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Symptom determinant of two Taiwan strains of cucumber mosaic virus is on RNA 3

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Abstract. Two cucumber mosaic virus (CMV) strains, NT9 which causes severe mosaic in *Nicotiana tabacum* L. cv. Van-Hicks and M48 which causes symptomless infection in same host, were used to construct the pseudorecombinants for genetic analysis of symptom formation. Pseudorecombinant $N_1N_2M_3$ was constructed by mixing gel separated RNAs 1 and 2 from NT9 with RNA 3 from M48 and $M_1M_2N_3$ by mixing gel separated RNAs 1 and 2 from M48 with RNA 3 from NT9. Each construction was inoculated onto *Chenopodium quinoa,* cloned by serial single-lesion passages through this host and propagated in tobacco. The true nature of the pseudorecombinants was checked by gel analysis of RNAs, peptide mapping of capsid proteins and reverse constructions to restore the parental virus strains. The symptoms on most of host species inoculated with $N_1N_2M_3$, $M_1M_2N_3$ and their parental strains gave evidence for an effect of CMV RNA 3 on symptoms.

Key words: Cucumber mosaic virus; Pseudorecombinants; RNA 3; Symptom determinant.

Introduction

The genome of cucumber mosaic virus (CMV) consists of three single-stranded, positive-sense RNAs designated as RNAs 1 to 3 in order of decreasing sizes, RNA 1 is 3387 nucleotides (Rezaian *et al.,* 1985), RNA 2 is 3035 nucleotides (Rezaian *et al.,* 1984) and RNA 3 is 2193 nucleotides long (Gould and Symons, 1982). All these RNAs are required for infectivity (Peden and Symons, 1973; Lot *et al.,* 1974). Another encapsidated subgenomic RNA, RNA 4, has a sequence of 1,027 nucleotides identical to that of 3'–terminus of RNA 3 (Gould and Symons, 1982) and codes for CMV coat protein (Schwinghamer and Symons, 1977). In addition, there are the fifth encapsidated single-stranded RNAs of 334–339 nucleotides, termed satellite RNA or cucumber mosaic virus-associated RNA 5 (CARNA5), present in some strains of CMV and dependent on the latter for replication. (Kaper *et al.,* 1976; Kaper and Waterworth, 1977; Gould *et al.,* 1978; Murant and Mayo, 1982; Francki, 1985).

Genetic analyses of cucumovirus strains are facilitated by a divided genome of the viruses. Pseudorecombinants constructed by exchanging one or more of the genomic RNAs have been employed for assessing genetic functions of the various RNA species in CMV and the related tomato aspermy virus (TAV) (Habili and Francki, 1974; Marchoux et *al.,* 1974; Mossop and Francki, 1977; Hanada and Tochihara, 1980; Rao and Francki, 1982;

Edwards *et al.*, 1983). In this paper, we report the construction of pseudorecombinants by exchanging RNAs between two local CMV strains and the resultant symptom expression.

Materials and Methods

Virus and Purification

CMV-NT9, originally isolated from tomato (*Lycopersicum esculentum*), and CMV-M48, originally from mungbean (*Vigna radiata*), were kindly supplied by Dr. Sylvia K. Green (The Asian Vegetable Research and Development Center, Tainan, Taiwan). Prior to use, three single-lesion passages were made for each virus using *Chenopodium quinoa* as a local lesion host. Both strains were propagated in tobacco (*Nicotiana tabacum* cv. Van-Hicks) in which NT9 causes severe mosaic and M48 causes symptomless infection. They were purified essentially by the procedures of Lot *et al.* (1972). The final purified virus preparations were in borate buffer (10 mM sodium borate, pH 7.5, containing 1 mM EDTA), and were either used immediately or stored at −70°C until used.

Isolation and Fractionation of RNA Species

RNA was extracted from purified virus preparations by the SDS-phenol method (Peden and Symons, 1973; Hsu and Brakke, 1985). The extracted RNA samples were denatured with 6% formaldehyde or by heating at 65°C for 10 min. They were then subjected to electrophoresis in 1.5% agarose tube gels at 3 mA/tube for 2.5 h (Shirako and Brakke, 1984). For preparing RNA inocula, 5pg RNA samples were first partially denatured by 7 M urea at 65°C for 10 min, and then electrophoresed in a 1.5% agarose/7 M urea tube gel at 6 mA/tube for 2.5h. The buffer system used in either case consisted of 20 mM Na_2HPO_4, 10 mM NaH_2PO_4 and 1 mM EDTA. RNA bands were visualized by staining with Stains-All (Dahlberg *et al.*, 1969).

In vitro Construction and Isolation of Pseudorecombinants

The RNA inocula were prepared by excising and mixing the genomic RNA bands from unstained gels located by referring to a stained gel from a simutaneous electrophoretic run. To effect the elution of RNAs from gel, bands were homogenized in 3ml of an inoculation buffer consisting of 0.1 M glycine, 0.05 M K_2HPO_4, pH 9.3, 100 μg/ml bentonite and 10 mg/ml celite (Jackson and Brakke, 1973). The slurry was then incubated on ice for an hour, and centrifuged to remove the gel debris. The supernatant in ten-fold serial dilutions was used to inoculate local lesion host *Chenopodium quinoa*. Each pseudorecombinant was cloned by three more serial single-lesion passages before being propagated in tobacco.

Characterization of Pseudorecombinants

All pseudorecombinants were purified by the method used for purifying the parental isolates. The RNA composition of each pseudorecombinant was analyzed by electrophoresis of formaldehyde- or heat-denatured preparations of RNAs in 1.5% agarose gel (Shirako and Brakke, 1984). Peptide mapping of viral coat protins was performed as previously described (Cleveland *et al.*, 1977). *Staphylococcus aureus* V–8 protease was used for limited proteolysis. Protein digests were analyzed by electrophoresis in an SDS 8–20% polyacrylamide gel (PAG). The gel was stained with silver nitrate (Morrissey, 1981).

Mechanical Inoculation and Symptomatology

All plant seedlings were grown in a glasshouse at 25–30°C. Test seedlings were inoculated mechanically with extracts from infected tobacco leaf tissue. Symptoms were recorded one month postinoculation.

Results

Electrophoretic Patterns of M48 and NT9 RNAs

Examination of formaldehyde-denatured RNAs following electrophoresis in 1.5% agarose gels revealed that RNAs 1 to 4 of M48 and NT9 had indistinguishable mobilities, with the former containing an additional RNA 5 (Fig. 1A). Electrophoresis of heat-denatured RNAs in same agarose gel as above could distinguish M48 from NT9. Under this condition RNAs 1 and 2 of M48 did not resolve into separate bands but RNAs 1 and 2 of NT9 did (Fig. 1B). In the partial denaturing agarose-urea gel, RNAs 1 to 4 of both strains migrated as separate bands with mobilities, however, indistinguishable between strains (Fig. 1C).

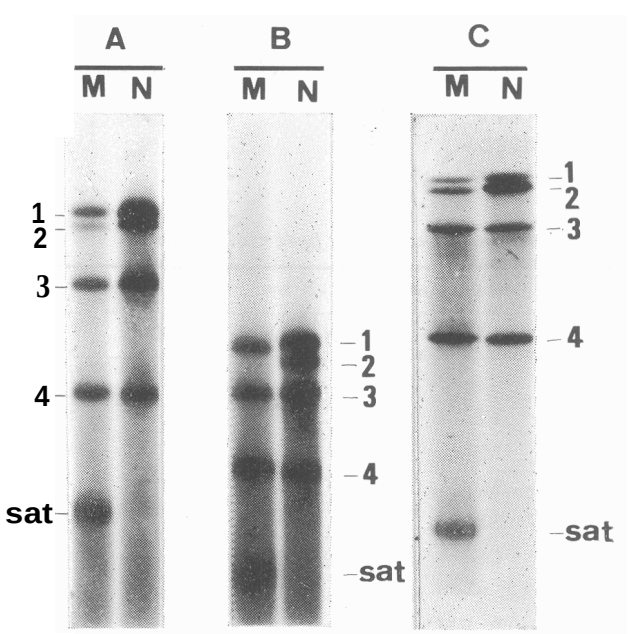

Fig. 1. RNA patterns of CMV-M48 (M) and NT9 (N). RNAs were denatured by 6% formaldehyde (A) or by heating at 65°C for 5min (B) before electrophoresis at 3 mA/gel for 2.5 h in 1.5% agarose gels. In (C) RNAs were denatured by 7 M urea and heating at 65°C for 10 min followed by electrophoresis at 6 mA/gel for 2.5 h in 1.5% agarose-7 M urea gels. Numbers and sat. marked at both sides indicated the RNA species and the satellite RNA, respectively.

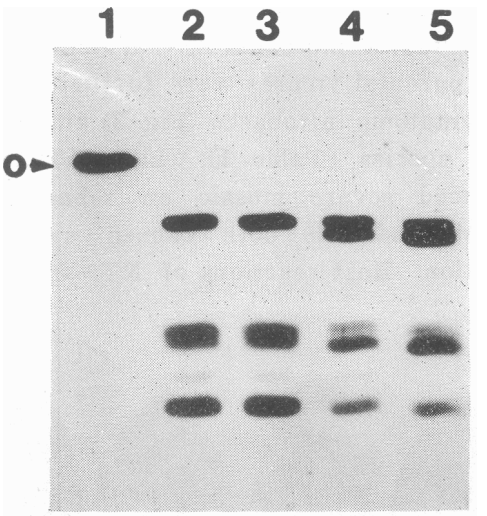

Fig. 2. Peptide maps of coat proteins of CMV-M48, NT9 and their pseudorecombinants $M_1M_2N_3$ and $N_1N_2M_3$. Purified virions were digested with V-8 protease and analyzed on an SDS 8-20% polyacrylamide gel. Undigested M48, and V-8 digested M48, $N_1N_2M_3$, NT9 and $M_1M_2N_3$ are shown in lanes 1, 2, 3, 4 and 5, respectively.

Characteristics of Pseudorecombinants

Pseudorecombinants $N_1N_2M_3$ and $M_1M_2N_3$ were constructed by exchange of RNA 3 between M48 and NT9. To verify the parental source for RNA 3 in the pseudorecombinants, their coat proteins were partially digested with V–8 protease and the resultant peptides were analyzed by electrophoresis in a PAG. Digestion of coat protein preparations from parental strains and pseudorecombinants produced the peptide maps as shown in Fig. 2. Peptide map of $N_1N_2M_3$ (Fig. 2, Lane 3) was the same as that of M48 (Fig. 2, Lane 2), but differed from that of $M_1M_2N_3$ (Fig. 2, Lane 5), the latter being indistinguishable from NT9 (Fig. 2, Lane 4).

RNAs of both $N_1N_2M_3$ and $M_1M_2N_3$ prepared by formaldehyde denaturation were resolved electrophoretically in 1.5% agarose gel into 4 bands representing RNAs 1 to 4. In heat denatured samples, RNAs 1 and 2 from both $N_1N_2M_3$ and NT9 were resolved as separate bands, but RNAs 1 and 2 of $M_1M_2N_3$ and M48 were not separated

under identical condition (data not shown).

Pseudorecombinants $N_1N_2M_3$ and $M_1M_2N_3$ and their parental viruses were further compared for symptoms in tobacco (Fig. 3) and other 12 plant species (Table 1). Like NT9, $M_1M_2N_3$ produced severe mosaic on tobacco, while $N_1N_2M_3$ and M48 both caused symptomless infection. Host reactions of NT9 and $M_1M_2N_3$ in other 12 test species were also indistinguishable. Likewise, $N_1N_2M_3$ and M48 induced the same reactions on the 12 plant species tested. From data given in Table 1, it appears that the exchange of RNA 3 between NT9 and M48 has a decisive effect on symptom expression.

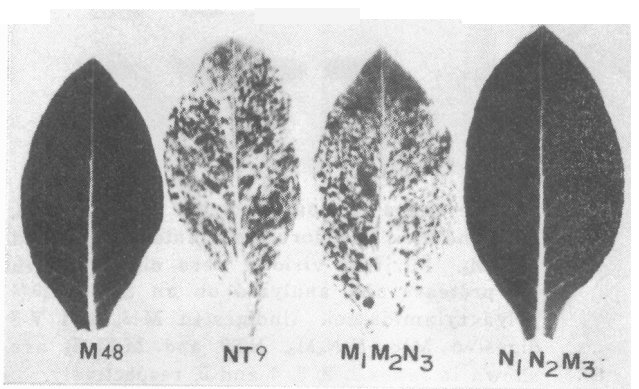

Fig. 3. Symptoms induced by CMV pseudorecombinants $M_1M_2N_3$ and $N_1N_2M_3$ and their parental virus strains, M48 and NT9 in *Nicotiana tabacum* cv. Van-Hicks at 20 days postinoculation.

We also constructed pseudorecombinants $N_1M_2M_3$ and $M_1N_2N_3$ by exchanging RNA 1, and pseudorecombinants $M_1N_2M_3$ and $N_1M_2N_3$ by exchanging RNA 2 between M48 and NT9. Symptoms induced by these pseudorecombinants on tobacco were indistinguishable from those of the parental RNA 3-donor strains. Thus, the pseudorecombinants $N_1M_2M_3$ and $M_1N_2M_3$, which had their RNA 3 derived from M48, caused symptomless infection on tobacco like M48, while $M_1N_2N_3$ and $N_1M_2N_3$ which had their RNA 3 derived from NT9 induced severe mosaic as did NT9. These results indicate that neither RNA 1 nor RNA 2 had significant effect on symptom formation of M48 and NT9 in tobacco.

Table 1. *Symptoms induced by CMV pseudorecombinants and their parental strains*

Plants tested	symptoms* caused by			
	NT9	$M_1M_2N_3$	$N_1N_2M_3$	M48
Phaseolus vulgaris	—	—	—	—
Lycopersicum esculentum cv. Rutgers	FL, N	FL, N	—	—
Capsicum frutescens	M	M	—	—
Capsicum annum	M	M	—	—
Nicotiana tabacurn cv. Van-Hicks	SM	SM	—	—
Datura stramonium	CS	CS	—	—
Zea mays	—	—	—	—
Cucumis sativus	*R*, SM	R, SM	—	—
Chenopodium quinoa	LL	LL	SLL	SLL
Vigna sinensis cv. Black eye	PS	PS	PS, MM	PS, MM
Vigna sinensis cv. KH.	PS	PS	PS, MM	PS, MM
Cucurbita moschata	R, M	R, M	—	—
Luffa cylindrica	*R*, SM	*R*, SM	—	—

+Abbreviations: Symptomless (—); Fern Leaf (FL); Necrosis (N); Mosaic (M); Severe Mosaic (SM); Chlorotic Spot (CS); Rugose (R); Local Lesion (LL); Systemic Local Lesion (SLL); Purple Spot (PS); Mild Mosaic (MM).

Restoration of Parental Strains from Pseudorecombinants

The true nature of the pseudorecombinants was further checked by reverse constructions, namely, by adding RNA 3 from $M_1M_2N_3$ to electrophoretically separated RNAs 1+2 from NT9 or by adding RNA 3 from $N_1N_2M_3$ to electrophoretically separated RNAs 1+2 from M48, to restore the parental strains. The restored viruses were compared with the original parental strains by RNA patterns, peptide maps and symptoms on differential hosts. In all examinations, the restored viruses were indistinguishable from the original parental virus strains, except that restored M48 lacked detectable RNA 5.

Discussion

After examining a number of CMV isolates, NT9 (a severe isolate) and M48 (a mild isolate) were selected as suitable materials for the construction of pseudorecombinants. These two strains differ significantly in symptoms they produce on tobacco and other test plants. Analysis of the RNA composition revealed that M48, but not NT9, contains a satellite RNA, in addition to the virus genomic RNAs 1-3 and subgenomic RNA 4. The satellite RNAs of CMV have been found either to induce new disease symptoms (Kaper and Waterworth, 1977; Takanami, 1981; Gonsalves *et al.*, 1982) or to reduce the severity of CMV symptoms (Kapper and Waterworth, 1977; Waterworth *et al.*, 1979) in various hosts. In our experimental systems, the M48-associated RNA 5 has been shown to exert little or no effect on symptoms of M48 or NT9 (Hu, 1987). Thus, the determinant(s) for symptom induction appeared to reside wholly in genomic segment(s) of the virus. Therefore, we further constructed strain hybrids by interchanging RNA 3 between M48 and NT9 to study the phenotypic expression of the virus after such genomic interchanges. Two pseudorecombinants, $M_1M_2N_3$ and $N_1N_2M_3$, were the major *in vitro* construction products.

Peptide mapping has been proved to be a reliable and sensitive technique for analyzing the coat proteins of CMV isolates (Edwards and Gonsalves, 1983). Using V-8 protease for protein digestion, we obtained peptide patterns that could distinguish the coat proteins of M48 and NT9 (Fig. 2). These patterns became a useful marker to trace the origins of RNA 3 in the two pseudorecombinants, $M_1M_2N_3$ and $N_1N_2M_3$. Furthermore, M48 and NT9 had their own characteristic mobilities of RNAs 1 and 2 on agarose gel when samples for electrophoresis were prepared by heat denaturation (Fig. 1B). This provides a criterium for assigning RNAs 1 and 2 of the two pseudorecombinants to their origins. The true nature of the pseudorecombinants was further checked by reverse constructions to restore parental strains. By all these criteria we confirm that $N_1N_2M_3$ and $M_1M_2N_3$ had indeed the respective RNA derivation as expected, i.e., $N_1N_2M_3$ had its RNAs 1 and 2 derived from NT9 and its RNA 3 from M48, while $M_1M_2N_3$ had its RNAs 1 and 2 derived from M48 and its RNA 3 from NT9.

Genetic analysis has been made for the various RNAs of several CMV strains (Hanada and Tochihara, 1980; Rao and Francki, 1982; Edwards *et al.*, 1983; Lakshman and Gonsalves, 1985). These studies led to the notion that either RNA 1 or RNA 2, or both determine the host reaction. RNA 3 which contains the coat protein gene determines the serological specificity (Habili and Francki, 1974; Mossop and Francki, 1977; Hanada and Tochihara, 1980) as well as aphid transmissibility (Mossop and Francki, 1977). RNA 3 alone (Marchoux *et al.*, 1974; Rao and Francki, 1982) or in interaction with RNA 2 to affect host reactions has also been reported (Rao and Francki, 1982). Our results show that the symptoms on all of 13

selected plant species inoculated with pseudorecombinants $N_1N_2M_3$, $M_1M_2N_3$ or their parental strains are determined by the origin of RNA 3. Interchanging of RNA 1 or RNA 2 between M48 and NT9 had no such effect. The results precluded RNA 1 or RNA 2 or both of our CMV strains from a role of controlling the symptoms.

Working with Q strain of CMV, Schwinghamer and Symons (1977) demonstrated that in addition to coat protein, RNA 3 is also capable of specifying another polypeptide under conditions for *in vitro* translation. The complete nucleotide sequence of CMV-Q RNA 3 has been determined, with indication for RNA 3's being a bicistronic messenger (Gould and Symons, 1982). We so far have no idea as to which one of the two cistrons or both are regulating the symptoms.

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胡瓜嵌紋病毒RNA3影響病徵的表現

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利用胡瓜嵌紋病毒 **(cucumber mosaic virus, CMV)** 兩分離株 NT9 和 **M48** 從事擬重組體之製備，以供病毒之病徵表現，作遺傳分析。NT9 在菸草植物 **(Nicotiana tabacum cv. Van-Hicks)** 上引起嚴重嵌紋病徵，而 **M48** 則在相同植物上引起無病徵感染。擬重組體 $N_1N_2M_3$ 是將從電泳膠體中分離而來之 **NT9 RNA 1** 和 **RNA 2** 與 **M48** 之 **RNA3** 混合而成，而 $M_1M_2N_3$ 則由 膠體中分離之 **M48 RNA 1** 和 **RNA 2** 與 **NT 9** 之 **RNA 3** 之混合而成，每一個擬重組體都接種到白藜 **(Chenopodium quinoa)**，經過連續單一病斑的純化，而增殖於菸草上。各擬重組體，均經 **RNA** 之膠體電泳和鞘蛋白之胜腱圖譜分析，以及回復親本病毒株後之基因體特性分析，加以確認。接種$N_1N_2M_3$，$M_1M_2N_3$ 以及親本病毒之寄主植物所顯現病徵，得知 **CMV RNA 3** 影響病徵之表現。