EXPRESSION AND SILENCING OF COWPEA MOSAIC VIRUS TRANSGENES

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Expression and Silencing of Cowpea Mosaic Virus Transgenes

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STELLINGEN

1. Integratie van extra sequenties in het genoom kan in plantencellen een afweermechanisme induceren dat werkt via specifieke afbraak van de geproduceerde RNA molekulen.

   Dit proefschrift.

2. "RNA-mediated virus resistance" duidt niet op een toestand maar op een proces; de term "antiviral state" hiervoor is derhalve ongelukkig gekozen.

   Lindbo et al., 1993. Plant Cell 5, 1749-1759.

3. Als in planten post-transcriptionele gen-suppressie sterker wordt na homozygotering, kan dit betekenen dat dit proces een rol speelt bij inteelteffecten.

   Goodwin et al., 1996. Plant Cell 8, 95-105.

   Elmayan and Vaucheret, 1996. Plant Journal 9, 787-797.

4. Het zogenaamde cyclische model voor co-suppressie van het chalcone synthase gen in Petunia is voorbarig, daar in de bijbehorende experimenten voorbij gegaan wordt aan de invloed die structuren, ontstaan door intramoleculaire baseparing van RNA en cDNA molekulen, kunnen hebben op primer-extensie experimenten en PCR analyses.

   Metzlaff et al., 1997. Cell 8, 845-855.

5. Hoewel bij de transporteiwitten van verschillende plantenvirussen het vermogen om buisvormige structuren te vormen het meest in het oog springt, zijn specifieke affiniteit voor plasmodesmata en virale eiwitten of nucleïnezuren evenzeer belangrijke eigenschappen.

6. Het voorkomen bij plant-pathogene bacterieën van eiwitten met signalen die deze eiwitten naar de plantenkern dirigeren, impliceert dat manipulatie van de gastheer belangrijk is in de pathogenese van deze organismen en dat manipulatie niet alleen kan plaatsvinden via overdracht van bacteriële genen naar het genoom van de plant.

   Van den Ackerveken et al., 1996. Cell 87, 1307-1316.

   Yang et al., 1995. MPMI 8, 627-631.

7. De waarneming dat prion-ziekten veroorzaakt worden door infectieuze eiwitten, geeft aan dat eigenschappen van organismen niet alleen bepaald worden door het genetische materiaal.

Prusiner et al., 1996. Trends Biochem. Sci. 21, 482-487.

8. Polycomb groep eiwitten zijn opwindende eiwitten, nu ook voor plantenbiologen.

Goodrich et al., 1997. Nature 386, 44-51.

9. Het consumeren van produkten die genetisch gemodificeerd zijn met humane genen, kan worden beschouwd als pseudokannibalisme en biotechnologen dienen de discussie hierover niet uit de weg te gaan.

10. Het wordt hoog tijd voor een onderzoeksproject naar de psychische en sociale effecten van het projectmatig financieren van wetenschappelijk onderzoekers.

11. Positieve kritiek is geen *contradictio in terminis* en kan derhalve gerust gegeven worden.

Van Dale, Groot woordenboek der Nederlandse taal.

12. Een goede fles wijn heeft niet alleen een ziel maar ook een geest.

13. Alle begin is moeilijk, loslaten nog moeilijker.

Stellingen behorende bij het proefschrift:
"Expression and Silencing of Cowpea Mosaic Virus Transgenes"
door Titia Sijen, te verdedigen 12 juni 1997.

In herinnering aan mijn vader, die mij de schoonheid van de natuur liet zien,
Voor mijn moeder, altijd op zoek naar het positieve,
En voor Matthieu, die de liefde in het leven zo mooi gestalte geeft.

VOORWOORD

Augustus 1991; een leeg bureau, een projectvoorstel en een zakje cowpea bonen: regenereren maar! Een half jaar later toch geen verdere financiering maar wel een nieuw project en dus: replicatie. Replicatie? Resistentie, uiteindelijk, na veel omzwervingen. En dan nu een proefschrift. Het is af.

Dat was nooit gelukt zonder de hulp van een aantal mensen die ik op deze plaats dan ook heel graag wil bedanken. Allereerst de promotor, Ab van Kammen. Ab, ik waardeer ten zeerste de warme belangstelling waarmee je al die jaren de proeven hebt gevolgd en ook de tijd en rust die je nam om uit te leggen waarom dingen zo niet opgeschreven konden worden. Heel erg bedankt, al heeft je handschrift mij wel eens verkrampte ogen bezorgd zeker op de hobbeligere treingedeeltes van het traject Wageningen - Amsterdam. Ook Joan Wellink, de co-promotor, wil ik bedanken voor de vriendelijke begeleiding die ik in mijn eigenwijsheid en felheid wellicht niet altijd even goed kon waarderen.

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Het onderzoek heeft zich echter niet alleen op Molbi afgespeeld; vele uurtjes heb ik doorgebracht in de viruskas en het halen van de kas-sleutels op virologie was een niet onbelangrijk bijbehorend ritueel; Marcel, ik heb intens genoten van onze soms erg wilde maar altijd enthousiaste discussies over experimenten en modellen, ik vond het wetenschap op z'n best. In de kas heeft Bert Essenstam heel belangrijke taken vervuld in het verzorgen van de planten. Bert, je zult regelmatig je hoofd hebben geschud wanneer je keek naar de door mij tamelijk rommelig opgepotte plantjes die zo'n schril contrast vormden met jouw strakke rijtjes; ontzettend bedankt voor al je groene zorgen en ook voor je betrokkenheid die zelfs zo ver ging dat je me thuis opbelde om te zeggen dat mijn plantjes een week eerder rijp waren voor inoculatie omdat de zon flink geschenen had. Verder wil ik van de vakgroep virologie nog in het bijzonder Rob Goldbach bedanken: Rob, ontzettend bedankt voor je belangstelling vooral op dat moment, toen alles niet zo goed ging; ik word weer even stil wanneer ik eraan terugdenk.

Ook de sfeer op het lab is belangrijk geweest voor het tot stand komen van het boekje en ik denk met veel plezier terug aan de diverse en regelmatig wisselende labgenoten en labcontacten. In mijn begintijd waren dat vooral Sacco en zijn penen die mij zo liefdevol adopteerden; allemaal hardstikke bedankt voor de gezelligheid en de zonnigheid; ik beschouw de term 'halve peen' nog steeds als een eer! We werden wreed gescheiden toen de CPMV-groep naar beneden, naar 'paviljoenen' 0 en 1, moest verhuizen, maar och, dat was eigenlijk ook best heel leuk: JanHo's krullenbol tussen de flessen door (immer aan de vooravond van een grote doorbraak), JanV's diverse geluidjes, het gevloek-vanuit-de-tenen van Sander (alleen maar heel soms,

hoor), the enthusiastic and lively Polish teachings from Alicja (and the wonder of being able to work together on a very small kitchen bench in Montpellier as a result of sharing a lab bench in Wageningen for a year), de wat stillere fransozen Jean-Michel en Jean-Baptiste en natuurlijk ook Hans, Heleen, Peter en studenten zoals Annemarie; allemaal bedankt voor het bewerkstelligen van een goede sfeer. En later kwam Tony, eigenlijk om braaf, rustig en stilletjes te sequencen en in z'n sequencing-hok is ie ook wel rustig, maar daarbuiten moet ie óf plagen óf hij hoort kamelen rare dingen doen en enkel midden in de nacht gaat ie voor een rustig kopje thee; maar Tony, je vormde een heerlijke uitlaatklep en de humor die met de lunches over tafel vloog zal ik nooit vergeten. Ook de morele en vloeibare steun van de overige AIO's zoals die op z'n best naar voren kwam tijdens de diverse AIO-weekenden zal ik niet gauw vergeten. Gelukkig kijken ook mijn nieuwe collega's in Amsterdam met een flinke dosis humor tegen het (lab)leven aan en hebben ze begrip voor het feit dat zo nu en dan sommige 'post-docs moeten zwemmen'.

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Tot slot nog wat woorden voor wellicht de twee liefste mensen in mijn leven. Mam, ik heb zoveel ontzag voor jouw levenskracht waarmee je altijd zo op zoek bent naar de positieve kanten van alles en iedereen; ik hoop dat ik dat ook een klein beetje mag kunnen en dat jij nog veel zult genieten, want dat kun je zo mooi. En Matthieu, ik zal je er wel nooit van kunnen overtuigen dat een virus een veel slimmer pathogeen is dan een schimmel, maar ik hoop het nog heel lang te mogen blijven proberen, want het leven is zo goed met jou.

Titia

CONTENTS

OUTLINE

At any stage during growth, plants can fall victim to infection by various pathogens and viruses are a special class of pathogens as they can only multiply within a suitable host. At the department of molecular biology of the Wageningen Agricultural University, we are interested in gaining more insight in the molecular properties of cowpea mosaic virus (CPMV) and the functioning of CPMV genes during plant infection. Transgenic plants that express one viral function can be very helpful to study the role of individual viral genes during infection. In this thesis, this approach was applied for genes of CPMV.

In Chapter 1, the various processes that occur in plants during infection with CPMV are described. Furthermore, this chapter illustrates how transgenic plants can be used not only to study the functioning of viral genes during plant infection but also to generate resistance to viral diseases. In addition, an overview is presented of processes that can affect expression of a transgene.

A prerequisite for utilising transgenic plants, is the availability of a method for plant regeneration and transformation. CPMV's natural host is the tropical legume cowpea or *Vigna unguiculata*, a species known, like other grain legumes, to be recalcitrant at regeneration. In Chapter 2, the progress that was made for developing a reliable regeneration and transformation system for cowpea is described. An efficient protocol, however, was not obtained.

Because tobacco, which is also a host for CPMV, can be regenerated and transformed very efficiently, this plant species was preferred to overexpress genes of CPMV. Chapter 3 describes experiments on overexpressing the CPMV movement protein (MP) genes in tobacco. In plants, viruses have to pass the cell walls to spread through a host. To overcome this barrier, plant viruses encode MPs which can modify the plant plasmodesmata. By overexpressing MPs in plants, the way by which these proteins mediate the passage of viral genomes to adjacent cells can be studied. The CPMV MP genes were not only expressed transgenically in tobacco plants, but also from recombinant potato virus X (PVX) genomes. This latter approach appeared to be the most promising.

In studying CPMV movement processes by overexpressing the MPs, a CPMV mutant that is defective in cell to cell movement, would be a useful tool. By molecular analyses, that are presented in Chapter 4, it was examined whether CPMV mutant N123, which produces both in cowpea and in 'Pinto' bean plants a markedly reduced infection, could be used for that. In addition, analysis of this mutant could reveal information on molecular aspects of the functioning of viral products during virus movement.

The transgenic tobacco plants that were carrying CPMV specific genes, did not appear very useful for studying the functioning of the CPMV proteins. However, in several lines an effect of the transgenes was found to occur as the plants exhibited a resistant phenotype upon inoculation with CPMV. This resistance, which is an example of pathogen derived resistance, is characterised in Chapter 5.

The mechanism underlying the resistance was investigated in more details in experiments described in Chapter 6. Resistance was found to be RNA-mediated and to involve a post-transcriptional RNA turnover process that is primarily aimed at the transgene mRNAs but to which also incoming viral RNA molecules can fall victim if they carry sequences homologous to

the transgene. Further analyses revealed some factors that are involved in inducing the mechanism and also some details on how the process is brought about.

In Chapter 7, the conclusions that can be drawn from the results presented in this thesis are summarised. Furthermore, it is discussed why the process of post-transcriptional RNA turnover would be existing in plants and how this process could be efficiently exploited to engineer virus resistance or to study plant gene expression.

CHAPTER 1

GENERAL INTRODUCTION

Plant viruses and responses of plants to virus infections

At any stage during growth, plants can fall victim to infection by various pathogens. Plant viruses are among the smallest pathogens but nevertheless they are able to cause severe losses in numerous crops world-wide. Viruses have a remarkably simple structure and are composed of a single type of nucleic acid (RNA or DNA) which is protected by a coat of proteins that in some cases is surrounded by a lipid envelope. Viral genomes carry only limited information and all components for building progeny viruses are provided by the biosynthetic processes of the host cells. The functions encoded by a viral genome are merely involved in regulating virus multiplication. As viruses are fully dependent on their hosts for their survival, they have been classified as obligate intracellular parasites. Viruses are historically not regarded as living organisms but rather as individually operating genetic material. Hence, not only viral pathogenesis but also virus multiplication has to be studied in relation to the host. On the other hand, studies on plant viruses can provide information on the cellular and molecular processes within plants as viruses exploit the plant machinery. In addition, viral genomes have been manipulated to serve as tools for the expression of heterologous genes, which can help in gaining knowledge on the functions encoded by these genes (Chapman et al., 1992; Donson et al., 1991).

Various interactions between plants and viruses can be distinguished. If the virus can not replicate at all in the plant cell it has entered, this non-host relationship is called true immunity. In case virus replication remains restricted to the initially infected cell, the plant-virus interaction is designated a subliminal infection. If the virus does not spread beyond the initially infected leaf, the infection is denoted a local infection, while in case of a systemic infection the virus spreads throughout the plant. In tolerant plants, systemic virus infections result in milder or absence of symptoms, although virus replication may be unaffected. Inefficient systemic spread of the replicating virus can allow plants to outgrow the viral infection.

Plants have developed complex defense systems against infections by viruses and other pathogens. These differ strongly from defense mechanisms in animals as plants lack a circulatory system and are not able to produce antibodies (Dangl, 1992). Some of these defense mechanisms are constitutively present and an example of such a defense mechanism is the presence of proteins, that can inactivate ribosomes, in the cell walls of some plant species, such as the *Phytolacca* species pokeweed and endod. Upon a virus infection, these proteins are thought to selectively enter the cell disrupt cellular protein synthesis and provide a local cell suicide by which virus replication is prevented (Bonness et al., 1994).

Beside constitutive defense mechanisms, plants have evolved inducible systems. The latter can be specifically activated when a product of a pathogen, denoted avirulence factor, is recognised either directly or indirectly by the product of a host resistance gene. Some virus resistance genes result in inhibition of the cellular viral replication which is denoted extreme resistance (Bendamahne et al., 1995). Other resistance genes (Santa Cruz and Baulcombe, 1993; Witham et al., 1994; Dinesh-Kumar et al., 1995; Weber et al., 1993) effect a hypersensitive response (HR) which restricts viruses to the primary infection site. HR is a complex plant defense mechanism, that involves a cascade of events which are rather aspecific

and irrespective of the kind of invading pathogen. Usually HR involves both local and systemic activities (reviewed by Baker and Orlando, 1995; Staskawicz et al., 1995, Chasan, 1994; Lamb, 1994; Ausubel et al., 1995; Hammond-Kosack and Jones, 1996). The local reactions include localised necrosis (actual HR) by rapid programmed cell death (apoptosis) of the challenged cells (Dangl et al., 1996; Greenberg, 1996). In this way, the plant can confine the pathogen to the primary site of infection. Concomitantly, the walls of cells around the infection site are reinforced by processes of chemical crosslinking. This could help to immobilise microbial pathogens or inhibit their cellular ingress and improve the effectivity of HR. Besides, a variety of antimicrobial compounds and other protecting metabolites are synthesised. In addition, when the avirulence factor is recognised, reactive oxygen intermediates are rapidly produced, a process that is denoted oxidative burst. These active oxygen species probably play a central role in arranging and inducing the defense responses (Tenhaken et al., 1995; Chasan et al., 1994).

The inducible defense responses can by accompanied by systemic events and resistance that evolves from this is generally known as systemic acquired resistance (SAR). SAR may defend the plant against secondary pathogen attack and is brought about by the onset of a wide variety of genes which encode various antipathogenic effects (Ryals et al., 1994; Dong, 1995; Dempsey and Klessig, 1994; Ryals et al., 1996). It has been found that the presence of salicylic acid (SA) is essential for SAR development (Chasan, 1995; Bi et al., 1995; Neuenschwander et al., 1995), but it remains controversial (Vernooij et al., 1994; Shulaev et al., 1995; Moelders et al., 1996) whether SA is the systemic signal that communicates the local presence of a pathogen to other parts of the plant.

Cowpea mosaic virus

Cowpea mosaic virus (CPMV) is one of the, more than twenty, viruses found to infect cowpea (Latunde-Dada, 1990; Thottapilly et al., 1995). CPMV causes systemic mosaic symptoms that severly affect plant growth and seed production. Cowpea or *Vigna unguiculata* has been cultivated for many centuries in the developing world, particularly Africa. The crop is well adapted to the stressfull growing conditions of the tropics, by for instance drought tolerance, and has excellent nutritional qualities. Under natural conditions, CPMV is transmitted to uninfected plants by beetles, mostly the cowpea leaf beetle *Ootheca mutabilis*. The host range of CPMV is very limited and mainly restricted to a number of legume species. CPMV has been studied at the Wageningen Agricultural University since the early sixties (Agrawal, 1964), and the extensive studies on the molecular properties of the virus illustrate very well what functions viral genes have in the infection process.

CPMV has divided its genetic information over two single stranded RNA molecules of positive (messenger-sense) polarity that are separately encapsidated in identical icosahedral capsids. These viral capsids are built up of 60 copies of each of the two coat proteins (CPs) which are both encoded by the smaller of the two CPMV segments. The genomic RNA molecules, denoted RNA1 and RNA2 with a length of 5889 nt and 3481 nt respectively, contain a small viral protein (VPg; viral protein genome linked) covalently linked to the 5' ends and a poly(A)

tail at the 3' ends (Figure 1; for review and references see Goldbach and Wellink 1996; Peters, 1994; Van Bokhoven, 1993c). Successful systemic infection of plants with CPMV depends on the replication and expression of both RNA1 and RNA2, but there is a clear distinction in the function of the proteins encoded by each of the genomic RNAs. While RNA2 codes for the two viral capsid proteins (VP23 and VP37) and the proteins involved in viral cell to cell movement, all proteins encoded by RNA1, have a role in viral replication. Consequently, RNA1 is able to replicate independently of RNA2, while RNA2 is dependent on replication of RNA1 and expression of RNA1-encoded proteins for its replication.

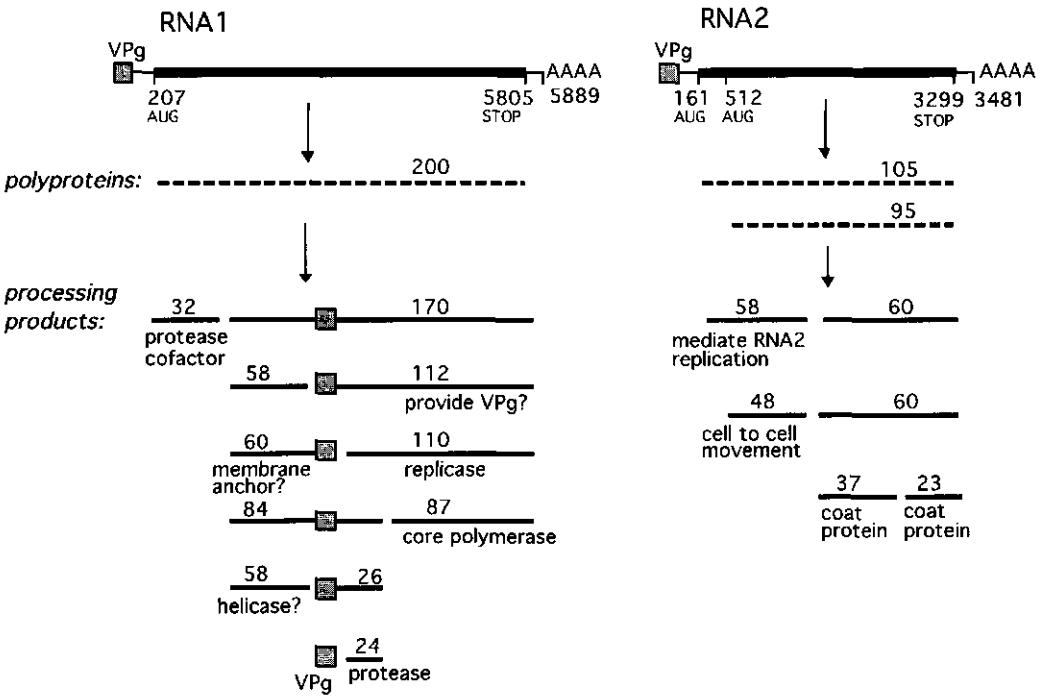

Figure 1. Genetic organisation and expression strategy of cowpea mosaic virus (CPMV).

CPMV consists of two positive stranded RNA molecules that carry a VPg molecule at the 5' end and a poly(A) tail at the 3' end. For further details see text. Both RNA1 and RNA2 contain a single large open reading frame which is represented by a solid bar. RNA2 can be translated into two overlapping polyproteins, due to internal initiation at a second AUG codon. Positions of start and stop codons are indicated. Through proteolytic cleavages by a viral protease, various proteins are generated that are indicated by their apparent molecular weight in kDa. The putative or known functions are indicated as well.

The two genomic RNA molecules each contain a single large open reading frame which is translated into polyproteins. From these polyproteins, smaller proteins are derived by

proteolytic cleavages catalysed by an RNA1-encoded protease (Figure 1). The consequence of this strategy of gene expression is that there is a direct quantitative relation between proteins encoded by one RNA segment. However, proteins encoded by each of the two RNA segments do not have to be produced in equimolar amounts. Such a division of the genetic information over two genomic segments may allow a virus to regulate the expression of its genes effectively and for example for CPMV, such a strategy can be very useful, because, theoretically, the proteins encoded by RNA2 need to be produced in 30 times larger amounts than the proteins encoded by RNA1. For, to generate one infectious unit of CPMV, RNA1 needs to be expressed four times to provide VPg molecules to the genomic and the replicative intermediate RNAs of both RNA1 and RNA2, while 120 molecules of the RNA2-encoded capsid proteins are needed for encapsidation of a molecule of each RNA1 and RNA2 into a viral capsid consisting of 60 copies of the coat proteins.

CPMV is assumed to be replicated in membrane-associated replication complexes that consist of both viral proteins and host factors (Van Bokhoven, 1993c). A virus-encoded RNA-dependent-RNA-polymerase (RdRp) provides the function of viral RNA synthesis, which could be primed by a VPg molecule that occurs covalentely bound to the 5' ends of both the genomic and replicative intermediate RNA molecules. The replication functions are encoded by RNA1 (Figure 1) and thus, RNA1 can replicate *in cis* while replication of RNA2 occurs *in trans*. The N-terminal part of the protein encoded at the 5' end of RNA2 (the 10kDa region of the 58kDa protein, Figure 1) is involved in directing the RNA1-encoded replicase towards the RNA2 molecules. Strikingly, replication of CPMV RNAs is tightly linked to translation; RNA1 only functions as a template for replication following the synthesis of the replicative proteins from the same RNA molecule, and RNA2 can only act as a template if the 10kDa region is expressed from the same RNA molecule.

In plants, cells are contained by a cell wall which forms a structural barrier for the spread of plant viruses through the host. It is generally accepted that plant viruses circumvent this cell wall by modifying the existing intercellular connections between plant cells, the plasmodesmata, in such a way that virus translocation can occur. These adaptations are established by virus-encoded movement proteins (MPs) (reviewed by Carrington et al., 1996). Electron microscopical studies on CPMV-infected plants have revealed the presence of tubules through plasmodesmata-like structures within the cell wall (Van Lent et al., 1990). The tubular structures can be labeled with antiserum raised against the CPMV RNA2-encoded 48kDa MP and are densely packed with virus particles (Van Lent et al., 1990). These observations suggest that CPMV transports its progeny genomes as encapsidated virus particles to adjacent cells through the plasmodesmata-channelling, MP-containing tubules. Consistently, CPMV has been found to require both the presence of the MPs and the two CPs for cell to cell movement of its progeny genomes (Wellink and Van Kammen, 1989). The assembly of tubular structures is not dependent on the presence of intact plasmodesmata or cell wall as tubules were also found on the surface of infected protoplasts (Van Lent et al., 1991). It seems likely that rather large amounts of MPs are needed to channel the plasmodesmata with these MP-containing tubular movement structures. The genetic organisation of CPMV establishes that the MPs are produced coordinately with the CPs of which many copies are needed to encapsidate progeny CPMV

genomes, and thus relatively large amounts of MPs are provided as well. CPMV RNA2 encodes, overlapping with the 48kDa MP, a 58kDa protein (Figure 1). This protein could, in addition to the previously described function in replication of RNA2, also have a role in movement processes, but this has sofar remained unclear.

For CPMV, several mutants have been generated and studied which revealed that changes in both the RNA1- and the RNA2-encoded functions can alter pathogenicity and symptom development (De Jager, 1978). Interestingly, for some mutants the extent of the effects varied for different hosts (De Jager, 1978), which strenghtens the idea that plant components, which can vary between plant species, have important roles during virus infections. Viruses that have a limited host range, like CPMV, might carry more specific requirements towards plant components than viruses that are able to infect a wide range of different plant species. In non-host plant species either the appropriate plant factors could be absent or the virus could hold a limited efficiency for gathering or exploiting the necessary host components. Protoplast studies have revealed that for several plant species in which CPMV does not establish an infection, the virus can replicate in the cell and induce tubular movement structures (Huber et al., 1977; Wellink et al., 1993), indicating that absence of infection can also occur from deficiencies in the virus transport processes.

Although the use of cellular and molecular biological techniques have extensively contributed to the knowledge on viruses like CPMV, many aspects of the viral life cycle still remain unsolved. For example, much remains to be elucidated on the processes of virus encapsidation and disassembly; which factors determine release of the viral RNA molecules from the capsids after infection of a cell, how the assembly of the capsids takes place and what determines the specific encapsidation of viral RNA molecules by viral coat proteins. More information on the replication of CPMV could be generated by the identifying and analysing of the host factors assumed to be involved in this process. This may also reveal why virus replication can occur in some plants but not in other, non-host plant species. Also, many aspects of virus pathogenicity remain unsolved; how symptoms are induced and what plant responses are triggered or what plant reactions can be shut off upon a virus infection. Analysis of the processes of viral cell to cell and long distance movement would provide a major contribution to the understanding of these highly intruiging processes and, in addition, probably also of the trafficking of various plant metabolites to adjacent or more distant cells (Citovsky, 1993; Lucas and Wolf, 1993; Mezitt and Lucas, 1996).

During the last years, several novel techniques have been developed which could prove to be useful for generating information on how viruses function, by example methods designed to find matching protein partners like the two-hybrid system and the phage display technique (Allen et al., 1995). Both methods use a target protein, for instance a viral protein, to identify interacting partners from an expression library of, for example, a host plant. The phage display method is an attractive approach for easily handling large libraries. The method exploits the fact that fusion proteins may be incorporated into the coat of phages, which allows selection and analysis of specific recombinant phages that are able to bind to the immobilised target proteins. The two-hybrid system is yeast-based and utilises hybrid genes to detect interacting proteins via the activation of reporter gene expression.

Transgenic plants to study viral functioning

A general, widely used strategy in studying viral processes, involves the introduction of mutations in the viral genome and analysis of the effects of these mutations *in vivo* (for CPMV for example De Jager, 1978; Lekkerkerker et al., 1996; Peters 1994). However, this approach does not allow investigation of the different viral functions individually. In cell free or *in vitro* systems single viral functions can be studied and this strategy has been applied successfully for the processes of virus encapsidation and uncoating (Gallie et al., 1987; Hwang et al, 1994). Also for the complex process of replication, *in vitro* assays have generated detailed information, like for example for alfalfa mosaic virus (AlMV) for which by these assays both the requirements of the RNA templates and the role of the AlMV coat protein could be determined (for details see De Graaff, 1996). These cell free replication systems were containing plant factors (De Graaff, 1996), which is consistent with the assumption that plant components have essential roles in virus multiplication. The work of Janda and Alquist (1993) has demonstrated that the function of plant factors in viral replication can also be fulfilled by yeast components as brome mosaic virus was found to be able to replicate in this organism. However, *in vitro* replication assays could not be established for several other viruses including CPMV (Van Bokhoven, 1993c), which illustrates the need for other approaches.

Transgenic plants in which individual viral functions are expressed separate from the other viral genes, could provide a useful tool to study viral processes. A crucial basis for utilising the potential of transgenic plants has been the development of efficient regeneration and transformation systems for various plant species. The value of transgenic plants expressing replicative proteins in studying the process of viral replication is well illustrated with AlMV. The transgenically produced replicative proteins were able to form active replication complexes and these transgenic plants allowed the complementation of AlMV-RNA segments not capable of replication themselves (Van Dun et al., 1988; Taschner et al., 1991). Using these plants, various aspects of the replication strategy of this virus could be determined (for details see Van der Vossen, 1996) and in addition, from these plants fractions could be derived that facilitated *in vitro* replication assays (De Graaff, 1996).

Transgenic plants could also be very helpful to study viral movement processes because intercellular translocation involves a role of plant plasmodesmata. For various viruses, it has been shown that MPs produced in transgenic plants are functional and enable the transport of movement-deficient mutants of the virus (Wolf et al., 1991; Cooper et al., 1996). In these plants, MP expression was accompanied by a permanent increase in the plasmodesmatal permeability, indicating that the MPs are, without the presence of other viral proteins, directed to the plasmodesmata and establish modification of these plant structures (Wolf et al., 1989; Poirson et al., 1993; Vacquero et al., 1994; Citovsky, 1993; Lucas and Wolf, 1993). Cellular localisation studies revealed that MPs co-localise with the cytoskeleton (Heinlein et al., 1995; Gail McLean et al., 1995), which might have a role in getting the MPs to the plasmodesmata.

Transgenic plants expressing viral coat proteins have shown their merit mostly in studies on the eliciting of specific plant responses like HR (Culver and Dawson, 1991) or in engineering resistance (Powell-Abel et al., 1986).

Transgenic plants to generate virus resistance

When viral sequences are expressed in transgenic plants, other scenarios than obtaining plants in which a single viral function is operating apart from the other viral products, can be envisaged as well. The expression of viral transgenes could disturb specific balances or interactions that occur either among viral factors or between viral products and host components. Such an interference might result in resistance against virus multiplication. Substantiation of this idea can be found in the phenomenon of cross protection, which implies that a plant can become protected against a virus by prior infection with a milder strain of the virus (reviewed by Valle et al., 1988). Actually, from the phenomenon of cross potection the idea of pathogen derived resistance (PDR) developed (Sanford and Johnston, 1985), which refers to resistance of plants against virus infection if the plants contain a viral transgene. It was envisaged that the expression of the pathogen-derived gene products is inhibitory to the viral infection process.

The development of biotechnological procedures has strongly stimulated research on engineering resistance against virus infections in genetically modified plants by various approaches (see reviews by Wilson, 1993; Scholthof et al., 1993; Hull and Davies, 1992; Fitchen and Beachy, 1993; Lomonossoff, 1995 and the references therein). Transgenic plants that carry a ribosome inactivating protein were found to be resistant to several viruses (Lodge et al., 1993). These highly toxic proteins are thought to be positioned in the cell wall and enter the cell together with a penetrating virus. Then they cause a local cell suicide that prevents multiplication of the virus. Protection against virus infections was also observed in transgenic plants carrying antisense inhibition of the expression of the plant S-adenosylhomocysteine gene. The product of this gene has a key function in transmethylation reactions which are known to be important for the 5' capping of mRNAs and many viral RNAs (Masuta et al., 1995). Further, transgenic plants expressing a functional single-chain Fv antibody against artichoke mottled crinckle virus were found to exhibit some protection against infection by the virus (Taviadoraki et al., 1993). Furthermore, it has been shown that the effects of some viruses can be reduced by the production of RNAs in transgenic plants that can act as satellite RNAs (satRNAs) or defective interfering RNAs (DI RNAs) (Gerlach et al., 1987; Harrison et al., 1987; Jacquemond et al., 1988). Both satRNAs and DI RNAs depend on a helpervirus for their replication; DI RNAs are truncated forms of the helpervirus, while satRNAs are not related by sequence similarity. These transgene mRNA molecules are assumed to act as parasitic RNAs and result in protection of the transgenic plant to an infection of the helpervirus. However, it has been reported that pathogenic variants can develop from transgenically expressed satRNAs (Palukaitis and Roossinck, 1996).

Most successes for biotechnologically engineering resistance against viruses have been achieved using a wide variety of sequences derived from the viral pathogen (reviewed by Baulcombe, 1996b), upon following the concept of PDR which was described above. The first success was achieved (Powell-Abel et al., 1986) when the coat protein gene of tobacco mosaic virus (TMV) was expressed in transgenic tobacco plants. Research showed that the presence of the coat protein itself was needed to generate the resistant phenotypeand that the protection

occurred from inhibition of the disassembly of infecting virions which is needed to release the viral RNA prior to replication (Powell-Abel, 1990). This type of resistance has been denoted coat protein mediated protection and nowadays it has been applied for many viruses (see reviews by Wilson, 1993; Scholthof et al., 1993; Hull and Davies, 1992; Fitchen and Beachy, 1993; Lomonossoff, 1995).

Also, the expression of other types of viral proteins, like movement or replicase proteins, resulted in resistance in transgenic plants. PDR was observed in plants expressing dysfunctional movement proteins (Lapidot et al., 1993; Malyshenko et al., 1993; Cooper et al., 1995; Beck et al., 1994). Resistance was not observed in transgenic plants expressing the functional version of these MPs (Wolf et al., 1991; Cooper et al., 1996), suggesting that the resistance occurs from aberrant interactions that the modified MPs have with a host component essential for cell to cell movement of the infecting virus. Interestingly, it was found that even the cell to cell translocation of unrelated viruses could be restricted (Lapidot et al., 1993), which suggests that the plant structures or host factors that are occupied by the dysfunctional movement proteins are utilised by a wide range of plant viruses.

Inhibition of virus multiplication was also found in transgenic plants expressing viral replicative proteins (reviewed by Baulcombe, 1994). It has been reported as well that resistance was specifically generated when mutated replicase proteins were expressed (Donson et al., 1993) as that resistance was only established in case the wild-type form of a virus replicase component was produced (Audy et al., 1994). The resistance occurring from expression of the intact replicase component could result from defects in the assembly of the multimeric replication complex due to presence of an excess amount of one of the components. The resistance occurring from the expression of defective (truncated) replicase proteins, might be caused by modified interactions of the truncated proteins with plant factors that result in the occupying or capturing of an essential host factor. Such a host factor could be common for the replication processes of various unrelated viruses because Donson et al. (1993) described that the resistance is acting against a broad range of unrelated viruses.

These examples illustrate that the mechanisms underlying these different cases of PDR are depending on the natural function of the viral protein that is expressed. While the transgenically produced CPs are inhibiting the virus disassembly, the MPs are impeding the process of cell to cell transport and the replicase proteins are interfering with the cellular viral replication processes. A common feature that may be assumed for all these different cases of PDR, is that a correlation should occur between the level of resistance and the level of transgene expression. Irrespective of what type of viral protein is directing resistance and irrespective of whether a defective or an unmodified version of the protein is involved, the interference with the normal viral life cycle will be stronger if more proteins are expressed. In addition, for all these cases of PDR, no resistant phenotype should be found in case plants that are transformed with a similar transgene from which no protein can be translated (untranslatable versions of the transgene). Such transgenes can be obtained by deleting for example the start codon from the coding sequence or by introducing immediately downstream of the start codon a stop codon.

Several researchers met with observations in conflict with these assumptions. Both Lindbo et al. (1992) and Van der Vlugt et al. (1992) found that plants transformed with

untranslatable versions of a potyvirus CP gene showed a resistant phenotype, just like the transgenic plants that contained a transgene encoding a unmodified CP. The resistance resulted in full immunity to virus multiplication in the cell (Lindbo et al., 1992) and occurred also when high inoculum doses were used of both encapsidated and unencapsidated viral genomes. The mechanism of this type of resistance must, for these reasons, differ from that of coat protein mediated protection. This resistance was denoted RNA-mediated PDR to be distinguished from protein-mediated PDR. Strikingly, no correlation was observed between the levels of untranslatable transgene mRNAs produced and the level of resistance. This observation indicated that the resistance is not occurring because host components needed for virus replication are being taken by the transgene mRNA molecules. Otherwise, this process of RNA-mediated virus resistance might be due to direct interactions between transgene RNA molecules and viral RNA strands that prevent viral replication. For all RNA viruses, RNA molecules of both polarities occur because genomic RNAs are replicated via strands of opposite polarity. Thus, transgene mRNA molecules could form double stranded RNA molecules with strands carrying the sequence of these mRNAs in the antisense orientation. However, for such an antisense inhibition, a correlation is expected between the level of resistance and the expression level of the transgene mRNAs, which was not observed (Van der Vlugt et al., 1992). Furthermore, sense-antisense interactions seem rather unlikely to generate full and inoculum dose independent inhibition of the cellular viral replication because viral RNA molecules usually replicate to very high copy numbers.

For these reasons, another explanation for this type of PDR had to be found. RNA-mediated PDR acts with high efficiency and specificity as the resistance results in full cellular immunity and is only active against viruses that are by sequence homology strongly related to the virus of which the transgene was derived (for reviews see Baulcombe 1996a; Prins et al., 1997a). To examine whether a specific, cytoplasmic RNA turnover mechanism underlies the resistance, the transgene transcription rates were determined by nuclear run-on assays and compared to the transgene mRNA steady state levels, for both sensitive and resistant lines (Lindbo et al., 1993a). These experiments revealed that specifically in the resistant plants, the transcription rates of the transgene were high in relation to the mRNA steady state levels (Lindbo et al., 1993a; and also Smith et al., 1994; Mueller et al., 1995; English et al., 1996; Pang et al., 1996; Prins et al., 1996). Thus, it appears that an RNA turnover process is induced post-transcriptionally on the transgene mRNAs to which also incoming viral RNAs that contain sequences highly homologous to the transgene sequence, fall victim. In this way, replication of the virus is prevented and full cellular immunity to the virus is established. As the mechanism is RNA-mediated the transgene can be derived from various types of viral genes and both functional or defective versions can be used.

Such a specific, rapid, cytoplasmic RNA turnover process, could also underlie the phenomenon of co-suppression (reviewed by Dougherty and Parks, 1995). Co-suppression or sense-suppression refers to the process in transgenic plants that mediates, in addition to the suppression of a sense-oriented transgene, the suppression of homologous endogenous genes and it was first observed in petunia plants in which transformation of flavonoid genes, rather suprisingly, resulted in suppression of homologous endogenous genes (Van der Krol et al.,

1990; Napoli et al., 1990). It was suggested that sense-suppression would occur from a post-transcriptional regulation process (de Carvalho et al., 1992) and comparative nuclear run-on assays and northern blot analyses indeed showed that post-transcriptional turnover of mRNA molecules underlies co-suppression (Van Blokland et al., 1994; reviewed by Stam et al., 1997). The turnover process is specific for the mRNAs of the transgene and of homologous endogenous genes. Recently, English and coworkers (1996) have linked sense-suppression and RNA-mediated virus resistance by showing that transgenic, sense-suppressed plants are resistant to a virus at which sequences homologous to the suppressed transgene were incorporated. Thus, more understanding of the mechanism of RNA-mediated virus resistance and of the factors that determine inducing of the RNA turnover process in a plant, may also develop from studies on co-suppression. The term homology-dependent post-transcriptioanl gene-silencing has been chosen to refer to the process underlying both co-suppression and RNA-mediated virus resistance.

Transgenic plants and the processes that can occur

When plants are transformed, one or more copies of the transgene are integrated at various positions of the plant genome. At different positions of the genome, the transcriptional activity can vary as a result of the local chromatin structure or due to the presence of enhancer or repressor elements. Consequently, structural features at the genomic integration site can influence the expression level of the transgene both positively and negatively. In addition, it has been found that transcription of transgenes can be prevented by transcriptional silencing (reviewed by Matzke and Matzke, 1995a), which is assumed to occur from methylation at promoter regions that inhibits the binding of elements of the transcription machinery. The methylation is thought to be induced when homologous promoter sequences interact for instance by ectopic pairing (Meyer, 1995a), which can happen in case during transformation several transgene copies are integrated. The expression level of a transgene is not only determined by the rate of transcription, but also by post-transcriptional processes. The above described process of homology-dependent post-transcriptional gene-silencing results in rapid turnover of the transgene mRNAs and other homologous RNAs which can also be incoming viral RNAs (RNA-mediated virus resistance).

Thus, the intruiging question arises why in some cases viral transgenes are expressed stably and produce viral proteins to levels at which they can form a functional unit, while in other cases the transgene mRNA molecules are rapidly and specifically eliminated and RNA-mediated virus resistance occurs. Remarkably, it has been observed in several cases that in the same transformation procedure plants are obtained that accumulate the viral proteins ('expressor' plants) as well as plants in which RNA-mediated resistance occurs ('suppressor' plants). For example, expression of a full-length replicase gene of cymbidium ringspot virus in transgenic *Nicotiana benthamiana* plants resulted in both highly resistant plants and in plants that supported the replication of a defective interfering (DI) RNA (Rubino and Russo, 1995; Rubino et al., 1993). In this latter type of transgenic plants the transgenically produced viral proteins can evidently function in replication processes, because DI RNAs are dependent on the

replicase proteins of the helpervirus. In the resistant plants the protection was found to carry characteristics of RNA-mediated resistance. Also for tomato spotted wilt virus (TSWV) both 'expressor' and 'suppressor' plants were obtained in one transformation procedure. Prins and coworkers (1996) transformed *Nicotiana tabacum* plants to carry the putative TSWV movement protein gene (NS_m). They obtained plants of two different phenotypes. Some plants were highly resistant to TSWV and it was determined that this resistance was RNA-mediated and caused by an RNA turnover mechanism (Prins et al., 1996), while other plants were found to be severly reduced in their growth (Prins et al., 1997b). In these plants, NS_m proteins were accumulating in the plasmodesmata which presumably caused the growth retardation of the plant by hindering of the intercellular communication between the plant cells (Prins et al., 1997b). Similarly, transformation of *Nicotiana benthamiana* (Vaira et al., 1995) or lettuce (Pang et al., 1996) plants with the TSWV nucleocapsid protein (N) gene gave rise to two types of plants that could be regarded 'suppressor' and 'expressor' plants, respectively. Plants that contained only limited amounts of N gene transcripts exhibited a strong, TSWV specific resistance shown to be due to a sense-suppression mechanism (Pang et al., 1996). Other plants produced high levels of N-protein and showed delayed and reduced symptoms when inoculated with TSWV or related tospoviruses. This type of resistance should probably be ascribed to a mechanism resembling coat protein mediated protection. From these three examples it can be concluded that rather specific details than obvious differences have a role in inducing sense-suppression of a transgene instead of allowing high and stable expression of the encoded proteins. Thus, neither the plant species, the viral transgene, the promoter sequences, nor the transformation procedure (including the *Agrobacterium* strain, the binary vector or the procedure itself) have to be of decisive importance, although these factors could influence indirectly the process obtained in the transgenic plants. Comparison of various aspects of the 'suppressor' and 'expressor' plants that carry the same transgene could reveal the crucial points, that determine whether a transgene is expressed or suppressed.

CHAPTER 2

REGENERATION OR TRANSFORMATION OF COWPEA, *VIGNA UNGUICULATA*

Titia Sijen, Jeanine Hendriks, Joan Wellink and Ab van Kammen

Several approaches were explored for generating transgenic cowpea (Vigna unguiculata) plants. Stably transformed cowpea callus and root cultures could be obtained upon PEG-mediated direct gene transfer of plasmid DNA to cowpea protoplasts or upon Agrobacterium tumefaciens or Agrobacterium rhizogenes-mediated transformation of cowpea explants. Fertile cowpea plants could be regenerated from thin cell layer segments that were excised over the cotyledonary nodes. A mass of shoot initials formed after more than six weeks of floating the explants on liquid medium. The excised shoots maturated into elongated and rooted plantlets upon subculturing on solidified medium during a period of at least six months. As plant regeneration is the major limiting factor in producing transgenic cowpea plants, the described regeneration procedure could provide a basis for further studies towards an efficient and reliable transformation system for cowpea.

INTRODUCTION

To utilise the full potential of plant biotechnology, efficient and reliable plant transformation and regeneration systems are essential. Grain legumes like cowpea (*Vigna unguiculata*), have generally been found to be recalcitrant to regeneration of transgenic plants (reviewed by Christou, 1994). Although most grain legumes have been shown to be transformation competent, the transformation competence of the regenerative cell types is in many cases low (Puonti-Kaerlas, 1993). Accordingly, for cowpea either stably transformed callus tissue, transgenic root cultures or chimeric transgenic plants have been obtained. Transgenic callus cultures were obtained upon *Agrobacterium tumefaciens*-mediated leaf-disc transformation (Garcia et al., 1986) and transformed roots were generated upon transformation with *Agrobacterium rhizogenes* (Suzuki et al., 1993). Chimeric transgenic cowpea plants were obtained by several procedures, like cocultivation of seed-derived embryos with *A. tumefaciens* (Penza et al., 1991), electroporation or imbibition of mature embryos in the presence of plasmid DNA (Akella and Lurquin, 1993; Penza et al., 1993), electroporation of nodal meristems that had first been injected with plasmid DNA and lipofectin (Chowrira et al., 1995) or microprojectile bombardment or coculture with *A. tumefaciens* of embryonic axes and cotelydonary base explants (Kononowicz et al., 1995). Besides, procedures for regeneration of untransformed cowpea shoots and plants have been described including direct shoot regeneration from apex-containing stem explants (Cheema and Bawa, 1992), stimulated shoot bud formation by gamma ray seed irradiation (Amitha et al., 1992), multiple shoot formation by the germination of mature seeds in the presence of thidiazuron (Ciardi et al., 1995), plant regeneration from protoplasts derived from immature cotelydons (Wei and Xu, 1993) and plant regeneration from primary leaf explants (Muthukumar et al., 1995).

In developing a reliable and efficient plant transformation system for cowpea, first emphasis goes to the finding of cells that are competent for both transformation and regeneration. In this study we have established that both cowpea mesophyl protoplasts and cells at wounded edges of cowpea leaf or stem explants are highly competent for transformation by PEG-mediated direct gene transfer or cocultivation with *A. tumefaciens* or *A. rhizogenes*, respectively. Cells at the axillary bud region of the cotelydonary nodes were found to be competent to regeneration. However, the regeneration of fertile cowpea plants was found to be time-consuming because a long maturation period was needed prior to elongation and rooting of the shoots. Regeneration is the most limiting factor in obtaining transgenic legume plants. Therefore, the regenerative nodal thin cell layer (TCL) segments might prove an effective explant source for generating transgenic plants if they will prove to be competent to *Agrobacterium*-mediated transformation or direct gene transfer methods.

RESULTS

Stable transformation of cowpea

Protoplasts were prepared from the primary leaves of ten days old *in vitro* cultured cowpea

seedlings. Immediately after isolation, protoplasts were subjected to polyethyleneglycol (PEG)-mediated transformation using the plasmids pGL2 and pMONGUS that carry a hygromycin resistance gene and a ß-glucoronidase (GUS) reporter gene, respectively. Both genes are under the control of an enhanced CaMV 35S promoter. Protoplasts were embedded into an alginate matrix (Damm and Willmitzer, 1988) and incubated for three days in a medium containing a high concentration of mannitol to induce cell wall regeneration and initial cell divisions. To obtain further growth, the medium was exchanged for a medium providing glucose as the main carbon source. After ten days of culture, hygromycin selection was initiated on the microcalli and after five weeks hygromycin resistant macrocalli were identified and subcultured during at least three weeks and then analysed for GUS activity. On average, 35% of the hygromycin resistant calli were showing GUS expression, indicating that both the co-transformation of the two plasmids (pGL2 and pMONGUS) and the hygromycin selection of the microcalli were efficient. GUS activity was observed in all cells of a callus and the expression of the GUS transgene were found to be stable in the transgenic calli during at least eight months of culture.

Freshly excised leaf, epicotyl and hypocotyl explants of ten days old *in vitro* cultured cowpea seedlings were cocultivated with either the disarmed *A. tumefaciens* strain LBA4404 or *A. rhizogenes* strain LBA9402 which were both carrying plasmid p35SGUSINT. This plasmid contains a GUS reporter gene with an intron sequence under the control of a CaMV 35S promoter (Vancanneyt et al., 1990). After *A. tumefaciens*-mediated transformation, kanamycin resistant callus cultures were obtained that showed GUS expression in all cells. When the various types of explants were cocultivated with *A. rhizogenes* and grown on kanamycin-containing selection medium, multiple fast-growing hairy roots developed on most explants. On average 80% of these roots displayed strong GUS activity (Figure 1A). The transgenic hairy roots showed stable GUS expression in all cells of a root during at least three months of subculturing.

Shoot regeneration on thin cell layer segment of the cotyledonary node

From 10 days old sterile cowpea seedlings, cotyledons and visible axillary buds were removed as described by Nauerby et al. (1991). Nodal thin cell layer (TCL) explants of approximately eight cell layers were obtained by cutting twice over each cotyledonary node. The explants were floated on nylon gauzes in liquid UK medium supplemented with 1.1 mg/l zeatine and 0.05 mg/l IBA. After six weeks, a mass of multiple shoot initials, that ranged in number from 5 to 20 shoots per explant, was formed on approximately 15% of the explants (Figure 1B). No significant difference in regeneration frequency were observed for the first or second nodal explant that was excised over a cotyledonary node.

The origin of the explants was found to be an essential parameter of regeneration since no shoot induction was found on TCL explants that were excised from epicotyl segments. Also, the composition of the medium was found to be of great importance because regeneration did not occur when either MS (Murashige and Skoog, 1962) or S (Nauerby et al., 1991) medium was used. However, the combination of hormones was found to be flexible since regeneration was obtained on approximately 10% of the explants in case UK medium containing 1.1 mg/l BAP and 0.05 mg/l NAA was used. The number of shoot initials per explant was similar to the number obtained with 1.1 mg/l zeatine and 0.05 mg/l IBA. The floating on liquid medium was identified

as an important parameter because no regeneration was observed when the explants were cultivated on UK medium (supplemented with 1.1 mg/l zeatine and 0.05 mg/l IBA) that was solidified with 0.8% plant agar. Less efficient regeneration was obtained in case the explants were not floated on nylon gauzes.

The shoots were excised and cultured on hormone free MS20 medium for maturation. After a minimum of six months of culture shoots started to elongate. Elongated shoots were rooted on MS20 medium containing 1 mg/l IAA and transferred to the greenhouse where they developed into fertile cowpea primary regenerants (Figure 1C). In total, during twelve months 98 plants were regenerated from 288 explants from three independent experiments.

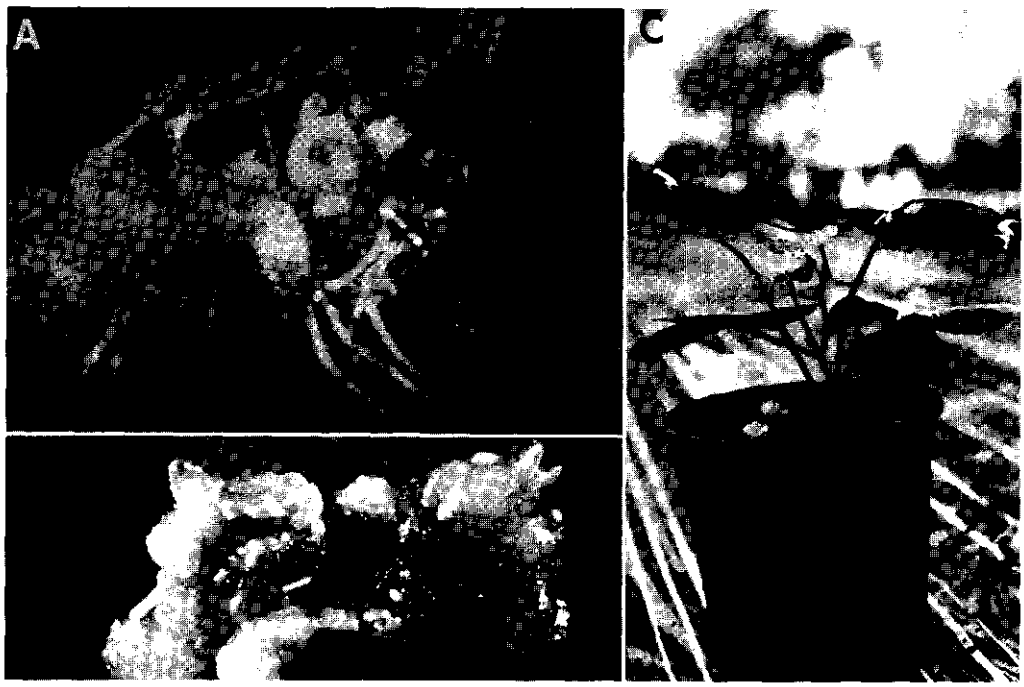

Figure 1. Transformation or regeneration of cowpea.

Panel A shows GUS expressing, transgenic hairy roots obtained upon *A. rhizogenes*-mediated transformation of hypocotyl explants. Panel B shows the induction of multiple shoot initials on nodal thin cell layer segments, six weeks after excision of the explant. Panel C shows a fertile cowpea regenerant.

DISCUSSION

In this study, progress was made for establishing a procedure for transformation and regeneration of cowpea, one of the major grain legume crops. Transgenic, GUS-expressing

callus and root cultures were efficiently obtained either upon PEG-mediated co-transformation of protoplasts or by *A. tumefaciens* or *A. rhizogenes*-induced transformation of different cowpea explants. *Agrobacterium*-based transformation of cowpea has previously been reported by Garcia et al. (1986) and Suzuki et al. (1993). Selection of transformed cowpea tissue could effectively be performed by hygromycin or kanamycin. Shoot initials or somatic embryos could not be induced on the callus or root cultures, eventhough various hormone treatments on different media for varying time periods (extended hormone treatment or application of hormone shocks) were tested. The treatments only resulted in the formation of roots or callus of different types. The use of *A. tumefaciens* "shooter" strains (Steffen et al., 1986) that harbour mutations in the T-DNA genes which are involved in the induction of plant tumors, was also found not to be effective in regenerating cowpea shoots.

The cotyledonary nodes could be successfully used as an explant source for the regeneration of fertile cowpea plants and 98 plants were regenerated from 288 explants. To stimulate the time-consuming process of maturation of the shoot initials, several culture media and hormone treatments, including the application of gibberellic acid or abscicic acid, have been tested but none proved to be effective. To examine whether the regenerative explants would be competent for transformation, explants were cocultivated with *A. tumefaciens*. However, the viability and also the regeneration efficiency were found to be severely affected by the cocultivation and the subsequent culturing in the presence of the antibiotics which is necessary for elimination of the bacteria. Next, it was studied whether electroporation of cowpea TCL explants in the presence of DNA (D'Halluin et al., 1992; Dekeyser et al., 1990; Dillen et al,. 1995) would be an effective transformation method. Epicotyl TCL segments were used in these experiments as on average five times more epicotyl than nodal TCL explants can be obtained from one seedling. The segments were electroporated in the presence of a plasmid carrying a GUS reporter gene under various conditions that differed in buffer composition, pulse strength, pulse time and number of subsequent pulses. However, at 2 to 5 days post electroporation no transient GUS activity could be detected under any of the examined conditions.

Plant regeneration is the major limiting factor in establishing an efficient and reliable transformation system for grain legumes like cowpea. Consequently, for regeneration often multicellular explants of complex tissues are used which are complicated to transform. Accordingly, for cowpea mainly chimeric transgenic plants have been obtained upon various procedures of transformation of intact apical or nodal meristems (Akella and Lurquin, 1993; Chowrira et al., 1995; Penza et al., 1993; Penza et al., 1991; Kononowicz et al., 1995). The procedure described in this study involves regeneration from excised explants of the region of the cotyledonary node from which visible meristems had been removed (Nauerby et al., 1991) which might reduce the chance on the formation of chimeric plants upon transformation. Recently, generation of transgenic cowpea plants has been reported after *A. tumefaciens*-mediated transformation of de-embryonated mature cotelydons (Muthukumar et al., 1996). The cells that resulted in regeneration were situated at the proximal side of the explants, which is the previous position of the nodal meristems. Southern blot analyses showed the presence of the transgene in the regenerated plants, but it was not examined whether the plants were chimeric or not and whether the transgene could be stably transferred to the progeny.

In the preliminary studies that we performed on the suitability of the nodal TCL segments for *A. tumefaciens* mediated transformation, the explants suffered severely from the cocultivation. However, TCL explants of *Nicotiana plumbaginifolia* (Hahn Trinh et al., 1987) and *Brassica napus* (Charest et al., 1988) have been shown to endure *A. tumefaciens* mediated transformation. Whether the regenerative cells within the multicellular nodal TCL segments would be accessible and competent for *A. tumefaciens* mediated transformation in case the conditions for cocultivation could be improved, is unclear. Interestingly, the generation of genuine non-chimeric transgenic plants upon *A. tumefaciens*-mediated transformation of nodal regions has been reported for pea (Grant et al., 1995; Davies et al., 1993; Schroeder et al., 1993). In addition, several alternatives to *A. tumefaciens*-mediated transformation could be examined. The electroporation procedures might be open to improvement for instance by the application of lipofectin as has been demonstrated by Chowrira et al. (1995). Besides, particle bombardment or biolistic DNA delivery might prove a useful procedure for transforming the TCL segments because this method can deliver DNA to cells at several cell layers inside an explant. Particle bombardment of shoot meristems has been shown to be effective in generating stably transgenic plants for the legumes soybean, common bean and peanut (Sato et al., 1993; Russell et al., 1993; Brar et al., 1994). Also, cowpea leaves and embryonic axes have been reported to become transiently or stably transformed upon particle bombardment (Finer et al., 1992; Kononowicz et al., 1995). Furthermore, novel plant transformation techniques are being developed which might prove very useful for the transformation of recalcitrant crops, such as the recently described method of "agrolistics" (Hansen and Chilton, 1996), in which the advantages of *Agrobacterium* transformation are combined with the high efficiency of biolistic DNA delivery. Possibly, cowpea cultivars other than California Blackeye could be more efficient in the suggested regeneration or transformation procedures.

Genetic engineering of the important grain legumes is not yet performed routinely (Christou, 1994). However, for several legume crops important progress has been made that will probably soon allow crop improvement utilizing the tools of molecular plant breeding. Nothwithstanding the gradual advancement in plant regeneration and transformation, it remains unclear why some plant species, like grain legumes, are recalcitrant while other plants species, like many of the family of *Solanaceae*, are highly competent for regeneration and transformation procedures. Both the physiological and the molecular processes in the plant could determine recalcitrance or competence. Physiological determinants could be, for instance, the internal hormone balance of a plant or the sensitivity of the plants to externally applied hormones or other stimuli. Also, cellular, molecular and nuclear processes could play a role like processes that are involved in determining dedifferentiation of a cell, which has to precede regeneration. Factors involved in dedifferentiation processes have, however, not yet been identified.

MATERIALS AND METHODS

Plant material

Cowpea seeds (*Vigna unguiculata* [L.] Walp. cv California Blackeye) were sterilised in concentrated

sulfuric acid for 25 min under moderate shaking. After removal of the sulfuric acid, seeds were transferred to one liter of sterilised water precooled at 4°C and rinsed 3 more times with sterilised water. Seeds were germinated on half strength MS medium (AM medium) (Murashige and Skoog, 1962) containing 10 g/l sucrose at 25°C under 16h light - 8h dark regime.

Bacterial strains and plasmids

For direct gene transfer to protoplasts the plasmids pGL2 and pMONGUS were used. pGL2 carries the coding region of the hygromycin phosphotransferase gen (hpt) under the control of an enhanced CaMV 35S promoter and a CaMV 35S terminator. In pMONGUS the ß-glucuronidase gene is present under the control of an enhanced 35S promoter and a nos-terminator. In further transformation experiments *Agrobacterium tumefaciens* strain LBA4404 or *Agrobacterium rhizogenes* strain LBA9402 were used to which the binary vector p35GUSINT (Vancanneyt et al., 1990) was conjugated. This plasmid contains on the T-DNA region a neomycin phosphotransferase II (nptII) gene, that allows selection of transformed cells by kanamycin, and a ß-glucuronidase gene, in which an intron sequences is present, under the control of a 35S promoter and 35S terminator (kindly provided by Dr. L. Willmitzer).

Protoplast preparation, transformation and culture

Protoplasts were prepared and isolated according to Damm and Willmitzer (1988). After the first washing, pelleted protoplasts were resuspended in 8 ml 0.5 M sucrose and 1 ml of W5 solution was placed on top. After 5 min of centrifugation at 700 rpm, protoplasts at the interphase were collected and washed with 10 ml of W5 solution. PEG-mediated direct gene transfer to protoplasts was performed as described in Damm et al. (1989) using 10 µg of pGL2 and 10 µg of pMONGUS. Protoplasts were further washed and embedded in alginate as described in Damm and Willmitzer (1988). Protoplasts were cultured in 3 ml of V47 medium (Binding, 1974) supplemented with 0.5 mg/l BAP (6-benzylaminopurine), 0.5 mg/l kinetine and 0.4 mg/l 2.4-D (2,4-dichlorophenoxyacetic acid), at 25°C in the dark for 3 to 5 days to establish cell wall regeneration and to initiate the first cell divisions. For further divisions the medium was exchanged for UKM medium (hormone-free KM1p medium (Kao and Michayluk, 1975) supplemented with 45.6 g/l mannitol, 0.25 g/l xylose, 250 mg/l glutamine, 250 mg/l asparagine) containing 0.5 mg/l BAP, 0.5 mg/l kinetine and 0.4 mg/l 2.4-D. Protoplasts were kept at 25°C in the dark and after 7 days the medium was exchanged for similar medium supplemented with 15 µg of hygromycin for selection of transformed microcalli. After 10 days microcalli were transferred to UKM medium supplemented with 15 µg hygromycin, 0.25 mg/l BAP, 0.25 mg/l kinetine and 0.1 mg/l NAA (naphtaleneacetic acid) and cultivated for two times 10 days under 16h light - 8h dark regime at 25°C. Macrocalli were released from the alginate layer using citrate as described in Damm and Willmitzer (1988). Macrocalli were analysed for GUS activity or cultured further on UKM medium, of which the glucose content had been reduced to 20 g/l, containing 0.25 mg/l BAP, 0.25 mg/l kinetine and 0.1 mg/l NAA, solidified with 0.8% plant-agar.

Transformation of cowpea explants with *Agrobacterium*

Freshly excised leaf, epicotyl or hypocotyl explants were cocultivated with fresh, overnight cultures of either *A. tumefaciens* or *A. rhizogenes* for 2 days on MS20 medium (Murashige and Skoog, 1962), solidified with 0.8% plant-agar. For explants that were cocultivated with *A. tumefaciens*, the medium was supplemented with 2 mg/l BAP and 1 mg/l NAA. After 2 days, the explants were transferred to similar medium which contained 500 mg/l cefotaxime. Three days later, the explants were transferred to medium further supplemented with 50 mg/l kanamycin and cultured during two weeks. Callus, obtained upon *A. tumefaciens*-mediated transformation was excised and subcultured on MS20 medium containing 0.8% plant-agar, 2 mg/l BAP, 1 mg/l NAA, 200 mg/l cefotaxime and 75 mg/l kanamycin. Hairy roots, obtained upon *A. rhizogenes*-mediated transformation were excised and cultured on MS20 medium containing 0.3% phytagel, 200 mg/l cefotaxime and 75 mg/l kanamycin.

GUS activity assays

Protoplasts-derived macrocalli and callus and root cultures obtained upon *A. tumefaciens* or *A. rhizogenes*-mediated transformation were tested for the presence of GUS as decribed by Jefferson (1987).

Regeneration of fertile cowpea plants from nodal thin cell layer segments

From sterile cowpea seedlings, 10 to 12 days old, nodal thin cell layer (TCL) explants were obtained by cutting twice longitudinally with a razor blade over the cotyledonary node from which the cotyledons and visible axillary buds had been removed (Nauerby et al., 1991). The TCL explants have a size of approximately 10 mm long, 3 mm wide and 1 mm (6 to 8 cell layers) thick. The explants were floated in 24 well plates (Greiner) on sterilised, nylon gauzes. Each well contained one explant floating on 1.5 ml UK medium (UKM medium, see protoplasts culturing, in which the mannitol concentration had been reduced to 0.25 g/l) supplemented with 1.1 mg/l zeatine and 0.05 mg/l IBA (indole-3-butyric acid). The explants were cultured at 25°C in 16h light - 8h dark regime for six weeks. The multiple shoot initials or, if possible, individual shoots were excised and transferred to hormone-free MS20 medium solidified with 0.4% phytagel and subcultured monthly for maturation during six to twelve months. Shoots that started to elongate were transferred to MS20 medium containing 0.8% plant-agar and 1 mg/l IAA (indole-3-acetic acid) for rooting. Shoots that contained several well-growing roots were transferred to the greenhouse and developed into seed-bearing primary regenerants in two months.

CHAPTER 3

TOWARDS FUNCTIONAL STUDIES ON THE COWPEA MOSAIC VIRUS MOVEMENT PROTEIN *IN PLANTA*

Titia Sijen, Joan Wellink, Maarten T. Penning, Jan Verver and Ab van Kammen

To move from cell to cell, plant viruses are assumed to exploit the existing intercellular connections between plant cells, the plasmodesmata. This process is facilitated by movement proteins (MPs) that are encoded by the virus. The MPs of cowpea mosaic virus (CPMV) are present in tubular structures that occur, during a CPMV infection, in plasmodesmata and that are densely packed with virus particles. By expressing the CPMV MPs in plant cells separate from the other viral proteins, more information on the role of the MPs in virus transport processes might be obtained. Transgenic tobacco plants were raised that constitutively expressed the CPMV MP genes. In none of the transgenic lines accumulation of the MPs was detected. Rather, in several lines the expression of the transgene appeared to be post-transcriptionally silenced, which resulted in resistance against a CPMV infection. When the CPMV MP genes were expressed in transgenic Nicotiana tabacum plants under the control of a tetracycline inducible promoter, high levels of the transgene mRNA could be induced by application of tetracycline. But even then, no accumulation of MPs was detected. Expression of the CPMV MP genes in plant cells from the potato virus X (PVX)-based expression vector, did result in accumulation of the MPs. The MPs were detected in the form of tubular structures extending from the surface of protoplasts into the medium. The accumulation of the CPMV MPs did not restrain the PVX derivatives from establishing a systemic infection in tobacco plants. Hence, these PVX derivatives could provide an effective tool for studying the functioning of the CPMV movement proteins in plants separate from the other CPMV proteins.

INTRODUCTION

After entering a host plant cell, viruses generate the structural and nonstructural proteins that are needed for replication and production of progeny virus particles. Further infection of the plant requires translocation of viral genomes to adjacent cells over the host cell wall. Plant viruses are assumed to exploit the existing intercellular connections between plant cells, the plasmodesmata, for this cell to cell translocation and they encode specific proteins, movement proteins (MPs), that assist in this process. For the various plant viruses, at least two distinct schemes for cell to cell spread exist (Deom et al., 1992; Gail McLean et al., 1993; Lucas and Wolf, 1993; Carrington et al., 1996).

The process of cell to cell movement of tobacco mosaic virus (TMV) has been well studied using both TMV-infected plants (Tomenius et al., 1987; Heinlein et al., 1995) and transgenic plants expressing the TMV MPs (Atkins et al., 1991; Ding et al., 1992; Moore et al., 1992). These studies revealed that the only viral proteins that TMV needs for cell to cell movement of the viral genomes, are the MPs. For systemic virus spread to the higher leaves (long distance movement), TMV also requires the coat proteins (CPs) (Hilf and Dawson, 1993). The TMV MPs are localised to plasmodesmata and increase the plasmodesmatal size exclusion limit (SEL) (Tomenius et al., 1987; Ding et al., 1992; Atkins et al., 1991; Moore et al., 1992; Wolf et al., 1989; Padgett et al., 1996). In the cytoplasm, TMV MPs associate with the cytoskeleton (Gail McLean et al., 1995; Heinlein et al., 1995; Padgett et al., 1996). Furthermore, *in vitro*, purified TMV MPs can bind single stranded nucleic acids and form thin extended structures (Citovsky et al., 1992). Thus, the current view is that for cell to cell movement of TMV the plasmodesmata are transiently opened by the viral MPs to allow transport of a ribonucleoprotein complex consisting of viral RNA and viral MPs to the adjacent cell. The ribonucleoprotein complex is assumed to have been moved to the plasmodesma via cytoskeletal elements.

Cowpea mosaic virus (CPMV) employs a different method for cell to cell transport of its genetic information which requires both the MPs and the CPs (Wellink and van Kammen, 1989). The plasmodesmata appear rather drastically modified as inferred from electron microscopical studies of CPMV-infected tissues that revealed tubular structures that extend from the cytoplasm of one cell, through a plasmodesma, into the cytoplasm of an adjacent cell. These tubular structures appeared densely packed with CPMV virions (Van Lent et al., 1990). The tubular structures could be labeled with antiserum raised against CPMV MPs (Van Lent et al., 1990), suggesting that MPs are an important component of tubular structures. Similar tubular structures were found on CPMV-infected protoplasts on which the tubules extend from the surface into the medium (Van Lent et al., 1991). Presumably, for cell to cell movement of CPMV the plasmodesmata are modified by the viral MPs that assemble into a tubular structure, through which or with which virus particles move to adjacent cells.

Hence, various interactions are expected to occur for these CPMV MPs in achieving virus cell to cell movement. For tubule assembly association of the MPs is required and possibly also some host factors take part in this process. Assembly of tubular structures was observed upon transient overexpression of the CPMV 48kDa MP gene in protoplasts of plant species that are

both hosts and nonhosts for CPMV (Wellink et al., 1993). Furthermore, tubules were formed when the MP gene was expressed in cells of the insect *Spodoptera frugiperda* (Kasteel et al., 1996). These results indicate that tubule assembly results primarily from features of the 48kDa MP, but involvement of a conserved host factor can not be excluded. To enable specifically and efficiently the passage of CPMV virions to the adjacent cells, the CPMV MPs have to interact with the CPMV CPs. A mutational analysis of the CPMV 48kDa MP gene suggested that the C-terminal region of the MP is involved in the interaction with the virions (Lekkerkerker et al., 1996), because MPs that lacked this region assembled into tubules which did not contain virus particles. In addition, the MPs must probably connect with cellular components of the host to target the MPs to the plasmodesmata and accomplish modification of the plasmodesmata.

The genome of CPMV consists of two single stranded RNA molecules of positive polarity. RNA1 encodes the viral replicase, while RNA2 encodes the CPs and two overlapping proteins of 58kDa and 48kDa. The smaller protein is the result of initiation of translation at a second, in frame AUG-codon (Holness et al., 1989). RNA1 can replicate independently of RNA2, while replication of RNA2 is dependent on expression of the RNA1 encoded replicase gene. The unique N-terminal domain within the RNA2-encoded 58kDa protein is involved in regulating the *trans* replication of RNA2 (van Bokhoven et al., 1993a). Although expression of the 48kDa MP gene is sufficient to form tubules on protoplasts, a role for the 58kDa protein in CPMV cell to cell transport in plants can not be excluded. In this study both the 48kDa and the 58/48kDa genes are regarded MP genes.

To obtain more insight in CPMV movement processes, the functioning of the CPMV MPs in intact plant cells and tissues should be examined. Transgenic plants in which the MP genes are expressed separate from the other viral genes, might provide useful tools for these studies. Therefore, both the CPMV 48kDa and 58/48kDa MP genes were expressed in transgenic tobacco plants under the control of the constitutive enhanced CaMV 35S promoter. We used both *Nicotiana benthamiana* plants, which are a systemic host for CPMV, and *Nicotiana tabacum* cv Samsun NN plants, in which a CPMV infection remains restricted to the inoculated leaf.

Besides, the CPMV MP genes were brought under control of a tetracycline inducible CaMV 35S promoter (Gatz et al., 1991, 1992) and expressed in transgenic tobacco plants. This inducible 35S promoter (triple-Op-promoter) is modified to contain three tetracycline operator (tetOp) sites around the TATA-box. Expression from this promoter is strongly repressed when tetracycline repressor proteins (tetR) are bound to the tetOp sites. Transgenic plants of *N. tabacum* cv Winsconsin line TET1.1 (Gatz et al., 1991) express high levels of tetR proteins. When these plants are retransformed to carry a transgene under the control of a triple-Op-promoter, transgene expression is repressed but can be derepressed when tetracycline (Tc) is applied.

Finally, the CPMV MPs genes were expressed using the potato virus X (PVX)-based expression system (Chapman et al., 1992). This system involves recombinant PVX genomes that can express heterologous sequences from an additional subgenomic mRNA generated from a duplicated PVX coat protein promoter. When PVX derivatives that contained the 58/48kDa or 48kDa CPMV MP gene replicated in *N. benthamiana* protoplasts, the formation of CPMV MP

specific tubular structures was induced on the surface of the protoplasts. The presence of the CPMV MPs did not restrain the PVX derivatives from establishing systemic infection of tobacco plants. Thus, these recombinant PVX viruses might provide suitable tools for studying the effects of the CPMV MPs in plant cells.

RESULTS

Constitutive expression of the CPMV MP genes in transgenic plants

The two tobacco species *N. benthamiana* and *N. tabacum* cv Samsun NN were transformed using *Agrobacterium tumefaciens* to carry either the 48kDa or the 58/48kDa MP gene under the control of an enhanced CaMV 35S promoter and nos terminator (Sijen et al., 1995). In each case, ten primary transformants were identified that contained the transgene DNA and expressed the transgene mRNA as was determined by PCR and RT-PCR analyses (Sijen et al., 1995). All transformants showed a normal phenotype.

The primary transformants of both plant species were analysed for the presence of transgenically produced MPs. Leaf protoplasts were prepared, cultivated for 40 h and examined in immunofluorescence assays with an antiserum specific for the 48kDa MP (α48 serum). Protoplasts of leaves of untransformed plants were used as a control. In none of the protoplast samples a specific fluorescent signal was observed. In addition, leaf homogenates were prepared of both the primary transformants and untransformed plants and assayed by western blot analysis. Using the α48 serum, no accumulation of MPs could be detected for any transgenic plant.

The primary transformants were selfed to obtain an R1-generation progeny. Plants of the R1-generation of ten lines (three lines of *N. benthamiana* carrying the 48kDa MP gene, three lines of *N. benthamiana* carrying the 58/48kDa MP gene, two lines of *N. tabacum* carrying the 48kDa MP gene and two lines of *N. tabacum* carrying the 58/48kDa MP gene) were examined for pathogen derived resistance (Wilson, 1993). Full resistance against CPMV infection was detected in plants of one of the *N. benthamiana* lines carrying the 48kDa MP gene and in two of the *N. benthamiana* lines carrying the 58/48kDa MP gene (Sijen et al., 1995). None of the analysed *N. tabacum* plants showed resistance. The resistance in the three *N. benthamiana* lines was found to be the result of a post-transcriptional silencing mechanism that affected the expression of the transgene mRNAs (Sijen et al., 1996). Nuclear run-on transcription experiments revealed that in the resistant lines the nuclear transcription rate of the MP transgene was several folds higher than in the sensitive lines. However, as the result of the post-transcriptional silencing mechanism the steady state levels of transgene mRNAs, as determined by northern blot analyses, were several folds lower in the resistant lines than in the sensitive lines (Sijen et al., 1996). In the three sensitive *N. benthamiana* lines that had the highest steady state levels of transgene mRNAs, no accumulation of MPs was detected, neither by immunofluorescence assays on cultivated protoplasts nor by western blot analyses of leaf homogenates.

Tetracycline-induced expression of the CPMV MP genes in transgenic plants

Since transgenic plants expressing the CPMV MP genes from a constitutive promoter did not accumulate MPs to detectable levels, it was tested whether expression from an inducible promoter would be more effective. Induced expression might circumvent lethal effects, like for example malfunctioning of plasmodesmata, that abundantly expressed MPs might produce during shoot regeneration, which could determine that only plantlets containing low MP expression levels are regenerated. In addition, this approach might prevent the induction of a post-transcriptional RNA turnover mechanism, which also results in low MP expression levels. Besides, induced expression is more comparable to the natural way MPs are expressed during a virus infection. MPs are not expressed continuously in cells but only in the relatively short period, after the virus has entered the cell, during which virus replication and gene expression occur. In this way, induced expression might support the stability or the functioning of the MPs.

Accordingly, the 58/48kDa MP gene was cloned under the control of a tetracycline inducible 35S promoter, the triple-Op-promoter, in the binary vector pBINHygTX (kindly provided by Dr. C. Gatz). Sequence analysis of the construct pBINHygTX.58 showed that no mutations in the 58/48kDa MP gene sequence had occurred in the cloning procedure. pBINHygTX.58 was transferred to *A. tumefaciens* for transformation of leaf explants of *N. tabacum* cv Winsconsin line TET1.1, that is expressing tetracycline repressor (tetR) proteins at high levels (Gatz et al., 1991). Hygromycin resistant shoots were regenerated and primary transformants containing the 58/48kDa MP sequence were identified by PCR-analyses.

Leaves of ten primary transformants and of plants of non-transformed line TET1.1 were vacuum infiltrated with Tc and subsequently incubated on Tc-containing medium for two days. As a control, leaves of three of the primary transformants and of line TET1.1 were infiltrated with a medium without Tc. Total RNA was extracted from the leaves and analysed on a northern blot using a MP gene specific probe. In seven of the ten primary transformants high levels of transgene mRNAs were induced when Tc was applied (Figure 1). Next, it was determined whether in any of these seven transgenic plants, treatment with Tc would induce accumulation of the 58/48kDa MPs to detectable levels. Leaves were detached, kept for one day on Tc-containing medium and protoplasts were prepared and cultured for two days in the presence of Tc. As a control, for all plants the procedure was followed using solutions without Tc. Immunofluorescence assays were performed on the protoplast samples using α48 serum. For none of the plants specific fluorescing signals were detected in the protoplasts to which Tc had been applied. Another 19 primary transformants containing the 58/48kDa MP gene were identified by PCR-analyses and from these plants protoplasts were prepared and treated and analysed as described above. Neither for these transgenic plants accumulation of CPMV MPs was detected after treatment with Tc.

Potato virus X-based expression of CPMV movement proteins in tobacco cells

When cells become infected with CPMV, the viral genomes are replicated in the cytoplasm and the MP-encoding RNA molecules (the RNA2 molecules) are generated rapidly in high numbers

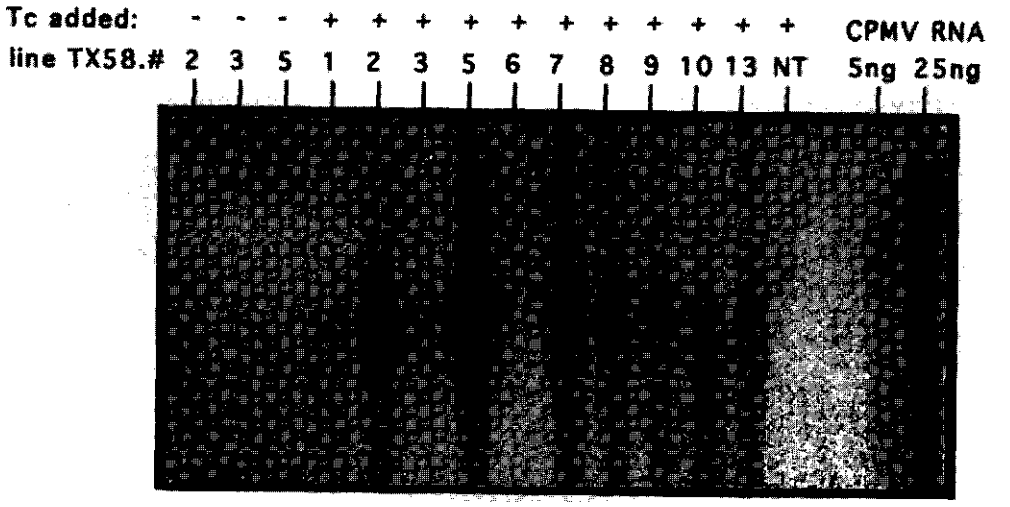

Figure 1. Induction of the CPMV MP transgene mRNA upon application of tetracycline in transgenic plants in which the 58/48kDa MP gene is present under the control of a tetracycline inducible promoter.

Total RNA was extracted from leaves that had (+) or had not (-) been treated with tetracycline (Tc) and for each sample 15µg was used for northern blot analysis. Lines carrying the 58/48kDa MP gene under the control of a tetracycline inducible promoter comprise TX58.1, TX58.2, TX58.3, TX58.5, TX58.6, TX58.7, TX58.8, TX58.9, TX58.10 and TX58.13. As a control line TET1.1 was used which is not transformed (NT) with the CPMV MP gene. For positive control 5 or 25 ng of CPMV RNA were analysed.

per cell. This results in the production of MPs in amounts that are easily detected by immunofluorescence assays on protoplasts in the form of MP-containing tubular structures that extend from the surface of the protoplasts into the medium. These tubules could be structures in which the stability of the MPs is increased. Both the level and the timing of expression might stimulate tubule formation. In addition, besides the MPs several other viral products are generated. These viral factors could have various effects that could increase accumulation of the MPs, like stimulating efficient translation of the viral RNA molecules, stabilising the translated MPs or enhancing of tubule formation. This way of expressing MPs in a cell could be mimiced when a heterologous virus is used for expression of the CPMV MPs in plants, such as recombinant PVX viruses that are obtained from the PVX expression vector (Chapman et al., 1992).

Accordingly, both the 58/48kDa and the 48kDa MP genes were cloned into the PVX-based vector pPC2S (kindly provided by Dr. D. Baulcombe) which is a derivative of pCG3 (Chapman et al., 1992) and contains convenient, unique restriction sites behind the sequences that correspond to the duplicated PVX CP promoter. Sequence analysis of the constructs pPVX48 and pPVX58ΔNTR showed that no mutations at the MP sequences had appeared during cloning procedures. *In vitro* transcripts of both wildtype PVX (wtPVX) and the PVX derivatives PVX48 and PVX58ΔNTR were generated and used to infect *N. benthamiana* protoplasts. Immunofluorescence assays using an antiserum specific for the PVX CP, revealed that for all

three infections viral genomes were replicating in 10-20% of the protoplasts. Immunofluoresence assays using α48 serum detected both in the PVX48 and in the PVX58ΔNTR infected protoplasts CPMV MP specific tubular structures on approximately 10% of the protoplasts (Figure 2). The tubular structures looked like the tubules obtained upon transient expression of the 48kDa and 58/48kDa MP genes from an enhanced CaMV 35S promoter in cowpea protoplasts (Wellink et al., 1993). Apparently, expression of the CPMV MP genes from heterologous viral genomes results in accumulation of MPs, detected in the form of tubular structures on protoplasts that are labelled with an anitserum specific to the CPMV MPs.

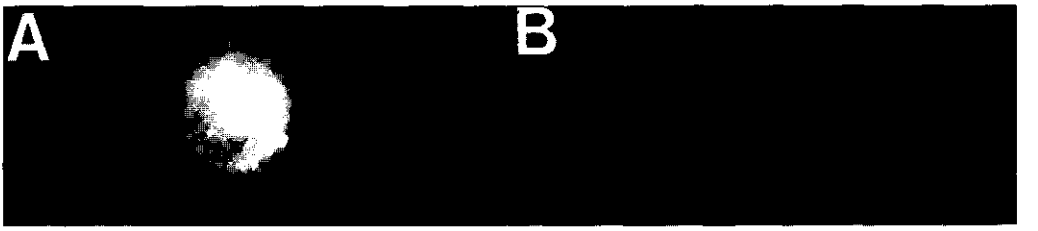

Figure 2. Detection of CPMV MP specific tubular structures on *Nicotiana benthamiana* protoplasts transfected with recombinant potato virus X genomes that are expressing the CPMV 48kDa MP.

Immunofluorescent images of *N. benthamiana* protoplasts transfected with PVX derivative PVX48 (Panel A) or PVX58ΔNTR (Panel B) which were obtained using an antiserum specific for the CPMV MPs.

Subsequently, transcripts of pPVX48 and pPVX58ΔNTR were used to inoculate *N. benthamiana* and *N. clevelandii* plants. In both tobacco species the recombinant PVX genomes induced systemic mosaic symptoms at 6 to 8 days post inoculation (dpi), which is similar to the time span needed for obtaining a systemic infection when inoculating *in vitro* transcripts of wtPVX. RT-PCR analyses using a primer pair that is flanking the insertion site (as described in Sijen et al., 1996), detected in the systemically infected leaves at 7 dpi only the presence of the recombinant PVX genomes (results not shown). Thus, presence of the CPMV MPs seems not to interfere with cell to cell transport of PVX genomes.

The approach of using the PVX expression system for *in planta* overexpression of the CPMV MPs was followed in experiments that had the aim to study the effects of mutations in the MP. Thus, we expressed a truncated 48kDa MP (48ΔX), from which the 29 C-terminal amino acids (aa) were removed and replaced by the 237 aa of the jelly fish green fluorescent protein (GFP) as a translational fusion (48ΔXGFP). *In vitro* transcripts of the PVX derivative encoding this fusion protein (PVX48ΔXGFP) were introduced into *N. benthamiana* protoplasts after which the protoplasts were cultured for 2 days to allow replication of the recombinant viral genomes. Fluorescence light microscopy revealed tubular structures extending from the surface of the infected protoplasts into the medium. The tubules showed strong green fluorescence (Figure 3A), indicating that the fusion protein carries both the capacity of tubule formation, similar to the CPMV MP, and the characteristic of GFP to give green fluorescent signals. Bright green fluorescence was also detected at the periphery of the protoplasts. When *in vitro* transcripts of

this PVX derivative were inoculated onto *N. benthamiana* plants, no PVX specific mosaic symptoms developed.

The green fluorescing signals occurring from the fusion proteins were exploited to examine the infection process of this PVX recombinant in the inoculated leaves and fluoresence light microscopy was used to identify infected cells. These studies revealed that cell to cell movement of the PVX genomes (PVX48ΔXGFP) was strongly inhibited. At 3 dpi the recombinant PVX48ΔXGFP had established infection in approximately 8 adjacent cells which had spread at 5 dpi to approximately 25 adjacent cells (Figure 3B). The PVX derivative PVXGFP (Baulcombe et al., 1995), on the other hand, which is expressing unfused GFP, had established at 3 dpi infection in at least 200 adjacent cells (Figure 3C) which had spread at 5 dpi to a systemic infection of the higher leaves. Evidently, presence of the 48ΔXGFP fusion protein interferes with efficient cell to cell movement of the PVX genomes, and thus with the functioning of the PVX MPs.

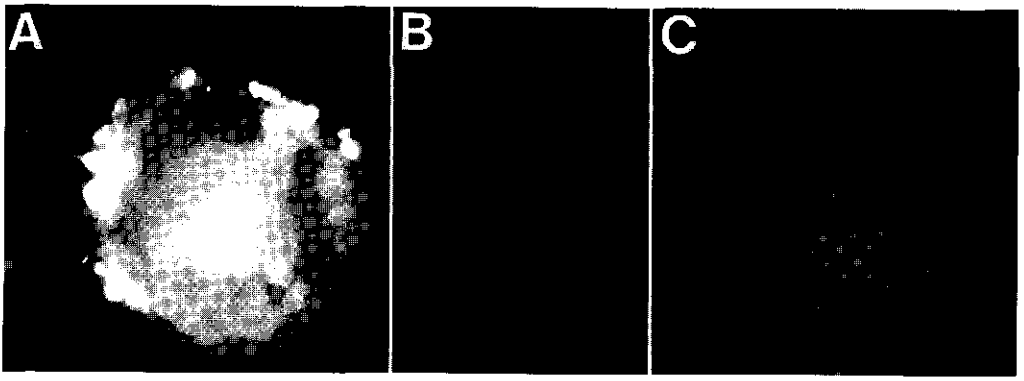

Figure 3. Detection of green fluorescing signals occurring from a truncated CPMV MP and GFP fusion protein or a non-fused GFP which are expressed from the recombinant PVX genomes PVX48ΔXGFP and PVXGFP respectively.

Panel A shows green fluorescing tubules extending from the surface of PVX48ΔXGFP-infected *N. benthamiana* protoplasts. Panel B shows the presence of green fluorescing signals in approximately 25 adjacent cells in *N. benthamiana* plants infected with PVX derivative PVX48ΔXGFP at 5 dpi. Panel C shows the presence of green fluorescing signals in approximately 200 adjacent cells in *N. benthamiana* plants infected with PVX derivative PVXGFP at 3 dpi.

DISCUSSION

By several approaches we have tried to obtain plants in which CPMV MPs accumulated, separate from the other CPMV proteins, to detectable levels. Such expression of MPs could allow to study the functioning of the CPMV MP in intact plant cells, particularly the interactions occurring

between the MPs and host components such as the plasmodesmata. The test for the accumulation of MPs involved immunofluoresence assays of protoplasts, because the lack of the cell wall reduces the possibilities for aspecific background interactions upon using the MP-specific antiserum.

The first approach involved constitutive expression of the CPMV MP genes in transgenic plants of two tobacco species. In this way, stable expression to detectable levels has been obtained for MP genes of several viruses like TMV (Atkins et al., 1991; Wolf et al., 1991; Ding et al., 1992), alfalfa mosaic virus (AlMV) (Poirson et al., 1993) and cucumber mosaic virus (CMV) (Vaquero et al., 1994; Cooper et al., 1996). For these viruses, this approach revealed that the MPs are located in the plasmodesmata where they bring about an increased gating capacity (SEL). However, for CPMV this method did not result in the production of detectable amounts of MPs in any of the transgenic plants. The transgenic *N. benthamiana* plants that contained the highest nuclear transcription rate of the transgene showed to be resistant to infection with CPMV. This resistance was found to be the result of a post-transcriptional RNA turnover mechanism that was primarily aimed at the transgene mRNAs and to which also homologous incoming viral RNA molecules fall victim (Sijen et al., 1996).

Because expression of a CPMV MP transgene from a constitutive promoter did not result in accumulation of the MPs to detectable levels, a second approach was tried. This method involved expression by means of an inducible promoter. By induced expression possible lethal effects of abundantly expressed MPs in regenerating shoots, like for instance malfunctioning of plasmodesmata (Prins et al., 1997b), might be circumvented. Besides, this mode of expression does more resemble the natural situation of a CPMV infection, in which MPs are expressed after a virus has entered a developed, non-meristematic cell. The time point of expression during cell or plant development could be of importance in MP tubule formation or increase the stability of the MPs. In addition, expression of the MP genes from an inducible promoter might prevent the induction of a post-transcriptional RNA turnover process. When the CPMV 58/48kDa MP gene was expressed in transgenic tobacco plants under the control of a tetracycline inducible 35S promoter (Gatz et al., 1991; 1992), transcription of transgene mRNAs was indeed induced when Tc was applied and the MP gene mRNAs accumulated to levels which easily allowed detection. However, in none of the plants accumulation of MPs was found.

Consequently, a third approach was explored for expressing the CPMV MPs in plants. This approach involved expression of the CPMV MP genes from the PVX-based expression vector (Chapman et al., 1992). The replicating recombinant PVX genomes generate, similar to CPMV, high numbers of MP-encoding RNA molecules in the cytoplasm of infected cells, which probably results in rapid production of large amounts of MPs. This timing of expression could stimulate the formation of the MP-containing tubular structures in which the stability of the MPs could be increased. The presence of other viral factors, either from CPMV or PVX, could act to stabilise the MPs, or the MP-encoding RNA molecules, or stimulate tubule formation. However, these viral products, seem not of great importance since stable MP expression and efficient assembly of tubular structures was also observed when the CPMV MPs were expressed in protoplasts from transient expression vectors (Wellink et al., 1993). When recombinant PVX genomes carrying either the 48kDa or the 58/48kDa MP gene were inoculated into *N.*

benthamiana protoplasts, accumulation of MPs was clearly observed. The proteins functioned in forming tubular structures, which extended from the surface of infected protoplasts into the medium. It has not been determined whether these tubular structures are able to support the cell to cell movement of CPMV virions. Interestingly, presence of the CPMV MPs did not restrain the PVX genomes from establishing a systemic infection in plants and therefore, this approach could be very valid for *in planta* studies on the functioning of the CPMV MPs.

Thus, in this study, we only succeeded in expressing in plants the CPMV MPs to detectable levels when we used the PVX-based expression vector and not when we generated transgenic tobacco plants in which MP expression was either controlled by a constitutive or by an inducible 35S promoter. In all experiments tobacco species were used, so the host plant seems not to be of large importance in determining the success of the approach. We could demonstrate (Sijen et al., 1996 and Figure 1) by northern blot analyses that in both types of transgenic plants the MP transgenes were actively transcribed. In general, for the plants with the inducible promoter the MP mRNAs were more easily detected, but we have not made a direct comparison of the MP gene steady state mRNA levels between the plants with the constitutive or the inducible 35S promoter. In none of these transgenic plants, the MP gene transcripts were translated to levels of MPs detectable in immunofluorescence assays on protoplasts. The reasons why these transgene mRNAs are not translated into functional MPs that can form tubular structures are unclear. Strikingly, a transient expression vector that contains the same expression cassette as the binary vector that was used to generate the transgenic plants with the constitutive 35S promoter, has successfully been used to express CPMV proteins in protoplasts (Wellink et al., 1993). Using this transient expression vector, the 48kDa MPs were produced in protoplasts and found in tubular structures. Thus, these studies proved that other viral proteins are not needed for the assembly of tubular stuctures. Therefore, the absence of other viral proteins in the transgenic plants does not appear to be the reason why accumulation is not detected. It rather seems that in the transgenic plants the MPs are expressed to amounts below the level of detection or that they are very unstable and rapidly degraded. Our results contrast with the findings with several other viruses for which accumulation of MPs could be detected in transgenic plants that were generated with a similar transformation approach and that carried expression cassettes with a similar promoter (Atkins et al., 1991; Wolf et al., 1991; Ding et al., 1992; Poirson et al., 1993; Vaquero et al., 1994; Cooper et al., 1996). For these viruses, possibly less of the MPs is needed to enable detection, or these MPs are more stable or the detection methods, mostly using specific antisera, are more efficient for these MPs.

In the PVX-based expression system, for which we showed that it is effective for expressing the CPMV MPs, the MP-encoding mRNAs are derived from replicating viral genomes. This can result in both a level and a timing of expression comparable to CPMV-based MP expression, and perhaps also to the expression occurring from transient expression vectors. The results give the impression that rapid, and abundant, expression is essential for the formation of MP-containing tubular structures on protoplasts. The exact levels of MP-encoding subgenomic RNA molecules that are generated from the recombinant PVX genomes, have not been determined and compared to the MP gene mRNA expression levels that occur from the constitutive and inducible 35S promoters in transgenic plants. Three major complications

would occur in the interpretation of such a comparison, firstly the fact that the PVX-produced subgenomic RNA molecules carry many additional PVX sequences that could affect the stability or the translation efficiency of these mRNAs, secondly the fact that in a PVX-infected plant not all cells carry PVX genomes and thirdly the fact that steady state RNA expression levels do not reveal whether expression occurred rapidly in a short time span or gradually during a longer period. Especially this latter aspect could be of major importance for stable CPMV MP expression.

The PVX derivatives expressing the CPMV MPs may prove very useful for studying the effects of the MPs on intact plant cells. Electron microscopical studies on plants infected with the PVX derivatives PVX48 or PVX58ΔNTR, may provide information on the localisation of the MPs in the cytoplasm, the process of tubule formation and the plasmodesmatal modifications. To determine whether the PVX-based expression of CPMV MPs in plants can result in biologically functional modification of the plasmodesmata, the PVX recombinants PVX48 and PVX58ΔNTR could be tested for their ability to complement CPMV mutants that are defective in cell to cell movement. A CPMV mutant in which the 48kDa gene has been replaced by the gene encoding GFP (Jan Verver, unpublished result) will be a useful tool in such studies. In this way, it could eventually be possible to determine whether the 48kDa MP is sufficient for establishing CPMV cell to cell movement or whether the presence of the 58kDa protein is also required.

A complication that accompanies the use of the PVX-based expression system for studies on the CPMV MPs, is formed by the presence of the MPs of PVX, because these also cause plasmodesmatal modifications (Angell et al., 1996). In addition, the PVX CPs have a role in the PVX cell to cell movement processes (Oparka et al., 1996). Therefore any effect in PVX48 or PVX58ΔNTR-infected plants, will not necessarily be specific to the CPMV MPs, but can result from the CPMV MPs and/or the PVX MPs and CPs. PVX and CPMV can simultaneously systemically infect *N. benthamiana* plants (result not shown) which could suggest that the two viruses can move from cell to cell, undisturbed by interactions possibly occurring between the two types of MPs. Thus, it could be possible to analyse the specific effects of the CPMV MPs in plants infected with PVX48 or PVX58ΔNTR using plants infected with wtPVX as a control. A disadvantage of the PVX-based expression system is that the length of heterologous sequences that can be expressed without inhibiting the replication of the recombinant PVX genomes is limited (D. Baulcombe, personal communication). In addition, insertion of long sequences into PVX genomes triggers deletion of the inserts by homologous recombination incited by the duplication of the coat protein promoter (Chapman et al., 1992). This recombination process can result in plants infected with a mixture of recombinant and wtPVX genomes of which the wtPVX genomes will replicate more efficiently. Nevertheless, the PVX-based expression system has strong advantages. The method is very fast, does not require the generation of transgenic plants with the accompanying problems of position effects and transgene-silencing and it can be applied on all plant species that support replication of PVX genomes. Consequently, the approach appears very useful for analysing the effects of mutations within the CPMV MP genes. To study then the subcellular localisation of the altered proteins, it is very convenient to fuse a marker protein, like GFP, to the MP (Santa Cruz et al., 1996; Heinlein et al., 1995; Oparka et al., 1996; Padgett et al., 1996).

The approach of employing the PVX-based expression system for *in planta* expression of the CPMV MPs was used in some initial studies on the effects of mutating the MP gene. The modified CPMV MP consisted of a translational fusion between a truncated CPMV MP, that lacked the C-terminal 29 aa, and GFP. In protoplasts it was found this replacement did not affect the ability of the MP to induce the assembly of tubular structures. However, strikingly, *in planta*, the cell to cell transport of the PVX genomes was found to be strongly inhibited. This effect is specific for this CPMV MP-GFP fusion protein, since both a recombinant PVX expressing free GFP (Baulcombe et al., 1995) and a PVX derivative expressing the full-length CPMV MP (described in this study), are able to systemically infect plants. The length of the inserted sequences which encode this fusion protein, appears not to be the cause of the inhibition of the cell to cell movement of the PVX genomes, because PVX derivatives carrying inserted sequences of similar length but encoding a different protein, produce a systemic infection efficiently (PVX.S.58, Sijen et al., 1996). Apparently, the modified and enlarged CPMV MP-GFP fusion protein interferes with induction of the specific plasmodesmatal modifications needed to enable cell to cell movement of PVX genomes. This could be due either to aberrant modifications of the plasmodesmata by the fusion proteins or to the occupation of cellular or plasmodesmatal factors that are common for the two viral MPs. Evidently, the cellular effects that occur when unmodified CPMV MPs are expressed from the PVX-vector are different because the expression of these proteins alone does not limit the cell to cell spread of the PVX genomes. Microscopical studies on the few infected cells could provide information on the cellular modifications induced by these fusion proteins and on the localisation of the factors used both by the CPMV and PVX MPs.

MATERIALS AND METHODS

Construction of plasmids

The binary vectors pBINM48 and pBINM58/48 that were used to obtain transgenic tobacco plants that are constitutively expressing the CPMV MP genes, were constructed as described in Sijen et al. (1995). pBINHygTX.58 was made by cloning a 2.1 kb XbaI-SmaI fragment of pMM58/48, that contains the sequence of the 58/48kDa MP gene and the sequence of the nos-terminator, into XbaI-SalI-digested vector pBINHygTX (kindly provided by C. Gatz), of which the SalI-overhang was blunted with Klenow. As a result of this cloning the 58/48kDa MP gene is under the control of the tetracycline inducible 'triple-Op'-promoter and followed by both a nos-terminator and a ocs-terminator. To obtain the constructs pPVX48, pPVX58ΔNTR and pPVX48ΔXGFP, the PVX-expression vector pPC2S was used, which is a derivative of pGC3 (Chapman et al., 1992), in which the GUS-gene has been replaced by restriction sites to allow convenient cloning. For construction of pPVX48 a 1.1 kb NspV-StuI fragment of pM58S (Kasteel et al., 1993) was cloned into ClaI-EcoRV-digested pPC2S. Construct pPVX58ΔNTR was obtained by exchanging a 1.5 kb AflII-SpeI fragment of pPVXS58 (Sijen et al., 1996) by a 1.1 kb AflII-SpeI fragment of pPVX48, as a result of which the 3' non-translated region (NTR) of 400 bp of the 58/48kDa MP gene in pPVXS58 is deleted. For the construction of pPVX48ΔXGFP a 1.7 kb NspV-SalI fragment of pTM48ΔXGFPΔCP was cloned into ClaI-SalI-digested pPC2SΔS. pTM48ΔXGFPΔCP contains the coding region of the 48 kDa MP gene, except for the nucleotides encoding the 29 C-terminal aa which are replaced by the coding region of GFP in the way that a translational fusion is obtained.

pPC2SΔS is a derivative of pPC2S which lacks a 40 bp SstI-SphI fragment (Jan Verver, unpublished results).

Plant transformation

Constructs pBINM48, pBINM85/48 and pBINHygTX.58 were transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating with also *E. coli* strain RK2013 and used for transformation of leaf explants and regeneration of transformed shoots as described in Sijen et al. (1995). In the various transformations either leaf explants of *Nicotiana benthamiana*, *Nicotiana tabacum* cv Samsun NN or *Nicotiana tabacum* cv Winsconsin line TET1.1 (Gatz et al., 1991) were used. For selection of transformed shoots either 150 mg/l kanamycin or 25 mg/l hygromycin was used.

Nucleic acid analyses and preparation of PVX transcripts

To identify primary transformants that contain transgene DNA sequences, DNA was isolated from regenerated shoots and analysed by PCR as described in Sijen et al. (1995). For identification of transgene mRNA expressing transformants, RNA-isolation and RT-PCR analyses were also performed as described in Sijen et al. (1995). Northern blot analysis was performed as described in Sijen et al. (1996).

PVX transcripts were prepared by in vitro transcription of (derivatives of) construct pPC2S as described in Sijen et al. (1996). Prior to protoplast transfections, transcripts were purified by extracting the transcription mixture once with phenol/chloroform and once with chloroform, which was followed by precipitation with an equal volumn of 4M LiCl for 1 h at 0°C. After centrifugation, the pellets were washed with 70% of ethanol and resuspended in water.

Protoplast preparation, transfection and immunofluorescence assays

Protoplasts were essentially prepared as described in Sijen et al. (1995). Leaves of *N. benthamiana* plants were incubated for 1 h on the enzyme solution while leaves of *N. tabacum* plants were incubated for 2 h on this solution. The protoplasts were transfected with approximately 10 µg of in vitro transcripts of PVX-derivatives as described in van Bokhoven et al. (1993b). After either 1 or 2 days of cultivation of the protoplasts, immunofluorescence assays were performed as described by Sijen et al. (1995).

Induction of the triple-Op-promoter by the application of tetracycline

Prior to northern blot analysis, tetracycline (Tc) was applied to leaves of plants transformed with pBINHygTX.58 by vacuum infiltrating the leaves for 5 min in water containing 2 mg/l Tc and subsequent culturing of the leaves on water-agar (1% of agar) containing 2 mg/l Tc for 2 days. Prior to immunofluorescence assays on cultivated protoplasts, tetracycline was applied by spraying water containing 5 mg/l Tc on detached leaves and incubating the leaves on water containing 10 mg/l Tc for 1 day. Protoplasts were prepared in the presence of 5 mg/l Tc and subsequently cultured in medium containing 5 mg/l Tc for 2 days.

Plant inoculations and ELISA analyses

To examine transgenic plants transformed with either pBINM48 or pBINM58/48 for pathogen derived resistance against CPMV, 20 individual plants of R1-generation lines were inoculated with CPMV-virions containing plant sap as described by Sijen et al. (1995).

To test whether the PVX derivatives expressing the CPMV MP genes were able to systemically infect tobacco plants, *N. benthamiana* or *N. clevelandii* were inoculated with PVX-transcripts as described in Sijen et al. (1996).

Western blot analyses

Leaf homogenates of transgenic plants were prepared in the extraction buffer described by Dorssers et al. (1982). The homogenates were divided into a pellet and a supernatant fraction after centrifugation for 15 min at 3000 rpm at 4°C. The protein fractions were analysed by Western blotting assays after SDS-PAGE according to Van Bokhoven et al. (1990).

Detection of green fluorescent protein

Protoplasts or detached leaves were viewed directly for fluoresence occurring from the jellyfish green fluorescent protein by the Nikon Optiphot microscope with epifluoresence-optic using either the UV-1A filterset (excitation filter 365/10 nm, dichromatic mirror 400 nm, barrier filter 400 nm) or the B-2A filterset (excitation filter 450-490 nm, dichromatic mirror 510 nm, barrier filter 520 nm).

CHAPTER 4

THE DEFECTS IN CELL TO CELL MOVEMENT OF COWPEA MOSAIC VIRUS MUTANT N123 ARE NOT SOLELY DUE TO MUTATIONS IN THE VIRAL MOVEMENT PROTEINS

Titia Sijen, Tony van Kampen, Jan Verver, Joan Wellink, Cees de Jager and Ab van Kammen

The nitrous acid-induced cowpea mosaic virus (CPMV) mutant N123 is defective in systemically infecting cowpea plants and induces much smaller and more necrotic lesions on 'Pinto' bean plants than wild-type CPMV (wtCPMV). In cowpea protoplasts the mutant appears to behave just like wtCPMV and replicates efficiently, forms virions and induces the assembly of movement protein (MP)-containing tubular structures. These observations suggest that the mutation(s) of N123 have affected the ability of the virus to spread efficiently through the plant. Previous studies have shown that the phenotype of N123 is the result of mutation(s) in RNA2, the RNA segment encoding the MPs and the two coat proteins (CP). Molecular analyses, presented in this study, revealed that RNA2 of N123 carries at least 14 nucleotide changes, five of which result in amino acid (aa) substitutions; two located in the MP (M1 and M2) and three in the small CP (M6, M7 and M8). Analyses showed that the second aa substitution in the MP (M2) is associated with the appearance of local lesions on 'Pinto' bean plants that are partly of a N123 like phenotype (small and necrotic) and partly of wt-phenotype (large with necrotic veins). A quintuple CPMV mutant, that carries mutations M1 and M2 in the MP gene and M6, M7 and M8 in the small CP gene, was found to cause mainly small necrotic lesions on 'Pinto' bean plants. When this mutant was inoculated onto cowpea plants, systemic infection did not occur or was strongly delayed. Although the symptoms induced by this mutant appeared slightly different from those brought about by N123, basically the quintuple mutant showed similar defects as N123 in virus spread on both 'Pinto' bean and cowpea plants. Also during further passages, this mutant was stable in producing N123-resembling symptoms. Interestingly, a triple CPMV mutant, in which only the three aa alterations in the CP (M6+M7+M8) are present, was found to cause on both cowpea and 'Pinto' bean plants the wtCPMV characteristic phenotype. These results suggest that the defects in virus translocation of N123 result from alterations in both the MP and the small CP, which implies that CPMV movement is a complex process requiring specific features of the MP as well as the small CP, other than those involved in tubule and virion formation.

INTRODUCTION

Nitrous acid is an effective agent to mutate viruses (Mundry and Gierer, 1958) as it deaminates bases in nucleic acids. Consequently, cytosine is converted to uracil and adenine to hypoxanthine. Because hypoxanthine acts like guanine during replication and base pairs with cytosine, adenines are eventually replaced by guanines. Guanine is changed into xanthine which during replication acts similar to guanine and consequently, no mutation is effectuated. Uracil and thymine do not contain free amino groups and are therefore not altered. For the plant virus cowpea mosaic virus (CPMV) various nitrous acid-induced mutants have been described (De Jager, 1978). One of these mutants is N123 (De Jager, 1976), and this mutant was analysed at the molecular level in this study.

CPMV is a bipartite virus and its genome consists of two positive stranded RNA molecules. All proteins encoded by RNA1 are involved in replication, whereas RNA2 encodes the two capsid proteins of 37kDa and 23kDa and the two overlapping 58kDa and 48kDa proteins. The 48kDa protein is a viral movement protein (MP) that induces the assembly of MP-containing tubular structures. In infected plants, these tubules appeared to occur in plasmodesmata like structures and in infected protoplasts, they were found to extend from the surface of the protoplast into the medium (Van Lent et al., 1990; 1991). The N-terminal 10kDa region specific for the 58kDa protein, is involved in directing the replication of RNA2 (Van Bokhoven et al., 1993a). Yet, it can not be excluded that, in addition, the 58kDa protein holds functions in viral cell to cell or long distance movement. Both the MPs and the CPs are needed for cell to cell spread of CPMV (Wellink and Van Kammen, 1989). Hence, CPMV is assumed to move to adjacent cells by channelling plasmodesmata with the MP-containing tubular structures through which, or with which, the virus moves its genome encapsidated in virus particles (reviewed by Gail McLean et al., 1993; Carrington et al., 1996).

In comparison to wild-type CPMV (wtCPMV), mutant N123 shows a markedly reduced infection. On 'Pinto' bean plants, the N123-induced lesions are much smaller in size and have a more necrotic appearence. On cowpea plants, the infection of the inoculated primary leaves is strongly reduced and systemic infection of the higher leaves is not established (De Jager, 1976). On the other hand, on cowpea protoplasts, N123 behaves just like wtCPMV and replicates efficiently, forms virions and induces the assembly of MP-containing tubular structures (Taliansky et al., 1993). These observations suggest that N123 is defective in virus translocation and not able to move efficiently from cell to cell in plants. Supplementation and reassortment tests have shown that the mutation(s) that result in the characteristic N123 phenotype, are located on RNA2 (De Jager, 1976). This supports the hypothesis that the phenotype of N123 is due to defects in virus spread as all viral proteins required for cell to cell movement are encoded by RNA2. Systemic infection of cowpea plants by N123 has been achieved by co-infection with the unrelated tobamovirus sunn hemp tobamomosaic virus (SHMV) (Taliansky et al., 1993). This is a remarkable observation since tobamoviruses employ a strategy of cell to cell movement differing from that of CPMV. Tobamoviruses do not require the CP and are assumed to move the viral genomes as an elongated, non-virion, RNA-protein-complex. To enable passage of this complex through plasmodesmata, the tobamovirus' MPs act to

increase the size exclusion limit of the plasmodesmata (for reviews see Gail McLean et al., 1993; Carrington et al., 1996). Cell to cell movement of a CPMV mutant lacking the MP gene is complemented by SHMV to only a very limited extent (Taliansky et al., 1993), which indicates that translocation of non-encapsidated CPMV genomes in a tobamovirus characteristic mode is limited. Hence, for achieving systemic infection of N123 in cowpea by complementation, SHMV presumably provides a specific helper function that effects translocation of N123 in the CPMV characteristic mode. Such helper function might involve the mobilisation of a host factor required for CPMV characteristic cell to cell transport, or the suppression of a host factor involved in a defensive host response (Taliansky et al., 1993).

The phenotype of N123 and especially the complementation by SHMV suggested that detailed molecular analyses of mutant N123 could provide understanding of various aspects of CPMV cell to cell movement. We have determined in which nucleotides the sequence of RNA2 of N123 differs from that of wtCPMV. Fourteen nucleotide changes were identified, five of which result in amino acid (aa) substitutions of which two were located in the 58/48kDa MPs and three in the smaller 23 kDa CP. We analysed the effects of the aa substitutions by introducing the corresponding mutations, separate as well as together, into the genome of wtCPMV. The mutants were inoculated onto cowpea and 'Pinto' bean plants and the infection process and symptoms were studied. Strikingly, reduced infection that resembled strongly an infection by N123, only occurred when mutations had been introduced in both the MP and the small CP. This result indicates that CPMV cell to cell movement is a complex process requiring specific features in the MP as well as the small CP, that have other roles than tubule or virion formation, respectively.

RESULTS

Determination of the nucleotide sequence of RNA2 of N123

Mutant N123 infects both cowpea and 'Pinto' bean plants to very low levels (De Jager, 1976) which makes it very difficult to isolate virus particles from infected leaves. For that reason, total RNA was isolated from leaf disks carrying typical, small and necrotic, N123 lesions on 'Pinto' bean plants and cDNA of RNA2 was prepared using primer M3450-, that is complementary to the 3' non-translated region (NTR) of RNA2 of CPMV. Using different combinations of several RNA2 specific primers, which are listed in materials and methods, various partly overlapping fragments were amplified by PCR. The fragments were purified and submitted to Taq-polymerase based sequence analyses (cycle-sequencing) using RNA2 specific primers. RNA2 consists of 3482 nucleotides (nt) of which nt 1 to 160 form the 5'NTR, nt 161 to 3298 the coding region and nt 3299 to 3482 the 3'NTR (Van Wezenbeek et al., 1983). Four viral proteins are encoded by RNA2, the 58kDa protein is encoded by nt 161 to 1537, the 48kDa MP by nt 512 to 1537, the 37kDa CP by nt 1538 to 2659 and the 23kDa CP by nt 2660 to 3298. For N123, the sequence of RNA2 between nucleotide positions 89 and 3398 was determined which includes the complete coding region. In this region, fourteen nucleotide

changes were observed (Table 1) compared with RNA2 of wtCPMV (Van Wezenbeek et al., 1983). Four of the nucleotide changes involved a change from cytosine to uracil or from adenine to guanine (Table 1) and might therefore be attributed to the mutagenic treatment with nitrous acid which generated N123. Five of the nucleotide changes will result in aa substitutions in the encoded proteins. Two of these alterations (M1 and M2; Table 1) occurred in the 58/48kDa MP gene and the other three changes (M6, M7 and M8; Table 1) in the 23kDa CP gene. The mutations M1 and M2 could have been caused by treatment with nitrous acid, but not the mutations M6, M7 and M8 (Table 1).

Table 1. Sequence analysis of RNA2 of CPMV mutant N123

regions of RNA2[ab]	positions of N123 mutations (nt)	nucleotide[b] substitution	nitrous acid[c] mutation	amino acid[d] substitution	name mutation
5'NTR	-	-	-	-	-
MP genes (MP58)	-	-	-	-	-
" (MP48)	651	C → U	yes	ser → phe	M1
" "	744	C → U	yes	thr → met	M2
" "	805	A → G	yes	silent	-
" "	1012	C → G	no	silent	-
" "	1153	U → C	no	silent	-
CP genes (VP37)	1921	U → C	no	silent	-
" "	1936	G → A	no	silent	-
" "	1951	U → A	no	silent	-
" "	2513	C → U	yes	silent	-
" "	2614	G → A	no	silent	-
" (VP23)	2702	A → U	no	met → leu	M8
" "	2738	G → A	no	val → leu	M6
" "	3104	G → A	no	val → leu	M7
3'NTR	3334	U → A	no	no	-

[a] The full-length RNA2 of CPMV comprises 3482 nucleotides (nt). The region of RNA2 of N123 that was sequenced covers the nucleotides between the positions 89 and 3398.

[b] The 5'NTR (non-translated region) spans from nt 1 to nt 160, the 58/48kDa MP (movement protein) gene from nt 161 to nt 1537, the 48kDa MP gene from nt 512 to nt 1537, the 37kDa CP (coat protein) gene (VP37) from nt 1538 to nt 2659, the 23kDa CP gene (VP23) from nt 2660 to nt 3298 and the 3'NTR from nt 3299 to nt 3482.

[c] Treatment with nitrous acid can result in two kinds of nucleotide substitutions; either a change from cytosine to uracil or a change from adenine to guanine.

[d] In case the nucleotide alteration does not result in an amino acid substitution the alteration is denoted silent. Ser is an abbreviation of serine, phe of phenylalanine, thr of threonine, met of methionine, leu of leucine and val of valine.

Analysis of the effects of specific nucleotide substitutions related to N123

N123 appears to be defective in virus translocation, a process for which the MPs and CPs are required. Thus, it seems plausible that the phenotype of N123 is caused by aa changes in one of these viral proteins rather than by nucleotide substitutions in the viral RNA. Therefore, we focussed our analyses on the mutations that will result in aa substitutions in the MPs or CPs (M1, M2, M6, M7 and M8; Table 1). The first experiments concentrated on the mutations M1 and M2 located in the MP gene because these two nucleotide substitutions could have been brought about by mutagenesis by nitrous acid (Table 1). By site-directed mutagenesis (Kunkel, 1985) M1 and M2 were introduced in the full length wtRNA2-cDNA clone, either separately or together. The presence of each of the mutations was confirmed by sequence analyses. Mutation M1 could also be detected by restriction analysis as this nucleotide change introduces an additional AhaIII-site in cDNA corresponding to RNA2. Cowpea protoplasts were then infected with a mixture of in vitro transcripts from cDNAs of wildtype RNA1 and either wildtype RNA2 or RNA2 containing mutations M1, M2 or both M1 and M2. The different RNA-inocula were equally infectious and established in approximately 5% of the protoplasts replication viral of RNAs, production of CPs and formation of MP-containing tubular structures as revealed by immunofluorescence assays using various antisera. Extracts made from the infected protoplasts were inoculated onto cowpea plants and eight days later systemic mosaic symptoms developed on the plants inoculated with wtCPMV as well as on the plants inoculated with all three N123-related mutants *M1*, *M2*, *M12* that carry M1, M2 or M1+M2, respectively (Table 2). When the protoplast extracts were inoculated onto 'Pinto' bean plants, *M1* induced local lesions of wt-phenotype while both *M2* and *M12* induced two types of local lesions namely small necrotic lesions resembling N123 characteristic lesions and large lesions with necrotic veins that were similar to wtCPMV-induced lesions (Figure 1C) (Table 2). At eight days post inoculation (dpi), sap inoculum was prepared from both the primary and the secondary leaves of the systemically *M12*-infected cowpea plants and also from both small and large lesions induced by *M12* on 'Pinto' bean plants. With all four inocula, cowpea plants became systemically infected within seven dpi and on 'Pinto' bean plants mainly large and only few small local lesions developed (Table 2). In addition, sap inoculum was prepared from secondary leaves of cowpea plants systemically infected with *M1* or *M2* and used to pass the *M1* and *M2* mutants on to cowpea and 'Pinto' bean plants. Both inocula induced systemic infections in cowpea but on 'Pinto' bean plants *M1*-containing inoculum induced only large local lesions while *M2*-containing inoculum induced, besides many large local lesions, also few small local lesions (Table 2).

The observation that *M2* and *M12* produce on 'Pinto' bean plants a mixture of large, wtCPMV like local lesions and small, N123-resembling lesions raised the question whether the virus inocula used in the experiments were contaminated with wtCPMV. Therefore, we tested the purity of virus in the secondary leaves of systemically infected cowpea plants that had been inoculated with either wtCPMV, *M1* or *M12* by analysing the virus in these leaves for presence of mutation M1. Total RNA was isolated from these leaves and by means of RT-PCR, the sequence corresponding to the MP gene was amplified. The amplified fragments were analysed for the presence of an additional AhaIII restriction site which corresponds to the presence of mutation

Table 2. Characteristics of infections of cowpea or 'Pinto' bean plants with mutants related to N123

Mutants and controls	First passage[ab] from cowpea protoplasts		Second passage[ab] from 'Pinto' beans		Second passage[ab] from primary cowpea		Second passage[ab] from secondary cowpea	
	on 'Pinto'	on cowpea	on 'Pinto'	on cowpea	on 'Pinto'	on cowpea	on 'Pinto'	on cowpea
wtCPMV	large[c]	systemic[d]	large	systemic	large	systemic	large	systemic
N123	small[e]	not systemic	small	not systemic	small+ few large	not systemic[f]	no symptoms	no symptoms
M1	large	systemic	nd[g]	nd	nd	nd	large	systemic
M2	large+small (60%+40%)	systemic	nd	nd	nd	nd	mainly large +few small	systemic
M12	large+small (70%+30%)	systemic	mainly large +few small[h]	systemic	mainly large +few small	systemic	mainly large +few small	systemic
M678	large[i]	systemic	large	systemic	large	systemic	large	systemic
M12678	small[j]	not systemic[k]	small	not systemic	small	not systemic[k]	one single large lesion	no symptoms
Water	no symptoms	no symptoms	no symptoms	no symptoms	no symptoms	no symptoms	no symptoms	no symptoms

[a] The inoculated plants were studied during 6 to 12 dpi.
[b] In the first passage, an extract of infected cowpea protoplasts was inoculated on one 'Pinto' bean and one cowpea plant. In the second passage, sap inocula that were prepared from these plants at 8 dpi, were inoculated on to three 'Pinto' bean and three cowpea plants.
[c] Large refers to large local lesions with a diameter of 1-3 mm and with necrotic veins.
[d] A systemic infection of the higher leaves is accompanied by large chlorotic lesions on the inoculated leaves.
[e] Small refers to small local lesions with a diameter of 0.3-1 mm that are fully necrotic.
[f] On the inoculated leaves, small chlorotic lesions occurred. Occasionally the infection does spread systemically to the higher leaves at many dpi (*eg* 3 weeks), to which has been referred as reverting of the mutant (De Jager, 1976).
[g] nd refers to not determined.
[h] No difference was observed for inocula that were either derived from small or from large local lesions.
[i] The large local lesions appeared to contain less vein necrosis than the wtCPMV-induced local lesions.
[j] The local lesions appeared slightly smaller and more necrotic than N123-induced local lesions.
[k] On the inoculated leaves, medium-sized chlorotic lesions occurred. In several plants the infection spread to the systemic leaves at around 11 dpi.

M1. For wtCPMV inoculated plants, the amplified fragment could not be digested with AhaIII, while for both the *M1* and the *M12* inoculated plants the amplified fragments were fully digested (results not shown). This indicates that the systemic mosaic symptoms resulted fully from mutant CPMV and that no contamination with wtCPMV had occurred in the *M1* and *M12*-inoculated plants. For mutation M2, such a test was not available, but presumably the *M2*-inoculated plants were neither contaminated with wtCPMV. Therefore, the appearance of two types of local lesions, N123 like lesions and wtCPMV like lesions, on 'Pinto' bean plants inoculated with *M2* or *M12*, seems to be charactersitic of the presence of mutation M2. Apparently, *M2* can cause on 'Pinto' bean leaves reduced infection that results in small, N123-resembling local lesions, but not at all sites of infection.

Next, the mutations M6, M7 and M8 that are present in the small CP gene and that represent the remaining aa substitutions of N123, were analysed for their involvement in causing the N123 specific phenotype. Since neither of these mutations can be attributed to mutagenesis with nitrous acid, they were introduced all three together in both the RNA2-cDNA clones of wtCPMV and mutant *M12* (Kunkel, 1985). By sequence analyses the presence of the three mutations was confirmed. Cowpea protoplasts were then inoculated with a mixture of in vitro transcripts from the cDNAs of wtRNA1 and of RNA2 that contained either all five mutations M1, M2, M6, M7 and M8 or the three mutations M6, M7 and M8. Both the quintuple mutant (*M12678*) and the triple mutant (*M678*) replicated in approximately 5% of the infected protoplasts as was determined in immunofluorescence assays. Extracts were made from the protoplasts and inoculated onto both cowpea and 'Pinto' bean plants. At eigth dpi, *M678* induced systemic symptoms on cowpea plants and only large lesions with partial vein necrosis, which is slightly different from the vein necrosis in wtCPMV lesions, on 'Pinto' bean plants (Figure 1E) (Table 2). On the other hand, *M12678* did not establish a systemic infection of cowpea plants and induced only very small, fully necrotic lesions on the 'Pinto' bean plants (Figure 1D) (Table 2). At eight dpi, sap inoculum was prepared from both the (inoculated) primary and the secondary leaves of the cowpea plants and from local lesions induced on the 'Pinto' bean plants, and used to inoculate both cowpea and 'Pinto' bean plants. All inocula derived from *M678*-infected plants resulted in a systemic mosaic on the cowpea plants and large lesions with partial vein necrosis on the 'Pinto' bean plants (Table 2). Apparently, *M678* behaves like wtCPMV and the mutations M6, M7 and M8 do not affect the ability to systemically infect cowpea or to alter strongly the lesion type induced on 'Pinto' bean leaves. For the quintuple mutant *M12678*, the inocula derived from primary cowpea leaves and the 'Pinto' bean lesions induced only very small necrotic lesions on 'Pinto' bean plants and no systemic infection in cowpea plants. (Table 2). The inoculum prepared from the non-inoculated secondary cowpea leaves did not give rise to symptoms (Table 2), indicating that *M12678*, in contrast to *M678*, had not systemically infected cowpea plants at eigth dpi.

Thus, *M12678* showed a markedly reduced infection on both cowpea and 'Pinto' bean plants. The local lesions brought about by *M12678* on 'Pinto' bean leaves, appeared to be somewhat smaller than N123-induced lesions (Figure 1B and D). Also in cowpea plants, an infection by *M12678* might be somewhat different from an infection by N123 because *M12678* causes, in contrast to N123, but similar to wtCPMV, chlorotic spots on the inoculated leaves. In

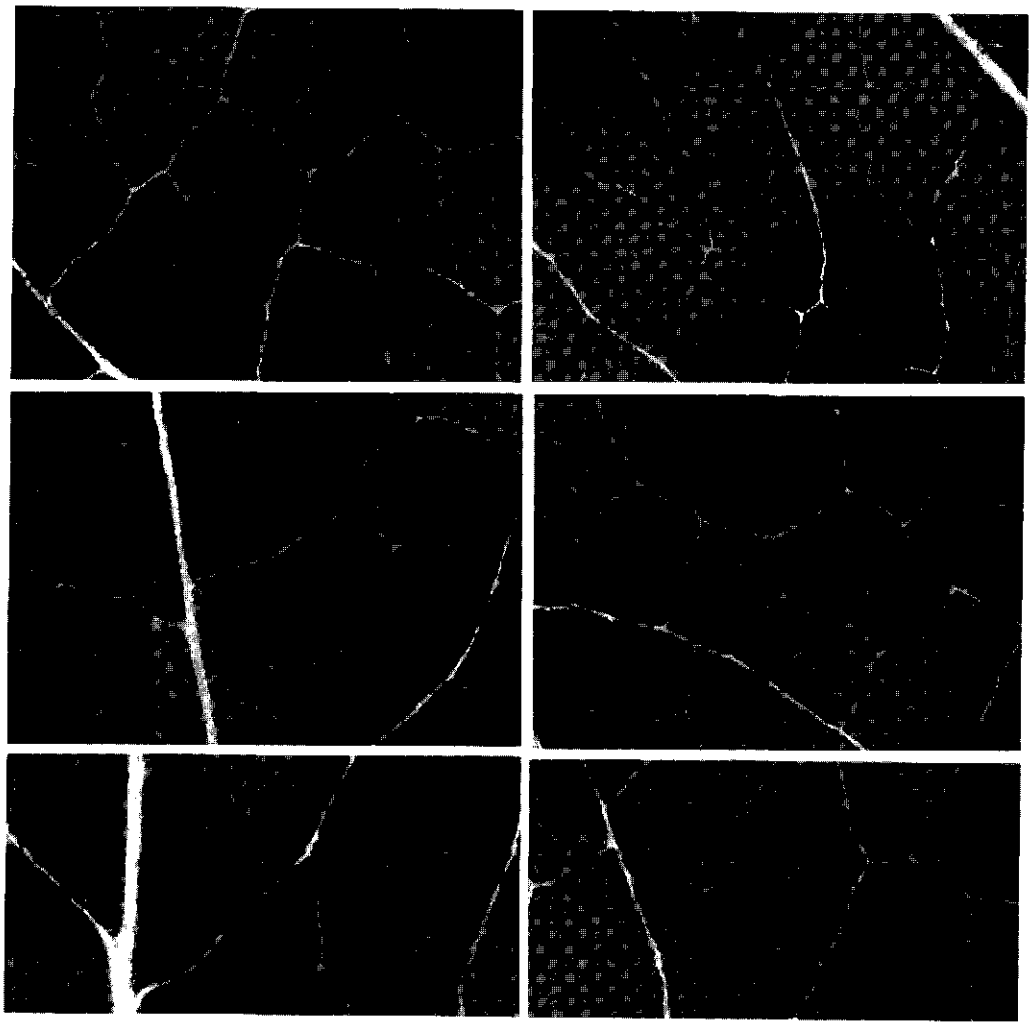

Figure 1. Local lesions induced on 'Pinto' bean plants by various N123-related CPMV mutants.

Various CPMV mutants carrying N123-related mutations, were inoculated on to primary leaves of 'Pinto' bean plants and the lesions induced by these mutants were photographed at 8 dpi. Panel A shows typical large local lesions with necrotic veins induced by wtCPMV. Panel B shows characteristic small, fully necrotic local lesions induced by mutant N123. Panel C shows lesions induced by mutant M_{12}, which partly resemble the N123 phenotype and partly the wtCPMV phenotype. Panel D shows lesions induced by mutant M_{12678} which resemble lesions induced by N123, although they appear to be somewhat smaller. Panel E shows lesions induced by mutant M_{678} which resemble wtCPMV-induced lesions, although the vein necrosis appears to be less profound. In panel F water was used as inoculum and no lesions occurred.

addition, after 11 dpi, several *M12678*-inoculated cowpea plants started to develop systemic symptoms. For N123, only occasionally such delayed systemic infections occur and they mostly appear at longer periods after inoculation. Thus, although mutant *M12678* seems to cause slightly different symptoms than N123, it basically resembles N123 as it results on both cowpea and 'Pinto' bean plants in a markedly reduced infection. Remarkably, this reduction of infection was only observed for this mutant, that carried both aa substitutions in the MPs and in the small CP, and not for the mutant that contained either the aa substitutions in the MPs or the substitutions in the CP.

DISCUSSION

By treatment with nitrous acid a CPMV mutant, N123, was generated that shows a markedly reduced infection on both cowpea and 'Pinto' bean plants (De Jager, 1976). In protoplasts on the other hand, N123 behaves just like wtCPMV which suggested that the mutant carries defects in virus translocation (Taliansky et al., 1993). In previous studies, the mutation(s) responsible for the N123 specific phenotype were shown to be localised on RNA2 (De Jager, 1976), the RNA segment encoding the viral MPs and CPs. In this study, we analysed RNA2 of N123 at the molecular level and identified fourteen nucleotide changes of which five will result in aa substitutions. Two of these aa changes are located in the 58/48kDa MP and three in the 23kDa CP. The sequence analyses of RNA2 of N123 were performed by cycle-sequencing of fragments which had been amplified by RT-PCR. By this approach, occasional mistakes generated during PCR amplification are averaged out and a reliable sequence is obtained. In addition, the fragments were sequenced in both directions and, moreover, the fragments were partially overlapping.

The three mutants, *M1*, that contains a serine to phenylalanine substitution in the 58/48kDa MPs, *M2*, that carries a threonine to methionine substitution in the MPs, and *M12*, that carries both these aa substitutions, were all found to efficiently induce a systemic infection of cowpea. Interestingly, when these three mutants were inoculated on to 'Pinto' bean plants, the mutants *M2* and *M12* produced a phenotype that differed from that of wtCPMV, while mutant *M1* induced wtCPMV characteristic symptoms. Both M2-containing mutants produced a mixture of large, wtCPMV like local lesions and some small, N123 like local lesions. When the virus present in *M2*- or *M12*-induced lesions was passed on to 'Pinto' bean plants, both mutants gave mainly large lesions and a few small local lesions. This mixture of lesions was obtained irrespective of whether the inoculum had been prepared from small or large local lesions. Systemically infected cowpea plants that had been inoculated with *M12* or *M1*, were analysed by AhaIII-digestion of fragments amplified by RT-PCR for the presence of mutation M1. These analyses showed that the inocula had not been contaminated with wtCPMV and that mutation M1 was well maintained during the first eight days of the infection. Although we can not rule out that, extremely rapid after inoculation, mutation M2 was reverted or that second site reversions occurred, we believe that the mixture of two types of lesions induced by the *M2* and *M12*-inocula is genuinely characteristic for the presence of mutation M2. At some sites of

infection, mutation M2 results in a reduced infection that gives, on 'Pinto' bean leaves, rise to small, N123-resembling local lesions while at other sites the infection resembles wtCPMV and large local lesions are induced. A more detailed analysis using for instance various inoculum doses, might reveal under which conditions these mutants induce small lesions.

Clearly, the two aa alterations in the MPs are not the decisive determinants for the limitation in systemic spreading that is apparent with N123. Neither was the combinantion of the two mutations sufficient to explain the N123 specific phenotype. For that reason, it was examined whether the aa changes identified for the small CP of N123 had a role in establishing the N123 characteristic phenotype. Two of these aa substitutions involved a change from valine to leucine while the other mutation was a change from methionine into leucine. The corresponding mutations were introduced into RNA2 of either wtCPMV or mutant *M12*, generating mutants *M678* and *M12678*, respectively. Mutant *M678*, was found to be able to systemically infect cowpea plants and induced large local lesions on 'Pinto' bean leaves that hardly differed in appearance from wtCPMV-induced lesions. However, the quintuple mutant *M12678*, in which the aa substitutions in the MPs and the small CP are combined, was found to induce a markedly reduced infection on both cowpea and 'Pinto' bean plants. On cowpea plants, *M12678* gave no, or a strongly delayed, systemic infection and on 'Pinto' bean plants, *M12678* induced necrotic local lesions that appeared a little smaller than the lesions induced by N123 (Figure 1B and D). These symptoms were stably maintained during further passage of mutant *M12678.* Thus, although the symptoms induced by this mutant appear slightly different from those brought about by N123, basically mutant *M12678* carries defects in virus spread that are similar to the defects of N123. The difference in the symptoms induced by *M12678* and N123 could be due to the presence of at least 9 additional nucleotide substitutions in RNA2 of N123 that did not result in an aa substitution. These nucleotide changes could affect the stability or the efficiency of replication and translation of RNA2 and in that way influence symptom development. Alternatively, mutations in RNA1 of N123 might have a role in regulating the infection process and the symptoms. It remains to be tested whether, as for N123, the defects in virus translocation of mutant *M12678* can be complemented by co-inoculation of cowpea plants with SHMV (Taliansky et al., 1993).

The mutations M1 and M2 are, in contrast to the mutations M6, M7 and M8, consistent with changes that can occur when nucleic acids are treated with nitrous acid. N123 has been obtained from a single small necrotic lesion on a 'Pinto' bean leaf directly after treatment of unfractionated virus particles with nitrous acid to a 50% survival rate (De Jager, 1978). Presumably, the mutations M6, M7 and M8 existed prior to the mutagenic treatment that added the mutations M1 and M2 and resulted in N123. Further molecular analysis of the individual mutations M6, M7 and M8 in the CP gene together with particularly mutation M2 of the MP gene is required to provide further information on the exact molecular basis of the N123 specific phenotype. De Jager (1976) has described that N123 can regain spontaneously the ability to systemically infect cowpea plants and we have confirmed the appearence of such reversions during N123-infections in these studies, especially when using high inoculum doses. The rapid appearance of large lesions and systemic cowpea infections with the N123-related mutants *M2*, *M12* and to some extent also *M12678*, might indicate that reversion of N123 is rather due to

specific conditions at the site of infection than to alteration in the N123 genome. Interestingly, analyses of the nucleotide sequence of RNA2 of CPMV genomes that were present in higher cowpea leaves that became systemically infected several weeks after inoculation with N123, so putatively genomes of a N123 revertant, did not reveal any other nucleotide change than the 14 alterations observed for N123 (results not shown). This might substantiate the hypothesis that a nucleotide substitution is not required to obtain a systemic infection of cowpea with N123-related mutants or N123 itself but that rather specific, but unknown, conditions are deciding. Alternatively, it could be that a nucleotide substitution did occur in a N123 genome and that this true revertant enables both translocation of the reverted genomes and the original N123 genomes. If such a complementation and therefore mixed infection is occurring, sequence analyses on cDNA generated on total RNA extracted from systemically infected leaves will not reveal the mutations.

None of the N123-related aa substitutions resulted in a major alteration of the charge of the proteins and only mutation M7 resulted in a change of an aa that is conserved for various comoviruses (Shindo et al., 1993). Accordingly, the five aa substitutions did not abolish infectivity and did not affect the processes of tubule or virion formation in itself. Rather, other, more subtle, viral functions that are important for virus translocation are influenced by these mutations. The mutated aa in the small CP are all three situated at the capsid exterior (Chen et al., 1990; Le Gall et al., 1995) which indicates that these aa could be involved in interactions with either viral or host factors. Possibly, the interactions between MP-containing tubules, plasmodesmata and virus particles that are needed for efficient translocation of CPMV virions to adjacent cells, are altered by the presence of the mutations. Electron microscopical studies on N123-infected protoplasts or on cells at and around typical N123 lesions on 'Pinto' bean plants might reveal information on this aspect. Alternatively, both the MPs and the small CPs function in mobilising a host-factor needed for CPMV cell to cell movement or act to suppress a plant factor that is involved in a host defense response (Taliansky et al., 1993). The complementation of systemic spread of N123 in cowpea by the unrelated tobamovirus SHMV, suggests that rather an interaction with a specific host factor is affected than the interaction among CPMV proteins. However, this complementation has not yet been tested with *M12678*. A role for host factors in virus movement is also suggested from the observation that complementation of systemic spread occurs for several unrelated viruses (Atabekov and Taliansky, 1990).

Changes in virus movement as a result of a few aa substitutions in viral proteins has been noted before. Drastic effects have been reported for aa changes in viral MPs of brome mosaic virus (De Jong et al., 1995), turnip yellow mosaic virus (Tsai and Dreher, 1993) or squash leaf curl gemini virus (Ingham and Lazarowitz, 1993) and in the viral CPs of turnip crinckle virus (Laakso and Heaton, 1993) or brome mosaic virus (Flasinski et al., 1995). Some reports show that the aa changes affect viral movement in only some hosts, implying that an interaction with specific host factors has an important role in viral movement. Ingham and Lazarowitz (1993) showed that a single aa substitution in the MP affects the infectivity of squash leaf curl virus on *N. benthamiana* but not on pumpkin or squash. Experiments by Flasinski et al. (1995) showed that various regions in the brome mosaic virus CP differentially affect virus spread in barley and *Chenopodium hybridum*. Interestingly, De Jong et al. (1995)

showed that four individual aa substitutions in the MP of brome mosaic virus all influenced the adaptation of the virus to cowpea, whereas barley was a systemic host irrespective of any of these four aa substitutions, which also indicates that aa substitutions can have multiple interactive effects. Inoculation of N123 on a broad range of different host plants, like *Nicotiana benthamiana* or *Arabidopsis thaliana*, could reveal whether the identified mutations should be regarded as host determinants (Dawson and Hilf, 1992) which could indicate how conserved a putative host factor would be among various plant species.

MATERIALS AND METHODS

RNA isolation, RT-PCR, sequencing and primers

Total RNA was isolated as described in Sijen et al. (1996) from leaf disks of 'Pinto' bean plants with N123 characteristic local lesions. RT-PCR analyses were performed as described in Sijen et al. (1995). Amplified PCR-fragments were purified to remove salts, free nucleotides and oligo nucleotides using Qulagen quickspin colums (Westburg) as described by the manufacturer.

Sequencing was performed using Taq-polymerase (cycle-sequencing kit, Pharmacia) and sequence analyses were done on poly acryl amide gels using the ABI-automatic sequence apparatus. On average the sequence of 600 nt, starting 50 nt from the 3' end of the primer was obtained.

All primers used for PCR and sequencing had a length of 21 to 23 nt and were either fully homologous to the sequence of RNA2 or contained few mismatches (indicated "). Forward primers (indicated +) are specific to the viral plus-strand while reverse primers (indicated -) are specific to the viral minus-strand. The numbers indicate the nucleotide position in RNA2 (indicated as M in the primer name) that corresponds to the 5' end of the primer. The primers used are: M44+, M197+, M512+ (= D1*), M1648+, M1985+, M2559+, M3040+, M504-, M884"- (= T3-Ti), M1537- (= D2*), M1787-, M2680"-, M3322- and M3450-. The primers M1537- and M3450- were used to generate cDNA used in RT-PCRanalyses.

Introduction of mutations in RNA2

Bacteriophage M13-derivatives containing cDNA corresponding to the RNA2 plus-strand from either nt 1 to nt 1540 (M13M1+) or nt 1540 to nt 3482 (M13M3+) were used for site-directed mutagenesis as described by Kunkel et al. (1985). All primers used for mutagenesis had a length of 22 nt or 23 nt, contained one mismatch (") in the primer at the site of the desired mutation (Table 1) and were specific to the RNA2 minus-strand (reverse orientation, -). The primers are denoted N123M1 (= M660"-), N123M2 (= M754"-), N123M8 (= M2714"-), N123M6 (= M2747"-) and N123M7 (= M3108"-). After mutagenesis M13M1+ derivatives containing either the N123-related mutations M1 or M2 or M1+M2 and M13M3+ derivatives containing either the N123-related mutations M6 or M7 or M8 were obtained. DNA of the replicative form was isolated from these M13-derivatives and digested with the appropriate restriction enzymes to exchange similar fragments in plasmid pTM2074 that contains the cDNA clone corresponding to RNA2 of wtCPMV (Lekkerkerker et al., 1996) for the fragments that carried the N123-related mutations. The mutations M1, M2 or M1+M2 were exchanged as SalI-BamHI fragments to result in pTM2074M1, pTM2074M2 and pTM2074M12. From pTM2074M12 and pTM2074 a BamHI-ClaI fragment was replaced by a BamHI-NheI fragment carrying mutation M8, a NheI-NcoI fragment carrying mutation M6 and a NcoI-ClaI fragment carrying mutation M7 to result pTM2074M12678 and pTM2074M678, respectively. Because in plasmid pTM2074 and its derivatives the cDNA of RNA2 is present downstream of a T7 RNA polymerase promoter, *in vitro* transcripts can be produced as described in Vos et al. (1988).

Protoplast infections and plant inoculations.

Cowpea protoplasts were prepared and transfected with a mixture of 10 µg in vitro synthesised wtCPMV RNA1 and 10 µg of in vitro transcripts of RNA2 of the various N123-related mutants, as described by Van Bokhoven et al. (1993b). Replication and subsequent expression of viral proteins was examined by immunofluorescence assays using antisera specific for either the viral MPs, the CPs or the RNA1 encoded replicase proteins, essentially as described in Maule et al. (1980). Protoplasts were collected upon centrifugation at 3000 rpm (eppendorf centrifuge) and for plant inoculations extracts were prepared by adding 50 µl 10 mM sodium phsphate buffer (pH 7.0) and vortexing for 1 min. Primary leaves of 7 to 10 days old cowpea or 'Pinto' bean plants were inoculated as described by De Jager (1976). At 8 dpi, sap-inoculum was produced from cowpea leaves or 'Pinto' bean leaf disks containing local lesions as described by De Jager (1976). Plants were grown in the greenhouse at approximately 25°C and analysed for the appearance of symptoms between 6 and 12 dpi.

CHAPTER 5

REPLICATION OF COWPEA MOSAIC VIRUS RNA1 OR RNA2 IS SPECIFICALLY BLOCKED IN TRANSGENIC *NICOTIANA BENTHAMIANA* PLANTS EXPRESSING THE FULL-LENGTH REPLICASE OR MOVEMENT PROTEIN GENES

Titia Sijen, Joan Wellink, Jeanine Hendriks, Jan Verver and Ab van Kammen

Nicotiana benthamiana plants were transformed with either the cowpea mosaic virus (CPMV) RNA2-derived movement protein gene or the RNA1-originating replicase gene. For both types of genes, half of the R2-generation lines showed complete resistance when challenged with CPMV. Experiments using protoplasts revealed that the resistance operates at the single cell level by specifically preventing replication of the RNA segment from which the transgene was derived. In both cases, the resistance acts against wild-type strain CPMV-Sb and the very homologous CPMV-S1 and CPMV-S8 strains, but not to other comovirus species including cowpea severe mosaic virus (CPSMV). These data and the inability to detect transgene-encoded proteins, suggest an RNA-mediated nature of the resistance.

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INTRODUCTION

Cowpea mosaic virus (CPMV) possesses a genome consisting of two messenger-sense, single-stranded RNA molecules, which are separately encapsidated. Upon translation of both segments, referred to in this paper as RNA1 and RNA2 polyproteins are produced from which functional proteins are generated by a defined pathway of proteolytic cleavages. All proteins necessary for viral replication originate from the RNA1-encoded 200kDa polyprotein. The proteins involved in virus movement and encapsidation are encoded by the smaller RNA2. This RNA segment is translated into two carboxy-coterminal polyproteins as a result of additional initiation at a second in-frame AUG-codon. From these two polyproteins beside the two capsid proteins overlapping 48kDa and 58kDa proteins are derived (Vos et al., 1984, Eggen and Van Kammen, 1988, Holness et al., 1989). Various CPMV mutants provided genetic evidence that both the capsid proteins and the 48kDa protein are needed for cell to cell movement of the virus, while these proteins are dispensable for virus replication (Wellink and Van Kammen, 1989, Kasteel et al., 1993). Electron microscopical studies revealed that for cell to cell movement CPMV generates tubular structures that are presumably penetrating plasmodesmata and contain virus particles (Van Lent et al., 1990). As similar tubular structures were found on protoplasts that transiently express the RNA2-encoded 48kDa protein (Wellink et al., 1993), this protein was identified as a movement protein (MP). Though the unique N-terminal domain within the 58kDa protein is involved in replication of RNA2 (Van Bokhoven et al., 1993a), it has not been excluded that this 58kDa protein has an additional function in movement in the plant tissue, for instance by interacting with the plasmodesmata or by mediating long distance movement.

More insight in the functioning of viral proteins in the processes of viral movement and replication can be obtained by studies that involve transgenic plants expressing viral genes, as demonstrated for example with the MP gene of tobacco mosaic virus (TMV) (Wolf et al., 1989, Deom et al., 1990, Ding et al., 1992, reviewed by Wolf and Lucas, 1994), sunn-hemp mosaic virus (SHMV) (Deom et al., 1994), alfalfa mosaic virus (AlMV) (Poirson et al., 1993) and cucumber mosaic virus (CMV) (Vaquero et al., 1994) or the replicase protein genes of AlMV and brome mosaic virus (BMV) (Van Dun et al., 1988, Mori et al., 1992). On the other hand, introduction of viral sequences into the genome of a host plant has frequently resulted in pathogen-derived resistance (PDR) (reviewed by Wilson, 1993). The first report on PDR described coat protein (CP)-mediated protection against TMV in tobacco plants (Powell Abel et al., 1986). Since then, numerous cases of PDR have been described for viruses of at least 13 different taxonomic groups (Hull and Davies, 1992, Kunik et al., 1994). These examples demonstrated that PDR can be accomplished in various ways. Not only CP genes were found to confer resistance to transgenic plants, but also MP genes (Lapidot et al., 1993) and replicase genes (reviewed by Carr and Zaitlin, 1993). Even, mutated or truncated versions of viral genes were shown to be capable of inducing resistance in transgenic plants (Anderson et al., 1992, Braun and Hemenway, 1992, Longstaff et al., 1993, Donson et al., 1993, Lapidot et al., 1993, Audy et al., 1994). In some of these cases the resistance was exhibited only against viruses, closely related to the virus from which the transgene originates, while in other cases the resistance was extended to less related viruses (Donson et al., 1993). It appears that the

presence of the viral proteins or nucleic acids disturbs the subtle balance in amount, time and place of viral- and host-factors, needed for a systemic infection of a host plant with a virus. The fact that PDR can be achieved in a variety of ways and can differ considerably in spectrum, implies that distinct molecular mechanisms may underlie different cases of PDR. How exactly the resistance is established, remains to be demonstrated.

In this study we have transgenically expressed both the CPMV 200kDa replicase and 48kDa and overlapping 58/48kDa movement protein genes by transforming *Nicotiana benthamiana* plants. These plants did not allow functional studies of the viral transgenes, but, instead, exhibited a complete resistance when inoculated with CPMV virions or CPMV RNA.

RESULTS

Three types of transgenic plants

The binary constructs pBINB200, pBINM48 and pBINM58/48 (Figure 1) were designed to

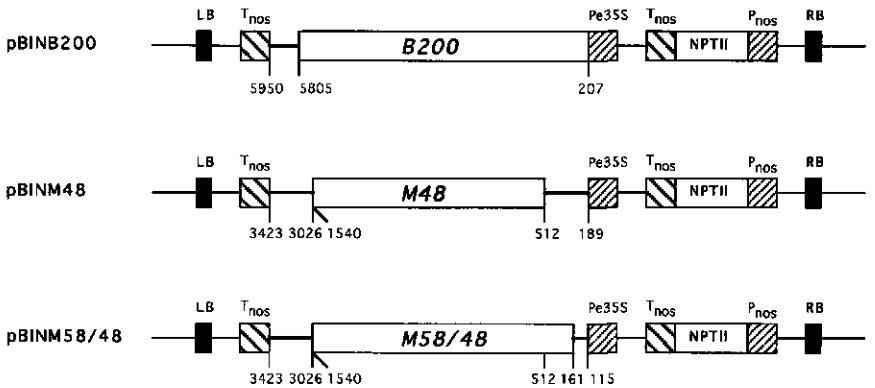

Figure 1. The binary vectors that were used to generate transgenic plants.

Diagrammatic representation of the plant transformation vectors pBINB200, pBINM48 and pBINM58/48 which were used to transfer the CPMV 200kDa replicase and 48kDa and 58/48kDa movement protein genes, respectively, to *N. benthamiana* plants. In plasmid pBINB200, the replicase coding sequence from RNA1 nt 207 to 5805 is present, followed by the RNA1-derived 3' non-translated region from nt 5805 to 5950. Plasmid pBINM48 contains as a leader the RNA2-derived sequences from nt 189 to 512, followed by the 48kDa coding sequences from the AUG codon at position 512 to the stop codon at nt 1540 and a 3' non-translated region comprising the RNA2-derived nucleotides 3026 to 3423. Plasmid pBIN58/48 contains as a leader the RNA2 region from nt 115 to 161, succeeded by the 58/48kDa coding sequences from the AUG codon at position 161 to the stopcodon at nt 1540 and followed by a 3' non-translated region comprising the RNA2-originating nt 3026 to 3423. LB and RB refer to the left border and right border sequences of the T-DNA of *A. tumefaciens*, respectively. Tnos refers to the nopaline synthetase terminator sequences, Pnos to the nopaline synthetase promoter sequences, Pe35S to the enhanced CaMV 35S promoter sequences and NPTII to the kanamycin resistance gene.

express the CPMV replicase and MP genes in plants under the control of an enhanced 35S promoter and a NOS terminator. The genes are derived from the plasmids pMB200, pMM48 and pMM58/48, that have previously been shown to express functional proteins upon transient expression in protoplasts (van Bokhoven et al., 1993b and Wellink et al., 1993).

Upon *Agrobacterium tumefaciens*-mediated transformation, kanamycin resistant shoots (the R0-generation plants) were selected. By PCR on DNA and RT-PCR on cDNA preparations of kanamycin resistant plants (Figure 2), plants that were expressing the transgenic mRNA were identified and for each construct 10 positive plants were selfed to give R1-generation-lines.

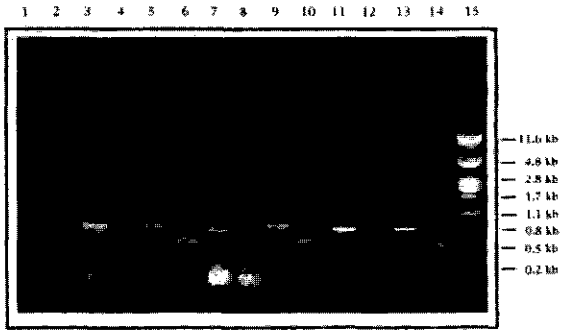

Figure 2. Analysis of plants for presence of the transgene and the transgene mRNAs.

Agarose gel electrophoresis of the products of the PCR amplifications on DNA, RNA or cDNA isolated from non-transformed plants (lanes 1 and 2), or plants transformed with pBINM48 (lanes 3, 4, 5 and 6), pBINM58/48 (lanes 7, 8, 9 and 10) or pBINB200 (lanes 11, 12, 13 and 14). Amplified gene fragments are present in the PCR reactions on preparations of genomic DNA (lanes 3, 7 and 11) and cDNA (lanes 5, 6, 9, 10, 13 and 14). 48kDa and 58kDa coding sequences were detected by primer combination D1*-D2*, which gives rise to a 1kb fragment, that can be specifically digested with *AccI* into 400bp and 600bp fragments (lanes 6 and 10). 200kDa coding sequences were detected by primer pair T_1-T_2, resulting in a 800bp fragment, which can be specifically digested with *HaeIII* into 300bp plus 500bp fragments (lane 14). In genomic DNA preparations of non-transformed plants (lanes 1 and 2) and in total RNA extracts of the transgenic plants (lanes 4, 8 and 12), no amplified fragments can be detected neither with primer pair D1*-D2* (lanes 1,4 and 8), nor with primer combination T1-T2 (lanes 2 and 12). Lane 15 shows λ-DNA digested with *PstI* as a marker. Especially in lanes 3, 7 and 8 low molecular weight, so-called primer-dimer bands, are visible, which are non specific PCR products.

Transgenic R1- and R2-generation plants are resistant to CPMV

Ten R1-generation lines were chosen and assayed for segregation on kanamycin and resistance against CPMV inoculation. These 10 lines included four lines transformed with the CPMV replicase gene and three lines transformed with each of the MP gene constructs. Segregation on kanamycin and analysis of these segregation data (Table 1) revealed that in two plants single T-DNA insertions had occurred (plants BII-9 and 3B) and that eight plants contained two insertions (for instance plant 76.3), that in some cases were linked (plants BII-3 and 5.4). These integration events were confirmed by southern blotting of *BamHI* (for MP gene

insertions) or *Xbal* (for replicase gene insertions)-digested genomic DNA and hybridisation with either a MP or replicase gene specific probe (results not shown).

For each line, 19 plants were inoculated with plant sap containing CPMV and one plant was inoculated with buffer as a control. Eight days post inoculation, all non-transformed plants displayed characteristic mosaic symptoms, while buffer-inoculated plants remained symptom-free. Interestingly, within some of the transgenic lines (Table 1) besides infected plants, plants free of symptoms were observed (Figure 3). These mosaic-free plants were analysed for CPMV replication in both the inoculated and higher located leaves by ELISA and twelve days post-inoculation these ELISAs were repeated. This resulted in the identification of completely resistant plants in two of the four pBINB200, two of the three pBINM58/48 and one of the three pBINM48 transgenic lines (Table 1). According to the kanamycin resistance segregation analysis, all these resistant lines contained two insertions of the transgene (Table 1; plant lines C2-6, BII-3, 76.4, 76.3 and 5.4). From all resistant R1-plants per plant R2-seeds were harvested and germinated on kanamycin. In this way, resistant R1-plants giving rise to a homozygous R2-progeny could be identified for the lines BII-3, 76.4, 76.3 and 5.4 (results not shown). Additionally, this approach revealed that the resistant phenotype was not only displayed

Table 1. Assessment of segregation of kanamycin resistance in the R1-progeny and resistance to CPMV and CPSMV in the R1- and R2-generations of MP gene- and replicase gene-transformed *N. benthamiana* plants.

		Kanamycin resistance[a]		Resistance to virus infection[b]				
Plant line	Transgene	R1, km^S:km^R	Total no.	R1+CPMV	R2+CPMV	R2+S1	R2+S8	R2+CPSMV
Ben	-	1:0	223	12/12	4/4	4/4	4/4	4/4
C2-4	200	1:14	350	19/19	ND[c]	ND	ND	ND
C2-6	200	1:13	378	12/17	2/8	0/7	1/8	8/8
BII-3	200	1:11	730	10/19	0/7	0/6	0/6	6/6
BII-9	200	1:3	304	19/19	ND	ND	ND	ND
76.4	48	1:14	362	5/18	0/6	0/6	0/6	6/6
3B	48	1:2.5	479	19/19	ND	ND	ND	ND
B4-1	48	1:16	223	19/19	ND	ND	ND	ND
76.3	58/48	1:15	919	7/14	0/4	0/4	0/4	4/4
5.4	58/48	1:10	493	3/16	0/4	0/4	0/4	4/4
4.13	58/48	1:15	200	18/18	ND	ND	ND	ND

[a] Since on one T-DNA insert both a viral gene and a kanamycin resistance gene are present, the number of transgene loci can be determined by assessment of the segregation ratio for kanamycin resistance in R1-generation plants. After germination and cultivation of R1-generation plantlets on kanamycin for 2 weeks, the proportions of sensitive and resistant plants were determined.

[b] Resistance is determined as the number of infected plants out of the total number of inoculated plants, as was assayed by symptom occurrence and ELISAs. The plants were challenged not only with the wild-type CPMV-Sb strain, but also with the mutant strains S1 and S8.

[c] ND = not determined.

by R1-plants homozygous for the insertions, but also by heterozygous R1-plants. Eight R2-progeny plants of each of the five resistant lines were tested for resistance against CPMV. As expected, plants of all five lines (C2-6, BII-3, 76.4, 76.3 and 5.4) displayed the resistant phenotype (Table 1). However, in line C2-6 beside resistant plants also susceptible plants were present. This segregation for resistance was anticipated since for this line no resistant homozogous R1-plant was identified.

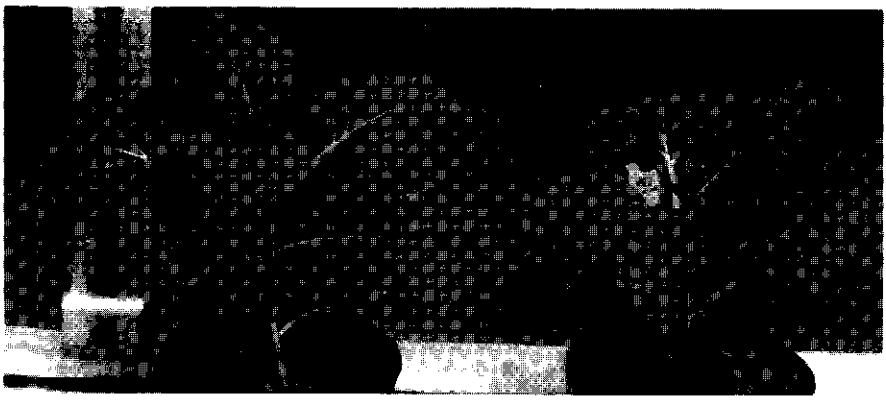

Figure 3. Resistance of transgenic *N. benthamiana* plants to CPMV.

Within one line, in the segregating R1-generation both plants susceptible (right) and resistant (left) to CPMV inoculation can be detected, as shown here for the replicase transformed line C2-6.

The resistance does not hold against a heterologous comovirus

The R2-progeny of the five resistant lines was also tested for resistance against the CPMV strains S1 and S8 that are, in contrast to the wild type Sb strain, capable of systematically infecting *V. unguiculata* cv. Early Red (De Jager and Wesseling, 1981). In addition, another comovirus species, cowpea severe mosaic virus (CPSMV) (Chen and Bruening, 1992a and 1992b), which shares for RNA1 50% and for RNA2 44% nucleotide sequence homology to CPMV, was tested. All lines resistant to CPMV-Sb were found to be resistant to the strains S1 and S8, but not to CPSMV (Table 1). Again not all plants of line C2-6 exhibited the resistant phenotype.

The resistance acts by preventing the replication of the donor RNA segments

In the resistant lines transformed either with the replicase gene or with the MP gene, CPMV could not be detected by ELISA at 8 or 12 days after inoculation. This result indicated that the lines have complete immunity to CPMV infection. To establish whether the resistance acts at the single cell level, protoplasts of R2-generation transgenics were transfected with CPMV RNA. After 18 h of cultivation, replication of RNA1 and RNA2 were separately monitored in immunofluorescence assays by using the two different antisera, α110 and αCPMV, that

specifically recognise RNA1 and RNA2 derived proteins, respectively. In the non-transgenic protoplasts both CPMV RNA segments were replicated in 80% of the protoplasts (Table 2).

Table 2. Assessment of the percentages of immunofluorescing protoplasts prepared from R2-generation transgenic plants, transfected with CPMV RNA.

		Fluorescing protoplasts (% of total)[a]	
Plant line	**Transgene**	α110[b]	**αCPMV**[b]
Ben	-	80%[b,c]	80%
C2-6	200	5%	10%
BII-3	200	0%	0%
76.4	48	80%	0%
76.3	58/48	65%	0.4%
5.4	58/48	80%	0.2%

[a] Protoplasts were transfected with either 1 µg or 2.5 µg of CPMV RNA per million protoplasts. The percentage reflects the average of the two doses, which did not differ significantly (results not shown).
[b] The α110 antiserum detects RNA1-encoded replicase proteins, while the αCPMV antiserum detects the capsid proteins, which are encoded by RNA2.

In protoplasts from the MP gene resistant plants RNA1 was still capable of replication but the replication of RNA2 was strongly inhibited; no RNA2 specific proteins were detected in on average 99.8% of the protoplasts, while in 0.2% of the protoplasts fluorescence signals at wild-type infection level were observed. However, in the replicase resistant protoplasts no replication of any CPMV RNA at all was found, except for line C2-6, which showed a reduced percentage of immunofluorescing protoplasts with both antisera (Table 2). The incomplete resistance in line C2-6 is attributed tot he presence of protoplasts derived from susceptible plants; the R2-progeny of this line were segregating for resistance to CPMV infection (Table 1). Since replication of RNA2 is dependent on replication of RNA1, inhibition of replication of RNA1 prevents the synthesis of RNA2 specific proteins. No differences were observed in the percentages of fluorescing protoplasts between the two different CPMV RNA-inoculum concentrations that were used.

Absence of accumulation of transgene-encoded proteins

All plants tested for resistance expressed transgenic mRNA, as was shown by RT-PCR (Fig 2). Despite several attempts (Chapter 3), no accumulation of CPMV specific proteins could be detected in any transgenic R0-plant; neither in plants generating susceptible lines nor in plants that gave a resistant progeny. Furthermore, accumulation of CPMV MPs was found to be absent

in homozygous R2-generation plants of the resistant line 5.4. Both the α48 serum and the α110 serum, a serum that specifically recognises RNA1-derived proteins, cross-react with several tobacco proteins, which reduced the specificity of the antisera for the transgene-encoded proteins.

DISCUSSION

The bipartite nature of the CPMV genome enabled us to show that in *N. benthamiana* plants a resistance against CPMV can be engineered, that is specifically directed against the viral RNA segment from which the transgene is derived. In plants transformed with the RNA1-derived full-length replicase gene, replication of RNA1 and consequently RNA2 was blocked, while in plants expressing the RNA2-originating full-length MP gene, the replication of RNA2, but not of RNA1, was prevented. For replicase genes, resistance obtained by integration of full-length constructs has been described previously (Audy et al., 1994, Rubino et al., 1993, Braun and Hemenway, 1992, Mueller et al., 1995). The results on the 48kDa and 58/48kDa transgenics, on the other hand, show for the first time that PDR can be obtained by transformation with a full-length MP gene. Engineered resistance against CPMV has neither been described before; transgenic expression of the 60kDa precursor of the two CPMV capsid proteins of 37kDa and 23kDa was shown not to induce PDR (Nida et al., 1992). However, it should be noted that in these experiments only four lines were tested which were selected on high accumulation levels of the CP precursor protein and that *Nicotiana tabacum*, which is a nonsystemic host of CPMV, was used for transformation.

Resistance was observed in half of the R1-generation lines, transformed with each of the three constructs, and occurred as complete immunity to CPMV infection. In all cases, the resistance was retained in the R2-generation. The resistance was maintained against the CPMV mutants S1 and S8, but it did not hold against the heterologous comovirus CPSMV, which is homologous to CPMV at nucleotide sequence level in both RNA1 (50%) and RNA2 (44%). This specificity of resistance implies a rather narrow range of the resistance. Furthermore, the resistance was shown to act at the single cell level as shown by Carr and Zaitlin (1991) for resistance to TMV. This was analysed by immunofluorescence assays on protoplasts transfected with CPMV RNA, using antisera detecting proteins translated from either RNA1 or RNA2. In addition, these experiments revealed that, in all protoplasts, a transgenic replicase gene can reduce the replication of RNA1, and consequently RNA2, to levels that are below detection when using immunofluorescence. However, in protoplasts derived from MP gene-transformed plants, RNA1 replication was still allowed at wild-type infection levels, while RNA2 replication was inhibited. In most protoplasts no RNA2 specific proteins were detected, but up to 0.4% of the protoplasts were showing fluorescence signals at a level comparable to wild-type infected protoplasts. The observation, that this leakage of resistance is specific for MP gene-transgenics, suggests that a transgene specific mechanism underlies this effect. In these experiments the difference between the replicase and the MP gene-transformed plants may be due to replication and translation of RNA1. This process would have taken place in cells

transformed with RNA2, but not with RNA1-derived genes. There would consequently have been an excess of replication complex formed that could compete with the resistance-mediating factors for the access to RNA2.

For several cases of PDR, it is known that resistance is mediated either by the transgenic proteins or the transgenic mRNAs. For example, it was shown that a truncated TMV replicase protein can reduce the accumulation of several tobamoviruses (Donson et al., 1993) and that the full-length TMV 54kDa protein, a putative component of the replicase, and the pea early browning virus equivalent of this protein can confer a resistance at the cellular level (Golemboski et al., 1990, Carr et al., 1992, MacFarlane et al., 1992). In these cases the transgenically expressed proteins could compete with factors involved in functioning or formation of the replicase complex or they could interfere with a possible regulatory role of the protein. A role for the transgenic mRNA was assigned in the case of resistance, obtained by the integration of either mutated or full-length replicase genes of PVX (Mueller et al., 1995). Here, resistance coincided with a low mRNA expression level of the transgene. Crossing of resistant, low transgene mRNA expressing plants with susceptible, high mRNA-expressing plants revealed that resistance was dominant over susceptibility and that low transgene mRNA expression was dominant over high expression (Mueller et al., 1995). These results indicate that a low expressed, probably silenced, transgenic mRNA can act *in trans* to suppress the expression of a high expressed transgene or infecting viral RNAs. Since in the CPMV replicase resistant plants the 200kDa polyprotein could not be detected, an RNA-based mechanism is also assumed.

Lapidot and coworkers (1993) have shown that a truncated, inactive MP of TMV can reduce the spread of several tobamoviruses, a phenomenon not found in transgenic plants expressing the wild-type TMV MP (Holt and Beachy, 1991). These results suggest that the truncated MP molecules can preoccupy certain cellular host factors needed for cell to cell movement of the virus, making these cellular target sites inaccessible to the MPs of the infecting virus (Lapidot et al., 1993). In contrast, a transgenically expressed CPMV 48kDa MP gene, that has no function in viral replication (van Bokhoven et al., 1993a), can inhibit specifically the replication of RNA2 at the cellular level. It is therefore likely that resistance from expression of CPMV and TMV MP genes operates through different mechanisms, with the CPMV MP resistance being RNA-based. It is not known whether in the resistant plants carrying the 58/48kDa MP transgene, the presence of the 58kDa MPs is involved in inducing resistance. Since the 58kDa protein does have a function in replication of RNA2, the protein might have a role in generating resistance, although we were not able to detect the 58kDa protein in resistant plants.

The rather high percentage of resistant lines as found in our experiments, plus the fact that the transformation plasmids were derived from functionally active expression vectors (Van Bokhoven et al., 1993b and Wellink et al., 1993) make it highly improbable that the resistance is due to a dominant negative effect of the insertion of the sequences as mutated forms, although we can not entirely exclude this. In conclusion, we suppose an RNA-based resistance for all the three types of transgenic plants, although this needs to be tested in more detail.

A model on how RNA-mediated resistance might operate, was introduced by Lindbo et al. (1993) and extended by Smith et al. (1994) and Dougherty et al. (1994). They propose that transgene mRNA levels that exceed a certain threshold level activate a cytoplasmatic process that targets these mRNAs for inactivation in a sequence specific way. Incoming viral RNAs and/or their replicated progeny will also be affected by this degradation system and thus result in a resistant phenotype. Therefore, RNA-based resistance is inversely correlated with mRNA expression levels, is present at the cellular level (Lindbo and Dougherty, 1992) and acts only against very homologous strains. Additionally, it can be induced by a viral infection since the viral RNAs can elevate the mRNA levels beyond the threshold level (Lindbo et al., 1993). Grafting studies revealed that this RNA degradation activity is a programmed cell response which is not induced by a diffusible signalling molecule (Dougherty et al., 1994).

In general it can be said, that when full-length viral genes are inserted in plant genomes, no predictions either on the occurrence of resistance, or on the ability of performing functional studies, can be made. Much remains to be elucidated of the mechanisms leading to pathogen derived resistance and the many factors involved in this process.

MATERIALS AND METHODS

Plants and viruses

Vigna unguiculata cv. Blackeye (cowpea) was used for the propagation of the CPMV strains Sb, S1 and S8 (kindly provided by Dr. C. P. de Jager, Dept. of Virology, WAU) and CPSMV (Chen and Bruening, 1992a and 1992b). At the nucleotide level CPSMV RNA1 is 50% and RNA2 is 44% homologous to CPMV RNA1 and RNA2, respectively. Inoculum was freshly prepared from CPMV infected cowpea plants by grinding one leaf in 1.5 ml 50 mM NaPi-buffer pH 7.2. The CPMV sequences that were used for the construction of the plant transformation vectors, were derived from cDNA clones of strain CPMV-Sb. Strains S1 and S8 are mutants of strain Sb, which are able to systemically infect *V. unguiculata* cv. Early Red, a local lesion host for strain Sb. The mutations are located on RNA1 (De Jager and Wesseling, 1981). *Nicotiana benthamiana*, a systemic host for CPMV, was used for the production of transgenic plants.

Construction of plasmids

The 200kDa replicase and the 58/48kDa MP coding sequences are present under the control of an enhanced CaMV 35S promoter and a NOS terminator in the plasmids pMB200 (Van Bokhoven et al., 1993b) and pMM58/48 (Wellink et al., 1993) respectively. In this vector the 35S promoter is enhanced by duplication of the -352 to -90 region (Kay et al., 1987). The expression cassette of pMB200 was isolated as a *SmaI-NotI* fragment and subcloned into *SmaI-NotI*-digested *E. coli* BlueScript vector (Stratagene), resulting in plasmid pBSMON200. Plasmid pBINB200 was constructed by cloning the expression cassette of pBSMON200 as two fragments (*SalI-BglII* and *BglII-HindIII*, respectively) into *SalI-HindIII*-digested binary vector pBIN19 (Bevan, 1984). The expression cassette of pMM58/48 was subcloned in two fragments (*SmaI-XbaI* and *XbaI-HindIII*, respectively) in *SmaI-HindIII*-digested BlueScript vector which resulted in plasmid pBSMON58/48. From this plasmid the 48kDa coding sequence and NOS terminator were isolated as a *BglII-SalI* fragment and cloned together with the 35S promoter as a *HindIII-BglII* fragment into *SalI*- and *HindIII*-digested pBIN19, giving rise to the plasmid pBINM48. Plasmid pBINM58/48 was constructed similarly as pBINM48, except that not the 48kDa but

the 58/48kDa coding sequence was used which was present on a 2.1 kb fragment that arose after *SalI*- and partial *BglII*-digestion of pBSMON58/48.

Plant transformation

Plasmids pBINB200, pBINM48 and pBINM58/48 were conjugated into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Rogers et al., 1986). *Nicotiana benthamiana* leaf explants were transformed as described by Horsch et al. (1985) and shoots were regenerated on MS20-medium containing 0.8 mg/l benzylaminopurine and 0.1 mg/l 2.4-dichlorophenoxyaceticacid in the presence of 500 mg/l cefotaxim and 150 mg/l kanamycin. Shoots were excised and rooted on hormone-free medium in the presence of 100 mg/l kanamycin. R0-generation plantlets were transferred to soil and maintained in the greenhouse, where R1-generation seeds were harvested. Of this segregating R1-generation, 19 plants were tested for resistance against CPMV. Resistant plants were identified and from each of these plants R2-generation seeds were harvested per plant. These seeds were germinated on MS20-medium containing 150 mg/l kanamycin and the seedlings were cultivated on the same medium for 2 weeks; the first week the plantlets were kept in the dark and the second week under day-night regime. When possible, homozygous R2-generation plants were selected.

Nucleic acid analysis

Prior to the isolation of nucleic acids, it was ensured that the plant material was free of *A. tumefaciens* by cultivation of the transgenic plantlets for at least two weeks without cefotaxim.

DNA was isolated from a leaf disc using 100 µl DNA extraction buffer of 65°C containing 0.14 M Sorbitol, 0.22 M Tris pH 8.0, 0.022 M EDTA, 0.8 M NaCl, 0.8% CTAB and 1% sarkosyl. After grinding, 40 ml of chloroform was added and after mixing the samples were incubated for 5 to 30 min at 65°C. From the aqueous phase the DNA was precipitated using isopropanol and subsequently dissolved in 30 µl water.

RNA was isolated according to Verwoerd et al. (1989) and purified from contaminating DNA by a DNase treatment. After phenol-chloroform extractions and ethanol precipitation, cDNA was prepared by incubating a mixture of 1 µl (2 µg) of RNA, 1 µl (1 µg) of the appropriate primer (T2 or D2* that are respectively complementary to the RNA1 nt 1851-1833 or the RNA2 nt 1538-1518) and 8 µl annealing buffer (250 mM KCl, 10 mM Tris pH 8.3, 1 mM EDTA) for 3 min at 83°C, followed by 30 min incubation at 42°C. After addition of 15 µl cDNA buffer (24 mM Tris pH 8.3, 16 mM $MgCl_2$, 8 mM DTT, 0.4 mM dNTPs) and 0.1 µl AMV Reverse Transcriptase (2.5 U/ml, Gibco), the incubation at 42°C was extended for 90 min.

On 5 µl of either the DNA, RNA or cDNA preparation, PCR was performed at alternating temperatures of 94°C, 50°C and 72°C for 32 cycles. For the replicase gene primers T_1 and T_2 were used, which are complementary to the RNA1 nt 1019-1039 and 1851-1833 and generate a 800bp fragment. For the MP gene primer pair D1* and D2*, complementary to the RNA2 nt 512-530 and 1538-1518 were used which should result in a 1kb fragment. For southern blot analysis, DNA was isolated using an urea buffer (7 M urea, 0.3 M NaCl, 20 mM EDTA pH 8.0, 50 mM Tris pH 8.0, 1% sarkosyl) followed by 3 phenol/chloroform extractions, an isopropanol precipitation, again 3 phenol/chloroform extractions and an ethanol precipitation. For each plant, 20 mg of DNA was digested with either *BamHI* or *XbaI*, for MP or replicase gene transformants respectively. The fragments were separated by electrophoresis on a 1% agarose gel in TAE-buffer (Sambrook et al., 1989), transferred to a nylon membrane (GeneScreen, NEN) and hybridised with RNA2 or RNA1 specific probes (Sambrook et al., 1989).

Plant virus resistance assay

Approximately 4 to 5 weeks after sowing, two expanded leaves of the transgenic and control *N. benthamiana* plants were sprayed with carborundum powder and inoculated with plant sap from CPMV

infected cowpea plants. In the segregating R1-generation 19 plants per line were tested with CPMV-Sb inoculum, while in the homozogous R2-generation eight plants per line were tested using plant sap containing CPMV-Sb, CPMV-S1, CPMV-S8 or CPSMV virions. One week after inoculation, plants were scored for symptoms and analysed for the presence of virus by ELISA. Both inoculated and higher situated leaves were tested. Two weeks after inoculation symptom free plants were checked again by ELISA. ELISA was performed as described by Taliansky et al. (1993), with the modification that the plates were coated with plant sap, followed by an incubation with 1000-fold diluted polyclonal antiserum against CPMV particles and an incubation with 2,500-fold diluted goat anti rabbit alkaline phosphatase (GARAP).

Protoplast preparation and transfection

Protoplasts were prepared from fully expanded, smooth leaves of greenhouse-grown R2-generation *Nicotiana benthamiana* plants, as described for cowpea by Van Bokhoven et al. (1993b), with the modification that the leaves were subjected to enzyme treatment for 1 hour. Protoplast transfection and cultivation were performed as described by Van Bokhoven et al. (1993b), using inoculum concentrations of 1 or 2.5 µg CPMV RNA per approximately one million of protoplasts.

Immunofluorescence assay

Approximately 18 h after transfection, protoplasts were tested for replication of both RNA2 and RNA1, by using the indirect immunofluorescence assay described by Hibi et al. (1975) and Maule et al. (1980). Replication of RNA2 was monitored using antiserum against CPMV particles. Replication of RNA1 was assessed using antiserum against the 110kDa protein which is a portion of the viral replicase.

Protein analysis

Transgenic plants were fractionated according to Dorssers et al. (1982). Fractions P, F1, F3 and F4 were analysed for proteins by western blotting assays which were performed as described by Sambrook et al. (1989) and Van Bokhoven et al. (1990).

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CHAPTER 6

RNA-MEDIATED VIRUS RESISTANCE: ROLE OF REPEATED TRANSGENES AND DELINEATION OF TARGETED REGIONS

Titia Sijen, Joan Wellink, Jean-Baptiste Hiriart, and Ab van Kammen

Resistance to cowpea mosaic virus (CPMV) in transgenic Nicotiana benthamiana plants is RNA-mediated. In resistant CPMV movement protein (MP) gene-transformed lines, transgene steady state mRNA levels were low, whereas nuclear transcription rates were high, implying that a post-transcriptional gene silencing mechanism is at the base of the resistance. The silencing mechanism can also affect potato virus X (PVX) RNAs when they contain CPMV MP gene sequences. In particular sequences situated in the 3' part of the transcribed region of the MP transgene direct elimination of recombinant PVX genomes. Remarkably, successive portions of this 3' part, that can even be as small as 60 nucleotides, all tag PVX genomes for degradation. These observations suggest that the entire 3' part of the MP transgene mRNA is initial target of the silencing mechanism. The arrangement of transgenes in the plant genome has an important role in establishing resistance because the frequency of resistant lines increased from 20% to 60% when transformed with a transgene containing a direct repeat of MP sequences, instead of a single MP transgene. Interestingly, we detected strong methylation in all plants containing directly repeated MP sequences. In sensitive lines only the promoter region was found heavily methylated, whereas in resistant lines, only the transcribed region was strongly methylated.

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INTRODUCTION

Transgenic plants expressing virus-derived sequences can be resistant to plant virus infections. Pathogen-derived resistance is mediated either by the protein encoded by the transgene or by the transcribed mRNA (Wilson, 1993; Lomonossoff, 1995). Protein-mediated resistance generally offers moderate protection against a broad range of viruses, whereas RNA-mediated resistance results in immunity in the cell for closely related viruses.

RNA-mediated virus resistance has been interpreted as an example of homology-dependent gene silencing (Flavell, 1994; Smith et al., 1994; Dougherty and Parks, 1995; Meyer, 1995a; Mueller et al., 1995; English et al., 1996; Prins et al., 1996). Gene silencing can operate transcriptionally or post-transcriptionally (Finnegan and McElroy, 1994; Jorgensen, 1995; Matzke and Matzke, 1995a; Meyer and Saedler, 1996) and is detected as either the absence of nuclear transcription or as decreased accumulation of a specific mRNAs, respectively. For RNA-mediated virus resistance, post-transcriptional silencing seems plausible, because no homologous endogenous gene is present and viral RNA replication is merely restricted to the cytoplasm. This was indeed demonstrated in several cases of viral resistance (Lindbo et al., 1993a; Smith et al., 1994; Mueller et al., 1995; Swaney et al., 1995; Prins et al., 1996) in which low transgene mRNA steady state levels relative to transcriptional activity in resistant lines was determined by nuclear run-on assays.

RNA-mediated virus resistance is a highly specific process. The mechanism is induced in only some of the transgenic lines and only very specific sequences are a target for the resistance mechanism. To explain sequence-specific cytoplasmic RNA degradation, either a role for antisense RNAs formed in the nucleus or for complementary RNAs formed in the cytoplasm by a plant RNA-dependent RNA polymerase (RdRp) has been suggested (Flavell, 1994; Dougherty and Parks, 1995; Meyer, 1995a). The duplex RNA structures, which are consequently formed, would serve as a target for nucleolytic cleavage by a cellular factor, after which exonucleases could continue the degradation process (Dougherty and Parks, 1995). Various factors have been suggested to play a role in triggering the silencing mechanism; for example, exceeding an RNA threshold level (Lindbo et al., 1993a; Smith et al., 1994), the formation of aberrant mRNAs (Dougherty and Parks, 1995; Meyer, 1995a; English et al., 1996), or the presence of an unknown genomic feature at or near the silencing transgene locus (Mueller et al., 1995). This genomic feature could relate to a complex organization of T-DNA insertions at a locus (Van Blokland et al., 1994) or the methylation of the transgene (Smith et al., 1994; English et al., 1996). These aspects are not mutually exclusive and might cause aberrations in processes such as mRNA transcription, processing, or export that could trigger gene silencing (Flavell, 1994).

Previously we have obtained engineered resistance to cowpea mosaic virus (CPMV) in *Nicotiana benthamiana* (Sijen et al., 1995). CPMV consists of two genomic RNA molecules of which RNA1, encoding the viral replicase, is able to replicate independently, whereas RNA2, which encodes the movement protein (MP) and the coat proteins (CP), is dependent on expression of RNA1 for its replication. When the viral replicase and MP genes were expressed, several transgenic plants carried resistance that specifically blocked the cellular replication of

the viral segment from which the transgene was derived (Sijen et al., 1995). These characteristics suggested, for the MP gene-containing plants in particular, that an RNA rather than a protein-based mechanism underlies resistance.

The RNA-based nature of the resistance is confirmed in this study for both MP and replicase-mediated protection. In transgenic plants expressing defective versions of the MP or replicase genes, a similar type of resistance is detected. In addition, we show that a CPMV CP gene can confer cellular, segment-specific resistance, supporting the idea that transgenic expression of a CPMV RNA sequence rather than expression of a specific CPMV protein is involved in generating this type of resistance to CPMV. In resistant MP-transformed lines, the steady state transgene mRNA levels were low relative to the high nuclear transcription rates, thus supporting the concept of RNA-mediated viral resistance as a sequence-specific post-transcriptional RNA degradation process. To obtain more information about the mechanism of post-transcriptional silencing, some aspects of the recognition of the sequences that are targeted for degradation as well as some features of the resistance-conferring locus were examined in the experiments described in this report.

In MP gene-resistant plants, not only RNA2 but also heterologous RNA molecules can be subject to the resistance mechanism if they contain the sequence corresponding to the MP gene sequences of the transgene. The genomic RNA of potato virus X (PVX) (Chapman et al., 1992) was used as the recipient heterologous RNA molecule. The PVX expression system was further exploited to determine whether the resistance mechanism is targeted to a specific region, sequence, or structure of the transgene mRNA. By studying the fate of recombinant PVX genomes containing sequences corresponding to different parts of the transcribed region of the MP transgene, we show that in three independent resistant MP-transformed lines, sequences representing the 3' part of the transcribed region are specific targets of resistance, as has been shown previously for a post-transcriptionally silenced β-glucuronidase (*GUS*) transgene (English et al., 1996). We generated PVX derivatives that contained sequences corresponding to various smaller or larger portions of the 3' part of the transcribed region. When inoculated on resistant MP-transformed plants, these PVX derivatives revealed that the entire 3' part is a target for the resistance mechanism rather than a specific sequence or structure within this 3' part.

To examine more directly whether integration of transgenes as repeated sequences can trigger post-transcriptional silencing, we constructed transgenic plants expressing transgenes with either inverted or direct repeats of the MP gene sequences. These transgenic plants revealed not only that transgenes with directly repeated sequences at the transcribed region can confer resistance at increased frequency, but also that methylation at the transcribed region of the transgene could have a role in establishing resistance. A refined model (Dougherty and Parks, 1995; English et al., 1996) on the mechanism of RNA-mediated virus resistance taking into account these novel observations will be discussed.

RESULTS

Transgenic plants and the resistance phenotype

Resistance to CPMV in transgenic plants expressing full-length CPMV MP or replicase genes is present in the cell and is specifically directed against the accumulation of the viral segment of which the transgene was derived (Sijen et al., 1995). This observation suggests an RNA-based rather than a protein-based mechanism. To test this hypothesis, transgenic *N. benthamiana* plants were generated expressing defective versions of the MP and replicase genes that produce the full-length mRNAs but only truncated proteins (Table 1, MP48ΔN and Rep200ΔX). The defective versions were made by introducing frameshifts downstream of the AUG codon, leaving reading frames for 71 of the 342 amino acids of the MP and 233 of the 1866 amino acids of the replicase, respectively. The genes were under the control of an enhanced cauliflower mosaic virus (CaMV) 35S promoter and a nopaline synthase 3' termination signal.

Table 1. Transgenic plants examined for resistance to CPMV

	Plant[a]		Protoplast[b]	
Transgene[c]	R_0	R_1	RNA1	RNA2
MP48ΔN	4/19	4/19	+	-
Rep200ΔX	3/26	3/26	-	-
MP48AS	0/15	0/15	nd	nd
MP58/48AS	0/15	0/15	nd	nd
Rep200AS	0/16	0/16	nd	nd
MEX48	0/20	nd	nd	nd
MP48PL	0/24	0/6	nd	nd
VP23	nd	1/16	+	-
VP37	0/18	0/5	nd	nd
VP23 cotransformed VP37	6/20	3/6[d]	+	-

[a]The values represent the number of resistant R_0 plants or R_1 lines of the total number tested for resistance to CPMV.

[b]From plants, that appeared to carry a resistant phenotype, protoplasts were made and transfected CPMV RNA. In subsequent immunofluorescence assays, two different antisera were used to monitor the replication of the two RNA segments, RNA1 and RNA2, individually. +, replication of the examined CPMV RNA segment; -, inhibition of replication; nd, not determined.

[c]The viral sequences used to transform *N. benthamiana* were a defective version of the 48kDa MP gene (MP48ΔN), a defective version of the 200kDa replicase gene (Rep200ΔX), a promoterless 48kDa MP gene (MP48PL), and full-length versions of 37kDa and the 23kDa viral CP genes (VP37), (VP23).

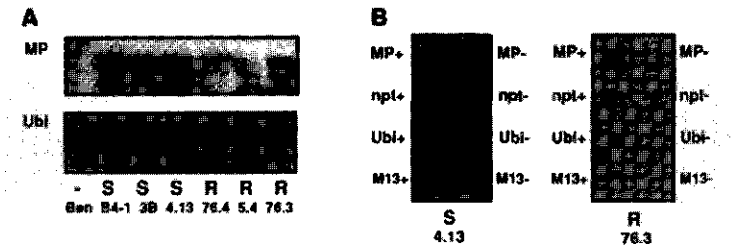
[d]The six R_0 generation plants that were selected for examination of resistance in the R_1 generation comprised three resistant and three sensitive plants, which were scored as three resistant and three sensitive lines in the R_1-generation.

Plantlets that contained the transgene sequence, as determined by polymerase chain reaction (PCR) analysis, were transferred to the greenhouse and inoculated with CPMV. When a plant remained free of symptoms 10 days post inoculation (DPI), protoplasts were prepared

and infected with CPMV RNA to examine resistance in the cell. When protoplasts are infected with CPMV RNA, it is possible to monitor the replication and subsequent expression of the two viral segments (RNA1 and RNA2) individually by using two distinct and specific antisera in immunofluorescence assays. Several transgenic plants expressing the defective MP or replicase genes revealed a resistant phenotype similar to the phenotype observed in resistant transgenic plants expressing full-length sequences (Table 1; Sijen et al., 1995). Resistance is complete in the plant, maintained in the cell and specifically directed against the replication of the viral segment from which the transgene was derived. These results confirm an RNA-based nature of resistance.

RNA gel blot blot analysis of uninfected plants carrying a full-length MP transgene (Sijen et al., 1995) revealed relatively low steady state transgene expression levels for the resistant lines (Figure 1A). However, nuclear run-on experiments (Figures 1B and 1C) showed that the nuclear transcription rates of the MP transgene normalized to the transcription rates of a constitutively expressed ubiquitin gene (Figure 1C) were not lower in the resistant lines than in sensitive lines. In resistant lines, the standardized transgene transcription rates were found to be two- to sixfold higher (Figure 1C). These results support the concept that post-transcriptional RNA degradation underlies RNA-mediated virus resistance. The RNA degradation process is primarily directed toward the transgene mRNAs, but homologous, incoming viral RNA molecules can also be eliminated, resulting in a resistant phenotype. For both resistant and sensitive lines, low levels of antisense transgene transcripts were detected (Figure 1B) that are barely above the background level, indicating that in resistant and sensitive lines, genomic enhancer sequences around the integration site do not result in significant levels of antisense transcripts.

Because the resistance is RNA-mediated, we were interested whether antisense versions of the CPMV MP- or replicase-genes could also confer resistance to transgenic plants, as has been previously shown for potyviruses (Lindbo et al., 1993b). However, none of the transgenic plants expressing antisense versions of CPMV replicase or MP genes showed resistance when inoculated with CPMV (Table 1, MP48AS, MP58/48AS and Rep200AS). RNA blot analyses revealed low transgene mRNA levels in all plants (results not shown), probably due to either low expression or low stability of these antisense mRNAs. The antisense constructs were made in the vector pMEX001, which is different from the vector pBIN19 used for the full length and defective constructs (Sijen et al., 1995; Table 1). Consequently, the antisense sequences were under the control of a non-enhanced CaMV 35S promoter (instead of a duplicated 35S promoter), had different T-DNA borders and were transferred to plants by a different *Agrobacterium tumefaciens* strain (GV3101 instead of LBA4404), with a different *vir*-region. To examine the possible role of the binary vector that was used in the transformation, *N. benthamiana* plants were transformed with pMEX001 binary vector containing the FL CPMV MP gene. None of the transformed plants showed a resistant phenotype (Table 1, MEX48FL), and therefore it is not possible to ascribe absence of resistance in the plants expressing antisense sequences to the antisense orientation of the genes, since effects of the non-enhanced promoter, the binary vector and the *A. tumefaciens* strain (De Block and Debrouwer, 1991) can not be excluded.



C

Plant line	B4-1	3B	4.13	76.4	5.4	76.3
Phenotype	S	S	S	R	R	R
MP+/Ubi+	3.0 ± 1.0	5.0 ± 1.2	2.8 ± 2.0	15.3 ± 8.0	18.4 ± 9.7	11.3 ± 5.0

Figure 1. RNA gel blot analysis (**A**) and nuclear run-on analysis (**B**, **C**) to determine steady state transgene mRNA levels and transcription rates in both resistant and sensitive full-length MP gene-transformed plants.

(**A**) RNA gel blot analysis was performed with total RNA extracted from nontransformed (Ben) plants, full-length MP-transformed resistant (R) lines 76.4, 5.4, and 76.3 and sensitive (S) lines B4-1, 4.13 and 3B (Sijen et al., 1995). For each line, approximately 18 µg of RNA was used, and the blot was probed with a double-stranded probe specific for either the MP transgene (MP) or the constitutively expressed plant ubiquitin gene (Ubi).
(**B**) and (**C**) Run-on transcription was performed with isolated nuclei of the same lines as those given in (**A**). The synthesized, ^{32}P-labeled RNAs were hybridized to a membrane containing spots of 1 µg of various single stranded phagemid DNAs, containing sequences specific for the sense (+) or antisense (-) sequence of the MP transgene (MP), the kanamycin resistance selection marker (npt), the constitutively expressed plant ubiquitin gene (Ubi) or the M13mp18/19 vector (M13). The hybridized ^{32}P-labeled transcripts were quantified using a PhosphorImager (Molecular Dynamics, Sunnydale, CA). For each line the sense (+) MP transgene transcription rates were normalized to the sense (+) ubiquitin transcription rate. (**B**) Typical results of run-on experiments with either sensitive (S) line 4.13 or resistant (R) line 76.3. (**C**) The average results of three or four independent run-on analyses are represented by the value MP+/Ubi+ and the concomitant standard deviation.

When transformed with a construct containing a promoterless MP gene, none of the 24 transgenic plants showed any resistance (Table 1, MP48PL), indicating that transcription is likely to be essential for establishing RNA-mediated resistance to CPMV. This would be in agreement with the assumption of a post-transcriptional basis of the resistance.

To investigate whether the other viral genes in addition to the replicase and MP genes could confer resistance of a similar type to transgenic plants, the two viral CP genes, encoding a 23- (VP23) or a 37kDa (VP37) protein, respectively, were separately cloned into binary vectors. Transgenic plants were generated that expressed the two CP genes either separately or together. Double transformants could be easily obtained by cotransformation because the CP genes were present on two different binary vectors, pBIN19 and pMEX001, containing different plant selection markers (kanamycin or methotrexate). As shown in Table 1 (VP23, VP37 and VP23 cotransformed VP37), resistance to CPMV was observed both in transgenic plants carrying the VP23 gene alone and in the transgenic plants containing both the VP23 and VP37 genes. Resistance was maintained cellularly and was specifically directed against the replication

of RNA2, also suggesting an RNA-based nature of the resistance for this gene. Since for transfer of the VP37 gene vector pMEX001 was used, absence of resistance in the transgenic plants carrying the VP37 gene, can not evidently be attributed to the gene itself as the binary vector that was used could have a role, as described above.

Sequences corresponding to the 3' part of the transcribed region of the MP transgene are the target of the resistance mechanism

The full-length MP-transformed lines 76.4 and 5.4 (Sijen et al., 1995), for which homozygous resistant R_2 lines were obtained, were chosen to investigate the RNA-mediated resistance to CPMV in more detail. In addition, plants of the nonhomozygous resistant R_2 line 76.3 were used in some experiments. Resistant plants of this line were first identified by examining protoplasts from one leaf of a plant for resistance to infection with CPMV RNA.

In the MP-transformed plants, only RNA molecules highly homologous to the transgene mRNAs are eliminated, particularly the CPMV RNA2 molecules. Accordingly, the plants are not resistant to the distinct potexvirus PVX. However, when the sequences corresponding to the CPMV MP gene sequences of the full-length MP transgene are inserted into the genome of PVX (Chapman et al., 1992; Figure 2), and infectious transcripts of this PVX derivative are inoculated onto resistant MP-transformed plants, the plants remained free of PVX specific symptoms (Figure 3, insert 1). Nontransformed *N. benthamiana* plants, inoculated with recombinant PVX full-length MP RNA, showed clear mosaic symptoms 8 DPI. This represents a

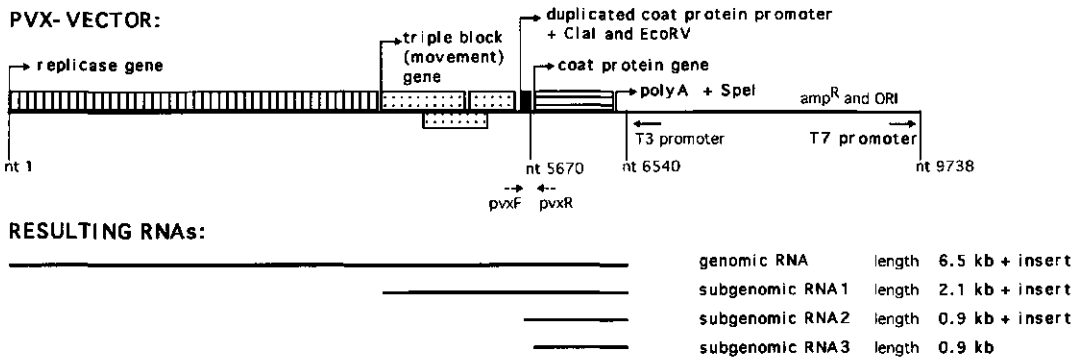


Figure 2. The organisation of the PVX vector used to obtain recombinant PVX genomes.

The PVX vector is a plasmid containing a cDNA copy of the genome of PVX in which the subgenomic CP promoter is duplicated between the triple block MP gene and the CP gene. The duplicated CP promoter is followed by two unique cloning sites, ClaI and EcoRV in which heterologous sequences can be inserted (Chapman et al., 1992). This duplicated promoter will result in an extra subgenomic RNA from which the inserted sequences can be expressed (subgenomic RNA 2). Upon linearisation of the plasmid with SpeI and transcription by T7 RNA polymerase, infectious transcripts are obtained. The primers pvxF and pvxR are situated around the insertion site with an 300 nt interval, as indicated in the figure.

2-day delay in symptom appearance as compared to wildtype PVX. This delay is probably due to the increased length of the genome (from 6500 to 8300 nucleotides) as described previously by Chapman et al. (1992).

The apparent resistance of the plants transformed with the full-length MP gene for the PVX full-length MP derivative was confirmed by reverse transcriptase-PCR (RT-PCR) analyses at 12 DPI. RT-PCR provided no amplified fragment specific for the recombinant PVX molecules (results not shown) when a primer pair (pvxF and pvxR) specific to sequences flanking the integration site was used. For the nontransformed plants, RT-PCR analyses demonstrated that at 12 DPI, not only recombinant PVX molecules but also wildtype PVX molecules were present (results not shown). These molecules presumably resulted from the deletion of the inserted sequences due to homologous recombination between the duplicated CP promoters in the recombinant PVX RNA (Chapman et al., 1992). These experiments revealed that a heterologous RNA molecule can become victim of the silencing mechanism if it harbors sequences homologous to the transcribed region of a transgene that is post-transcriptionally silenced (Figure 3, insert 1).

MP transgene: 5' AUG — STOP — 3'

Insert no.	Insert name	Length in nucleotides	Untransformed plants	Three resistant lines 76.4	5.4	76.3
1	Sense full length	1820	S	R	R	R
2	Sense 5'Nde	600	S	S	S	S
3	Sense 3'Bal	1130	S	R	R	R
4	Antisense 5'Bam	1390	S	R	R	S
5	Antisense 5'Nde	600	S	S	S	nd
6	Antisense NdeBam	820	S	R	R	nd
7	Antisense 3'Afl	640	S	R	R	R

Figure 3. Recombinant PVX genomes tested as targets for resistance on resistant full-length MP gene-transformed plants.

In addition to the sequence homologous to the full-length MP transgene, sequences corresponding to smaller parts of the MP gene were inserted into the genome of PVX. The sequences were inserted in either the sense or the antisense orientation. The full-length MP transgene contains an open reading frame of 1380 nucleotides (from AUG to STOP-codon) encoding the CPMV 48kDa MP, a 5' leader sequence of 45 nucleotides and a 3' nontranslated region of 400 nucleotides. Untransformed *N. benthamiana* plants and three individual resistant MP-transformed lines (76.4, 5.4 and 76.3) were inoculated with infectious transcripts of the recombinant PVX genomes. For each line at least six plants were tested. Sensitive plants (S) developed severe mosaic symptoms 6 to 8 DPI. Resistant plants (R) either remained free of symptoms, or developed symptoms later than 12 DPI, which was shown in RT-PCR analyses to be due to wild-type PVX as a result of recombination. nd, not determined.

Furthermore, we examined whether minus strands of PVX would be accessible to the resistance mechanism. Viral minus strands play an essential role in viral replication and are thought to occur only in viral replication complexes. To obtain PVX minus strands containing sequences representing the transcribed region of a sense CPMV MP transgene, sequences corresponding to the MP gene were cloned in the antisense orientation into the PVX vector. The designed RNA molecules will be produced when this PVX derivative replicates. As shown in Figure 3, insert 4, the MP-transformed plants were also resistant to these recombinant viruses, and the resistance appeared similar to that to the PVX derivative carrying the sequence of the MP gene in the sense orientation. This suggests that for PVX, the genomic plus strands as well as the minus strands can serve as targets for a sense suppression mechanism existing in resistant lines, provided that they contain sequences representing the transcribed region of the transgene.

Surprisingly, in contrast to plants of resistant lines 76.4 and 5.4, plants of resistant line 76.3 did not resist to inoculation with the PVX recombinant carrying sequences of the CPMV MP gene in an antisense orientation (Figure 3, insert 4). RT-PCR analyses (Figure 4A), using a combination of primers of which one is specific to PVX and the other to MP gene sequences, confirmed this different response for plants of lines 76.3 and 76.4. In this PVX recombinant, the inserted sequences represent a partial MP gene that is lacking the extreme 3' 400 nucleotides. We were prompted to examine whether in line 76.3 resistance would occur against a PVX derivative containing sequences corresponding to the 3' terminal 640 nucleotides of the MP gene sequences present in the transgene. All three lines displayed resistance to this PVX derivative (Figure 3, insert 7). These results suggest that only RNA sequences corresponding to a limited region of the transgene can specifically direct the elimination of recombinant PVX genomes (Figure 3, line 76.3, inserts 4 and 7).

This hypothesis was analyzed in more detail with additional PVX recombinants containing sequences representing either only the 5' or the 3' part of the MP gene in the sense orientation. Plants of all three tested lines (5.4, 76.4, and 76.3) were sensitive to a PVX recombinant containing sequences homologous to the 5' part of the MP gene, but resistant to the PVX recombinant carrying sequences corresponding to the 3' part (Figure 3, inserts 2 and 3). Thus, elimination seems restricted for RNA sequences corresponding to the 3' part of the transcribed region of the transgene, indicating that recognition for the degradation process primarily occurs at the 3' part of the transgene mRNA molecules. This 3' part can be different for individual lines carrying the same transgene (Figure 3, insert 4, lines 76.3 and 5.4). The results with PVX derivatives containing sequences representing the 5' or middle part of the transcribed region of the transgene in the antisense orientation further support this observation (Figure 3, inserts 5 and 6).

Fate of heterologous RNAs containing small insertions homologous to the transcribed region of the transgene

In line 76.3, the sequences of the transgene mRNA that are specific targets of the degradation process appear to differ from the sequences specifically targeted in lines 5.4 and 76.4. For the

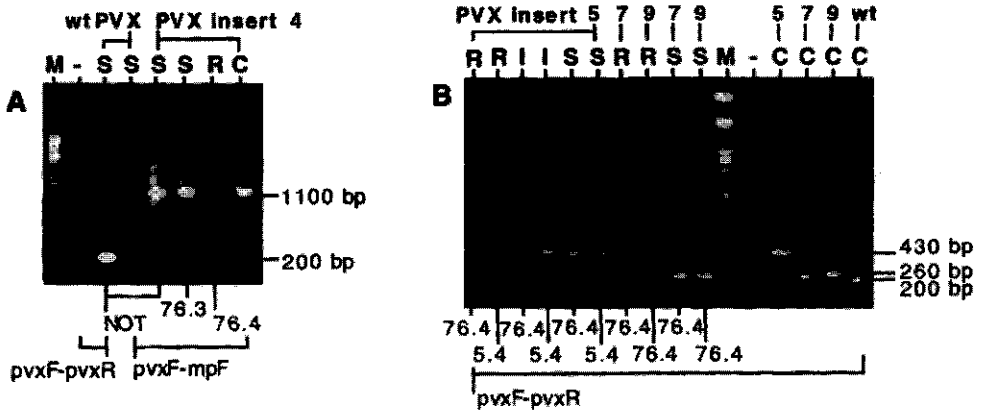


Figure 4. RT-PCR analyses of transgenic plants inoculated with in vitro transcripts of recombinant PVX genomes carrying sequences homologous to regions of the MP transgene.

(**A**) RT-PCR analyses on total RNA isolated from plants inoculated with wild-type PVX (wtPVX) or PVX Antisense 5'Bam (PVX insert 4 as in Figure 3, insert 4) at 9 DPI. Either untransformed *N. benthamiana* plants (NOT) or plants of MP-transformed lines 76.4 or 76.3 were used. Plants were denoted either resistant (R), or sensitive (S). Water (-) was used as a negative control. Plasmid DNA (C) was used as a positive control. A combination of primers was used (pvxF-mpF) of which one is specific to sequences in the PVX genome, upstream of the insertion site (pvxF) and the other is specific to sequences of the MP gene (mpF). This primer pair results in a fragment of 1100 bp for PVX insert 4 genomes, while no fragment is amplified for wtPVX. Primer pair pvxF-pvxR (Figure 2) is specific to sequences flanking the insertion site and results in a fragment of 200 bp for wtPVX genomes. The approximate sizes of the amplified fragments are indicated. A marker (M) is provided that presents fragments of 11kb, 5kb, 2.8kb, 2.4kb, 2.1 kb, 2.0kb, 1,7kb, 1.1kb, 1.0kb, 0.8kb, 0.5kb, 0.45kb, 0.3kb, 0.2kb, 0.15kb and 0.1kb.
(**B**) RT-PCR analyses on total RNA isolated from plants inoculated with wild-type PVX (wtPVX), PVX Sense 3'Nae (PVX insert 5 as in Figure 5, insert 5), PVX Sense XhoBam (PVX insert 7 as in Figure 5, insert 7), or PVX Antisense XhoBam (PVX insert 9 as in Figure 5, insert 9) at 13 DPI with transcripts. Either plants of MP-transformed lines 76.4 or 5.4 were used. Plants were denoted either resistant (R), intermediate resistant (I), or sensitive (S). Controls of water (-), plasmid DNA (C), and a marker (M) are as in (**A**). Primer pair pvxF-pvxR was used which results in amplified fragments of 200 bp for wtPVX, 430 bp for PVX insert 5 and 260 bp for PVX insert 7 or 9. The approximate sizes of the amplified fragments are indicated.

transgene mRNA molecules in line 76.3, either sequences corresponding to a less extended 3' part, or specific sequences located more to the extreme 3' end seem to be the specific targets. To examine in more detail to which portion of the 3' part of the transgene mRNA the degradation mechanism was specifically aimed, we inserted sequences corresponding to different portions of the 3' part into recombinant PVX genomes and examined degradation of these PVX derivatives. The inserted region representing the 3' part of the full-length MP transgene was defined into portions in two ways. The first approach involved a stepwise reduction in the size of the insert corresponding to the extreme 3' part of the MP gene from 1130 to 230 nucleotides (Figure 5, inserts 1, 2, 3, 4, 5, 10, and 11). In the second approach, small fragments, ranging in size from 60 to 140 nucleotides, corresponding to small internal sequences within the 3' part of the MP gene, were inserted in PVX and examined (Figure 5, inserts 6, 7, 8 and 9). All these newly

generated PVX derivatives, showed to be as infectious as wild-type PVX when inoculated on untransformed *N. benthamiana* plants. Furthermore, the stability of these recombinant PVX RNAs strongly increased because of reduction of the length of the insert and deletion of inserted sequences by homologous recombination was not detected in infected plants 2 weeks after inoculation. Consequently, for the PVX derivatives described in Figure 5, reliable sap inoculum could be obtained from transcript-inoculated nontransformed plants at 7 DPI that contained

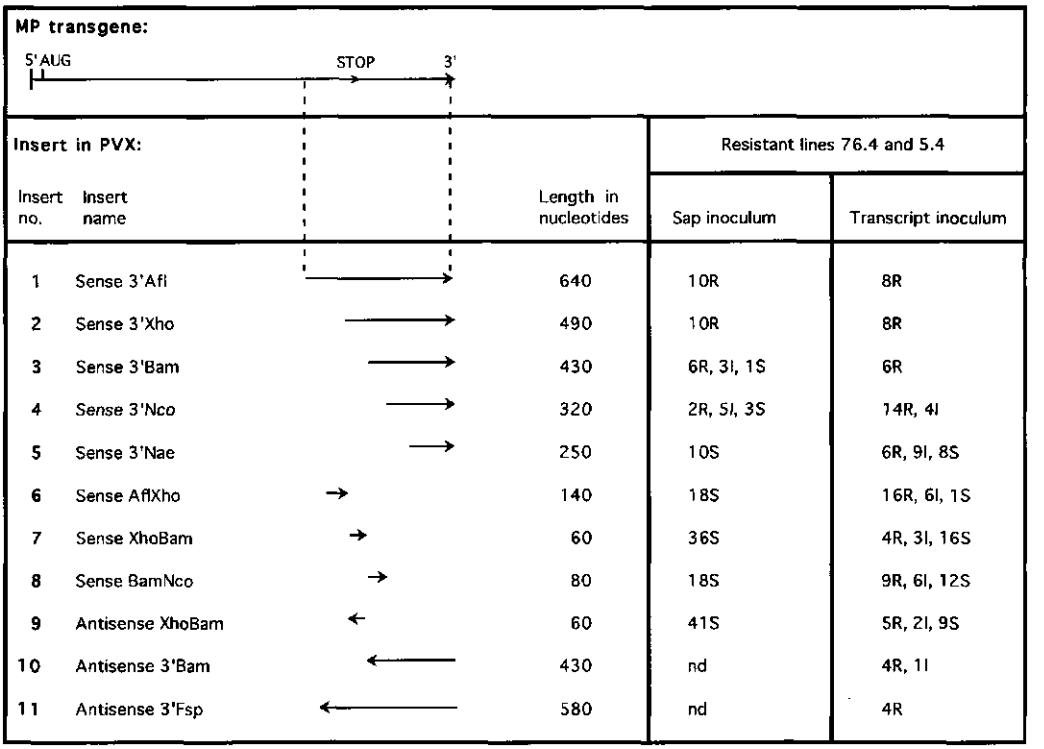


Figure 5. Recombinant PVX genomes containing small inserts homologous to the 3' part of the MP transgene, tested as targets for resistance on resistant full-length MP gene-transformed plants.

Two resistant full-length MP gene-transformed lines (76.4 and 5.4) were inoculated with various recombinant PVX genomes containing small portions of the 3' part of the transcribed region of the MP transgene (see legend Figure 3), either in the sense or in the antisense orientation. Untransformed *N. benthamiana* plants were inoculated as a control, and indicated that all PVX derivatives were as infectious as wild-type PVX (results not shown). Because the two resistant lines responded similarly, the results are recorded together. Plants were inoculated either directly with transcripts or with sap obtained from a systemically infected nontransformed plant at 7 DPI with transcripts. When systemic mosaic symptoms appeared 5 to 7 DPI with transcript inoculum or 4 to 6 DPI with sap inoculum, plants were denoted sensitive (S). When no symptoms were observed, plants were called resistant (R). Plants showing a delay in symptom appearance, with symptoms occurring 8 to 11 DPI with transcript inoculum or 7 to 10 DPI with sap inoculum, were denoted intermediate resistant (I). The values indicate the number of plants showing a specific response.

only virions of the recombinant PVX and no PVX molecules that had lost the insert, as was confirmed by RT-PCR-analyses.

The sap inoculum was used to test the plants for resistance to higher inoculum concentrations of the PVX recombinants. At these high inoculum doses, systemic mosaic symptoms emerged very rapidly (4 to 5 DPI) and, thus, the observations using sap inoculum were done within a period in which PVX derivatives carrying these small insertions are stable. Because lines 76.4 and 5.4 responded similarly to the inoculated PVX derivatives, the responses of plants of these lines are recorded together in Figure 5. For line 76.3, no homozygous line was obtained and, therefore, individual plants had to be examined for resistance at the protoplast level prior to inoculation with PVX derivatives. Consequently, not enough plants of line 76.3 were examined to obtain data sufficiently reliable to be included in Figure 5.

Remarkably, when inoculated with PVX derivatives containing inserts as listed in Figure 5, some plants of the homozygous lines 76.4 and 5.4 became rapidly infected whereas other plants appeared resistant. A third type of response was also observed. Some plants showed a delay in the appearance of symptoms of several days and became systemically infected only 8 to 11 DPI, whereas all nontransgenic control plants showed systemic mosaic symptoms at 5 to 7 DPI. Such a delay in symptom appearance can be regarded as an intermediate resistance response (Figure 5). Plants carrying phenotypes denoted either resistant, sensitive, or intermediate resistant were examined by RT-PCR analyses using a primer pair specific to sequences in the PVX genome flanking the integration site (pvxF and pvxR), as shown in Figure 4B for plants inoculated with transcripts of the PVX derivatives with inserts 5, 7, or 9 (Figure 5). As shown in Figure 4B in lanes 1, 2, 7, and 8, plants that were designated resistant were truly resistant to PVX recombinants because no amplified fragment was obtained. In plants denoted sensitive (Figure 4B; lanes 5, 6, 9, and 10) or intermediate resistant (Figure 4B; lanes 3 and 4), recombinant PVX genomes were replicating, and because for all plants only a fragment specific for the PVX recombinants is amplified, it is evident that the symptoms observed for the sensitive or the intermediate resistant plants are the result of an infection with only recombinant PVX genomes. The frequency at which sensitive and intermediate resistant plants were found, appeared to depend both on the length of the inserted sequence (Figure 5, transcript inoculations) and the inoculum concentration (Figure 5, transcript to sap inoculations).

The variability in responses of plants of one homozygous line suggests that a variability in recognition of the inoculated recombinant PVX RNA molecules occurs in infected plants or cells. The intermediate phenotype should probably be regarded as the result of a difference in the response of individual cells of one plant to the infection with a PVX derivative. The efficiency with which recombinant PVX RNAs are targeted by the resistance mechanism appeared to decrease when the size of the inserted region was reduced or when the inoculum dose was increased. However, the presence of only 60 nucleotides homologous to sequences within the 3' part of the transcribed region of the full-length MP transgene seems sufficient to tag a heterologous RNA, presumably even both PVX plus strands and PVX minus strands, for elimination by the silencing mechanism present in the resistant plants transformed with the full-length MP gene (Figure 5, inserts 7 and 9, transcript inoculations).

A transgene containing a direct repeat of CPMV MP gene sequences induces resistant lines with high frequency

Van Blokland et al. (1994) reported a correlation between post-transcriptional silencing and integrations of multiple T-DNA copies, preferably arranged in a complex locus containing an inverted repeat. To examine the T-DNA arrangement in our transgenic plants, two resistant and two sensitive transgenic lines carrying the full-length MP gene (Sijen et al., 1995) were subjected to DNA gel blot analyses (results not shown). The sensitive line 4.13 contained two transgenic loci each with a single copy T-DNA insertion. Sensitive line 3B contained one locus harboring two T-DNAs arranged in an inverted repeat. Resistant line 76.3 contained two transgenic loci, one having a single T-DNA insertion and one having two T-DNAs inserted as a direct repeat. Resistant line 76.4 contained four T-DNA copies at two loci. Two of the T-DNAs were inserted as a direct repeat, and another two were integrated as an inverted repeat. Although we tested only a limited number of lines, our results are consistent with the general finding that transgenic plants that carry gene silencing processes often contain multiple copies of the transgene that are often arranged in complex loci.

To examine the possible significance of repeated transgenic sequences more directly, transformation vectors were constructed in which CPMV MP gene sequences were cloned as repeated sequences within the expression cassette formed by an enhanced CaMV 35S promoter and a nopaline synthase 3' termination signal. We used a CPMV MP gene with a 3' deletion of 436 nucleotides (400 nucleotides of the 3' nontranslated region and the 36 nucleotides encoding the 12 C-terminal amino acids) (MP48ΔB). This truncated MP48ΔB transgene conferred resistance to CPMV in approximately 20% of the transgenic lines (three of 14) (Table 2, MP48ΔB). This frequency is comparable to the 20% of resistant lines (four of 19) that is obtained when transforming plants with a defective version of the MP gene (Table 1, MP48ΔN). A construct carrying two inverted repeats of the MP gene (denoted IR transgene) situated within the expression cassette, with the orientation sense followed by antisense was made. Also, a construct containing two direct tandem repeats of a MP gene (denoted TR transgene) located within the expression cassette, both orientated in the sense direction, was constructed. DNA gel blot analyses revealed that the TR transgenes were transferred properly into the plant genome by Agrobacterium but that the IR transgenes had recombined in different ways during or after integration in the plant genome. However, still at least a partially invertedly repeated MP gene was left. In some plants a portion of the antisense oriented MP gene was deleted (IRΔ transgene). In other plants the transgene occurred in an integration of 3 successive MP genes oriented sense, antisense, sense, within the expression cassette, which can be regarded as an inverted plus inverted repeat (IIR transgene) (data not shown).

From the 20 transgenic plants that contained an IR transgene, either as an IRΔ or an IIR transgene, only one plant showed resistance to CPMV infection (Table 2, MP48IR). On the other hand, when plants containing a TR transgene were tested for resistance, resistance occurred in approximately 60% (19 of 31) of the lines, which is a strong increase compared to the frequency of 20% (3 of 14) at which resistant plants occur when transformed with a single CPMV MP transgene (Table 2, MP48ΔB and MP48TR). For all the lines, cellular resistance was

confirmed in protoplast experiments and found to be specifically directed against the accumulation of CPMV RNA2 (Table 2), which is indicative of an underlying post-transcriptional silencing mechanism. Strikingly, DNA gel blot analysis on IR-transformed plants revealed that the one resistant plant, carries a transgenic locus consisting of two, directly repeated T-DNA insertions, whereas none of the seven sensitive lines examined contained such a complex locus. Interestingly, two TR-transformed lines (TR6 and TR12) both carried two independently integrated, single T-DNA inserts, while another transgenic line (TR11) carried one single T-DNA insertion. Thus, a TR-transformed plant can show a resistant phenotype when a single copy of the TR transgene is integrated, as the plants were tested in the hemizygous state.

Table 2. Transgenic plants with repeated sequences tested for resistance to CPMV

	Plant[a]		Protoplast[b]	
Transgene[c]	R_0	R_1	RNA1	RNA2
MP48ΔB	3/14	3/14	+	-
MP48IR	1/20	1/8	+	-
MP48TR	19/31	5/8	+	-

[c]Transgenes containing repeats of CPMV MP gene sequences were used for transformation of *N. benthamiana*. The used single MP gene sequences were encoding a truncated MP (MP48ΔB). Both an inverted repeat (MP48IR) and a direct tandem repeat (MP48TR) of these sequences were used.
[ab]See the footnotes [ab] in Table 1.

We assume that a transgene with a transcribed region of two identical directly repeated sequences can be regarded to carry two copies in the genomic DNA, that is transcribed into one RNA molecule. This suggests that the mechanism underlying RNA-mediated resistance to CPMV does not follow simply a model based on exceeding of a threshold level of transgene mRNA model, because this does not explain the increased frequency of CPMV-resistant lines when plants are transformed with a TR transgene instead of a single MP transgene. This observation rather suggests that the arrangement of the integrated transgene sequences is of importance in generating RNA-mediated resistance to CPMV.

Methylation at the transcribed region of the transgene strongly correlates with resistance

DNA gel blot analyses of genomic DNA of both resistant and sensitive full-length MP gene-transformed plants, that was digested with Sau3AI, BamHI, or XhoI, indicated that bands due to partial digestion of the DNA were specifically present in the resistant lines (results not shown). This partial digestion was only observed for methylation-sensitive restriction sites

within the MP gene sequence, mainly for sites situated in the middle and 3' part of the transcribed region. This partial digestion could be due to limited methylation at cytosine residues in the DNA sequences.

This correlation between resistance and methylation of transgene sequences was examined further by DNA gel blot analyses of the transgenic plants containing transgenes with repeated MP genes (IIR , IRΔ , TR transgenes). In the one IIR and the four TR-transformed resistant lines that were analyzed, restriction analysis of the four HindIII sites in the sequence of the repeat-carrying transcribed region revealed extensive methylation (Figure 6A, for TR-transformed lines). The HindIII site flanking the expression cassette was barely methylated (digestion of the flanking EcoRI site is not sensitive to methylation at the C residue). In the five IR- , the two IRΔ- and three TR-transformed sensitive lines that were analyzed, no or only very limited methylation either of the transcribed sequences or of the sequences flanking the expression cassette was observed (Figure 6A, for TR-transformed lines). Accordingly, in DNA gel blot analyses of TR-transformed lines (Figure 6A), for the three sensitive lines that were analyzed mainly two bands of 1100 and 1300 bp (Figure 6C), representing full digestion of each MP gene, were observed. For all four resistant lines that were analyzed mainly one band of 3400 bp, characteristic of the entire expression cassette (Figure 6C) was detected.

Resistant and sensitive IR- and TR-transformed plants were also analyzed for methylation of sequences within the promoter region. Surprisingly, in this AluI restriction analysis, extensive methylation at the three AluI sites in the promoter region was found, only for the three sensitive plants containing a TR transgene (Figure 6B). In the resistant TR-transformed plants, two bands of 280 and 300 bp characteristic of fully digested promoter sequences (Figure 6C) were clearly detected, although some methylation of the promoter region was observed as a hybridizing fragment of approximately 3400 bp, representing the entire expression cassette. This latter observation strengthens the finding that for resistant lines specific strong methylation at transcribed transgene sequences occurs, because full methylation was found for 10 AluI sites within the transcribed region. For the IR transgene-transformed lines, neither the resistant nor the sensitive lines displayed methylation at the promoter region (*results not shown*).

Methylation in the promoter region is known to be involved in establishing transcriptional silencing (Meyer, 1995a). RNA gel blot analysis of poly(A)$^+$ RNA of sensitive TR-transformed plants in which specifically methylation in the promoter region was found (Figure 7, TR5 and TR9; TR13 result not shown) confirmed that no transgene mRNA could be detected, implying that transcriptional silencing occurred. In the resistant TR-transformed plants (Figure 7, TR12; TR2 and TR6 results not shown), transgene mRNAs could be detected,albeit at low levels. The amounts of MP-transformed mRNAs detected in Figure 7 are not directly indicative of the relative steady state levels of the transgenic mRNAs of each line because variable quantities of poly(A)$^+$ RNA could be isolated and analyzed for the lines. For line TR12 approximately four times less poly(A)$^+$ RNA and for lines TR5 and B4-1 approximately 10 times less poly(A)$^+$ RNA were analyzed than for lines TR9 and 76.4, as determined by hybridization of the blot with a ubiquitin probe (results not shown). Taking into account the variable amounts of poly(A)$^+$ RNA that were analyzed, the blot (Figure 7)

confirmed the relatively low steady state level of transgene mRNAs for the resistant full-length MP-transformed line 76.4 compared to the sensitive full-length MP-transformed line B4-1 (Figure 1A).

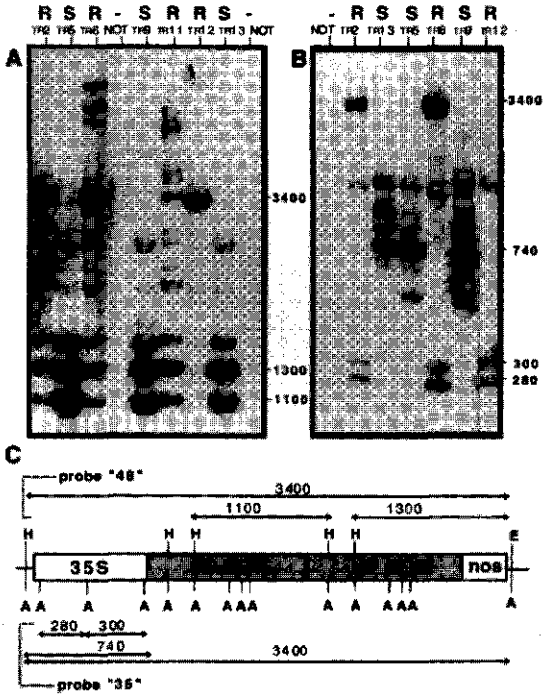


Figure 6. DNA gel blot analysis of genomic DNA of plants containing a transgene of a directly repeated CPMV MP gene.

(**A**) DNA gel blot of genomic DNA of the transgenic lines TR2, TR5, TR6, TR9, TR11, TR12, and TR13, carrying a direct tandem repeat of the CPMV MP gene within the transgene expression cassette and of nontransformed plants (NOT) digested with EcoRI and HindIII and probed with a fragment specific for the MP gene.
(**B**) DNA gel blot of genomic DNA of a nontransformed plant (NOT) and the transgenic lines TR2, TR13, TR5, TR6, TR9, and TR12 digested with AluI and probed with a fragment specific for the the CaMV 35S promoter. Resistant (R) are the lines TR2, TR6, TR11, and TR12. Sensitive (S) are lines TR5, TR9, and TR13. Untransformed plants do not contain a transgene (-).
(**C**) Diagram showing the positions of the AluI (A), EcoRI (E) and HindIII (H) sites. Major fragments, resulting from either full or partial digestion, that are hybridizing with either a probe specific for the CaMV 35S promoter (probe "35") or specific for the CPMV MP gene (probe "48") are indicated here and in (**A**) and (**B**). nos, nopaline synthase.

In summary, the striking observation is that in all of the analyzed plants containing a transgene carrying two directly repeated MP genes, methylation was detected. In all three sensitive plants, methylation was found specifically at all three examined sites in the promoter region. In all four resistant plants, methylation was detected specifically at all 10 examined

sites in the transcribed region. Furthermore, in all of these plants, silencing of transgene expression had occurred. In sensitive plants it occurred transcriptionally, and in resistant plants, it occurred post-transcriptionally.

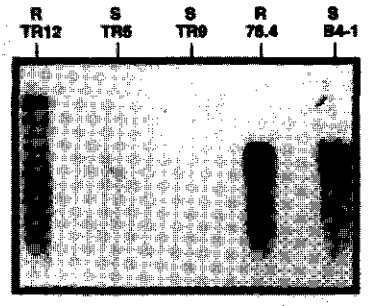


Figure 7. RNA gel blot analysis of both transcriptionally and post-transcriptionally silenced lines containing a TR transgene.

Poly(A)$^+$ RNA was isolated from the lines TR12 (TR transgene, resistant (R), extensively methylated specifically at transcribed transgene sequences), TR5 and TR9 (both TR transgenes, sensitive (S), extensively methylated specifically at promoter sequences), 76.4 (full-length MP transgene, resistant), and B4-1 (full-length MP transgene, sensitive). The blot was hybridized using a MP gene-specific probe. The size of a full-length MP transgene transcript is approximately 1750 nucleotides, and the size of a TR transgene transcript is 2700 nucleotides. No MP gene specific transcript was detected in the sensitive lines TR5 and TR9. The amounts of MP-transformed mRNAs detected are not directly indicative of the relative steady state levels of MP-transformed mRNAs of each line because variable quantities of poly(A)$^+$ RNA were isolated and analyzed for the lines. For lines TR12 approximately four times less and for lines TR5 and B4-1 approximately 10 times less poly(A)$^+$ RNA was analyzed than for lines TR9 and 76.4. This was determined when the blot was hybridized using a ubiquitin probe (results not shown).

DISCUSSION

Resistance to CPMV and post-transcriptional silencing

Resistance to CPMV has been observed in transgenic *N. benthamiana* plants expressing either a full-length CPMV replicase, MP or CP gene or a defective version of the replicase or MP gene. The resistance is complete in the plant and is maintained cellularly. In the cell, the resistance is found to be specifically directed toward the viral segment from which the transgene was derived. Specifically, in resistant MP-transformed plants, relatively low steady state transgene mRNA levels are found, whereas the nuclear transcription rates of the transgene are high. These characteristics imply that resistance to CPMV can be regarded as RNA-mediated virus resistance that is based on a post-transcriptional silencing mechanism (English et al., 1996) primarily directed at the transgene mRNA. In addition, the silencing mechanism can eliminate incoming, homologous viral RNA molecules. Hence, transcription of the transgene is essential

for inducing virus resistance, and indeed no resistance was observed in 24 lines transformed with a promoterless MP transgene.

This observation is at first sight in contrast to the results of Van Blokland et al. (1994) who found that in transgenic petunia plants carrying chalcone synthase (*chs*) coding sequences without promoter sequences, cosuppression of the endogenous *chs* gene occurred. However, an essential difference between the two systems should be noted; in the case of cosuppression using *chs* coding sequences an endogenous *chs* gene is present in the nucleus, whereas in the case of RNA-mediated virus resistance no endogenous homologous gene is present. A *chs* promoterless transgene might impose an effect on the endogenous gene that is under the control of an endogenous promoter. Thus, in case of a promoterless transgene, post-transcriptional silencing could be induced upon transcription of the endogenous gene, that has been affected, in a yet unidentified manner, by the promoterless transgene.

The resistant full-length MP-transformed plants showed higher nuclear transcription rates of the transgene than the sensitive lines, suggesting that the rate of transgene transcription could have a role in inducing post-transcriptional silencing. The observed difference between the transcription in sensitive and resistant plants can be partly attributed to the presence of more copies in the genome of the resistant lines (lines 76.3 and 76.4 contain three or four copies, respectively) than in the sensitive lines (lines 3B and 4.13 both contain two copies). Previously, a role of the level of RNAs in inducing resistance has been assumed from the finding of a recovery phenotype among transgenic plants displaying RNA-mediated resistance to potyviruses (Lindbo et al., 1993a; Dougherty et al., 1994; Smith et al., 1994; Swaney et al., 1995). This recovery phenotype suggested that viral RNA molecules contributed to raising the level of RNAs above a threshold required for establishing resistance. On the other hand, Van Bt al. (1995), and English et al. (1996) did not find a significant correlation between the nuclear transcription rates of transgenes and the establishment of post-transcriptional gene silencing.

The silencing mechanism is directed toward a defined region of the transgene mRNA and can act with variable efficiency

By using the PVX vector system (Chapman et al., 1992), we show that heterologous RNA molecules are target of the resistance mechanism in full-length MP-transformed plants if they contain sequences homologous to the MP gene sequences of a post-transcriptionally silenced CPMV MP transgene. Furthermore, in all three examined lines, the silencing mechanism was found to be specifically targeted at the 3' region of the MP transgene mRNA. These results are in agreement with the findings of English et al. (1996) who have shown that in three different types of transgenic plants in which transgenes (carrying either *GUS*, polygalacturonase, or neomycin phosphotransferase coding sequences) are post-transcriptionally silenced, the accumulation of PVX molecules that harbor insertions homologous to these transgene coding regions is inhibited. The silencing of the *GUS* transgene was also found to be targeted specifically to the 3' region of the transgene mRNA. In our system, the extent of the 3' region that is the target of the resistance mechanism was found to differ for individual transgenic lines, because

one of the three examined lines showed no resistance to recombinant PVX genomes containing sequences representing the transcribed region of the transgene lacking the 3' terminal 400 nucleotides. Furthermore, we showed that a small insert of only 60 nucleotides, corresponding to a sequence within the 3' region of the transcribed region of the transgene, was sufficient to tag a recombinant PVX molecule for the elimination process.

PVX derivatives containing small inserts of 140, 60, 80, or 320 nucleotides corresponding to successive, nonoverlapping sequences within the 3' terminal 600 nucleotides of the MP gene, are all prone to elimination by the resistance mechanism present in full-length MP-transformed plants. This result suggests that recognition of the elimination process can happen at various sites within the 3' region of the transgene mRNA and that not a specific sequence or structure within the 3' region of the transgene mRNA is of predominant importance in the initiation of degradation. This interpretation goes against the suggestion of Goodwin et al. (1996) who proposed that selected sequences or structures of the transgene mRNA are sites of initial cleavages. They detected in RNA gel blot and primer extension analyses smaller internal fragments of the transgene mRNA specifically in resistant lines. These smaller fragments might, however, reflect specific products of further degradation than specific sites of initial cleavage.

Recombinant PVX RNAs containing MP gene sequences in the antisense orientation were also found to be eliminated in resistant full-length MP plants. Strikingly, no essential differences were observed for the fate of recombinant PVX genomes carrying sequences homologous to various parts of the transcribed region of the transgene either in the sense or in the antisense orientation. This indicates that the same mechanism underlies resistance to the PVX derivatives containing MP gene sequences in the sense and in the antisense orientations. This suggests that viral minus strands, even though they are presumably located in viral replication complexes, are accessible to the suppression mechanism. Otherwise, this observation could suggest that the elimination mechanism recognises sequences corresponding to either polarity of the transgene mRNA in the PVX plus strand. In either case, the result invites the suggestion that the RNA-mediated resistance conferred by antisense expression of viral genes, such as the tobacco etch virus CP gene (Lindbo and Dougherty, 1992) could be regarded as sense suppression of viral minus strands rather than as antisense inhibition of the viral genomes. Antisense inhibition has been proposed to result from direct interaction of the transgene antisense transcripts with the viral RNA molecules.

Remarkably, we observed that individual plants of the same homozygous line responded differently to inoculation of recombinant PVX genomes containing small inserts corresponding to the 3' region of the MP gene. Some plants were completely resistant, some plants were fully sensitive, and other plants displayed an intermediate resistant phenotype. In these cases, when inoculated with higher inoculum concentrations, more plants were found to be sensitive, suggesting dose-dependent resistance. These results imply that the resistance mechanism is variable in its efficiency of eliminating RNA molecules. Whether all RNA molecules are eliminated, resulting in full resistance, depends on both the length of the target region present in the RNA molecule, and the concentration of RNA molecules in the inoculum. Thus, the resistance mechanism holds quantitative aspects. A positive correlation between the level of

resistance and the length of the insert, corresponding to sequences of the transcribed region of the transgene that are present in a heterologous RNA molecule, was also reported by Hellwald and Palukaitis (1995). Resistance to cucumber mosaic virus was studied using chimeric constructs of two cucumber mosaic virus strains, which can be regarded as a homologous and a heterologous virus (similar to CPMV and PVX in our studies) because resistance only holds against the strain of which the transgene was derived.

Direct repeats of CPMV MP gene induce resistance at high frequency and show strong methylation

DNA gel blot analyses of several resistant and sensitive full-length MP-transformed plants indicated that the resistant lines contained more transgene copies than the sensitive lines. The multiple transgene copies are partly present in complex loci. When *N. benthamianan* plants were transformed with a direct repeat of the CPMV MP gene, placed within the expression cassette under the control of the same promoter as the single full-length MP transgene, we found a frequency of 60% of resistant lines. This frequency is considerably higher than the frequency of 20% of resistant lines that is found when plants were transformed with a single MP transgene. For one of the resistant TR lines, we showed that one single T-DNA insertion was integrated. The primary transformant of this line was inoculated with CPMV and found to be completely resistant. For hemizygous plant carried two copies of the CPMV 48kDa sequences in the genomic DNA but these two copies are transcribed into one RNA molecule. These observations suggest that a direct tandem arrangement of CPMV MP gene sequences is important for inducing the resistance mechanism. Thus, it appears that establishing of RNA-mediated resistance can be influenced by qualitative traits of the transgene mRNA rather than only by quantitative aspects. Remarkably, plants containing a inverted MP gene repeat (IRΔ transgene or IIR transgene) showed resistance at a lower frequency than did plants carrying a single MP gene. This is in contrast to the TR-transformed plants that showed resistance at a higher frequency than plants containing a single MP gene. This strengthens our suggestion that specific qualitative aspects have a role in inducing the resistance mechanism, because both TR and IR transgenes result two copies of the MP gene in the genomic DNA that, when transcribed, are present on one mRNA molecule. Furthermore, this finding might suggest that mRNAs containing inverted repeat sequences are less able to generate RNA-mediated resistance. This could be due to their feature of being (partially) double stranded RNAs that could be prone to degradation by cellular double-stranded RNases and deliver high instability to the IR transgene transcripts.

If the arrangement of transgenic sequences in the plant genome has a role in establishing resistance, absence of resistance in transgenic plants that were generated upon using binary vector pMEX001 (the transgenes MEX48, MP48AS, MP58/48AS, Rep200AS and VP37 as described in Table 1), might be due to absence of specific arrangements of integrated T-DNAs. The mode of integration of T-DNAs can namely be influenced by features of the binary vector or by the *A. tumefaciens* strain used for transformation of the plants (De Block and Debrouwer, 1991).

Resistant plants containing a CPMV MP gene displayed specific methylation at several sites of the transcribed region of the transgene. This methylation was not detected in sensitive full-length MP-transformed plants. It is known that multiple copies, integrated either as concatamers or at ectopic positions, are preferred targets for methylation (Meyer, 1995a). In resistant full-length MP-transformed plants, more copies of the transgene were present than in sensitive lines. Methylation of the restriction sites that were examined in these plants was found to be limited to some integrated copies or to some cells, because the inhibition of cleavage by the restriction enzymes was not complete. In resistant TR MP gene-transformed plants, that all carry in the genome at least twice the MP gene, however, the examined restriction sites in the transcribed transgene sequences were found to be methylated at all integrated copies of all cells of the plants. It would be interesting to confirm the specific methylation for resistant TR-transformed lines by studying methylation at several more sites within the transgene sequence for the lines studied in this paper and by adding more TR-transformed lines in these analyses. Methylation of the C residue in the HindIII and AluI sites is not at CG or CXG sequences, the symmetrical target sequences for maintenance DNA methylation in plants, and can thus be regarded as de novo methylation.

Recently, Hohn et al. (1996) have clearly shown that methylation at the coding region alone can lead to a reduction of reporter gene expression in transiently transformed plant protoplasts. For various cases of post-transcriptional silencing of transgene mRNAs, a correlation with methylation of transcribed transgene sequences has been reported previously (Hobbs et al., 1990; Ingelbrecht et al., 1994; Smith et al., 1994). English and coworkers (1996) have even connected the region of the transcribed transgene sequences that is methylated to the region of the transgene mRNA that is the specific target of the RNA elimination process. Several groups have not detected a correlation between post-transcriptional gene inactivation and transgene methylation (Hart et al., 1992; Van Blokland et al.,1994; Judelson and Whittaker, 1995; Goodwin et al, 1996). However, it should be noted that in these cases, methylation could have occurred either in only a limited number of the integrated copies of the transgene (as suggested above for the full-length MP transgenes) or was restricted to a limited region of the transgene (English et al., 1996). We have tried to determine whether methylation at transcribed sequences of the transgene has a direct role in establishing RNA-mediated resistance. We germinated and cultivated seedlings of resistant TR lines on 5-azacytidine (Weber et al., 1990; Bochardt et al., 1992; Palmgren et al., 1993). However, at concentrations of 5-azacytidine (20 to 60μM) at which plants just survived, only very limited demethylation had occurred (DNA gel blot analyses not shown) and the plants remained resistant.

In conclusion, the striking observation from our study is that in all TR MP-transformed plants examined, methylation at introduced sequences correlates with silencing of transgene expression. In all of the sensitive lines tested, only the promoter sequences were methylated, resulting in transcriptional silencing. In the resistant lines examined, methylation specifically at the transcribed transgene sequences occurred together with post-transcriptional silencing. This striking concurrence of methylation and silencing could imply that de novo methylation occurs to repress expression of repeated transgene sequences. If promoter regions are affected

by triggered de novo methylation, transcriptional silencing is obtained (Matzke et al., 1989) and the plants remain sensitive. Plants carrying transcriptional silencing are analogous to the transgenic plants containing a promoterless MP transgene, of which we found none to be resistant. If sequences at the transcribed region of the transgene are affected by methylation, post-transcriptional silencing occurs (English et al., 1996), and the plants show resistance, when inoculated with a homologous virus. Remarkably, we have not come across lines in which both the promoter and the transgene sequences were extensively methylated, suggesting that methylation of either the promoter or the transcribed transgene sequences is sufficient to silence expression of a directly repeated CPMV-MP.

A refined model for RNA-mediated virus resistance

A model on RNA-mediated virus resistance (Figure 8) should explain all features of the process. The most striking characteristic of the resistance mechanism is the high specificity in recognition of sequences that are target of the degradation process. The viral RNA molecules are degraded in the cytoplasm while for initiating the resistance process active transcription of the introduced transgene is required. The resistance mechanism is specifically aimed at sequences corresponding to a defined region of the transcribed sequences of the transgene. Integration of transgenes carrying a transcribed sequence of direct repeats of CPMV MP gene sequences can stimulate the induction of resistance and the appearance of resistance is accompanied by extensive methylation of sequences of the transcribed region of the transgene.

The most widely held assumption is that complementary RNA molecules (cRNAs) are involved in accomplishing the specificity of the RNA degradation (Dougherty and Parks, 1995; Meyer, 1995a; English et al., 1996). The cRNAs could arise as antisense RNA molecules formed in the nucleus upon transcription from promoter sequences near the genomic integration site. Alternatively, the cRNAs might be produced in the cytoplasm by the action of a plant-encoded RdRp (Lindbo et al., 1993a) shown to exist in plants (reviewed by Fraenkel-Conrad, 1986) and capable of unprimed transcription at the 3' terminal nucleotidess of single-stranded RNA templates (Schiebel et al., 1993). We favor the hypothesis involving a role of a cellular plant RdRp over the nuclear production of antisense RNAs because in the nuclear run-ons presented in this study, the transcription of antisense transgene RNAs is hardly above background and thus does not outnumber the transcription of sense transgene RNAs. The cRNAs can either act in cis on their template RNAs or in trans on nontemplate transgene mRNAs or incoming viral RNAs. Subsequently, duplex structures might by formed by binding of the cRNAs to target RNAs which could effect a nucleolytic cleavage by a double-stranded RNase allowing subsequent further degradation by cytoplasmic exonucleases (Dougherty and Parks, 1995).

In this study we showed that plants of one homozygous line can respond in a different way to inoculation with recombinant PVX genomes containing inserts corresponding to various parts of the transcribed sequences of a MP transgene. Therefore, we propose that the RdRp produces a pool of small cRNA molecules (Figure 8). For individual cells this pool can vary in composition, availibility or efficieny of interaction with the target RNA molecules. If the target region in a heterologous RNA molecule is made larger or the inoculum dose is increased, the chance

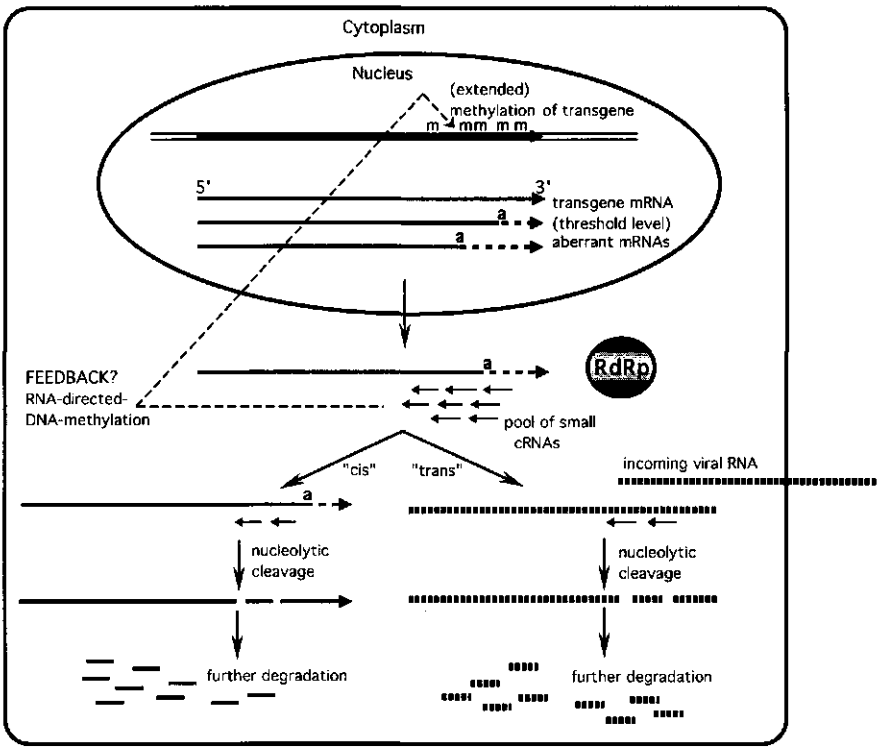


Figure 8. Model on RNA-mediated virus resistance.

This refined model (adapted from Dougherty and Parks, 1995) of RNA-mediated virus resistance could also account for cases of post-transcriptional silencing of transgenes or endogenous genes. We propose that in the nucleus aberrant (a), abnormal, or tagged RNA molecules are formed, possibly due to (limited) methylation (m) of the transgene sequence. Methylation could be due to integration of transgenes or T-DNAs in a special arrangement, for instance as a direct repeat as described in this paper. The form of an aberrant RNA remains to be determined. The presence of an amount of aberrant RNAs above a certain threshold level could have a role in the efficiency of the process that follows. The aberrant RNAs can serve, in the cytoplasm, as a template for a plant-encoded RdRp, which produces a pool of small cRNA molecules. Per cell, this pool can vary in composition, availability or efficiency of interaction with the target RNA molecule. The cRNAs can form duplex structures either in cis on the template mRNAs or in trans on homologous mRNAs or incoming viral RNAs. Such a duplex structure could induce a nucleolytic cleavage which would render RNA molecules accessible for further degradation by exonucleases. Possibly the cRNAs have an additional feedback effect in the nucleus, like a previously described process of RNA-directed DNA methylation (Wassenegger et al., 1994), which could lead to an extended methylation of the transgene sequence.

increases that all the RNA molecules interact with the cRNAs and are eliminated, resulting in full resistance. Thus, the resistance mechanism definitely acts with quantitative features. As the cRNAs have to act in trans on viral RNA molecules, we follow Dougherty and Parks (1995) in their assumption that the cRNA molecules are rather small in size although large enough to be sequence-specific. In *Caenorhabditis elegans* a role of small cRNAs of a length of 22 or 61

nucleotides in down-regulating the translation has been described (Lee et al., 1993), which illustrates that small cRNAs can have specific regulatory effects.

In inducing the specific RNA turnover (Figure 8), both quantitative (Lindbo et al., 1993a; Smith et al., 1994; Jorgensen, 1995) and qualitative (Meyer, 1995a; English et al., 1996) aspects of transgene mRNAs have been suggested to play a role because both high levels of transcription of the transgene (Lindbo et al., 1993a; Smith et al., 1994) and methylation at transcribed transgene sequences (Smith et al., 1994; English et al., 1996) were found to correlate with resistance. These aspects are not mutually exclusive, and in our study resistant full-length MP-transformed plants show both specific methylation at sequences of the transcribed region of the transgene and high transgene transcription rates. Also, our experiments using the various PVX derivates indicated quantitative aspects of the resistance mechanism. However, the increased frequency of resistant lines that is observed when a transgene carrying directly repeated MP gene sequences at the transcribed region is integrated into a plant genome, strongly suggest that also qualitative traits of the transgene mRNAs can have a role. This observation, therefore, seems to argue against a simple threshold model in our system. Nevertheless it would be interesting to study whether the use of a double enhanced CaMV 35S promoter (Elmayan and Vaucheret, 1996) to control expression of a CPMV MP TR transgene will further stimulate the frequency of generating resistant lines. Likewise it would be of interest to determine whether duplication of the number of direct repeats of the CPMV MP gene, expressed from a single enhanced CaMV 35S promoter, will increase the frequency of resistant lines above the 60% reported in this paper. In addition, it would be interesting to develop experiments in which methylation is either stimulated or prevented and thus study whether methylation can indeed have a causative role in post-transcriptional silencing. In conclusion, both quantitative and qualitative aspects of the transgene mRNAs might define these molecules as preferred templates for the RdRp and effect the synthesis of cRNA molecules (Dougherty and Parks, 1995) which then results in a post-transcriptional correction of the expression of the foreign transgenes. The proposed pool of small cRNAs can be produced either as a result from a divergent pool of template RNAs or because of an intrinsic character of the RdRp.

The increased frequency of resistant lines if the CPMV MP gene was integrated as a direct repeat within an expression cassette, suggests that establishing resistance is influenced by the arrangement of the transgenes in the genome. Furthermore, all TR-transformed lines, both the sensitive and the resistant lines, show concurrence of extensive methylation at transgene sequences and silencing of transgene expression. This observation might suggest that methylation of the transgene sequences is cause rather than consequence of the resistance (Figure 8) as was suggested previously by English et al. (1996), who showed for a silenced *GUS* transgene that the region in the transgene corresponding to the target sequence for the silencing mechanism falls together with the region of the transgene at which methylation occurred.

In mouse cells, methylation of genomic DNA has been shown to alter an active chromatin structure (Keshet et al., 1986; Kass et al., 1993) into a type of structure that resulted in transcriptional inactivation (Keshet et al., 1985). Dorer and Henikoff (1994) showed in Drosophila, that integration of transgene repeats could induce heterochromatin formation what

led to gene silencing. In the plant genome, methylation of transgene sequences might also affect chromatin structure as previously suggested by Ingelbrecht et al. (1994). Ten Lohuis et al. (1995) showed that in petunia repetitive DNA that carries a hot spot for methylation induces variegated expression of adjacent sequences, which was suggested to be due to heterochromatin-mediated gene inactivation. Genes in inactive or heterochromatin domains are considered to be not accessible to the regulation, either activation or repression, that is afforded by DNA elements in promoters (Lewin, 1994; Rivier and Pillus, 1994).

However, in the process of RNA-mediated virus resistance, transcription is not inhibited, as was demonstrated in our experiments and earlier by Lindbo et al. (1993a), Mueller et al. (1995), and English et al. (1996), and the resistance process acts post-transcriptionally. So the question is how a methylated DNA sequence in the transcribed region of a gene can render an RNA molecule tagged in such a way that it is recognized as a molecule to be eliminated or, in our model, as a template for the RdRp. If methylation can induce an alteration of chromatin structure, a similar alteration may be achieved independent of whether the DNA has a function as a coding or a promoter region. Furthermore, several proteins are known to bind specifically to methylated DNA (Zhang et al., 1989; Lewis et al., 1992). It is conceivable that both an altered chromatin structure and the binding of methylated DNA binding proteins could affect normal transcription by RNA polymerase II. Barry et al. (1993) and Colot et al (1996) have shown that methylation in *Ascobolus immersus*, results in a low level of smaller transcripts due to premature transcription termination and low stability of the transcripts. Lang et al. (1994) have proposed that in yeast a relatively non-specific signal can cause RNA polymerases to pause. In this system the pausing is not sufficient for the termination of transcription, although pausing is a prerequisite in the process of release of the transcript. Possibly alteration of the chromatin structure or specific binding of methylated DNA binding proteins impedes RNA polymerase II, which could tag the produced mRNA molecule, either by inducing a specific sequence or structure on the mRNA molecule or by triggering another process that modifies the mRNA molecule. Possibly, also features other than methylation at transcribed transgene sequences could result in the formation of truncated, modified or aberrantly structured transgene mRNA molecules, as for several cases of post-transcriptional gene silencing no concurrence with methylation has been reported (Hart et al., 1992; Van Blokland et al., 1994; Judelson and Whittaker, 1995; Goodwin et al., 1996).

In the model presented in Figure 8, a role of a possible feedback mechanism from cytoplasm to nucleus is included (Dougherthy and Parks, 1995; English et al., 1996). Wassenegger et al. (1994) have shown that upon RNA-RNA replication of viroid genomes, occurring when introduced transgene sequences are expressed, the corresponding DNA sequences become methylated (RNA-directed DNA metylation). In our model, the cRNAs might have such a feedback effect on de novo methylation of the transgene DNA, in addition to their role in RNA degradation and amplify or extend an initially limited methylation by RNA-directed DNA methylation activity thereby reinforcing the resistance mechanism.

The presented model also applies for cases of transgene induced post-transcriptional silencing of endogenous genes (cosuppression). In these cases no incoming, viral RNA molecules

but mRNAs transcribed from a homologous endogenous copy of the transgene are eliminated in trans.

Although much remains to be elucidated on the mechanism of post-transcriptional gene silencing, deliberate integration of repeated transgenes might provide a general strategy for efficiently down-regulating the expression of endogenous genes or for engineering virus resistance.

METHODS

Construction of transgenes and transgenic plants

Plasmid pBINM48ΔNde, containing a defective version of the CPMV MP gene, was constructed by NdeI-digestion of pBINM48 (Sijen et al., 1995), followed by blunt-end formation when treated with the Klenow fragment of DNA polymerasel and religation. The binary plasmid pBINB200ΔXba, containing a defective version of the cowpea mosaic virus (CPMV) replicase gene was constructed similarly by XbaI digestion of pBINB200 (Sijen et al., 1995). Plasmid pBINM48ΔB, encoding a truncated CPMV movement protein (MP), was obtained when of a BamHI fragment of 450 bp was deleted. This 450 bp fragment was encoding the last C-terminal 12 amino acids of the MP gene and 400 nucleotides 3' nontranslated region, from plasmid pBINM48 (Sijen et al., 1995). The plasmids pBINM48IR and pBINM48TR, containing within the expression cassette inverted or direct repeats of the truncated CPMV MP gene respectively, were made when a 1.1 kb-BglII-BamHI fragment was ligated with BamHI-digested pBINM48ΔB in either the antisense or the sense orientation.

Plasmid pMP48AS, containing the 48kDa MP gene in the antisense orientation, was generated by ligating a 1.1 kb BglII-BamHI fragment encoding a truncated 48kDa MP gene into BamHI-digested pMEX001, a binary vector carrying on the T-DNA region a methotrexate resistance gene and a multiple cloning site that is flanked by a non enhanced 35S promoter and a 35S 3' termination signal. Plasmid pMP58/48AS was generated by ligating a 1.8 kb XbaI-BamHI fragment encoding the 58/48 kDa MP gene in the antisense orientation into XbaI-BamHI-digested pMEX001. Plasmid pRep200AS was generated by ligating a 5.7 kb BglII-StuI fragment containing the CPMV replicase-gene in the antisense orientation into BamHI-SmaI-digested pMEX001. Plasmid pBINM48PL, containing a promoterless MP gene, was obtained a BglII-SstI fragment of 1.5 kb containing the full-length MP gene was ligated with into BamHI-SstI-digested binary vector pBIN19.

pBINM23 encodes the small viral coat protein (CP), and it was made by replacing the HindIII-BamHI fragment containing the enhanced cauliflower mosaic virus (CaMV) 35S promoter and CPMV replicase gene of plasmid pBINB200 with a 1.2 kb-HindIII-BamHI fragment from pMMVP23 (Wellink et al., 1996) containing an enhanced 35S promoter and the small CP gene. pMEXM37, containing the large viral CP gene, was made by ligating a 1.1 kb-BglII-XbaI fragment from pMMVP37 (Wellink et al., 1996) containing the large CP gene, with BamHI-XbaI-digested pMEX001.

The pBIN19-derived binary plasmids were transferred to Agrobacterium LBA4404 with the aid of *Escherichia coli* RK2013. The pMEX001-derived plasmids were electroporated into competent cells of Agrobacterium GV3101.

Transformation of explants of in vitro cultivated *Nicotiana benthamiana* plantlets was performed as described in Sijen et al. (1995). pBIN19-transformed shoots were selected on 150 mg L^{-1} kanamycin, pMEX001-transformed shoots were identified by selection on 0.05 mg L^{-1} methotrexate. Double transformants were selected on medium containing both 150 mg L^{-1} kanamycin and 0.05 mg L^{-1} methotrexate.

Regenerated shoots were cultivated on medium without cefotaxim for approximately 2 weeks, to ensure that no Agrobacteria were present. DNA from one leaf DNA was isolated as described by Sijen

et al. (1995), and polymerase chain reaction (PCR)-analysis was performed to identify transgenic shoots. Positively identified plantlets were transferred to the greenhouse and placed into soil.

Inoculation of transgenic plants and testing of resistance to CPMV

Within one week after the transfer of primary transformants to the greenhouse, the plants were inoculated with the sap of CPMV-infected tissue. If no symptoms occurred 10 days after inoculation, protoplasts were prepared from the transgenic plants as described by Sijen et al. (1995). The protoplasts were infected with 2 µg of CPMV RNA, and approximately 18 hr after infection, immunofluorescence assays were performed (Sijen et al., 1995). Two different antisera were used to allow seperate monitoring of the replication and subsequent expression of RNA1- and RNA2-specific proteins.

The primary transformants were selfed to obtain R_1 generation plants. In cases in which R_1 lines were tested for resistance, 20 individual plants were analyzed for the occurrence of symptoms after inoculation with sap of CPMV-infected tissue.

Construction of recombinant PVX genomes and testing on plants

Potato virus X (PVX) mutants were cloned into the PVX expression vector pPC2S, a derivative of pGC3 (Chapman et al., 1992) in which the ß-glucuronidase (*GUS*) coding sequence was replaced by unique restriction sites to allow convenient cloning. The vector was generously provided by D. Baulcombe (Sainsbury Laboratory, Norwich, UK). The CPMV MP gene was cloned in the sense orientation into the ClaI-EcoRV-digested PVX vector as an 1.8-kb ClaI-SstI fragment (the SstI overhang was blunted with the Klenow fragment) derived from pMM58/48 (Wellink et al., 1993) resulting in pPVXS58. The 5' region of 600 bp of the CPMV MP gene was cloned in the sense orientation as a ClaI-NdeI fragment (the NdeI overhang was blunted with the Klenow fragment) into ClaI-EcoRV-digested PVX vector resulting in plasmid pPVXS58Nde5'. A truncated CPMV MP gene was cloned in the antisense orientation into ClaI-EcoRV-digested PVX vector as an 1.1 kb-ClaI-BglII fragment (the ClaI site was obtained when the BamHI site was filled in and the BglII overhang was blunted with the Klenow fragment), resulting in plasmid pPVXAS48Bam5'.

To clone the extreme 3' region of 640 bp antisense into the PVX vector, a AccI-AflII fragment (the AflII overhang was blunted with the Klenow fragment) from pPVXS58 was ligated into ClaI-EcoRV-digested PVX vector (the AccI overhang of the insert is compatible to the ClaI overhang of the vector) to result in plasmid pPVXAS48Afl3'. Plasmids pPVXAS48Fsp3' and pPVXAS48Bam3' were cloned similar to pPVXAS48Afl3' using a FspI or blunted BamHI overhang instead of a blunted AflII overhang. The constructs containing smaller CPMV inserts were made from plasmid pPVXS58 or pPVXAS48Bam5' (or the subsequent derivatives of these plasmids) by the exchange of fragments containing CPMV and PVX sequences with fragments containing fewer CPMV and the same PVX sequences. In the PVX vector-derived sequences, the unique ClaI, ApaI, or SstI site was used. In the CPMV derived-sequences, the NdeI, BalI, AflII, XhoI, BamHI, NcoI, or NaeI site was used. The plasmids that were generated in this way were pPVXS58Bal3', pPVXS58Afl3', pPVXS58Xho3', pPVXS58Bam3', pPVXS58Nco3', pPVXS58Nae3', pPVXS58AflXho, pPVXS58XhoBam, pPVXS58BamNco, pPVXAS48Nde5', pPVXAS48NdeBam, and pPVXAS48XhoBam.

Infectious transcripts were produced from these clones as described by Chapman et al. (1992), with minor modifications. The DNA was isolated by Quigen Quick Spin Columns (Westburg, Leusden, the Netherlands), and linearization with SpeI was performed during the transcription reaction. No purification steps were performed and prior to plant inoculations, bentonite and sodium phosphate, pH 7.2, were added to concentrations of 5 µg μL^{-1} and 40 mM, respectively, to the transcription reaction mixture. Plants were inoculated with approximately 5 µg of infectious transcripts, distributed over three leaves.

When plants were infected with PVX transcripts, systemic mosaic symptoms occur about 5 to 7 days post inoculation. Especially after inoculation with recombinant PVX genomes containing large

inserts, at several days post inoculation RNA was extracted from the diseased plants and reverse transcriptase PCR (RT-PCR) analyses (Sijen et al., 1995) were performed. The primers that were used were corresponding to sequences around the insertion site in PVX or to the MP gene. In this way it could be established that the symptoms were indeed due to the replication of the recombinant PVX genomes, not due to wild-type PVX that could occur when the inserted sequences had been deleted.

Nucleic acid analyses of transgenic plants and nuclear run-on analysis

Total RNA was extracted from ground leaf material with a 1:1 mixture of phenol and extraction buffer (100 mM LiCL, 100 mM Tris, pH 8.0, 10 mM EDTA and 1% SDS) preheated to 80°C. After the addition of half a volume of chloroform isoamyl alcohol (24:1), the samples were centrifugated 15 min at 4°C . RNA was precipitated from the aqueous phase with one volume of 4M LiCl (upon incubation for 1h at -70°C), collected by centrifugation for 30 min at 4°C and washed with 70% ethanol. Poly(A)$^+$ RNA was isolated from total RNA using Qiagen Oligotex mRNA columns (Westburg). RNA was separated by electrophoresis on 1% w/v agarose formaldehyde gels, blotted on GeneScreenPlus (New England Nuclear, Boston, MI) according to standard procedures (Sambrook et al., 1989) and hybridized using ^{32}P labeled PCR-amplified fragments as probes (Sambrook et al., 1989).

Genomic DNA was purified by Qiagen Genomic Tips using the isolation protocol provided by the manufacturer (Westburg). Digestion of the genomic DNA was performed by incubation twice for 2 hr in the presence of 1 μM of spermidine. Gel electrophoresis, DNA gel blotting, and hybridisation procedures were performed according to standard procedures (Sambrook et al., 1989). Nuclear run-on analysis was essentially performed as described by Van Blokland et al. (1994).

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CHAPTER 7

GENERAL DISCUSSION

This thesis; goals and results

The aim of the research described in this thesis, was to study the functioning of individual genes of cowpea mosaic virus (CPMV), in transgenic plants of cowpea (*Vigna unguiculata*), the natural host of CPMV. Cowpea is one of the major grain legume crops and has excellent nutritional qualities. Cowpea plants are well adapted to the stressfull growing conditions in the tropics and particularly in Africa they make an important food crop. Grain legumes are notoriously difficult for obtaining transgenic plants, which is mainly due to their recalcitrance in shoot regeneration. This was the first major problem that we had to deal with to be able to study the functioning of CPMV specific genes in transgenic cowpea plants. An efficient regeneration and transformation protocol would not only be useful for research purposes, but would also allow the utilisation of the great potential of biotechnology for crop improvement, including control of plant diseases and improvement of various qualitative traits (Colmar 2nd Symposium for Biological Sciences Abstractbook, 1996). Unfortunately, we have not succeeded in establishing a procedure for efficiently generating transgenic cowpea plants, although some progress was made, mainly in the finding that cowpea shoots can be regenerated from nodal thin cell layer explants (Chapter 2).

Notwithstanding this lack of success for regenerating transgenic cowpea plants, the interest remained for studying the functioning of the CPMV genes in plants. Our special interest went to the CPMV movement protein (MP) gene. The CPMV MPs carry the striking ability of forming tubular structures that can channel plasmodesmata and enable efficient passage of CPMV virions to adjacent cells. Studying the interactions of these MPs with cellular plant factors, like the plasmodesmata, might result in information on the CPMV specific movement processes and also reveal some general aspects of intercellular communication between plant cells (Mezitt and Lucas, 1996). For that reason, the CPMV MP genes were introduced into the genomes of various tobacco species (Chapters 3, 5 and 6). However, no accumulation of MPs was detected in any of the transgenic tobacco plants, irrespective of whether the expression of the transgene was controlled by an enhanced or an inducible 35S promoter (Chapter 3). Only when the CPMV MP genes were expressed from recombinant PVX genomes, accumulation of MPs was observed. In protoplasts infected with the PVX derivatives, the CPMV MPs were found to accumulate in the form of MP-containing tubular structures that extended from the surface of the protoplasts into the medium. Similar tubular structures had been observed on CPMV-infected protoplasts and on protoplasts in which the MPs are expressed from transient expression vectors (Van Lent et al., 1991; Wellink et al., 1993). To explain these findings, it was proposed (Chapter 3) that for tubule assembly either specific timing of MP gene expression and rapid production of MPs is required, or a high expression level is needed which is only obtained if the MPs are expressed from viral genomes or from transient vectors. For other viruses, like tobacco mosaic virus (TMV), it has been reported that expression of the MP gene in transgenic plants results in the accumulation of MPs to detectable levels. In several of the TMV MP expressing plants, the expression levels were not only sufficient for detection using specific antibodies, but in addition enabled the cell to cell movement of transport defective TMV mutants (Wolf et al., 1991; Cooper et al., 1996). TMV is assumed to transiently increase the gating capacity of the plasmodesmata,

while CPMV is thought to modify the plamodesmata more drastically by channeling them with MP-containing tubular structures. Thus, it is conceivable that CPMV requires larger amounts of the MPs than TMV to establish cell to cell virus translocation. During a CPMV infection the MPs can be generated in large amounts since the MPs are expressed coordinately carrying the viral coat proteins (Chapter 1), of which 60 molecules are needed to encapsidate each viral RNA segment. Clearly, it should now be determined whether the recombinant PVX genomes with the CPMV MP gene can induce the assembly of tubular structures in plants. Then it should be tested whether the tubules can enable the translocation of CPMV virions, for instance the virus particles of CPMV mutants that have defects in movement protein functioning.

In Chapter 4 it was examined whether the nitrous acid-induced CPMV mutant N123 would be suited for such complementation assays. These studies revealed that the defective cell to cell movement of N123 is not brought about by mutations only in the MP genes but that additional mutations in the small coat protein gene have a role. Thus, complementation studies should not be done employing N123. Rather, a CPMV mutant in which the MP gene has been replaced by the gene encoding the fluorescent marker protein GFP, as has recently been generated (Jan Verver, unpublished results), could be useful for such studies. The spread of the green fluorescence over adjacent cells would be indicative for complementation.

Although none of the transgenic tobacco plants showed accumulation of CPMV specific proteins (Chapters 3 and 5), several plants were highly resistant to CPMV infection. The characteristics of the resistance were studied to attempt to understand the underlying mechanism (Chapters 5 and 6). It was concluded that this engineered resistance against CPMV is RNA-mediated and occurs from a highly specific cytoplasmic RNA turnover process that is primarily directed at the transgene mRNAs and to which also incoming, homologous viral RNAs fall victim.

It is by no means clear how the cytoplasmic degradation of the transgene mRNAs and the incoming viral RNAs is brought about. To analyse the specificity of the elimination mechanism in more detail, recombinant PVX genomes that contained various inserts corresponding to different regions of the transgene, were inoculated onto the CPMV resistant transgenic tobacco lines (Chapter 6). These experiments revealed that the degradation mechanism specifically targets sequences that correspond to a defined region, the 3' part, of the transgene. Further studies revealed that within the targeted region several small, non-overlapping stretches can be targeted when present in recombinant PVX genomes. These observations support the hypothesis of a role for specific complementary RNA molecules in this highly (sequence) specific RNA degradation process.

As only some of the transgenic lines were found to be resistant and other lines not, it was examined which factors could have a role in the induction of the RNA turnover process resulting in resistance. Plants of resistant lines had higher nuclear transgene transcription rates than susceptible lines (Chapter 6). This could imply that quantitative aspects of transgene transcription are important in mediating resistance (Lindbo et al., 1993). In addition, it was found that all resistant lines carried a direct repeat of T-DNA insertions in the plant genome. Furthermore, when a transgene with a directly repeated CPMV MP gene was transferred to tobacco plants, the frequency at which resistant lines appeared strongly increased. Strikingly,

it was further observed that resistance concurred with methylation of the transgene at the transcribed region (Chapter 6; English et al., 1996). This suggests that factors at the DNA level have a role in mediating resistance.

Transgenes; expression or gene-silencing?

The transgenic plants that were generated during the studies described in this thesis, clearly showed that a transgene can undergo different fates in individual transgenic plants. Besides expression, which does not always result in stable and detectable accumulation of the encoded proteins (Chapter 3), gene-silencing can occur. Two different processes of gene-silencing have been distinguished of which so far the process of transcriptional silencing is best understood. In this process transcription of a gene is prevented presumably by methylation at promoter sequences (Matzke and Matzke, 1995b; Meyer, 1995a; Mittelsten Scheid, 1995). The methylation could prevent essential interactions of the promoter region with elements of the transcription machinery. Recently, nuclease protection assays on isolated nuclei of transgenic *A. thaliana* plants have shown that transcriptional transgene silencing can be accompanied by an altered, more condensed, chromatin configuration at the transgene locus, which could have a direct role in inhibiting transcription of the transgene (Ye and Singer, 1996). Silencing at the level of transcription was also found in some of the transgenic plants generated during our studies, and it occured together with methylation at promoter sequences (Chapter 6).

The other process of gene-silencing occurs at the post-transcriptional level and does not involve prevention of transcription of the transgene but reduces the cytoplasmic accumulation of transgene mRNAs. Thus, the mechanism involves a specific RNA turnover process like that occurring in the transgenic plants displaying RNA-mediated virus resistance against CPMV (Chapters 5 and 6). A similar RNA degradation mechanism is underlying events of co-suppression, which refers to transgenic plants in which an endogenous gene is suppressed by the presence of a homologous transgene. The common process is denoted homology dependent post-transcriptional gene-silencing (English et al., 1996) and will be discussed in more details below.

In many of the CPMV transgenic plants, the expression of the transgene appeared unaffected by the silencing processes, but CPMV specific proteins were not detected (Chapters 3 and 5). Often, transgenic plants are generated to study the functioning the proteins encoded by the transgenes. In these cases high transgene expression levels are desired and then it is of importance to determine factors that can increase or stabilise transgene expression levels. Expression levels can theoretically be increased by using a promoter in combination with strong or multiple enhancer sequences to control transgene transcription, such as a duplicated 35S promoter (Elmayan and Vaucheret, 1996). However, there are indications that especially a high level of transcription or transcription above a certain threshold level can have a role in inducing post-transcriptional RNA turnover mechanisms that result in low steady state levels of transgene mRNAs (Lindbo et al., 1993, Elmayan and Vaucheret, 1996). Otherwise, it might be possible to stabilise and increase transgene expression by utilising SARs (scaffold attachment regions) or MARs (matrix attachment regions). SARs and MARs are thought to insulate

sequences from the influence of surrounding chromatin presumably by binding to compounds of the nuclear scaffold and thus create separate transcriptional units. Indeed, the presence of MARs around a transgene has been reported to reduce variegation of transgene expression in stably transformed plant cells (Mlynárová et al., 1995; Mlynárová et al., 1996). Allen and coworkers (1993; 1996) have shown that expression of a reporter gene in transformed plants can also be increased when transgenes are flanked by SARs. Interestingly, this increased transgene expression (Allen et al., 1996) was only obtained when less than approximately 10 copies of the transgene had integrated, suggesting that these SARs can not protect against particular gene-silencing events that occur upon integration of more than 10 transgene copies.

These observations illustrate how strong gene-silencing events can interfere with stable and high gene expression. Thus, the necessity of understanding why and how gene-silencing phenomena are established, is clear. It appears that small details of the transformation events can be of decisive importance in determining the fate of a transgene because it has been found that in the same transformation procedure both plants containing detectable expression of the proteins and plants carrying post-transcriptional gene-silencing were obtained (Rubino et al., 1993; Rubino and Russo, 1995; Vaira et al., 1995; Pang et al., 1996; Prins et al., 1996; Prins et al., 1997a; Prins et al., 1997b; see also in Chapter 1). Also, in one transformation procedure both plants displaying transcriptional transgene silencing and plants featuring post-transcriptional transgene silencing were obtained (Chapter 6). Detailed comparisons of these plants that are genetically similarly modified but display such a different phenotype, might reveal some factors that determine the fate of a transgene which might, in the future, be utilised to regulate transgene expression.

An alternative approach to understanding gene-silencing processes, could be to identify and analyse elements that actively determine the higher order chromatin configuration at a locus (Felsenfeld et al., 1996; Van Holde and Zlatanova, 1996). These elements might contribute in establishing a chromatin configuration at a transgene locus that stimulates transcription or protects the transgene from processes that cause transgene instability. In animal systems and yeast, locus control regions and transcriptional activators have been identified (*eg* Festenstein et al., 1996; Côté et al., 1994; Tamkun, 1995; Milot et al., 1996) that can establish an open chromatin configuration and thus stimulate transcription. For plants, such elements are unknown. Neither, plant specific boundary elements have been isolated that, like in animals (*eg* Chung et al., 1993; Gdula et al., 1996), can protect genes from position effects that are regulated by chromatin complexes such as heterochromatin (Pirotta, 1996).

The model on RNA-mediated virus resistance revisited

The observations on the mechanism underlying engineered resistance against CPMV, prompted us to postulate a refined model for RNA-mediated virus resistance in Chapter 6. This model might also apply to co-suppression as it has been proven that the same mechanism can underlie both phenomena (English et al., 1996). Briefly, the model suggests that resistance acts through a mechanism, induced by and directed to the transgene mRNAs, that recognises incoming homologous viral RNAs as well. It is proposed that degradation occurs when specific, small

complementary RNA molecules (cRNAs) interact with the transgene mRNAs, or incoming viral RNAs, where upon dsRNAs could occur that putatively form a target for dsRNases. The cRNAs are proposed to be produced by a cytoplasmic, plant-encoded RNA-dependent RNA-polymerase (RdRp) on transgene mRNA molecules, that carry aberrant features and are therefore denoted aberrant RNA molecules (abRNAs). The aberrant features could comprise an unusual quantity of the RNAs or a modification of nucleotides, structure or sequence in the RNAs and these features might originate in the nucleus as a consequence of the transgene transcription rate or the genomic organisation at the transgene integration site or methylation at transgene coding sequences (Fig. 8, Chapter 6).

One of the most intruiging questions with regard to post-transcriptional silencing concerns the induction of the process. Various observations imply that the quantitative level of transgene transcription is of importance. These observations refer to intermediate resistant plants with delayed appearance or less strong symptoms (Chapter 6; Pang et al., 1996), to plants which recovered from virus infection and developed virus-free shoots fully resistant to secondary infection (Lindbo et al., 1993a; Smith et al., 1994; Goodwin et al., 1996; Beck et al., 1996), and flowers of co-suppressed plants showing various patterns in pigmentation (Jorgenson et al., 1996; Cluster et al. 1996). For several of these cases, it has been determined that post-transcriptional silencing increased as the number of transgene copies was enlarged upon selfing hemizygous plants (Goodwin et al., 1996; Pang et al., 1996; Jorgensen et al., 1996). On these grounds, it was suggested that the quantitative levels of transgene RNAs have a decisive role in inducing the RNA turnover mechanism (Lindbo et al., 1993; Smith et al., 1994), and accordingly models were proposed postulating that this specific RNA turnover starts when the amounts of RNA are above a specific threshold level. However, in other studies no correlation was found between post-transcriptional silencing and high transgene transcription rates (Mueller et al., 1995; Van Blokland et al., 1994). Thus, even though the post-transcriptional silencing process most likely holds quantitative aspects, these are presumably not the only factors that determine whether RNA turnover is induced. Besides, other aspects have been found to correlate with post-transcriptional silencing such as the organisation pattern of the transgene (Chapter 6, Van Blokland et al., 1994; Jorgensen et al., 1996; Cluster et al., 1996) and methylation at transgene sequences (Chapter 6; English et al., 1996). Detailed experiments are yet required to conclusively establish whether these factors are cause or consequence of the silencing process (Baulcombe, 1996a) and how they could cause the induction of the silencing process. Possibly, multiple mechanism for inducing RNA turnover exist which might reinforce one another and determine that transgene mRNAs become degraded.

The strongest argument that mRNA turnover is induced after a threshold level of RNA molecules is exceeded, is the recovery phenotype found for several cases of RNA-mediated virus resistance (Lindbo et al., 1993; Smith et al., 1994; Goodwin et al., 1996; Beck et al., 1996; Guo and Garcia, 1997). This phenotype refers to resistance that occurs in the higher leaves of transgenic plants only after the lower leaves have been supporting virus infection. It has been proposed that during the initial infection the replicating viral RNAs supplement the level of transgene mRNAs to the level of RNAs that is required to induce resistance. Because the recovery phenotype has never been observed for non-transgenic plants infected with these viruses, this

recovery seems to specifically occur in those transgenic plants in which a silencing process has been induced but at too low level to block viral replication. Remarkably, this recovery phenotype has so far only been observed in plants with RNA-mediated resistance against members of the potyvirus group. Potyviruses are a wide-spread family of plant viruses, they have a positive stranded RNA genome that is encapsidated to flexuous filamentous particles. During potyvirus infections both in the cytoplasm and in the nucleus inclusion bodies can be formed of which the cytoplasmic inclusion bodies have a very characteristic structure, denoted pinwheels (reviewed by Van der Vlugt, 1993). So, it can be envisaged that other factors than the potyviral RNA molecules contribute to the silencing induction of the silencing process, which could be features or factors specific for potyviral infections. Developmental and environmental aspects have been reported to be able to influence silencing processes (Pang et al., 1996; Flavell et al., 1995), so possibly, potyvirus infections result in specific cellular conditions that assist or stimulate the RNA turnover mechanism. Also, the plasmodesmatal modifications induced by the putative potyviral MPs might enable cell to cell translocation of factors involved in the specific RNA turnover after which the cellular amounts of these factors could be increased. Whatever the contribution of these viruses to the silencing process, the stable resistant state of the recovered tissue which has not been in contact with the virus, points to a cellular feedback mechanism and an imprinting which determines that in all new cells resistance occurs without further potyviral contribution. In the model presented in Chapter 6, RNA-directed DNA-methylation (RdRM; Wassenegger et al., 1994; Saenger et al., 1996) has been suggested as a candidate for this feedback process. Thus, another possibility could be that specifically during potyvirus infections such a feedback process occurs with high efficiency.

Once post-transcriptional turnover of the transgene mRNAs has been induced and established in the cells of a plant, degradation of incoming, homologous viral RNAs can only occur if the viral molecules are accessible for the proposed cRNAs. RNA replication intermediates, but for some viruses (like tospoviruses) also the genomic RNAs, are assumed to occur at most time associated with proteins which could severely reduce the accessibility for an RNA degradation mechanism. The results presented in Chapter 6 suggest that for potato virus X (PVX) both the genomic RNAs, the plus-strands, and the replication intermediates, the minus-strands, are accessible for the degradation process since resistance to recombinant PVX in sense MP gene transgenic plants, is independent of whether CPMV sequences are inserted into the PVX genomes in the sense or in the antisense orientation. At the other hand, it has not been excluded that besides cRNAs also ccRNAs, *i.e.* RNA molecules complementary to the cRNA molecules, can be produced by the RdRp upon using the cRNAs as templates. In that case in the sense MP transgenic plants, cRNAs could be responsible for degradation of plus-strands of recombinant PVX carrying MP gene sequences in the sense orientation and the ccRNAs for the degradation of plus-strands of the PVX recombinants with the CPMV sequences in the antisense orientation, while the PVX minus-strands will not be affected. The possible occurrence of such ccRNAs is also suggested from the results by Prins et al. (1996), who observed that RNA-mediated resistance against the negative stranded tospovirus tomato spotted wilt virus (TSWV) only occurs for sequences corresponding to genes of which the expression is essential for virus multiplication in plants. This result suggests that not the viral genomes as such, which are for

this tospovirus at most times covered with nucleocapsid proteins, are degraded by the resistance mechanism because in that case transgenic expression of all viral genes should result in resistance. It rather appears that the viral mRNAs are the target of the degradation process because resistance is only occurring for transgenes that correspond to TSWV genes, which produce mRNAs encoding proteins essential for viral multiplication in the plant. Strikingly, transgenes carrying sequences corresponding to these viral mRNAs both in the sense and in the anti-sense orientation were able to induce RNA-mediated resistance. As explained above, this suggests the presence of both ccRNAs and cRNAs.

A further question is how essential the integration of the transgene into the genome of the plant is for establishing the mechanism. In *N. benthamiana* plants infected with recombinant TMV genomes carrying sense-oriented sequences corresponding to a region of the phytoene desaturase (PDS) gene, in the noninoculated higher situated leaves bleaching occurred (Kumagai et al., 1995), and in the bleached tissues the accumulation of endogenous PDS mRNAs was strongly decreased. This indicated that specific cytoplasmic turnover of PDS sequences was taking place and that for the induction of this process only replication of the recombinant TMV genomes but no genomic integration of a transgene was required. It was not reported whether also the recombinant TMV genomes were affected by the turnover process. In *N. benthamiana* plants infected with wild-type TMV genomes, no degradation of TMV RNAs was observed in the higher systemically infected leaves. These features resemble aspects of the recovery phenotype observed for transgenic plants carrying potyviral genes as described above (Lindbo et al., 1993; Smith et al., 1994; Goodwin et al., 1996; Beck et al., 1996), in particular as recovery is established in the systemic leaves and it does not occur when wild-type plants are infected with (wild-type) virus. This could indicate that genomic DNA homologs, of the silenced RNAs need to be present, either in the form of endogenous genes (like the PDS gene) or as transgenes (like the potyvirus genes) for effective, stable RNA turnover to occur. Both the efficiency and the timing of RNA degradation could be more effective if the DNA homologs are present. As for the recovery phenotype, this implies that in the plants infected with recombinant TMV genomes carrying PDS sequences, a feedback signal goes from the RNAs in the cytoplasm to the DNA homologs, the PDS gene, in the nucleus. Another possibility could be that the wild-type TMV genomes do not trigger induction of the RNA turnover mechanism, while the recombinant TMV genomes do stimulate the process, because the RNAs that carry the PDS sequences serve as abRNAs which provide efficient templates for the proposed RdRp.

The model presented in Chapter 6 requires the presence and functioning of various enzymes and the identification of these enzymes and their possible role could be an objective of further research on the mechanism of the RNA turnover. Plants have been shown to contain an RdRp, which has been purified (Schiebel et al., 1993a; Schiebel et al., 1993b), but the function of the protein in the plant has remained unclear. It has been shown in cowpea that upon a CPMV infection the host RdRp activity is increased at least 20-fold and can, in inoculated leaves, represent 95% of the total RdRp activity, leaving 5% for the viral RdRp (Van der Meer et al., 1983; Van der Meer et al., 1984; Dorssers et al., 1982). Yet, the host RdRp is not involved in viral RNA replication. Neither did the increased RdRp activity result in resistance to the viral pathogen. Cloning of the RdRp gene would enable more direct approaches to determine

whether the RdRp indeed has a role in post-transcriptional silencing. In the model it is proposed that the RdRp generates cRNA molecules. Thus, it can be examined whether there are indeed such cRNAs, which should especially exist for the region of a transgene that is the primary target of the RNA degradation process (Chapter 6, English et al., 1996). Another enzyme proposed to have a crucial in the RNA turnover process is the dsRNase and it could be attempted to purify the dsRNase and identify the corresponding gene. Otherwise, mutational analyses or tagging experiments could be started to revert resistant plants to a sensitive state and identify the genes involved. It is conceivable, however, that disruption of genes involved in this process will strongly affect plant growth, because the gene products, like DNA-methylases (Ronemus et al., 1996) or the RdRp or the dsRNase, probably have important roles in plant development.

The phenomenon of post-transcriptional gene-silencing; contemplations on why would it exist

The process of post-transcriptional gene-silencing that we have come across in the research described in this thesis, is a cytoplasmic mechanism of very efficient turnover of specific RNA sequences. The process was discovered in transgenic plants in which sequences had been introduced into the genome. However, it seems rather unlikely that this process only occurs for introduced transgenic sequences (Matzke and Matzke, 1995a). Thus, intriguing questions arise like, when, why and how, post-transcriptional RNA turnover may happen for naturally occurring RNA molecules. It seems not so likely that the process should be regarded as a general mechanism for controlling stability (reviewed by Ross, 1996), and in that way the cellular amounts of mRNAs of endogenous genes, because the mechanism degrades RNA molecules with high specificity. Furthermore, the RNA degradation acts very drastically and reduces steady state mRNA levels very strongly, which seems a too radical approach for regulating generally the abundance of endogenous mRNAs.

Interestingly, post-transcriptional silencing has only been found in plants and possibly also in fungi (Romano and Macino, 1992; Cogoni et al., 1996), but has not been reported for animals. Maybe, post-transcriptional silencing is a different approach to a surveillance system, like the animal immune system, that eliminates specifically, drastically and efficiently foreign elements. It is clear that the way in which these two processes operate, is very different. Post-transcriptional silencing acts in the cytoplasma against RNA molecules, and thus this process can only tackle pathogens that invade the cytoplasm, like for instance viruses. Support for this hypothesis can be found in the observation that kohlrabi plants carry a natural resistance against cauliflower mosaic virus which involves recovery from viral infection which is associated with post-transcriptional degradation of the viral RNAs (Covey et al., 1997). Also, this hypothesis might explain the occurrence of non-host relationships between plants and viruses or the presence of not-infected patches in the mosaic patterned symptoms that accompany many virus infections.

Alternatively, it can be speculated that post-transcriptional gene-silencing is one of the mechanisms of complex organisms to specifically reduce expression of inappropriate genes. As pointed out by Bird (1995), an increased biological complexity which coincides with an

increased gene number, should be accompanied by an increased efficiency of repression of inappropriate genes. Thus, he compared processes and components occurring in organisms of different biological complexity and proposed for several features that they could function to repress inappropriate genes. The nuclear envelope could for instance be considered as a filter that prevents that spurious transcripts become translated. Furthermore, an important function of histones and chromatin structure could be to prevent or inhibit the transcription of inappropriate genes. Transcriptional repression of inapproriate genes could also occur from DNA methylation at regulatory promoter elements. In line of this discussion, post-transcriptional gene-silencing might be regarded as an additional way to regulate the expression of inappropriate endogenous genes.

Post-transcriptional silencing as a general approach to control virus diseases?

The engineered resistance against CPMV described in this thesis illustrates well how effectively virus infections can be resisted in genetically modified plants by utilising the post-transcriptional gene-silencing process. Despite the great potential, transgenic plants meet with difficulties in public acceptance, partly for rational reasons. New virus diseases might be generated (Falk and Bruening, 1994) for instance upon recombination between a viral genomic RNA molecule and a transgenic mRNA molecule (Schoelz and Wintermantel, 1993; Green and Allison, 1994). Also, transgenically expressed RNAs might spontaneously change to pathogenic variants, as shown with the cucumber mosaic virus satellite RNA (Palukaitis and Roossinck, 1996). Proteins expressed from a transgene might stimulate the infection process of a heterologous virus by for instance complementation of the transport functions or the encapsidation in a new coat. All these risks are minimal when virus resistance is engineered through a post-transcriptional RNA turnover process because with this approach the transgenic mRNAs are not replicating, they do not have to encode a protein and the steady state levels of transgene mRNAs are very low which minimises the possibilities of recombination events.

Since, RNA-mediated resistance has great potential for engineering resistance to major virus diseases in crops, it would be useful to identify strategies by which post-transcriptional gene-silencing is induced with high efficiency. This would be especially of importance in controlling viral diseases in plant species that are not highly competent for regeneration and transformation, like for instance grain legumes (Chapter 2). The results described in Chapter 6 indicate that introduction of a transgene with directly repeated (CPMV MP gene) sequences increases the frequency at which resistant lines occur strongly (from 20% to 60%) compared to transformation of a transgene containing a single CPMV MP gene. Thus, transformation of directly repeated transgenes might be a general approach for increasing the efficiency of the induction of resistance. However, for other transgenes other genomic organisations might be more effective; post-transcriptional silencing of a chalcone synthase gene in petunia has been found to specifically correlate with invertedly repeated arrangement of integrated transgene sequences (Van Blokland et al., 1994). As it might be time-consuming to establish for each transgene the most effective organisation of integration, it may be more effective to try to

exploit the known abilities of directly repeated CPMV MP gene sequences. It could be examined whether transgenes that contain directly repeated CPMV MP gene sequences that are intervened with sequences of another gene, are able to induce efficiently not only RNA turnover of the MP gene sequences but also of sequences homologous to the gene placed between the MP gene sequences. If this approach would prove successful, a general 'knock-out' vector could be developed which would be a binary vector, comprising a 35S promoter, directly repeated CPMV MP gene sequences, several unique restriction sites, again directly repeated CPMV MP gene sequences and a nos-terminator. The optimal number of direct repeats, the optimal length of repeated MP gene sequences and the optimal length of the sequence of the inserted gene should of course be experimentally established. Such a vector could be a useful tool both for engineering RNA-mediated resistance against many viruses and for studies on plants in which the effects of suppression of a specific gene or sequence with unknown function are investigated.

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SUMMARY

Plant viruses are interesting pathogens because they can not exist without their hosts and exploit the plant machinery for their multiplication. Fundamental knowledge on viral processes is of great importance to understand, prevent and control virus infections which can cause drastic losses in crops. In this thesis, cowpea mosaic virus (CPMV) was studied. This virus consists of two, icosahedral particles that each carry a distinct single stranded RNA molecule of positive polarity. Several years of research have revealed much information on the genomic organisation, the strategy of gene expression and the multiplication processes of CPMV, which are described in Chapter 1, but also many aspects remain to be elucidated.

To study individual viral processes, like replication, encapsidation or cell to cell movement, transgenic plants can be generated that express individual viral genes like the replicase, coat protein or movement protein gene. A prerequisite in this approach is the presence of an efficient and reliable plant regeneration and transformation system. CPMV's natural host is the tropical grain legume cowpea, Vigna unguiculata, a plant species that is recalcitrant at regeneration. Although in experiments described in Chapter 2 fertile plants could be regenerated from nodal thin cell layer segments, the explants were not competent for Agrobacterium-mediated transformation. Possibly in further studies, these nodal explants could prove suited for another transformation method.

Therefore, tobacco, which is also a host for CPMV and highly competent for regeneration and transformation, was preferred as the species to generate transgenic plants carrying CPMV specific genes. Especially the CPMV movement proteins (MP) genes appealed to us for overexpression studies. CPMV cell to cell movement is enabled by the CPMV MPs that act to modify plasmodesmata. They are assumed to channel plasmodesmata with MP-containing tubular structures and through or with these tubules virus particles are transported to adjacent cells. To obtain more information on the plasmodesmatal modifications brought about by the MPs, transgenic tobacco plants were generated that carried the MP gene under the control of either a constitutive or an inducible 35S promoter. However, in none of these plants the MPs were expressed to detectable levels (Chapter 3). Using the potato virus X (PVX)-based expression vector, accumulation of CPMV MPs was observed in the form of tubular structures extending from the surface of infected protoplasts into the medium. These PVX-derivatives look promising for providing effective tools in future studies on the effects of the CPMV MPs in plants.

Studies on MP functioning could involve complementation experiments with a CPMV mutant that is defective in cell to cell movement. In experiments described in Chapter 4 is was analysed by a molecular approach whether the CPMV mutant N123, that was first described in 1976, could be used to this effect. As the basis of the N123 specific phenotype was found not only to rest in the movement protein gene but also in one of the two coat protein genes, this mutant seemed not very suitable for complementation studies. Presumably a recently developed CPMV mutant in which the MP gene has been replaced by the fluorescent marker protein GFP (green fluorescent protein), will be a more appropriate tool.

Transgenic Nicotiana benthamiana plants that were expressing either the CPMV MP or the replicase gene under the control of a constitutive promoter, were found to exhibit a resistant phenotype when inoculated with CPMV (Chapter 5). Protoplast studies revealed that the

resistance occurred as full immunity and was maintained in the cell. Resistance was specific to viruses highly homologous to CPMV, and in addition it was found to be specifically directed against the replication of the CPMV segment of which the transgene was derived (Chapter 5). Pathogen derived resistance can be mediated either by the protein encoded by the transgene or by the transcribed mRNA. Protein-mediated resistance generally offers moderate protection against a broad range of viruses, while RNA-mediated resistance results in immunity at the cellular level. Resistance obtained in transgenic plants transformed with defective genes confirmed that an RNA-based mechanism was underlying the highly specific transgenic resistance against CPMV (Chapter 6).

Specifically in the resistant lines, the transgene mRNA steady state levels were low compared to the relative transgene nuclear transcription rates (Chapter 6). This indicated that resistance occurs from a specific, cytoplasmic RNA turnover mechanism. This process can be regarded as a post-transcriptional gene-silencing process, that is primarily induced on the transgene mRNAs but to which also incoming, homologous CPMV genomes fall victim. In addition, heterologous RNA molecules, like PVX genomes, that contain the sequences corresponding to the transgene, are eliminated (Chapter 6). By inserting sequences homologous to only parts of the transgene in the genome of PVX and studying the fate of these recombinant genomes, it was shown that the degradation process is primarily targeted to a defined region of the transgene mRNA, the 3' region. Further analyses revealed that degradation can occur at various sites within this 3' region and that not a specific sequence or structure is of predominant importance. We observed that small inserts, like of only 60 nucleotides, can tag recombinant PVX molecules for the elimination process, albeit with reduced efficiency, which suggested that the RNA turnover process carries quantitative features.

On the intruiging question why post-transcriptional gene-silencing is induced in only some of the transgenic lines, we revealed (Chapter 6) that the organisation of integrated transgene sequences has an important role. Transformation with a transgene containing a directly repeated MP gene, increased the frequency at which resistant lines arise to 60%, compared to 20% of resistant lines that occur upon transformation with a transgene with a single MP gene. Thus, the resistance process seems influenced by qualitative features of the integrated transgenes. Also, it was observed that resistance concurred with extensive methylation at the transcribed transgene sequences (Chapter 6), which could indicate an essential role of methylation at transcribed sequences in obtaining RNA-mediated pathogen derived resistance.

From these observations and from data described in literature, a model for RNA-mediated virus resistance was made and presented in Chapter 6. In Chapter 7, the post-transcriptional gene-silencing phenomenon is discussed in more details and in addition an approach is presented by which the process could be exploited to efficiently engineer virus resistance or study plant gene expression.

SAMENVATTING

Planten kunnen tijdens hun groei aangetast worden door allerlei ziektenverwekkers, waardoor aanzienlijke schade kan optreden in een gewas. Plantenvirussen vormen een speciale klasse van ziektenverwekkers die niet kunnen bestaan zonder hun gastheer. Virussen zijn opgebouwd uit één of enkele strengen genetisch materiaal welke worden omgeven door een beschermende mantel van eiwit. Het genetisch materiaal omvat enkele genen, die elk zorg dragen voor de regulering van een gedeelte van de levenscyclus van het virus. Virussen gebruiken voor hun vermeerdering de bouwstenen van de plant in plaats van deze zelf te synthetiseren en feitelijk reguleren virussen dus enkel dat hun vermenigvuldiging door de plantecel wordt uitgevoerd. Bij het bestuderen van plantenvirussen en infecties door deze pathogenen kan derhalve de rol van de plant niet buiten beschouwing worden gelaten. Wanneer men in een plant het functioneren van afzonderlijke virale genen in detail wil bestuderen, dan kunnen plantecellen getransformeerd en vervolgens geregenereerd worden tot een transgene plant die in het genoom een integratie heeft van een enkel viraal gen. Deze benadering van onderzoek staat in detail beschreven in Hoofdstuk 1. Om dergelijke planten te verkrijgen, dient men niet alleen beschikking te hebben over het geïsoleerde virus gen, maar ook over een methode om uit een enkele plantecel een nieuwe, transgene plant te regeneren. Vervolgens dient de transgene plant dan het virale gen stabiel en in sterke mate tot expressie te brengen.

Het virus dat gebruikt werd in de studies die beschreven staan in dit proefschrift, is het cowpea mozaiek virus, afgekort CPMV. De natuurlijke gastheer van CPMV is cowpea (latijnse naam Vigna unguiculata). Cowpea is in de tropen, vooral in Afrika, een belangrijk voedselgewas en de planten zijn goed aangepast aan de zware tropische groeiomstandigheden. Een CPMV infectie veroorzaakt op de bladeren van deze planten een mozaiekpatroon en deze symptomen kunnen de groei van de cowpea planten sterk belemmeren en de opbrengst van cowpea bonen flink reduceren. CPMV is een virus waarvan de genetische informatie verdeeld is over twee segmenten die afzonderlijk zijn verpakt in een identieke eiwitmantel. Deze twee segmenten bestaan uit RNA molekulen en deze RNA ketens zijn van een zogeheten positieve polariteit, wat betekent dat ze direct als boodschapper RNAs kunnen dienen voor de synthese van de diverse virus specifieke eiwitten. CPMV codeert eiwitten die drie typen functies hebben in de levenscyslus van het virus, te weten de vermenigvuldiging ofwel replicatie van de virale RNA ketens, het inpakken van deze genomen in de eiwitmantels en het transport van de virusdeeltjes door de plant waardoor de infectie zich uit kan breiden naar nog niet geïnfecteerde cellen. Tussen plantecellen zijn kanalen aanwezig, plasmodesmata, waardoor uitwisseling van stoffen kan plaatsvinden. Deze plasmodesmata zijn echter niet wijd genoeg om virus door te laten. Daarom coderen plantenvirussen speciale eiwitten, zogenaamde transporteiwitten, die ervoor zorgen dat de plasmodesmata aangepast worden zodat cel naar cel transport van het virus kan plaatsvinden. Eerder onderzoek heeft aangetoond dat de transporteiwitten van CPMV kunnen assembleren tot buizen, die door plasmodesmata kunnen heensteken en waardoorheen virusdeeltjes vervoerd kunnen worden. Het aanvankelijke doel van het onderzoek beschreven in dit proefschrift was om de modificaties van plasmodesmata die door deze transporteiwitten van CPMV bewerkstelligd worden, in detail te bestuderen door enkel het transporteiwitgen van dit virus in transgene planten tot expressie te brengen.

Omdat cowpea de natuurlijke gastheer van CPMV is, ging in eerste instantie de voorkeur uit naar het transformeren van deze plant met het transporteiwitgen. Het regeneren van een nieuwe plant uit een enkele (getransformeerde) cel is echter voor cowpea, net als voor vele anderen vlinderbloemigen, een moeizaam proces. Derhalve werd geprobeerd om een efficiënte transformatie en regeneratie methode voor cowpea te ontwikkelen. Hoewel enige voortgang werd geboekt, hetgeen staat beschreven in Hoofdstuk 2, slaagden we er niet in een bruikbaar protocol te ontwikkelen.

Daarop werd besloten een andere plantensoort te gebruiken voor het genereren van transgene planten. Omdat tabaksplanten, in het bijzonder van de soort Nicotiana benthamiana, zowel een goede gastheer zijn voor CPMV als ook zeer gemakkelijk te transformeren, werd deze plantensoort gekozen. Er werden transgene tabaksplanten gemaakt waarin de expressie van het CPMV transporteiwitgen ofwel werd gereguleerd door een zogeheten constitutieve promoter die vrijwel in alle cellen van de plant gedurende de gehele groeiperiode aktief is, ofwel door een induceerbare promoter die alleen aktief is indien een bepaalde stof, in dit geval tetracycline, aanwezig is. Echter, in geen van deze transgene planten kon ophoping van het transporteiwit worden aangetoond. Daarom werd er nog een andere benadering uitgeprobeerd om toch expressie van het CPMV transporteiwitgen in planten te verkrijgen. Deze methode betrof expressie vanaf het genoom van een ander, heteroloog, virus, te weten het aardappel X virus (in het engels potato virus X, afgekort PVX), dat gemodificeerd was zodat het PVX genoom het CPMV transporteiwitgen bevatte. Deze methode bleek zeer effectief en resulteerde in duidelijk waarneembare ophoping van de CPMV transporteiwitten, hetgeen staat beschreven in Hoofdstuk 3. De op deze manier geproduceerde CPMV transporteiwitten bleken prima in staat de bovengenoemde buizen te assembleren, maar door tijdgebrek kon niet meer worden onderzocht of deze buizen ook inderdaad biologisch aktief zijn, in de zin dat ze het cel naar cel transport van CPMV mutanten die deficiënt zijn voor deze functie, kunnen complementeren.

Voor dergelijke complementatie studies dient men uiteraard te beschikken over een CPMV mutant die niet in staat is tot virus transport en waarvan het defect enkel wordt veroorzaakt door mutaties in het transporteiwitgen. In het onderzoek beschreven in Hoofdstuk 4, werd bestudeerd of de CPMV mutant N123 geschikt was voor dit doel. Hiertoe werd van deze mutant het gedeelte van het genoom dat de virale eiwitten codeert die van belang zijn voor virus transport, geanalyseerd op het moleculaire niveau. Hieruit bleek dat mutant N123, in vergelijking met CPMV van het wilde type, verscheidene mutaties in dit deel van de RNA ketens bevatte welke na vertaling ook resulteren in veranderingen in de virale eiwitten. Door deze mutaties afzonderlijk en gecombineerd te analyseren, bleek dat bij het defecte virus transport van N123 zowel mutaties in het transporteiwitgen als in een manteleiwitgen een rol spelen. Hoewel dit een interessante waarneming is die aangeeft hoe complex het proces van virus transport is, bleek hierdoor mutant N123 niet geschikt voor complementatie studies. Gelukkig werd er recentelijk in onze onderzoeksgroep een mutant ontwikkeld, welke zeer goed geschikt lijkt te zijn voor deze studies omdat hierin het transporteiwitgen is vervangen door een gen dat codeert voor een fluorescerend markereiwit.

Zoals eerder genoemd, werden er verscheidene transgene tabaksplanten gemaakt die het CPMV transporteiwitgen bevatten, onder de controle van een constitutieve promoter. Ook werden er transgene planten gemaakt die andere CPMV genen bevatten zoals het replicatiegen en de

manteleiwitgenen. Hoewel in geen van deze planten de CPMV eiwitten ophoopten tot detecteerbare hoeveelheden, was er in een flink aantal van deze planten toch een effect van het CPMV transgen waar te nemen. De planten bleken namelijk resistent te zijn tegen infectie met CPMV, en dit staat beschreven in Hoofdstuk 5. In de literatuur was al eerder melding gemaakt van het verkrijgen van virusresistentie door planten te transformeren met genen van hetzelfde virus. Ook is gebleken dat dit soort resistentie kan voortkomen uit twee verschillende processen, waarvan het ene resistentieproces werkt via het virale eiwit dat in de transgene planten tot expressie komt, en het andere door de boodschapper RNA molekulen die overgeschreven worden van het geïntroduceerde gen. Om te bestuderen welk mechanisme in onze transgene planten tot resistentie leidde, werd via een nieuwe reeks transgene planten bestudeerd of het nodig was dat de transgene boodschapper RNA molekulen vertaald konden worden in functionele CPMV eiwitten. Het bleek dat de aanwezigheid van de transgene RNA molekulen zelf voldoende was om resistentie te bewerkstelligen en daarmee valt deze resistentie onder RNA-gebaseerde resistentie. Een belangrijk kenmerk van dit type resistentie is dat de resistentie helemaal volledig is en er in geen enkele cel enige virusvermeerdering plaatsvindt. Wel is de resistentie alleen effectief tegen infecties met virussen die sterk verwant zijn aan het virus waarvan het transgen is afgeleid.

Hoewel verschillende gevallen van dit type resistentie reeds eerder zijn beschreven, is het nauwelijks bekend hoe het resistentieproces op gang wordt gebracht en hoe het precies functioneert. Daarom zijn we deze resistentie in detail gaan karakteriseren in onze resistente planten en de resultaten van deze proeven staan beschreven in Hoofdstuk 6. Het bleek dat de resistentie het gevolg is van een mechanisme dat, in het cytoplasma van de cel, selectief en zeer efficiënt de transgene boodschapper RNA molekulen afbreekt. Binnendringende virale RNA ketens, waarvan gedeeltes dezelfde basevolgorde hebben, worden ook door dit mechanisme herkend en afgebroken, waardoor virusvermeerdering wordt voorkomen. Het is verwonderlijk dat zo'n proces plaatsvindt omdat het inefficiënt lijkt dat RNA molekulen eerst geproduceerd worden en dan heel efficiënt worden afgebroken. De hoge mate van selektiviteit van het afbraakproces geeft wellicht aan dat dit proces dient om, in een later stadium, de productie van speciale, vreemde of verkeerde RNA molekulen te corrigeren. Vervolgens werden proeven gedaan om te proberen te begrijpen waarom dit proces alleen in sommige van de transgene planten op gang wordt gebracht, en in andere niet. Een aantal aspecten van het transgen bleken te correleren met het aanwezig zijn van resistentie. Zo werden in de resistente planten in het algemeen in de kern meer boodschapper RNA molekulen van het transgen overgeschreven. Tevens waren in de resistente planten meerdere kopieën van het transgen geïntegreerd in het plantegenoom, steeds als een tweevoudige, gelijkgeörienteerde integratie van het transgen op één insertieplaats. Toen met opzet een gelijkgeörienteerde duplicatie van het CPMV transporteiwitgen werd geïnserteerd in een nieuwe serie transgene planten, bleek de frequentie van resistente planten sterk toe te nemen. Verder bleek dat in de resistente planten de DNA molekulen van de geïntegreerde transgenen gemodificeerd waren en extra methylgroepen bevatten. Deze waarnemingen wijzen erop dat eigenschappen van het transgen in de kern van grote invloed zijn op het bewerkstelligen van cytoplasmatische afbraak van transgene boodschapper RNAs en tevens van binnenkomende virale RNAs als die gedeeltes met dezelfde basevolgorde bevatten.

Hoewel we nog verre zijn gebleven van het geheel begrijpen van het resistentiemechanisme, stellen we een model voor dat beschrijft hoe dit op RNA-gebaseerde resistentieproces zou kunnen worden geïnduceerd en hoe specifieke cytoplasmatische afbraak van de transgene boodschapper RNAs en de virale RNAs tot stand zou kunnen komen. Het is een boeiende vraag of dit proces, dat tot op heden alleen nog is waargenomen in transgene planten, ook optreedt gedurende groei en ontwikkeling van normale planten en welke functie het dan heeft. In Hoofdstuk 7 wordt hierover gefilosofeerd. Wat de normale functie van dit proces ook moge zijn, het is zeer effectief voor het bewerkstelligen van resistentie tegen virusinfecties in het laboratorium en daarmee kan het wellicht een zeer bruikbare methode zijn om ook verschillende landbouwgewassen te beschermen tegen virusziektes.

CURRICULUM VITAE

Titia Sijen werd als Laetitia Maria Theodora Sijen geboren te Sittard op 26 september 1967. Na het behalen van het Gymnasium β diploma aan de Scholengemeenschap "Serviam" te Sittard in 1985, ging zij in hetzelfde jaar plantenveredeling studeren aan de Landbouwuniversiteit Wageningen. Tijdens deze studie voerde zij een stage uit voor de vakgroep plantenveredeling op het International Crops Research Institute for the Semi-Arid Tropics te Hyderabad, India, onder de begeleiding van dr. H. van Rheenen en dr. D.V.R. Reddy. De afstudeervakken betroffen moleculaire virologie (dr. R. Goldbach en dr. P. de Haan), moleculaire fytopathologie (dr. P. de Wit en dr. J van Kan) en moleculaire genetica (dr. M. Koornneef), waarvan deze laatste werd uitgevoerd op het Institut für Genbiologische Forschung te Berlijn, onder begeleiding van dr. L. Willmitzer. In augustus 1991 studeerde zij af en begon als toegevoegd onderzoekster op de vakgroep moleculaire biologie van de Landbouwuniversiteit Wageningen aan een onderzoek naar regeneratie en transformatie van cowpea, dat echter niet langer dan 7 maanden gefinancierd kon worden. Daarom startte ze in maart 1992 als OIO op dezelfde vakgroep een promotieonderzoek, gefinancierd door de afdeling Scheikundig Onderzoek Nederland van de Nederlandse organisatie voor Wetenschappelijk Onderzoek. Dit onderzoek heeft geleid tot dit proefschrift getiteld 'Expression and silencing of cowpea mosaic virus transgenes' Sinds september 1996 is ze als wetenschappelijk medewerkster werkzaam aan de afdeling genetica van de Vrije Universiteit Amsterdam in de groep van dr. J. Kooter en dr. J. Mol aan een project aangaande het ontwikkelen van strategieën om voorspelbare en stabiele expressie van transgene te bereiken en dit project wordt gefinancierd door de Stichting Technische Wetenschappen.