OCCURRENCE OF *CUCUMBER MOSAIC VIRUS* IN ORNAMENTAL PLANTS AND PERSPECTIVES OF TRANSGENIC CONTROL

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To my Mom, my wife, my daughter and

In memory of my Dad

Preface

On the very first day, February 15, 1998, when I arrived in the Netherlands, the sun was shining and the temperature was 15˚C. Dr. Huub Löffler picked me up at Schiphol and on the way to Wageningen he told me: “it’s exceptional, the weather”. And, like the weather that day, the five years following it have been EXCEPTIONAL in my life.

It is very gratifying to conclude the time spent in pursuing my PhD by acknowledging the people whose invaluable contributions have helped accomplishing this work.

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do hope Lam and Ah-Chin's restaurant will rise from its ashes as the phoenix did.

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Last but not least, I thank my wife Yih-Yun and my daughter Shyu-Ann for everything they have done for me during this EXCEPTIONAL period of time in our lives. Bedankt, mij liefje en schatje.

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Contents

Chapter 1

Introduction

With rapid international expansion of trade, horticultural production of ornamental plants has become a global industry with commercial production of flowers and bulbs in many countries (Table 1.1). Many of the ornamental crops are asexually propagated and a few major propagators supply most of the plants to growers over very large geographic areas. These conditions make the ornamental crop production industry conducive to the proliferation and distribution of diseases. Indeed, ornamental crop production has frequently been affected by various kinds of plant pathogens including viruses (Loebenstein *et al.*, 1995). Not only have virus diseases caused severe losses to the ornamental crop industry itself, but ornamental crop plants and plant materials have also served as a source of introducing viruses into other crops and other countries. Unlike most fungal and bacterial diseases, which can effectively be controlled by chemical pesticides, control of virus diseases has focused on producing virus-free propagation materials. For this purpose it has been necessary to set up elaborate and costly virus screening programs linked to certification of export products by national and international authorities.

The use of disease-resistant cultivars is commonly used as a strategy for controlling plant viruses in many crop species. Though successful to limit the crop losses, this has not always led to virus-free propagation of plants as they are tolerant rather than resistant to viral diseases and certification remains necessary. A second drawback of conventional plant breeding strategies for general use in ornamental crops is the diversity of involved plant species, the overriding importance of horticultural and quality traits, and the difficulty in identifying virus resistance genes. Hence, novel approaches, that are generally applicable in breeding for resistance to viruses in ornamental plants, such as pathogen-derived resistance (PDR), have been developed since the concept had been proposed by Sanford and Johnston (1985). To date, many PDR approaches have been successfully developed for a multitude of transformable crop plants.

Table 1.1. The top 10 ornamental crops for cut flowers in some areas

	Netherlands	Taiwan	United States
1	Rose	Chrysanthemum	Rose
2	Tulip	Rose	Gladiolus
3	Chrysanthemum	Gladiolus	Lily
4	Gerbera	Lily	Iris
5	Lily	Gerbera	Tulip
6	Alstroemeria	Carnation	Gerbera
7	Freesia	Dahlia	Snapdragon
8	Carnation	Anthurium	Delphnium
9	Iris	Tuberose	Carnation
10	Gypsophila	Lisianthus	Alstroemeria

CMV infections in ornamental crops

Many important ornamentals fall into the broad host range *of Cucumber mosaic virus* (CMV), which is known to infect more than 1000 species of plants. These include alstroemeria, anemone (*Anemone coronaria*), aster (*Aster* spp.), crocus (*Crocus* spp.), dahlia, freesia, gladiolus, hippeastrum (*Amaryllis*), hyacinth, iris, lily (*Lilium* spp.), narcissus, nerine (*Nerine bowdenii* and *N. sarniensis*), tulip, *phlox (Phlox drummondii*), orchids (*Dendrobium* spp.), carnation (*Dianthus caryophyllus*), lisianthus (*Eustoma granduflorum*), asclepias (*Asclepias syriaca* and *A. tuberosa*), geranium (*Pelargonium hortorum*), impatiens (*Impatiens wallerana*), hydrangea (*Hydrangea macrophylla*), African daisy (*Dimorphotheca sinuata*), Calla lilies (*Zantedeschia* spp.), sweet william (*Dianthus barbatus*), cyclamen (*Cyclamen persicum*) and begonia (*Begonia tuberhybrida* cv. Multiflora) (Loebenstein *et al.* 1995). Although single infection of ornamental crops by CMV is often not a major limiting factor in producing plants such as alstroemeria, lily and tulip, cases of complex infection with CMV and other viruses are common and result in severely enhanced symptom expression.

Cucumber mosaic virus

One of the most widespread viruses in ornamental crops *is Cucumber mosaic virus* (CMV). CMV is the type species of the *Cucumovirus* genus in the family of *Bromoviridae* (Rybicki, 1995; van Regenmortel *et al.*, 2000), of the superfamily of alphavirus-like viruses (Goldbach, 1987; Koonin and Dolja, 1993). CMV was first reported in 1916 as the causal agent of plant diseases (Doolittle, 1916). It has a broad host range, a worldwide distribution and a severe impact on cultivated crops. These characteristics make it one of the economically most important plant viruses in commercially grown crops (Palukaitis *et al.*, 1992). CMV expresses three genomic RNAs, designated RNA 1, RNA 2, and RNA 3, and at least two subgenomic RNAs, RNA 4 and RNA 4A, which are transcribed from the 3' portions of RNA 3 and RNA 2, respectively (Ding *et al*, 1994; Peden and Symons, 1973) (Fig. 1.1). RNAs 1 and 2 encode the proteins of the replication complex, while RNA 3 encodes the movement protein (MP) (Suzuki *et al.*, 1991) and the coat protein (CP) (Schwinghamer and Symons, 1977). Subgenomic RNA 4 allows the translation of the CP, while the RNA 2-derived RNA 4A encodes the 2b protein that is involved in the suppression of gene silencing, long-distance movement, and expression of systemic symptoms (Ding *et al.*, 1995; Brigneti *et al.*, 1998). Based on phylogenetic analysis of the CP ORF and rearrangements in the 5' nontranslated region (NTR) of RNA 3, CMV strains can be divided into three subgroups: IA, IB, and II (Anderson *et al.*, 1995; Palukaitis and Zaitlin, 1997; Quemada *et al.*, 1989; Roossinck *et al.* 1999). More recently, a complete phylogenetic analysis of nucleotide sequences of 15 CMV strains showed that the tree estimated for ORFs located on the different RNAs were not congruent and did not completely support the subgrouping indicated by the CP ORF. This indicates that different RNAs may have independent evolutionary histories and reassortment plays an important role in the evolution mechanism of CMV (Roossinck, 2002).

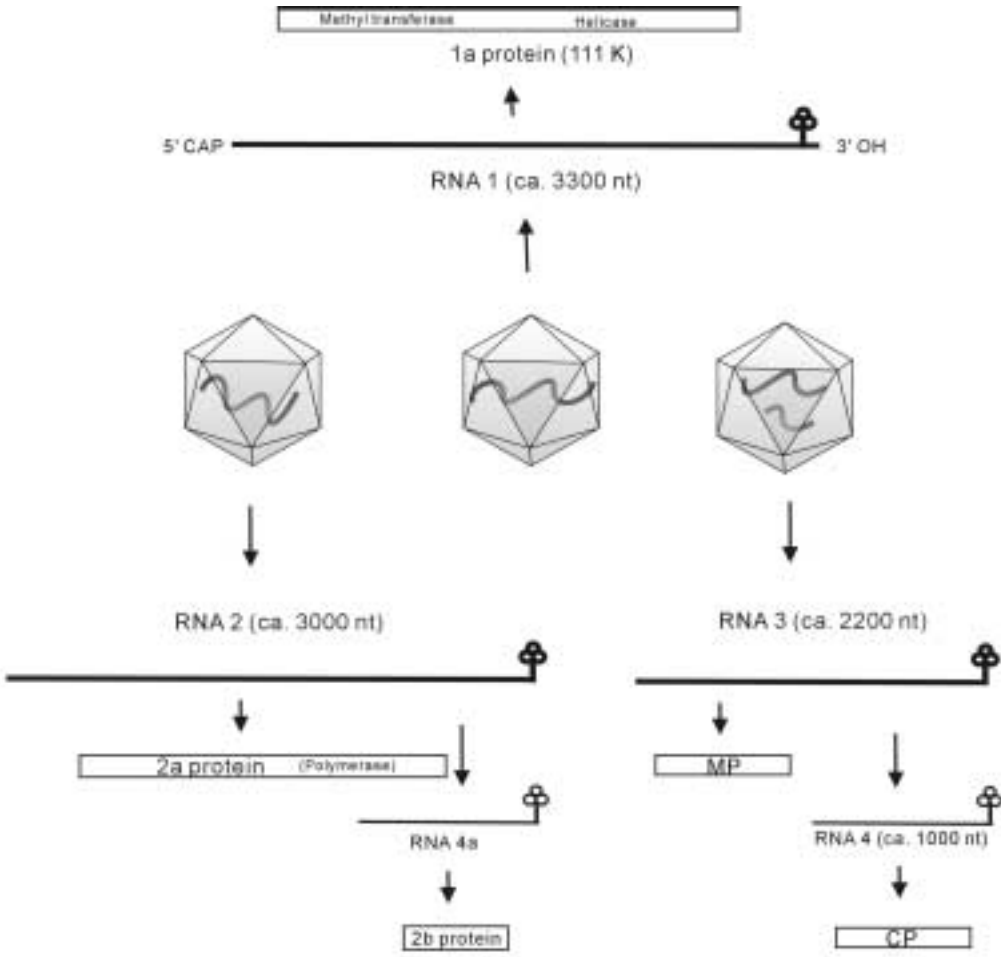

Fig. 1-1. Genome organization and expression of tripartite RNA genome of *Cucumber mosaic virus* (CMV).

Pathogen-derived resistance to plant viruses

The concept of pathogen-derived resistance (PDR) arose from the phenomenon of cross protection (Hamilton, 1980) and suggested the possibility of inducing resistance by transforming a susceptible host plant with genes derived from the pathogen (Sanford and Johnston, 1985). As it was reported that the coat protein of *Tobacco mosaic virus* (TMV) was responsible for cross protection (Sherwood and Fulton, 1982), the first PDR approaches involved this gene product.

Indeed, tobacco plants transformed with the TMV coat protein genes were capable of delaying the expression of virus symptoms when inoculated with TMV (Powell-Abel *et al.*, 1986). Since the first successful demonstration of PDR by using the TMV coat protein gene, it has been found that also other parts of viral genome, both coding and non-coding are capable of conferring resistance. To date, protein-based and RNA-based PDR has been successfully applied to protect plants against 14 genera of plant viruses with positive-strand RNA genomes, as well as the ambisense RNA tospoviruses and the single-stranded DNA-containing geminiviruses (Walsh, 2000).

Coat protein-mediated resistance

The viral coat protein gene is the most frequent source of PDR. Following the first example of TMV, coat protein-mediated resistance (CPMR) has been effectively conferring resistance against at least 30 plant viruses. However, the mechanism that is responsible for imparting the resistance to the host is not well understood in all cases. It is thought that the resistance results from (an) early event(s) in the infection process (Nelson *et al.*, 1987; van Dun *et al.*, 1987). The proposed mechanisms include the inhibition of virion disassembly in initially infected cells and the re-encapsidation of viral RNA by coat protein molecules expressed by the transgene. There appears to be a correlation between the expression level of coat protein and the efficiency of CPMR; the more protein expressed, the more resistance obtained (Loesch-Fries *et al.*, 1987; Hemenway *et al.*, 1988; Powell *et al.*, 1990; Okuno *et al.*, 1993a; Okuno *et al.*, 1993b). Although CPMR, in general, not only operates against closely related virus strains but also against less homologous ones (Hammond and Kamo, 1995; Hefferon *et al.*, 1997; Hassairi *et al.*, 1998), point mutations in coat proteins resulted in breakdown of resistance in some cases (Wilson, 1993). In addition to this limitation, CPMR can be overcome by high concentrations of viral inoculum and viruliferous vectors in the field (Ploeg *et al.*, 1993). Moreover, heterologous encapsidation, which involves the encapsidation of infecting viral genomes by transgenically expressed coat protein, has been observed in several instances of transgenic plants

containing viral coat protein gene (Walsh, 2000). The application of CPMR against CMV was first demonstrated in tobacco (Cuozzo *et al.*, 1988) and has been applied effectively to mainly vegetable crops, such as tomato (Fuches *et al.*, 1996; Gielen *et al.*, 1996; Kaniewski *et al.*, 1999; Provvidenti and Gonsalves, 1995; Xue *et al.*, 1994;), cucumber (Gonsalves *et al.*, 1992), squash (Tricoli *et al.*, 1995), and melon (Gonsalves *et al.*, 1994) using the CP genes of various CMV strains.

Replicase-mediated resistance

The application of plant viral replicase (or/and polymerase) genes for the transformation of host plants, which leads to the generation of plant lines resistant to the donor virus, is termed replicase-mediated resistance and has been shown effective in several cases (Carr and Zaitlin, 1993; Palukaitis and Zaitlin, 1997). Replicase-mediated resistance is often stronger than CPMR as it is effective even when plants are challenged with high levels of virus and infectious viral RNAs. Despite the fact that the resulting transgenic plants were highly resistant to the virus of which the replicase gene was derived, they were not resistant even to closely related viruses. Full-length replicase genes can confer high levels of resistance for *Potato virus X* (Braun and Hemenway, 1992), but not for *Alfalfa mosaic virus* (van Dun *et al.*, 1988) and *Brome mosaic virus* (Mori *et al.*, 1992). Defective forms of replicase genes also conferred resistance (e.g. Gal-On *et al.*, 1998). The effectiveness of various constructs derived from CMV replicase genes has been demonstrated in tobacco (Carr *et al.*, 1994; Hellwald and Palukaitis, 1995; Suzuki *et al.*, 1996; Wintermantel *et al.*, 1997; Gal-On *et al*., 1998). Mechanisms for explaining the observed resistance phenomena conferred by various constructs of replicase genes leads to RNA-mediated, homology-dependent resistance (Baulcombe, 1996) (see also next paragraph). Replicase-mediated resistance scores over CPMR in that it appears to be stronger in reducing symptoms and virus titers and can not lead to transencapsidation. However, high strain-specificity restricts the application of this strategy.

RNA-mediated resistance

In addition to the sequences representing diverse functional viral proteins, defective or truncated versions of these genes, either expressed in sense or antisense orientations, can confer resistance. These types of resistance have been found to operate completely at the RNA level (de Haan *et al.*, 1992; Lindbo and Dougherty, 1992; van der Vlugt *et al.*, 1992), and are referred to as RNA-mediated virus resistance (RMVR). This type of resistance needs no expression of transgenic protein and is often associated with high nuclear transcription rates combined with low steady state levels of transgene mRNA. Plants showing RMVR were either completely resistant to virus infection or recovered from the initial infection, while the resistance is limited to closely related virus strains (Waterhouse *et al.*, 1999). The characteristics of RMVR share great similarity with the post-transcriptional gene silencing (PTGS) or co-suppression phenomenon (Lomonossoff, 1995; Baulcombe, 1996; Prins and Goldbach, 1996; Vaucheret *et al.*, 1998; Hammond *et al.*, 2001). PTGS is a highly sequence specific process in which RNA with homology to the transgenic inducer RNA is specifically degraded in the cytoplasm. Similar phenomena have also been observed in other eukaryotic organisms, such as fungi (*Neurospora crassa*) (Romano and Macino, 1992), nematodes (*Caenorhabditis elegans*) (Kelly and Fire, 1998), insects *(Drosophila melanogaster*) (Pal-Bhadra *et al.*, 1997), and vertebrates (Bahramian and Zahbl, 1999). Various terms have been used to refer to these phenomena, such as co-suppression and PTGS for plants, quelling for fungi, and RNA interference (RNAi) for animals (Cogoni and Macino, 2000). The term of RNA silencing has been proposed to refer to these collective phenomena (Baulcombe, 1999; Ding, 2000).

Several mechanisms have been proposed to explain how this sequence-specific RNA degradation system might be activated, these include levels of transgene mRNA exceeding a ‘threshold’ to induce the degradation system (Dougherty and Parks, 1995), the methylation of the transgenes resulting in prematurely terminated aberrant RNAs to initiate the degradation mechanism (Wassenegger and Pelissier, 1998), and the formation of double stranded RNAs

that are able to induce and direct RNA degradation (Waterhouse *et al.*, 1998). A four-step model has been proposed to envision the RNA silencing pathway (Hutvagner and Zamore, 2002). Recent genetic evidence raises the possibility that the RNA silencing pathway is branched and that the branches converge in the production of double-stranded RNA (dsRNA). The dsRNAs molecules can be produced by host-encoded or viral-encoded RNA-dependent RNA polymerases, by transcription from converging promoters, or from rearranged loci (Dalmay *et al.*, 2000; Mourrain *et al.*, 2000; Sijen *et al.*, 2001). The long molecules of dsRNA are processed into short RNA molecules of 21 – 25 nucleotides, termed small interfering RNAs (siRNAs), by a RNase III-like dsRNA-specific endonuclease called DICER (Bernstein *et al.*, 2001). The siRNAs, with both sense and antisense strands of the target RNA, are then incorporated into a multi-subunit endonuclease, termed RISC: RNA-induced silencing complex (Voinnet, 2002) or RNA interference specificity complex (Baulcombe, 2002), that is guided to its target RNA by base paring and thus ensures that it specifically cleaves RNA that shares sequence similarity with the inducing dsRNA. Small interfering RNAs (siRNAs), have not only been found to associate with PTGS in plants (Hamilton and Baulcombe, 1999) but also with a similar process termed RNA interference (RNAi) in *Caenorhabditis elegans* and are used as markers for RNA silencing (Bernstein *et al.* 2001). Viruses are both targets and inducers of PTGS (Marathe *et al.*, 2000) and may carry PTGS suppressors (Brigneti *et al*., 1998; Voinnet *et al*., 2000). This suggests that PTGS acts as a natural defense mechanism for protecting plants from invading viral RNA (Ratcliff *et al.* 1997; Covey *et al.* 1997) or DNA sequences such as transposable elements or T-DNA of *Agrobacterium*. A silencing suppressor that is encoded by an insect-infecting virus has been reported recently (Li *et al.*, 2002), suggesting the possibility that a similar strategy could also be employed in animal cells.

Current status of the application of pathogen-derived resistance against virus infection in ornamental crops

The current technical limitation for generating transgenic virus resistant floral crops is not so much the viral resistance strategy but rather the gene delivery (transformation) system that must be developed for each crop. To date, by using *Agrobacterium*-mediated or by microprojectile bombardment gene-delivery methods, successful transgenic ornamentals have been obtained for: alstroemeria (Lin *et al.*, 2000), chrysanthemum (Ledger *et al.*, 1991), carnation (Lu *et al.*, 1991), gerbera (Elomaa *et al.*, 1993), lisianthus (Deroles *et al.*, 1995), rose (Firoozabady *et al.*, 1994), alstroemeria (Lin *et al.*, 2000), lily (Watad *et al.*, 1998), tulip (Wilmink *et al.* 1995) and gladiolus (Kamo *et al.* 1995). Nevertheless, except transgenic chrysanthemun plants expressing N gene of TSWV (Sherman *et al.*, 1998), no practical applications of PDR in ornamental crops have been reported. This is most likely due to the combination of low efficiency of transformation protocols and the relatively large numbers of transgenic lines that are required for selection of resistant lines. Improvement on one or both of these lines of research is therefore needed to make genetic transformation a more feasible option for the production of resistant ornamental crops against viral infections.

Scope of this thesis

By using genetic engineering techniques, the application of PDR in improving host resistance overcomes the source limitation of natural resistance to viral infections. However, in addition to the possible limiting factors mentioned above, only a relatively low percentage of resistant lines can be obtained from the construct containing only a single copy of inserted fragment. Although it has been identified in many ornamental crops, the grouping of ornamental-infecting *Cucumber mosaic virus* has not been clearly characterized at the nucleic acid level, which is necessary for broad application of RNA-mediated virus resistance (RMVR). The aim of this research was to characterize a range of ornamental-infecting CMV strains and to develop novel transgene constructs to improve the efficiency of obtaining resistant transformants which is essential for

most ornamental plants, especially the monocotyledonous ones, that are difficult to transform and regenerate.

RMVR was the principal strategy to use to compensate the low efficient transformation and regeneration because it results in virus free material, in contrast to CPMR that leads to reduced virus titers. However, in order to prevent the transformants from the disadvantage of highly strain-specific of RMVR, the genetic diversity among ornamental-infecting CMV strains has to be considered. Therefore, nucleotide sequence-based grouping of ornamental-infecting CMV was carried out in the beginning of this research (Chapter 2). During the investigations described in Chapter 2, an unexpected recombination was found in alstroemeria-infecting CMV isolates. This recombinant and its improved biological fitness are described in Chapter 3 and Chapter 4.

It has been demonstrated that the transgenic expression of coat protein and replicase genes of CMV conferred protection against CMV infection (Cuozzo *et al.*, 1988; Carr *et al.*, 1994). A survey of the viral genome for RNA or protein based resistance demonstrated that resistance against CMV was feasible (Chapter 5). Several lines of evidence indicate that RNA in a double-stranded form is the key trigger of RNA-mediated resistance (post-transcriptional gene silencing, PTGS). Double-stranded RNA can be produced via the transcription of an inverted repeat to generate hairpin mRNAs. Inverted repeated sequences based on RNA 2 and the region of RNA 3 spanning the CP ORF of CMV have been constructed and transformed into model plants. These plants were subsequently successfully tested for high efficiency resistance (Chapter 6) opening the way for the use of these transformation constructs for the production of virus resistant ornamental plants.

Chapter 2

Characterization of *Cucumber mosaic virus* isolated from ornamental crops based on the nucleotide sequences of the coat protein region

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Abstract

For classification of *Cucumber mosaic virus* (CMV) isolates from ornamental crops of different geographical areas, these were characterized by comparing the nucleotide sequences of RNAs 4 and the encoded coat proteins. Within the ornamental-infecting CMV viruses both subgroups were represented. CMV isolates of alstroemeria and crocus were classified as subgroup II isolates, whereas 8 other isolates, from lily, gladiolus, amaranthus, larkspur, and lisianthus, were identified as subgroup I members. In general, nucleotide sequence divergence correlated well with geographic distribution, with one notable exception: the analyzed nucleotide sequences of 5 lily isolates showed remarkably high homology despite different origins.

INTRODUCTION

Cucumber mosaic virus (CMV), the type species of the genus *Cucumovirus* in the family of *Bromoviridae* (Rybicki, 1995), is one of the most widespread plant viruses in the world. The genome of CMV consists of three capped plus-sense single stranded RNAs (Peden and Symons, 1973). Proteins translated from RNAs 1 and 2 are associated with viral genome replication (Nitta *et al.*, 1988), while the bicistronic RNA 3 encodes the 3a protein, shown to be involved in virus movement (Suzuki *et al.*, 1991), and the viral coat protein (Schwinghamer and Symons, 1977). The coat protein (CP) is translated from the subgenomic RNA 4 which is encoded by the 3'-half of RNA 3 (Gould and Symons, 1982). In addition to the four major viral genes, a small overlapping gene (2b), encoded by RNA 2, was discovered, which is most likely expressed from a second subgenomic mRNA (RNA 4A) (Ding *et al.*, 1994). The product of the 2b gene is involved in the virulence of the virus, possibly by suppressing gene silencing (Brigneti *et al.*, 1998).

CMV has an extraordinary wide host range and a large number of CMV isolates have been described. The complete nucleotide sequences of the genomic RNAs of several CMV isolates have been reported and numerous isolates of CMV have been classified into two major subgroups - I and II - on the basis of biological and serological properties and nucleotide sequence homology (Quemada *et al.*, 1989; Palukaitis *et al.*, 1992; Anderson *et al.*, 1995). New subgrouping of subgroup I into subgroups IA and IB, has been proposed recently on the basis of the nucleotide sequence of the 5' nontranslated region of RNA 3 (Palukaitis and Zaitlin, 1997; Roossinck *et al.* 1999). Moreover, the RT-PCR products obtained from the 3' halves RNA 3, encompassing the coat protein (CP) gene of 44 CMV isolates revealed distinguishable patterns on electrophoresis gel after digested with restriction enzyme *Msp*I. Based on the RFLP of 44 tested CMV isolates, six subgroups, designated subgroup I, I-α, I-β, I-γ, II, and nonI-nonII, were proposed (Anonymous, 1998). This method provides an easy screening method for large amounts of novel virus isolates.

CMV infections, although rarely disastrous by themselves, cause great

problems in the growing of ornamental crops in combination with other viruses. Flower crops greatly depend on clean practice or virus resistant varieties, as minor infection damage leads to greatly reduced market value. Limited availability of natural resistance sources has led to the development of successful transgenic forms of resistance. Often these forms of resistance are highly sequence specific (reviewed e.g. in Prins and Goldbach, 1996). Before attempting to create RNA-mediated resistance in notoriously difficult-to-transform ornamental crops, it is therefore essential to first establish sequence variation within CMV isolates infecting these plants.

The current study reports the nucleotide sequences of the CP-encoding RNAs 4 from 13 CMV isolates that were collected from ornamental plants originating from different geographic areas (Table 2-1).

Table 2-1. Names and origins of tested *Cucumber mosaic virus* strains

Name	Original host	Geographic origin
CRY	Crocus	Netherlands
S	Pumpkin	USA
D	Bean	USA
GPP	Gladiolus cv. Peter Pears	Netherlands
LICK	Lily Asiatic hybrid cv. Connecticut King	Netherlands
LISR	Lily Asiatic hybrid cv. Sun Ray	Netherlands
LILY	Lily Asiatic hybrid cv. Polyanna	France
LINB	Lily Oriental hybrid cv. New Butterfly	Taiwan
LITW	Lily	Taiwan
AMA	Amaranthus	Taiwan
DEL	Larkspur	Taiwan
EUS	Lisianthus	Taiwan

MATERIALS AND METHODS

Virus isolates

All 13 CMV isolates were maintained in *Nicotiana benthamiana* by mechanical inoculation during the experimental period. Eight isolates were obtained from the Bulb Research Center (LBO-LNV), Lisse, The Netherlands, two isolates isolated from lily (LITW and LINB) were gifts from Dr. C.A Chang of Taiwan Agricultural Research Institute and Dr. T.C. Yang of Taiwan Seeds Service, respectively, and three isolates from non-bulbous crops (DEL, AMA, and EUS) were described previously (Chen and Hu, 1999; Chen *et al.*, 1995a; Chen *et al.*, 1995b). The names and origins of the virus isolates are listed in Table 2-1.

RNA isolation, cDNA synthesis and cloning

Total RNA was extracted from leaf tissue of CMV-infected *N. benthamiana* by the method of Seal and Coates (1998). Approximately 5 μg of total RNA extracted from CMV-infected *N. benthamiana* plants was used as template for cDNA production. First strand cDNA synthesis was initiated with primer CMVCP-5 (5'-CCCCGGATCC*TGGTCTCCTT* -3'), complementary to the conserved ultimate 3' terminal 10 nucleotides of all CMV RNA 3. Second strand cDNA synthesis was primed with degenerated primer CMVCP-1 (5'-CCCCGGATCC*ACATCAYAGTTTTRAGRTTCAATTC*-3'), corresponding to nucleotide 1102 to 1126 of RNA 3, just upstream of the RNA 4 subgenomic promoter. Both primers contained a *BamH*I site (underlined) at the 5' end for cloning purposes. The RT reaction was carried out with M-MLV reverse transcriptase (Gibco-BRL) and subsequent PCR amplification with *Taq* polymerase (Gibco-BRL). The cDNA was amplified for 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 7 min. The RT-PCR fragments were analyzed on 0.8% agarose and then digested with *BamH*I restriction enzyme prior to cloning into standard vectors.

Sequence analysis

Nucleotide sequences were analyzed using the University of Wisconsin GCG package. PILEUP and GROWTREE options were used to construct the phylogenetic tree. Sequence data for Fny-CMV, NT9-CMV, Q-CMV and Trk7-CMV were obtained from Owen *et al.* (1990), Hsu *et al.* (1995), Davies and Symons (1988) and Salanki *et al.* (1994), respectively.

Analysis of RT-PCR products

Amplified cDNA products were separated on 0.8% agarose gel and, if required, eluted with glass beads and digested with 2- 4 units of *Msp*I restriction endonuclease. A 1.5% agarose electrophoresis followed after digestion at 37°C for 2 hr to determine the pattern of restriction fragments.

RESULTS

RNA 4 nucleotide sequences of ornamental plant-infecting CMV isolates

The size of all cDNA fragments obtained from RT-PCR were as expected (approximately 1100 nt), except for isolate ALS (Fig. 2-1).

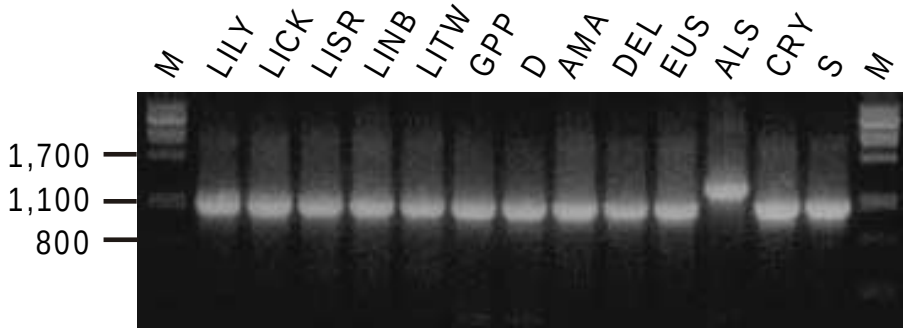

Fig. 2-1. Agarose gel electrophoresis of RT-PCR fragments amplified from total RNA of tobacco leaves infected with 13 CMV isolates. Lanes containing CMV RT-PCR products are indicated. Lambda DNA digested with restriction enzyme *PstI*, was used as size marker. The numbers on the side indicate the size of fragments in base pairs.

The nucleotide sequences of the cDNA clones of the 3'-half of RNA 3 encoding the RNA 4 subgenomic messenger from 13 isolates of CMV as determined and will be available in the EMBL database with accession numbers AJ131615 through AJ131627 (Table 2-2). Sequences of RNA 4 and putative amino acid sequences of the CP ORF of all isolates used in this study were compared with those of isolates Fny (Owen *et al.*, 1990) from subgroup IA, NT9 (Hsu *et al.*, 1995) from subgroup IB and isolates Q (Davies and Symons, 1988) and Trk7 (Salanki *et al.*, 1994) from subgroup II. The length of the RNA 4 encoding cDNAs of tested CMV isolates were 1113 nt to 1123 nt except isolate ALS which contained an additional 218 nt sequence in the central part of the 3' nontranslated region (3' NTR). The length of the 5' NTRs, CP ORFs, and 3' NTRs of tested isolates were nearly identical to those of previously described members of both subgroups, i.e. Fny-, NT9-, Q-, and

Trk7-CMV (Table 2-2).

The sequences 5' to the CP start codon consisted of 81 or 82 nt of intercistronic region and 55 to 77 nt of RNA 4 5' leader sequence. The conserved internal control region-like motif (ICR2 / 5'G$^{G}/_{A}$TTCAATTCC3') resided between positions 16 to 26 of all isolates. The homology at the nucleotide levels were greater than 97% between ALS-CMV, CRY-CMV as well as S-CMV and other subgroup II isolates, whereas only 70.4 to 75.4% homology was observed between these 3 isolates and all other examined isolates that all belong to subgroup I. At the amino acid level, ALS, CRY and S shared more than 95% homology with other subgroup II CMV isolates, but less than 88.5% homology with subgroup I CMVs. In contrast, the homology at both nucleotide and amino acid levels between the other 10 isolates tested and three subgroup II isolates are less than 75.4% and 84.9%, respectively. Homologies greater than 88% were

Table 2-2. Length of the coding and non-coding regions of RNA 4 of the CMV isolates included in this report

Subgroup	CMV strains	5' NTR	CP ORF	3' NTR	Full length	Accession number	Reference
I	Fny	155	657	303	1115	D10538	Owen *et al*., 1990
	D	155	657	301	1113	AJ131624	this study
	GPP	155	657	303	1115	AJ131623	this study
	LICK	155	657	310	1122	AJ131616	this study
	LILY	155	657	311	1123	AJ131615	this study
	LISR	155	657	311	1123	AJ131617	this study
	LINB	153	657	311	1121	AJ131618	this study
	LITW	155	657	310	1122	AJ131619	this study
	AMA	159	657	300	1116	AJ131625	this study
	DEL	158	657	300	1115	AJ131626	this study
	EUS	158	657	300	1115	AJ131627	this study
	NT9	150	657	302	1110	D28780	Hsu *et al*., 1995
II	Q	134	657	321	1112	J02059	Davies and Symons, 1988
	ALS	137	657	539	1333	AJ131622	this study
	CRY	141	657	321	1119	AJ131621	this study
	S	141	657	321	1119	AJ131620	this study
	Trk7	145	657	321	1123	L15336	Salanki *et al*., 1994

found among these 10 isolates and previously published CMV isolates (Table 2-3). We therefore conclude that CMV isolates of ALS, CRY, and S are to be classified as subgroup II and CMV isolates denoted D, GPP, LILY, LICK, LISR, LINB, LITW, AMA, DEL, and EUS should be classified as subgroup I isolates (Fig. 2-2).

Table 2-3. Sequence homology (%) of RNA 4 and CP between CMV isolates

Nucleotide Sequence of RNA 4

	Fny	D	GPP	LICK	LITW	LILY	LISR	LINB	AMA	DEL	EUS	NT9	Q	S	CRY	ALS	Trk7
Fny		98.9	96.1	94.0	94.5	94.5	94.4	94.6	89.4	87.8	88.8	92.9	72.7	71.8	71.9	70.6	71.4
D	99.5		96.2	94.3	94.7	94.6	94.5	94.9	88.6	88.3	89.1	92.4	72.8	72.5	72.1	70.8	71.4
GPP	98.2	97.7		94.2	94.7	94.6	94.5	95.0	88.3	88.3	89.3	91.7	72.9	73.2	72.4	71.1	72.3
LICK	96.8	96.3	95.9		99.1	98.9	98.7	99.2	87.6	87.8	88.3	88.3	72.9	72.6	72.6	70.4	71.8
LITW	99.1	98.6	97.3	96.8		99.5	99.2	99.5	88.1	88.2	88.8	90.7	73.3	73.1	72.9	70.9	72.2
LILY	99.5	99.1	97.7	97.3	99.5		99.4	99.3	88.1	88.2	88.7	91.6	73.3	73.2	73.3	71.3	72.3
LISR	99.1	98.6	97.3	96.8	99.1	99.5		99.2	88.1	88.3	88.9	90.9	73.7	72.9	73.1	70.9	72.9
LINB	99.1	98.6	97.3	96.8	99.1	99.5	99.1		88.5	89.2	89.9	91.1	73.7	73.6	73.4	71.2	72.9
AMA	98.6	98.2	96.8	95.4	97.7	98.2	97.7	97.7		98.6	96.8	92.6	74.7	74.3	74.6	72.8	74.0
DEL	98.6	98.2	96.8	95.4	97.7	98.2	97.7	97.7	99.1		97.4	92.5	75.4	74.4	75.4	72.5	74.6
EUS	97.7	97.3	95.9	94.5	96.8	97.3	96.8	96.8	98.2	98.2		92.3	74.9	74.1	74.8	72.1	74.1
NT9	99.5	99.1	97.7	96.3	98.6	99.1	98.6	98.6	98.2	98.2	97.3		75.4	74.0	74.9	73.1	74.6
Q	88.5	88.0	87.1	86.6	88.5	88.9	88.5	88.9	87.6	87.6	86.6	88.0		97.5	98.5	98.2	97.8
S	88.1	87.6	86.7	86.2	87.6	88.5	87.6	88.5	87.2	87.2	86.2	87.6	98.2		97.0	97.5	97.1
CRY	88.1	87.6	86.7	86.2	88.1	88.5	88.1	88.5	87.2	88.1	86.2	87.6	99.5	97.7		98.8	97.9
ALS	86.2	85.8	84.9	84.9	86.2	86.7	86.2	86.7	85.3	86.2	84.4	85.8	97.7	95.9	98.2		97.5
Trk7	86.7	86.2	85.3	84.9	86.7	87.2	86.7	87.2	85.8	85.8	84.9	86.2	97.7	95.9	97.3	95.4	

CP Peptide Sequence

The percentage of nucleotide sequence homology between 5' NTRs of all isolates tested showed high divergence between isolates of different subgroups and even between isolates of different geographical origins within the same subgroup (Table 2-4). This divergence made it possible to divide the subgroup I isolates into two further subgroups: D-CMV, GPP-, LILY-, LICK-, LISR-, LINB- and LITW-, like Fny-CMV, can be classified as isolates of subgroup IA, while AMA-CMV, DEL-, and EUS-, like NT9-CMV, belong to subgroup IB, despite the lack of dataon the 5' termini of RNA 3 on which this subdivision was originally proposed

(Palukaitis and Zaitlin, 1997; Roossinck *et al.*, 1999). When compared at the nucleotide level, sequences derived from the three non-bulbous isolates, i.e. AMA, DEL, and EUS, are over 92% homologous to one another as well as to another isolate of reported Asian origin (NT9-CMV). Less than 90% homology was observed with any of the other subgroup I isolates, suggesting a geographic isolation of these CMV stains.

Table 2-4. Sequence homology (%) of 5' and 3' NTRs of RNA 4 between CMV isolates

5' NTR

	Fny	D	GPP	LICK	LITW	LILY	LISR	LINB	AMA	DEL	EUS	NT9	Q	S	CRY	ALS	Trk7
Fny		98.6	95.9	98.6	98.6	98.6	98.6	98.6	53.2	56.0	56.0	76.4	45.3	43.3	53.3	49.1	41.3
D	98.0		93.7	90.5	90.5	90.5	90.8	90.8	85.7	87.7	90.7	89.7	41.9	45.0	53.3	47.2	40.5
GPP	93.1	93.7		94.6	94.6	94.6	94.5	94.4	62.2	56.8	56.8	76.4	43.2	43.3	55.0	47.2	43.2
LICK	88.5	90.5	88.5		100	100	100	98.6	60.8	55.4	55.4	75.3	41.9	45.0	53.3	49.0	40.5
LITW	88.5	90.5	88.5	100		100	100	98.6	60.8	55.4	55.4	76.3	41.9	45.0	49.0	49.0	40.5
LILY	88.5	90.5	88.5	99.4	99.4		100	98.6	60.8	55.4	55.4	75.3	41.9	45.0	53.3	49.0	40.5
LISR	88.9	90.8	88.5	99.0	99.0	99.0		98.6	60.8	55.4	55.4	75.3	41.9	45.0	53.3	49.0	40.5
LINB	88.9	90.8	89.2	99.0	99.0	99.0	99.4		65.3	56.9	56.9	76.1	44.4	45.0	45.0	43.6	43.1
AMA	86.7	85.7	86.0	85.0	85.1	85.8	83.7	85.1		98.7	98.7	59.7	42.9	45.0	48.3	43.4	42.9
DEL	87.0	87.7	85.7	85.7	85.8	85.8	84.1	85.4	98.0		100	69.7	44.7	45.0	48.3	55.5	44.7
EUS	90.0	90.7	89.4	88.1	88.1	87.8	87.8	87.8	92.3	93.0		69.7	44.7	45.0	48.3	55.5	44.7
NT9	89.7	89.7	87.4	86.4	86.4	89.9	86.4	88.5	93.0	93.3	92.3		49.1	55.0	48.3	50.9	46.0
Q	65.1	66.2	64.5	67.7	67.7	67.5	67.8	67.8	66.1	65.4	65.1	65.5		83.0	90.6	90.6	84.9
S	65.4	67.9	64.6	67.9	68.6	68.5	68.8	67.9	65.7	65.4	65.4	65.8	98.4		78.3	83.6	86.7
CRY	64.7	65.5	62.7	66.3	66.3	67.0	67.3	67.3	65.7	65.0	64.7	65.1	98.1	98.4		94.5	90.0
ALS	53.5	53.3	52.7	55.9	55.3	55.7	55.5	55.7	55.7	55.7	55.3	57.1	64.8	65.4	65.5		89.1
Trk7	65.1	65.2	62.4	66.0	66.0	66.7	67.0	66.7	66.0	65.4	65.0	65.8	98.1	98.4	98.7	68.2	

3' NTR

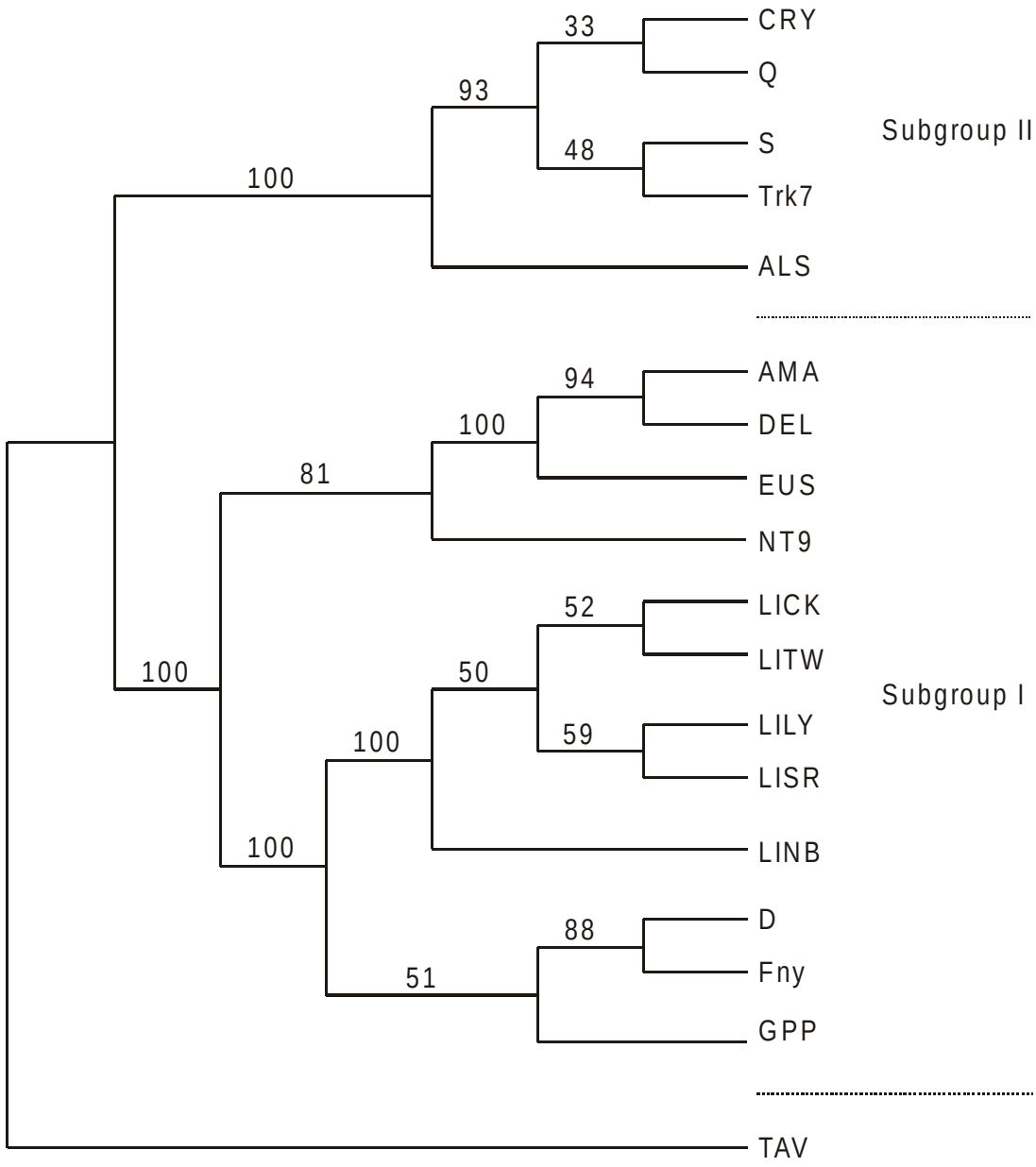

Fig. 2-2. Phylogenetic dendrogram of CMV strains based on the nucleotide sequences of RNA 4. Neighbor-joining and subsequent PAUP bootstrap analysis was used to create the dendrogram. Names of the virus isolates are as indicated in Table 2-1. The RNA 4 sequence of *Tomato aspermy virus* (TAV), another *Cucumovirus* (O'Reilly *et al*., 1994) (accession number S72468), was used as outsource.

Comparison of nucleotide and peptide sequences of RNA 4 between tested CMV isolates

As shown in Table 2-3, the homology in nucleotide and amino acid sequence level of the isolates within a subgroup is much higher than that of the isolates between subgroups. When compared, the nucleotide sequences of CMV isolates within a subgroup showed 87.6% to 99.5% homology between subgroup I isolates and 97.0% to 98.8% between subgroup II isolates. However, nucleotide sequence homologies between isolates of different subgroup are much lower (70.4% to 75.4%). Comparison of the amino acid sequences of the CMV isolates of the same subgroup showed 94.5% to 99.5% conservation between isolates of subgroup I and 95.4% to 98.2% conservation between subgroup II isolates, but only 84.4% to 88.5% conservation between isolates of different subgroups. Among tested subgroup I isolates, nucleotide sequence homology between isolates of the same geographic area is much higher than that between isolates of different areas. Interestingly, the nucleotide and amino acid sequences of isolates isolated from lily plants, i.e. LILY-, LICK-, LISR-, LINB- and LITW-CMV, are highly conserved in spite of the different geographic origins and horticultural varieties.

The length of CP ORF for all tested isolates is 657 nt and thus encodes a coat protein of 218 amino acids. The nucleotide sequence homology of the ORF is more than 92% (up to 99%) and 74% (up to 78%) for isolates within and between subgroups, respectively. This translates to more than 94% and 84% within and between subgroups for the deduced CP amino acid sequence.

Sequence analysis of 3' NTRs

The 3' NTRs contained 300 to 311 nt for subgroup I isolates, 321 nt for two subgroup II isolates (CRY and S) and 539 nt for the ALS isolate. Sequences of 3'-NTR in all tested isolates are less divergent than that of the 5' NTR and more than 83% (up to 100%) homology can be observed between isolates within the same subgroup. In contrast, less than 69% sequence homology can be observed between isolates in different subgroups. When a comparison was made between the 3' NTR of ALS-CMV and other CMV isolates, the homology percentage

decreased to less than 58% and 66% between and within subgroup isolates, respectively. A remarkable insertion of 218 nucleotides in the central part of the 3' NTR of ALS-CMV RNA 3, seems to be derived from RNA 1 or 2 by recombination and has not been observed in any previously reported RNAs 3.

RFLP of the cDNA products

Digestion of the PCR-amplified cDNA of CMV CP and its flanking regions with *Msp*I produced four different restriction patterns that are in accordance with the results obtained from the sequence analysis (Table 2-5). The first pattern consisted of two restriction fragments of 600 bp and 513 or 515 bp. This pattern was produced from Fny-CMV, D-CMV, and GPP-CMV and is similar to the *Msp*I patterns produced by other subgroup II isolates (Rizos *et al.*, 1992) and *Msp*I type SI isolates (Anonymous, 1998). The second pattern was composed of two larger restriction fragments of 603/604 bp and 484 bp and a smaller fragment of only 28 bp. This pattern was produced from three isolates that isolated from non-bulbous crops and was similar to that of type SI-α (Anonymous, 1998). All five isolates that isolated from lily plants consisted of a third pattern of restriction fragments of 179-181 bp, 193 bp, 329-331 bp, and 419 bp. A fourth pattern was produced from those subgroup II isolates (*MspI* type I), i.e. Q-CMV, S-CMV, CRY-CMV, which consisted of six restriction fragments of 28, 158, 173-180, 197, 248, and 308 bp. The ALS-CMV also should be grouped with these isolates with the 368 bp fragment increased to 526 bp due to the aforementioned 218 nt insertion in the 3’ NTR.

Table 2-5. CMV phylogeny by *Msp*I digestion

CMV subgroup	CMV isolate	*Msp*I restriction map (nt)	*Msp*I group*	Reference
IA	Fny	537, 337	SI	Quemada *et al.*, 1989
IA	Fny	600, 515	SI	Owen *et al.*, 1990
IA	D	600, 513	SI	this study
IA	GPP	600, 515	SI	this study
IB	AMA	604, 484, 28	SI-α	this study
IB	DEL	603, 484, 28	SI-α	this study
IB	EUS	603, 484, 28	SI-α	this study
IA	LINB	419, 331, 193, 179	nonI-nonII	this study
IA	LITW	419, 329, 193, 181	nonI-nonII	this study
IA	LICK	419, 329, 193, 181	nonI-nonII	this study
IA	LILY	419, 330, 193, 181	nonI-nonII	this study
IA	LISR	419, 330, 193, 181	nonI-nonII	this study
II	Q	248, 197, 158, 126, 110, 28	SII	Quemada *et al.*, 1989
II	Q	308, 248, 197, 173, 158, 28	SII	Davies and Symons, 1988
II	S	308, 248, 197, 180, 158, 28	SII	this study
II	CRY	308, 248, 197, 180, 158, 28	SII	this study
II	ALS	526, 248, 197, 176, 158, 28	SII	this study

*: Anonymous, 1998

DISCUSSION

Cucumber mosaic virus isolates have been classified as two subgroups based on visual symptoms, serology and nucleic acid hybridization tests (Palukaitis *et al.*, 1992). The sequence homology of coat protein gene ranges between 96.3% and 99.5% within a subgroup and between 76.0% and 77.5% for isolates of different subgroups. Homologies in CP peptide sequences are 94.0% to 99.2% and 79.5% to 83.2%, respectively. In accordance with this, the isolates tested in this study can be classified into two subgroups. ALS-CMV, CRY-, and S- are subgroup II isolates, while the remaining nine isolates (D-CMV, GPP-, LILY-, LICK-, LISR-, LINB-, LITW-, AMA-, DEL-, and EUS-) belong to subgroup I. Based on the divergence found on the nucleotide sequence of the 5' NTR of CMV RNA 3, the subgroup I CMV isolates can be further subdivided into Subgroup IA and IB. Interestingly, all the IB isolates are of Asian origin, like all other previously reported isolates of this group (Roossinck *et al.*, 1999). The conserved hexanucleotide sequence (5'-TCGTAG-3') upstream of the CP start codon, shown to be involved in 18S ribosomal RNA binding, was previously reported in subgroup I isolates (Yamaguchi *et al.,* 1982). In the isolates studied here, this conserved sequence was only found in the subgroup IA isolates, including all 5 viruses isolated from lily plants (position 137 to 142) but not in any of the subgroup IB isolates.

RT-PCR has been proven to be a useful and sensitive tool for detecting plant viruses, including CMV, which can be effectively detected with different sets of degenerate primers (Hu *et al.*, 1995; Singh *et al.*, 1995; Choi *et al.*, 1999). The set of primers used in this study amplified the CP ORF and flanking regions of CMV isolates successfully and therefore can be used as an alternative for CMV detection. Combining the RT-PCR and RFLP of PCR products, CMV isolates can be divided into two major subgroups (Rizos *et al.*, 1992) or even further subdivided into six groups (Anonymous, 1998). The restriction fragments obtained from *Msp*I-digested PCR products of the CMV isolates reported here reveals not two but four types of patterns and can be classified into four of the six subgroups previously described, i.e. SI, SI-α, SII, and nonI-nonII (Anonymous, 1998). Both

complete sequence determination and RFLP of RT-PCR products have been performed in this study. Our results indicate that the use of *MspI* pattern characterization is a simple and powerful strategy for phylogenetic characterization of large numbers of CMV isolates and can be an effective tool for fast and simple characterization of CMV in even greater numbers than reported here.

In general, the variation between CMV isolates prevalent in geographic regions as well as those in a specific crop has proven to be rather limited. Due to this observation, the use of CMV sequences for the introduction of transgenic RNA-mediated resistance has been supported rather than discouraged. Further, more detailed studies will be needed to verify the exact tolerance of RNA-mediated resistance for micro variation in viral gene sequences of the transgene target region and subsequently which region of the CMV genome would be the most suitable for using in a durable resistance strategy in ornamental and other crops.

Interestingly, two isolates isolated from lily in Taiwan are extremely homologous to other lily isolates of distinct geographic origin as well as to those isolated from other lily varieties. Two possible reasons can be indicated for this observation: recent dispersal of contaminated bulbs through trade or genuine host specificity. Lily plants have been mechanically inoculated with all 13 tested CMV isolates and only the CMV isolates originating from lily were able to re-infect lily plants successfully (data not shown), which would favor the host specificity hypothesis that lily plants are susceptible only to subgroup I CMV isolates.

Chapter 3

Alstroemeria-infecting *Cucumber mosaic virus* isolates contain additional sequences in the RNA 3 segments

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Abstract

The coat protein (CP) genes and flanking regions of three alstroemeria-infecting *Cucumber mosaic virus* isolates (CMV-ALS), denoted ALS-LBO, ALS-IPO, and ALS-NAK, were cloned and their nucleotide sequence determined and compared at both nucleic acid and deduced protein level with the published sequences of CMV RNA 3. The sequences of these isolates showed more than 95% nucleotide and peptide sequence homology to each other and to other members of subgroup II CMV. Strikingly, an additional sequence of 218 nt was found in the central region of the 3' non-translated region (3' NTR) of RNA 3 of two out of three isolates. The additional sequence appeared to have originated from RNAs 1 or 2. A subgroup-specific DIG-labeled probe has been developed from this additional sequence and applied to detect subgroup II CMV strains in dot blot hybridization and to differentiate the Alstroemeria isolates containing additional sequences in Northern blot hybridization.

INTRODUCTION

Alstroemeria has become an important ornamental crop during the past decade in the Netherlands and all over the world (van Zaayen, 1995). Several viruses infecting alstroemeria have been described, including *Alstroemeria mosaic virus* (AlMV), *Alstroemeria carla virus* (AlCV) (Phillips and Brunt, 1986), *Alstroemeria streak virus* (AlSV) (Wong *et al.,* 1992; van Zaayen *et al.*, 1994), *Tomato spotted wilt virus* (TSWV) (Hakkaart and Versluijs, 1985). Also *Cucumber mosaic virus* (CMV) (Hakkaart, 1986) has been reported in *Alstroemeria*. However, very little was reported on the interaction between CMV and alstroemeria plants because of its sporadic occurrence. Since CMV in alstroemeria usually was reported together with other viruses (van Zaayen, 1995), the symptoms caused by CMV-infection were not clear and not described until 1996. Bellardi and Bertaccini (1996) reported that CMV-infected alstroemeria showed necrosis on the leaves of *Alstroemeria* cv. Red Sunset and malformed flowers on *Alstroemeria* cv. Rossella. In addition, chlorotic mosaic on the leaves and stunting also were observed in *Alstroemeria* Red Sunset. The combination of CMV with other alstroemeria viruses can induce different symptoms (necrosis, red stripes, and yellowing) which could depend upon environmental conditions, alstroemeria cultivars and the growth stage at which the infection occurred (Bellardi and Bertaccini, 1997). Seed transmission of CMV in alstroemeria was reported as well (Bellardi and Bertaccini, 1997). Antisera against alstroemeria CMV strains have been developed and have been applied to routine inspection.

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus* in the family of *Bromoviridae* (Rybicki, 1995) and consists of isometric particles with a diameter of about 28 nm. Its genome consists of three functional single-stranded RNA molecules designated as RNA 1, 2, and 3, which are required for infection. RNA 1 and 2 encode components of the viral RNA-dependent RNA polymerase (Nitta *et al*., 1988), while RNA 3 encodes the movement protein (MP) (Suzuki *et al*., 1991) and coat protein (CP) (Schwinghamer and Symons, 1977). The CP is translated from the subgenomic RNA 4 which is encoded by roughly the

3’-half of the RNA 3 (Gould and Symons, 1982). A small overlapping gene (2b), encoded by RNA 2, was discovered more recently and is most likely expressed through subgenomic RNA 4A (Ding *et al*., 1994). The product of the 2b gene is involved in the virulence of the virus, possibly by suppression of gene silencing (Brigneti *et al*., 1998). The complete nucleotide sequences of the genomic RNAs of several CMV strains have been reported and have been classified into two major subgroups I and II (Anderson *et al*., 1995; Palukaitis *et al*, 1992; Quemada *et al*., 1989). Moreover, a further grouping of subgroup I, designated as IA and IB, has been proposed based on the nucleotide sequences of 5’ NTRs of RNA 3 (Palukaitis and Zaitlin, 1997; Roossinck *et al*. 1999). CMV is one of the most widespread plant viruses in the world and has a host range of over 1,000 species of plants (Palukaitis *et al*, 1992). Also alstroemeria has been reported to be a host of CMV (Hakkaart, 1986).

In a previous survey on CMV strains infecting ornamental crops, we characterized 13 CMV strains of ornamental crop origins on the basis of nucleotide sequence of RNA 4 and flanking regions (Chapter 2; Chen *et al*., 2001). The sequence data showed that an additional sequence of 218 nucleotides, with similarity to RNA 1 or 2 of the homologous virus, resided in the central region of 3’ NTR of the RNA 3 of the alstroemeria-infecting CMV isolate (ALS-LBO) (Chapter 2). To assure that the occurrence of the additional sequence in ALS-LBO is not incidental, two additional sources of alstroemeria CMV (ALS-IPO and ALS-NAK) were used to confirm the results of our previous studies. In this study, we report the cDNA sequences of the CP gene and its flanking regions of three alstroemeria-infecting CMV isolates obtained from different sources and compare these sequences at nucleotide and protein level. Moreover, the additional sequence is used as a template for the production of a specific probe to distinguish alstroemeria CMV isolates in Northern analysis.

MATERIALS AND METHODS

Virus sources and plant materials

The first isolate of alstroemeria-infecting CMV (ALS-LBO) was obtained from the Bulb Research Center (LBO-LNV), Lisse, the Netherlands. The second, ALS-IPO, was a stock isolate (code: N-11) of the Department of Virology, Plant Research International (PRI), Wageningen, The Netherlands. The third source (ALS-NAK) was obtained from the Inspection Service for Floriculture and Arboriculture (NAKB), Den Haag, The Netherlands. ALS-LBO and ALS-IPO were originally maintained in *Nicotiana clevelandii* and as dried material of *Cucumis sativa*, respectively, whereas ALS-NAK was isolated from a CMV-infected alstroemeria plant. All tested isolates were mechanically inoculated to *Chenopodium quinoa* and the local lesions were mechanically inoculated to *N. benthamiana* for maintenance. Alstroemeria plants (clone VV024-6) (Lin *et al*, 1998) were kindly offered by Dr. M. de Jeu of Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University and were used for pathogenicity tests.

Virion and RNA purification and total RNA extraction

All three isolates were propagated on *N. tabacum* cv. White Burley *or N. benthamiana* for virion purification, which were carried out as described by Lot *et al.* (1972). Viral RNA was isolated from the purified virions by the method of Palukaitis and Symons (1980). Total RNA was extracted from leaf tissue of inoculated *N. benthamiana* with the method of Seal and Coates (1998).

RT-PCR

The degenerated oligonucleotide primer pair for RT-PCR to amplify CMV coat protein gene and flanking regions was designed according to some of the determined sequences of CMV RNA 3. BamHI sites were added for cloning purposes. The sequence of the forward primer (CMVCP-1) is 5'-CCCCGGATCCACATCAYAGTTTTRAGATTCAATTC-3' and that of the reverse

primer (CMVCP-5) is 5'-CCCGGATCCTGGTCTCCTT-3'. The predicted length of the amplified DNA fragment is 1111 to 1122 bp for subgroup II CMV strains. The forward and reverse primers, denoted as ALSEXT-F and ALSEXT-R, to amplify the additional fragment of 218 nucleotides were 5'-CGGGTATCGCCTGTGG-3' and 5'-CAAGGGTACCTCGACAACCC-3', respectively. Total RNA of infected plants was used to synthesize first strand cDNA using MMLV-reverse transcriptase with primer CMVCP-5. PCR amplifications of cDNA were carried under the following conditions: denaturation for 3 min at 94ºC and then 35 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 52ºC, and extension for 1 min at 72ºC. The final extension was a 7-min incubation at 72ºC. PCR products were analyzed by electrophoresis on a 1% agarose gel and the DNA bands were visualized by ethidium bromide staining and by UV transilluminator.

cDNA cloning and sequencing

PCR products of the expected size were eluted from agarose gel (Concert Matrix, GibcoBRL) and precipitated with ethanol. Purified DNA was ligated to the pGEM-T vector (Promega) and transformed to *E. coli*. The plasmids were denoted as pLBOCP51, pIPOCP51, pNAKCP51 for the gene and flanking regions of ALS-LBO, ALS-IPO, ALS-NAK, respectively, and pALS218 for the additional sequence. The cloned sequences of all three tested CMV isolates were compared with that of subgroup I and subgroup II CMV strains, whose sequences were available in the literatures and in GenBank, i.e. Fny-CMV (Owen *et al*., 1990; D10538) and NT9-CMV (Hsu *et al*., 1995; D28780) for subgroup I CMV, and Q-CMV (Davies and Symons, 1988; J02059) and Trk7-CMV (Salanki *et al*., 1994; L15336).

Preparation of digoxigenin-labeled RNA probe

RNA probes were prepared from the plasmid DNA of pALS218 using SP6 or T7 RNA polymerase. One μg of the plasmid DNA was added to a 25 μl T7 or SP6 RNA transcription mixture containing 1x DIG RNA labeling mixture, 1x T7/SP6 RNA polymerase buffer, 10mM DTT, 0.5 μl RNasin and 1.5 μl T7 or SP6 RNA

polymerase (GibcoBRL), and incubated at 37ºC for 1 hr. Transcribed DIG-labeled probe RNAs were ethanol precipitated and dissolved in 50 μl distilled water. DIG-labeled RNA probes were checked by electrophoresis on a 1% agarose gel.

Dot blot and Northern hybridization

For dot blot analysis, total RNA extracted from healthy or virus-infected leaf tissue was diluted with 20x SSC to a final concentration of 0.2 μg/μl. One μg was spotted onto a nylon membrane and baked at 80ºC for 2 hr. For Northern blot analysis, purified viral RNA or extracted total RNA was separated on a 0.8% agarose gel in a 1x methyl mercuric hydroxide buffer system, transferred to nylon membrane by capillary force and baked at 80ºC for 2 hr. Hybridization was carried out as described in Webster and Barker (1998) by using a DIG-labeled RNA probe and Goat-anti-DIG-AP conjugated complex both in a dilution of 1:2500.

RESULTS

Pathogenicity tests

Using mechanical inoculation, all CMV-ALS isolates were capable of systemically infecting *N. benthamiana* and *N. tabacum* cv. White Burley. *Chenopodium quinoa* could be infected locally only. *Alstroemeria* plants (clone VV024-6) could also be inoculated by all three isolates, however, the infection rates of ALS-LBO and ALS-NAK weres much higher than that of ALS-IPO, with an average infection rate of 60% for ALS-LBO and ALS-NAK, but only 5% for ALS-IPO. All isolates infected tobacco at an infection rate of 100%.

RT-PCR and sequence analysis

The designed degenerated primers, CMVCP-5 and CMVCP-1, amplified an expected single DNA band of about 1,100 bp for ALS-IPO; however, a DNA band with a length of about 1,300 bp was produced from ALS-LBO and ALS-NAK (Fig. 3-1). These products were cloned and their sequences determined. Sequencing data showed the precise length of 1,118 nt of the amplified ALS-IPO cDNA and 1,333 nt for both ALS-LBO and ALS-NAK. This size difference between the 3 isolates appeared to be caused by an additional sequence of 218 nucleotides in the 3' NTR (see Fig. 3-2). This additional sequence made the length of RNAs 3 and 4 in both isolates longer than that of ALS-IPO the isolate (Fig. 3-3). Flanking the inserted sequence, a pair of repeated sequences of 16 nucleotides (5'-TGTCGAGGTACCCTTG - 3'), were observed in ALS-LBO and ALS-NAK. In fact, this 16-nt sequence is a congenital sequence and resides directly upstream of the 3' terminal tRNA-like structure of all CMV strains.

The partial RNA 3 sequences of both ALS-LBO and ALS-IPO are available in the EMBL database with accession numbers AJ131622 and AJ672587, respectively. ALS-NAK has not been submitted.

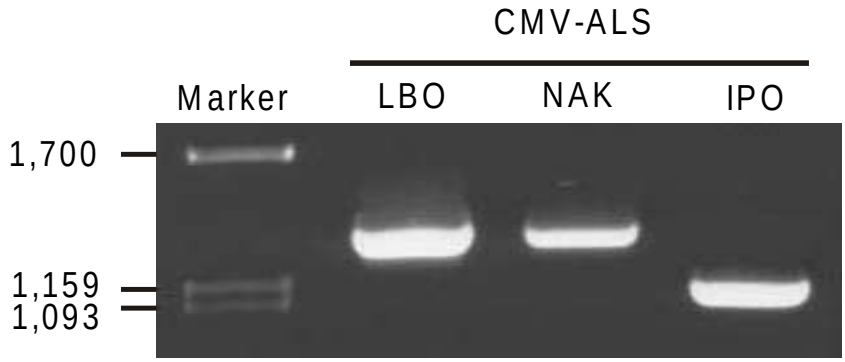

Fig. 3-1. Amplified DNA fragments of the CMV-ALS isolates obtained by RT-PCR. Primers CMVCP-1 and CMVCP-5 were used to perform RT-PCR on total RNA isolated from tobacco leaves infected with alstroemeria CMV isolates (CMV-ALS), LBO, NAK, and IPO. Lambda DNA digested with restriction enzyme PstI is used a size marker.

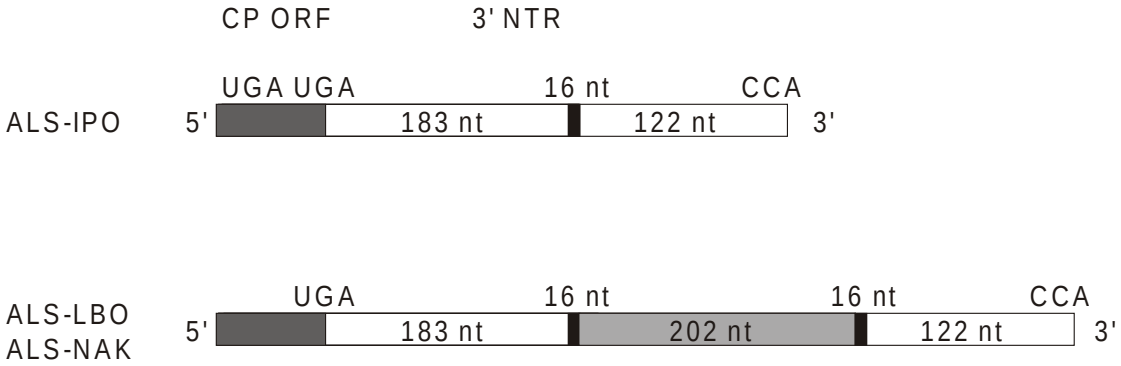

Fig. 3-2. Schematic diagram of 3' non-translated region (3' NTR) of RNA 3 of alstroemeria-infecting *Cucumber mosaic virus* isolates. An additional sequence of 218-nt (light gray box), which is flanked by a repeated sequence of 16-nt (solid boxes), resides in the central region of 3' NTR of ALS-LBO and ALS-NAK but not in ALS-IPO. Dark gray boxes indicate the coat protein open reading frame (CP ORF) and the numbers indicate the length of nucleotides.

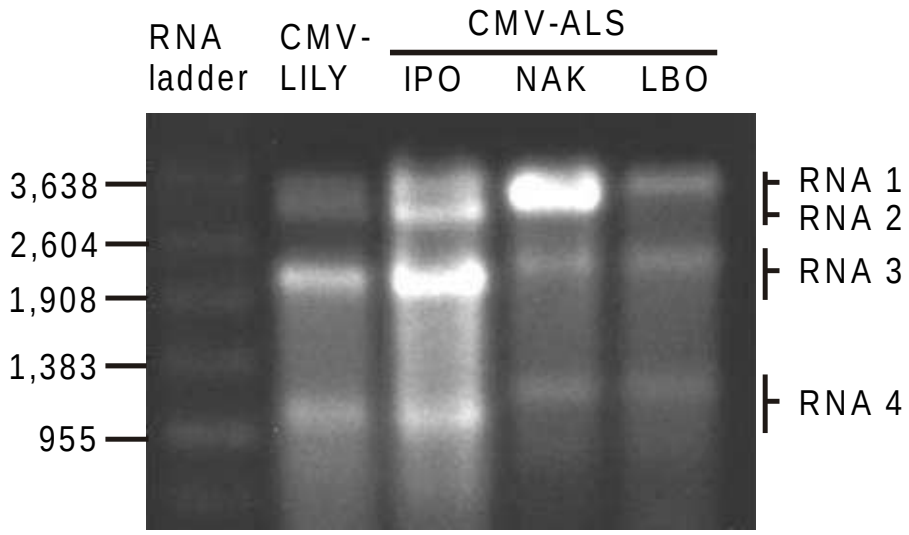

Fig. 3-3. RNA pattern of CMV strains in 0.8% agarose gel. The addtinal sequence which resides in the 3'-half of RNA 3 made the RNAs 3 and 4 of ALS-LBO and ALS-NAK longer than that of ALS-IPO and CMV-LILY.

Comparison of nucleotide and deduced amino acid sequences

The nucleotide sequences of the 3'-halves of RNA 3 and the putative amino acid sequence of the CP ORFs of the three alstroemeria CMV isolates were compared with those of some strains in both subgroups of CMV, i.e. Fny (D10538), NT9 (D28780), Q (J02059) and Trk7 (L15336). The homologies at the nucleotide level are more than 97% between tested isolates and subgroup II CMV strains, whereas only 70 to 75% homology between tested isolates and subgroup I strains was observed (Table 3-1). The nucleotide sequences of RNA 3 are identical between ALS-LBO and ALS-NAK and, apart from the 218 nt additional sequence, share 99% homology with that of ALS-IPO. The differences are 7 nt in the 5'-NTR of the CP ORF and 3 nt in the CP ORF itself. The sequence of the 218 nt inserted fragment, which was found only in the RNAs 3 and 4 of ALS-LBO and ALS-NAK, is highly homologous to sequences of RNAs 1 and 2 of subgroup II CMV isolates (more than 83% homology), but neither to RNAs 3 nor 4 (less than 70% homology). Also no homology was observed to any segment of subgroup I CMV isolates or related viruses (Table 3-2).

Table 3-1. Sequence homology (%) of the 3'-halves of RNA 3 and the CP protein between the tested alstroemeria isolates and some other CMV strains

Nucleotide sequence							
CMV strains	ALS-LBO	ALS-NAK	ALS-IPO	Q	Trk7	Fny	NT9
ALS-LBO		100	99.5	98.2	97.5	70.6	73.0
ALS-NAK	100		99.5	98.2	97.5	70.6	73.0
ALS-IPO	98.2	98.2		98.6	97.8	72.2	75.2
Q	96.8	96.8	98.6		97.8	72.2	75.4
Trk7	94.5	94.5	96.8	97.7		71.4	74.6
Fny	85.3	85.3	87.2	88.5	86.7		92.9
NT9	84.9	84.9	86.7	88.0	86.2	99.5	
Peptide sequence of CP							

Table 3-2. Sequence homology (%) between the 218 nt additional sequence and genomic RNAs of some virus strains of CMV and related *Bromoviridae*

	RNA 1	RNA 2	RNA 3	Accession Number*
ALS-LBO	84.9	94.5	100	AJ276584, AJ276585, AJ131622
ALS-NAK	84.9	94.5	100	AJ304404, AJ304395, AJ304398
ALS-IPO	83.0	89.5	69.6	AJ304405, AJ276586, AJ276587
Q-CMV	83.8	84.3	69.2	X02733, X00985, J02059
Trk7-CMV	83.0	88.1	70.0	AJ007933, AJ007934, L15336
Fny-CMV	56.3	60.7	59.0	D00356, D00355, D10538
NT9-CMV	55.4	62.4	60.3	D28778, D28779, D28780
PSV	58.2	54.0	57.3	U33145, U83332, U31366
TAV	68.2	65.9	64.7	D10044, D10663, D01015
AlMV	36.2	42.7	40.1	L00163, K02702, X00819
BMV	44.2	39.0	44.5	X02380, X1678, V00099
TSV	43.1	41.3	39.0	U80934, U75538, X00435

Dot-blot and Northern blot hybridization

DIG-labeled RNA probes developed from the 218 nt additional sequence of ALS-LBO were used in dot-blot hybridization assays to assess specificity. This probe specifically hybridized with total RNA extracted from alstroemeria and tobacco that were infected with subgroup II CMV strains including the alstroemeria CMV isolates. None of the RNAs of subgroup I CMV isolates cross-reacted to the probe (Fig. 3-4). Alstroemeria CMV isolates that contain additional sequences, i.e. ALS-LBO and ALS-NAK, could be distinguished from other subgroup II CMV strains in Northern hybridization by their deviant hybridization pattern (Fig. 3-5).

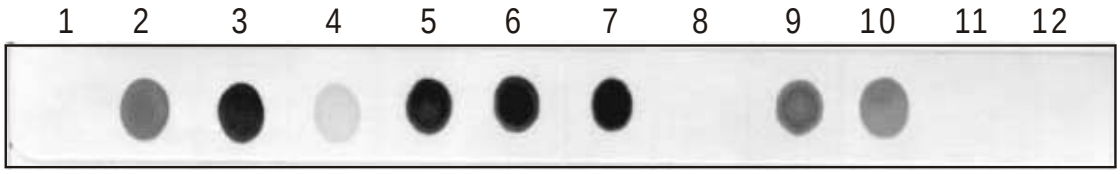

Fig. 3-4. The DIG-labeled RNA probe derived from the additional sequence of CMV-LAS isolates reacts with total RNA of subgroup II CMV strains but not with subgroup I CMV. Dots 1- 4 are RNA extracted from alstroemeria leaves and dots 5 - 12 are from *Nicotiana benthamiana*. 1: healthy control, 2: ALS-IPO, 3: ALS-LBO, 4: ALS-NAK, 5: ALS-IPO, 6: ALS-LBO. 7: ALS-NAK, 8: healthy control, 9: CMV-CRY, 10: CMV-S, 11: CMV-LILY, 12: CMV-GPP. CMV-CRY and CMV-S are subgroup II strains, CMV-LILY and CMV-GPP are subgroup I strains.

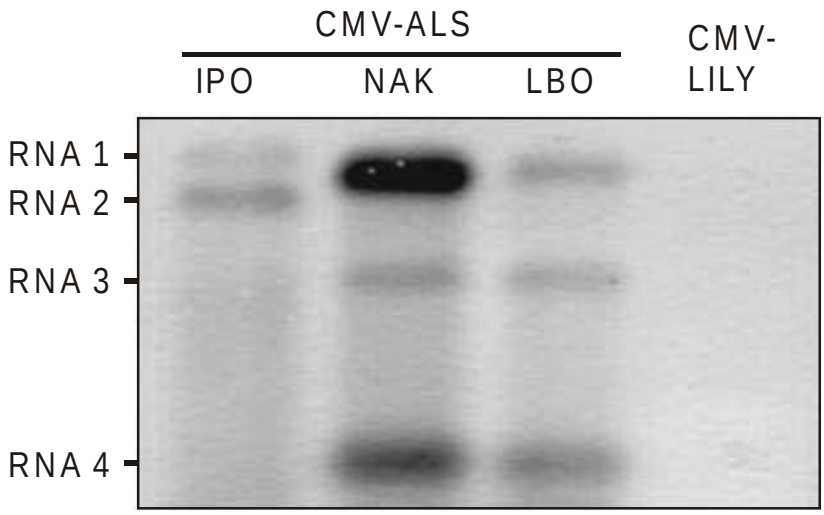

Fig. 3-5. Hybridization of the 218 nt probe to CMV RNAs.The DIG-labeled RNA probeconsisting of the additional sequence of 218 nt of the LBO isolate hybridizes to RNA 1 and 2 of subgroup II (CMV-ALS), but not subgroup I (CMV-LILY), isolates and RNAs 3 and 4 of the NAK and LBO isolates. Sizes of the genomic RNAs are indicated on the left.

DISCUSSION

RT-PCR can be used to rapidly and sensitively detect plant viruses. Indeed, the RT-PCR method has proven to be more sensitive than ELISA and dot-blot hybridization (Hu *et al.*, 1995). Also *Cucumber mosaic virus* (CMV) can be effectively detected with different sets of degenerate primers (Choi, *et al.* 1999; Hu et al, 1995; Singh *et al.*, 1995). The set of primers used in this study amplified the CP ORF and flanking regions of CMV isolates from ornamental crops successfully. In addition, it allows screening for additional sequences observed in alstroemeria-infecting CMV isolates.

Strains of *Cucumber mosaic virus* can be classified into two major subgroups (I and II) on the basis of host range, serological relationships, peptide mapping, and nucleic acid hybridization (Palukaitis *et al.*, 1992). Strains in the same subgroup share 91-100% homology in nucleotide and putative amino acid sequences, while only 76-84% homology can be observed between strains of different subgroups (Owen *et al.*, 1990; Quemada *et al.*, 1989). Based on the nucleotide sequence of the 3'-half of RNA 3 and deduced amino acid sequences of the coat protein, three studied isolates infecting alstroemeria plants in the Netherlands were classified as subgroup II CMV.

A nonradioactive RNA probe, derived from the additional sequence residing in RNA 3 of ALS-LBO, is highly specific to subgroup II CMV strains. In combination with the degenerated primer set and specific probe developed in this study, the detection and subgroup differentiation of CMV strains can be achieved. Moreover, the probe can be efficiently used for differentiating isolates of CMV in alstroemeria when applied in Northern blot hybridizations.

To date, the occurrence of RNA recombination has been established in several members of the Bromoviridae family, *Brome mosaic virus* (BMV) (Bujarski and Kaesberg, 1986) and *Cowpea chlorotic mosaic virus* (CCMV) (Allison *et al.*, 1990). The additional sequence in the 3' NTR of RNAs 3 and 4 of ALS-LBO and ALS–NAK shares high homology with the RNAs 1 and 2 of the homologous virus and other subgroup II CMV strains, suggesting a RNA recombination event may

have occurred between RNA 3 and RNA 2 or RNA 1.

Due to its limited incidence in the crop, *Cucumber mosaic virus* infecting alstroemeria has not been studied as intensive as CMV occurring in other crops (van Zaayen, 1995). The routine indexing of CMV in alstroemeria plants is carried out mainly by serological methods, which are sufficiently sensitive to detect CMV infection in alstroemeria (Maat, 1980). However, serological methods cannot distinguish CMV-ALS isolates that contain additional sequences outside the CP ORF. This might be one of the reasons that CMV isolates containing additional sequences in the 3' NTR have not been reported previously, neither in alstroemeria nor in other crops. Consequently, recombination events may be much more common among plant-infecting viruses, but are simply not detected due to the common use of serological methods rather than RNA-based detection systems.

The biological relevance of the additional sequences to CMV in alstroemeria requires further elucidation. For *Tobacco mosaic virus* (TMV) (Leathers *et al.*, 1993) and BMV RNA 3 (Gallie and Kobayashi, 1994) it is described that the 3' NTR, not only the structure but also the primary sequence, enhances the translation rate. The pseudoknots located just upstream of the tRNA-like structure of TMV and BMV RNA 3 are necessary for RNA replication and participate in replication and/or translation (Leathers *et al.* 1993; Lahser *et al.*, 1993). The insertion of an additional sequence in the RNA 3 of ALS-LBO and ALS-NAK alters the primary sequence and structure of their 3' NTR and make it different from those of common CMV strains. An altered 3' NTR may subsequently change the biological function of the RNA resulting in increased infectivity or stability in the host. Mechanisms causing the recombination, the role of the 16-nt repeated sequences flanking the additional sequence and the biological effects of the additional sequence are unclear at present and warrant further detailed study.

Chapter 4

Adaptation of *Cucumber mosaic virus* to *Alstroemeria* involves both inter- and intramolecular RNA recombination

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Abstract

In four distinct alstroemeria-infecting *Cucumber mosaic virus* isolates, additional sequences of various lengths were observed in the 3’ NTRs of both their RNAs 2 and 3. The nucleotide sequence and tentative secondary structure of these segments suggest intra- and inter-molecular RNA recombinations. Competition experiments revealed that recombined RNA 2 and 3 segments increase the biological fitness of the virus in alstroemeria. To our knowledge this is the first report that directly relates the occurrence of RNA recombination to host-specific fitness of naturally occurring virus isolates.

INTRODUCTION

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus* in the family *Bromoviridae* (Rybicki, 1995; van Regenmortel *et al.*, 2000) and has been extensively studied (for review, see Palukaitis *et al.*, 1992). The CMV genome consists of three functional single-stranded RNA molecules, of which RNA 1 and RNA 2 encode components of the viral RNA-dependent RNA polymerase (Nitta *et al.*, 1988), while the bicistronic RNA 3 encodes the movement protein (MP) (Suzuki *et al.*, 1991) and coat protein (CP) (Schwinghamer and Symons, 1977). The CP is translated from the subgenomic RNA 4, which is encoded by the 3'-half of the RNA 3 (Gould and Symons, 1982). A small overlapping gene (2b), encoded by RNA 2, was discovered more recently and is most likely expressed through a second subgenomic RNA (Ding *et al.*, 1994). The product of the 2b gene is involved in the virulence of the virus, probably by suppression of gene silencing (Brigneti *et al.*, 1998). In addition to its structural role, the CP also encompasses determinants for symptom induction and vector transmission (Perry *et al.*, 1994; Shintaku *et al.*, 1992). The complete nucleotide sequences of the genomic RNAs have been reported for several CMV isolates and these viruses have been classified into two major subgroups I and II (Quemada *et al.*, 1989; Palukaitis *et al.*, 1992; Anderson *et al*., 1995). CMV is one of the most widespread plant viruses in the world and has a host range of over 1,000 plant species (Palukaitis *et al.*, 1992). Also the ornamental plant alstroemeria has been reported a host of CMV (Hakkaart, 1986), although little data has become available over the years on the interaction between CMV and this host.

Evidence from nucleotide sequence data suggests that natural RNA recombination has occurred in satellite RNA associated with Y-CMV (Masuta *et al.*, 1992) and defective RNAs of Fny-CMV (Garves and Roosinck, 1995). Recombination has also been observed in the artificial pseudo-recombinant viruses composed of CMV and *Tomato aspermy virus* (TAV) RNAs (Masuta *et al.*, 1998). However, systematic search of available CMV nucleotide sequences could not conclude potential recombination events among numerous analyzed CMV

strains (Candresse *et al.*, 1997). No natural mixed infection and no genetic exchange was found between CMV strains of different subgroups (Fraile *et al.*, 1997), indicating that most heterologous genetic recombinations seem to be at a competitive disadvantage (Garcia-Arenal *et al.*, 2000), although a case of natural interspecies recombination between CMV and TAV has been reported (Aaziz and Tepfer, 1999). More recently, a homologous recombination between RNA 1 and RNA 2 or RNA 3 has been demonstrated *in vivo* in subgroup I CMV when using transgene sequences as donor (Canto *et al.*, 2001).

RNA recombination represents one of the major forces that favor the evolution and adaptation of RNA viruses (Strauss and Strauss, 1988; Dolja and Carrington, 1992; Lai, 1992; Simon and Bujarski, 1994; Roossinck, 1997). Most of the data collected from different experimental systems suggest that RNA recombination prefers replicase (RNA-dependent RNA polymerase)-driven template switching models or copy-choice mechanisms (Simon and Bujarski, 1994; Bujarski and Nagy, 1996; Nagy and Simon, 1997). Alternatively, the breakage-and-ligation mechanism has been suggested and proven as a possible means for *in vitro* RNA recombination as well (Chetverin *et al.*, 1997). RNA recombination has been classified into three classes: homologous recombination, aberrant homologous recombination, and non-homologous recombination (King, 1988; Lai, 1992). Based on the components of the recombination machinery, the putative structure of recombination intermediates, and the end products of recombination events, Nagy and Simon (1997) proposed a new classification into three classes: similarity-essential recombination (class 1), similarity-nonessential recombination (class 2), and similarity-assisted recombination (class 3). In similarity-essential recombination events, sequence similarity between the parental RNA strands is required and is the major determinant of the events. The end products can either be precise or imprecise (aberrant). Similarity-nonessential recombination includes those events that do not require sequence similarity between the nascent and acceptor strands, but other features of the RNAs, such as RdRp binding sequences, secondary structure elements and heteroduplex formation between parental RNAs. The class 3 recombination combines the features of both class 1

and class 2 recombination.

In our previous work we described an alstroemeria-infecting subgroup II CMV isolate (ALS-CMV) that contains an additional sequence of 218 nucleotides in the 3' non-translated region (3' NTR) of RNAs 3 and 4 (Chapter 3; Chen *et al.*, 2002b). This additional sequence appears to be identical to parts of the 3' NTRs of RNAs 1 and 2 of the homologous virus, suggesting an RNA recombination event has occurred between RNA 3 and RNA 1 or RNA 2. To investigate the universality of additional sequences in the 3' termini of alstroemeria-infecting CMV isolates the genomic RNAs of five distinct ALS-CMV isolates have been analyzed. Furthermore, the biological relevance of the recombination events encountered was tested by competition experiments.

MATERIALS AND METHODS

Virus isolates, viral RNA and plant materials

Cucumber mosaic virus strains that independently isolated from alstroemeria (ALS-CMV) were stock material of the Bulb Research Center (LBO-LNV), the Inspection Service for Floriculture and Arboriculture (NAKtuinbouw), and the Department of Virology, Plant Research International (PRI) all in the Netherlands. The isolates were collected in the Netherlands and were designated as ALS-0, ALS-1, ALS-2, ALS-3, and ALS-4 (Table 4-1). The ALS-0 isolate, although originally isolated from alstroemeria, had been maintained on tobacco for extended periods of time, the four others were passaged on alstroemeria. All five CMV-ALS isolates were mechanically inoculated to *Chenopodium quinoa* and the local lesions subsequently inoculated to *Nicotiana benthamiana* for short-term propagation. *Nicotiana tabacum* White Burley plants were used as a host for virion purification and alstroemeria plants (clone VV024-6) (Lin *et al.*, 1998) were used for pathogenicity assays. Purifications of virions, viral RNA, and total RNA were carried out as described by Lot *et al.* (1972), Palukaitis and Symons (1980) and Seal and Coates (1998), respectively.

Cloning and sequencing

The degenerate oligonucleotide primer sets for RT-PCR amplification of CMV genomic RNAs were specific for RNA 1 (corresponding to nucleotides 1-23 / 1098-1108, 1074-1087 / 2238-2255, and 2238-2248 / 3307-3391), RNA 2 (corresponding to nucleotides 1-22 / 1105-1118, 1105-1118 / 2062-2075, and 1937-1951 / 3025-3039), or RNA 3 (corresponding to nucleotides 1-34 / 1120-1131 and 1087-1111 / 2195-2204). Typically, approximately 5 μg of total RNA extracted from CMV-infected *N. benthamiana* plants was used as template for cDNA production. The RT-PCR conditions were as described in Chapter 2 (Chen *et al.*, 2001). Products of RT-PCR were purified by agarose gel electrophoresis and cloned into pGEM-T easy (Promega). Sequences of the inserts were determined in both directions. Programs of the UW-GCG package were used for sequence

analysis. Sequences are available in GenBank (codes AJ304393 through AJ304405; Table 4-1)

TABLE 4-1. Lengths in nucleotides and accession numbers of genomic RNAs of alstroemeria-infecting cucumber mosaic viruses (ALS-CMV)

	ALS-0*	ALS-1*	ALS-2*	ALS-3	ALS-4
RNA 1					
Full length	3391	3442	3391	ND	ND
3' NTR	317	317	317	ND	ND
Accession No.	AJ304405	AJ304393	AJ304404	ND	ND
RNA 2					
Full length	3039	3334	3289	1398**	1261**
3' NTR	424	719	674	719	582
Accession No.	AJ304396	AJ304394	AJ304395	AJ304400	AJ304401
RNA 3					
Full length	2204	2419	2419	1413**	1415**
3' NTR	321	539	539	616	616
Accession No.	AJ304399	AJ304397	AJ304398	AJ304402	AJ304403

ND: not determined
* : CMV isolates ALS-0, -1, -2 were designated as ALS-IPO, -LBO, and -NAK, respectively, previously (Chapter 3).
**:partial sequences.

Competition inoculation tests

Purified virions of isolates ALS-0 and ALS-2 were mechanically inoculated to *N. benthamiana*, *N. tabacum*, and alstroemeria in a ratio of 1/0, 1/10, 10/10, 10/1, and 0/1 (μg/μg). Systemically infected leaves were collected and the competitiveness of the isolate was detected by RT-PCR amplification of the 3' half of RNA 3 using degenerate primers (Chapter 2).

To further pinpoint the timeframe in which the competition occurred, purified ALS-0 and ALS-2 virions were inoculated in a ratio of 10/1 to alstroemeria and 1/10 to tobacco, favoring the predicted least fit species by a factor 10 in either case. Here, in addition to the aforementioned RT-PCR method, Northern blot hybridization was carried out to detect the relative abundance of the RNAs of the

competing isolates. RNA was extracted from inoculated leaves of tobacco (2,4,8,12 and 16 days post infection) and alstroemeria (1,2,3,4,5,6 weeks post infection). RNA samples were transferred to Hybond-N membranes after electrophoresis on a 0.8% agarose gel and hybridized to DIG-labeled cDNA probes specific for the CP ORF of subgroup II CMV. The DIG-labeled probe was PCR amplified with a pair of degenerate primers CMVCP-2 and CMV-ALS-CP-R (corresponding to positions1227 to 1240 and 1866 to 1883 on RNA 3 of the ALS-0 isolate, respectively).

RESULTS

Alstroemeria CMV isolates contain additional sequences in both RNA 2 and 3

Nucleotide sequences obtained from cDNA clones of all ALS-CMV genomic RNAs are highly homologous (>97%) to that of reported subgroup II CMV strains. However, the genomic RNA molecules, particularly in the 3' NTR of RNA 2 and RNA 3 molecules were found to be variable in length (Table 4-1). No significant differences were observed in the RNA 1 molecules of the three CMV isolates tested (ALS-0, ALS-1 and ALS-2). All comprise of a 5' NTR of 95 nucleotides, a 1a ORF of 2979 nucleotides and a 3' NTR of 317 nucleotides. A tandemly repeated sequence of 51 nucleotides was found only within the 1a ORF (position 3046–3096) of ALS-1. The RNA 2 molecules contain identical nucleotide numbers in their 5' NTR (92 nt), 2a ORF (2523 nt), and 2b ORF (303 nt), but vary considerably in length in their 3' NTRs (Table 4-1). Also the RNA 3 molecules reveal similar lengths in their 5' NTR (96 nt), 3a ORF (840 nt), intercistronic regions (287–290 nt) and CP ORF (657 nt), but are notably different in the length of the 3' NTRs (Table 4-1). The RNA patterns shown in Figure 4-1, indicate that the observed additional sequences are not a PCR artifact, as the RNAs 3 and 4 of ALS-CMV strains 1 to 4 are visibly larger than those of the ALS-0 isolate and an other subgroup II CMV isolate from crocus. The size addition to RNA 2 makes this RNA indistinguishable in size from RNA 1 in a Northern blot (Fig. 4-1). As ALS-0 has been maintained for several years on *N. benthamiana,* it cannot be excluded that it also originally contained additional sequences.

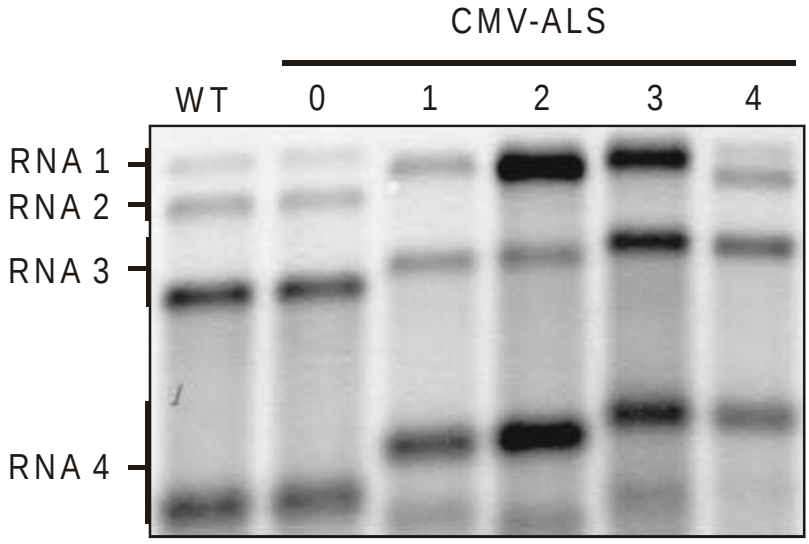


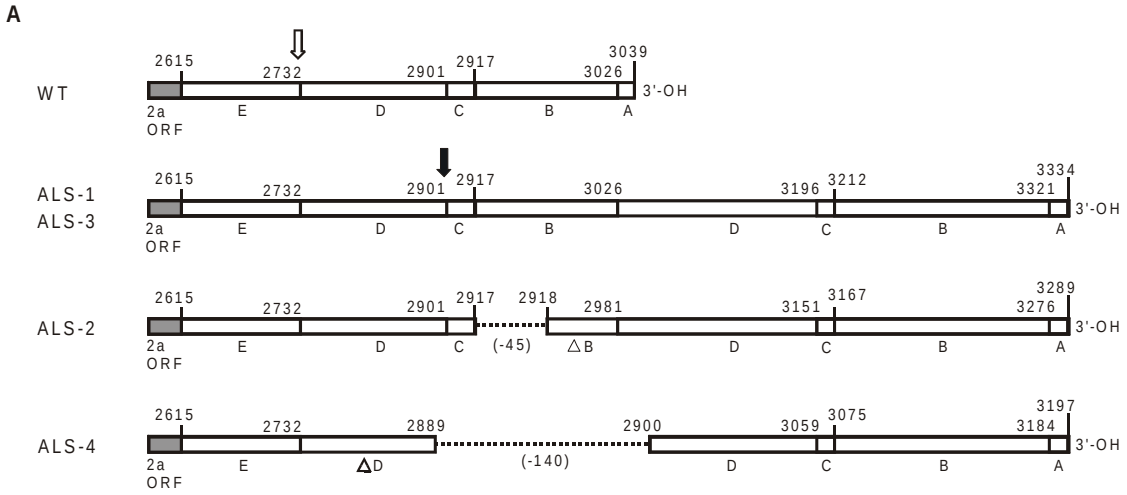
Fig. 4-1. RNA patterns of the five alstroemeria-infecting CMV strains. Northern blot analysis by DIG-labeled cDNA probes specific for the CP ORF of subgroup II CMV in combination with DIG-labeled RNA probes specific for the RNA 2 derived 218 nucleotides in RNA 3 of ALS-1 and ALS-2 (Chapter 3). WT is RNA of a subgroup II CMV isolated from Crocus (Chapter 2), used as a size reference for common CMV isolates. Size ranges of the genomic RNAs (and sgRNA 4) are indicated to the left.

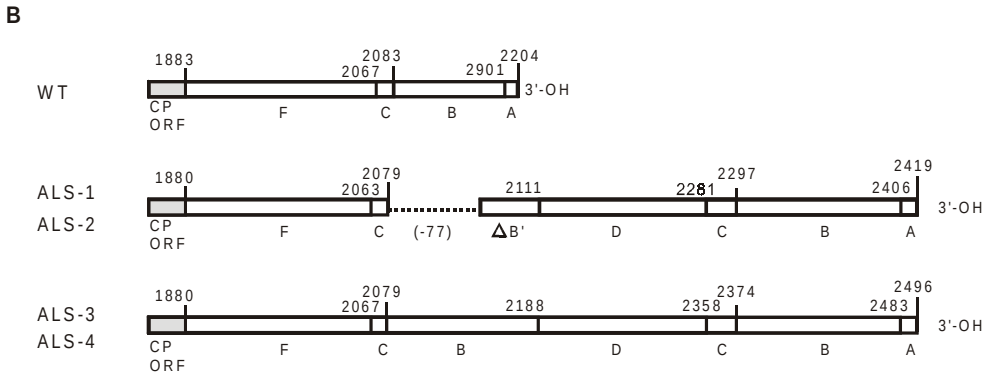
Composition of the 3' NTR sequences suggests several consecutive intra- and intermolecular recombination events in ALS-CMV

In the ALS-0 CMV isolate, the length of the 3' NTRs of RNA 2 (424 nt) and RNA 3 (321 nt) are in accordance with those of reported subgroup II CMV strains. These regions can be arbitrarily divided into five regions in RNA 2 (A, B, C, D, E) (Fig. 4-2A) and four regions in RNA 3 (A, B, C, F) (Fig. 4-2B). Regions denoted A, B and C are common to all reported CMV segments and can be folded into a tRNA-like structure that is involved in viral minus-strand synthesis (Boccard and Baulcombe, 1993). The lengths of the regions A through F are 13, 109, 16, 170, 116, and 183 nucleotides, respectively. In the ALS-0 isolate, regions containing secondary structures consisting of stem-loop configuration are predicted between regions C and D (position 2868 to 2906) (Fig. 4-2C) and at the 3' end of region E

bordering region D (position 2698 to 2731) (Fig. 4-2C). Interestingly, additional sequences of different length were observed in the central region of the 3' NTRs of both RNA 2 and RNA 3 in all four other ALS-CMV isolates. The additional sequences in the RNA 2 of both ALS-1 and ALS-3 are 295 nt and are an exact duplicate of regions D, C and B (Fig. 4-2A). Shorter additional sequences of 250 nt and 158 nt were found in the RNA 2 of ALS-2 and ALS-4, respectively, and seem to have arisen from deletions following the original duplication that resulted in the RNAs 2 of ALS-1 and ALS-3.

In the 3' NTR of RNA 3, ALS-CMV isolates harbor an additional sequence either of 218 nt in ALS-1 and ALS-2 or of 295 nt in ALS-3 and ALS-4 (Fig. 4-2B). Strikingly, the additional 295 nt inserted in the RNA 3 of ALS-3 are completely identical to those of the RNA 2 of the same virus, both in length and in nucleotide sequence (Fig. 4-2).


A
WT
2615
2732
2901
2917
3039
3026
3'-OH
2a
ORF
E
D
C
B
A
ALS-1
ALS-3
3334
3321
3196
3212
ALS-2
2918
2981
3151
3167
3289
3276
(-45)
△B
ALS-4
2889
2900
3059
3075
3197
3184
(-140)
ΔD


B
WT
1883
2083
2067
2204
CP
ORF
F
C
B
A
ALS-1
ALS-2
1880
2079
2063
2111
2281
2297
2419
2406
(-77)
ΔB'
ALS-3
ALS-4
2188
2358
2374
2496
2483

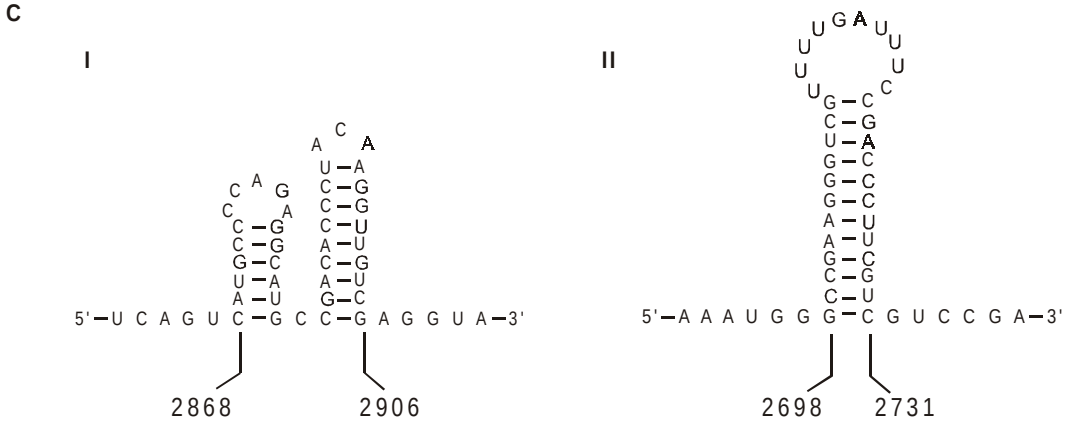

C
I
II
5'—U C A G U C—G C C—G A G G U A—3'
2868
2906
5'—A A A U G G G—C G U C C G A—3'
2698
2731

Fig. 4-2. Schematic representations of the 3'-NTR of the RNA 2 (A) and RNA 3 (B) of alstroemeria-infecting cucumber mosaic viruses. Secondary structures of stem loop can be predicted between nucleotides 2868 – 2906 (I) and nucleotides 2698 – 2731(II) of wt RNA 2 (C). The solid frames in black and gray indicate part of the open reading frames of 2a and CP genes, respectively. The numbers above the empty frames indicate the nt number of the various regions relative to the 5' terminus. The capitalized letters under the diagrams indicate the arbitrarily divided regions of nucleotide sequences in various lengths: A=13 nt, B= 109 nt, C= 16 nt, D= 170 nt, E=116 nt, and F=183 nt. Regions represented with in the same letters are identical in nucleotide sequence and length. The dotted lines indicate deleted parts and their length. Δ indicates (partially) deleted regions. The empty arrowhead indicates stem loop structure II (C) where replication of RNA 2 is halted prior to restarting downstream of that position to form a recombinant RNA 2. The solid arrowhead indicates the position of stem loop structure I shown in (C) where replication termination is thought to occur prior to reinitiation on RNA 3.

Competitive inoculation assays reveal that RNA recombinations are an adaptation of the virus to the host

Both alstroemeria and tobacco plants (*N. benthamiana* and *N. tabacum*) are susceptible to CMV, either with or without recombinant genomic RNAs. However, based on visual observations, all isolates containing recombinant RNAs 2 and 3 (ALS-1 through 4) are more virulent than isolates lacking these repetitions when inoculated to alstroemeria, suggesting a greater fitness on this host. To verify this observation, tobacco and alstroemeria plants were inoculated with mixtures of various ratios of purified virions of 'wild-type' ALS-0 and one of the recombinant-type isolates ALS-2. In systemically infected leaves of tobacco plants, only non-recombinant sequences could be amplified in subsequent RT-PCR analysis, except when ALS-2 was inoculated alone. In alstroemeria plants,

however, only RNAs of recombinant ALS-2 were detected in plants inoculated with all mixed inocula used (Fig. 4-3). These results demonstrate a higher replicative ability of the non-recombinant ALS-0 over ALS-2 when infecting tobacco plants, while the recombinant ALS-2 isolate was more competitive in infecting alstroemeria.

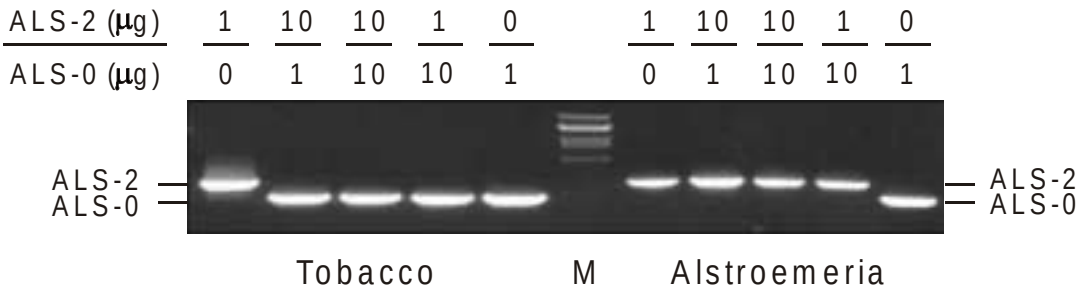


Fig. 4-3. Agarose gel electrophoresis of RT-PCR fragments amplified from total RNA of tobacco or alstroemeria leaves infected with CMV isolates ALS-0 and ALS-2. Various ratios of inoculated virion amounts are indicated on top of the lanes. The degenerated primers amplify the intercistronic region, CP ORF, and 3' NTR of RNA 3. cDNA fragments of 1.1 kb (ALS-0; wt) and a 1.3 kb (ALS-2) are indicated. Lambda DNA digested with *PstI* was used as size marker (M).

Selecting the host-dependent fitness of CMV isolates is a rapid process

To further delineate the difference of replication speed, tobacco and alstroemeria plants were inoculated with mixtures of virus isolates in which the least fit virus was added in 10-fold excess. Viral RNA replication was monitored in the inoculated leaves by Northern blot analyses and RT-PCR assays (Fig. 4-4). The enlarged RNAs 3 and 4 of ALS-2 isolate can only be detected until the fourth day-post-inoculation in samples from tobacco on Northern blots and RT-PCR while those of the ALS-0 isolate can be detected from the first after inoculation and the signals further increased over time accumulated. However, on alstroemeria, the ALS-0 isolate, even though inoculated in 10-fold excess, remained undetectable in inoculated leaves. This experiment indicates that ALS-0 and ALS-2 are

outcompeted during the early stages of infection on alstroemeria or tobacco, respectively, in spite of the initial molecular excess over the competitor. Therefore, the additional sequences in the 3' NTRs of RNAs 2 and 3, which are the only differences observed between these isolates, are responsible for the enhanced fitness of the virus in alstroemeria, but produced an adverse effect in tobacco plants.

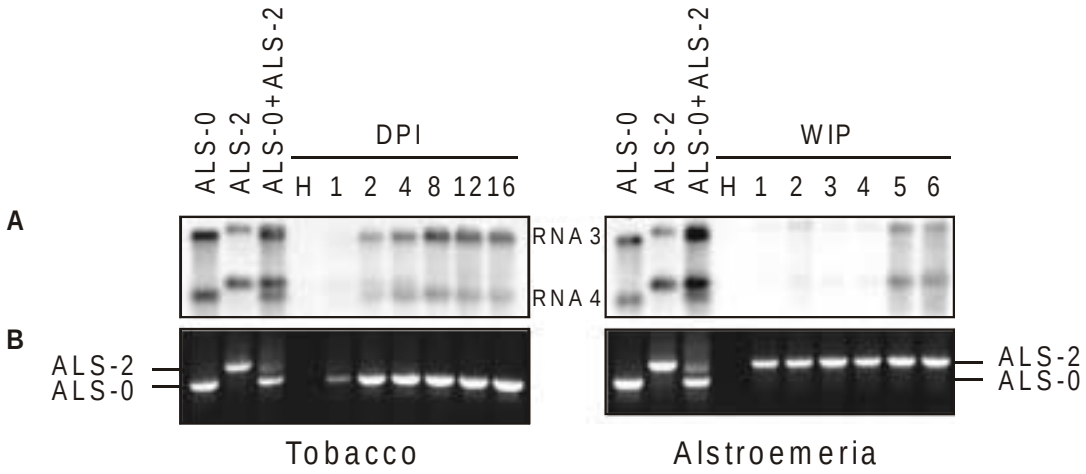


Fig. 4-4. Competition between ALS-CMV recombinant (ALS-2) and non-recombinant (ALS-0) isolates upon mixed infection of tobacco and alstroemeria. RT-PCR (A) and Northern hybridization (B) of RNA extracted at indicated days (DPI) or weeks (WPI) post-inoculation from tobacco and alstroemeria inoculated with CMV ALS-0 and ALS-2 isolates.

DISCUSSION

For all CMV isolates that have been sequenced to date, the sizes of full-length genomic RNA 1, RNA 2, and RNA 3 molecules are approximately 3.3 kb, 3.0 kb, and 2.2 kb, respectively. In this study, additional sequences of various lengths were found in the 3' NTRs of RNA 2 and RNA 3 of four distinct alstroemeria-infecting CMV isolates. Though many cases of recombinations between RNA segments of viruses or between viral RNAs and their satellite RNAs have been described (e.g. Bruyere *et al.*, 2000; Masuta *et al.*, 1998; Nagy *et al*., 1999) naturally occurring stable recombinations are rare (Fraile *et al.*, 1997). An increase of the biological fitness of a virus by recombination within or between its segments has not been reported previously under natural conditions.

We have shown that out of five alstroemeria isolates of CMV, four contain similar large additions to the 3' NTR regions of their RNAs 2 and 3. A fifth isolate did not show this remarkable feature, perhaps because it had been maintained on tobacco for considerable time. To verify the biological significance of these additional sequences to the CMV genome, mixed inocula of a recombinant-type isolate (ALS-2) and a 'wild-type' isolate (ALS-0) lacking these recombinations were tested for replicative fitness on alstroemeria as well as tobacco. Strikingly, The relative biological fitness of these isolates appeared to be completely depended on the host to which the mixed inoculum was applied. In alstroemeria, the ALS-2 isolate rapidly outcompeted ALS-0, while on tobacco, the situation was reverse. This clearly demonstrates the biological relevance of the additional sequences in isolate ALS-2 and furthermore suggests that maintenance of ALS-0 on tobacco may have lead to adaptation to that host, concomitant with the lack of additional sequences.

How the recombinations may have arisen remains subject to further study. Some clues, however, can be obtained from the position of two stem-loop structures (Fig. 4-2), in combination with the previously implicated involvement of the CMV RdRp in template switching (Kim and Kao, 2001). The template switching mechanism has been commonly accepted as one of the major means for the

generation of recombinant RNAs (Simon and Bujarski, 1994; Bujarski and Nagy, 1996; Nagy and Simon, 1997; Chetverin, 1999; Worobey and Holmes, 1999). The RNAs 2 of the recombinant isolates show the duplication of a contiguous internal 3' NTR segment. The same RNA 2-derived sequence was found to be incorporated in the 3' NTR of the RNAs 3 of the recombinant alstroemeria CMV isolates, of which some seem to have undergone further deletions. A complex secondary structure consisting of a stable hairpin (II in Fig. 4-2C) may have caused the RdRp to halt and disengage during minus strand synthesis of the recombinant RNA 2. Subsequent restarting of replication at the 3' end of a second RNA 2 template resulted in the initial duplication (Fig. 4-2A). While replicating the 3' end of the recombinant RNA 2, the viral replication complex may have halted and separated at hairpin loop I (Fig. 4-2C). Subsequently, the RdRp continued replication on the highly homologous 3' NTR of RNA 3, resulting in an RNA 3 molecule with a duplicated 3' NTR of RNA 2 origin. A model has been proposed for the formation of recombinant RNAs 2 and 3 (Fig. 4-5).

Recombinational events leading to mutant viruses as observed here may be commonplace for CMV, as was already suggested by Canto and co-workers (2001). These authors were readily capable to generate recombinant CMV RNAs in a transgene setting, selecting for such events. Nonetheless, the natural occurrence of recombinant viruses is very limited, most likely due to replicative fitness selection in favor of wt CMV (Fraile *et al*., 1997). Our observations in the mixed infections of tobacco plants are in agreement with that notion. Alstroemeria plants, however, seem to form an exception to that rule sustaining the presence of specific recombinant viruses. Additional sequences observed in the ALS-CMV isolates are all duplicated 3' NTRs. These 3' ends of positive strand viral RNAs contain the origin of minus strand synthesis and are the presumed site of the promoter elements that control this synthesis *in cis* (Dreher, 1999). Duplication within the NTRs viruses infecting alstroemeria could be explained by the recruiting of host proteins involved in the viral replication complex. If binding of these components in alstroemeria is less efficient than in most other plant species, it may be beneficial to duplicate the regions involved in recruiting these host factors.

Although the inserted sequences might interrupt the sequence and secondary structure of the 3' termini, the alteration offers biological advantages on alstroemeria-infecting CMV isolates. In addition, the slow infection process of CMV in alstroemeria and low accumulation of viral RNA (results not shown) could indicate that duplications in viral sequences are tolerated in this host, while in other hosts these viruses are increased in their sensitivity to host defense responses, such as gene silencing. The competitive inoculation tests demonstrated the merit of these recombination events that enhance the fitness of ALS-CMV when infecting alstroemeria. This does not only explain the fact that all studied ALS-CMV isolates that had been maintained on this host contain additional sequences at the 3' termini of RNAs 2 and 3, but also shows that RNA viruses can benefit from the recombination events (Worobey and Holmes, 1999) as an adaptation to differing circumstances in specific host plants.

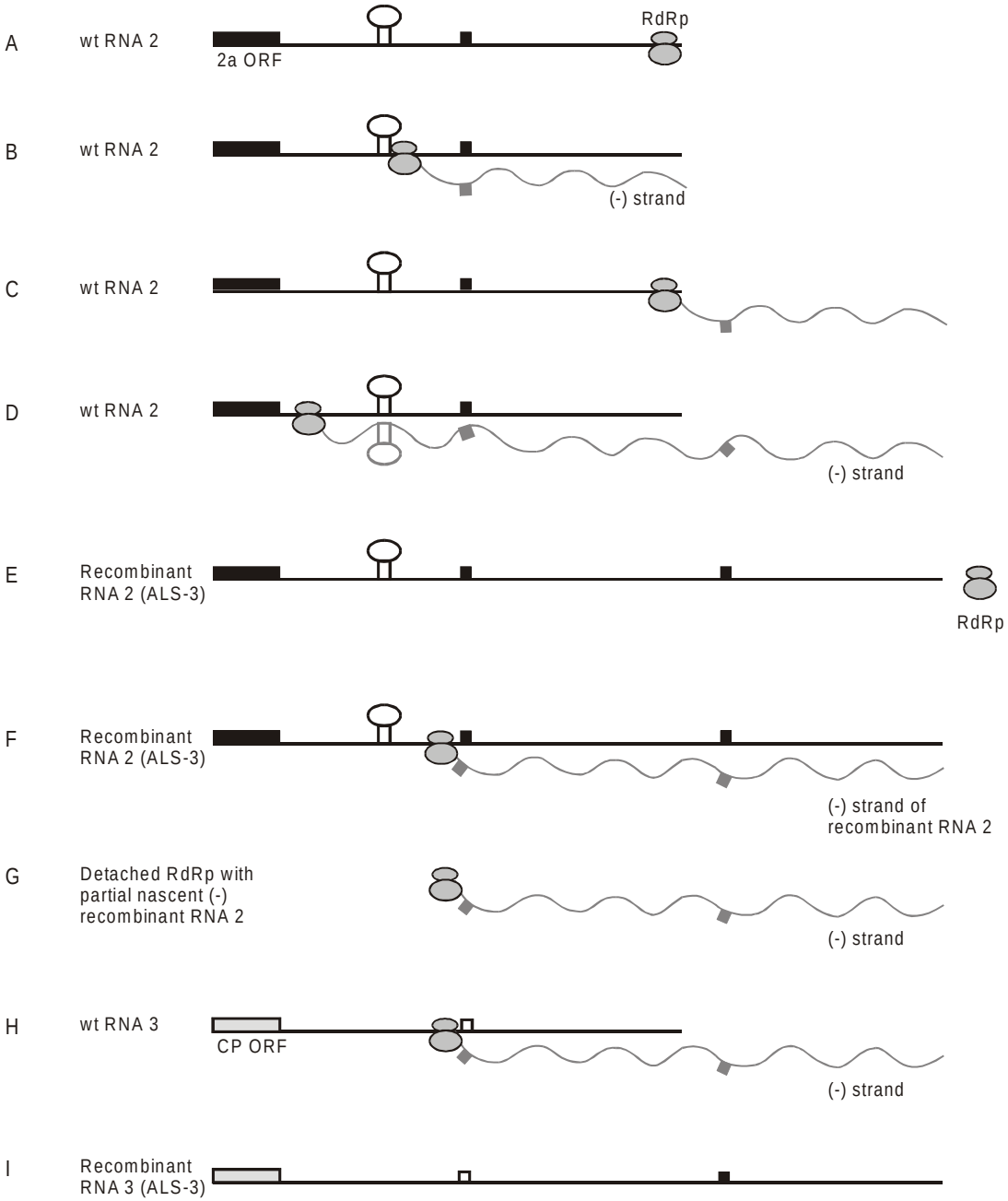

A
wt RNA 2
2a ORF
RdRp
B
wt RNA 2
(-) strand
C
wt RNA 2
D
wt RNA 2
(-) strand
E
Recombinant
RNA 2 (ALS-3)
RdRp
F
Recombinant
RNA 2 (ALS-3)
(-) strand of
recombinant RNA 2
G
Detached RdRp with
partial nascent (-)
recombinant RNA 2
(-) strand
H
wt RNA 3
CP ORF
(-) strand
I
Recombinant
RNA 3 (ALS-3)

Fig. 4-5. A proposed model to explain how the recombinant molecules may have arisen by using the RNAs 2 and 3 of CMV-ALS. Initially, the RdRp uses the RNA 2 of a wild type CMV as a template to initiate synthesis of the minus strand (Fig. 4-5A). A complex secondary structure consisting of a stable hairpin (position 2698 to 2731) causes the RdRp to halt and disengage just downstream of region E (Fig. 4-5B). Subsequent restarting of the RdRp from the promoter of replication in the 3' end (Fig. 4-5C) of a second RNA 2 template (processive mechanism) or from the promoter of the original template (non-processive mechanism)(Jarvis and Kirkegaard, 1991) results in the duplication of regions D, C and B (Fig. 4-5D), thereby creating the recombinant RNA 2. The recombinant RNA 2 is then used as a template for the formation of the RNA 3 recombinant (Fig. 4-5E). The viral RdRp complex, while replicating the 3' end of the recombinant RNA 2 is stopped at a hairpin loop at the 5' end of the second region C (Fig. 4-5F) and separates from the RNA 2 template (Fig. 4-5G). Since the nucleotides in the 3' end of CMV genomic RNAs are highly homologous, a displaced nascent minus strand RNA 2 can hybridize to positive sense RNA 3 strands (Fig. 4-5H). Subsequently, the RdRp continues replication using RNA 3 as template, resulting in an RNA 3 molecule with a part of the (duplicated) 3' NTR of RNA 2 (Fig. 4-5I).

Chapter 5

Assessing coat protein- and RNA-mediated resistance as options for host resistance against *Cucumber mosaic virus*

Yuh-Kun Chen, Tony de Rover, Annemarie Westerbroek, Marcel Prins, Rob Goldbach and Peter de Haan

Abstract

Cucumber mosaic virus (CMV) ranks among the most important and widespread viruses of both field-grown and greenhouse crops and ornamentals. Since for most of these crops limited sources of natural resistance are available which are suitable for breeding programs, novel strategies to obtain alternative forms of CMV resistance in plants are needed. Based on the concept of pathogen-derived resistance (PDR) alternative approaches such as transgenic expression of viral coat protein (CP) or (defective) replicases in plants to confer resistance against CMV infections have been reported over the years, albeit with sometimes very different outcomes. In view of the opposing results reported for engineered forms of host resistance to CMV, here we have assessed the practical value of such approaches, using a number of cDNA constructs derived from CMV RNAs 2 and 3. Using tobacco as model host plant only limited protection to CMV was obtained upon transformation with the CP gene, the S_1 progeny of CP-expressor plants showing only a delay in systemic disease symptom development and the homozygous S_2 progeny still only prove to be partially protected. With respect to RNA-mediated resistance, (non-translatable) RNA 3-derived sequences were not capable to induce resistance but sequences derived from RNA 2 conferred high levels of resistance. In the latter case the S_1 progeny of a number of transgenic lines showed high levels of resistance, while the homozygous S_2 offspring of these lines were completely protected. The results underscore that, though not all genomic sequences seem equally suitable, RNA-mediated resistance is a better option for introducing transgenic resistance to CMV than CP-mediated resistance.

INTRODUCTION

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus* within the Family *Bromoviridae* (Rybicki, 1995; Van Regenmortel *et al.*, 2000). Of the tripartite CMV genome RNAs 1 and 2 are separately encapsidated and encode the viral polymerase subunits 1a and 2a respectively. RNA 2 moreover encodes an additional protein, 2b, expressed from a subgenomic mRNA (Ding *et al*., 1994). This 2b protein is capable to suppress the host RNA silencing mechanism and thereby this protein is also involved in symptom severity (Brigneti *et al*., 1998). Also RNA 3 is bicistronic, coding for the viral movement protein (MP), and the viral coat protein (CP). Like the 2b protein, the CP is expressed via the formation of a subgenomic RNA molecule, denoted RNA 4, which is encapsidated in a single virus particle together with RNA 3 (Palukaitis *et al*., 1992). CMV has an extremely wide host range and many different strains have been identified (Douine *et al*., 1979; Kaper & Waterworth, 1981). Based on phylogenetic analysis of the CP ORF and rearrangements in the 5' nontranslated region (NTR) of RNA 3, CMV strains can be divided into three subgroups: IA, IB, and II (Anderson *et al.*, 1995; Palukaitis and Zaitlin, 1997; Quemada *et al.*, 1989; Roossinck *et al.* 1999). Subgroup I isolates of CMV are the most predominant and of major economic importance. Outbreaks of diseases incited by CMV infections have caused significant yield losses in many economically important crops (Tomlinson, 1987). Unfortunately, for most crops, suitable sources of natural resistance to CMV have not become available for breeding (Watterson, 1993). Hence, to date, most crops can only be protected from CMV infections by taking phytosanitary measures, by early detection and by using virus-free starting material.

Transgenic host resistance has been proposed as a mean to protect plants from viruses. The concept of pathogen-derived resistance (PDR) (Sanford and Johnston, 1985) was first demonstrated in plants by using the CP gene of *Tobacco mosaic virus* (TMV) in tobacco plants to confer resistance against its homologous virus (Powell Abel *et al*. 1986). Transgenic expression of viral CP in plants confers

protection against the homologous virus and sometimes to closely related viruses. This strategy, referred to as CP-mediated protection, has been widely tested and shown to protect plants against infections by certain viruses (reviewed by Beachy *et al*., 1992). CMV is one of the viruses for which CP-mediated resistance has been aimed. The various reports on this subject however indicate very different, and sometimes even opposing outcomes of this approach, which might be partly due to different levels of CP accumulation obtained, the use of different gene constructs and/or different host systems. For all reported successful cases of CP-mediated resistance against CMV high expression levels of transgenic CP were reached (Cuozzo *et al*., 1988; Gonsalves *et al*., 1992; Gonsalves *et al*., 1994; Kaniewski *et al*., 1999; Nakajima *et al*., 1993; Namba *et al*., 1991; Okuno *et al*., 1993a; Okuno *et al.*, 1993b; Quemada *et al*., 1991; Xue, *et al*., 1994; Yie, *et al.,* 1992; Yoshioka *et al*., 1993), though it is known that this does not at all guarantee a resistant phenotype (Jacquemond *et al*., 2001). The resistance responses in the different CMV-host combinations seem to vary considerably, in some cases only a temporal delay in symptom appearance was obtained, in other cases milder or attenuated expression of symptoms were reported, and in yet other cases no symptoms at all were observed suggesting full resistance.

Another form of transgenic resistance is based on transcriptional expression of viral transgenes which apparently induce a (RNA) sequence-specific defense mechanism in plants generally referred to as post-transcriptional gene silencing (PTGS) or simply “RNA silencing” (Baulcombe, 1999; Ding, 2000; Chicas and Macino, 2001; Hammond *et al.*, 2001; Vance and Vaucheret, 2001; Waterhouse *et al.*, 2001; Baulcombe, 2002). RNA silencing involves the recognition of double-stranded (ds)RNA by an RNase III type enzyme (called Dicer) and the generation of small RNA molecules of 21 to 25 nucleotides (small interfering RNA, siRNA) in both sense and antisense orientations (Hamilton and Baulcombe, 1999; Bass, 2000). RNA-mediated resistance as a form of transgenic resistance has been reported for a growing number of virus-plant combinations (Matzke *et al.*, 2001; Voinnet, 2001; Waterhouse *et al.*, 2001). In case of CMV, however, the reported results have been mostly described as “replicase-mediated” resistance,

explaining the resistance observed by interference by a transgenically expressed (mutant) replicase subunit (Anderson *et al.*, 1992; Carr *et al.*, 1994). In retrospective, however, the reported cases of replicase-mediated resistance most probably were based on RNA silencing as in none of the reported cases transgenically expressed replicase proteins could be detected and the resulting resistant phenotype fit that of RNA silencing (full immunity) (Dougherty *et al.*, 1994; Mueller *et al.*, 1995).

In view of both seemingly opposing results with respect to CP-mediated resistance, and the confusing reports with respect to replicase-mediated versus RNA-mediated resistance, we have tried to validate both CP-mediated resistance and RNA-mediated resistance side by side, as potential options for engineering host resistance to CMV.

MATERIALS AND METHODS

Viruses and plant materials

CMV isolate ZU (Gielen *et al.*, 1996), a subgroup I member closely related to strain Fny, was stored as desiccated leaf material at 4°C in the presence of $CaCl_2$, or as fresh leaf material in liquid nitrogen. Before use in the cloning and inoculation experiments, CMV was multiplied in *Nicotiana tabacum* cv. SR1. This tobacco variety was also used as recipient in the transformation experiments. The transgenic tobacco plants were grown under certified greenhouse conditions according to Dutch legislation (VROM/COGEM GGO 02-095).

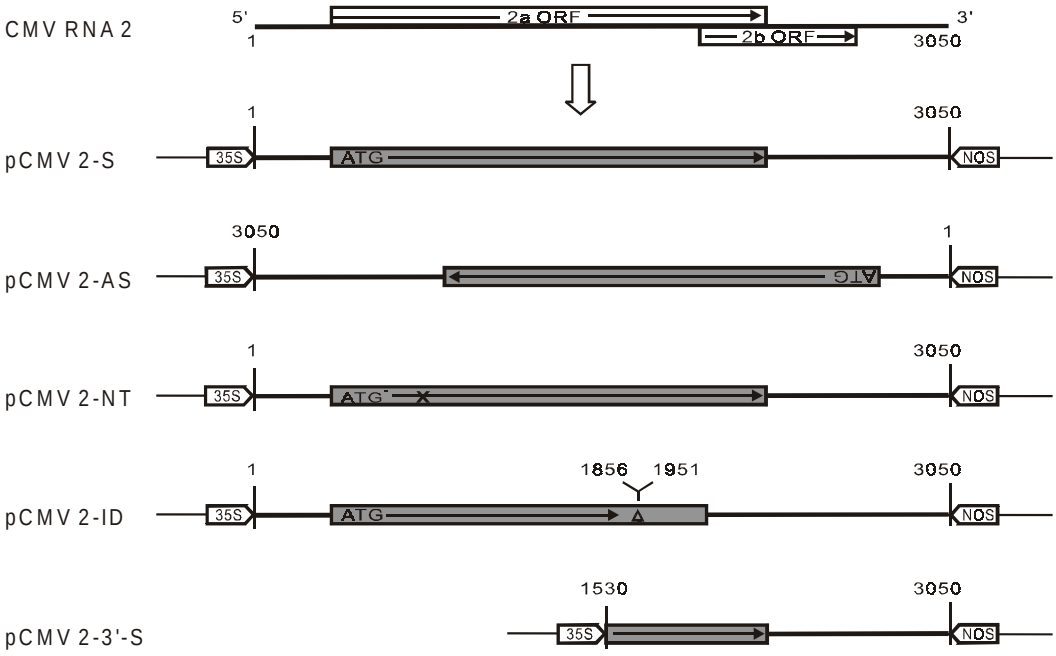
Construction of plant transformation vectors

All manipulations involving DNA were essentially performed according to standard procedures (Ausubel *et al*., 1987; Sambrook *et al.*, 1989). Plant expression vectors pZU-A and pZU-B have been described previously (Gielen *et al*., 1991). The CMV-ZU CP gene was amplified by PCR and subsequently cloned in sense form in pZU-B and pBIN19 respectively as described by Gielen and co-workers (1996). This recombinant binary vector has been denoted pCMV3-CP-S. In a similar way the CMV movement protein (MP) gene was PCR amplified and cloned in pZU-B and pBIN19. This vector has been denoted pCMV3-MP. The RNA 2 and RNA 3 segments of CMV were PCR-amplified using synthetic oligonucleotides homologous or complementary to 15 nucleotides at the 5' and 3' termini of both RNA molecules. The full-length cDNA clone of CMV RNA 2, pCMV2-ZU, was cloned in sense and antisense orientation in pZU-A. The resulting plasmids were denoted pCMV2-S and pCMV2-AS, respectively. The 3’ halves of RNA 2 molecules were amplified by a set of 15-nt primers complementary to positions 1530 to 1544 and the 3’ ultimate terminus of RNA 2 and were cloned in sense orientation in pZU and pBIN19. The resulting plasmids were denoted as pCMV2-3’-S. Plasmid pCMV2-S of ZU strain was digested with *Sal*I, the ends were made blunt-ended with T4 DNA polymerase and religated, yielding pCMV2-NT, a non-translatable RNA 2 gene cassette. An RNA 2 gene

cassette with an internal deletion, according to Anderson and co-workers (1992), was produced by digestion of plasmid pCMV2-S of CMV-ZU strain with *Nco*I and *Bst*EII. After creating blunt end termini with T4 DNA polymerase the vector was religated, yielding pCMV2-ID. Plasmid pCMV3, a full-length cDNA clone of CMV-ZU RNA 3 was also cloned in sense and antisense orientation in pZU-A, resulting in plasmids pCMV3-S and pCMV3-AS respectively. A non-translatable RNA 3 gene cassette was produced by digestion of pCMV3-S with *Xcm*I. The termini were made blunt-ended with T4 DNA polymerase and religated, yielding pCMV3-NT. All RNA 2 and 3 derived gene cassettes were released as *Sac*II/*Sma*I fragments. The ends were blunt ended with T4 DNA polymerase and the fragments were cloned in the *Sma*I site of pBIN19 (Bevan, 1984). The schematic diagrams of the constructs are shown in Fig. 5-1.

By using pRK2013 as a helper plasmid (Ditta *et al*., 1980), the resulting transformation vectors were transferred to the non-oncogenic *Agrobacterium tumefaciens* strain LBA4404 (Ooms *et al*., 1981) by means of triparental mating. The recombinant *A. tumefaciens* strains were checked for the integrity of the transformation vectors by Southern blot analysis.

A


CMV RNA 2
5'
1
2a ORF
2b ORF
3'
3050
pCMV 2-S
35S
ATG
NOS
pCMV 2-AS
pCMV 2-NT
pCMV 2-ID
A
1856
1951
pCMV 2-3'-S
1530

B

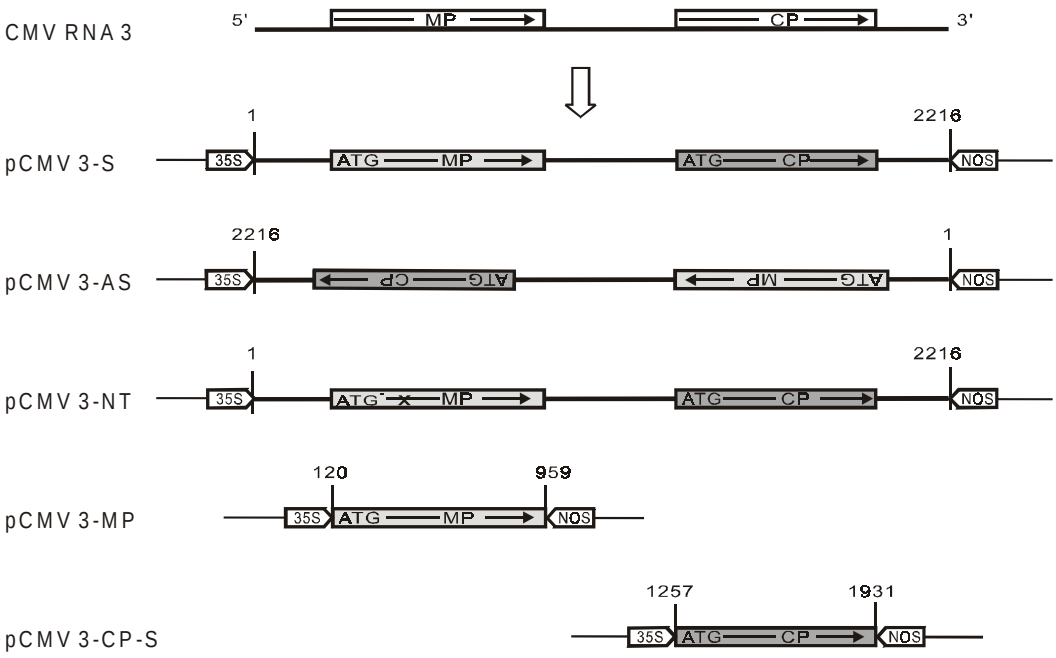

CMV RNA 3
MP
CP
pCMV 3-S
pCMV 3-AS
pCMV 3-NT
pCMV 3-MP
120
959
pCMV 3-CP-S
1257
1931

Fig. 5-1. Schematic representation of the DNA constructs used to transform tobacco. All constructs were provided with a 35S CaMV promoter (containing a TMV translational enhancer) and a nopaline synthetase (nos) terminator sequence. CMV sequences are indicated as thick black lines, with the ORFs shown as shaded areas. Numbers refer to nucleotide positions on the original CMV genomic RNA sequences.

Transformation of tobacco

Transformation and regeneration of *in vitro* grown *Nicotiana tabacum* cv. SR1 were performed by the leaf disk method, essentially according to Horsch *et al*. (1995). Transgenic tobacco shoots, selected for resistance to kanamycin (100 *μ*g/ml), were rooted, potted in soil and transferred to the greenhouse. Schematic representations of the expressed viral RNA from the transformation vectors and the number of regenerated transformants are indicated in Fig. 5-1 and Table 5-1, respectively. To detect the accumulation of CMV CP, transgenic tobacco plants harbouring pCMV3-CP were analyzed by a double antibody sandwich (DAS) ELISA using an antiserum raised against gel-purified CMV CP, which was produced in *E. coli* (Gielen *et al*., 1996).

Table 5-1. Number of transgenic *Nicotiana tabacum* SR1 lines (R_0) transformed with various constructs derived from CMV RNAs 2 or 3

Constructs	Number of transgenic lines produced
PCMV2-S	30
PCMV2-AS	29
PCMV2-NT	24
PCMV2-ID	22
PCMV2-3'-S	22
PCMV3-S	20
PCMV3-AS	20
PCMV3-NT	24
PCMV3-MP	18
PCMV3-CP-S	30

Determining transgenic CP production in tobacco

Transgenic coat protein expression was determined by DAS-ELISA using rabbit anti-CMV immunoglobulin. Leaf extracts were applied to microtitre plates coated with rabbit anti-CMV IgG and washed after incubation. Samples were then incubated with a 1/2500 dilution of alkaline phosphatase-conjugated anti-CMV IgG. After washing, the nitrophenylphosphate substrate was added and absorbance at 410 nm was monitored.

Analysis of protection to CMV infection

Tobacco seedlings of six weeks old were analyzed for resistance to the ZU isolate of CMV using mechanical inoculation (Gielen *et al*., 1991; de Haan *et al*., 1992; Gielen *et al*., 1996). The amount of virus present in the inocula was quantified by DAS-ELISA, using known amounts of purified CP as internal standards. Inoculated plants were monitored daily for the development of characteristic systemic mosaic disease symptoms.

RESULTS

Construction of CMV gene cassettes and transformation of tobacco

All constructs made and tested in this study are shown in Figure 5-1. The cDNA clones of the CMV CP gene and of full-length genomic RNA 2 and RNA 3, were placed in both sense (S) and antisense (AS) orientation between the *Cauliflower mosaic virus* (CaMV) 35S promoter and the nopaline synthase (*nos*) terminator sequence using pZU plasmids (Gielen *et al*., 1991). The CaMV promoter was modified by fusing the TMV 5' untranslated leader sequence which has translation enhancing activity (Gallie *et al.*, 1987) immediately downstream of the transcription initiation site.

Non-translatable (indicated NT, see Fig. 5-1) gene cassettes were made by distortion of the translational reading frames immediately after the AUG start codons. Also a CMV RNA construct was made containing a large internal deletion (construct pCMV2-ID) and potentially encoding a truncated 2A replicase protein. All DNA constructs were transferred to the tobacco genome using *A. tumefaciens*. For each construct at least eighteen independent primary transformants were produced (Table. 5-1). All transformants had normal phenotypes and were fertile. Plants were self-pollinated in the greenhouse and the S_1 progeny seedlings were subsequently analyzed for resistance against CMV infection.

Analysis of CP gene transformed tobacco for resistance against CMV infections

Thirty primary tobacco (*N. tabacum* SR1) plants transformed with a translatable CMV CP transgene were analyzed for the accumulation of viral CP by DAS-ELISA. Only 4 transgenic *N. tabacum* SR1 accumulated detectable amounts of CP, i.e. transformant R_0 plants CP-3, 5, 6 and 14. The amounts of transgenically produced CP, however, were low and did not exceed 0.05% of the total soluble protein content (results not shown). The other 26 tobacco plants did not accumulate detectable amounts of CP, despite sequence-confirmed correctness of

the constructs used.

The four expressor lines CP-3, 5, 6 and 14 were tested for their response to CMV infection. To this end, 30 plants of each line were mechanical inoculated with CMV, non-transgenic plants serving as susceptible controls. The inoculum, containing approximately 5 μg virus per ml as determined by DAS ELISA, was applied on a carborundum-dusted first true leaf, approximately six weeks after germination. All of the non-transformed plants and the S_1 progeny plants of all non-expressor lines developed symptoms characteristic for CMV infection three to five days post inoculation. Of the 4 expressor lines, lines CP-5 and -6 became symptomatic with an average delay of one day, whereas lines CP-3 and -14 showed a delay in the development of systemic disease symptoms of approximately two days (see Fig. 5-2). As a next step to identify lines with good levels of CP-mediated resistance, the S_2 plants of all four expressor lines were tested (Table 5-2). Only line CP-3 yielded S_2 progeny lines with delayed symptom development upon mechanical inoculation. The delay in symptom development was even more pronounced at lower inoculum pressure. In the latter case both lines CP-3 and CP-14, both having the highest transgenic CP expression, gave S_2 lines which showed systemic disease symptoms four days later than the non-transformed controls and the non-expressor transgenics. At lower inoculum pressure, not all control plants became infected and results obtained were not further analyzed.

The homozygous S_2 plants were re-screened for protection against CMV with inocula of approximately 0.5 μg/ml and 5 μg/ml of virus respectively. The homozygous lines descending from line CP-5 and 6 again showed a delay in systemic symptom development, but no full resistance. Resistance in the CP-3 and CP-14 S_2 lines was also only expressed as delay of symptoms, although resistance levels were higher (Table 5-2). In general, when higher titers of virus were inoculated to plants belonging to these lines, the number of plants that developed CMV symptoms increased. Hence, in summary it can be concluded that CP-mediated resistance turns out to be difficult to achieve. Not only few, low level expressor lines could be selected out of 30 primary transformants, but moreover,

these few expressor lines showed a limited level of resistance to CMV, i.e. the infection process was delayed.

Table 5-2. Percentage of virus-resistant S_2 plants of transgenic tobacco lines expressing CMV coat protein

CP-S S_1 lines	CP-S S_2 lines	Inoculum	
		5 *u*g/ml	0.5 *u*g/ml
3	3-1	25	30
	3-2	30	30
	3-4	20	45
5	5-1	0	0
	5-3	0	0
	5-4	0	0
6	6-1	0	0
	6-2	0	0
	6-3	0	0
14	14-2	0	20
	14-3	0	15
	14-4	0	10
Control (Non-transgenic)		0	0

Testing CMV RNA 3 constructs for inducing RNA-mediated resistance

To test whether sequences of the genomic RNA 3 segment of CMV could induce RNA-mediated resistance a series of transgenic constructs were made and transformed to tobacco. These constructs contained either the full-length copy of RNA3, in sense (S) or antisense (AS) orientation, either with its ORFs intact or made non-translatable (NT). Also a construct was made containing only the MP ORF (Figure 5-1B). For each construct thirty S_1 progeny plants of each of at

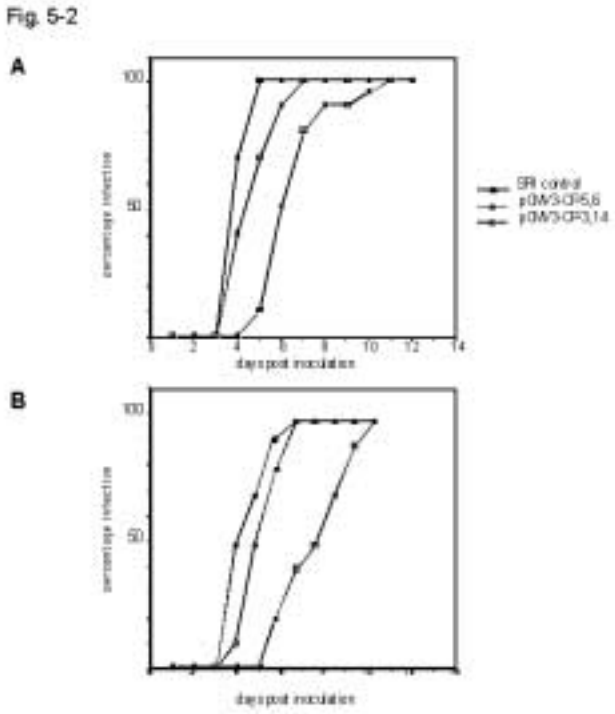


Fig. 5-2. Analysis of the S_1 progeny of four CMV CP-expressing transgenic tobacco plants. Panel A, Symptom development of plants inoculated with 5 μg/ml of virus; panel B, plants inoculated with 0.5 μg/ml of virus.

least 18 independent transgenic lines (see Fig. 5-1) were inoculated with a 5 μg/ml virus suspension and an equal number of plants were inoculated with 0.5 μg/ml of virus. As indicated in Table 5-3 all transgenic plants developed systemic disease symptoms four to five days post inoculation, similar to the non-transgenic SR1 controls.

Testing RNA 2 sequences for inducing RNA-mediated resistance

When the S_1 progeny plants of transgenic tobacco plants containing a full-length CMV RNA 2 cDNA construct in either sense (S) or antisense (AS) orientation, in a non-translatable format (NT), or with an internal deletion (ID), were inoculated with virus (5 *u*g/ml), lines with various levels of resistance were identified. The observed levels of resistance could be arbitrarily grouped into low

(level B), medium (level C) or high (level D) (Table 5-3). The plants that became infected upon inoculation developed mosaic symptoms four to five days post inoculation, just as the non-transgenic SR1 control plants. Lines containing a sense CMV RNA 2 copy exhibited a higher frequency of high levels of resistance than lines containing a NT or ID version of this copy. Lines containing the antisense RNA 2 construct only expressed low or moderate levels of resistance, while transgenic plants expressing only a partial clone of RNA 3 (CMV3-MP) did not show any resistance against CMV at all. Next, the S_2 progeny of the 'level D' resistant plants harboring the full-length sense or full-length non-translatable RNA 2 clone were assayed for resistance (Table 5-4). A number of homologous S_2 lines proved completely resistant against CMV: even at higher inoculum pressures the plants never showed symptoms and virus could not be detected. Some of the other lines showed segregation of the transgene in the S_2 generation and hence did not show 100% resistance.

Table 5-3. Resistance levels of transgenic lines harboring transgenes derived from CMV RNA 2 or RNA 3

DNA constructs	Number of S_1 lines				
	Tested	Resistance levels*			
		A	B	C	D
pCMV2-S	30	11	4	5	10
pCMV2-AS	29	11	16	2	0
pCMV2-NT	24	12	3	5	4
pCMV2-ID	22	6	6	6	4
pCMV2-3'-S	22	22	0	0	0
pCMV3-S	20	20	0	0	0
pCMV3-AS	20	20	0	0	0
pCMV3-NT	24	24	0	0	0
pCMV3-MP	18	18	0	0	0
pCMV3-CP-S	30	30	0	0	0

*, Resistant level A, less than 5% of the plants remained free of symptoms; level B, 5 – 40%; level C, 41-70% and level D, more than 70% of the plants remained free of symptoms.

Table 5-4. Analysis of the resistance in S_2 progeny of transgenic *Nicotiana tabacum* containing DNA constructs of pCMV2-S and pCMV2-NT

S_1 lines	S_2 lines	% Resistance
pCMV2-S-10	10-1	100
	10-2	85
	10-3	90
	10-4	85
	10-6	100
pCMV2-S-11	11-1	80
	11-2	80
	11-3	90
	11-5	80
	11-6	100
pCMV2-NT-6	6-1	100
	6-2	75
	6-3	35
	6-4	50
	6-5	55
pCMV2-NT-13	13-1	50
	13-2	15
	13-3	100
	13-5	15
	13-6	40

DISCUSSION

In order to assess and compare the effectiveness and practical value of different forms of pathogen-derived resistance against CMV, transgenic tobacco plants were produced expressing different parts of the viral genome. The primary transformants were self-pollinated, seeds were collected and progeny S_1 plants were screened for resistance against CMV infection by mechanical inoculation. It was found that progeny of transgenic plants expressing detectable amounts of CP only showed a limited delay (one or two days) of disease symptom expression compared to non-transgenic controls. S_2 lines, homozygous for the CP transgene, were also only partially resistant against CMV infection. Furthermore, this limited resistance was overcomed by higher inoculum pressures. Previously published data suggest that the levels of CP-mediated resistance against CMV are correlated with the amounts of transgenically produced CP (Cuozzo *et al.*, 1988; Namba *et al*., 1991; Okuno *et al.*, 1993b; Rizos *et al*., 1996; Kaniewski *et al*., 1999). Our data fit these conclusions as for all four CP expressor lines only low CP accumulation levels were found (less than 0.05% of total soluble proteins) and provided less applausive resistance in terms of delayed symptom expression. Previous studies have reported estimated CP levels of 0.16% and 0.2% of total soluble protein in resistant CP-transformed tobacco plants (Quemada *et al.*, 1991; Okuno *et al*., 1993b). It cannot be excluded that our approaches to aim for CP-mediated expression were sub-optimal, resulting in too low levels of CP expression to provoke resistance. Nonetheless, it is clear that transformation using the CP gene to obtain virus-resistant host plants is far from guaranteed to be a successful approach. When high levels of CP expression are indeed crucial for obtaining operational levels of resistance it is a laborious technique as high CP expressor lines have always been observed to form a small minority (Kaniewski *et al.*, 1999), requiring the transformation and testing of large numbers of lines. Apparently, 30 independent tobacco lines were not sufficient to select one single high expressor line. Studies published in the course of our experiments (Kaniewski *et al.*, 1999) revealed that the use of the *Figwort mosaic virus* (FMV) major

promoter to replace the CaMV 35S promoter may lead to a 20-fold enhanced accumulation of transgenic CP corresponding to enhanced resistance levels as well. Hence, this might be one way to improve the methodology for CP-mediated resistance, albeit that the result may turn out to be host dependent.

The mechanism by which CP-mediated resistance operates is not fully understood yet, making it even more difficult to further improve this approach. Experiments in transgenic TMV CP-expressing tobacco protoplasts revealed that an early step in virus replication is blocked, possibly co-translational disassembly (Osbourn, *et al*., 1989). In addition, it has been suggested that CP-mediated resistance is based on abortive replication of the invading viral RNAs by the viral RdRp, due to the presence of large amounts of CP in the transgenic plant cells (de Haan, 1998). Several reported negative results with respect to CP-mediated resistance despite high transgenic CP expression levels indicate that the coordination between host and CMV strain could interfere with the applicability of CP-mediated resistance to CMV (Kaniewski *et al*., 1999; Jacquemond *et al*., 2001).

Although not all genomic sequences of CMV seem equally suitable, our results indicate that RNA-mediated resistance may be a much better option. Formally, in the presented experiments with RNA 2, RNA silencing as an underlying mechanism for the observed resistance has not been demonstrated, but the fact that similar high levels of resistance were found with a non-translatable version of this genome segment forms a strong indication. Full immunity, which could not be broken at higher inoculum pressure, was obtained when full length RNA 2 sequences were used, either with the ORF being intact (constructs S), distorted (construct ID) or expressed as antisense (AS). Although this result may not seem very novel, it should be noted that despite the many reports of RNA-mediated host resistance for a diversity of viruses, reports on this type of resistance to CMV have thus far been scarce.

CMV resistance has been reported before for transgenic plants harboring CMV RNA 2 sequences (Anderson *et al.*, 1992; Carr *et al*., 1994) but it was generally thought that the synthesis of a mutated viral replicase protein was

interfering with the normal interaction between virus and host. In line with this a correlation between transgenic 2a protein expression and resistance has previously been found (Carr *et al.*, 1994; Wintermantel *et al.*, 1997; Wintermantel and Zaitlin, 2000) suggesting the resistance found was “replicase-mediated”. The reported cases of this proposed type of resistance indicate it to be stronger and more effective than CP-mediated resistance and sequence specific (Palukaitis and Zaitlin, 1997). However, as in most reported cases there is no apparent correlation between resistance and the accumulation of the transcripts or protein products of the transgene, the resistance may act on RNA- instead of protein-level. The studies presented in this chapter demonstrate that pathogen-derived resistance against CMV can be obtained by interfering the normal interaction between virus and host via transgenic expression of (full-length) RNA 2 but not by expression of RNA 3 sequences.

This is the first report in which an antisense construct of RNA 2 was shown to be capable to confer resistance against CMV. Possibly, the expressed antisense RNAs as described in some previous reports were too short in length (Rezaian *et al*., 1988). Remarkably, the frequency of CMV-resistant transgenic plants expressing antisense or non-translatable RNA 2 derived constructs is lower than that of plants expressing a translatable (sense) RNA 2 derived sequence. An explanation for this may be that the translatable transcripts, in contrast to non-translatable or antisense transcripts, are covered with ribosomes and as a consequence may have a higher stability than that of the unprotected RNAs, thereby increasing the possibility to induce the sequence-specific RNA degradation mechanism (Jorgensen, 1992).

Since RNA-mediated virus resistance is highly sequence-specific, *Cucumber mosaic virus* subgroup II isolates are able to overcome the resistance against subgroup I isolates (Zaitlin *et al*., 1994; Hellwald and Palukaitis, 1994). Although it is difficult to predict the type of CMV isolates that will occur in a certain area at a particular time (Garcia-Arenal *et al*., 2000), the occurrence of subgroup I CMV isolates is largely predominant. This makes the RNA-mediated resistance approach highly attractive to combat CMV in crops grown in fields and greenhouse.

Approaches based on inducing the dsRNA-producing constructs into plants to efficiently confer RNA-mediated resistance have been demonstrated (Smith *et al*., 2000; Wang *et al*., 2000). The application of CMV RNA 2 sequences for transgenic production of CMV-specific dsRNA could be a potential way to produce CMV-resistant crops and ornamental plants effectively and efficiently.

Chapter 6

High frequency induction of RNA-mediated resistance against *Cucumber mosaic virus* using inverted repeat constructs

Yuh-Kun Chen, Dick Lohuis, Rob Goldbach and Marcel Prins

Abstract

The application of RNA-mediated resistance against *Cucumber mosaic virus* (CMV) by using single transgene constructs usually results in only a small portion of resistant individuals. Inverted repeat constructs encoding self-complementary double-stranded (ds)RNA have been demonstrated to be a potential way to obtain RNA-mediated resistance at high efficiency. To test this observation as a possible method for high frequency induction of CMV resistance *Nicotiana benthamiana* plants were transformed with transgenes designed to produce dsRNA containing CMV RNA 2 or coat protein (CP) gene sequences. Seventy-five percent of the tested R_0 lines transformed with an RNA 2 derived inverted repeat construct (of 1534 nt repeat unit) showed extreme resistance to CMV while a lower percentage of resistance (30%) was observed in R_0 lines transformed with a similar construct of a shorter viral sequence (490 nt). The resistance level conferred by CP sequences was also greatly improved by using a dsRNA construct, reaching a level of 50%. Self-pollinated (S_1) progenies obtained from most resistant R_0 plants showed a resistance level of 100%. The results indicate the use of inverted repeat constructs to be an efficient approach to obtain a high frequency of CMV resistant transgenic plants that can compensate the low efficiency protocols for the transformation of ornamental crops.

INTRODUCTION

In the previous Chapter it has been investigated whether host resistance to *Cucumber mosaic virus* (CMV) based on RNA-mediated resistance can be achieved by transformation of tobacco using (non-translatable) viral sequences. From these analyses it appeared that a transgenically expressed full-length copy of genomic RNA segment 2 could induce RNA-mediated resistance. Though the frequency at which transgenic plant lines were obtained was rather low. In case the transgenic construct contained a full RNA 2 copy only 10 out of 30 lines (33.3%) exhibited high levels of resistance in the S_1 stage. Using a construct encompassing only the 3' half of the RNA 2, out of 22 lines not even a single resistant line was obtained, despite the fact that the size of the viral transgene exceeded reported minimal sequences required to provoke RNA-mediated resistance (Pang, *et al.*, 1997; Jan *et al.*, 2000). Given that these analyses were done using an easy to transform cultivar of tobacco (*Nicotiana tabacum* var. SR1), these results indicate that the frequency of resistance induction is a major bottleneck in the procedure. The problem of this low frequency induction of RNA-mediated resistance will be even more cumbersome for crops and ornamentals that are difficult to transform. Hence to introduce transgenic resistance to CMV in ornamental plants like lily and other monocotyledons, it is required to first optimize the resistance induction frequency.

Indeed, from a practical point of view, transforming plants with a single sense or antisense construct typically results in only a small proportion of individuals with silenced (in case of viral transgenes: resistant) phenotypes (Smith *et al.*, 2000). Several lines of evidence indicate that double-stranded RNAs (dsRNAs) or self-complementary hairpin RNAs (hpRNAs) that were produced during the intermediate steps of viral genome replication are the key triggers of the RNA degradation mechanism (Bass, 2000; Hamilton *et al.*, 1998; Waterhouse *et al.*, 1998). Based on this suggestion, it was shown that using dsRNAs or hpRNAs as an inducer of RNA mediated resistance can be much more effective than simple sense or antisense constructs (Smith *et al.*, 2000; Wang *et al.*, 2000; Wang and Waterhouse, 2000; Wesley *et al.*, 2001; Giordano *et al.*, 2002). The spectacular

silencing effect of hpRNA constructs has been demonstrated in protecting hosts from *Potato virus Y* and *Barley yellow dwarf virus* (Smith *et al.*, 2000; Wang *et al.*, 2000; Wesley *et al*., 2001). Recent studies by the group of Waterhouse (Smith *et al.*, 2000; Wang *et al.*, 2000; Wesley *et al.*, 2001) have indicated that inverted repeat (IR) constructs of transgenes can effectively induce RNA silencing to a very high level and at high frequency. These experiments also included repeated viral transgene sequences that are shown to lead to high resistance frequencies, even in (heterozygous) R_0 plants.

To obtain, for instance, a sufficient amount of virus resistant transformants for monocotyledonous ornamental crops, large numbers of single sequence expressing transgenic lines have to be produced and screened. The application of cDNA constructs of viral sequences which can form double-stranded or hairpin structure RNAs in such low efficient transformation protocols presents a welcome opportunity to increase the chances to generate virus-resistant ornamental plant lines. Using the information obtained in Chapter 5, it is now tested whether the frequency of resistance induction can be improved by using inverted repeat sequences derived from this CMV RNA 2 segment or from the RNA 3 located coat protein gene.

MATERIALS AND METHODS

Viruses and plants

The lily and alstroemeria isolates of *Cucumber mosaic virus* (CMV-LILY and CMV-ALS, Chapter 2; Chen *et al.*, 2001) were maintained on *Nicotiana benthamiana* plants. These plants were also used for transformation experiments.

Cloning and construction of inverted repeat (IR) constructs derived from RNA 2 of CMV-lily

Routine nucleic acid cloning methods (Sambrook *et al*., 1989) were used throughout the construction process. The full-length cDNA of CMV-LILY RNA 2 was prepared by RT-PCR with a pair of degenerated primers (R2-F and R2-R) complementary to the 5'-terminal nucleotides 1 to 15 and 3'-terminal nucleotides 3048 to 3061, respectively. The RT-PCR fragments were cloned into the pGEM-T vector (Promega) and designated pCMV2-LILY. The nucleotide sequence has been submitted to the EMBL database (accession number AJ276583).

Using pCMV2-LILY as template, long (L) and short (S) cDNA fragments covering the ultimate 3' 1534 or 490 nucleotides, respectively, were amplified by various primer combinations (Table 6-1) with *Bam*HI/*Bam*HI or *Bam*HI/*Not*I restriction sites and cloned into the pGEM-T easy vector (Promega) as pCMV2-3'L–B/B, pCMV2-3'L-B/N and pCMV2-3'S-B/N, respectively.

Table 6-1. Constructs derived from CMV-LILY cDNA sequences and position of primers used

Plasmid designation	CMV sequence	Positions / Length (nt)	Primers*
pCMV2	full length RNA 2 (AJ276583)	1 – 3061 / 3061	R2-F: 5'-ccc**ggatcc**gtttattctcaagagcgtatggttcaacccctgcc R2-R-BamHI: 5'-ccc**ggatcc**tggtctccttgtgg
pL	3' half RNA 2	1528 –3061 / 1534	R2-MD-F: 5'-ccc**ggatcc**gcttccagagatgcc R2-R-BamHI: 5'-ccc**ggatcc**tggtctccttgtgg
pLIR	3' half RNA 2	1528 –3061 / 1534	R2-MD-F: 5'-ccc**ggatcc**gcttccagagatgcc R2-R-NotI: 5'-ccc**gcggccgc**tggtctccttgtgg
pSIR	3' terminus of RNA 2	2572 –3061 / 490	R2-D-F: 5'-ccc**ggatcc**ccgcttcctacc R2-R-NotI: 5'-ccc**gcggccgc**tggtctccttgtgg
pCPIR	CP ORF and part of the 3' NTR of RNA 3	156 – 945**/ 790	CP-IR-F: 5'-ccc**ggatcc**atggacaaatctg CP-IR-R: 5'-ccc**gcggccgc**gtaagctggatggac
pINT	Actin2 intron of *Arabidopsis thaliana*	449 nt	Act2-F: 5'-ccc**ggatcc**aagGTaataggaactttc Act2-R: 5'-ccc**ggatcc**gagCTgcaaacacacaaaaag

*, The sequences of restriction sites are in bold and underlined. The capitalized nucleotides in primers for actin2 intron indicate the splicing sites.
**, The nt position shown are based on the pCMV-LILY-CP51 sequence (EMBL Acc. No. AJ131615) (Chapter 2; Chen *et al.*, 2001).

For the constructs consisting of a single copy transgene, the pCMV2-3'L-B/B was digested with *Bam*HI, cloned between a CaMV 35S promoter and a nos terminator cassette in sense or antisense orientations, which was further cloned into the *Xba*I site of binary vector pBIN19 (Bevan, 1984), resulting in pL-s and pL-as, respectively (Fig. 6-1-A)

For the constructs containing inverted repeats (IR), the CMV cDNA fragments were separated by the actin2 intron (449 nt) of *Arabidopsis thaliana* (An *et al*., 1996). The intron was inserted with *Bam*HI restriction sites. The *Bam*HI-digested intron fragments were cloned into *Not*I-digested pBluescript SK together with *Bam*HI and *Not*I double digested CMV2-3'L-B/N or CMV2-3'S-B/N fragments to produce inverted repeat constructs yielding pCMV2-3'-LIR or pCMV2-3'-SIR, in the order antisense-intron-sense to prevent possible translation and resulting complications in the resistance analysis by a putative translation product. The plasmids pCMV2-3'-LIR and pCMV2-3'-SIR were then digested with *Not*I and cloned into pRAP (Schouten *et al.*, 1997) that contains a 35S-*Not*I-nos cassette. The proper clones with introns in sense or antisense orientations, designated pLIR-INTs, pLIR-INTas, pSIR-INTs and pSIR-INTas, respectively, were further cloned into binary vector pBINPLUS (van Engelen *et al.*, 1994) after *Asc*I and *Pac*I digestion (Fig. 6-1-A). The CP ORF fragments for IR constructs were amplified with PCR by using pCMV-Lily-CP51 (EMBL AJ131615) (Chapter 2; Chen *et al.*, 2001), cloned into binary vector with the strategies identical to that for RNA 2 fragments and were designated as pCPIR-INTs and pCPIR-INTas (Fig. 6-1-B).

Figure 6-1

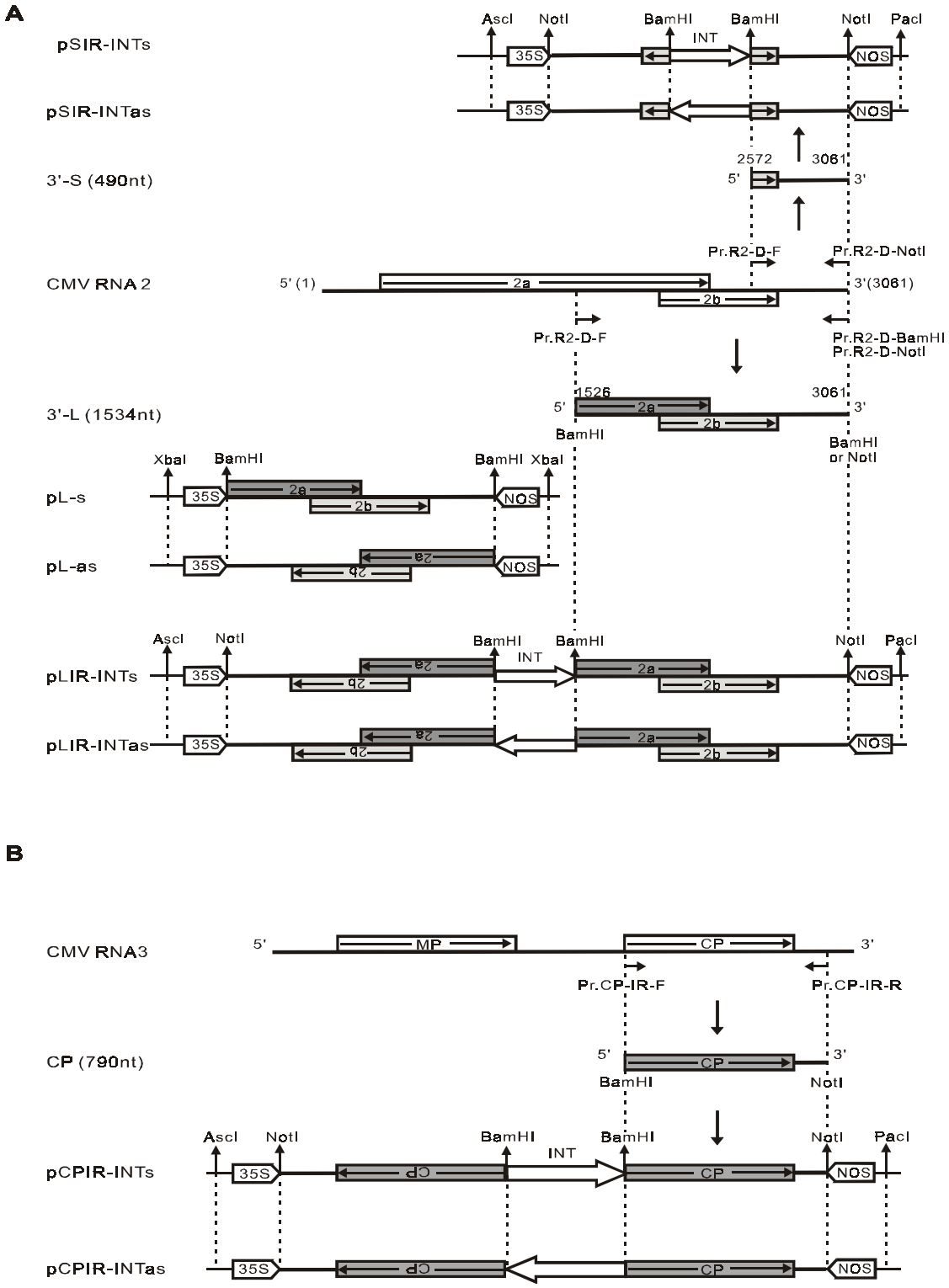

A
pSIR-INTs
pSIR-INTas
3'-S (490nt)
CMV RNA 2
3'-L (1534nt)
pL-s
pL-as
pLIR-INTs
pLIR-INTas
AscI
NotI
BamHI
BamHI
INT
NotI
PacI
35S
NOS
2572
3061
5'
3'
Pr.R2-D-F
Pr.R2-D-NotI
5' (1)
2a
2b
3'(3061)
Pr.R2-D-F
Pr.R2-D-BamHI
Pr.R2-D-NotI
1526
3061
BamHI
BamHI
or NotI
XbaI
BamHI
BamHI
XbaI
B
CMV RNA3
MP
CP
Pr.CP-IR-F
Pr.CP-IR-R
CP (790nt)
BamHI
NotI
pCPIR-INTs
pCPIR-INTas

Fig. 6-1. Engineering simple and inverted repeat constructs derived from CMV RNAs 2 and 3 sequences. RNA 2 (A) and RNA 3 (B) cDNA fragments were amplified by primer pairs as described in Table 6-1. L: long RNA 2 fragment (1534 nt); S: short RNA 2 fragment (490 nt); CP: coat protein gene; s: sense orientation; as: antisense orientation; IR: inverted repeats; INT: actin2 intron of *Arabidopsis thaliana*; INTs: intron in sense orientation; INTas: intron in antisense orientation; 35S: cauliflower mosaic virus 35S promoter; and NOS: nopaline synthase terminator.

Transformation of *Nicotiana benthamiana*

The pBIN19-derived vectors, pL-s, pL-as, pLIR-INTs, pLIR-INTas, pSIR-INTs, pSIR-INTas, pCPIR-INTs and pCPIR-INTas, were introduced into *Agrobacterium tumerfaciens* strain LBA4404 (Ditta *et al.*, 1980) by triparental mating by using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid. *Nicotiana benthamiana* plants were transformed and regenerated by standard methods (Topping, 1998). After co-cultivation with *Agrobacterium*, leaf discs of *N. benthamiana* were incubated on MS media containing hormones (BAP, 1 mg/l, and NAA, 0.1mg/l) and antibiotics (carbenicillin, 250 mg/l, and kanamycin, 100 mg/l) in a growth chamber for shoots formation. Regenerated shoots were cut from explants and were transferred to hormone-free MS media containing the antibiotics kanamycin (100 mg/l) and cefotaxime (250 mg/l) for rooting. Rooted small plants in their six leaf stage were then transferred to pots and grown in a temperature-controlled greenhouse.

Analysis of intron splicing in transgenic plants by RT-CR

Total RNA was extracted and purified as described by Worrall (1998). RT-PCR was used to detect the transcription of the transgenes and the correct splicing of the intron. Prior to RT-PCR, the total RNA (10 μg) was treated with DNaseI at 37 ^{o}C for 60 min. to remove the genomic DNA of transgene insertions. DNaseI was inactivated at 65 ^{o}C for 30 min, followed by phenol/chloroform extraction. The first strand synthesis was primed with intron primers (either Act2-F 5’-CCCGGATCCAAGGTAATAGGAACTTTC-3’ or Act2-R 5’-CCCGGATCCGAGCTGCAAACACACAAAAAG 3’ depending on the orientation of the intron). PCR of this DNaseI-treated RNA without RT was used as control. To

amplify fragments of the Act2 intron PCR was carried out using primer sets indicated in Table 6-1 on the cDNA.

Plant inoculations and virus resistance assays

Original (R_0) transformants harboring inverted repeat constructs were challenged with CMV-lily by mechanical inoculation at the six leaf stage, while the transformants of single copy transgene (pL-s and pL-as) were inoculated only as S_1 progeny plants as the R_0 plants were expected to be resistant at a (too) low frequency. Fresh leaves obtained from CMV-infected *N. benthamiana* (10-15 dpi) were ground in a 0.1 M phosphate buffer (pH 7.2) in a w/v ratio of 1/10 and were used as inoculum for mechanical inoculation. A second inoculation was carried out on each plant 5 days after the first one to ensure the effectiveness of inoculation. Plants were scored as infected when the upper leaves showed mosaic symptoms, for susceptible plants these usually occurred 7 days after inoculation. All the resistant lines and some of the susceptible lines were kept until self-pollinated (S_1) seeds were formed. Some of the lines were further tested in the S_1 progeny to confirm the stability of the observed resistance. The S_1 seeds of each R_0 plant were sown on selective MS media containing kanamycin (100 mg/l) after surface sterilization with commercial bleach. The S_1 plants were mechanically inoculated and symptoms were monitored on a daily basis. The visually healthy plants were checked weekly by ELISA using polyclonal antiserum against CMV, until 6 weeks after inoculation to confirm the absence of viral infection.

Detection of small interfering RNAs (siRNAs)

Small interfering RNAs were extracted from leaves of transgenic plants of about 8 weeks old after sowing on kanamycin containing MS medium. The extraction was performed according to Papaefthimiou *et al*. (2001). RNAs were separated in a 12% polyacrylamide gel containing 7 M urea and 0.5 mM TBE buffer and were transferred to Nylon membrane.

Dig-labeled CMV-specific DNA probes were synthesized by PCR with primers amplifying the 3' half of RNA 2 (primers R2-MD-F and R2-R, Table 6-1) or

CP (primers complementary to the 5' and 3' ends of the CP ORF, respectively). Prehybridization and hybridization were carried out in 5XSSC at 42 ^{o}C. Membranes were washed twice after hybridization with 2XSSC and 0.2% SDS at 42 ^{o}C and the siRNAs were detected with chemoluminesce as described by Webster and Barker (1998).

RESULTS

Transforming *Nicotiana benthamiana* plants with CMV RNA 2 or CP gene sequences

In the previous Chapter it was established that RNA 2 sequences were able to induce RNA-mediated CMV resistance in transgenic plants. Notwithstanding the success of the use of RNA 2 sequences for resistance, only a small proportion of the transgenic plants showed resistance in the S_1 generation. Since the transgene construct derived from the 3' half of CMV RNA 2 did not yield any virus resistant plant line (Chapter 5), we decided to use this construct as a starting point (expected resistance frequency 0%) to generate transgenes containing invertedly repeated cDNA fragments of two different lengths, i.e. with a 1534 and a 490 nucleotides long repeat unit (Fig. 6-1A). In order to stabilize the inverted repeat constructs during the cloning and transformation process, the *Arabidopsis* actin2 intron was inserted to separate the invertedly repeated cDNA fragments. To test the effectiveness of the presence of the spliceable intron versus 'random' inserted DNA on resistance, the intron was cloned in both sense (functional) and antisense (dysfunctional) orientations. As the viral coat protein (CP) gene was also known to act poorly in resistance induction (Chapter 5) the use of an inverted repeat construct of this gene (Fig. 6-1-B) was also included in this research.

Via *Agrobacterium*-mediated transformations, a total of 41 and 150 transgenic plants respectively were produced containing a single transgene (L-s and L-as) or one of the inverted repeat constructs (LIR, SIR and CPIR). Twenty-four (L-s) and 17 (L-as) R_0 plants were obtained containing the single transgene constructs in sense and antisense orientation, respectively. Similar numbers of R_0 transformants were obtained for each of the 6 different inverted repeat constructs (Table 6-2).

Table 6-2. Resistance levels of transgenic *Nicotiana benthamiana* plants expressing sequences of CMV RNA 2 or CP

Transgene construct [1]	R_0 lines				S_1 lines		
	# Transformed plants	# Inoculated	# Resistant	% Resistant	# Inoculated[2]	# Resistant	% Resistant
Single copy							
L-s	24	NT[1]	NT	NT	24	5	21
L-as	17	NT	NT	NT	17	2	11
Inverted repeat							
LIR-INTs	39	39	29	74.4	7	7	100
LIR-INTas	32	32	24	75.0	5	5	100
SIR-INTs	25	25	8	32.0	1	1	100
SIR-INTas	12	12	4	33.3	2	2	100[3]
CPIR-INTs	30	30	12	40.0	4	4	100
CPIR-INTas	12	12	6	50.0	2	2	100
Non-transformed		36	0	0	1	0	0

[1] L: 3' 1534 nt of RNA 2; S: 3' 490 nt of RNA 2; CP: CP ORF; IR: inverted repeat; s : sense orientation; as : antisense orientation; INTs: Intron in sense orientation; INTas, Intron in antisense orientation; NT: not tested.
[2] Ten plants per line were tested. In each series of inoculations 10 control plants were used.
[3] Tolerant phenotype. Symptoms appeared similar to nontransformed control plants in the first two weeks but disappeared later, though virus remained detectable by ELISA.

Resistance frequencies of transgenic lines

In view of the low resistance frequencies expected, the lines harboring single copy transgenes (L-s and L-as) were not tested in the R_0 stage but only as S_1 progeny (Table 6-2). As for plants containing IR constructs, these were tested at the R_0 level. All of the transformed R_0 plants tested appeared normal and were mechanically inoculated. A standard second inoculation was carried out 5 days after the first inoculation to minimize random escape of infection. All non-transformed controls and susceptible transgenic lines showed typical CMV

disease symptoms in their systemic leaves 7-8 days after inoculation, while the resistant lines remained symptomless.

With respect to the non-repeat transformants (L-s and L-as), resistant (S_1) lines were obtained with a frequency of 21% and 11%, respectively (Table 6-2). Thus, the percentage of resistance induction in case of a non-repeat transgene turned out to be higher than predicted based on the results of Chapter 5. Moreover, 4 out of 5 resistant L-s lines (lines 2, 3, 4 and 22) showed an excellent 100% resistant phenotype while the 2 resistant L-as lines (lines 4 and 9) had resistance levels of 60%.

Transformants containing the long inverted repeat transgene (LIR-INTs and LIR-INTas) revealed a remarkable 75% resistance on the R_0 level, irrespective of the intron being functionally inserted or not (Table 6-2). Using the short inverted repeat (SIR) constructs a still considerable but lower percentage of resistance (30%) was observed in the R_0 lines (Table 6-2).

The S_1 seeds collected from 15 resistant and 3 susceptible R_0 plants of LIR and SIR, either with functional or non-functional intron, were sown under kanamycin selection. Ten plants of each S_1 line were inoculated with the homologous isolate of CMV. Twelve out of 15 tested S_1 lines that were resistant in the R_0 showed 100% resistance to the homologous virus. One line (LIR-INTs-5) showed 60% resistance and two lines (SIR-INTas-2 and SIR-INTas-3) showed a tolerant phenotype (Table 6-3). The tolerant plants initially showed the same symptoms as non-transformed control plants but these symptoms disappeared after two weeks, although the virus remained detectable by ELISA during the whole experiment (6 weeks). The S_1 progeny plants obtained from 3 susceptible R_0 lines remained susceptible. All the tested S_1 lines were also challenged with a subgroup II CMV isolate (CMV-ALS) and proved fully susceptible (Table 6-3), confirming the previously demonstrated high sequence specificity of RNA-mediated resistance.

A considerable part (40-50%) of the CP-IR-transformed R_0 plants turned out to be resistant to CMV (isolate LILY, subgroup I) irrespective whether the intron within the transgene construct was inserted in sense or in antisense (Table 6-2).

Among the 24 susceptible R_0 lines, 6 lines showed a delay in symptom expression of 6 up to 10 days. The S_1 progeny of resistant R_0 lines were 100% resistant while that of the susceptible R_0 lines remained fully susceptible. All the tested CP-IR transformed lines were susceptible to subgroup II CMV (CMV-ALS), which shares approximately 70% sequence homology (Table 6-3).

Table 6-3. Resistance levels of transgenic *Nicotiana benthamiana* plants harboring inverted repeat constructs to homologous (CMV-LILY; subgroup I) and heterologous (CMV-ALS; subgroup II) viruses

Lines	% resistant S_1 plants (CMV-LILY)	% resistant S_1 plants (CMV-ALS)
LIR-INTs		
5	60	0
6	100	0
7	100	0
8	100	0
9	100	0
14	100	0
16	100	0
LIR-INTas		
1	100	0
5	100	0
6	100	0
7	100	0
8	100	0
16	0	0
SIR-INTs		
6	0	0
23	100	0
SIR-INTas		
1	0	0
2	100*	0
3	100*	0
CPIR-INTs		
2	100	0
3	0	0
9	100	0
22	0	0
Nontransformed *N. benthamiana*	0	0

*Tolerant plants: initial wild type symptoms disappeared two weeks later, although the virus remained detectable by ELISA.

To test whether the resistance prevents transgenic plants from viral infection or prevents viruses from systemic movement, inoculated and systemic leaves of R_0 LIR and CPIR lines were sampled 3 weeks post inoculation and possible presence of virus was monitored. Though CMV could be readily detected in susceptible plants, it was absent in both inoculated and systemic leaves of resistant lines as determined by ELISA (data not shown) and Western blots (Fig. 6-2), indicating that these plants exhibited full immunity. A time course analysis provided further confirmation that CMV infection only occurred in susceptible lines but not in resistant lines (Fig. 6-3).

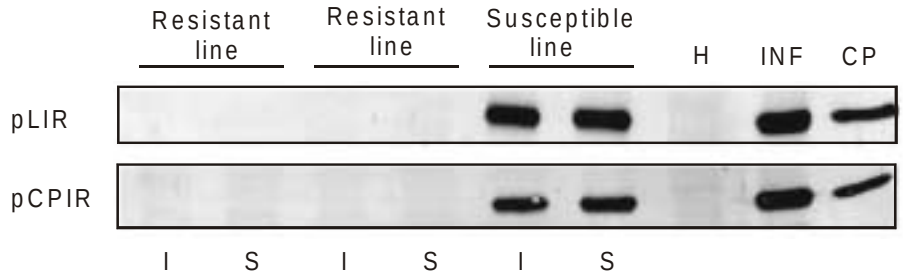


Fig. 6-2. Western blots showing the infection occurred in inoculated (I) and systemic (S) leaves of resistant and susceptible transgenic *Nicotiana benthamiana* lines transformed with pLIR or pCPIR at 14 days post inoculation. H and INF indicate healthy and CMV-infected non-transformed control plants, respectively. CP indicates the lane of purified CMV CP.

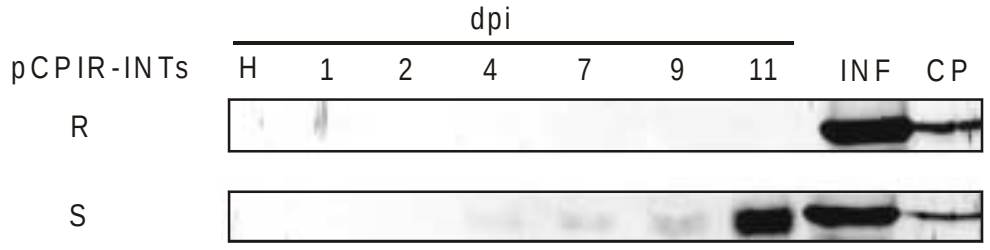


Fig. 6-3. Western blot showing a time course after CMV inoculation in resistant (R) and susceptible (S) lines of pCPIR-transformed *N. benthamiana*. Inoculated leaves were tested. H and INF indicate the healthy and infected (non-transformed) control plants and CP indicates the position of purified viral coat protein.

Analysis of transcription and intron splicing in R_0 transformants

After transcription and pre-mRNA processing of the inverted repeat transgenes, stem-loop or hairpin structures are expected to form. In order to detect these sequences, (RT-)PCR amplification was first attempted using primers within the inverted repeat region. This proved unsuccessful, probably due to the stability of the complementary duplex and the inability of the primers to properly anneal and be elongated. This feature was also observed previously when testing these constructs in bacteria. As the intron sequence in the LIR-INTas and SIR-INTas constructs still should be included in the transcripts, amplification of the (antisense) intron sequences was used as an alternative.

The functional intron (sense orientation) was expected to be spliced out from the mRNA transcripts and an RT-PCR fragment of 450 nt was not expected in these lines, only from inverted repeat constructs with a non-functional (antisense) intron. As shown in Figure 6-4, a DNA fragment of the size of the Actin2 intron was amplified by RT-PCR from all inverted repeated constructs, while this fragment was not amplified without a prior RT reaction. This indicates that unspliced sense oriented intron sequences are present in transgenic plants. Possibly only part of the transcripts was spliced, or the intron is not functioning properly at all. In favor of the latter scenario (no splicing at all) is the observation that similar resistance frequencies were obtained in plants transformed with 'functional' and non-functional intron insertions, whereas in a previous report (Smith *et al*., 2000) a further increase of frequency was reported for functional introns. An unanticipated observation can also be made in Fig. 6-4. Even though the DNase treated samples did not show a PCR amplified fragment, both forward (act2-F) and reverse (act2-R) primers of the actin2 intron are consistently able to prime the synthesis of first strand. This suggests that both sense and antisense transcripts of the intron region are present in all plants tested. A possible explanation that leaky termination of the selection marker gene (NPTII) would result in a long antisense transcript containing the opposite intron can be excluded as the nos promoter driving the NPTII gene is directed to the right border, while the IR transgene is inserted near the left border. Perhaps plant promoters near the T DNA integration sites can be

responsible for low level of antisense readthrough transcripts. Due to the sensitive RT-PCR analysis used here this signal is amplified. Northern analysis will have to confirm the relative amounts of sense and antisense RNA present.

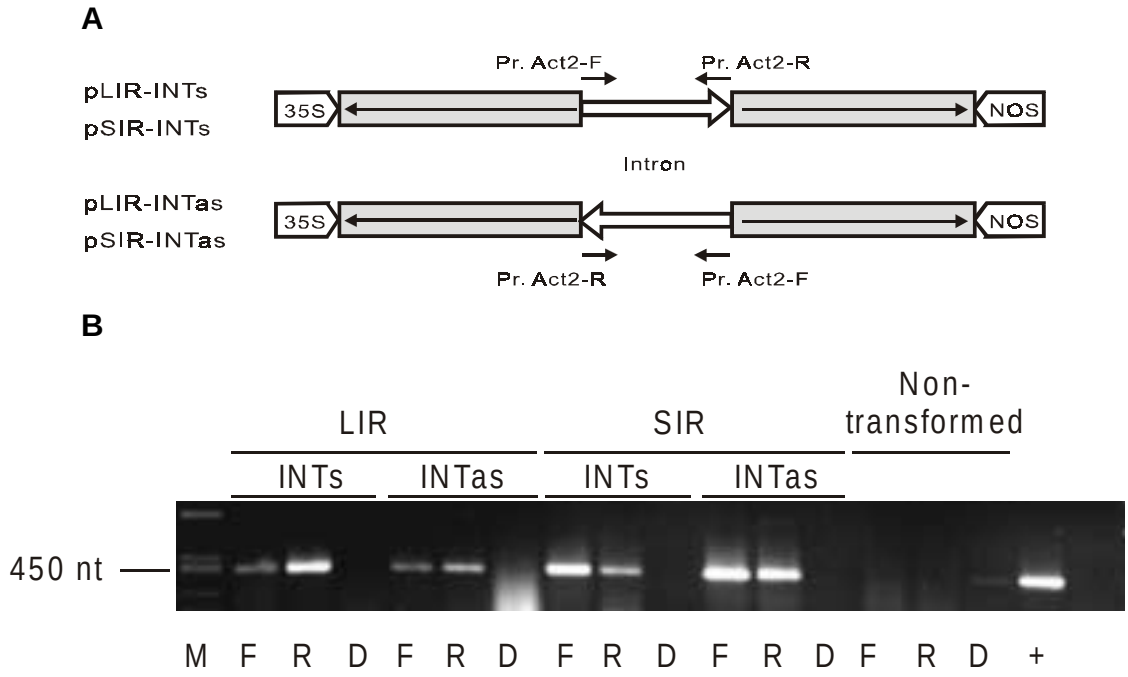


Fig. 6-4. A. The schematic diagram shows the position and orientations of the intron in the inverted repeat constructs and that of the primers (Act2-F and Act2-R) used. B. RT-PCR pattern from the inverted repeat constructs containing CMV RNA 2 sequences and Arabidopsis actin 2 intron. Total RNA extracted from transgenic *Nicotiana benthamiana* plants harboring inverted repeat constructs of pLIR and pSIR containing intron sequences in sense (INTs) and antisense (INTas) orientations was PCR amplified with intron specific primers after reverse transcription on total RNA. All the RNA samples were digested with DNaseI before reverse transcription reaction to remove genomic DNA. F and R indicate the primers Act2-F and Act2-R, respectively, which were used to prime the first strand synthesis followed by PCR with primers F and R. D indicates PCR directly on DNaseI treated material. M is the DNA size markers (*Pst*I digested λ DNA) and "+" indicates the PCR-amplified cloned intron sequence.

Occurrence of CMV-specific small RNA (siRNA) correlates with resistant phenotypes

To test the correlation between the resistant phenotypes and the formation of siRNAs as a hallmark of RNA silencing, the leaves of S_1 plants of a limited number of transgenic lines were harvested before inoculation and examined for the presence of siRNAs. Results shown in Figure 6-6 indicate that siRNAs can only be detected in resistant lines expressing either RNA2 or CP inverted repeat constructs (Fig. 6-5-A and Fig. 6-5-B) and that they are absent in susceptible (non)transgenic plants. Though based on a thus far rather small number, the perfect correlation between a resistant phenotype and the presence of siRNAs confirms that the observed resistance is indeed based on RNA silencing, which is most likely triggered by dsRNA produced from the inverted repeat transgene.

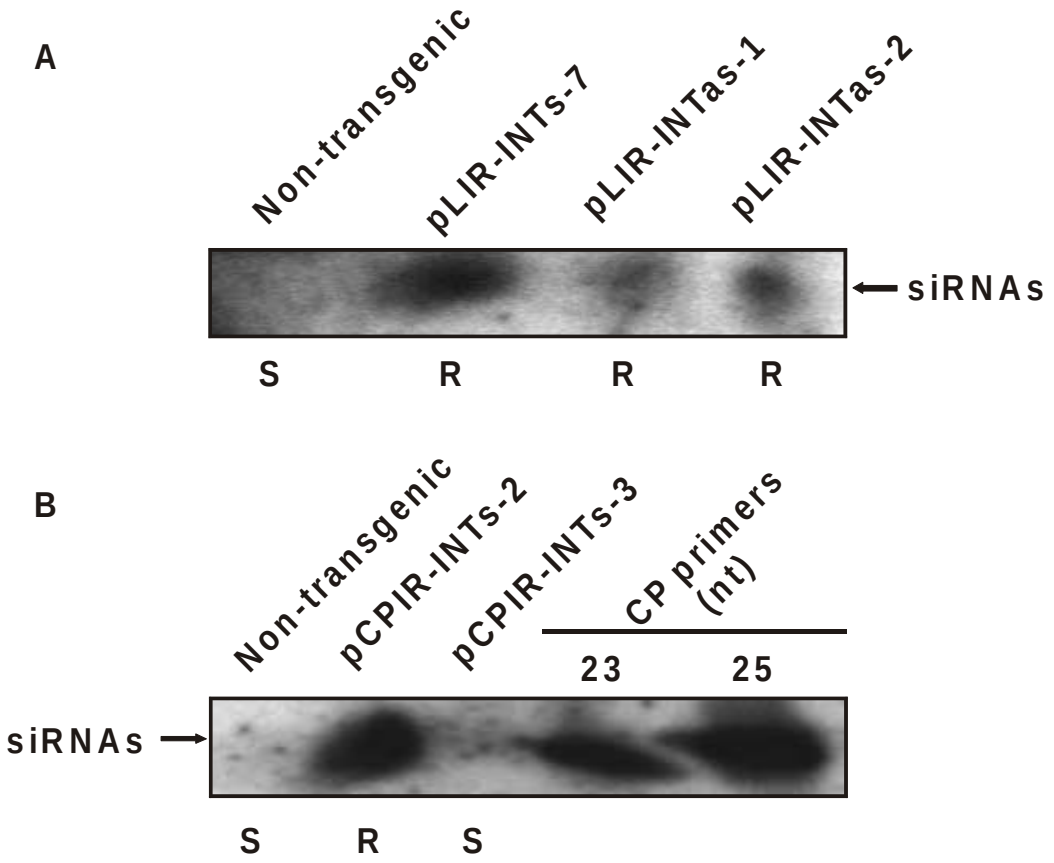


Fig. 6-5. Detection of CMV-specific small interfering RNAs (siRNAs) in non-inoculated S_1 transgenic plants. Small interfering RNAs extracted from S_1 plants harboring inverted-repeat constructs derived from CMV RNA 2 (pLIR-INT) and CP (pCPIR-INT) sequences and were detected with both sense and antisense specific probes against CMV RNA 2 (A) and CP (B) sequences. R and S indicate resistant and susceptible phenotypes. RNA extracted from non-transgenic plants was used as control. DNA primers of 23 and 25 nt were used as size markers.

DISCUSSION

To introduce resistance against viruses in plants that lack efficient transformation protocols, such as many ornamental and/or monocotyledonous plant species, an optimized approach inducing resistance at high frequency is desperately needed. In general, all previous applications of pathogen-derived resistance against CMV infection showed either a partial (protein-mediated) or narrow spectrum of (RNA-mediated) resistance to the virus (Beachy, 1997), but neither of these approaches ever reached high efficiencies.

In Chapter 5, a survey of the CMV genome for sequences suitable to confer resistance in transgenic plants was described. In this survey, RNA 2-derived sequences proved to be the most potent to confer resistance against CMV, albeit at a low frequency. Though the use of CP sequences in single transgene constructs was reportedly successful against CMV in several cases (Cuozzo *et al.*, 1988; Gonsalves *et al.*, 1992; Yie *et al.*, 1992; Nakajima *et al.*, 1993; Okuno *et al.*, 1993a; Gonsalves *et al.*, 1994; Tricoli *et al.*, 1995; Rizos *et al.*, 1996), in our hands it proved to be marginal at best (Chapter 5), a view supported by other reports (Kaniewski *et al.*, 1999; Jacquemond *et al.*, 2001).

Recently, the introduction of double-stranded homologous RNA sequences into plants has been demonstrated to be an efficient method to obtain a high ratio of resistant transgenic lines (Smith *et al.*, 2000; Wang *et al.*, 2000). As the CMV RNA 2 sequences were shown to be suitable candidate genomic fragments to provoke RNA-mediated resistance against CMV infection, the application of inverted repeat sequences derived form this viral genome segment may be a suitable approach for obtaining CMV resistance at a high frequency.

Indeed it could be demonstrated that hairpin RNA-encoding constructs containing such reiterated sequences are able to induce transgenic resistance at high (up to 75% in the R_0 stage) frequency. This is a significant improvement compared to resistance efficiencies reached with single transgene constructs containing the same sequence (20% or less at the S_1 level). Likewise, for CP transgene constructs even an improvement from 0% (Chapter 5) to 50% was

obtained. These results confirm the suggestion that the effectiveness of hpRNA structures can be applied in a wide range of circumstances (Wesley *et al.*, 2001). A high frequency (75%) of resistant lines can be induced by the IR constructs comprising the 3' 1534 nt sequence of RNA2 (pLIR) and a lower, but still considerable, frequency was obtained with the IR constructs consisted of only the 3' terminal 490 nt of RNA 2 (pSIR). Possibly, characteristics like (viral) sequence specificity, configuration, size and position of the transgene, and/or chromosomal positions of the transgene insertion are important in defining the transgene to be targeted by gene silencing (Hutvagner *et al.*, 2000).

It has been demonstrated that different parts of a transgene have different effects in terms of becoming a target for posttranscriptional gene silencing (PTGS). The target region for RNA degradation often resides in the 3' region of the transgene coding sequence as demonstrated for the movement protein gene of *Cowpea mosaic virus* (Sijen *et al.*, 1996), the CP gene of *Sweet potato feathery mottle virus* (Sonoda *et al.*, 1999), the UidA (GUS) gene (English *et al.*, 1996; Hutvagner *et al.*, 2000; Braunstein *et al.*, 2002), and the endogenous ACO1 [1-aminocyclo-propane-1-carboxylate (ACC) oxidase 1] gene (Han and Grierson, 2002). In concert with these observations, small RNA molecules (siRNAs) are not generated from either the 5' coding region or the 3' noncoding region of the GUS mRNA (Hutvagner *et al.*, 2000), while the 5' and 3' nontranslated regions of the beta-1,3-glucanase mRNA, were inefficient RNA silencing targets (Jacobs *et al.*, 1999).

The 3' terminal 180 nucleotides in all 3 CMV genomic RNAs are similar in sequence and can be folded into a tRNA-like structure (Palukaitis *et al.*, 1992). As the RNA 2 sequences of all IR constructs used in this study were cloned in an 'outward pointing direction' (5' antisense-intron-sense 3'), the 5' termini of the viral complementary RNA 2 sequences were synthesized first during the transcription of the transgenes and may thus have formed tRNA-like structures during (and after) transcription of the transgenes. These structures may have interfered with the formation of a genuine dsRNA transcript. Corrected for this complication the SIR transgenes might therefore yield a dsRNA molecule of only 300 bp, which could be

less efficient in generating RNA silencing.

The current model envisions that post-transcriptional gene silencing (PTGS) and RNA interference (RNAi) are initiated by the cleavage of dsRNA fragments into 21-25 nucleotides double-stranded RNA molecules, termed small interfering RNAs (siRNAs) (Hamilton and Baulcombe, 1999; Zamore *et al.*, 2000; Elbashir *et al.*, 2001), by the enzyme DICER, a member of the RNase III family of dsRNA-specific endonuclease (Bernstein *et al.*, 2001). These siRNAs duplexes are then incorporated into a protein complex called RISC (RNA-induce silencing complexes) (Hammond *et al.*, 2000) and activated the RISC by the siRNA duplexes unwinding. Then, the activated RISC can recognize and cleave target RNA complementary to the unwound strand of the siRNA (Hutvagner and Zamore, 2002). Theoretically, about 45 siRNA molecules can be produced from a dsRNA fragment of 1 kilobase after DICER digestion. The length of transgenic dsRNA therefore determines the amount of siRNA molecules produced after DICER cleavage. Thus, the production of siRNAs from a longer dsRNA (pLIR in this case) could be higher than from a shorter one (pSIR), and would provide higher amplification and more efficient RNA degradation. The variation of sequence position and size between pLIR and pSIR could be used as one of the explanations for the different efficiency on conferring resistance by these two RNA 2-derived inverted repeat constructs, although the preference of discrimination in fragment size and sequence position were not observed in other studies (Ruiz *et al.*, 1998; Wesley *et al.*, 2001).

Another possible factor contributing to the better performance of pLIR compared to pSIR could be that pLIR -but not pSIR - includes the full-length coding sequence of the viral 2b gene. As the 2b protein is responsible for long distance movement of CMV and, perhaps more importantly, acts as a silencing suppressor (Brigneti *et al.*, 1998), the degradation of the 2b gene mRNA of invading CMV could be more effective in pLIR transformants than in pSIR transformants and make the establishment of CMV infection more difficult in pLIR transformants.

One of the characteristics of RNA-mediated resistance is its immune-like response upon infection. CMV RNA 2 encodes a subunit of the viral

RNA-dependent RNA polymerase (RdRp) (Nitta *et al.*, 1988) which has to function during early stages of infection. A stem-loop structure which interacts with the replicase to initiate the synthesis of minus-strands of RNA 2 has been found in the 3' end of CMV RNA 2 (Sivakumaran *et al.*, 2000), indicating that the 3' part of CMV RNA 2 may have an important role in the early event of RNA 2 replication. Therefore, the RNA 2 could be a potent candidate as a target for RNA degradation to prevent initiation of CMV infection and confer RNA-mediated viral resistance in transgenic plants. The extreme resistance observed in the resistant transgenic plants transformed with RNA2-derived IR-constructs (Fig. 6-2) indicates that the virus indeed completely fails to initiate infection, though protoplast infections to verify this assumption have not been performed yet.

RNA-mediated resistance was shown to occur in plants transformed with CP sequences of potyviruses (Lindbo and Dougherty, 1992; van der Vlugt *et al.*, 1992) but in our hands not in plants transformed with CP gene RNA sequences of CMV (Chapter 5). Resistance conferred by CMV CP sequences generally seemed to depend on the (high level of) expression of transgenic coat protein (Cuozzo *et al.*, 1988; Okuno *et al.*, 1993b; Jacquemond *et al.*, 2001). Given the positive effects of using IR sequences on resistance frequency for RNA 2-derived constructs, it was also investigated here whether the application of CP sequences in inverted repeat constructs would add to resistance induction frequency. Indeed, using CP sequences in inverted repeat constructs, a significant percentage (up to 50%) of resistant R_0 line was obtained (Table 6-2), suggesting that CP gene sequences can be used for RNA-mediated resistance as long as they can form a dsRNA structure.

It has been shown that an inverted repeat construct containing an effectively spliced intron yielded 96% PVY-resistant lines while similar constructs without proper intron splicing only yielded 65% resistant lines (Smith *et al.*, 2000). This result indicates that there is still gain to be expected for transgenic IR constructs containing a functional intron, which is removed after transcription. RT-PCR analysis on pLIR-INTs and pSIR-INTs transgenics showed that the introns in sense orientation, like the non-functional antisense introns (pLIR-INTas and pSIR-INTas)

were either only partially spliced, or not sliced at all (Fig. 6-4). This may explain why no difference in efficiency was observed among lines with presumed functional and non-functional intron insertions. The accuracy of splicing is mediated by conserved sequences in the pre-mRNA and by the spliceosomal snRNPs and other factors that recognize these sequences (Brown and Simpson, 1998). The actin2 intron sequence that was used in this study has only three nucleotides between the inverted repeat regions and the splicing sites (Table 6-1). These flanking sequences may be too short to provide a proper binding site for spliceosomes in the double stranded context of the transcript. Alternatively, mutations could have been introduced in and around the splicing consensus sequences in the cloning process. However, due to the inverted repeat structure of the DNA surrounding the intron sequence, attempts to sequence the splicing sites were unsuccessful. Finally, the possibility that the Arabidopsis intron sequence is not recognized in *N. tabacum* has to be considered.

Although the detailed mechanism(s) of the improved inverted repeat-induced resistance still need to be explored, our results demonstrate that the application of such constructs appears to be a highly efficient way to produce CMV resistant plants. The high efficiency also made the testing of resistance on R_0 transformants rather than their progeny possible, thus saving considerable time. Hence, the use of inverted repeat CMV sequences is highly recommended in low efficiency transformation/regeneration protocols, as often is the case for ornamental plant species.

Acknowledgement

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Chapter 7

Summary and concluding remarks

During the past two decades various approaches, based on the concept of pathogen-derived resistance and transformation technologies, have been applied to obtain plant resistance against viruses. In these novel approaches the development of virus resistant ornamental plants lags behind that of other crops because the methods for transgenesis of ornamental plants, especially the monocotyledonous ones such as *Lilium* and *Gladiolus,* have still remained cumbersome.

The works presented in this thesis is the initiating part of a longer-term project to obtain, through transformation approaches, lily cultivars resistant to *Cucumber mosaic virus* (CMV) and less susceptible to damage caused by other viruses whose symptoms are aggravated by CMV co-infection. Of the various available approaches, RNA-mediated transgenic resistance was regarded to be potentially the best option to reach this goal, and hence this strategy has been tested in this Ph.D. thesis. An important aspect of the application of RNA-mediated resistance is that it has been demonstrated to be only effective against the virus from which the transgene has been derived and that it allows limited sequence variation between transgene and invading virus. In view of this, first the diversity in RNA sequence of ornamental plant-infecting CMV strains was determined. Of 11 CMV isolates from ornamental crops originating from different geographical areas, the nucleotide sequence of their RNAs 4 (containing the coat protein gene) was determined and compared. This revealed that both subgroup I and II isolates are represented in ornamental plants. Two isolates (of alstroemeria and crocus) were classified as subgroup II isolates, whereas the other 9 isolates, from gladiolus, amaranthus, larkspur, lisianthus and five from lily, were identified as subgroup I members. In general, nucleotide sequence divergence correlated well with geographic distribution, with one notable exception: the analyzed nucleotide and encoded amino acid sequences of RNA 4 in 5 lily isolates showed remarkably high homology despite their different origins (Chapter 2). This finding strengthened the feasibility of the application of RNA-mediated resistance in transgenic lily.

During the comparative analysis of CMV isolates, a CMV isolate from alstroemeria was shown to contain an additional sequence of 218 nucleotides in

the 3’ non-translated region (3’ NTR) of its RNA 3. After this finding, the RNA sequences of three additional alstroemeria-infecting CMV isolates were also analyzed and this revealed that the observed recombinative nature seems a common phenomenon for alstroemeria-infecting CMV isolates, as additional sequences in both RNA 3 and RNA 2 were found in all of them. Using this information, a specific DIG-labeled probe was developed and applied to detect subgroup II CMV strains in dot blot hybridization and to differentiate the alstroemeria isolates containing these additional sequences by Northern hybridization from the ones lacking them (Chapter 3). The nucleotide sequence and tentative secondary structure suggested that both intra- and inter-molecular RNA recombination events occurred during viral RNA replication, possibly via a template-switching mechanism- thus achieving the recombinant RNA 2 and RNA 3, respectively. The biological fitness of the recombinant relative to wild type virus was subsequently shown to be higher in alstroemeria, while in tobacco plants this was reversed (Chapter 4). Inoculating host plants with various combinations of infectious transcripts synthesized from genomic clones of wild-type and alstroemeria CMV strains might give further insight in the proposed template-switch process in future resarch.

As the different alstroemeria isolates, though all having extra sequences in their RNAs 2 and 3, are not identical, reassortment of their genome segments during a mixed infection may lead to further variants with possibly altered fitness again. Hence, it may turn out that within the broad host range of CMV some host plants may have a relatively high impact on the evolutionary adaptability of CMV.

Based on the concept of pathogen-derived resistance (PDR) alternative approaches such as transgenic expression of viral coat protein (CP) or (defective) replicases in plants to confer resistance against CMV infections have been reported over the years, albeit with rather differing outcomes. The coat protein gene region of the CMV genome has been shown to confer effective resistance in some earlier studies; however, negative results with respect to CP-mediated resistance despite high transgenic CP expression levels have also been reported (Kaniewski *et al*., 1999; Jacquemond *et al*., 2001). A positive correlation between

(defective) replicase expression and transgenic resistance has been reported, suggesting the resistance was “replicase-mediated” (Carr *et al*., 1994; Wintermantel *et al.*, 1997; Wintermantel and Zaitlin, 2000). However, as in most other reported cases, no apparent correlation between resistance and the accumulation of transgenic protein was observed hence it can not be excluded that transgenic “replicase-mediated” resistance against CMV may act at the RNA- rather than the protein-level. In view of the aforementioned opposing results reported for engineered forms of host resistance to CMV, the practical value of such approaches was assessed by transforming a model host plant, *Nicotiana tabacum*, with a series of viral cDNA constructs derived from CMV RNAs 2 or 3 (Chapter 5). Only limited protection to CMV was obtained upon transformation with the CP gene, the S_1 progeny of CP-expressor plants showing only a delay in systemic disease symptom development and the homozygous S_2 progeny still only being partially protected. With respect to RNA-mediated resistance, (non-translatable) RNA 3-derived sequences were found to be uncapable to induce resistance but sequences derived from RNA 2 conferred high levels of resistance. In the latter case the S_1 progeny of a number of transgenic lines showed high levels of resistance, while the homozygous S_2 offspring of these lines were completely protected. The results underscore that, though not all genomic sequences seem equally suitable, RNA-mediated resistance is a better option for introducing transgenic resistance to CMV than CP-mediated resistance.

While RNA-mediated resistance may potentially be a very good strategy to obtain resistant host plants, still the efficiency of inducing this type of resistance is rather low. Typically, one has to regenerate and test dozens of independent transgenic lines to select a fully resistant line at the S_2 level. This low efficiency presents a real bottleneck in case of - transformation - recalcitrant plant species such as monocotyledonous ornamentals. Therefore it was investigated whether transgenes containing viral inserts as invertedly repeated sequences, which upon transcription could form dsRNA, would provoke RNA-mediated resistance at significantly higher efficiency. If indeed, as can now be anticipated, the observed RNA-mediated resistance against CMV is based on post-transcriptional gene

silencing or RNA silencing, than the transgenic production of dsRNAs might be very effective in triggering the defense mechanism. It is known that dsRNA forms are the key triggers of RNA silencing mechanisms. Indeed, during induction of the RNA silencing mechanisms, short (21 to 23 nt-long) small interfering RNAs (siRNAs) are generated from dsRNA by digestion with an RNaseIII-like enzyme (DICER). The siRNAs are proposed to subsequently guide an endonuclease complex (RNA-induced silencing complex, RISC) to RNA that shares sequence similarity with the inducing dsRNA and ensures that this RNA is specifically degraded (for reviews on this topic see Baulcombe, 2002 and Voinnet, 2002). To test whether high frequency induction of RNA-mediated resistance against CMV could be achieved, inverted repeat constructs derived from the 3' halves of CMV RNA 2 and of RNA 3 (CP gene region) were made and transformed to *Nicotiana benthamiana*. A striking seventy-five percent of the tested R_0 lines transformed with an RNA 2-derived inverted repeat construct showed extreme resistance to CMV, while a lower, but still significant, percentage (30%) of resistant R_0 lines was obtained using a similar construct of shorter viral sequence. The resistance level conferred by CP sequences was also improved by using an inverted repeat construct, reaching a level of 50%. For most resistant lines self-pollinated (S_1) progenies were obtained with a resistance level of 100% (Chapter 6). These results indicate that the application of a dsRNA-producing transgene construct provides an efficient approach for obtaining virus resistant host plants. The use of this approach may compensate for the low efficiency protocols for the transformation of ornamental crops.

Both the outcome of the non-translatable constructs (Chapter 6) and the immunity of the transformants obtained confirm that the transgenic resistance is RNA-mediated. The molecular evidence is further provided by the existence of siRNAs specifically in resistant lines. However, the questions on the effects of size and region of the sequences used for constructing dsRNA-encoding plasmids on inducing silencing response remain open. It would be of interest to study the effect of dsRNA-inducing constructs containing sequences of various sizes and from genomic regions as to characterize the molecular basis of resistance induction in

more detail.

As both subgroup I and II isolates of CMV were shown to be present in ornamental plants (Chapter 2), the strategy for developing RNA-mediated resistance against subgroup I as described in Chapter 6 can also be applied to ornamental hosts infected by subgroup II strains such as alstroemeria and crocus. Combining the previous methods, the protocol could be further improved by using a transgenic construct that would produce a dsRNA transcript containing genomic sequences from both subgroups resulting in plants resistant to all CMV isolates.

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SAMENVATTING

Door gebruik te maken van het principe van pathogeen-afgeleide resistentie (engels: pathogen-derived resistance, PDR) zijn de afgelopen twee decennia verschillende benaderingen ontwikkeld om planten door middel van genetische modificatie virus-resistent te maken. De ontwikkeling van transgene virus-resistente sierplanten loopt achter bij die van andere gewassen, omdat de introductie van transgenen in sierplanten, in het bijzonder monocotylen zoals lelie en gladiool, zeer moeizaam is gebleken.

Het werk dat beschreven is in dit proefschrift is de eerste fase van een lange-termijn project met als doel het ontwikkelen van lelie cultivars die resistent zijn tegen het Komkommer-mozaiëk virus (*Cucumber mosaic virus,* CMV) en als gevolg daarvan ook minder vatbaar zijn voor schade veroorzaakt door andere virussen waarvan de symptomen versterkt worden door co-infectie met CMV. Van alle voor handen zijnde methodes leek de op specifieke expressie en afbraak van RNA gebaseerde transgene resistentie (*RNA-mediated virus resistance,* RMVR) het meest veelbelovend om dit doel te bereiken, en dus werd voor deze benadering gekozen .

Hoewel RMVR erg effectief is heeft het als nadeel dat het slechts werkt tegen het virus waarvan het transgen is afgeleid, dientengevolge is het belangrijk allereerst te inventariseren welke CMV isolaten sierplanten infecteren. Van 11 CMV isolaten uit sierplanten van verschillende geografische herkomst werd de nucleotidesequentie van het RNA 4 (coderend voor het manteleiwit) bepaald. Op basis van vergelijking van deze sequenties onderling en met bekende sequenties uit de databank zijn de isolaten in te delen in twee subgroepen. Twee isolaten, afkomstig van alstroemeria en crocus, konden worden ingedeeld in subgroep II, terwijl de andere 9 isolaten, afkomstig van gladiool, *amaranthus, larkspur, lisianthus* en lelie, bleken te behoren tot subgroep I. Over het algemeen was de variatie in sequentie een afspiegeling van de geografische verdeling, met één belangrijke uitzondering: de vijf lelie-isolaten vertoonden onderling een bijzonder hoge mate van homologie in RNA 4, ondanks hun uiteenlopende geografische

herkomst (Hoofdstuk 2). Deze bevinding geeft aan dat het toepassen van RMVR in transgene lelie een haalbare optie is.

Uit de sequentievergelijking van verschillende CMV isolaten (Hoofdstuk 2) bleek een CMV isolaat van alstroemeria een extra sequentie van 218 nucleotiden te bevatten in het 3' niet-coderende gedeelte (*nontranslated region*, NTR) van RNA 3. In een vervolgstudie (Hoofdstuk 3) werd aangetoond dat ook de RNA sequenties van drie andere CMV isolaten uit alstroemeria vergelijkbare inserties bevatten in zowel RNA 3 als RNA 2. Aan de hand van deze informatie werd een *DIG*-gelabelde probe ontwikkeld die gebruikt kan worden om door middel van dot blot hybridisaties de aanwezigheid van subgroep II CMV isolaten aan te tonen, alsmede om door middel van Northern hybridisatie onderscheid te maken tussen CMV isolaten die deze extra sequenties al dan niet bevatten (Hoofdstuk 3). De nucleotidesequentie en voorspelde secundaire structuur van het 3' uiteinde van de alstroemeria CMV RNAs 2 en 3 wezen erop dat tijdens virale RNA replicatie zowel intra- als intermoleculaire recombinaties hadden plaatsgevonden. In een replicatieanalyse werd vervolgens aangetoond dat in alstroemeria de replicatie-efficiëntie van de recombinant groter was dan die van het wildtype, terwijl in tabaksplanten juist het omgekeerde het geval was (Hoofdstuk 4). Aangezien de verschillende alstroemeria isolaten, hoewel ze allen extra sequenties bevatten in hun RNAs 2 en 3, niet identiek zijn, kan "pseudo-recombinatie" van hun genoom-segmenten tijdens een gemengde infectie wellicht leiden tot meer varianten met andere replicatie-efficiënties. Hierdoor is het mogelijk dat binnen het zeer brede gastheerbereik van CMV sommige plantensoorten een relatief grote invloed zouden kunnen hebben op de evolutionaire ontwikkeling van dit virus.

De afgelopen jaren zijn verschillende nieuwe benaderingen om resistentie tegen CMV te bereiken gerapporteerd. Deze zijn gebaseerd op het PDR concept, en behelzen o.a. transgene expressie van viraal manteleiwit of (defect) replicase. De uitkomsten van deze studies zijn echter sterk wisselend. Zo werd in een aantal van de oorspronkelijke studies aangetoond dat het manteleiwitgen van CMV effectieve resistentie kan bewerkstelligen, terwijl dit in latere studies niet herhaald

kon worden, ondanks een goed expressieniveau van transgeen manteleiwit (Kaniewski *et al*., 1999; Jacquemond *et al*., 2001). Daarnaast is een positief effect op resistentie gerapporteerd van transgene expressie van (defect) replicase, hetgeen duidt op een *"replicase-mediated"* resistentie (Carr *et al*., 1994, Wintermantel *et al*., 1997, Wintermantel en Zaitlin, 2000). Er werd echter net als in de meeste andere studies geen correlatie gevonden tussen resistentie en de accumulatie van transgeen eiwit, en dus zou de transgene *replicase-mediated* resistentie tegen CMV wellicht op RNA in plaats van eiwit-niveau kunnen werken. Gezien de tegenstrijdige resultaten van deze studies naar artificiële vormen van resistentie tegen CMV, werd de praktische waarde van een dergelijke benadering geëvalueerd door transformatie van een modelplant, *Nicotiana tabacum*, met een serie virale cDNA constructen afgeleid van CMV RNAs 2 en 3 (Hoofdstuk 5). Transformatie met het manteleiwitgen resulteerde in zeer beperkte bescherming tegen CMV, de S_1 nakomelingen van manteleiwit-expressieplanten vertoonden slechts een vertraging in de ontwikkeling van systemische ziektesymptomen terwijl ook de homozygote S_2 slechts gedeeltelijk beschermd was. Wat betreft RMVR bleek dat transgene (niet-vertaalbare) RNA 3 sequenties niet in staat zijn resistentie te induceren, RNA 2 sequenties daarentegen kunnen dat wel. Door transgene expressie van RNA 2 sequenties vertoonden de S_1 planten van een aantal transgene lijnen een hoge mate van resistentie, terwijl de homozygote S_2 nakomelingenpopulatie van deze lijnen volledig beschermd was. Deze resultaten benadrukken dat, hoewel niet alle genomische sequenties even geschikt zijn, het toepassen van RMVR de voorkeur heeft boven resistentie gebaseerd op manteleiwitexpressie.

Hoewel RMVR een zeer goede strategie kan zijn om virus-resistente waardplanten te verkrijgen, bleek de efficiëntie van de methode erg laag. Het verkrijgen van één volledig resistente lijn op S_2 niveau vereist het produceren en testen van tientallen onafhankelijke transgene lijnen. Deze lage efficiëntie zou een groot probleem zijn in het geval van moeilijk te transformeren planten zoals monocotyle sierplanten. De waargenomen RMVR tegen CMV is gebaseerd op een plant-eigen antiviraal verdedigingsmechanisme dat *"post transcriptional gene*

silencing" (PTGS) of *"RNA silencing"* wordt genoemd. In diverse studies is aangetoond dat de transgene productie van dubbelstrengs RNA (dsRNA) zeer effectief kan zijn in het induceren van dit afweermechanisme. Door het RNA silencing mechanisme worden 21 tot 23 nt kleine *"small interfering RNAs"* (siRNAs) gegenereerd middels het knippen van dsRNA door een RNAseIII-achtig enzym (DICER). Volgens de hypothese maken deze siRNAs deel uit van een multimeer endonuclease (*RNA-induced silencing complex*, RISC) en zorgen zij ervoor dat dit complex specifiek RNA kan knippen dat sequentiehomologie vertoont met het inducerende dsRNA. Om een hogere efficiëntie van inductie van *RNA-mediated* virus resistentie tegen CMV te bereiken, werden *inverted repeat* constructen, afgeleid van de 3' helft van CMV RNA 2 en RNA 3, gemaakt en getransformeerd naar *Nicotiana benthamiana*. Daar waar de transgene planten die enkelstrengs CMV tot expressie brengen een resistentiefrequentie van hooguit 5% van de S_1 lijnen halen, bleek maar liefst vijfenzeventig procent van de geteste R_0 planten die getransformeerd waren met een RNA 2-afgeleid *inverted repeat* construct extreem hoge resistentie tegen CMV te vertonen. Een lager maar nog steeds significant percentage (30%) resistentie in R_0 lijnen werd verkregen met een soortgelijk construct dat een kortere virale RNA 2 sequentie bevatte. Opvallend was dat ook de resistentie die bereikt werd met manteleiwit sequenties sterk kon worden verbeterd door gebruik van een *inverted repeat* construct, en wel tot een niveau van 50% van de origineel getransformeerde R_0 planten. Van de meeste resistente planten werden S_1 lijnen verkregen die volledig beschermd waren tegen virusinfectie (Hoofdstuk 6). Deze resultaten tonen aan dat de toepassing van een dsRNA-producerend transgen construct een bijzonder efficiënte benadering is om virusresistente planten te verkrijgen, wat zou kunnen compenseren voor de lage efficiëntie van transformatie van siergewassen.

Zowel de uitkomsten van de niet-vertaalbare constructen (Hoofdstuk 6) als de immuniteit van de verkregen transformanten, duiden er op dat de transgene resistentie door transgeen RNA geïnitieerd wordt. De moleculaire bewijzen hiervoor werden verder versterkt door het bestaan van siRNAs specifiek in resistente lijnen aan te tonen. In vervolgonderzoek zou het interessant zijn om het

effect te bestuderen van dsRNA-vormende transformatieconstructen met sequenties van verschillende lengtes en van verschillende delen van het virale genoom, om tot in meer detail te bepalen wat de moleculaire basis van sequentie specifieke resistentie-inductie zou kunnen zijn.

Aangezien zowel subgroep I als subgroep II isolaten van CMV aanwezig zijn in sierplanten (Hoofdstuk 2) kan de strategie voor de ontwikkeling van RVMR tegen subgroep I, zoals beschreven in hoofdstuk 6, ook toegepast worden voor gastheren die vatbaar zijn voor subgroep II stammen, zoals alstroemeria en krokus. Bovendien kan het protocol verder verbeterd worden door gebruik te maken van een transgeen construct dat een dsRNA kan produceren dat genomische sequenties van beide subgroepen bevat en op die wijze een brede bescherming zou kunnen bieden tegen alle isolaten van CMV.

觀賞植物之胡瓜嵌紋病毒及應用轉基因防治之展望
Occurrence of *Cucumber mosaic virus* in ornamental plants and perspectives of transgenic control

中文摘要

陳煜焜

胡瓜嵌紋病毒（*Cucumber mosaic virus*，CMV）為球型單股 RNA 病毒，可藉蚜蟲，種子和機械摩擦等方式傳播，其寄主範圍極廣，為害千種以上之植物。CMV 亦為百合和唐菖蒲的重要病原之一，且常與它種病毒複合感染產生協力作用加重病害造成之損失。

近二十年來，結合病原衍生型抗病性（pathogen-derived resistance，PDR）概念與基因轉殖技術所建構之轉基因抗病性（transgenic resistance），已被廣泛且有效地應用於多種植物以抵抗植物病毒之為害。然而，相較於它種經濟作物，這種新的抗病策略在花卉作物上的應用與發展顯然較為落後，單子葉球根花卉尤然，如百合和唐菖蒲。究其因，轉型（transformation）和再生（regeneration）效率低所致。

建構轉基因抗病性於百合以預防 CMV 之感染，減輕 CMV 本身或其與它種病毒複合感染協力所造成之損失為一長期性計劃。 本論文所述及者為此長程計劃之開端，即測試應用轉基因抗病策略於生產抗胡瓜嵌紋病毒之轉基因百合之可行性。在數種目前可行的方法中，RNA 中介式轉基因抗病性（RNA-mediated transgenic resistance）因能提供幾近於免疫的超強抗病能力而被認為是達成此目標之最佳選項。然而，RNA 中介式抗病性的高專一性使得其有效性僅能及於提供轉基因（或基因片段）的病毒本身及與該病毒類緣極近的它種病毒。因此有必要評估感染花卉作物之各 CMV 病毒株（CMV isolates）間的類緣關係，以確保將來釋出具抗 CMV 能力的轉基因百合之成果。質是之故，吾人首先著手調查感染百合及其他數種花卉植物的 CMV 差異性。來自 11 個不同來源的 CMV 病毒株，其 RNA 4（含病毒鞘蛋白基因）的核酸序列經解序並與已知核酸序列之 CMV 病毒株比對後發現：所調查的 CMV 病毒株分別屬於 CMV 第一亞群（subgroup I）或第二亞群（subgroup II）。其中，感染水仙百合（alstroemeria）和番紅花（crocus）者屬 CMV 第二亞群病毒株；其餘源自唐菖蒲（gladiolus）、紅莧（amaranthus）、飛燕草（larkspur）、洋桔梗（lisianthus）及百合（lily）者則屬第一亞群病毒株。一般而言，核酸序列的相異程度可對應於其地理分布之差異，亦即源自不同地域的 CMV 病毒株其核酸序列差異較大；反之，則較近似。但在所分析的 5 個感染百合的 CMV 病毒株之間，不論其

來源的地理分布，其核酸序列均極為近似（第二章）。此發現強化了應用 RNA 中介式轉基因抗病性於生產抗 CMV 的轉基因百合的構想，有助於生產抗病毒之轉基因百合。

在分析比對 CMV 病毒株的核酸序列過程中，於水仙百合 CMV 病毒株（alstroemeria CMV）RNA 3 的 3'-端非轉譯區（3' non-translated region，3' NTR）上發現一長為 218 nucleotides 的插入片段。經另外分析三個不同來源之水仙百合 CMV 病毒株之 genomic RNA 後發現，在 RNA 2 和 RNA 3 上均有額外的且長度不一的核酸片段插入於 3'-端非轉譯區，顯示在水仙百合 CMV 病毒株之間，此種因 RNA 重組（RNA recombination）而插入額外片段的現象極為普遍。應用此一發現，針對額外的插入片段發展出極具專一性的非放射性核酸探針（non-radioactive DIG-labeled probe）。該探針可應用於 dot-blot hybridization 以偵測第二亞群的 CMV 病毒株，亦可應用於 Northern hybridization 以有效地自第二亞群 CMV 病毒株之間區分出具額外插入片段的水仙百合 CMV 病毒株，或反之亦可行（第三章）。由已知的 RNA 序列和可能的二級結構（tentative secondary structure）推論之，在病毒 RNA 複製的過程中，發生 RNA 分子內重組（intra-molecular recombination），而形成重組型 RNA 2（recombinant RNA 2）；同時也發生 RNA 分子間重組（inter-molecular recombination）導致重組型 RNA 3 的形成。重組現象可能經由模本轉換機制（template-switch mechanism）完成。重組型水仙百合 CMV 病毒株和野生型 CMV 病毒株同時接種於水仙百合或同時接種於菸草，顯示前者較適應於水仙百合而後者較適應於菸草（第四章）。

在過去十數年間，CMV 的基因如鞘蛋白基因（coat protein gene，CP），複製酵素基因（replicase gene）等，均已被實際應用於病原衍生型抗病理論之實踐，然而成效參差不一。早期的研究顯示，轉殖 CMV 鞘蛋白基因於植物可有效地達到抗 CMV 的目的；然而近來也有研究顯示 CMV CP 雖可在轉基因植物內大量表現卻不能提供有效的抗性（Kaniewski *et al*.，1999；Jacquemond *et al*.，2001）。也有研究顯示，不論完整或有闕失的（defective）CMV 複製酵素基因轉殖於植物後，轉基因的表現與轉基因植物的抗性有關，故稱此等轉基因抗病性為複製酵素中介型抗病性（replicase-mediated resistance）。不過，在大多數已知的複製酵素中介型抗病案例中，由於轉基因植物的抗病性與轉基因蛋白質（複製酵素）於該轉基因植物內的累積並無必然之相關性，此種抗病機制作用於 RNA 層次（即轉基因的 RNA 錄本，RNA transcripts），而非蛋白質層次，的可能性並不能排除；異言之，複製酵素中介型抗病性的抗病機制有可能是 RNA 中介型（RNA-mediated），而非蛋白質中介型（protein-mediated）。基於有必要選用較易誘發抗性的病毒基因（片段），並擇取成效較佳的抗病機制應用於生產抗 CMV 之轉基因百合，吾人由 CMV 的 RNA 2 和 RNA 3 選取若干基因（或核酸片段），構築一系列含有病毒基因的 cDNA 載體，並轉殖於菸草（*Nicotiana tabacum*），以評估病毒基因（或核酸片段）和抗病機制（蛋白質中介型或 RNA 中介型抗病性）的實用性（第五章）。實驗結果顯示，CMV 鞘蛋白基因所提供的蛋白質中介型抗病性非常有限。僅有少數的轉基因菸草能表現病毒的鞘蛋白基因產物，且此等轉基因植株的自交第一代（S_1）子株所表現的抗性

只能延緩病徵之表現，而自交第二代（S_2）植株雖已是同型結合子的子代（homozygous progeny），也只有部份植株表現抗性。相對於蛋白質中介型抗病性，RNA 中介型抗病性雖不能利用不具轉譯功能的（non-translatable）RNA 3 片段來誘發，卻可由 RNA 2 片段來達成此目的。轉殖 CMV RNA 2 片段的轉基因菸草，其自交第一代（S_1）子株表現較高程度的抗病性，而此等抗病植株的自交第二代（S_2）子株則完全抗病。上述結果顯示：CMV 的各個基因片段並不是全適用於建構轉基因抗病性；而 RNA 中介型抗病性，相較於蛋白質中介型抗病性，則是建構轉基因抗病性於植物以抗 CMV 感染的較佳選擇。

在得知 RNA 中介型抗病機制是建構轉基因抗病性較佳的選擇後，所面臨的另一個問題是轉基因花卉作物的轉型與再生的成功率偏低，要由少數再生的轉基因植株中篩選得具有轉基因抗病性植株之比率顯然更低。一般而言，必須生產並測試大量具有轉基因的個別品系（independent transgenic lines）才能篩選出一個自交第二代（S_2）子株完全抗病的品系。對不易獲得轉基因植株的植物如單子葉的球莖花卉而言，更加重生產轉基因抗病植株的困難度。因此有必要探討利用病毒的基因片段構築出一個具有 inverted repeat sequences 的載體，使其能在轉錄時形成雙股核醣核酸（double stranded RNA，dsRNA）錄本，進而提高生產具有 RNA 中介型抗病機制的轉基因花卉植物效率之可能性。目前已知雙股形態的 RNA 是植物體啟動轉錄後基因消寂作用（post-transcriptional gene silencing， PTGS；或稱為 RNA silencing）的主要啟始因子。植物體內的 dsRNA 會受一種被稱為 DICER 的 RNase III-like enzyme 分解成長度只有 21 到 23 個 nucleotides，名為 small interfering RNA（siRNA）的小分子。雙股 RNA（dsRNA）分解而成的 siRNA 一部份是由 dsRNA 的 sense-strand 所構成，另一部份則是由 dsRNA 的 antisense-strand 所構成。此等 siRNA 分子經與一種被稱為 RISC （RNA-induced silencing complex or RNA interferencespecificity complex）的多單位內切酵素（multi-subunit endonuclease）合併，並引領該內切酵素透過 siRNA 與標的 RNA （target RNA, 指與 dsRNA 有相同或極近似的核酸序列的 RNA 分子，在此指病毒的 RNA）的鹽基配對（base paring）作用，對標的病毒的 RNA 進行專一性分解，從而產生抗病毒的效果。設若轉基因花卉植物抗 CMV 的機制是如預期地基於轉錄後基因消寂作用（PTGS or RNA silencing），則轉基因的 dsRNA 錄本應能有效地啟始轉基因抗病機制。為測試利用 dsRNA 有效啟始轉基因抗病機制的特性，以提高獲得抗 CMV 轉基因植物之可能性與所提高之比率，吾人選取 CMV RNA 2 位於 3'-端的基因片段（長度各為 1500 nt 和 490 nt）和位於 RNA 3 3'-端的鞘蛋白基因（CP gene）用以構築具 inverted repeat sequences 的載體，並轉殖於 *Nicotiana benthamiana*。由較長的 RNA 2 片段構築而成的 inverted repeat construct 經轉殖於 *N. benthamiana* 後，癒傷組織再生所得的植株（R_0 lines）中有 75% 的轉基因植株表現抗性；而由較短的 RNA 2 片段組成的同類型載體亦能誘使 30% 的轉基因植株（R_0 lines）表現抗 CMV 的性狀。鞘蛋白基因的核酸序列經構築成具 inverted repeat sequences 的載體並轉殖於植物後，亦可大幅提升獲得具轉基因抗病性植株（R_0 lines）的比率達 50% 。絕大多數表現轉基因抗病性的 R_0 植株，其自交第一代（S_1）子株均表現抗 CMV 的性狀（100% resistant）（第六章）。上述結果顯示，將一個會形成 dsRNA 的轉基因載體轉殖於植物，是一

個獲得具轉基因抗病性植物的有效途徑，而目前用以獲取轉基因花卉作物所使用的方法成功率偏低的缺點或可利用此策略以彌補之。

Curriculum Vitae

Yuh-Kun Chen was born December 21, 1958 in Po-Tzu, Chia-Yi, Taiwan. He obtained his bachelor degree in science in 1982 and a master degree in 1984 at the National Chung Hsing University, Tai-Chung, Taiwan. In 1986, he started his venture on plant virology and electron microscopy in the Institute of Botany, Academia Sinica in Taipei, Taiwan. In 1988, he returned to his *alma mater* and joined the Department of Plant Pathology where he focused his works on plant disease diagnosis, mainly plant virus diseases. In 1996, he was awarded a scholarship by the Taiwanese Ministry of Education to study in the Netherlands where he started his Ph.D. studies in the Laboratory of Virology of Wageningen University in February 1998. He will return to his home institute and continue his career of research and teaching there in February 2003.

Account

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