# Field Testing of a Satellite-Containing Attenuated Strain of Cucumber Mosaic Virus for Tomato Protection in Japan

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## ABSTRACT

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A potentially useful, attenuated cucumber mosaic virus (CMV), CMV-KO2, was isolated from a tomato field in Japan. The virus had an associated nonnecrogenic satellite RNA (SatRNA) containing 368 nucleotides with microsequence heterogeneity and a unique sequence in positions 79–87 compared to previously reported SatRNAs. Experimental infections in tomato plants showed that the concentration of CMV-KO2 was maintained stably at low levels, and it was not transmitted from tomato to tomato by *Myzus persicae*. A host-range study of 32 cultivars of 20 species from eight families showed that CMV-KO2, in general, caused either no symptoms or mild symptoms on those plants. No synergistic effect caused by mixed infections of CMV-KO2 and tobacco mosaic virus or potato virus Y was observed in tomato plants. Field tests during 1989 and 1990 revealed that tomato plants treated with CMV-KO2 had a 20–200% higher yield (20–40 t/ha) compared to nontreated plants in CMV-infested fields. Collectively, the results indicate that CMV-KO2 is useful and safe to use.

Cucumber mosaic virus (CMV) of tomatoes (*Lycopersicon esculentum* Mill.) has become increasingly important in Japan since the mid-1970s, particularly in tomato processing. The virus caused serious damage to tomato plants in the main production areas of Ibaraki, Tochigi, Gumma, Fukushima, and Nagano prefectures. The disease has gradually spread to the north, and production areas have been shifted to escape CMV infestation. A large number of farmers have stopped cultivating tomatoes, and in some areas, tomato production essentially has been terminated (1). Considerable research efforts have been expended through the collaboration of national and regional governmental institutes and the private sector in attempts to address various aspects of the problem. Although some progress has been made using insecticides and repellents to prevent aphid transmission, using preinoculation with attenuated virus, through changes in cultural practices, using resistant cultivars, and through the use of combinations of these methods, no conclusive measures for control of this disease have been found.

Control of CMV using satellite-attenuated CMV strains has been investigated extensively in France (4), China (22), Japan (24), the United States (19), and Italy (2), and satellite-mediated biocontrol has proved useful in protecting plants from CMV. However, satellite-mediated biocontrol has not been put to practical use, except in China where satellite-attenuated CMV has been applied successfully in several crops (7). Several factors contribute to the reluctance to utilize this control strategy: 1) Although a satellite may attenuate CMV-induced symptoms in the target crop, symptoms may be intensified in other crops (9). Because the virus is also transmitted by aphids in a nonpersistent manner, in practical use, the targeted host plants and many other agriculturally important plant species have to be tested for the symptoms induced by attenuated CMV. 2) Attenuated CMV, particularly its satellite RNA, could change properties during serial passage in some host plants (6). Therefore, it is extremely difficult to maintain stably a specific CMV and satellite-RNA combination. 3) CMV strains designated from greenhouse studies as attenuated may not exhibit similar effectiveness in the field (T. Sato, H. Sayama, and M. Kominato, *unpublished data*).

We studied some of these aspects in a new satellite-attenuated isolate of CMV, designated KO2. This paper describes CMV-KO2's characterization and demonstrates its practical usefulness for protecting and improving the fruit yield of tomato in the field.

## MATERIALS AND METHODS

**Virus source and maintenance.** The KO2 strain of CMV was isolated from a naturally infected tomato plant in Gumma Prefecture and was propagated in 10 serial passages on tomato cultivar TMK143 in the greenhouse. CMV-KO2 contains a satellite RNA (SatRNA), designated 57-SatRNA, and in this study, is referred to as the attenuated CMV. Satellite-free CMV-KO2 was obtained by single-lesion isolation on *Chenopodium quinoa* and was designated KO2G (because it contained only the KO2 genomic RNA). KO2G was propagated in tomato for use in protection experiments in the greenhouse. Two severe CMV isolates, CMV-853 and CMV-876, were propagated in tobacco (*Nicotiana tabacum* cv. Xanthi-nc) and were used in the greenhouse, in host-range studies (CMV-853), and in protection tests (CMV-876). CMV-853 does not contain any detectable SatRNA and causes stunting and severe mosaic symptoms in tomato; CMV-876 contains a SatRNA that causes lethal necrosis in tomato (M. E. Tousignant and J. M. Kaper, *unpublished data*). Tobacco mosaic virus (TMV) and potato virus Y (PVY), which were used for mixed-infection tests, were maintained in tomato cultivar Kyoryokugoko and in tobacco cultivar Xanthi-nc, respectively.

**Protection experiments in the greenhouse.** Five TMK143 tomato plants in the cotyledon stage were preinoculated with either CMV-KO2G or CMV-KO2, at a 50 $\mu$g/ml concentration, and were challenge inoculated 10 days later with CMV-853 or necrogenic CMV-876. Five tomato plants were also inoculated with only CMV-853 or CMV-876 isolates, at 50 $\mu$g/ml, as a control treatment. Inoculation buffer in all cases was 37.5 mM sodium phosphate buffer (pH 7.0).

**Protection experiments in the field.** During 1989, a small-scale test was conducted in a field in Nagano Prefecture, where 20 CMV-KO2 treated and 20 nontreated TMK143 tomato plants

were arranged in a randomized complete-block design with two replications. During 1990, a large-scale test was conducted in two fields in Nagano Prefecture and in a field in Gumma Prefecture, where 2,850 CMV-KO2 treated and 2,850 nontreated TMK143 tomato plants were arranged in a randomized complete-block design with three replications. For all field tests, tomato plants at the cotyledon stage were inoculated in the greenhouse with CMV-KO2, at a 50 $\mu$g/ml concentration. Two weeks later the upper leaves were analyzed by ELISA (enzyme linked immunosorbent assay) to verify infection by CMV-KO2 and were tested for ds57-SatRNA accumulation using total nucleic acid (TNA) analysis (23). About 40 days after inoculation, both the plants treated with CMV-KO2 and the control plants were transplanted to the field. Fruits were harvested weekly from early August to mid-September. In 1989, harvested fruits were classified in three groups: healthy fruits, diseased fruits with CMV symptoms, and other diseased fruits. The healthy fruits were weighed at each harvest and were counted to obtain the healthy fruit yield. However, in the diseased fruits with CMV symptoms, fruit yield was estimated from the number of diseased fruits multiplied by the average healthy fruit weight at each harvest, because CMV-diseased fruits were often decayed and direct weighing was not representative of their real weights. In 1990, only healthy fruits were measured.

**Virus concentration in leaves.** Twelve TMK143 tomato plants in the cotyledon stage were inoculated with CMV-KO2 or CMV-853, at a 50$\mu$g/ml concentration in 37.5 mM sodium phosphate buffer (pH 7.0). Twelve noninoculated plants were tested as a control treatment. In each treatment, the first primary leaves of three plants were sampled at 10, 20, 30, and 40 days after inoculation, were weighed, were frozen at −80 C, and were ground in 0.02 M sodium phosphate buffer (pH 7.2), at 1:50 tissue/buffer (w/v), with a mortar and pestle. Relative virus concentration was measured at 405 nm according to the absorbance calculated by ELISA, utilizing a microplate reader (BioRad Model 450). The antiserum was a gift from K. Hanada (National Agriculture Research Center, Tsukuba, Japan).

**Sequence determination.** 57-SatRNA was isolated from virions of CMV-KO2 by SDS/phenol extraction (12,17) followed by sucrose density-gradient ultracentrifugation and gradient fractionation (10). Five micrograms of ds57-SatRNA was annealed with an excess amount of synthetic oligonucleotide (5′GGGTCCTGT3′), which is complementary to the 3′ end of ds57-SatRNA. First-strand cDNA was synthesized at 42 C for 1 h in 30 $\mu$l of reaction mixture containing 50 mM Tris-HCl (pH 8.3), 25 mM KCl, 5 mM $MgCl_2$, 20 mM DTT, 1 mM dNTP, 20 U of RNasin (Promega, Madison, WI), and 30 U of AMV reverse transcriptase (Life Sciences Inc., St. Petersburg, FL). After SatRNA template removal in 250 mM NaOH at 70 C for 20 min, the single-stranded cDNA was annealed to the synthetic oligonucleotide (5′GAATTCGTTTTGTTTGTTAGAGAATT3′), which is complementary to the 3′ end of the negative strand and contains an *Eco*RI site, by boiling for 2 min and by incubating at 42 C for 20 min. Second-strand synthesis was carried out at 37 C for 1 h in 50 $\mu$l of 100 mM HEPES (pH 6.9), 10 mM $MgCl_2$, 2.5 mM DTT, 70 mM KCl, 0.5 mM dNTP, and 10 U of the Klenow fragment of DNA polymerase I. The incomplete 3′ ends were first filled by T4 DNA polymerase and then were ligated to *Eco*RI linkers (dCCGAATTCGG) using standard procedures (18). The full-length double-stranded cDNA was ligated into the *Eco*RI site of pUC118 (Takara) and was transformed into *Escherichia coli* JM101. Transformants were selected on media containing ampicillin and on recombinants containing the SatRNA sequences identified by colony blot hybridization with the synthetic oligonucleotide probe (5′GGGTCCTGT3′), which is complementary to the 3′ end of 57-SatRNA. The transformants were superinfected with the helper phage M13KO7 (Takara) to generate single-stranded cDNA. The nucleotide sequences of the cDNAs were determined with the Applied Biosystems model 370A DNA sequencing system, using the manufacturer's protocol.

**Host range.** CMV-KO2 and CMV-853 were inoculated into five plants each from 32 cultivars from 20 species from eight families, and their symptoms were compared. All inoculations were done with CMV, at a 50 $\mu$g/ml concentration in 40 mM sodium phosphate buffer (pH 7.0). Inoculated and uninoculated leaves were checked for the presence of cucumber mosaic virus using local lesion assay on cowpea (*Vigna sesquipedalis*) 14 days after inoculation. Symptoms were evaluated in inoculated and uninoculated leaves 14–20 days after inoculation.

**Aphid transmission.** TMK143 tomato plants were used as source plants (acquisition access) and test plants (inoculation access) in transmission tests. *Myzus persicae* (Sulzer) were reared on Xanthi-nc tobacco plants in an insectary in the greenhouse and were starved 2 h before transmission tests. Aphids fed on acquisition-access plants for 10 min, and 10 inoculation-access plants were used with five aphids per inoculation-access plant. The experiment was replicated twice, and tests plants were checked for CMV by immunosorbent electron microscopy (ISEM).

**Mixed infections of CMV-KO2 with other important viruses in tomato.** Sixteen TMK143 tomato plants were inoculated at the cotyledon stage with CMV-KO2 (first inoculum) and were inoculated 18 days later with triturated leaf tissue from either TMV or PVY infections diluted 1:1 (w/v) with 0.02 M sodium phosphate buffer (pH 7.2) (second inoculum). The control treatment consisted of eight tomato plants inoculated with triturated leaf tissue infected by TMV or PVY. Infections were confirmed in each plant by ELISA, using appropriate antisera. Symptoms were evaluated 20 days after the second inoculation.

**Stability of CMV-KO2 during passage in tomato.** CMV-KO2 was serially passaged 10 times in tomato TMK143. For each passage, triturated leaf tissue was diluted 1:1 tissue/buffer (w/v) with 0.02 M sodium phosphate buffer (pH 7.2) and was inoculated at the cotyledon stage into 25 tomato plants. Symptoms were observed for 20 days after inoculation. After each passage, the dsSatRNA in the first and second primary leaves of 10–25 plants was analyzed by TNA analysis (23). During the sixth passage, ds57-SatRNA and a larger dsSatRNA were detected in some plants. As a result, after this passage, in addition to random sampling, inoculum was selected carefully from those plants having only ds57-SatRNA.

**Tomato fruit-quality analysis.** Four fields that were free of CMV disease in Nagano and Gumma prefectures were selected to compare the effect of the CMV-KO2 treatment on fruit quality of tomato TMK143. The experiment was performed three times using healthy fruits sampled from the second, third, and fourth harvests during 1989 and 1990. For each determination, 20 fruits were selected at random and were weighed and blended in an ordinary blender. The raw juice was used in pH, soluble solids, titratable acidity, and color determinations. The analytical methods used have been described (16).

**Morphology of tomato plants.** In 1990, two fields in Nagano Prefecture that were free of CMV disease were selected for comparing the morphological effect of the CMV-KO2 treatment. The height of tomato plants was measured in late June and the fruit weight and the numbers of fruits per plant from five plants treated with CMV-KO2 and from five nontreated control plants in each field were counted at each harvest date.

## RESULTS

**Sequence of 57-SatRNA.** Twenty clones were sequenced and all satellite inserts had 368 bp with microsequence heterogeneity. The clones were subdivided into six groups with identical sequences: pKO2-sat1, -sat2, -sat3, -sat4, -sat5, and -sat6 representing 6, 6, 1, 1, 3, and 3 clones, respectively. Sequence heterogeneity was found at positions 13, 197, 250, 291, and 292, with one to four base changes among the clone groups (Fig. 1). In comparison with D-SatRNA (11), which is 335-nt long, 57-SatRNA had a large unique insertion region between positions 79 and 87, comprising three segments between positions 79 and 80, 83 and 84, and 86 and 87 (Fig. 1).

This insertion region has not been found in any other reported SatRNA (11). The sequence homologies of pKO2-sat1 with D-, Q-, Y-, R-, S-, 1-, G-, B1-, and WL1-SatRNA (5,11) were 80,

80, 81, 81, 79, 84, 78, 80, and 80%, respectively. In the "necrogenic region" between positions 282 and 297 (20), 57-SatRNA clones had one or three base changes compared to necrogenic prototype D-SatRNA (Fig. 1).

**Properties of CMV-KO2.** *Virus concentration of CMV-KO2 in tomato.* The titer of CMV-K02 generally was stable and low at all times after inoculation. The titer of CMV-853 was much higher at 10 days after inoculation and then gradually decreased (Fig. 2).

*Host range.* CMV-KO2 and CMV-853 (as control) were compared for symptomatology in different host plants. In general, CMV-KO2 induced milder symptoms than CMV-853. CMV-KO2 induced local lesions or was symptomless in plants of the Leguminosae, Cruciferae, Compositae, and Umbelliferae families and induced mild symptoms in plants of the Solanaceae, Cucurbitaceae, Chenopodiaceae, and Amaranthaceae families. Yellowing was observed in CMV-KO2 inoculated leaves of *Cucurbita moschata* (Table 1).

*Aphid transmission.* CMV-KO2 was not detected by ISEM in any of 20 tomato test plants exposed to five viruliferous *Myzus persicae* aphids each, suggesting that the virus was not transmitted from tomato to tomato by *Myzus persicae*.

*Mixed infections of CMV-KO2 with other viruses.* In Japan, tomato plants often are infected naturally by CMV and TMV and rarely by PVY (T. Sato, H. Sayama, and M. Kominato, *unpublished data*). Only mosaic and mild mosaic symptoms were observed in mixed inoculations of CMV-KO2 with TMV and PVY, respectively. These symptoms were characteristic of those induced by inoculation with TMV or PVY alone, suggesting that CMV-KO2 would not interact synergistically with the two viruses.

**Stability of CMV-KO2.** CMV-KO2 was transferred serially in tomato to assess the stability of the satellite. Only mild mosaic symptoms were observed in systemic leaves, and no symptom changes were detected after 10 passages. The 57-SatRNA was electrophoretically homogeneous until the fifth passage. During the sixth passage, two dsSatRNA electrophoretic bands were observed, representing 57-SatRNA and a larger SatRNA, designated 60-SatRNA. Sequence analysis of 60-SatRNA revealed a molecule 390–391 nt in length. The proportion of 60-SatRNA gradually increased with each passage, until the tenth passage, when 57-SatRNA was no longer detectable (Fig. 3). The replacement of 57-SatRNA by 60-SatRNA occurred when the inoculum was randomly collected for each passage. However, 57-SatRNA could be maintained stably when inoculum was obtained from

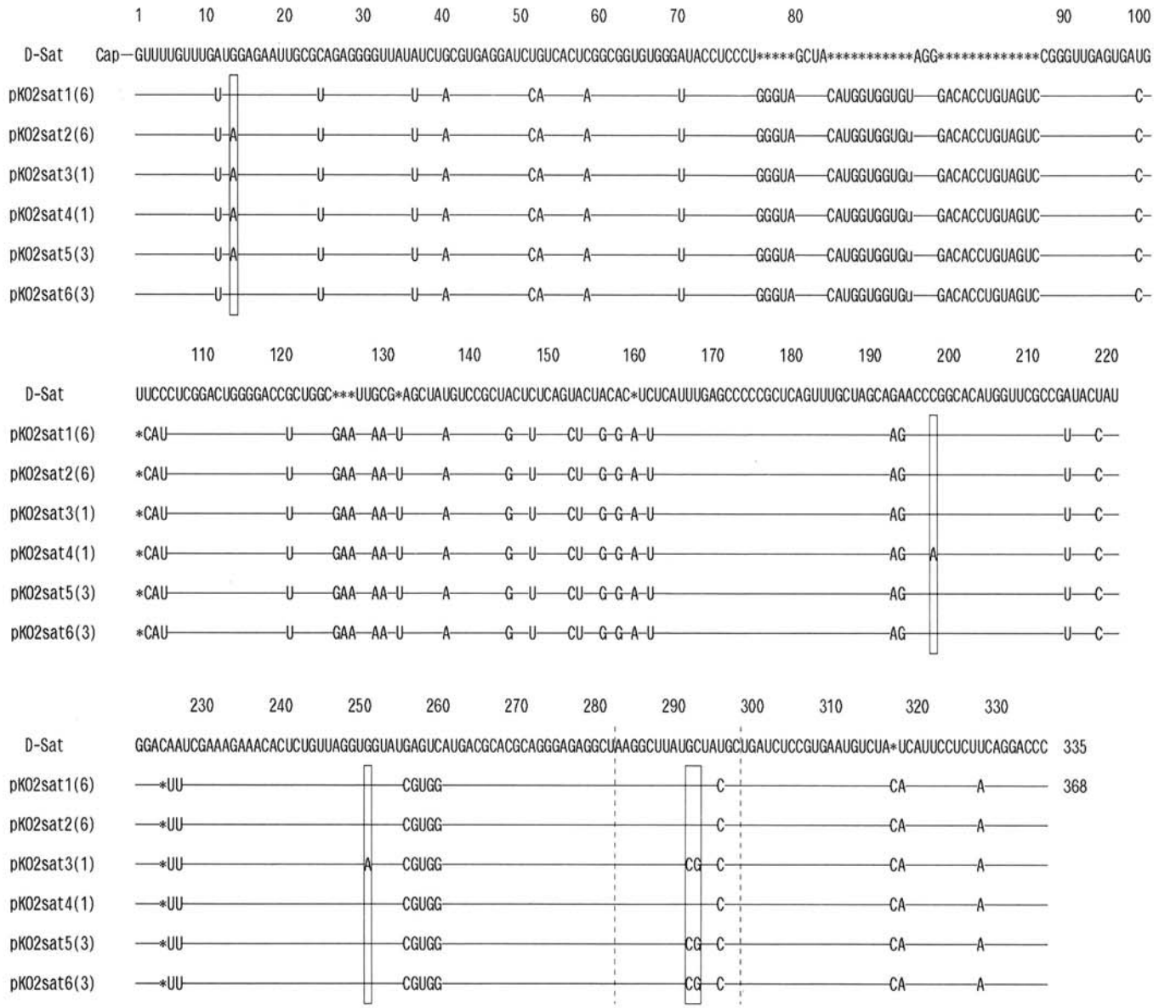


**Fig. 1.** Nucleotide sequences of cDNA clones of 57-SatRNA in comparison with D-SatRNA (11). Changes in the sequences are indicated by letters. Deletions are denoted by asterisks (*). Horizontal lines indicate no change. Boxed areas indicate microsequence heterogeneity in 57-SatRNA. The region between two vertical dotted lines denotes the "necrogenic region." Numbers in parentheses after each clone signify the numbers of independent cDNA clones with the same sequences.

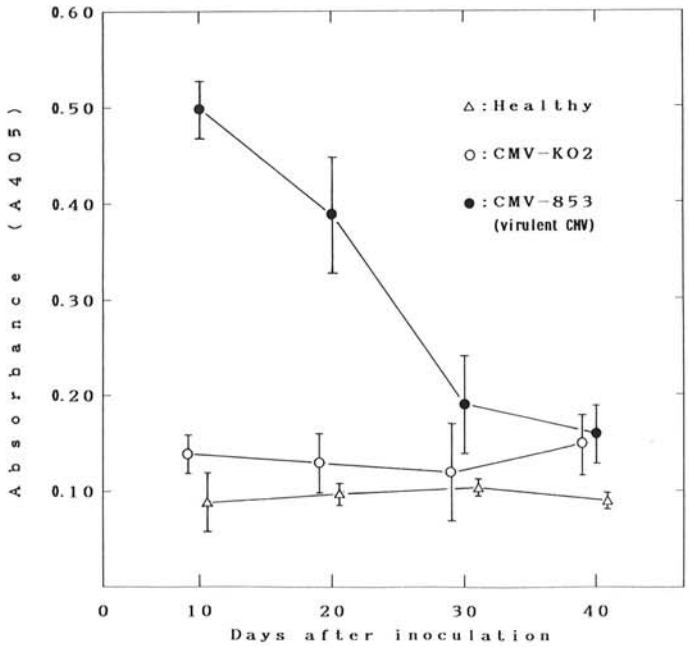


**Fig. 2.** Comparison of virus concentration in tomato plants inoculated with CMV-KO2 or CMV-853 (virulent strain). Relative virus concentration was measured by ELISA absorbance at 405 nm. Each data point represents the average ELISA reading of three plants. Vertical bars indicate standard errors.

plants having only ds57-SatRNA.

**Effects of CMV-KO2 preinoculation on fruit quality and plant growth.** Fruit quality of plants treated with CMV-KO2 was compared with the fruit quality of nontreated plants in four fields without CMV infestation. No statistical differences in pH, soluble solids, and fruit color were noted between plants treated with CMV-KO2 and nontreated plants. However, total acid was reduced by 4%, and the the CMV-KO2 treatment reduced the fruit weight by 12 g (Table 2).

CMV-KO2 treated plants were dwarfed 5–6% in height, resulting in a 10–20% reduction in the number of fruits per plant in the fields (data not shown). In the absence of CMV disease in the field, this could be a disadvantage of CMV-KO2 treatment. Preliminary experiments suggest, however, that this could be compensated for by increasing the plant density of plants in the field treated with CMV-KO2 by about 20% (data not shown).

**Protective effect of CMV-KO2.** Tomato plants inoculated with CMV-876 were stunted with mosaic symptoms on the leaves; plants preinoculated with CMV-KO2 (containing 57-SatRNA) or CMV-KO2G (free of 57-SatRNA) and then challenge inoculated with CMV-853 remained symptomless or had mild mosaic symptoms on the leaves. In contrast, when CMV-876, containing necrogenic SatRNA, was used for challenge inoculation, plants preinoculated with CMV-KO2 remained symptomless or had mild mosaic symptoms on the leaves, but plants preinoculated with CMV-KO2G became necrotic (Fig.4).

In 1989, ELISA detected CMV in all nontreated plants at the test field; however, no TMV, PVY, or potato virus X was detected. The plants infected with CMV showed mosaic and fern leaf

TABLE 1. Comparison of the symptoms[x] in inoculated and uninoculated leaves on different host plants inoculated with CMV-KO2 or CMV-853

| Plants | | CMV-KO2 | | CMV-853 | |
|---|---|---|---|---|---|
| | | Inoculated leaves | Uninoculated leaves | Inoculated leaves | Uninoculated leaves |
| Solanaceae | | | | | |
| *Lycopersicon esculentum* | (2)[y] | O (+)[z] | m (+) | O (+) | M, S (+) |
| *Nicotiana tabacum* | (3) | O (+) | m (+) | O (+) | M, S (+) |
| *Solanum melongena* | (1) | O (+) | m (+) | O (+) | M (+) |
| *Capsicum annuum* | (1) | O (+) | cs (+) | O (+) | CS (+) |
| Leguminosae | | | | | |
| *Vigna sesquipedalis* | (1) | L (+) | O (−) | L (+) | O (−) |
| *Phaseolus vulgaris* | (2) | O (−) | O (−) | O (−) | O (−) |
| | (1) | L (+) | O (−) | L (+) | O (−) |
| *Vicia faba* | (1) | L (+) | O (−) | L (+) | O (−) |
| Cucurbitaceae | | | | | |
| *Cucumis sativus* | (2) | O (+) | O (+) | O (+) | O (+) |
| | (1) | O (+) | m (+) | O (+) | M (+) |
| | (1) | O (+) | O (−) | O (+) | O (+) |
| *Cucurbita maxima* | (1) | O (+) | O (−) | O (+) | O (−) |
| | (1) | O (+) | O, m (+) | O (+) | m (+) |
| *C. moschata* | (1) | O (+) | O (−) | O (+) | O (−) |
| | (1) | Y (+) | y (+) | Y (+) | Y (+) |
| *C. pepo* | (1) | O (+) | m (+) | y (+) | M (+) |
| Chenopodiaceae | | | | | |
| *Spinacia oleraceae* | (3) | O (+) | m (+) | O (+) | N (+) |
| *Chenopodium quinoa* | (1) | L (+) | O (−) | L (+) | O (−) |
| *C. amaranticolor* | (1) | L (+) | O (−) | L (+) | O (−) |
| Amaranthaceae | | | | | |
| *Gomphrena globosa* | (1) | O, L (+) | m (+) | O, L (+) | M (+) |
| Cruciferae | | | | | |
| *Raphanus sativus* | (1) | O (+) | O (−) | O (+) | O (−) |
| *Brasica rapa* | (1) | O (+) | O (+) | O (+) | M (+) |
| Compositae | | | | | |
| *Lactuca sativa* | (1) | O (+) | O (+) | O (+) | M, R (+) |
| *Zinnia elegans* | (1) | O (+) | O (+) | O (+) | M (+) |
| Umbelliferae | | | | | |
| *Apium graveolens* | (1) | O (+) | O (+) | O (+) | M (+) |

[x]Symptom: O = symptomless; m = mild mosaic; M = mosaic; S = stunt; cs = light chlorotic spot; CS = chlorotic spot; L = local lesion; y = light yellowing; Y = yellowing; N = necrosis; R = rugose.

[y]Numbers in parentheses denote the number of cultivars tested in each species.

[z]+ or − in parentheses indicates that the virus was detected or was not detected, respectively, by back inoculation to *Vigna sesquipedalis*.

symptoms (necrosis on leaves, stems, and fruits) (Fig. 5). These fruits were variably deficient in lycopene, ranging from complete deficiency, with grayish spongy pericarp tissue, to partial deficiency, resulting in "yellow blotchy fruits." Fruit deformation and vascular browning in outer pericarp tissue were also observed in these CMV-infected fruits. Necrogenic SatRNA also was detected in all 13 samples of fruits from plants with CMV symptoms, however, 14 fruit samples from plants treated with CMV-KO2 had only 57-SatRNA, except for two samples in which both 57-SatRNA and necrogenic SatRNA were found. Overall, tomato plants preinoculated with CMV-KO2 produced about three times as many healthy fruits and significantly less CMV-diseased fruits than did nontreated plants in the field (Table 3).

In 1990, CMV disease was not as severe as in 1989. However, 80% or more of the nontreated plants were infected with CMV at three fields, and plants treated with CMV-KO2 produced about 30% more healthy fruits than did nontreated plants (Table 3).

## DISCUSSION

In four tomato fields infested with CMV, in 1989 and 1990, preinoculation of plants with CMV-KO2 significantly increased healthy fruit yield over the yield of nontreated control plants (Table 3). The protective effect of CMV-KO2 against natural CMV infection also was verified by the fact that KO2-treated plants produced only 0.2 t/ha of CMV-diseased fruits (0.3% of the healthy fruit yield) in the 1989 field test; the nontreated control plants produced 39.5 t/ha of CMV-diseased fruits (200% of the healthy fruit yield) (Table 3). Although the genomic RNA of CMV-KO2 could contribute to the protective effect in tomato plants, it would be unable to prevent lethal tomato necrosis caused by naturally occurring CMV that contains a necrogenic SatRNA (8). This makes it imperative that attenuated CMV used in preventive inoculation contain a nonnecrogenic SatRNA. In the field test with plants treated with CMV-KO2, 57-SatRNA and necrogenic SatRNA sometimes coexisted in the same plants, indicating that their competing replication in plant tissue probably directed the protection of the plants (21).


**Fig. 3.** Replacement of 57-SatRNA by 60-SatRNA during CMV-KO2 passages in tomato plants. The inoculum was randomly collected for each passage. Lane 1, third passage; lane 2, fifth passage; lane 3, sixth passage; lane 4, seventh passage; and lane 5, tenth passage.

TABLE 2. Comparison of fruit quality between tomato plants preinoculated with CMV-KO2 and nontreated control plants in four fields without CMV infestation[y]

| Treatment | Fruit weight (g) | pH | Soluble solids (%) | Total acid (%) | Color (Lb/a) |
|---|---|---|---|---|---|
| CMV-KO2 | 88 a[z] | 4.3 a | 6.3 a | 0.47 a | 10.9 a |
| Nontreated | 100 b | 4.3 a | 6.3 a | 0.51 b | 11.1 a |

[y] All values are averages of four locations.
[z] Different letters indicate a significant difference at the 5% level in each column.


**Fig. 4.** Protection effect of SatRNA against necrogenic CMV-876 in tomato. Left, control inoculated with necrogenic CMV-876; middle, preinoculated with CMV-KO2 followed by challenge inoculation with CMV-876; and right, preinoculated with CMV-KO2G (without SatRNA) followed by challenge inoculation with CMV-876.

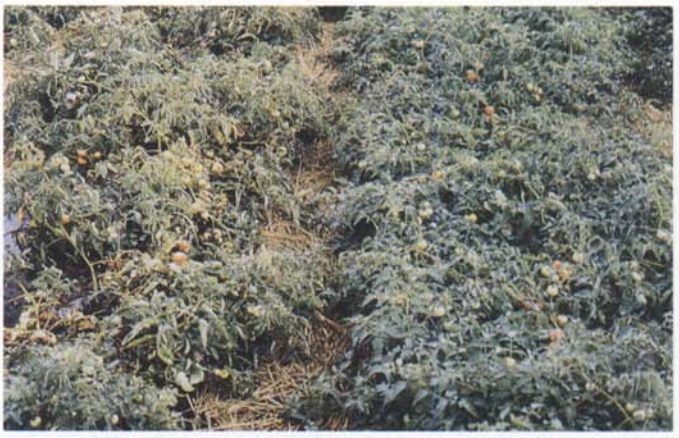

**Fig. 5.** Field test. Left row, nontreated control tomato plants diseased by natural CMV infection; right row, CMV-KO2 preinoculated plants with vigorous vines and symptomless leaves and fruits.

TABLE 3. Comparison of the fruit yield of tomato plants preinoculated with CMV-KO2 and nontreated plants in CMV-infested fields, in Japan

| Year[w] | No. of plants tested | Healthy fruits (t/ha) CMV-KO2 treated | Healthy fruits (t/ha) Nontreated | Fruits with CMV symptoms (t/ha)[x] CMV-KO2 treated | Fruits with CMV symptoms (t/ha)[x] Nontreated |
|---|---|---|---|---|---|
| 1989 | 20 | 58.4 a[y] | 19.5 b | 0.2 a | 39.5 b |
| 1990 | 2,850 | 80.3 a | 60.1 b | ND[z] | ND |

[w] Field experiments were conducted with two replications in 1989 and three replications in 1990.
[x] Fruit yield of fruits with CMV symptoms were estimated from the number of the diseased fruits multiplied by the average healthy fruit weight at each harvest.
[y] Different letters within the same year in healthy fruits and fruits with CMV symptoms denote a mean significant difference at the 5% level.
[z] No data.

In serial passages of CMV-KO2 in tomato, we observed a shift in proportions from 57-SatRNA to a larger SatRNA, designated 60-SatRNA. Kaper (6) reported a similar proportional shift of SatRNA during sequential passage in different host plants. Although the emergence of 60-SatRNA cannot be explained from our data, it seems unlikely that 60-SatRNA was derived from 57-SatRNA. The sequence homology between the two SatRNAs is only 73%. Furthermore, the unique insertion of 57-SatRNA between positions 79 and 87 is not present in 60-SatRNA (data not shown). Microsequence heterogeneity in SatRNAs can be induced even in infections of SatRNA cDNA transcripts during serial passages, particularly in tobacco plants (14,15). As a result, such heterogeneities are probably a natural feature of SatRNA, and their emergence is host-plant dependent (3). During serial passage of CMV-KO2 in tomato plants, mild mosaic symptoms were maintained stably. This implies that 57-SatRNA, in spite of its microsequence heterogeneity, had not mutated in a significant way to a necrogenic SatRNA. This was supported by the fact that none of the six 57-SatRNA clones isolated contained the "necrogenic region" between positions 282 and 297 (Fig. 1). These results indicate that to stably maintain attenuated CMV and produce it in large amounts for practical use, careful selection of host plants containing a specific SatRNA in each passage and the use of in vitro transcribed SatRNA are very important. With this purpose in mind, we succeeded in transcribing two clones of pkO2sat1 and pkO2sat6 in vitro (13) for possible future use.

CMV-KO2 might not be transmitted from tomato plant to tomato plant by *Myzus persicae*, although this result still has to be verified and checked in other plants and for different aphid species. It also is important to test whether this apparent difficulty in transmission by aphids could be related to the low-virus concentration of the tomato plants treated with CMV-KO2. In host-range studies, CMV-KO2 was symptomless or caused only mild symptoms in many important crop species. This fact and the absence of synergistic effects with other agriculturally important viruses indicate the potential of CMV-KO2 for practical use. Another factor encouraging its use by farmers as well as by tomato processors, for whom fruit-quality parameters are important, is that the levels of chemical components in tomato fruits were not affected by CMV-KO2 treatment, except for the slight reduction of total acid. The observed decrease in fruit size could be related to the inhibition of plant growth by CMV-KO2 treatment. This reduction in size could be compensated for with general agricultural practices, such as increasing plant density, using vigorous cultivars, increasing the amount of fertilizers applied, and combinations of the above.

The use of CMV-KO2 will contribute to several aspects of practical agriculture: 1) its use will assure stable tomato yields in fields in which CMV infestations are endemic, 2) tomato processors will reliably obtain from farmers the expected amounts of tomatoes with good fruit qualities, and 3) areas in which tomato cultivation has been discontinued as the result of CMV infestation will be recovered for production.

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