoll, including 50 μg tRNA per ml) of filters was at 42^{0}C for at least 5 h. Hybridisation with up to 10^7 cpm run-on transcripts was performed at 42^{0}C for 72 h. Filters were subsequently washed with 1*SSPE (0.1% SDS) at RT, 1*SSPE at 55^{0}C and 0.1*SSPE at 55^{0}C. A phosphorimager was used for detection and quantification of ^{32}P activity.

Preparation of Nicotiana tabacum protoplasts and purification of infectious TSWV particles

Protoplasts of *N. tabacum* plants were isolated essentially as described for *N. rustica* and *Vigna unguiculata* (Kikkert *et al.*, *submitted*). TSWV was isolated in sulfite containing buffers using a 10-40% sucrose gradient (Gonsalves and Trujillo, 1986). Purified virus was resuspended in water and 10 μg was used per inoculation of approximately one million protoplasts. Inoculation was accomplished by the addition of 40% polyethylene glycol (PEG) in 10 mM $CaCl_2$.

Acknowledgements

The authors wish to thank Cor Meurs for skilled technical assistance, Maike Stam and Jan Kooter for useful discussions and assistance in the run-on assays, Bert Essenstam for excellent maintenance of plants in the greenhouse and Drs. Richard Kormelink, Mart van Grinsven, Jan Gielen and Dick Peters for useful discussions and continuous interest in our work.

Chapter 8

TRANSGENIC TOBACCO PLANTS EXPRESSING THE PUTATIVE MOVEMENT PROTEIN OF TOMATO SPOTTED WILT VIRUS EXHIBIT ABERRATIONS IN GROWTH AND APPEARANCE

Within the *Bunyaviridae* virus family, members of the genus *Tospovirus* are unique in their ability to infect plants. A characteristic genetic difference between tospoviruses and the animal-infecting members of this virus family is the occurrence of an additional gene, denoted NS_M, located on the genomic M RNA segment. This gene has previously been implicated in the cell-to-cell movement of this virus during systemic infection. Transgenic tobacco plants have been obtained expressing the NS_M protein of tomato spotted wilt virus (TSWV), the type member of the tospoviruses, from a constitutive promoter. Detectable amounts of the NS_M protein could be observed in plants from nine different lines. The protein was only detectable in fractions enriched for cell wall material. More detailed immunogold labeling studies revealed specific association of NS_M protein with plasmodesmata. Plants accumulating the NS_M protein to detectable levels developed aberrations in growth, resulting in a significant reduction of size and accelerated senescence. In addition, these plants are restricted in their capacity to produce flowers. The results presented provide additional evidence that the NS_M protein, by modifying plasmodesmata, represents the cell-to-cell movement function of tospoviruses. Furthermore the phenotype of the NS_M transgenic plants suggests involvement of the NS_M gene product in TSWV symptom expression.

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INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus*, which encompasses the plant infecting members of the family *Bunyaviridae* (Goldbach and Peters, 1994; Murphy *et al.*, 1995).

The tripartite RNA genome of TSWV contains five open reading frames that specify six mature viral proteins. Typically for Bunyaviridae, the L RNA is of negative polarity and encodes the putative viral polymerase of 331.5 kD (De Haan *et al.*, 1991). In contrast, the M and S RNAs of tospoviruses are both ambisense (De Haan *et al.*, 1990; Kormelink *et al.*, 1992c). The S RNA codes for the nucleoprotein (28.8 kD) and a non-structural protein (NS_S) of 52.4 kD (De Haan *et al.*, 1990). The M RNA codes for the precursor to the envelop glycoproteins G1 and G2, and a non-structural protein (NS_M) of 33.6 kD, which represents the putative viral cell-to-cell movement protein (Kormelink *et al.*, 1994). When comparing the genome arrangements of the Bunyaviridae, tospoviruses are unique in having an ambisense M RNA segment, due to the presence of the NS_M gene which is lacking in the genomes of the animal-infecting members of the Bunyaviridae. Immuno-cytological detection of NS_M protein in TSWV infected leaf-material revealed a specific association with nucleocapsid aggregates and with tubular structures extending through plasmodesmata, suggesting involvement in cell-to-cell transport of the virus across cell walls (Kormelink *et al.*, 1994; Storms *et al.*, 1995).

For some positive strand RNA viruses of plants, detailed information concerning the functioning of the viral movement protein has been obtained by studying the effect of mutations in this protein using infectious cDNA clones (reverse genetics). For negative strand RNA viruses, like TSWV, such studies have been hampered by the impossibility to recover infectivity from cloned DNA copies. Since transgenic host plants are a potential source of information on the contribution of viral proteins in the virus infection cycle, we have transformed tobacco plants with the NS_M gene of TSWV. The observations made on such plants support the hypothesis that the NS_M protein is involved in the spread of tospoviruses through plant hosts and, moreover, is involved in the induction of tospoviral disease symptoms.

RESULTS

Transformation of tobacco plants with NS_M gene sequences

Two different DNA constructs containing cDNA sequences derived from the TSWV NS_M gene under the control of a CaMV 35S promoter were made, pTSWV NS_M-A, and pTSWV NS_M-B (Figure 8.1). Construct pTSWV NS_M-A contained the intact NS_M gene in a translatable form, whereas in the pTSWV NS_M-B construct the original ATG-start codon of the NS_M gene was replaced by CTG, creating a non-translatable form of this gene. The first alternative ATG codon is located out of frame and translation would result in a peptide of only 4 amino acid residues. In addition, the 5' leader sequence of TMV RNA (Gallie *et al.*, 1987) was cloned in front of the pTSWV NS_M-A gene construct, to enhance translation of the NS_M ORF. The two NS_M gene sequence expressor cassettes were cloned into the binary vector pBIN19, and transferred to the genome of *Nicotiana tabacum* var. SR1 plants, via *Agrobacterium tumefaciens*-mediated leaf disk transformation.

In total, 61 transformed plants were obtained that expressed NS_M-derived sequences, as verified by Northern blotting (data not shown). Of these plants, 35 contained NS_M-A sequences and 26 the NS_M-B insert. All original transformants were maintained for seed production.

Phenotype of transgenic plants

All plants transformed with the NS_M-B construct, original transformants as well as S1 and S2 progenies, had phenotypes that were indistinguishable from untransformed *N. tabacum* var. SR1 plants. Twenty-nine out of the 35 original transformants of the NS_M-A type developed normally. The other six pTSWV NS_M-A transformed plants (A14, A16, A26, A29, A31 and A35) developed extensive chlorosis of leaf tissue during their development (see Figure 8.2). Two of these plants (A26 and A31) were unable to set seed, therefore no progeny of these plants could be tested. As will be discussed later, no NS_M protein was produced in the NS_M-A plants that developed normally, in contrast to the plants with a deviating phenotype.

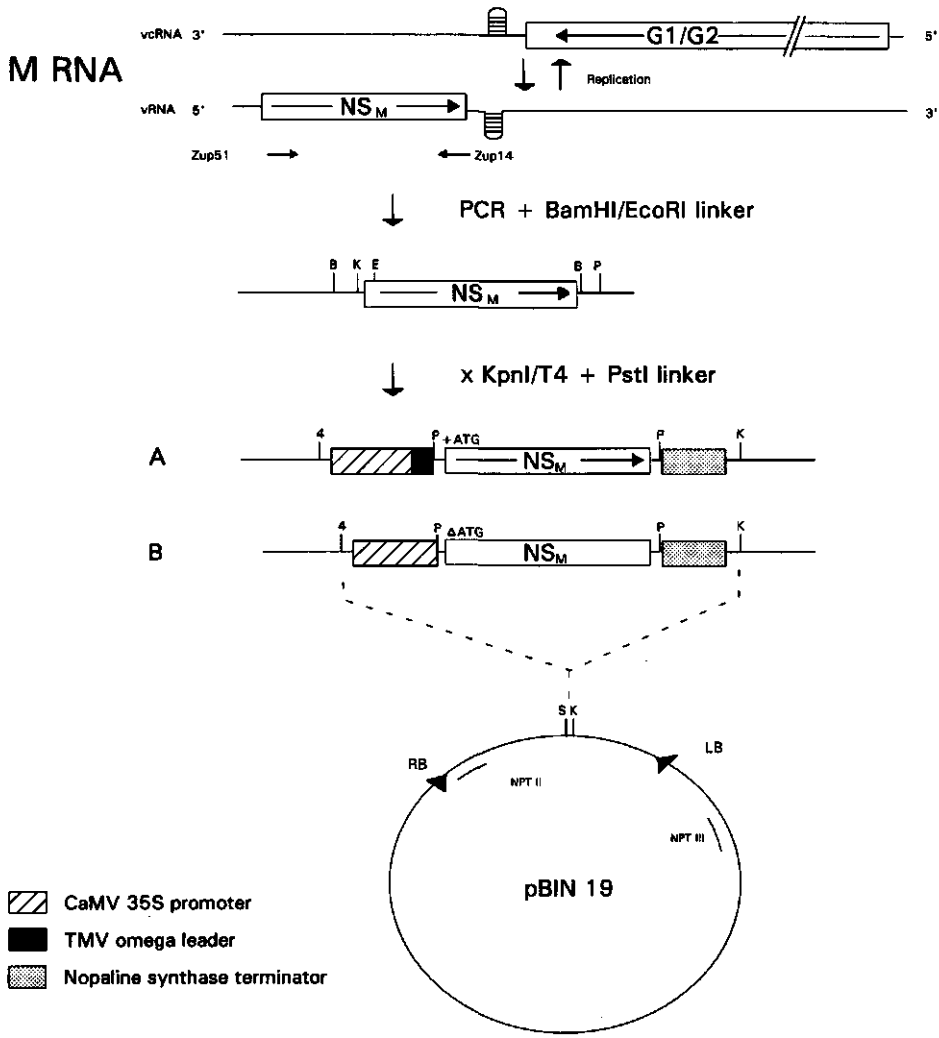


Figure 8.1: Construction of plant transformation vectors pTSWV NS_M-A and pTSWV NS_M-B 1: NS_M sequences are PCR amplified from a genomic cDNA clone of TSWV, using primers that add a BamHI restriction site to the 3' end of the gene (Zup014) and a EcoRI site immediately downstream of the original start codon (Zup051). 2: An oligonucleotide linker sequence containing an in frame start codon is ligated to the 5' end of the PCR fragment after BamHI digestion, thereby restoring the NS_M ORF. Two nucleotides upstream of the ATG a unique KpnI site is present. By treating KpnI linearised DNA differentially with T4 DNA polymerase, blunt ends are created either leaving the start codon intact (A) or destroying it (B). Additional ligation of PstI linkers allows cloning the NS_M gene in the pZU-A plasmid (Gielen *et al.*, 1991) also in antisense orientation (C). Finally, the NS_M constructs were cloned in the binary vector pBIN19. B=BamHI; E=EcoRI; K=KpnI; P=PstI; 4=blunt after treatment with T4 DNA polymerase. RB and LB are right and left border sequences, respectively. The ΔATG indicates removal of the ATG start codon.

Progeny plant lines derived from self-pollination of the original pTSWV NS_M-A transformants revealed a more complicated picture. Nine S1 lines (A2, A11, A13, A14, A20, A23, A25, A29, and A35) exhibited morphologically altered plants, in a ratio close to 1:3 or 3:1 relative to the morphologically unmodified plants within these lines. These ratios indicate the involvement of a single translationally active gene insertion in the aberrant phenotype. This was confirmed by kanamycin resistance studies (results not shown).

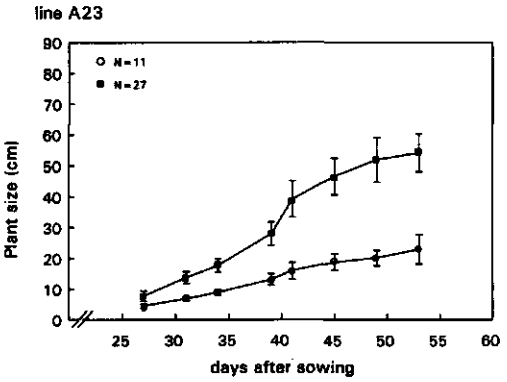
Among other symptoms such as necrosis and wilting, tobacco plants infected with TSWV show chlorotic symptoms and a marked delay in growth, which, based on the results obtained with the NS_M transgenic plants, may be related to NS_M expression.

Development and further characterisation of S1 progeny of lines A23 and A25

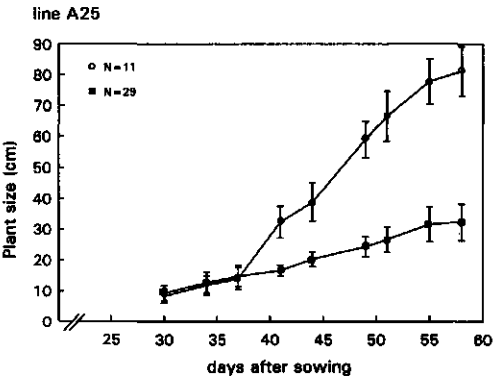
Growth of plants from S1 lines A23 (segregation ratio 1:3) and A25 (segregation ratio 3:1) was monitored during the development of these plants. Sizes of plants were measured twice a week for a period of eight weeks after sowing, by adding up the length of the two largest leaves plus the length of the entire stem. A dramatic difference in growth rate within segregating progenies of lines A23 and A25 was observed (Figure 8.3). Representative selections of plants of lines A23 and A25 are shown approximately 7 weeks after sowing (Figure 8.3). The presence or absence of TSWV NS_M protein in plants of lines A23 and A25 was verified by Western blot analysis of purified cell-wall material. In plants of line A25 the NS_M protein was only detected in small plants and not in the segregants with wild type phenotype (results not shown). The presence of the NS_M gene in the genome of plants of line A23 alone appeared not to be sufficient for inducing modifications in morphology. Only one out of four plants developed growth aberrations, indicating that in this line only plants homozygous for the NS_M gene could reach sufficient NS_M expression levels to result in retardation in growth. Indeed, kanamycin resistance, indicating the presence of the transgene, occurred in two-third of the plants that developed normally in this experiment. Somaclonal variation as a possible explanation for the observed phenomenon can be ruled out, since the occurrence of aberrations strictly coincides with the presence of the NS_M gene product. This view is confirmed by the observation that plants transformed with untranslatable NS_M-B sequences never exhibited aberrations in morphology.

A

B


line A23
N=11
N=27
Plant size (cm)
days after sowing

A


line A25
N=11
N=29
Plant size (cm)
days after sowing

B

C

D

Figure 8.2: **A)** Extensive chlorosis of developing transformant A14. Similar observations were made on transformants A16, A26, A29, A31 and A35. **B)** Close-up of a leaf of transformant A14.

Figure 8.3: Growth curves of S1 plants of two NS_M expressing *N. tabacum* var. SR1 lines displaying a segregation in size in two categories, i.e. those developing as wild type and those showing an aberrant phenotype. **A)** Sizes of 38 individual plants of line A23 were measured twice weekly and stopped when the first plants started flowering. Plants of this line display a 3:1 segregation ratio with respect to size. **B)** Of line A25, forty individual plants were monitored for development. This line displayed a 1:3 segregation ratio. **C)** and **D)** Representative selections of plant from lines A23 and A25, respectively, approximately seven weeks after sowing.

Expression of NS_M specific RNA and protein in transgenic plants

The presence of NS_M gene-specific transcripts in S1 lines was checked by Northern blot analysis of pooled leaf material, using a ^{32}P-dATP labeled double-stranded NS_M cDNA probe. Transgenically produced NS_M RNA could be detected in all lines tested (results not shown), albeit at low levels when compared to levels reached in TSWV infected plants.

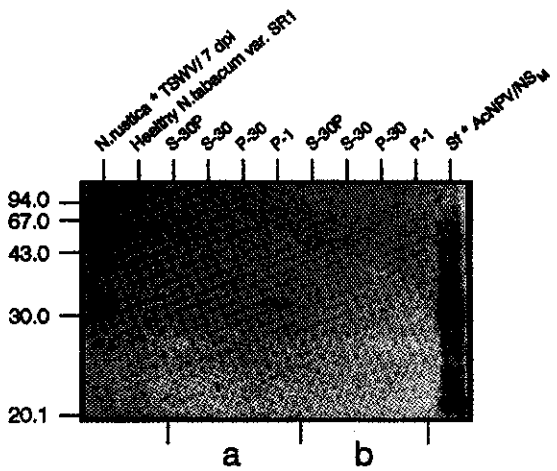


Figure 8.4: Western blot analysis of subcellular fractions of a morphologically aberrant plant, using NS_M specific antiserum. **A)** Morphologically aberrated plant of S1 line A25.
B) unmodified plant from the same line. Various subcellular fractions of crushed leaves as described by Kormelink *et al.* (1994), P-1: crude cell wall material collected at 1,000 *g*; P-30 pellet of previous supernatant after 30,000 *g* centrifugation; S-30 supernatant of this fraction; S-30P pellet of previous fraction after 125,000 *g* centrifugation through a 30% sucrose-cushion (reveals nucleocapsids in virus infected plants).

In leaf extracts from NS_M-A transformed plants, NS_M protein could neither be obser ved by Western blot analysis, nor when ELISA techniques were used. Only after fractionation of these extracts into cellular components, NS_M protein could be observed in cell wall-enriched fractions, but not in other fractions, indicating that this protein accumulated to low levels in cell wall material (Figure 8.4). This was further confirmed by immuno-cytological data.

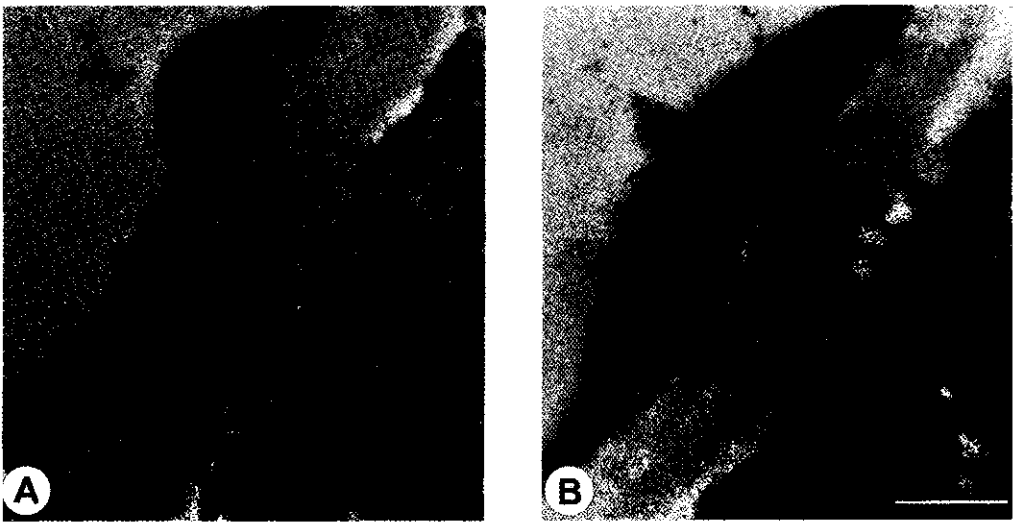


Figure 8.5: Immunogold decoration studies on approx. seven weeks old plant tissue, using gold labeled NS_M antibodies showing specific association of the transgenically expressed NS_M protein with plasmodesmata **(A)** and the branched morphology of these plasmodesmata **(B)**. Bar indicates 200 nm.

In situ analyses of NS_M protein transgenically expressed in tobacco.

To gain insight as to how the transgenically expressed NS_M protein could have led to the observed phenotypic aberrations, cytological analyses of leaf tissue of NS_M protein-expressor plants were performed at the electron microscopical level using immunocytochemistry as well as structural analysis of the tissue morphology. Ultrathin sections of transgenic leaf tissue probed with antiserum against the NS_M protein clearly illustrated that the NS_M protein was only detectable in the cell wall, confirming the

Western immunoblot analyses. More specifically, the protein was found in clear association with plasmodesmata (Figure 8.5A). A detailed quantification revealed that in the NS_M expressing lines, approximately 80% of the plasmodesmata contained NS_M protein, a majority of these NS_M-positive plasmodesmata clearly showing a branched morphology (Figure 8.5B). The transgenically expressed NS_M protein was detected along the entire length of the plasmodesma and never formed tubular structures as found in plasmodesmata of TSWV-infected tissue (Kormelink *et al.*, 1994). When analysing different tissues, NS_M-containing plasmodesmata were observed in mesophyll tissue, between mesophyll and bundle sheath cells as well as in vascular tissue.

DISCUSSION

Morphological aberrations have been observed in transgenic tobacco plants expressing the putative movement protein of tomato spotted wilt virus. Modifications in growth and appearance were only observed in plant lines expressing translatable NS_M RNA (NS_M-A lines). The majority of the NS_M-A transformed plants did not accumulate detectable amounts of NS_M protein. Plants that did express NS_M protein to a detectable level always developed morphological aberrations, indicating that accumulation of this viral protein has a negative effect on the growth of the plant, resulting in the deviating phenotype. The modified plants might be either the homozygous NS_M expressor plants (e.g. in line A23), expressing the protein to such a level that it influenced the morphology of the plant, or also hemizygous plants with a single, but more actively translated NS_M transgene (e.g. in line A25). In all cases tested, expression levels of the transgenic protein were low and protein was only observed in fractions enriched for cell wall material derived from stunted plants. Possibly, higher expression levels than those observed are lethal to plants, and therefore no such plants were recovered during the transformation and regeneration process. Immuno-cytological analyses of transgenic tissues revealed that the NS_M protein was specifically associated with plasmodesmata. Random distribution among plasmodesmata of leaf tissue was observed, illustrating that the NS_M protein was expressed in all leaf cell-types. This is not surprising considering the use of the CaMV 35S promoter. In addition, it can be concluded that NS_M protein

expressed in various tissues behaves in similar manner with regard to association with plasmodesmata. In contrast to the constitutive expression in transgenic plants, the level of NS_M expression during viral infection seems to be regulated. Expression levels of NS_M are low during the early stages of infection, in which the NS_M protein is probably associated with both viral nucleocapsids and plasmodesmata, and can be observed in both P1 and P30 fractions (Kormelink *et al.*, 1994). Higher expression levels are reached later in the infection process, coincidental with the formation of tubular structures in the plasmodesmata (Storms *et al.*, 1995). Since the NS_M protein in transgenic plants can only be detected in Western blot analysis after concentrating the cell wall fractions, the level of protein expression seems significantly lower than during virus infection. This could be the reason why in transgenic plants no tube-like extensions are observed in tissue. However, association of the NS_M protein with plasmodesmata as such, even without forming tubular structures, most likely underlies the inability of these plants to develop normally. Indeed, it is well recognized that plasmodesmata play an important role in development and supracellular organisation of plants (Lucas *et al.*, 1993).

In contrast to what has been found in transgenic tobacco plants expressing the 30K movement protein of TMV, the NS_M protein was observed along the entire length of the plasmodesma and not specifically associated with the central cavity of structurally modified secondary plasmodesmata (Ding *et al.*, 1992). Morphologic changes have not been reported for plants expressing the TMV movement protein, although an increase of the size exclusion limit was demonstrated, which is a clear indication of the modification of the plasmodesmata. Also transgenic plants expressing the 3a movement protein of CMV showed an increased size exclusion limit, while no change was observed in the morphology of the plant (Vaquero *et al.*, 1994). However, transgenic tobacco plants expressing the BL1 movement protein of the geminivirus SqLCV, like TSWV NS_M expressors, display a clearly visible change in the morphology of the plant. Plants expressing this viral protein exhibited symptoms comparable to viral infection, potentially caused by association of the expressed protein with plasmodesmata, because the protein was demonstrated to be associated with cell wall fractions (Pascal *et al.*, 1993). Since also the cell-to-cell movement of gemini viruses has been suggested to involve tubules (Kim and Lee, 1992), the aberrant development of transgenic plants might be characteristic of tubule-forming movement proteins.

The occurrence of aberrations in the morphology of plants expressing the TSWV NS_M protein, together with a clear association of this protein with plasmodesmata, present further evidence of the involvement of this protein in cell-to-cell movement of tomato spotted wilt tospovirus through modified plasmodesmata. Additional studies will be carried out to reveal the effect of this transgenically expressed protein on the size exclusion limit of plasmodesmata.

MATERIALS AND METHODS

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

Preparation of subcellular extracts of transgenic plants and western immunoblot analyses were according to Kormelink *et al.* (1994).

Viruses and plants

Tospovirus strains, BR-01 (TSWV), BR-03 (TCSV), and SA-05 (GRSV), have been described by Àvila *et al.* (1990, 1992 and 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America or *N. tabacum* var. SR1.

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

Construction of NS_M gene sequence expression vectors

NS_M gene sequences of TSWV (Kormelink *et al.*, 1992c), were modified using PCR in such a way that an EcoRI site was generated immediately downstream of the original start codon, using primer Zup051 (dGGGAATTCTTTTCGGTAACAAGAGGCC) locate d at position 108 to 129 of the viral M RNA and Zup014 (dCCCTGCAGGATCCGAAATT-TAAGCTTAAATAAGTG) located at position 1043 to 1023 of the viral complementary M RNA. The resulting PCR fragment was digested with EcoRI and a EcoRI/BamHI linker including an internal KpnI site and an in frame start codon

```
5' GATCCGGCAACGAAGGTACCATGGG     3'
3'     GCCGTTGCTTCCATGGTACCCTTAA 5'
   BamHI          KpnI NcoI EcoRI
```

was ligated. This slightly modified NS_M gene (starting with amino acid sequence Met.Leu.Ile... in stead of Met.Thr.Val...) was cloned in pMON999 and successfully checked for tubule inducing ability in transfected protoplasts (Storms *et al.*, 1995), although an effect of these single aminoacid changes on other functions of the protein can not be completely excluded. Simultaneously, the fragment was ligated in pUC18 as a BamHI restriction fragment. The resulting plasmid was linearised using KpnI, and PstI linkers were ligated after creating blunt ends using T4 DNA polymerase. In addition, the exonuclease activity of T4 DNA polymerase was used to create an untranslatable NS_M sequence. The resulting clones were checked by sequence analysis and beside a translatable clone, a clone was selected in which the original start codon was mutagenised to CTG. The PstI restriction fragments, were ligated in plant transformation vector pZU-A (Gielen *et al.*, 1991) between the CaMV 35S promoter and the nopaline synthase (nos) terminator. In addition, the translation enhancing TMV untranslated leader (Gallie *et al.*, 1987) was ligated in front of the translatable NS_M-A construct. Finally, the NS_M constructs were inserted in binary vector pBIN19 (Bevan, 1984), yielding pTSWV NS_M-A (translatable) and pTSWV NS_M-B (untranslatable). Details of this cloning schedule are presented in Figure 8.1.

Transformation of tobacco

The pBIN19-derived vectors pTSWV NS_M-A and NS_M-B were introduced into *A. tumefaciens* strain LB4404 (Ditta *et al.*, 1980) by triparental mating, using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid. *N. tabacum* var. SR1 plants were transformed and regenerated as described by Horsch and co-workers (1985).

Immunocytochemistry

Both transgenic *N. tabacum* plants expressing the TSWV NS_M protein as well as non-transgenic tobacco plants were used for immunocytological analyses at electron microscopy level. Leaf samples were fixed in 2% (w/v) paraformaldehyde/ 0.01% glutaraldehyde (w/v) in phosphate citrate buffer, dehydrated, imbedded in London Resin

Gold at -25^{0}C and UV-polymerized. Ultrathin sections were made and processed for immunogold labeling using antiserum against the NS_M protein (Van Lent *et al.*, 1990; Kormelink *et al.*, 1994). Specimens were examined using a Philips CM12 electron microscope.

Acknowledgements

The authors wish to thank Alie van Schepen for skilled technical assistance in the transformation of tobacco, Bert Essenstam for excellent maintenance of plants in the greenhouse, Drs. Dick Peters, Jan Gielen and Mart van Grinsven for useful discussions and continuous interest in our work.

Chapter 9

SUMMARY AND CONCLUDING REMARKS

Over the past two decades tomato spotted wilt virus (TSWV) has become increasingly important as a pathogen in many crops. This can be attributed to intensified world trade and concomitant spread of one of the most important vectors of the virus, the thrips *Frankliniella occidentalis*. Moreover, this vector species has become resistant to most insecticides. Efforts using conventional breeding to include resistance into major crops generally are laborious and time-consuming, and moreover, suitable sources of natural resistance against TSWV are very limited. Alternative strategies for conferring virus resistance to plants are therefore urgently needed.

The main topic of the research described in this thesis concerns the development and improvement of transgenic resistance in crop plants against tomato spotted wilt virus (TSWV) and related tospoviruses. From previous investigations it was known that resistance against TSWV could be obtained by expressing the nucleoprotein (N) gene of the virus in transgenic tobacco plants. In the initial hypothesis, the observed resistance was attributed to the expressed viral protein. In contrast to this general theory, however, plants expressing the highest amounts of protein were not most resistant. In Chapter 3 evidence is presented that the expression of the viral N protein in transgenic plants is not essential for resistance, since expression of a translationally defective N gene RNA results in plants with identical resistance phenotypes, indicating a novel, RNA-mediated, form of resistance.

The specificity of this RNA-mediated resistance appeared to be high and was only functional against the homologous virus (TSWV), not against the related tospoviruses TCSV and GRSV. By simultaneous expression of the three nucleoprotein genes from these viruses, it was demonstrated that it is possible to introduce a broad resistance against tospoviruses by expressing multiple sequences from a single insertion in the genome (Chapter 4).

To answer the question whether any part of the TSWV genome is capable of inducing RNA-mediated resistance in transgenic plants and thereby further expanding the possible

use of tospoviral sequences for transgenic resistance, a large array of viral genome parts was expressed in transgenic plants. This proved to be successful only when sequences derived from the previously mentioned N gene and the viral movement protein gene, NS_M, were used as transgenes (Chapter 5). In contrast, all other parts of the TSWV genome, when expressed in transgenic plants, did not induce resistance, suggesting gene-specific resistance induction (Chapter 6). More detailed studies revealed that nuclear transcription rates of transgenes in resistant and susceptible plants differed considerably, while their steady state cytoplasmic RNA levels were the same. This suggested that the expressed sequences were actively broken down in resistant plants by a mechanism that could also degrade incoming viral RNAs (with sequences identical to the transgene). This mechanism is similar to the "co-suppression" phenomenon observed in other transgenic plants where endogenous genes could be silenced by transgenes. By studying the effect of virus inoculation on protoplasts it appeared that virus replication could be blocked in N gene transgenic protoplasts, whereas this was not observed in protoplasts isolated from NS_M transgenic plants. Considering these plants are resistant at the tissue level, this implies inhibition of virus transport. Differences in resistance mechanisms at the cellular level support the explanation that the resistance specifically operates on the (N or NS_M) mRNA level in the respective transgenic plants (Chapter 7).

In Chapter 8 it is shown that transgenic expression of viral proteins can have unwanted side-effects, that are nonetheless informative for the biochemical activity and function of the expressed protein. Plants expressing the NS_M protein to detectable levels showed aberrations in growth, probably as a result from specific accumulation of this transgenically expressed protein in plasmodesmata, cytoplasmic channels connecting neighbouring cells. Specific association of this protein with plasmodesmata gave further evidence that this protein is involved in cell-to-cell transport of the virus, and moreover that part of the typical TSWV symptoms may be attributed to this protein.

Some of our preliminary data have shown that RNA-mediated resistance can compete with - and even beat - the scarcely available sources of natural resistance. Transgenic tomato plants expressing N gene sequences were challenged with a TSWV isolate capable of overcoming a natural source of resistance (*Sw-5*) in tomato (kindly provided

by Dr. G. Thompson, Pretoria, South Africa). It was shown that despite the capacity to break natural resistance genes in tomato plants, this TSWV isolate was unable to infect transgenic plants. Anticipating the breaking of transgenic resistance by mutant TSWV isolates, transgenic resistance provides a more flexible approach when compared to natural resistance, since genes derived from future resistance-breaking isolates can be swiftly and efficiently used to breed a new generation of resistant plants.

Exploiting transgenic resistance based on co-suppression-like RNA-mediated resistance as described in this thesis, minimizes chances of unwanted genetic exchange between transgenes and incoming viruses. First of all, no transgenic protein is produced, and second, the produced transgenic RNA is rapidly broken down already in the transgenic plant cell, reducing any possible recombination to the utmost minimum.

In conclusion, forms of transgenic resistance as described in this thesis, provide a useful tool to combat tospovirus diseases in crop plants in the future.

SAMENVATTING

Gedurende de afgelopen twee decennia is het tomatenbronsvlekkenvirus (Engels: tomato spotted wilt virus, TSWV) in toenemende mate belangrijk geworden als pathogeen van veel land- en tuinbouwgewassen. Dit kan onder andere worden toegeschreven aan geïntensiveerde wereldhandel en de daarmee samenhangende verspreiding van één van de belangrijkste vectoren van het virus, de trips *Frankliniella occidentalis*, die bovendien resistent is geworden tegen de meeste bestrijdingsmiddelen. Pogingen om met gebruik van conventionele veredelingstechnieken resistentie te verkrijgen in belangrijke gewassen zijn in het algemeen arbeidsintensief en tijdrovend, bovendien zijn er bijzonder weinig geschikte bronnen van natuurlijke resistentie tegen TSWV beschikbaar. Alternatieve strategieën voor het verkrijgen van virusresistentie zijn daarom dringend nodig.

Het hoofdonderwerp van het onderzoek beschreven in dit proefschrift betreft de ontwikkeling en verbetering van transgene resistentie in landbouwgewassen tegen het tomatenbronsvlekkenvirus en verwante tospovirussen. Uit voorgaand onderzoek was gebleken dat resistentie tegen TSWV verkregen kon worden door het nucleoproteïne (N) gen van TSWV tot expressie te brengen in transgene tabaksplanten. Aanvankelijk was de hypothese dat de waargenomen resistentie gebaseerd was op transgene expressie van het virale eiwit. In tegenstelling tot de algemene theorie bleek echter dat planten met de hoogste eiwitexpressie niet het meest resistent waren. In hoofdstuk 3 wordt aangetoond dat de expressie van het virale eiwit in de plant niet cruciaal is voor resistentie, omdat ook de expressie van onvertaalbare RNAs identieke resistentie fenotypes bewerkstelligt, wat wijst op een nieuw soort, op RNA gebaseerde, resistentie. De specificiteit van deze resistentie bleek hoog te zijn en slechts functioneel tegen het homologe virus (TSWV) en niet tegen de verwante tospovirussen TCSV en GRSV. Door gelijktijdige expressie van de nucleoproteïnegenen van deze drie virussen werd aangetoond dat het mogelijk is brede resistentie te introduceren tegen tospovirussen door meerdere sequenties tot expressie te brengen vanaf een enkele insertie in het genoom (hoofdstuk 4).

Om een antwoord te geven op de vraag of ieder willekeurig deel van het tospovirale genoom in staat is om resistentie te induceren, waardoor het mogelijk gebruik van tospovirussequenties verder zou worden uitgebreid, werd een verzameling van virale cDNA klonen, die samen 70% van het virale genoom omvatten, tot expressie gebracht in transgene planten. Opvallend genoeg bleken alleen sequenties van het eerder genoemde N gen en het gen coderend voor het virale transporteiwit, NS_M (hoofdstuk 5), in staat transgene resistentie te induceren en alle overige sequenties niet (hoofdstuk 6). Nader onderzoek gaf aan dat de kerntranscriptieactiviteit van het virale transgen aanmerkelijk hoger was in resistente planten ten opzichte van gevoelige planten, terwijl cytoplasmatische RNA concentraties vergelijkbaar waren. Dit suggereert dat tot expressie gebrachte transgene RNA sequenties actief worden afgebroken in resistente planten door een mechanisme dat ook in staat is binnendringende virale RNA moleculen (die dezelfde sequentie hebben) af te breken. Dit is vergelijkbaar met het "co-suppressie" fenomeen dat is aangetroffen in planten waarin expressie van (endo)genen kon worden stilgelegd door expressie van identieke transgenen. Door het effect van virusinoculatie op protoplasten te bestuderen bleek dat virusvermeerdering werd geblokkeerd in N gen transgene protoplasten, terwijl dat niet het geval was in protoplasten geïsoleerd uit NS_M transgene planten. Dat deze laatste planten toch resistent zijn duidt op een blokkering van virustransport op weefselniveau. De verschillen in resistentiemechanismen op cellulair niveau ondersteunen de verklaring dat de resistentie specifiek gericht is tegen virale (N of NS_M) boodschapper RNAs in de respectievelijke transgene planten (hoofdstuk 7).

Hoofdstuk 8 geeft aan dat transgene expressie van virale genen ook ongewenste verschijnselen kan veroorzaken, die niettemin informatief kunnen zijn over de biochemische activiteit en functie van het tot expressie gebrachte eiwit. Planten die NS_M eiwit tot meetbare hoeveelheden aanmaakten bleken altijd groeiafwijkingen te vertonen, waarschijnlijk als gevolg van specifieke ophoping van dit eiwit in plasmodesmata, de cytoplasmatische verbindingskanalen tussen aangrenzende cellen. Deze specifieke associatie gaf nieuwe aanwijzingen voor de functie van dit eiwit in het transport van het virus en gaf bovendien aan dat een deel van de typische TSWV symptomen kan worden toegewezen aan dit eiwit.

De mogelijke doorbreking van de transgene resistentie kan in de toekomst niet worden uitgesloten, maar in dit geval kunnen genen van dit resistentie doorbrekende isolaat gebruikt worden om snel en efficiënt een nieuwe generatie resistente transgene planten te produceren.

Het gebruik van transgene resistentie gebaseerd op het induceren van (virus)specifieke RNA afbraak in transgene planten, zoals beschreven in dit proefschrift, minimaliseert de kans op ongewenste interacties tussen transgene planten en infectieuze virussen. Ten eerste wordt geen transgeen eiwit geproduceerd en ten tweede wordt het transgene RNA al in de plant actief afgebroken, waardoor mogelijke recombinaties tot het absolute minimum worden beperkt.

Samenvattend kan gezegd worden dat vormen van transgene resistentie zoals beschreven staan in dit proefschrift een bruikbaar instrument vormen om tospovirusziekten in landbouwgewassen in de toekomst te kunnen bestrijden.

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Curriculum vitae

Op 15 juni 1968 werd ik in Hellendoorn geboren als Marinus Willem Prins, en mocht van mijn ouders verder door het leven als Marcel. Na de lagere school doorlopen te hebben in Hellendoorn en Castricum, begon ik aan mijn voorbereiding op het wetenschappelijk onderwijs in Castricum (Bonhoeffer College). In 1986 haalde ik mijn VWO diploma in Kampen aan het toenmalige Johannes Calvijn Lyceum, en begon in hetzelfde jaar aan mijn studie Moleculaire Wetenschappen aan de Landbouwuniversiteit in Wageningen. Het propaedeuse diploma volgde een jaar later en ik besloot me te gaan toeleggen op de Chemisch-Biologische en Biotechnologische afstudeeroriëntaties. Na afstudeervakken bij de vakgroep Moleculaire Biologie (Prof. Van Kammen) begeleid door Francine Govers en Virologie (Prof. Goldbach) begeleid door René van der Vlugt, liep ik stage bij het "Waite Agricultural Research Institute" in Adelaide, Australië (Prof. Symons). In Maart 1992 studeerde ik met lof af in beide orientaties en begon als assistent in opleiding bij de vakgroep Virologie aan een vier jarig project gefinancierd door S&G Seeds (toen nog Zaadunie). Het werk uit die periode betrof transgene resistentie tegen het tomatenbronsvlekken virus (TSWV). Het verbreden van de gevonden resistentie naar verwante virussen en het verdiepen van de kennis omtrent de moleculaire achtergrond van deze resistentie staan beschreven in dit proefschrift. Sinds Maart 1996 ben ik wederom in dienst van de vakgroep Virologie, ditmaal als post-doctoraal onderzoeker. In dit project, gefinancierd door de Europese Unie (FAIR-project), werk ik, samen met collega's in enkele binnenlandse en buitenlandse laboratoria aan het verkrijgen van resistentie tegen TSWV door gebruik te maken van transgene expressie van antilichamen gericht tegen dit virus.

Account

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