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Resistance against cucumber mosaic virus in plants expressing the viral replicon

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Abstract CMV RNAs 1 and 2 are considered to constitute the viral replicon. Tobacco plants were transformed with either RNA1 or RNA2 to produce plant lines V1 and V2, respectively. Plants homozygous for each of the RNAs were generated and crossed to produce V1V2 (V2V1) lines that expressed both RNA1 and RNA2. An RNase protection assay indicated that RNA1 and RNA2 multiplied in V1V2 (V2V1) plants. Surprisingly, V1V2 (V2V1) plants, unlike their parent lines, showed a remarkably high level of resistance to CMV; this resistance was more effective against RNA inoculation than against virion inoculation. Experiments using protoplasts showed that the resistance was expressed at the single cell level. All the data together suggested that the observed resistance does not fit the criteria for either 'RNA-mediated' or 'replicase-mediated' resistance.

Key words: CMV; Replicon; Resistance; Transgenic plant

1. Introduction

Cucumber mosaic virus (CMV) possesses a genome consisting of three plus-sense, single-stranded RNA molecules, designated RNA1, RNA2 and RNA3 in decreasing order of molecular weight. The 1a protein and 2a protein encoded by RNA1 and RNA2, respectively, are necessary for viral replication [1,2]. The 3a protein encoded by RNA3 is involved in virus movement, and a subgenomic RNA, RNA4, which is generated from the 3′ half of RNA3 serves as a messenger RNA for the viral coat protein.

CMV disease wreaks such enormous damage on agricultural crop production throughout the world that significant efforts have been made to create CMV-resistant plants by classical breeding techniques. There are now a number of studies that describe the establishment of CMV resistance in transgenic plants. For example, transgenic plants that express the CMV coat protein gene are often virus resistant [3,4], and plants highly resistant to CMV infection can be generated by expressing non-structural genes such as gene 2a [5–7]. This kind of resistance is called 'pathogen-derived' resistance. Recent experiments using other viruses suggest that transgenes expressing untranslatable sense RNAs can confer effective resistance; this is called 'RNA-mediated' or 'homology-dependent' resistance [8–12].

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Abbreviations: CMV, cucumber mosaic virus; CMV-Y, cucumber mosaic virus Y strain; PCR, polymerase chain reaction.

We obtained transgenic tobacco plants that simultaneously expressed intact forms of RNA1 and RNA2, and we expected that all the transformed cells from these plants would contain infectious RNA1 and RNA2. We also expected that these plants would support CMV RNA3 replication, instead we observed a high degree of resistance to CMV in these plants. The mechanism for the observed resistance seemed to be different from that of the resistance which have been reported as 'replicase-mediated' [5,6] or 'RNA-mediated'. This resistance and its relation to the ratio of accumulation of each of the viral RNAs during the establishment of CMV infection is discussed.

2. Materials and methods

2.1. Construction of a plant expression vector and generation of transgenic plants

Cauliflower mosaic virus 35S promoter in a plant expression vector, pBI121 (Clontech), was subcloned between the *Hin*dIII and *Bam*HI sites of pUC118 (Takara Shuzo, Japan). Oligonucleotide-directed mutagenesis was used to create a *Stu*I site at the transcription start site of the promoter [13]. A fragment containing the entire cDNA clone of RNA1 of CMV-Y, which was prepared by *Pst*I digestion and T4 DNA polymerase treatment followed by *Sac*I digestion of pCY1-T7KP [14], was inserted between the *Stu*I and *Sac*I sites of the modified vector. After double digestion with *Hin*dIII and *Sac*I, the 4.1 kb fragment containing the cDNA of RNA1 and the 35S promoter region was cloned between the *Hin*dIII and *Sac*I sites of pBI121 (Fig. 1). For convenience, the 5′ half of the RNA2 cDNA clone from CMV-Y (a *Bam*HI–*Hin*dIII fragment of pCY2-T7 [14]) and the 3′ half of pCY2-T7 (a *Hin*dIII–*Sac*I fragment) were ligated to create intact RNA2 after insertion between the *Bam*HI and *Sac*I sites of the modified vector. This construct contained a T7 promoter region upstream of the cDNA of RNA2. The T7 promoter region of pCY2-T7 was deleted by oligonucleotide-directed mutagenesis so that the viral 5′ end sequence could be linked at the transcription site of the 35S promoter. The recombinant plant expression vectors were introduced into *Agrobacterium tumefaciens* LBA 4404 which was used to transform tobacco (*Nicotiana tabacum* cv. BY-4) by the leaf disk method [15]. Plants transformed with either RNA1 or RNA2 were designated V1 and V2, respectively. The homozygous R_2 generations of the self-fertilized transgenic plants were crossed with each other to produce plants expressing both RNA1 and RNA2; these progeny plants were designated V1V2 or V2V1 depending on the maternal plant line.

2.2. PCR analysis of transgene

DNA samples were prepared from total nucleic acids extracted from tobacco plants by LiCl precipitation [14]. Each sample (0.5 μg) was subjected to PCR using an LA PCR Kit (Takara Shuzo). Three synthetic oligonucleotides were used as primers: 5′-GTTCAATACACCACAGTACTCGTAGCGG-3′ (complementary to the region from positions 3019 to 3046 of RNA1), 5′-ATAGCAATACTGCCAACTCAGCTCC-3′ (complementary to the region from positions 2792 to 2816 of RNA2) and 5′-GGATCTAACAGAACTCGCCG-3′ (about 500 nucleotides upstream from the transcription start site for the 35S promoter). The PCR lasted for thirty cycles of amplification with the following parameters: denaturation at 98°C for 15 s, annealing at 62°C for 15 s, and synthesis at 68°C for 5 min.

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2.3. RNase protection assay

The following restriction fragments of pCY1-T7 and pCY2-T7 [14] cDNA were subcloned into pBluescript II SK(−) and KS(−) (Stratagene), respectively, to generate ^{32}P-labeled antisense transcripts used as probes for RNase protection assay: (i) *Xba*I–*Eco*RI fragment (from nucleotide positions 382 to 851 in cDNA of RNA1). (ii) *Sal*I–*Eco*RV fragment (from nucleotide positions 145 to 598 in cDNA of RNA2). The resultant plasmids containing DNA fragments for RNA1 and RNA2 were linearized with *Xba*I and *Xho*I, respectively, and transcribed with T7 RNA polymerase containing [α-^{32}P]UTP. The specific activity of RNA probes was 1×10^8 to 2×10^8 cpm/μg. Guardian RNase Protection Assay Kit (Clontech) was used essentially as described [16]. Total nucleic acids were isolated from leaf tissue by the guanidinium isothiocyanate extraction followed by phenol/chloroform (1 : 1) extraction, and total RNAs were precipitated by LiCl as described [14]. Ten μg of RNA samples were mixed with the ^{32}P-labeled riboprobes. The mixed samples were treated with RNase mixture (1 : 1000) at 45°C for 12 h, precipitated by ethanol, and then electrophoresed in a 4% polyacrylamide-8 M urea gel. After drying, the gel was exposed to X-ray film.

2.4. Whole-plant inoculation experiments

CMV-Y has been maintained in our laboratory. Viral RNA was extracted as described previously [17]. Plants were dusted with carborundum, rub-inoculated with virus or viral RNA, and maintained in a greenhouse at 25–26°C under natural light conditions. Symptoms were noted about 1 month after inoculation.

2.5. Protoplast experiments

Preparation of protoplasts from transgenic and non-transgenic tobacco leaf tissue was described previously [18]. Virus and viral RNA were introduced into protoplasts in the presence of polyethylene glycol (PEG) and $CaCl_2$ [19]. Infected protoplasts were analyzed by Northern blot analysis. The inoculated protoplasts were stained with fluorescent polyclonal antibody against CMV-Y after incubation for 24 h at 28°C under continuous illumination with fluorescent lamps [20]. To evaluate the viral replication in the protoplasts, total RNAs were extracted from 2×10^4 protoplasts that had been incubated for 24 h. The samples were treated with buffer containing formaldehyde and formamide essentially as described previously [21]. The samples were then electrophoresed on a 1.5% agarose gel containing 0.66 M formaldehyde, transferred to a nitrocellulose membrane and hybridized with ^{32}P-labeled mixed probes. The probes were a mixture of following fragments: the 1,770 bases between the two *Xho*I sites of RNA1, the 1,750 bases between the *Sal*I and *Hin*dIII sites of RNA2, and the 1,870 bases between the two *Sty*I sites of RNA3. The probes were synthesized by the random primed method using a Prime-It II random primer labeling kit (Stratagene).

3. Results and discussion

3.1. Transgenic plants

The constructs of transgenes used in this study are shown in Fig. 1. Five V1 lines (R_0) transformed with the cDNA of RNA1 were obtained; only one V1 line contained a single T-DNA insertion as judged from the segregation data of the R_1 lines for kanamycin resistance. Six V2 (R_0) lines that were transformed with the cDNA of RNA2 were obtained; all the lines contained single T-DNA insertions. We have selected the transgenic lines harboring only a single cDNA copy so that the transgenic plants (R_1) homozygous for each of the cDNA of RNA1 and RNA2 should be easily selected based on the segregation data. The plants that were homozygous for the cDNAs of RNA1 and for RNA2 were crossed to generate V1V2 (V2V1), the plants which contained cDNA copies of both RNA1 and RNA2. The integration of the cDNAs from the respective viral RNAs into V1, V2, V1V2 and V2V1 plants was confirmed by PCR, by which the expected DNA fragments with almost full-length cDNA from RNA1 and RNA2 were amplified in all the preparations from the plants containing the transgenes (data not shown).

3.2. Transgene expression

When V1 plants were inoculated with in vitro synthesized RNA2 and RNA3, and when V2 plants were inoculated with in vitro synthesized RNA1 and RNA3, we observed typical CMV disease symptoms on those transgenic plants. The inoculation experiments showed that biologically active RNA1 and RNA2 molecules were actually generated from the corresponding transgenic plants. Although V1, V2 and V1V2 (V2V1) R_1 plants were initially examined for transgene expression by Northern blot analysis, the RNA bands with the expected sizes in V1 and V2 plants were hardly detected; but we observed faint bands in V1V2 and V2V1 plants. To determine the extent of accumulation of the expected transgene, we used an RNase protection assay, which is thought to be more sensitive than Northern analysis. As shown in Fig. 2, after RNase treatment the expected 469-nt and 453-nt bands for RNA1 and RNA2, respectively, were readily detected in the corresponding transgenic plants. In addition, the V1V2 (V2V1) plants contained bands that were more than 20-fold stronger than those detected in the preparations from their parents, suggesting that RNA1 and RNA2 had actually multiplied in the plants as a replicon. We do not know why the accumulation of the transgene transcripts were so low in the V1 and V2 plant lines.

3.3. Resistance against CMV infection

Transgenic plants were screened for viral resistance by mechanical inoculation with CMV virions or with CMV RNA (Table 1). Plants were monitored daily for the appearance of symptoms and scored one month after inoculation. The V1V2

Table 1
Assessment of resistance to CMV infection in transgenic tobacco plants

	Inoculum concentration (μg/ml)													
	CMV-Y virion					CMV-Y RNA								
Line	0.2	0.5	1.0	2.0	5.0	0.2	0.5	1.0	2.0	5.0	10.0	50	100	200
V1V2	2/10[a]	9/20	19/26	9/9	5/5	nt	0/5	1/8	1/17	7/17	6/13	4/10	8/9	2/2
V2V1	2/9	8/20	15/25	10/10	5/5	nt	0/5	1/8	1/16	4/17	5/13	2/4	4/8	5/5
BY-4	6/12	10/10	31/31	12/12	2/2	3/12	10/10	8/8	15/15	15/15	4/4	2/2	4/4	nt
V1	nt[b]	11/11	15/15	nt	nt	nt	6/7	17/17	nt	10/10	nt	nt	nt	nt
V2	nt	9/10	15/15	nt	nt	nt	6/7	17/17	nt	10/10	nt	nt	nt	nt

[a]Number of infected plants/number of inoculated plants.
[b]nt, not tested.

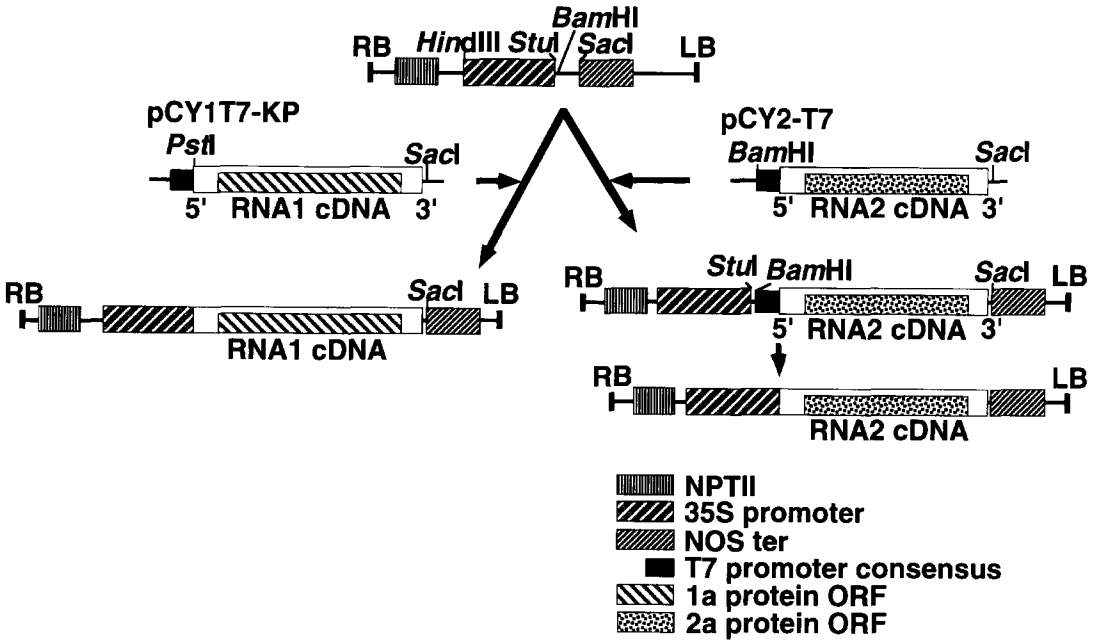

Fig. 1. Schematic representation of the gene constructs for CMV RNA1 and RNA2 used for tobacco transformation. The full-length cDNA clones of CMV RNA1 and RNA2 were inserted in expression cassettes in a binary vector, pBI121. NPTII refers to neomycin phosphotransferase II, 35S promoter to the 35S RNA promoter of cauliflower mosaic virus, NOS ter to the transcriptional terminator of nopaline synthase gene.

and V2V1 lines displayed some resistance to CMV while the V1 and V2 plants were all susceptible at concentrations of >1.0 μg/ml. Resistance was especially effective against inoculation with CMV RNA, and there was a marked delay in the appearance of systemic symptoms in V1V2 and V2V1 plants.

To determine if resistance is expressed at the single cell level, tobacco mesophyll protoplasts from the CMV-resistant transgenic plants were inoculated with CMV virions or CMV RNA. After incubation for 24 h, protoplasts were stained with fluorescent antibody against CMV. Total RNA was subjected to Northern blot analysis using ^{32}P-labeled probes that consisted of mixed RNA1, RNA2 and RNA3 fragments. As shown in Table 2, a marked suppression of CMV replication was observed after the inoculations not only with viral RNA but also with virions. The levels of CMV RNAs were also very low in RNA extracted from the CMV-inoculated protoplasts of the transgenic plants (V1V2, V2V1) whether the inoculum was virions or RNA (Fig. 3). These results suggested that resistance also occurred at the single cell level. This high level of resistance observed at the single cell level seemed to be different from the results observed in the intact plants inoculated with CMV virions.

3.4. *Possible mechanisms for resistance*

The integration of mutated replicase genes of CMV leads to a high level of resistance against CMV inoculation with virions and RNA; this type of 'pathogen-derived' resistance is especially called 'replicase-mediated' resistance [5,6]. The exact mechanism for this resistance remains unclear; however, it is generally thought that the synthesis of mutated viral replicase will interfere with normal interaction between virus and host. The resistance induced in this mode seems to be expressed only against the virus from which the transgene originated or against closely related viruses. In their previous papers describing this resistance, the authors found a correlation between the expression level of the transgene and the level of resistance [6]. However, recent studies suggest that there is no apparent relationship between resistance and the accumulation of transcripts or protein products of the transgene; resistance may, therefore, be RNA-mediated [9,12]. In a very recent report, Mueller et al. insisted that the so-called 'replicase-mediated' resistance should be grouped with the 'homology-dependent' resistance, which is RNA mediated [10]. The theory for the 'RNA-mediated' resistance is that transgene mRNA levels exceeding a certain threshold level activate the cytoplasmic machinery that targets the mRNAs for inactivation, possibly by RNA degradation, in a sequence specific manner. In V1V2 (V2V1) plants, 'RNA-mediated' resistance may have been triggered by amplification of CMV RNA1 and RNA2, which eventually exceeded the thresh-

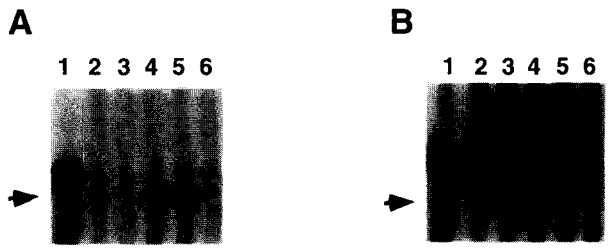

Fig. 2. RNase protection assay on expression of the transgene in transgenic plants. ^{32}P-labeled riboprobes complementary to RNA1 (A) and RNA2 (B) were hybridized to total RNA extracted from the transgenic plants, and subjected to RNase digestion. Lane 1, the undigested riboprobes; lanes 2–5, total RNA from the transgenic plant lines V1, V2, V1V2 and V2V1, respectively; lane 6, total RNA from the nontransformed BY-4 plants. The expected signal was detected (arrowheads).

old as a result of replication of RNA1 and RNA2. However, we think this resistance seems to be different from the 'replicase-mediated' and even 'RNA-mediated' resistance in several aspects. First, the CMV-resistant transgenic plants (V1V2 and V2V1) contained 20-fold more RNA1 and RNA2 than the susceptible transgenic lines (V1 and V2) that contained a very low amount of transgene transcripts. If the detected amounts of RNA1 and RNA2 in the V1V2 (V2V1) plants were much lower, an 'RNA-mediated' resistance mechanism might seem more feasible. Secondly, the transgenic plants exhibited a higher resistance when inoculated with RNA than with virions. If this is 'RNA-mediated', the resistance must be equally effective against inoculation with both CMV virions and CMV RNA. Thirdly, V1V2 (V2V1) plants were also resistant against the C strain of tomato aspermy virus (C-TAV), which is a distantly related virus to CMV (data not shown), and thus the resistance was not strictly sequence specific. Even though the resistance is 'RNA-mediated' or 'replicase-mediated', it should be sequence specific. Perhaps, in addition to 'RNA-mediated' resistance, another mechanism may exist. Taken together, these observations have led us to describe a new mechanism for the resistance observed in this paper. A plausible hypothesis is that very high level accumulation of RNA1 and RNA2 may affect the rate of encapsidation of RNA3 and RNA4; preexisting RNA1 and RNA2 might capture the newly synthesized coat proteins from RNA4 soon after they were produced, and genomic RNA3 and RNA4 would be subject to RNase attack before it could be encapsidated by a now depleted population of coat proteins. The observation in the protoplast experiment also supports this idea. After protoplast preparations, the cells must be surrounded by an increased level of RNase activity [22]. When the protoplasts are inoculated either with RNA or virions, the viral RNAs will be degraded before establishment of infection unless they are encapsidated as quickly as possible. We speculate that the inoculated viral RNAs were more easily degraded in the protoplasts than in the cells of the intact plant because the scavenging of coat protein by RNA1 and RNA2 will accelerate degradation of RNA3 and RNA4 from the challenge RNA whether or not the source of the challenge RNA was from inoculation with virion or RNA.

In summary, for our functional analysis of the CMV replicon, we inserted intact forms of viral RNAs in plant genomes.

Table 2
Susceptibility of protoplasts isolated from tobacco plants to infection with CMV, CMV RNA and TMV RNA

Inoculum (μg/ml)	% fluorescent cells[a]						
	CMV Virion			CMV RNA			TMV RNA
	20	40	100	5	10	40	5
Exp. 1							
BY-4	38.2	42.2		53.6	51.5		66.7
V1V2	2.8	1.4		0	0		55.9
V2V1	1.9	0.8		0	1.9		51.5
Exp. 2							
BY-4		26.0	29.5		49.9	62.7	
V1V2		0	0		0.5	1.1	
V2V1		0.2	0.5		0.2	0.6	

[a]Protoplasts were treated with the fluorescent antibody against CMV or tobacco mosaic virus (TMV) 24 h post inoculation and not fewer than 200 protoplasts were counted in each experiment. To check the transgenic plants are effective against a different virus, TMV was used as a control; note that no resistance was observed.

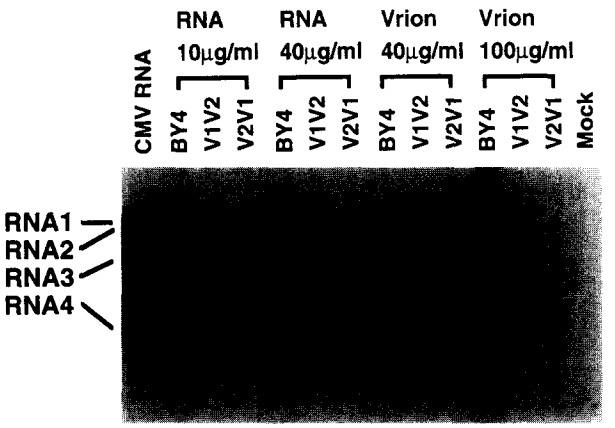

Fig. 3. Northern blot analysis of CMV RNAs in the infected protoplasts expressing the transgenes. Isolated mesophyll protoplasts were inoculated with CMV virions or with CMV RNA. After 24 h incubation, total RNA was extracted and examined for viral replication. The lanes labeled as CMV RNA and Mock contain 12.5 ng of CMV RNA and RNA from mock-inoculated BY-4 protoplasts, respectively. The inoculum concentrations and the inoculated plant lines are presented above each lane. Note that CMV RNA was hardly detected in the transgenic protoplasts.

Beyond our expectations, we observed the occurrence of resistance that could not be necessarily explained by the existing proposed mechanisms for engineered resistance against plant viruses. Although we considered a possible hypothesis for the new resistance, much remains to be understood about how it affects the basic features of viral infection such as regulation (switch) of replication, translation and encapsidation.

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