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Complementation of CMV subgroup IA strains in replicase-mediated resistant tobacco plants after co-inoculation with different cucumoviruses

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Abstract

Replicase-mediated tobacco plants are highly resistant to the Fny strain of *Cucumber mosaic virus* (CMV) and closely related subgroup IA strains. Two of these subgroup IA strains, Fny- and M-CMV, were co-inoculated with different resistance breaking cucumoviruses to nontransformed and transformed tobacco plants. RT-PCR analyses of single CMV RNAs were performed to study potential complementation of the subgroup IA strains by the resistance breaking cucumoviruses. After co-inoculation of M-CMV with PII-CMV, RNAs 1, 2 and 3 from M-CMV were detected in systemically infected leaves of control plants, whereas in noninoculated parts of replicase-mediated resistant plants only M-CMV RNAs 1 and 3 were found. Western blot studies confirmed the expression of M-CMV coat protein after co-inoculation with PII-CMV in leaves of transgenic plants. These plants also exhibited M-CMV typical yellow spots. M-CMV/TAV co-inoculated transgenic plants contained only M-CMV RNA 3, but no M-CMV RNAs 1 and 2. No M-CMV typical yellow spots were observed in these plants. Our data suggest different types of complementation of M-CMV in replicase-mediated resistant plants by PII-CMV and TAV in trans potentially leading to new RNA combinations in transformed plants compared to nontransformed plants.

Introduction

Cucumber mosaic virus (CMV) is a tripartite, single stranded plus sense RNA virus. CMV RNAs 1 and 2 code for proteins 1a and 2a, which form the viral replicase complex. RNA 3 contains two open reading frames coding for the movement protein and for the coat protein via subgenomic RNA 4 (Palukaitis et al., 1992). In addition RNA 2 encodes a protein 2b, which is assumed to be involved in viral movement (Ding et al., 1995). Numerous strains of CMV have been described with some of them causing severe diseases in different crops (Palukaitis et al., 1992). Whereas natural resistance is not well established in important CMV susceptible hosts, in the last few years pathogen-derived resistance has been proved to be a useful strategy against CMV. The use of plant viral replicase genes for the transformation of corresponding host plants leads in many cases to the generation of plant lines resistant to the donor virus. This phenomenon is called replicase-mediated resistance (for review, see Carr and Zaitlin, 1993; Palukaitis and Zaitlin, 1997). Anderson et al. (1992) used a defective polymerase gene on RNA 2 of CMV strain Fny to transform tobacco plants. Resistance obtained from these plants was shown to be highly specific, stable against high virus concentrations inoculated mechanically or by aphids, and effective at the protoplast level (Carr et al., 1994; Zaitlin et al., 1994). RNA 2 of Fny-CMV was identified as the target RNA for the resistance mechanism (Hellwald and Palukaitis, 1994; Zaitlin et al., 1994). In a survey to analyze specificity of replicase-mediated resistance against CMV, Zaitlin et al. (1994) found resistance against Fny-CMV and closely related subgroup IA strains of CMV, but not against strains of CMV subgroups IB and II.

CMV is distributed worldwide, and a survey performed in Spain has shown that strains of all

genetic subgroups can be isolated from one country (Fraile et al., 1997). Mixed infections of different CMV strains in one plant are well known for a long time (Price, 1934). Here we have addressed the question, whether mixed infections of cucumoviruses have any impact on the efficacy of replicase-mediated resistance. Evidence is presented that as a consequence of the RNA 2 target specific replicase-mediated resistance in tobacco plants, these plants might serve as a platform for the generation of new CMV RNA combinations. The results obtained are discussed in the background of risk assessment and virus resistant transgenic plants.

Materials and methods

Plants and viruses

Nontransformed *Nicotiana tabacum* cv. Turkish Samsun NN plants and the R3 progeny of the R2-2 tobacco plants described in Hellwald and Palukaitis (1995) transformed with a defective RNA 2 from Fny-CMV (Anderson et al., 1992) were used throughout this study. Tobacco plants were grown in 12 cm pots in the greenhouse under controlled environmental conditions at about 25/18 °C day/night cycle with 12 h supplemental light during the day. Fertilizer (Wuxal super) was applied to the plants once a week.

Fny-CMV was isolated in New York, USA (Roossinck and Palukaitis, 1990), M-CMV was isolated from a culture of Price's No. 6 strain in the United Kingdom (Mossop et al., 1976). K-CMV originates from China (Tien, 1982), and strain PII-CMV is a CMV subgroup II strain kindly provided by Rene van der Vlugt, IPO-DLO, Wageningen (Netherlands). Tomato aspermy virus (TAV) strain Ho is part of the virus collection at the Institute of Phytomedicine of the University of Hohenheim.

FKK-CMV was generated by using biologically active full-length clones of the corresponding CMV RNAs, Fny-CMV RNA 1 and K-CMV RNAs 2 and 3 (Rizzo and Palukaitis, 1990; Hellwald and Palukaitis, 1994; Roossinck et al., 1999). Except for M-CMV all cucumoviruses were purified by a procedure using differential centrifugation as described by Scott (1963) without a dialysis step. M-CMV was purified according to the method described by Mossop et al. (1976). Virus concentrations were determined in a UV-Spectrophotometer at a wavelength of 260 nm. Inoculations were carried out with a glas spatula at the 4 leaf stage of young tobacco plants. Ten microliters of purified virus solution per leaf in a concentration of 100 μg/ml virus were applied to the leaves treated with Celite as an abrasive.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used for the specific detection of single RNAs in co-inoculated tobacco plants. *Cucumovirus* sequences for RNAs 1, 2 and 3 were obtained from the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI), website: http://www.ncbi.nlm.nih.gov/BLAST. Multiple sequence alignment was performed with CLUSTAL W (Higgins and Sharp, 1988) Version 1.7. Specific primers were selected from these alignments for RNAs 1, 2 and 3 of Fny- and M-CMV (CMV subgroup IA), K-CMV (CMV subgroup IB), PII-CMV (CMV subgroup II) and Ho-TAV. Subgroup nomenclature is used according to a suggestion of Roossinck et al. (1999). The primers used are listed in Table 1. Primer nomenclature includes one letter for strain specificity and a number for RNA specificity. Different primers used for the detection of one specific RNA were distinguished by letters in alphabetical order. Fny-CMV specific primers were also able to detect M-CMV. Primer pf2a, for example, represents one primer specific for Fny-CMV RNA 2. For the generation of Fny-CMV RNA 2 specific primers it was required to avoid potential detection of the Fny-CMV RNA 2 derived transgene in transformed plants. As the transgene contained a deletion between nucleotides 1857 and 1950, this sequence region was chosen for the generation of an antisense primer pf2a. Primers used for specific detections are described in the text. In case only one specific primer is mentioned in Table 1, this primer represents a sense primer of the corresponding RNA used in combination with the antisense CMV-all primer, which is a general cucumovirus primer.

In case more than one primer is mentioned for one specific RNA, the primer located at the highest nucleotide position represents an antisense primer, the remaining primers were sense primers. Reverse transcription was performed with the Expand Reverse Transcription system obtained from Roche Molecular Biochemicals, Heidelberg, according to the manufacturer's instructions. Two microliters of the reverse transcription reaction were used for a 25 μl volume PCR. The PCR program used for all experiments included

Table 1. List of primers used to detect different CMV strains and TAV in co-inoculated transformed and nontransformed tobacco plants

Target RNA*	Name	Sequence
CMV-all		
3′ end of all cucumovirus RNAs		5′-AGCTGGATGGACAACCCGTTG-3′
Fny-CMV RNA 1		
nt 2184-2203	pf1a	5′-ACTGTCACGCGTTCTCTTCA-3′
Fny-CMV RNA 2		
nt 1922-1903	pf2a	5′-GATCATCGCCTGAGAATA-3′
nt 556-575	pf2b	5′-TCTACTACAGTGAAGAGTGT-3′
nt 49-69	pf2c	5′-TCCCTAGACTTAAATCTTTTC-3′
Fny-CMV RNA 3		
nt 1214-1197	pf3a	5′-AAACACTCTCTATATAGT-3′
nt 665-645	pf3b	5′-TTGCCAGTTACTACACACGCT-3′
nt 306-323	pf3c	5′- ACCCACGGTCGTATTGCT-3′
nt 595-614	pf3d	5′-CTGTCGTTATCGAAAGACAT-3′
PII-CMV RNA 1		
nt 3048-3067	pp1a	5′-TAATCGCTACTTGTATTCCG-3′
PII-CMV RNA 2		
nt 851-870	pp2a	5′-TTCTTATTCAAAGACCGAGG-3′
PII-CMV RNA 3		
nt 1926-1945	pp3a	5′-GGCGTCCGAAGACGTTAAAC-3′
Ho-TAV RNA 1		
nt 1635-1656	pt1a	5′-CGTCTGGACGAAACGACTCTTC-3′
Ho-TAV RNA 2		
nt 2630-2650	pt2a	5′-CCGATAATTCTTCAGATGAGG-3′
Ho-TAV RNA 3		
nt 1635-1656	pt3a	5′-CGTCTGGACGAAACGACTCTTC-3′

*All nucleotide positions of the CMV primers refer to Fny-CMV RNA sequences. In case, no second PCR primer is mentioned for a target RNA, CMV-all primer was used.

a 3 min initial denaturation step at 94 °C, followed by 30 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 54 °C and 2 min extension at 72 °C. PCR method was performed with a Biozym Multipac System. PCR products were separated on a 1% TAE agarose gel under 80 V constant voltage and stained with ethidium bromide.

Western blot analysis

Two hundred milligrams of fresh leaf material was homogenized in SDS extraction buffer according to Laemmli (1970). Samples were boiled for 3 min and then centrifuged for 5 min at 10,000 g. Ten microliters of the supernatant were subjected to SDS gel electrophoresis in a 10% polyacrylamide gel (Laemmli, 1970). Proteins were blotted onto a Fluoro Trans W membrane (Pall) by electroblotting. Color marker proteins (Sigma) were used for the determination of the molecular weights. Membranes were blocked with dry milk overnight and then probed with antibodies specific against CMV coat protein (supplied by Dr. A. Hamacher, University of Bonn). Alkaline phosphatase-labeled goat anti-rabbit IgG (Sigma) were used for the final detection. Bands were visualized by a mixture of Nitro blue tetrazolium salt and the p-toluidine salt of 5-Brom-4-chlor-3-indolylphosphate.

Results

In order to study complementation in replicase-mediated resistant plants, initial studies were based on biologically active full-length clones of subgroup IA strain Fny-CMV and subgroup IB strain K-CMV. As K-CMV RNA 1 was not available, only RNAs 2

and 3 in Fny-CMV were replaced by the corresponding RNAs from K-CMV, a strain with the ability to overcome replicase-mediated resistance. The resulting pseudorecombinant viruses FKK-CMV and Fny-CMV were co-inoculated to nontransformed and transformed tobacco plants in comparison to single inoculations of both viruses to determine the ability of the virus mixture to break replicase-mediated resistance. In Table 2 it is shown that FKK-CMV was able to infect replicase-mediated resistant plants after single inoculation as well as after co-inoculation with Fny-CMV.

A delayed symptom expression was observed in transgenic plants compared to control plants. It was shown before that Fny-CMV RNA 2 is the target RNA for the resistance mechanism in replicase-mediated resistant plants, whereas Fny-CMV RNA 3 is a non-target RNA. Experiments were set up to determine, whether replicase-mediated resistance was still effective against Fny-CMV RNA 2 after co-inoculation of this strain with a resistance breaking FKK-CMV. By using Fny-CMV RNA 2 and 3 specific primers in RT-PCR analysis, we were able to detect specifically the presence of these RNAs in systemically infected leaves of plants co-inoculated with Fny-CMV and FKK-CMV. Eight days after inoculation, samples were collected from systemically infected leaves and subjected to RT-PCR analysis. The results are shown in Table 3. In these experiments Fny-CMV RNA 2 was detected in 17 out of 20 control plants, but in none of the 20 transgenic plants tested indicating that Fny-CMV RNA 2 was still the target for the mechanism of resistance after co-inoculation with FKK-CMV. Fny-CMV RNA 3 was detected in 12 out of 12 control plants and in 18 out of 20 transgenic plants indicating that after co-inoculation with a resistance breaking virus Fny-CMV RNA 3 was able to enter noninoculated leaves in 90% of the plants. Our data indicated the generation of a new pseudorecombinant CMV in replicase-mediated resistant plants consisting of Fny-CMV RNAs 1 and 3 and K-CMV RNA 2. The mechanism of resistance still seemed to be active against Fny-CMV RNA 2, as we were not able to detect this RNA in systemically infected leaves of co-inoculated transgenic plants. Similar results were obtained with wild-type K-CMV strain instead of FKK-CMV (data not shown).

Table 2. Infectivity analysis of Fny-CMV, the pseudorecombinant FKK-CMV and a mixture of both viruses on nontransformed and transformed replicase-mediated resistant tobacco plants

	Number of infected plants/inoculated plants		
	Fny-CMV	FKK-CMV	Fny/FKK-CMV
Control plants	16/16	16/16	16/16
R3-2 plants	0/16	12/16	8/16

Table 3. Detection of single RNAs in control- and transgenic Fny-CMV resistant R3-2 tobacco plants after mixed infection with Fny-CMV and FKK-CMV in systemically infected leaves with specific RT-PCR

	CMV RNA 2*	CMV RNA 3*	Fny-CMV RNA 2	Fny-CMV RNA 3
Control	12/12	12/12	17/20	20/20
R3-2 plants	12/12	12/12	0/20	18/20

*General CMV RNA detection is based on oligonucleotides binding to all CMV subgroup I strains (Anonymous, 1999).

We have used the Fny-/K-CMV co-inoculation system for initial studies, as infectious *in vitro* transcripts were available for these viruses except for K-CMV RNA 1. In order to get more informations on the complementation phenomenon observed after co-inoculation experiments, an experimental setup was developed to study the production of viral proteins and subsequent symptom expression, and to perform an extended PCR analysis including CMV RNA 1. For different reasons we were not able to conduct these studies with the strains Fny- and K-CMV used before. Fny-CMV and K-CMV both belong to CMV subgroup I (subgroups IA and IB, respectively). Although the genetic differences between both strains were sufficient to generate Fny-CMV specific PCR primers, a differentiation of both strains by symptom expression or antibody specificity is not possible. As sequence data for K-CMV RNA 1 were not available, we were also not able to include a specific RNA 1 analysis in our PCR studies. For these reasons we decided to include additional cucumoviruses in our experiments.

M-CMV is another subgroup I strain that is not able to infect Fny-CMV derived replicase-mediated resistant plants systemically (Zaitlin et al., 1994). Whereas Fny-CMV causes green mosaic on nontransformed tobacco plants, M-CMV causes bright yellow mosaic in systemically infected leaves of these plants. The induction of yellow symptoms was mapped before to M-CMV RNA 3 (Shintaku et al., 1992). This strain therefore represented a suitable system for complementation studies with regard to symptom expression and was used for further studies. For the co-inoculation experiments K-CMV was substituted

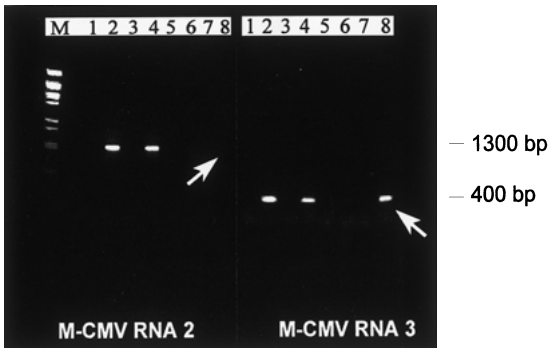

Figure 1. TAE-agarose gel electrophoresis of PCR products obtained after RT-PCR of CMV-infected leaf samples. Specific primer pairs (Table 1) pf2a/pf2b and pf3b/pf3c were used for the detection of M-CMV RNAs 2 and 3, respectively. M: marker; 1: control nontransgenic plants; 2: M-CMV from nontransgenic plants; 3: PII-CMV from nontransgenic plants; 4: M-/PII-CMV from nontransgenic plants; 5: control from transgenic plants. 6: M-CMV from transgenic plants; 7: PII-CMV from transgenic plants; 8: M-/PII-CMV from transgenic plants. Length of the PCR products is indicated. Arrows indicate the potential position of M-CMV RNA 2 (left panel, lane 8) and the position of M-CMV RNA 3 (right panel, lane 8) derived PCR products from M-/PII-CMV co-inoculated transgenic plants.

Table 4. Symptom expression in nontransformed and transformed tobacco plants after co-inoculation of M-CMV with different resistance breaking strains

Viruses	Nontransformed plants	Transformed plants
M-CMV	Bright yellow mosaic	No symptoms
PII-CMV	Very mild green mosaic	Very mild green mosaic
TAV	Green mosaic	Green mosaic
M-/PII-CMV	Bright yellow mosaic	Green mosaic, yellow spots
M-CMV/TAV	Bright yellow mosaic	Green mosaic*

*Nine out of 10 plants surveyed; one plant with yellow spot.

Table 5. PCR analysis of M-CMV and TAV RNAs 1, 2 and 3 in systemically infected leaves of M-CMV/TAV co-inoculated nontransformed and transformed tobacco plants 16 days after inoculation

RNA detected	Nontransformed plants	Transformed plants
M-CMV RNA 1	10/10	0/10
M-CMV RNA 2	10/10	0/10
M-CMV RNA 3	10/10	5/10
TAV-RNA 1	0/10	10/10
TAV RNA 2	0/10	10/10
TAV RNA 3	2/10	9/10

by the subgroup II strain PII-CMV and by TAV, both members of the genus *Cucumovirus*.

Like K-CMV (CMV subgroup IB), PII-CMV (subgroup II) and Ho-TAV also were able to overcome replicase-mediated resistance of transgenic R3-2 plants and caused green mosaic symptoms on these plants. As the primary molecular structure of PII-CMV and TAV is much more divergent from M-CMV sequence as compared to K-CMV, we were able to generate selective primers with higher efficacy for the detection of specific RNAs. In Figure 1 a PCR analysis of M-CMV and PII-CMV RNAs 2 and 3 is shown after co-inoculation of these strains to transformed and nontransformed plants. Although no M-CMV RNA 2 is detected in transgenic plants after co-inoculation, M-CMV RNA 3 was present in systemically infected leaves of these plants indicating a similar complementation phenomenon as observed with the Fny-/K-CMV system described before.

Co-inoculation experiments of M-CMV with either PII-CMV or Ho-TAV were performed to evaluate symptom expression in the corresponding plants. Data of evaluation of symptom expression in these co-inoculated plants are shown in Table 4. Control plants co-inoculated with PII-CMV/M-CMV exhibited M-CMV typical bright yellow mosaic symptoms, whereas transgenic plants showed only bright yellow spots within a green mosaic symptom. As RNA 3 is responsible for the induction of yellow symptoms by M-CMV, the yellow spots in transgenic plants indicated the presence of M-CMV RNA 3 in systemically infected leaves of these plants. A different result was obtained after Ho-TAV/M-CMV co-inoculation.

Nine out of 10 transgenic plants co-inoculated with M-CMV and TAV showed TAV typical green mosaic, and only one plant contained one leaf with a M-CMV typical bright yellow spot about three weeks after inoculation. The corresponding control plants showed typical M-CMV bright yellow mosaic. Back-inoculations from transformed plants to nontransformed tobacco using leaf area with green mosaic again caused green mosaic in these plants, whereas back-inoculation of the leaf area with the yellow spot resulted in a M-CMV like symptom expression (data not shown).

Systemically infected leaves of co-inoculated nontransformed and transformed tobacco plants were tested for the presence of RNAs 1, 2 and 3 of M-CMV using RT-PCR. Results of this PCR analysis after co-inoculation of M-CMV/TAV and M-CMV/PII-CMV are shown in Tables 5 and 6, respectively. In both cases we were not able to detect

Table 6. PCR analysis of M-CMV and PII-CMV RNAs 1, 2 and 3 in systemically infected leaves of M-CMV/PII-CMV co-inoculated nontransformed and transformed tobacco plants 16 days after inoculation

RNA detected	Nontransformed plants	Transformed plants
M-CMV RNA 1	7/8	4/8
M-CMV RNA 2	6/8	0/8
M-CMV RNA 3	8/8	6/8
PII-CMV RNA 1	8/8	6/8
PII-CMV RNA 2	8/8	6/8
PII-CMV RNA 3	8/8	4/8

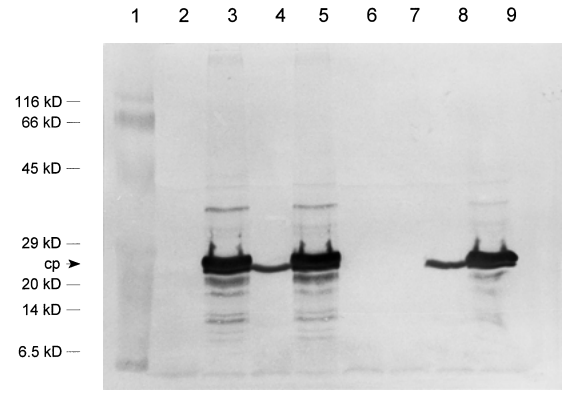

Figure 2. Western blot analysis of CMV coat protein in systemically infected tobacco leaves 8 days after inoculation of transformed and nontransformed plants. Samples 2–5 represent extracts from nontransformed plants, samples 6–9 represent extracts from transgenic plants. 1: marker proteins; 2,6: mock-inoculated; 3,7: M-CMV inoculated plant; 4,8: PII-CMV inoculated plant; 5,9: M-CMV/PII-CMV co-inoculated plant. The position of the CMV coat proteins (cp) is indicated by arrow.

M-CMV RNA 2 in systemically infected leaves of transgenic plants co-inoculated with the corresponding virus mixtures listed in Table 4.

In case of M-CMV/TAV co-inoculation (Table 5), 10 out of 10 control plants inoculated were infected with M-CMV, whereas TAV RNAs 1 and 2 could not be detected in these plants. In two plants TAV RNA 3 was detected. These data suggested that M-CMV invaded noninoculated tissue in control plants faster than TAV. This idea was supported by comparing single inoculations of TAV and M-CMV in control plants. Whereas M-CMV inoculation was followed by systemic symptom expression between day 4 and 5 after inoculation, TAV symptoms were not detected before 7 days after inoculation. In transgenic plants a different RNA pattern was found. In these plants nine out of 10 plants were infected with TAV, and the remaining plant still contained TAV RNAs 1 and 2, whereas M-CMV RNAs 1 and 2 were not detected in these plants. M-CMV RNA 3 was observed in five out of 10 plants inoculated. According to these observations at least five out of 10 plants contained a RNA combination consisting of TAV RNA 1, 2 and 3 and M-CMV RNA 3, whereas most of the control plants were infected solely by M-CMV. With M-CMV/PII-CMV co-inoculations we found M-CMV typical yellow spots in six out of eight co-inoculated transgenic plants. In two plants we did not detect any symptoms or viral RNA (Table 6). In the symptom bearing plants we were able to detect M-CMV RNAs 1 and 3 in transgenic plants, but not M-CMV RNA 2 (Table 6). After M-CMV/PII-CMV co-inoculations of control plants we were able to detect all three PII-CMV RNAs in systemically infected leaves indicating that PII-CMV was able to compete with a M-CMV infection.

Although coat proteins of both viruses reacted with a CMV coat protein specific antibody, we observed a slightly different migration pattern of M-CMV coat protein and PII-CMV coat protein in SDS gel electrophoresis. In Figure 2a, Western blot probed with coat protein antibody is shown from a M-CMV/PII-CMV co-inoculation experiment. In lane 3 M-CMV coat protein is represented by a dominant band, whereas PII-CMV coat protein in lane 4 showed a slightly different migration pattern. The M-CMV coat protein band is also visible in co-inoculated transgenic plants, but not in transgenic plants after single inoculation with M-CMV, which is not able to break replicase-mediated resistance.

Discussion

Replicase-mediated resistance derived from subgroup IA strain Fny-CMV (Anderson et al., 1992) is characterized by a high grade of specificity. Resistance can be overcome by CMV strains of subgroup IB or subgroup II and by TAV (Zaitlin et al., 1994; Hellwald and Glenewinkel, 1999). Single infections by defined strains of CMV will only randomly occur in the field, as surveys in various parts of the world revealed the presence of a great variety of CMV strains at different locations (Daniels and Campbell, 1992; Fraile et al., 1997; Varveri and Boutsika, 1999). Mixed infections of CMV in the field therefore can be considered a common event, which was confirmed by Fraile et al. (1997) in Spain.

Little is known about how occurrence and distribution of resistance breaking viruses will affect the efficiency of pathogen-derived resistance. Studies on the mechanism of resistance in these plants revealed a transgene sequence specific mechanism (Hellwald and Palukaitis, 1995). Therefore the target of the resistance mechanisms can be mapped to RNA 2 of CMV, as Anderson et al. (1992) used a defective Fny-CMV RNA 2 construct for the generation of resistant plants. Our studies showed that RNA 2 of Fny-CMV still serves as the target for the mechanism of resistance even in the presence of a resistance breaking strain.

In a previous report, it was shown that a limited amount of Fny-CMV RNA is accumulated in inoculated leaves of transgenic plants (Hellwald and Palukaitis, 1995). As, for example, FKK-CMV or PII-CMV replicate in transgenic plants, it seems difficult to explain why the small amount of Fny-CMV RNA 2 present in inoculated transgenic plants is not picked up by the replication machinery of these resistance breaking strains once established.

It has been proposed that pathogen-derived resistance involves a factor or unknown cellular or genomic feature capable of degrading specifically viral RNA thereby establishing the resistant status of these plants (Dougherty et al., 1994; Mueller et al., 1995) which is supposed to be part of a basic plant antiviral defense system (Brigneti et al., 1998). If such a degradation system would be active in Fny-CMV replicase-mediated resistant plants, one possible explanation would be that replication of Fny-CMV RNA 2 by K-CMV or PII-CMV replication complex in trans cannot cope with this degradation mechanism. Fny-CMV RNA 3 was picked up by the FKK-CMV replication machinery and systemic spread of this RNA was shown by RT-PCR detection in noninoculated leaves of mixed inoculated nontransformed and transformed plants. From this result it can be concluded that resistance breaking RNAs are able to support the replication of strains normally restricted by the resistance mechanism. Are there any consequences from this phenomenon? One important question is, whether RNAs gaining profit from this complementation have any impact on symptom expression of these plants. As different symptom determinants of CMV can be mapped to different CMV RNAs, we have studied the complementation of M-CMV RNA 3, which causes a bright yellow mosaic in tobacco, when accumulated systemically (Mossop et al., 1976).

M-CMV coat protein is responsible for the induction of bright yellow symptoms (Shintaku et al., 1992). In co-inoculations of M-CMV with PII-CMV we were able to observe M-CMV typical bright yellow spots in transgenic plants and also M-CMV coat protein was detected in these leaves by Western blot. These observations indicated that RNAs supported by a resistance breaking strain in transgenic plants are translated and can induce typical symptoms. In co-inoculations of M-CMV with PII-CMV, we detected M-CMV RNAs 1 and 3 in most of the systemically infected leaves of transgenic plants. In co-inoculations with M-CMV and TAV, we detected only M-CMV RNA 3, but no M-CMV RNAs 1 and 2 in systemically infected transgenic plants. These data could reflect the situation of incompatibility of CMV- and TAV-encoded replicase subunits, which has been shown before (Rao and Francki, 1981). Whereas 1a and 2a protein derived from CMV RNAs 1 and 2, respectively can easily be exchanged between CMV subgroup I and subgroup II strains, this appears not to be possible between CMV and TAV. Pseudorecombination between RNAs 1 and 2 of CMV and TAV was observed so far only under highly selective conditions, which involved the generation of a recombinant virus, a mutant RNA 1, and a formation of a quadripartite genome between CMV and TAV (Masuta et al., 1998). In wild-type plants we have observed a strong competitive advantage of M-CMV over TAV, as no TAV RNAs were detected after co-inoculations in these plants. In some experiments this was also observed before by Sackey and Francki (1990).

In replicase-mediated resistant plants, a reverse situation could exist. TAV could have a competitive advantage over M-CMV, as the M-CMV replication complex is affected by the mechanism of resistance. Besides a potential incompatibility between M-CMV and TAV replicase proteins, this advantage could also account for the fact that no M-CMV RNAs 1 and 2 are detected in these transgenic plants after co-inoculation.

Is there any impact of these studies on the discussion of risk assessment of transgenic, virus resistant plants? Our data indicate that the RNA composition in systemically infected replicase-mediated resistant plants after co-inoculation is different from the corresponding RNA composition of control plants. If Fny-CMV RNA 2 is eliminated, for example, during a Fny-/K-CMV co-inoculation by replicase-mediated resistance, K-CMV RNA 2 can substitute this function thereby generating pseudorecombinant viruses like FKF-CMV (RNAs 1 and 3 from Fny-CMV, RNA 2 from K-CMV) or FKK-CMV (RNA 1 from Fny-CMV, RNAs 2 and 3 from K-CMV). We have shown before that, for example, FKK-CMV causes a more severe phenotype

on pepper plants as compared to the corresponding wild-type viruses Fny-CMV and K-CMV (Hellwald et al., 2000). Under these conditions the generation of viruses with increased virulence facilitated by the transgenic plant seems possible. Two questions remain with regard to the significance of the data obtained in this report. First, the generation of new pseudorecombinant viruses strongly depends on the presence of all participating RNAs in the same plant cell, which in our studies could have been forced by mechanical co-inoculation, but may not reflect the situation during natural infections. Second, the stability and relative fitness of the potentially new generated pseudorecombinant viruses in comparison to the wild-type viruses is crucial for their ability to survive in the plant. Both questions are important for the final evaluation of our results and will be addressed in further studies.

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