Biological and Molecular Variability of Zucchini Yellow Mosaic Virus on the Island of Martinique

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ABSTRACT

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Zucchini yellow mosaic potyvirus (ZYMV) was isolated from zucchini squash on the island of Martinique for the first time in 1992, and it is now widespread on the island. Fourteen isolates collected from different cucurbit crops in 1992 and 1993 exhibited biological and antigenic variability as revealed by using differential hosts and a series of monoclonal antibodies. A partial sequencing of the coat protein and putative polymerase coding regions of the 14 isolates revealed that there was also some molecular variability, lower within the group of Martinique isolates than with ZYMV isolates from other geographical origins. This variability can result either from the introduction to the island of different ZYMV strains, or from a rapid evolution of an introduced ZYMV population with a narrow genetic base. The incidence of the observed variability will affect the development of efficient and durable control strategies.

Zucchini yellow mosaic virus (ZYMV), first described in Italy in 1973 (18), is responsible for major economic losses in cucurbit crops in many parts of the world (15,19). ZYMV belongs to the potyviruses, a group of plant viruses characterized by a monopartite, positive-sense, single-stranded RNA genome encapsidated in flexuous, filamentous particles. The RNA is translated into a single polyprotein cleaved by three viral proteases (26). The 36-kDa coat protein of ZYMV encapsidates the viral RNA and is also involved in aphid transmissibility of the virus (26). Strains of ZYMV from distinct geographic origins exhibit biological diversity (13), particularly concerning their host range, symptomatology, and aphid transmission.

The determination of variability within a virus group and the understanding of mechanisms and factors affecting this variability are of considerable agronomic significance, particularly for determining resistance gene deployment strategies, since natural resistance genes can be rapidly overcome by adapted virus strains. In addition, variability of virus strains, particularly within the capsid protein, raises a problem for the development of reliable diagnosis techniques, because most of them are based on the antigenic properties of the coat protein.

In Martinique, two viruses were reported to infect cucurbits: papaya ringspot potyvirus type watermelon (PRSV-W) and cucumber mosaic cucumovirus (CMV) (25). More recently, severe epidemics of ZYMV were observed (17). We used biological and serological methods, as well as sequence data, to characterize the variability of ZYMV in this ecosystem, in order to determine potential control strategies against this very destructive virus.

MATERIALS AND METHODS

Virus, plants, and aphid transmission. Fourteen isolates of ZYMV (MT1 to MT14) were collected from different cucurbits in several parts of Martinique in 1992 and 1993 (Fig. 1 and Table 1). They were tested by double antibody sandwich–enzyme-linked immunosorbent assay (DAS-ELISA, see later) to detect the presence of mixed infections with other cucurbit viruses: PRSV-W, CMV, watermelon mosaic potyvirus 2 (WMV2), squash mosaic comovirus (SqMV), and melon necrotic spot carmovirus (MNSV). Only five of the ZYMV isolates were present in mixed infections with PRSV-W. They were separated by inoculating *Pisum sativum* L., which supports local infection of ZYMV but not of PRSV-W (23). Isolate MT9.C3 was derived from MT9 by single local lesion passage through *Chenopodium amaranticolor* Coste and Reyn. The isolates were inoculated according to the standard methods used in our laboratory (11) on cotyledons of 10- to 15-day-old plantlets of muskmelon (*Cucumis melo* L.) cvs. Védrantais, Doublon, and PI 414723, cucumber (*Cucumis sativus* L.) cvs. Marketer and Taichung Mou Gua, squash (*Cucurbita moschata* (Duchesne) Duchesne ex Poir.) cvs. Musquée de Provence and Nigeria, and zucchini squash (*Cucurbita pepo* L.) cv. Diamant. Melon PI 414723, Taichung Mou Gua cucumber, and Nigeria squash were described as resistant to ZYMV (21–23), and Doublon melon possesses the *Fn* gene that induces a rapid and lethal wilting upon infection with some ZYMV isolates (13). Cross protection by the ZYMV-WK strain was tested as described in Lecoq et al. (12) for each isolate. Aphid transmissibility was established using five *Myzus persicae* Sulzer or *Aphis gossypii* Glover per plant, with five Védrantais melon plants per test and three independent repetitions for each isolate (11).

Generation of monoclonal antibodies (MAbs). Two 10-week-old female BALB/c mice were inoculated in the rear footpads with 50 µl of Freund's complete adjuvant containing 20 µg of purified virus (E9 strain of ZYMV) and were boosted 12 days later with the same amount of purified virus in incomplete adjuvant. At day 15, the popliteal and inguinal lymph nodes were removed and 1.7×10^8 cells were fused with the myeloma partner Sp2/0 using PEG 4000 according to a standard protocol (20). Selected hybridoma cells

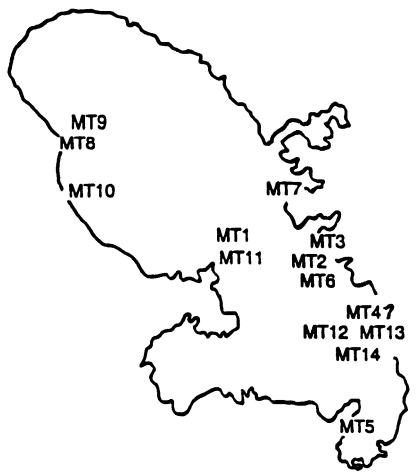

Fig. 1. Geographical origin of zucchini yellow mosaic virus isolates collected in Martinique in 1992 and 1993.

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were cloned and injected into the peritoneal cavity of BALB/c mice for bulk production of monoclonal antibodies (9).

ELISA. DAS-ELISA (4) with polyclonal antisera was used to check for the presence of ZYMV and the absence of other cucurbit viruses: PRSV-W, CMV, SqMV, WMV2, and MNSV. Triple antibody sandwich ELISA tests were also used. Plates were coated with the polyclonal antibody and virus incubated as for DAS-ELISA. Experiments were conducted with crude extract of ZYMV-infected zucchini squash plants ground 1/10 (wt/vol) in phosphate-buffered saline (PBS), or with purified virus preparations at the final concentration of 250 ng/ml in the same buffer. Negative and positive controls (extract from noninfected plants, and from plants infected by the E9 strain against which the MAbs were raised) were added on each plate. The MAbs (ascitic fluids diluted 1/1,000) and anti-mouse IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO), diluted 1/8,000, were incubated for 3 h at 37°C. Substrate incubation and reading of absorbance at 405 nm were as for DAS-ELISA.

Purification of virus and viral RNA. Purified virus preparations were obtained from infected zucchini squash plants as described (14) using a cesium-sulfate gradient method. The purified virus was heat-denatured in the presence of 2% sodium dodecyl sulfate, and the viral RNA was separated from viral proteins by centrifugation through a sucrose gradient, and isolated with an ISCO UA5 (ISCO Inc., Lincoln, NE) gradient fractionator (6).

Sequencing. Purified RNA from each virus isolate was reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) and the reverse (R) oligonucleotide TGCGTGGCAATGACAT as a primer. The cDNA was amplified by polymerase chain reaction (PCR) using the (R) and forward (F1): TATTTGCGCTGCGATG primers. The nucleotide sequence of the 432-nucleotide amplified cDNA fragment was determined by the dideoxynucleotide chain termination procedure of Sanger et al. (27), using an Applied Biosystems (Perkin-Elmer Corp., Foster City, CA) DNA sequencer 370A. The fragments were either sequenced directly with a PRISM Ready Reaction DyeDeoxy Terminator kit (Applied Biosystems) using the (R), (F1), and (F2): CCTACAAGCCCTCCATCAAG oligonucleotides as primers, or cloned into pBS (KS^-) plasmid (Stratagene, La Jolla, CA) and sequenced using the PRISM Ready Reaction Dye Primer -21M13 and M13 reverse kits (Applied Biosystems). Both strands were sequenced for each clone.

Sequence analysis. Sequence data were analyzed with the PileUp sequence comparison procedure of GCG (Genetic Computer Group, Madison, WI) (5). At least two fragments resulting from independent PCR reactions were analyzed for each isolate. The published sources for the nucleotide sequences used for comparison were: ZYMV-California (3), ZYMV-Connecticut (8), ZYMV- Florida (24), and ZYMV-Israel (7).

RESULTS

Biological variability. All isolates of ZYMV from Martinique induced typical symptoms of vein clearing, leaf deformation, and stunting on Diamant zucchini squash and Musquée de Provence squash. None of them induced systemic symptoms or was detected by DAS-ELISA in the apex of Nigeria squash. All isolates induced severe mosaic symptoms on Marketer cucumber but failed to induce systemic symptoms and were not detected by DAS-ELISA in the apex of Taichung Mou Gua cucumber. All isolates induced severe symptoms on Védrantais muskmelon. Eight out of 14 isolates induced a rapid, lethal wilting a few days after inoculation on Doublon melon possessing the *Fn* gene. This indicates that they belong to pathotype F (13). The six other isolates induced symptoms similar to those on Védrantais, indicating that they belong to pathotype NF (Table 1). The ratio of F/NF strains was similar to that observed for ZYMV strains from different parts of the world (13,16).

Six of the isolates belong to the pathotype 0, i.e., they induced no systemic symptoms on melon PI 414723 that has the *Zym* resistance gene to ZYMV (22). Five isolates induced systemic chlorotic or necrotic lesions, and are consequently classified in pathotype 1. Three isolates induced mosaic symptoms on some resistant plants and can be classified in pathotype 2, which overcome completely the resistance gene (Table 1).

As reported for other ZYMV isolates (13), there is no correlation between the pathotype on melon PI 414723 and the induction of the wilting reaction on Doublon.

Infection of Védrantais melon plantlets with the mild ZYMV-WK strain (12) efficiently protected the plants against chal-

Table 1. Origin and biological properties of zucchini yellow mosaic virus isolates from Martinique

Isolate	Original host	Date of sampling	Symptoms on Doublon[a]	Pathotype on PI 414723[b]	Aphid transmission[c]	
					Myzus persicae	*Aphis gossypii*
MT1	Zucchini	August 1992	YSt.n	1	9/15[d]	12/15
MT2	Zucchini	September 1992	W	1	12/15	13/15
MT3	Cucumber	September 1992	W	0	9/15	13/15
MT4	Zucchini	December 1992	W	1	13/15	12/15
MT5	Zucchini	December 1992	YSt	0	13/15	9/15
MT6	Zucchini	December 1992	W	2	10/15	9/15
MT7	Zucchini	December 1992	YSt	0	13/15	11/15
MT8	Cucumber	November 1992	YSt.n	0	11/15	7/15
MT9	Zucchini	November 1992	YSt.n	1	10/15	9/15
MT10	Zucchini	November 1992	W	2	9/15	10/15
MT11	Cucumber	April 1993	W	2	15/15	13/15
MT12	Muskmelon	April 1993	W	1	15/15	14/15
MT13	Muskmelon	April 1993	W	0	14/15	12/15
MT14	Muskmelon	April 1993	YSt	0	14/15	10/15

[a] W = wilt; YSt = yellowing, stunting, mosaic and leaf deformation; YSt.n = same as YSt but with some foliar necrosis.
[b] 0 = no systemic infection; 1 = systemic chlorotic or necrotic spots; 2 = systemic mosaic, yellowing, stunting, and leaf deformations.
[c] Five viruliferous aphids per test plant (cumulated data for three independent experiments with five test plants).
[d] Number of infected plants over number of inoculated plants.

Table 2. Serological variability of zuccini yellow mosaic virus isolates from the island of Martinique

Isolate	Reactivity with monoclonal antibody[a]					Polyclonal antiserum
	AB6	CC11	DD2	DE6	CE11[b]	
E9[c]	+++	+++	+++	+++	+++	+++
11 isolates[d]	+++	+++	+++	0	+++	+++
MT1	+	+++	0	0	+++	+++
MT7	+++	+	+++	0	+++	+++
MT9	+++	+	+++	0	+++	+++
MT9.C3	+++	0	+++	0	+++	+++

[a] Serological reactivity, measured as the absorbance at 405 nm (A) after 1 h of substrate incubation: 0 = A < 0.05; + = 0.05 < A < 0.5; ++ = 0.5 < A <1.5; +++ = A > 1.5.
[b] The results obtained for two other monoclonal antibodies, CE8 and CH10, were identical
[c] Reference strain from France, used for the production of the polyclonal and monoclonal antibodies.
[d] Martinique isolates MT2, MT3, MT4, MT5, MT6, MT8, MT10, MT11, MT12, MT13, and MT14.

lenge inoculation by any of the 14 isolates of ZYMV from Martinique.

All isolates were efficiently transmitted by the aphids *A. gossypii* and *M. persicae* (Table 1). Transmission rates using five aphids per test plant ranged from 60 to 100%, with a mean value of 80%, for *M. persicae*; and from 47 to 93%, with a mean value of 73%, for *A. gossypii.*

Antigenic variability. All ZYMV isolates reacted with a polyclonal antiserum raised against ZYMV-E9. However, monoclonal antibodies obtained against ZYMV-E9 revealed differential reactivities among the Martinique isolates (Table 2). The results were similar using crude plant extracts or purified virus at a concentration of 250 ng/ml as antigen. None of the Martinique isolates were recognized by the DE6 MAb, which reacted with most ZYMV isolates present in the laboratory collection (C. Desbiez and H. Lecoq, unpublished data). Variability among the Martinique isolates was detected using three other MAbs: AB6, CC11, and DD2. The MT1 isolate did not react with the DD2 MAb, and reacted weakly with AB6. The MT9.C3 variant from MT9 did not react at all with CC11, although MT9 reacted weakly, as MT7, with this antibody. Other variants obtained from MT9 by single local lesion cloning reacted either like MT9 or like MT9.C3 with the seven MAbs used in this study (data not shown).

There was no correlation between pathotype, serotype, and geographical or host origin of the isolates.

Molecular variability. Fourteen ZYMV isolates from Martinique, as well as MT9.C3 and strains E9 and E15 (15), were sequenced. Sequences were determined for 237 nucleotides (coding for 79 amino acids) of the putative polymerase coding region and 195 nucleotides (coding for 65 amino acids) of the coat protein coding region. These fragments were chosen for sequencing because the N-terminal part of the coat protein is known to be highly variable and to contain major virus-specific epitopes, due to its localization at the surface of the virion (29), whereas the polymerase is more conserved. At least two independent clones were sequenced for each isolate. The nucleotide sequences obtained with Dye Terminator and Dye Primer methods were identical.

Table 3 shows the nucleotide sequence identity percentage of different ZYMV isolates. The Martinique isolates had nucleotide sequence identities of 99 to 100%. Identities ranging from 93 to 98% were observed between the isolates from Martinique and isolates from other geographical origins, and 92 to 99% between independent isolates from different parts of the world. Nucleotide sequence identities were similar for the polymerase and capsid protein coding regions (data not shown). Amino-acid sequence identities (Fig. 2) between ZYMV isolates of different geographical origins ranged from 95 to 100% for the polymerase fragment, and from 80 to 100% for the N-terminal part of the coat protein. The Martinique isolates shared identities ranging from 96 to 100% for both protein fragments.

DISCUSSION

A significant biological and antigenic variability was observed among 14 isolates of ZYMV from the island of Martinique, collected from different hosts and locations within a 1-year period. Such a biological diversity has already been described for ZYMV either between isolates of different geographic origin, or for isolates kept for a long time in the greenhouse (13,16). In this study, variants were isolated from a limited geographical area soon after the first report of a ZYMV epidemic. The introduction of ZYMV to the island of Martinique, or at least the appearance of epidemics, must be recent, because no typical symptoms of ZYMV were noticed before 1992, and no virus was detected in ELISA tests in previous studies (B. Hostachy and H. Lecoq, unpublished). The introduction of ZYMV to the island may have occurred either through importation of infected plants or seeds, since ZYMV may be seed-transmissible in zucchini squash at a very low rate (28), or through migration of viruliferous aphids from neighboring countries where the virus is present, i.e., the Dominican Republic (H. Lecoq and H. Lot, unpublished) or Venezuela (10). Long-distance spread of potyviruses by viruliferous aphids has indeed been reported occasionally (30).

The biological, serological, and molecular variability observed for ZYMV on the island may be the consequence of a rapid evolution of the virus shortly after a single or limited number of introductions, or of multiple introductions of variable strains, probably coming from the same region because the molecular variability of ZYMV on Martinique remains limited. Sequence data indicate that the Martinique isolates share more homology with the Florida strain than with any other sequenced strain. This can be correlated with the relative geographical proximity of Florida and Martinique, and may suggest a common ancestor.

The molecular variability of the N-terminal end of the capsid of the Martinique isolates was low, although this region is known to be one of the most variable parts of the genome of potyviruses (29). The Martinique isolates shared more sequence homology among themselves than with the other strains, at the nucleotide and amino-acid level, which suggests a common origin. Six of the 14 isolates shared the same nucleotide sequence, and seven of 14 the same deduced amino-acid sequence (Fig. 2), in the 432-nucleotides fragment sequenced in this study. These seven isolates had the same reactivity toward the monoclonal antibodies tested. Nevertheless, they did not share the same biological properties: MT5 and MT8 induced no wilting on Doublon melon, whereas the other isolates did, and they were classified in pathotype 0 to 2 regarding the resistant melon line PI 414723. This indicates that the parts of the viral genome sequenced in this study have no direct effect on the symptom variations of ZYMV observed on muskmelon, and that other parts of the genome involved in these characters were also variable. All Martinique isolates had the amino-acid sequence Asp-Ala-Gly (DAG) at the N-terminal part of the coat protein and were efficiently transmitted by two aphid species. This is in agreement with the role established for the DAG amino-acid triplet in aphid transmission of potyviruses (2).

The limited molecular variability of ZYMV isolates from Martinique was not correlated with their biological variability, nor with their geographical origin on the island. Nevertheless, there was a good correlation between antigenic and molecular variability among ZYMV isolates. Mild digestion of purified ZYMV with trypsin indicated that the monoclonal antibodies that showed a variability among Martinique isolates, i.e., AB6, CC11, DD2, and DE6, seem to react with the external N- or C-terminal parts of the coat protein (C. Desbiez, unpublished). Most amino-acid variations in the N-terminal part of the coat protein could be tentatively associated with differential reactivity to these mono-

Table 3. Percentage of nucleotide sequence identity between fragments of the polymerase and coat protein coding regions of strains and isolates of zucchini yellow mosaic virus

	E9	E15	Florida	Connecticut	California	Israel	Martinique
E9 (France)	. . .	94[a]	92	94	94	99	93 to 94
E15 (France)		. . .	95	98	97	94	94 to 95
Florida			. . .	95	95	93	97 to 98
Connecticut				. . .	97	94	94 to 95
California					. . .	94	95 to 96
Israel						. . .	93 to 94
Martinique							99 to 100

[a] Percentage of nucleotide sequence identity for the fragment of 432 nucleotides sequenced (237 nucleotides of the C-terminal part of the putative polymerase coding region and 195 nucleotides of the N-terminal part of the coat protein coding region). The data for Martinique indicate the range of variation observed within the 14 isolates.

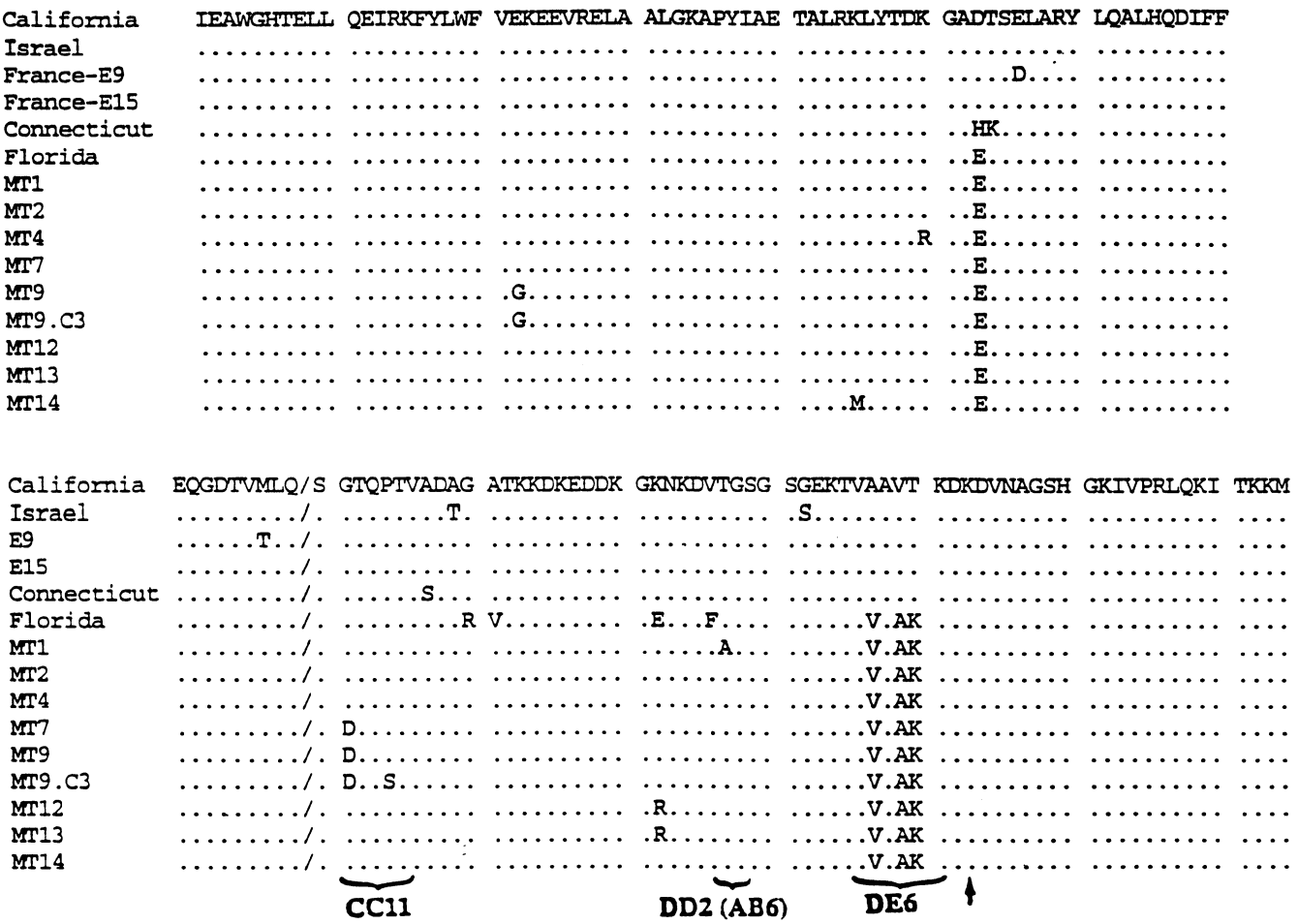

Fig. 2. Deduced amino-acid sequence alignment for the C-terminal part of the polymerase and the N-terminal part of the coat protein of zucchini yellow mosaic virus isolates. The consensus Q/S sequence identifies the cleavage site between the polymerase and the coat protein (26). The brackets indicate the amino-acid sequences differing between isolates specifically recognized by monoclonal antibodies, and thus may be involved in epitope formation. The arrow indicates the limit of the variable N-terminal part of the coat protein. MT3, MT5, MT6, MT8, MT10, and MT12 have the same amino-acid sequence as MT2.

clonal antibodies (Fig. 2). If this is confirmed by more accurate epitope mapping techniques, the monoclonal antibodies used in this study may be useful tools for revealing the molecular variability in this genome region. In some cases they may allow the detection of point mutations (as may be the case for DD2 and the MT1 isolate). In contrast, the high specificity of some of these MAbs restricts their usefulness for disease diagnosis.

The variability of the N-terminal part of the coat protein coding region, one of the most variable regions of the potyvirus genome, was low among ZYMV isolates from Martinique. If this is representative of the overall variability of the genome, we can theorize that the important phenotypic variability is related to a low number of point mutations, as recently reported for another plant virus (1). This suggests that variants with new biological properties may appear very easily within viral populations.

The ability of some isolates to overcome the *Zym* resistance gene of muskmelon suggests that durable control of ZYMV in melon will probably not be achieved using this gene. This emphasizes the need for additional sources of resistance derived by either classical breeding or pathogen-derived resistance. In contrast, resistance of Taichung Mou Gua cucumber and Nigeria squash seem to be effective and will probably control the virus in these crops. An alternative control method, until resistant cultivars with good agronomic characters are obtained, could be the use of cross protection with the mild ZYMV-WK strain that satisfactorily controls all Martinique isolates.

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