

Severe Chlorotic Spot Symptoms in Cucumber Mosaic Virus Strain Y-Infected Tobaccos Are Induced by a Combination of the Virus Coat Protein Gene and Two Host Recessive Genes

Hideki Takahashi and Yoshio Ehara

Faculty of Agriculture, Tohoku University, 1-1, Tsutsumidori Amamiyamachi, Aoba-ku, Sendai 981, Japan
Received 4 August 1992. Accepted 30 November 1992.

To investigate the genetic determinants of severe chlorotic spot symptoms in cucumber mosaic virus (CMV[Y])-inoculated tobacco (*Nicotiana tabacum* 'Ky57') leaves, symptom expression was compared among tobaccos (*N. tabacum* 'Ky57', *N. tabacum* 'Xanthi nc', and their hybrids) inoculated with CMV(Y), CMV(O), or pseudorecombinants of both strains of CMV. Xanthi nc showed very mild chlorotic spot symptoms in CMV(Y)-inoculated leaves. Both CMV(O)-inoculated Ky57 and Xanthi nc leaves showed no symptoms. In pseudorecombinant-inoculated Ky57 leaves, severe symptoms were determined by chimeric CMV(O) and CMV(Y) RNA3 transcripts, which contained most of the coat protein gene of CMV(Y). Genetic analysis of severe symptom formation in CMV(Y) or pseudorecombinant-inoculated tobaccos indicated that two recessive nuclear-coded host genes determined severe symptom formation in CMV(Y)-inoculated Ky57. The two recessive host genetic loci completely cosegregated with the two recessive burley loci (*yb1* and *yb2* genes) of Ky57, whose character is an apparent reduction in chlorophyll of the stems. When several cultivars of *N. tabacum* were inoculated with CMV(Y), all burley tobaccos clearly showed severe symptoms, but most other cultivars showed very mild symptoms. These results suggest that severe chlorotic spot symptom formation in CMV(Y)-inoculated Ky57 is induced by a combination of the virus coat protein gene and two recessive host genes that are closely linked to or identical to *yb* genes.

In virus-infected hosts, systemic symptoms are thought to result from complex interactions between virus and plant genes rather than a simple gene-for-gene relationship and to involve a large number of genes for symptom expression (Goodman *et al.* 1986; Matthews 1990). Recently, for some RNA and DNA viruses, the genomic and satellite RNA sequences necessary for symptom formation on virus-infected plants have been reported (Daubert 1988; Culver *et al.* 1991). However, little is known about host genes that determine systemic symptom formation on virus-infected plants.

Address correspondence to H. Takahashi.

MPMI Vol. 6, No. 2, 1993, pp. 182-189
© 1993 The American Phytopathological Society

Cucumber mosaic virus (CMV) pathogenesis genes have been analyzed extensively. It is well known that satellite RNAs change the systemic symptoms on tobacco and tomato when coinoculated with CMV strains that contain no satellite RNA. (Palukaitis 1988; Masuta and Takanami 1989; Devic *et al.* 1989; Kurath and Palukaitis 1989). Shintaku and Palukaitis (1990) recently reported that chlorotic or mosaic symptoms in noninoculated upper leaves of tobacco are caused by the CMV coat protein gene, judged by data from reciprocal recombinants between cDNA clones of RNA3 from a green mosaic strain, CMV(Fny) and a chlorotic strain, CMV(M). Furthermore, site-directed mutagenesis in the coat protein genes of CMV(Fny) and CMV(M) confirms that the local secondary structure surrounding amino acid 129 rather than a particular amino acid per se is involved in chlorosis induction (Shintaku *et al.* 1992).

Some reports have showed changes in the host protein population in CMV-infected plants in which systemic symptoms appeared (Camacho-Henriquez and Sanger 1982; Roberts and Wood 1981; Ziemi ecki and Wood 1975, 1976). One of these proteins, which is depressed by CMV infection, has been identified as a 23-kDa protein of the oxygen-evolving complex in photosystem II (Takahashi *et al.* 1991; Takahashi and Ehara 1992). Although some of these proteins may be associated with the primary molecular process of symptom expression, they cannot be identified as the sole cause of symptom expression. The host genes that determine symptom expression in CMV-infected plants have not been studied.

We have studied symptom expression in tobacco (*Nicotiana tabacum*) inoculated with two strains of cucumber mosaic virus, the yellow strain, CMV(Y), and the ordinary strain, CMV(O). CMV(Y) normally produces severe chlorotic spot symptoms in CMV(Y)-inoculated leaves of *N. tabacum* 'Ky57' and very mild chlorotic spot symptoms in *N. tabacum* 'Xanthi nc'. However, in noninoculated upper young leaves of both CMV(Y)-infected tobacco cultivars, severe systemic yellow/white mosaic symptoms are observed. CMV(O) induces no clear symptoms in inoculated leaves of either tobacco cultivar, although both CMV strains multiply to a similar extent (Takahashi and Ehara 1988). CMV(Y) contains a satellite RNA, but CMV(O) does not (Takanami *et al.* 1977). The satellite RNA does not cause the severe chlorotic spot symptoms in CMV(Y)-

Table 1. Infectivity of cucumber mosaic virus (CMV) RNAs and *in vitro* transcripts in *Chenopodium amaranticolor*

Inoculated RNA ^a				Pseudorecombinant	Number of local lesions per leaf ^b
CMV(O)	CMV(Y)	Plasmid ^c			
RNA1+2	O1O2-	0	
...	RNA1+2	...	Y1Y2-	0	
RNA3	-O3	0	
...	RNA3	...	-Y3	0	
RNA1+2	RNA3	...	O1O2Y3	19	
RNA3	RNA1+2	...	Y1Y2O3	13	
RNA1+2	...	Transcript pUCMVO3	O1O2O3tr	20	
RNA1+2	...	Transcript pUCMVYNX3	O1O2YNX3tr	16	
RNA1+2	...	Transcript pUCMVYSX3	O1O2YSX3tr	13	

^a 0.1 µg RNA1+2, 0.1 µg RNA3, and 5 µg each transcript RNA were used for mixed inoculations as described.

^b Each CMV RNA mixture was inoculated onto 10 leaves of *C. amaranticolor*. Numbers indicate the average number of local lesions per leaf of *C. amaranticolor*.

^c The plasmid constructs for the transcripts are shown in Figure 1.

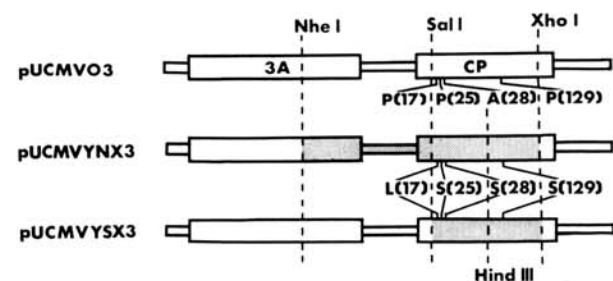


Fig. 1. Diagrams of the recombinant cDNA constructs of cucumber mosaic virus (CMV) RNA3. Rectangular boxes represent open reading frames of the 3A protein gene (3A) and the coat protein gene (CP). The region derived from cDNA of CMV(Y) is shadowed, that from CMV(O) unshadowed. Differences in the amino acid sequence and their positions are indicated. Restriction endonuclease cleavage sites are shown by dashed lines.

inoculated tobacco (*Nicotiana tabacum* 'Ky57') leaves (Nakashima and Ehara 1989). Ky57 belongs to White Burley tobacco, which originated in 1865, presumably as a mutation from the green-colored cultivar "Little Burley" (Mathewson 1912). The stems of Ky57 are clear white in contrast to the greenish-white stems of Xanthi nc. This White Burley character is determined by two recessive genetic loci, *yb1* and *yb2* (Henika 1932).

In this report, we identify virus and host genes that determine severe chlorotic spot symptom formation in CMV(Y)-inoculated tobacco (*N. tabacum* 'Ky57') leaves, using pseudorecombinant CMV containing either exchanged RNA3s between CMV(Y) and CMV(O), or an infectious RNA transcript from a chimeric cDNA of CMV(O) RNA3 with most of the CMV(Y) coat protein gene, and by genetic analysis of two cultivars of tobacco (*N. tabacum* 'Ky57' and 'Xanthi nc') following the severity of symptoms in CMV(Y)- or pseudorecombinant CMV-inoculated hybrid tobacco leaves.

RESULTS

Construction and verification of pseudorecombinants.

Preliminary experiments suggested that RNA3 of CMV(Y) was associated with the appearance of severe chlorotic spot symptoms in CMV(Y)-inoculated tobacco (*N. tabacum* 'Ky57') leaves (data not shown). Therefore, intact RNA1+2 and RNA3 of both CMV(O) and CMV(Y)

Table 2. Symptoms and amounts of viral coat protein in tobacco (*Nicotiana tabacum* 'Ky57') after inoculation with pseudorecombinant cucumber mosaic viruses

Virus ^a	Symptoms ^b	Amounts of coat protein ^c
CMV(O)	SL	NT ^e
CMV(Y) ^d	SCS	NT
Y1Y2O3	SL	0.572 ± 0.160
O1O2Y3	SCS	0.664 ± 0.260
O1O2O3tr	SL	NT
O1O2YNX3tr	SCS	0.697 ± 0.140
O1O2YSX3tr	SCS	0.732 ± 0.170

^a Notation of viruses is as in Table 1. Each virus was inoculated onto six tobaccos. Six days after inoculation, symptoms and amounts of viral coat protein in inoculated leaves were analyzed.

^b Symptoms are indicated as follows: SCS, severe chlorotic spots; SL, symptomless (Fig. 2A, B).

^c The average of ELISA absorbance values ($A_{405} \pm SD$).

^d CMV(Y) does not contain satellite RNA.

^e Not tested.

were completely separated, as judged by infectivity assay in *Chenopodium amaranticolor* leaves (Table 1).

To verify the construction of *in vitro* transcription vectors, pUCMVO3, pUCMVYNX3, and pUCMVYSX3 (Fig. 1), CMV cDNAs in these plasmids were sequenced. Sequencing showed that the CMV(Y) region in pUCMVYNX3 and pUCMVYSX3 completely matched the partial sequence of CMV(Y). However, in pUCMVO3, the phenylalanine codon (amino acid position 158 in the coat protein of CMV(O)) was changed to serine, which is the same amino acid at position 158 in the coat protein of CMV(Y) (data not shown). Therefore, there were only four amino acid differences (position 17, 25, 28, and 129) in the coat protein between pUCMVO3 and pUCMVYNX3 or pUCMVYSX3, and no differences in the 3A protein coding (Fig. 1). Infectivity tests of *in vitro* transcripts from vectors in *C. amaranticolor* leaves indicated that these transcripts were infectious (Table 1).

Severe chlorotic spot symptom formation in *N. tabacum* 'Ky57' leaves inoculated with pseudorecombinants.

In CMV(Y)-inoculated *N. tabacum* 'Ky57' leaves, early chlorotic spot symptoms usually appeared between 3 and 4 days after inoculation and matured to severe chlorotic spots in 5–6 days. CMV(O)-inoculated Ky57 leaves showed no symptoms (Table 2 and Fig. 2A). As systemic mosaic symptoms begin to appear in noninoculated young leaves

of CMV-infected tobacco at 9 days after inoculation (data not shown), tobaccos in Figure 2 do not show any mosaic symptoms.

In 5–6 days, all Ky57 leaves that were inoculated with O1O2Y3 (= CMV[O] RNA1+2 and CMV[Y] RNA3), showed severe symptoms, similar to CMV(Y)-inoculated leaves. However, Y1Y2O3 (= CMV[Y] RNA1+2 and CMV[O] RNA3)-inoculated Ky57 leaves did not show any symptoms (Table 2 and Fig. 2B). This indicates that RNA3 of CMV(Y) contains the sequence determining severe chlorotic spot formation in CMV(Y)-inoculated Ky57 leaves.

Since O1O2YNX3tr (= CMV[O] RNA1+2 and transcript from pUCMVYNX3) and O1O2YSX3tr (= CMV[O] RNA1+2 and transcript from pUCMVYSX3) induced severe symptoms in all leaves of six inoculated Ky57, but

O1O2O3tr (= CMV[O] RNA1+2 and transcript from pUCMVO3) did not (Table 2 and Fig. 2B), the determinant for severe chlorotic spot symptom formation is located within the sequence between the *Sall* and *Xho*I sites of pUCMVYNX3 and pUCMVYSX3 (Fig. 1). This portion contains about 88% of the coat protein gene of CMV(Y).

Restriction mapping and sequencing of cDNAs synthesized from each purified CMV RNA confirmed the multiplication of the pseudorecombinants in virus-inoculated Ky57 leaves. With O1O2Y3, out of 20 cDNA clones, five derived from CMV(O) RNA1, five from CMV(O) RNA2, and 10 from CMV(Y) RNA3 were identified by terminal sequencing. With Y1Y2O3, out of 20 cDNA clones, three from CMV(Y) RNA1, 11 from CMV(Y) RNA2, and six from CMV(O) RNA3 were identified in the same way (data not shown). The restriction

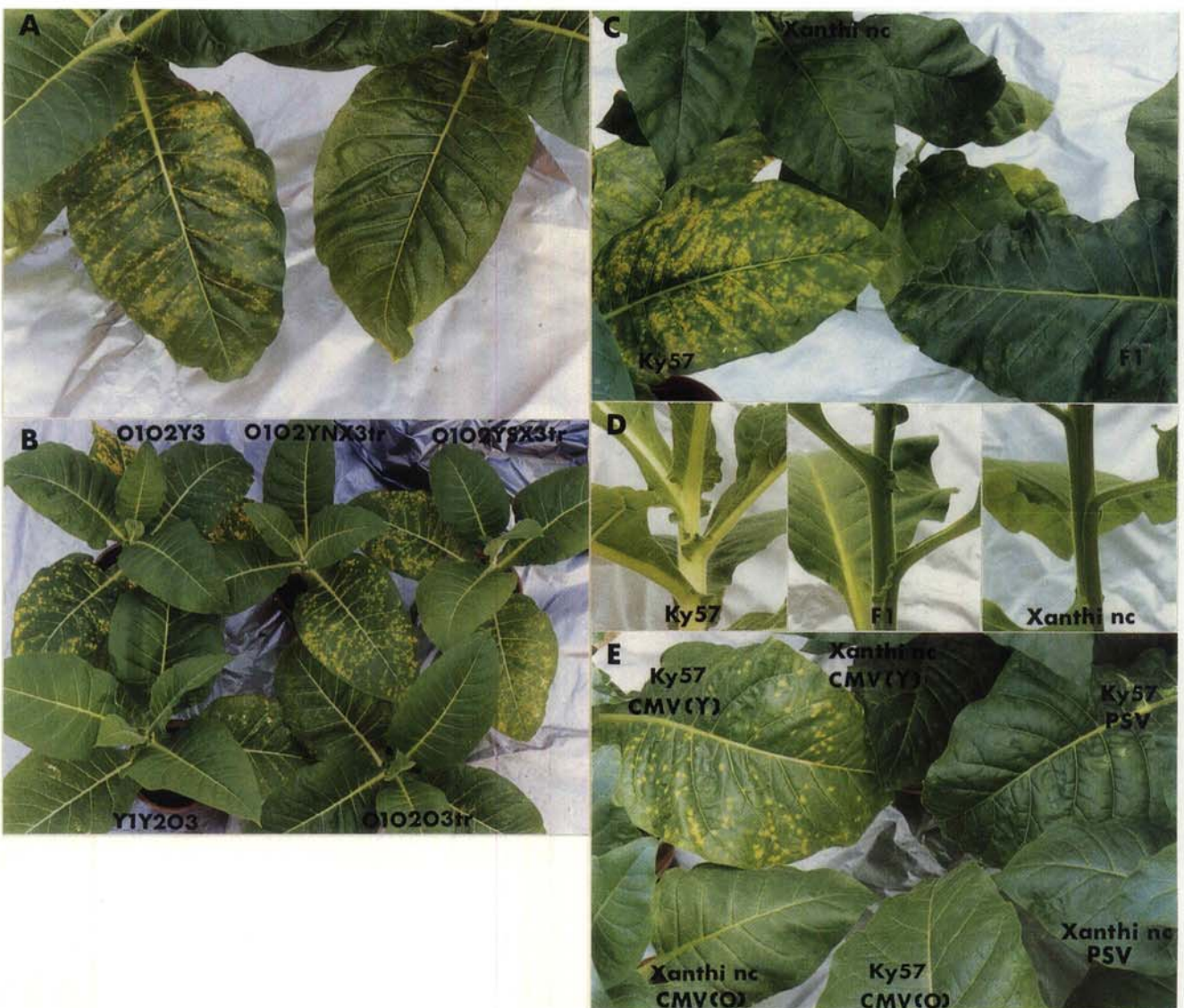


Fig. 2. Symptoms in cucumber mosaic virus (CMV)-infected tobaccos (*Nicotiana tabacum* 'Ky57', 'Xanthi nc' and their F₁ hybrid). **A**, Ky57 leaves inoculated with CMV(Y) (left) and CMV(O) (right) at 6 days. **B**, Ky57 leaves inoculated with pseudorecombinant CMVs at 6 days. Designation of pseudorecombinant CMVs is as described in Table 1. **C**, Ky57, Xanthi nc and F₁ hybrid (Ky57[♀] × Xanthi nc[♂]) leaves inoculated with CMV(Y) at 6 days. **D**, The stems of Ky57, Xanthi nc, and F₁ hybrid (Ky57[♀] × Xanthi nc[♂]). **E**, Ky57 and Xanthi nc leaves inoculated with CMV(Y), CMV(O), and PSV(J), respectively, at 6 days.

pattern of cDNAs to RNA3 of O1O2O3tr, O1O2YSX3tr, and O1O2YNX3tr showed the patterns expected from Figure 1 (data not shown). These results indicate that the expected pseudorecombinant CMV multiplied in the inoculated Ky57 leaves.

The amounts of coat protein in the pseudorecombinant-inoculated leaves were measured by ELISA. No significant difference was observed among Ky57 leaves inoculated with the different pseudorecombinants (Table 2). This indicates that the difference of the severity of symptoms in CMV-inoculated *N. tabacum* 'Ky57' leaves is not caused by a difference in the extent of virus multiplication.

Genetic analysis of severe symptom formation on CMV(Y)-inoculated tobaccos.

All reciprocal first-generation (F₁) hybrids (Ky57 × Xanthi nc) showed very mild symptoms in CMV(Y)-inoculated leaves, and all of their stems were greenish-white, similar to CMV(Y)-inoculated Xanthi nc (Table 3 and Fig. 2C,D).

When 47 of selfed generation (F₂) (Ky57 ♀ × Xanthi nc ♂) and 42 of F₂ (Xanthi nc ♀ × Ky57 ♂) were inoculated with CMV(Y), symptoms of both CMV(Y)-inoculated F₂ leaves indicated that the F₂ families segregated approximately 15 very mild symptoms/1 severe symptom (Table 3). The stems of all F₂, which showed severe symptoms in CMV(Y)-inoculated leaves, were clear white, whereas those of all F₂, which showed very mild symptoms in the inoculated leaves, were greenish-white. No F₂ were observed with severe symptoms in CMV(Y)-inoculated leaves and greenish-white stems, or with very mild symptoms in CMV(Y)-inoculated leaves and clear white stems, were observed (Table 3).

In backcrosses of F₁ to Ky57, the segregation of symptoms in CMV(Y)-inoculated leaves is, accordingly, three very mild symptoms/one severe symptom. The progenies

from backcrosses to Ky57, which showed severe symptoms in CMV(Y)-inoculated leaves, had the clear white stems, whereas those that showed mild symptoms had the greenish-white stems (Table 3). On the other hand, in backcrosses of F₁ to Xanthi nc, all CMV(Y)-inoculated progenies showed very mild symptoms, and their stems were greenish-white (Table 3).

Symptoms on 11 cultivars of CMV(Y)-inoculated *N. tabacum* were observed (Table 4). Six days after inoculation, all Burley tobaccos (*N. tabacum* 'Ky57', 'White Burley', 'Burley 21', and 'Shiro-Daruma') clearly showed severe symptoms in CMV(Y)-inoculated leaves, although other cultivars of tobaccos except for *N. tabacum* 'Oho-Daruma', which is a Japanese cultivar, showed very mild symptoms in CMV(Y)-inoculated leaves.

These results indicate that two recessive nuclear-coded genes of *N. tabacum* 'Ky57' determine severe chlorotic spot symptom formation in CMV(Y)-inoculated leaves. The response of several cultivars of *N. tabacum* to the CMV(Y) and the complete cosegregation of the severe chlorotic spot symptom formation in CMV(Y)-inoculated Ky57 and the clear white stems of Ky57 suggests that the two recessive genetic loci, which cause severe symptom formation in CMV(Y)-inoculated Ky57 leaves, are closely linked or identical to two recessive burley loci (*yb1* and *yb2* genes).

The amounts of coat protein in CMV(Y)-inoculated progenies from backcrosses of F₁ to Ky57 and in 11 cultivars of CMV(Y)-inoculated *N. tabacum* were measured by ELISA. When the average amounts of coat protein in seven CMV(Y)-inoculated leaves were compared between the progenies that showed severe symptoms and very mild symptoms in CMV(Y)-inoculated leaves, the absorbances at 405 nm were 0.745 ± 0.162 (severe symptoms) and 0.551 ± 0.125 (very mild symptoms), respectively. Among the 11 cultivars of *N. tabacum*, the amounts of coat protein did not correlate with severe symptom forma-

Table 3. Segregation of severe chlorotic spot symptoms and white stems in progenies of reciprocal hybrids (*Nicotiana tabacum* 'Ky57' × 'Xanthi nc')

Tobacco hybrid	Virus ^a	Phenotype ^b				Severe symptoms and white stems : mild symptoms and green stems		χ ²	χ ² 0.01
		Mild symptoms and green stems	Severe symptoms and white stems	Mild symptoms and white stems	Severe symptoms and green stems	Observed ratio	Expected ratio		
F ₁ [Ky57♀ × Xanthi nc♂]	CMV(Y)	11	0	0	0	11:0
F ₁ [Xanthi nc♀ × Ky57♂]	CMV(Y)	13	0	0	0	13:0
F ₂ [selfed F ₁ (Ky57♀ × Xanthi nc♂)]	CMV(Y)	44	3	0	0	14.7:1	15:1	0.001	6.635
F ₂ [selfed F ₁ (Xanthi nc♀ × Ky57♂)]	CMV(Y)	39	3	0	0	13.0:1	15:1	0.057	6.635
Backcross[Ky57♀ × (Ky57♂ × Xanthi nc♂)]	CMV(Y)	79	22	0	0	3.6:1	3:1	0.557	6.635
Backcross[Xanthi nc♀ × (Ky57♂ × Xanthi nc♂)]	CMV(Y)	10	0	0	0	10:0
Backcross[Ky57♀ × (Ky57♂ × Xanthi nc♂)]	O1O2YNX3	14	4	0	0	3.5:1	3:1	0.074	6.635
Backcross[Xanthi nc♀ × (Ky57♂ × Xanthi nc♂)]	O1O2YNX3	10	0	0	0	10:0

^a Notation of viruses is as in Table 1.

^b After 6 days of inoculation, symptoms were observed. Severe chlorotic spot symptoms are indicated as "Severe symptoms", mild chlorotic spot symptoms, "Mild symptoms".

tion in CMV(Y)-inoculated leaves (Table 4). These results indicate that severe chlorotic spot symptom formation in CMV(Y)-inoculated Ky57 leaves is not caused by a difference of virus multiplication among the cultivars of tobacco and that CMV accumulation and symptoms can be distinct.

When pseudorecombinant O1O2YNX3, which contains most of the coat protein gene of CMV(Y), was inoculated to progenies from backcrosses to Ky57 and from backcrosses to Xanthi nc, each segregation ratio of the stem color and symptom phenotypes was same as those of CMV(Y)-inoculated progenies from backcrosses to Ky57 and Xanthi nc, respectively (Table 3). On the other hand, both Ky57 and Xanthi nc, which were inoculated with CMV(O), showed no symptoms in their inoculated leaves (Fig. 2E). On both Ky57 and Xanthi nc, which were inoculated with another cucumovirus, PSV(J), there was no difference in the very mild chlorotic symptoms between their inoculated leaves (Fig. 2E). These results indicate that severe chlorotic spot symptom formation in CMV(Y)-inoculated *N. tabacum* 'Ky57' leaves is caused by the specific combination of the coat protein gene of CMV(Y) and the two recessive genes of Ky57.

DISCUSSION

We have identified two nuclear-coded recessive host genes that control the severity of chlorotic spot symptoms in CMV(Y)-inoculated tobacco (*N. tabacum* 'Ky57') leaves by genetic analysis of two cultivars of tobaccos: *N. tabacum* 'Ky57' (Burley tobacco), and 'Xanthi nc'. Symptom severity completely cosegregated with two recessive Burley loci (*yb1* and *yb2*). Furthermore, the response of several cultivars of *N. tabacum* to CMV(Y) indicated all 'burley'-type plants showed severe chlorotic spot symptoms in CMV(Y)-inoculated leaves. These data support the idea that two recessive

genes, which cause severe symptom formation in CMV(Y)-inoculated Ky57 leaves, are closely linked to *yb1* and *yb2* genes.

Although only relatively few plants have been tested for segregation of the stem color and symptom phenotypes, it cannot be ruled out that two recessive gene affecting symptoms in CMV(Y)-inoculated Ky57 leaves are identical to *yb* loci. Monosomic analysis demonstrated that the *yb1* gene is located on the B chromosome and *yb2* on the O chromosome (Clausen and Cameron 1944). The primary character of the *yb1* and *yb2* genes is an apparent reduction in chlorophyll of the stems (Henika 1932). Under good growing conditions, the color difference of the leaves between "Burley" tobacco and other green cultivars becomes less distinct. "Burley" tobacco loses much of its green color in the lower leaves with the approach of maturity. These Burley characters suggest that the *yb1* and *yb2* genes are likely to encode polypeptides that associate with chloroplasts. It has been reported that the mosaic or chlorotic symptoms in systemically virus-infected plants are attributable to chloroplast abnormalities (Goodman *et al.* 1986). These facts suggest that the *yb1* and *yb2* genes may be associated with the appearance of severe chlorotic spot symptoms in CMV(Y)-inoculated Ky57 leaves. Therefore, it is possible that the *yb* loci are identical to two recessive genes affecting symptoms in CMV(Y)-inoculated Ky57 leaves.

A comparative study of symptom formation in *N. tabacum* Ky57 and the progenies from backcrosses of F₁ to Ky57, which are inoculated with pseudorecombinants between CMV(Y) and CMV(O), indicated that a combination of the coat protein gene of CMV(Y) and two recessive genes determined severe symptom formation in CMV(Y)-inoculated Ky57 leaves. Because there is no difference in the severity of symptoms between Ky57 and Xanthi nc, which were inoculated with CMV(O) or another cucumovirus, PSV(J), the two recessive genes do not simply accelerate the senescence of all cucumovirus-inoculated tobaccos, but seem to determine specifically severe chlorotic spot symptom formation in CMV(Y)-inoculated Ky57 by interacting with the coat protein of CMV(Y).

Shintaku and Palukaitis (1992) did not address the question of chlorosis on CMV-inoculated tobacco leaves, but our pseudorecombinant experiment suggests the coat protein gene also determines symptom expression on the inoculated leaves. Although the mechanism(s) of coat protein induction of severe symptoms in CMV(Y)-inoculated Ky57 leaves is not understood, the secondary structure of the coat protein of CMV(Y) may play a important role in inducing symptoms, as with the coat protein of CMV(M) (Shintaku *et al.* 1992). Substitution of proline with serine at amino acid 129 was also observed between the coat protein genes of CMV(Y) and CMV(O) (Shintaku 1991).

Except for the two recessive genes that control severe chlorotic spot symptom formation in CMV(Y)-inoculated Ky57 leaves, there are, of course, many host genes that are necessary for symptom formation in virus-infected plants. But, with regard to CMV(Y) and CMV(O) and the two cultivars of tobacco (Ky57 and Xanthi nc) tested, just two recessive loci affect symptoms in virus-inoculated leaves. Therefore, to understand the mechanisms of symp-

Table 4. Symptoms and amounts of viral coat protein in cucumber mosaic virus strain Y [CMV(Y)]-inoculated *Nicotiana tabacum* cultivars

<i>N. tabacum</i> cv. ^a	Symptoms ^b	Amounts of coat protein ^c
Ky57	SCS	0.545 ± 0.033
White Burley	SCS	0.635 ± 0.096
Burley 21	SCS	0.769 ± 0.088
Shiro-Daruma	SCS	0.288 ± 0.032
<hr style="border-top: 1px dashed black;"/>		
Samsun NN	MCS	0.703 ± 0.134
Xanthi nc	MCS	0.408 ± 0.092
Havana 38	MCS	0.576 ± 0.122
Maryland Mammoth	MCS ^d	0.580 ± 0.095
Bright yellow 4	MCS ^d	0.576 ± 0.122
SR1	MCS ^d	0.813 ± 0.183
Oho-Daruma	SL	0.026 ± 0.043

^a Three tobaccos of each cultivar were inoculated with CMV(Y). Six days after inoculation, symptoms in inoculated leaves and the amounts of viral coat protein were analyzed. "Burley tobacco" are Ky57, White Burley, Burley 21 and Shiro-Daruma, which are divided to other cultivars of tobacco by dashed line.

^b Symptoms are indicated as follows: MCS, mild chlorotic spots; SCS, severe chlorotic spots; SL, symptomless.

^c The average ELISA absorbance values ($A_{405} \pm$ SD) are shown.

^d Mild chlorotic spot symptoms in Bright yellow 4, SR1, and Maryland Mammoth were a little more severe than in Samsun NN and Xanthi nc.

tom expression in CMV(Y)-infected tobaccos, it was necessary to clone the two recessive host genes and to analyze the functions of both gene products.

MATERIALS AND METHODS

Virus and plants.

The ordinary strain of cucumber mosaic virus (CMV[O]) and the yellow strain of cucumber mosaic virus (CMV[Y]) were kindly given by T. Hayakawa (Plantech Research Institute, Japan) and Y. Takanami (Kyushu University, Japan), respectively. Although a different isolate of CMV(Y) has been reported (Hayakawa *et al.* 1988), we used the original CMV(Y) (Nitta *et al.* 1988) for constructing pUCMVYNX3 and pUCMVYSX3. Peanut stunt virus strain J[PSV(J)] was kindly supplied by A. Karasawa (Tohoku University, Japan). Tobaccos and *C. amaranticolor* were grown in a growth cabinet at 24° C for a 14-hr light period (12,000 lx) and at 20° C for a 10-hr dark period. To compare systemic symptoms among CMV-inoculated tobacco cultivars, 11 cultivars of tobaccos were used: *N. tabacum* 'Ky57', 'White Burley', 'Burley 21', 'Shiro-Daruma', 'Oho-Daruma', 'Bright Yellow 4', 'Samsun NN', 'Xanthi nc', 'SR1', 'Havana 38', and 'Maryland Mammoth'.

RNA extraction and separation.

Fifty grams of CMV-infected tobacco leaves was homogenized with 100 ml of 0.1 M ammonium citrate buffer (pH 6.5) containing 10 mM EDTA and 0.1% (v/v) 2-mercaptoethanol, and 100 ml of chloroform. The homogenate was centrifuged at 7,000 rpm for 10 min (Hitachi RPR20-2 rotor), then the aqueous phase was recentrifuged at 40,000 rpm for 1.5 hr at 4° C (Hitachi RP50T rotor). The pellet was thoroughly resuspended in 10 ml of 0.1 M ammonium citrate buffer (pH 6.5) containing 10 mM EDTA, using a Teflon homogenizer kept on ice. After incubation at 4° C for 1 hr, the suspension was centrifuged at 10,000 rpm for 5 min at 4° C (Hitachi RPR20-2). The supernatant was added to 0.5 ml of 20% Triton X-100, mixed gently, and centrifuged at 40,000 rpm for 1.5 hr at 4° C (Hitachi RP50T rotor). The purified virus pellet was suspended in 4 ml of 0.5 M guanidium isothiocyanate containing 50 mM Tris-HCl (pH 7.5), 25 mM EDTA, 1% sodium laurylsarcosinate (SLS), 25 mM sodium citrate, and 5 mM 2-mercaptoethanol, layered on 0.5 ml of 2.5 M CsCl cushion containing 10 mM Tris-HCl (pH 8.0) and 1% SLS, and centrifuged at 45,000 rpm for 15 hr at 20° C (Hitachi RP80-AT rotor). The viral RNA pellet was washed with 70% (v/v) ethanol three times and dried thoroughly. The pellet was suspended in Tris-borate-EDTA buffer (TBE) (Peacock and Dingman 1968). To separate CMV RNAs to RNA1+2 and RNA3 by gel electrophoresis, 1 mg/ml CMV RNA was kept on ice for 1 hr, then denatured at 95° C for 5 min and cooled quickly on ice to avoid cross-contamination of CMV RNAs with each other. Ten micrograms of CMV RNA was added to 10 µl of TBE containing 20% sucrose and bromophenol blue, and layered on top of 10- × 1-cm diameter tube gels containing 1.5% agarose in TBE. The procedures of electrophoresis of RNA, staining of the gel, elution, and

purification of the CMV RNAs from the gel were performed by the method of Roossinck and Palukaitis (1990).

Construction of a chimeric cDNA clone of CMV RNA3.

Synthesis of cDNA was primed with a 15-mer oligonucleotide (5' TGGTCTCCTTTTGGGA3') complementary to the 3' end of RNA3 of CMV(Y) and CMV(O), and carried out according to the method of Gubler and Hoffman (1983). After treatment with T4 DNA polymerase, cDNA was cloned into *EcoRV*-digested pBluescript + SK (Stratagene, La Jolla, CA). cDNA clones to RNA3 were identified by determining the terminal sequences of several independent cDNA clones. The resulting plasmids, containing the cloned cDNA to RNA3 of CMV(O) or CMV(Y), were called pBS+SKCMO3 and pBS+SKCMY3, respectively. The T7 promoter sequence was introduced into pBS+SKCMO3 by the polymerase chain reaction (PCR) (Stoflet *et al.* 1988). Reaction mixtures (100 µl) contained 10 mM Tris-HCl (pH 8.9), 1.5 mM MgCl₂, 80 mM KCl, 0.5 mg/ml bovine serum albumin, 0.1% cholic acid sodium salt, 0.1% Triton X-100, 0.2 mM dNTPs each (dATP, dCTP, dTTP, and dGTP), 1 ng of pBS+SKCMO3 as a template, 25 pmoles of the first primer (15-mer) complementary to the 3' end of RNA3 from CMV(O) and 25 pmoles of the second primer (5'TGGCCACTGCAGTAATACGACTCACTATAGTAATCTTACCCTCGTGTGTGTGC3', with the viral sequence given in underline) containing a *PstI* restriction site, a T7 RNA polymerase promoter, and 25 nucleotides homologous to the known 5'-terminal sequence of RNA3 from CMV(O). Oligonucleotides were synthesized using an Applied Biosystems model 391A PCR-Mate EP DNA synthesizer (Foster City, CA). Four units of *Tth* DNA polymerase (TOYOBO, Japan) were added to the reaction mixture and cycled 30 times through the program: 94° C for 30 sec, 40° C for 2 min, and 72° C for 3 min. After extraction with phenol-chloroform, the PCR product was precipitated with ethanol. The purified DNA was suspended in 100 µl of 10 mM Tris-HCl (pH 7.8) containing 5 mM EDTA and 0.5% SDS, and added to 50 µg/ml proteinase K. After incubation at 37° C for 30 min, DNA was re-extracted with phenol-chloroform and reprecipitated with ethanol. After filling in both ends with T4 DNA polymerase, the DNA was digested with *PstI* and cloned into *PstI/SmaI*-digested pUC12. The resulting plasmid was called pUCMVO3.

The plasmid pUCMVYNX3 (containing 35% of the 3A protein gene, the intergenic region between the 3A protein gene and the coat protein gene, and 88% of the coat protein gene of CMV[Y]) was constructed by cloning a *NheI/XhoI* fragment of pBS+SKCMY3 to *NheI/XhoI*-digested pUCMVO3. pUCMVYSX3 (containing 88% of the CMV[Y] coat protein gene) was also constructed by cloning a *SalI/XhoI* fragment of pBS + SKCMY3 into *SalI/XhoI*-digested pUCMVO3. pUCMVO3, pUCMVYNX3, and pUCMVYSX3 are shown schematically in Figure 1. Each clone was identified by restriction mapping and sequencing (data not shown). cDNA clones to CMV RNA were sequenced using an Applied Biosystems model 373A DNA sequencer and/or the Sequenase kit (U.S. Biochemicals, Cleveland, OH) by

the method of Sanger *et al.* (1977). All incubations were done under conditions recommended by the suppliers.

***In vitro* transcription.**

To prepare the template cDNA for *in vitro* transcription, pUCMVO3, pUCMVYNX3, and pUCMVYSX3 were digested with *Sac*I, and then filled in with T4 DNA polymerase according to standard protocols (Sambrook *et al.* 1989). Reaction mixtures (100 μ l) contained 40 mM Tris-HCl (pH 8.0); 8 mM MgCl₂; 2 mM spermidine; 10 mM NaCl; 10 mM dithiothreitol; 0.5 mM each of ATP, CTP, and UTP; 0.05 mM GTP; 1 mM m7GpppG (Pharmacia); 35 units of RNasin (TAKARA, Japan); 70 units of T7 RNA polymerase; and 5 μ g of linearized template cDNA. After incubation at 37° C for 60 min, the transcribed RNA was purified by treatment with DNase I to remove the template, followed by phenol extraction and ethanol precipitation.

Construction of pseudorecombinants and inoculation.

First, 0.1 μ g of purified CMV RNA1+2 and CMV RNA3 were reassorted between CMV(Y) and CMV(O). Second, 0.1 μ g each of purified CMV RNA1+2 were mixed with 5 μ g of CMV RNA3 transcript, which was transcribed *in vitro* from either pUCMVO3, pUCMVYNX3, or pUCMVYSX3. These CMV RNA mixtures were inoculated onto *C. amaranticolor* leaves at the five- to seven-leaf stage. Four days after inoculation, individual local lesions were excised and reinoculated onto new plants. Local lesions from the third passage in *C. amaranticolor* were then inoculated onto tobaccos which were about 2 cm in height, for amplification and purification of virus. Fifty micrograms per milliliter of the resulting pseudorecombinants (listed in Table 1) was rub-inoculated to the approximately 30 cm of fully expanded leaves of six *N. tabacum* 'Ky57' and the six plants of each hybrid Ky57 \times Xanthi nc. At 6 days after inoculation, symptoms on virus-inoculated leaves were observed.

To confirm the multiplication of pseudorecombinants, virus was purified from inoculated leaves, and the RNA was extracted. For pseudorecombinants O1O2Y3 and Y1Y2O3 (Table 1), 20 independent cDNA clones were synthesized using total RNA of each pseudorecombinant by the method of Gubler and Hoffman (1983). Terminal sequences were determined and compared with the published sequences of RNA1, RNA2, and RNA3 of CMV(Y) and CMV(O) (Hayakawa *et al.* 1989a,b; Hase *et al.* 1992; Kataoka *et al.* 1990a,b; Nitta *et al.* 1988) (data not shown). For O1O2O3tr, O1O2YSX3tr, and O1O2YNX3tr, first-strand cDNA was synthesized using total viral RNA as the template and a 15-mer oligonucleotide complementary to 3' end of RNA3 as a primer. Full-length cDNA to RNA3 was synthesized using the first-strand cDNA product as a template and two oligonucleotides homologous to the 5' end of CMV(O) RNA3 and complementary to the 3' end of CMV(O) RNA3 as primers for the PCR. The condition of PCR was as described above. The PCR products were extracted with phenol-chloroform, precipitated with ethanol and resuspended. Pseudorecombinant cDNA were identified by digesting the full-length cDNA to RNA3 with

*Hind*III (data not shown).

Analysis of the hybrid *N. tabacum* 'Ky57' \times *N. tabacum* 'Xanthi nc.'

The first-generation reciprocal hybrid *N. tabacum* 'Ky57' \times *N. tabacum* 'Xanthi nc', and their backcrossed and selfed generations were inoculated with CMV(Y) or O1O2YNX3tr. Six days after inoculation, symptoms on virus-inoculated leaves were observed. The inheritance of two recessive *yb1* and *yb2* genes, which were contained in Ky57, were identified by the "white" stem character (Henika 1932).

Enzyme-linked immunosorbent assay.

To determine the extent of virus multiplication in virus-inoculated tobacco leaves, the amounts of coat protein in virus-infected leaves were measured by enzyme-linked immunosorbent assay (ELISA) (Koenig 1981).

ACKNOWLEDGMENTS

We thank T. Hayakawa, Plantech Research Institute, Japan; Y. Takanami, Kyushu University, Japan; and A. Karasawa, Tohoku University, Japan, for their gifts of CMV(O), CMV(Y), and PSV(J), respectively. We also thank T. Yamamoto, Y. Kawarabayashi, and members of the Gene Research Center at Tohoku University; T. Hashiba and M. Watanabe of the Faculty of Agriculture, Tohoku University for their valuable suggestions; and T. Suzuki for helpful technical discussions. We gratefully acknowledge Ilkka Havukkala of the National Institute of Agrobiological Resources, Japan, for revising the English and his valuable suggestions. We also express our thanks for financial support by grants-in-aid from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

- Camacho-Henriquez, A., and Sanger, H. L. 1982. Analysis of acid-extractable tomato leaf proteins after infection with a viroid, two viruses and a fungus and partial purification of the 'pathogenesis-related' protein p14. *Arch Virol.* 74:181-196.
- Clausen, R. E., and Cameron, D. R. 1944. Inheritance in *Nicotiana tabacum*. XVIII. Monosomic analysis. *Genetics* 29:447-477.
- Culver, J. N., Lindbeck, A. G. C., and Dawson, W. O. 1991. Virus-host interactions: Induction of chlorotic and necrotic responses in plants by tobamoviruses. *Annu. Rev. Phytopathol.* 29:193-217.
- Daubert, S. 1988. Sequence determinants of symptoms in the genomes of plant viruses, viroids, and satellites. *Mol. Plant-Microbe Interact.* 1:317-325.
- Devic, M., Jaegle, M., and Baulcombe, D. 1989. Symptom production on tobacco and tomato is determined by two distinct domains of the satellite RNA of cucumber mosaic virus (strain Y). *J. Gen. Virol.* 70:2765-2774.
- Fraser, R. S. S. 1990. The genetics of resistance to plant viruses. *Annu. Rev. Phytopathol.* 28:179-200.
- Goodman, R. N., Kiraly, Z., and Wood, K. R. 1986. *The Biochemistry and Physiology of Plant Disease*. University of Missouri Press, Columbia, MO, pp. 46-74.
- Gubler, U., and Hoffman, B. J. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263-269.
- Hase, S., Karasawa, A., Takahashi, H., and Ehara, Y. 1992. Nucleotide sequence of RNA2 of cucumber mosaic virus strain O. *Ann. Phytopathol. Soc. Jpn.* 58:789-793.
- Hayakawa, T., Hazama, M., Onda, H., Komiya, T., Mise, K., Nakayama, M., and Furusawa, I. 1988. Nucleotide sequence analysis of cDNA encoding the coat protein of cucumber mosaic virus: Genome organization and molecular features of the protein. *Gene* 71:107-114.
- Hayakawa, T., Mizukami, M., Nakajima, M., and Suzuki, M. 1989a.

- Complete nucleotide sequence of RNA3 from cucumber mosaic virus (CMV) strain O: Comparative study of nucleotide sequences and amino acid sequences among strains O, Q, D and Y. *J. Gen. Virol.* 70:499-504.
- Hayakawa, T., Mizukami, M., Nakamura, I. and Suzuki, M. 1989b. Cloning and sequencing of RNA-1 cDNA from cucumber mosaic virus strain O. *Gene* 85:533-540.
- Henika, F. S. 1932. The inheritance of the white burley character in tobacco. *J. Agric. Res.* 44:477-493.
- Kataoka, J., Masuta, C., and Takanami, Y. 1990a. Complete nucleotide sequence of RNA2 of cucumber mosaic virus Y strain. *Ann. Phytopathol. Soc. Jpn.* 56:495-500.
- Kataoka, J., Masuta, C., and Takanami, Y. 1990b. Complete nucleotide sequence of RNA1 of cucumber mosaic virus Y strain and evolutionary relationships among genome RNAs of the virus strains. *Ann. Phytopathol. Soc. Jpn.* 56:501-507.
- Koenig, R. 1981. Indirect ELISA methods for the broad specificity detection of plant viruses. *J. Gen. Virol.* 55:53-62.
- Kurath, G., and Palukaitis, P. 1989. Satellite RNAs of cucumber mosaic virus: Recombinants constructed *in vitro* reveal independent functional domains for chlorosis and necrosis in tomato. *Mol. Plant-Microbe Interact.* 2:91-96.
- Masuta, C., and Takanami, Y. 1989. Determination of sequence and structural requirements for pathogenicity of a cucumber mosaic virus satellite RNA (Y-satRNA). *Plant Cell* 1:1165-1173.
- Mathewson, E. H. 1912. The export and manufacturing tobaccos of the United States, with brief reference to the cigar types. U.S. Dept. Agric. Bur. Plant Indus. Bull. 244:100.
- Matthews, R. E. F. 1990. *Plant Virology* 3rd ed. Academic Press, New York. pp. 144-325.
- Nakashima, K., and Ehara, Y. 1989. Two satellite RNAs found in cucumber mosaic virus strain Y. *Ann. Phytopathol. Soc. Jpn.* 55:579-585.
- Nitta, N., Masuta, C., Kuwata, S., and Takanami, Y. 1988. Comparative studies on the nucleotide sequence of cucumber mosaic virus RNA3 between Y strain and Q strain. *Ann. Phytopathol. Soc. Jpn.* 54:516-522.
- Palukaitis, P. 1988. Pathogenicity regulation by satellite RNAs of cucumber mosaic virus: Minor nucleotide sequence changes alter host responses. *Mol. Plant-Microbe Interact.* 1:175-181.
- Peacock, A. C., and Dingman, C. W. 1968. Molecular weight estimation and separation of ribonucleic acid by electrophoresis in agarose-acrylamide composite gels. *Biochemistry* 7:668-674.
- Roberts, P. L., and Wood, K. R. 1981. Comparison of protein synthesis in tobacco systemically infected with a severe or a mild strain of cucumber mosaic virus. *Phytopathol. Z.* 102:257-265.
- Roossinck, M. J., and Palukaitis, P. 1990. Rapid induction and severity of symptoms in zucchini squash (*Cucurbita pepo*) map to RNA1 of cucumber mosaic virus. *Mol. Plant-Microbe Interact.* 3:188-192.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463-5467.
- Shintaku, M. H. 1991. Coat protein gene sequences of two cucumber mosaic virus strains reveal a single amino acid change correlating with chlorosis induction. *J. Gen. Virol.* 72:2587-2589.
- Shintaku, M. H., Zhang, L., and Palukaitis, P. 1992. A single amino acid substitution in the coat protein of cucumber mosaic virus induces chlorosis in tobacco. *Plant Cell* 4:751-757.
- Shintaku, M. H., and Palukaitis, P. 1990. Genetic mapping of cucumber mosaic virus. Pages 156-164 in: *Viral Genes and Plant Pathogenesis*. T. P. Pirone and J. G. Shaw, eds. Springer-Verlag, New York.
- Stoflet, E. S., Koeberl, D. D., Sarkar, G., and Sommer, S. S. 1988. Genomic amplification with transcript sequencing. *Science* 239:491-494.
- Takahashi, H., and Ehara, Y. 1988. Comparison of protein and mRNA syntheses in tobacco leaves infected with the yellow or the ordinary strain of cucumber mosaic virus. *Ann. Phytopath. Soc. Japan.* 54:164-173.
- Takahashi, H., and Ehara, Y. 1992. Changes in the activity and the polypeptide composition of the oxygen-evolving complex in photosystem II of tobacco leaves infected with cucumber mosaic virus strain Y. *Mol. Plant-Microbe Interact.* 5:269-272.
- Takahashi, H., Ehara, Y., and Hirano, H. 1991. A protein in the oxygen-evolving complex in the chloroplast is associated with symptom expression on tobacco leaves infected with cucumber mosaic virus strain Y. *Plant Mol. Biol.* 16:689-698.
- Takanami, Y., Kubo, S., and Imaizumi, S. 1977. Synthesis of single- and double-stranded cucumber mosaic virus RNAs in tobacco mesophyll protoplasts. *Virology* 80:376-389.
- Ziemięcki, A., and Wood, K. R. 1975. Changes in the soluble protein constitution of cucumber cotyledons following infection with two strains of cucumber mosaic virus. *Physiol. Plant Pathol.* 7:79-89.
- Ziemięcki, A., and Wood, K. R. 1976. Proteins synthesized by cucumber cotyledons infected with two strains of cucumber mosaic virus. *J. Gen. Virol.* 31:373-381.