

Characterization of genetic resistance to cucumber mosaic virus (CMV) in spinach (*Spinacia oleracea* L.)

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Short Report

Keywords: Cucumber mosaic virus, extreme resistance, hypersensitive resistance, spinach, *Spinacia oleracea*.

Posted Date: July 4th, 2024

DOI: <https://doi.org/10.21203/rs.3.rs-4575792/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Research Notes on November 12th, 2024. See the published version at <https://doi.org/10.1186/s13104-024-06987-2>.

Abstract

Objective

Cucumber mosaic virus (CMV) is a significant pathogen causing quality loss in spinach. Although host genetic resistance is the primary method of managing CMV infection in this crop, CMV resistance genes are not widely utilized in spinach breeding programs as the genetic and molecular mechanisms underlying resistance are not yet fully understood. CMV infections were therefore studied in different lines of spinach plants, and their progeny, to develop a model of the genetic basis of CMV resistance.

Results

Visual observations and RT-PCR assays revealed that three monoecious lines (03-258, 03-263, and 03-336) were susceptible to CMV, while three germplasm accessions (PI 699741, PI 699746, and PI 699758) and a near-isogenic line (NIL-M) exhibited resistance. A dioecious line (03-009) consisted of susceptible and resistant plants. Notably, resistant plants did not exhibit the lesions typical of the hypersensitive response; instead, they tended to show extreme resistance. Genetic analysis of progeny from the cross NIL-M × 03-336 indicated that a single dominant allele (designated *SRCm1*) controlled CMV resistance; analysis of sib-cross progeny populations derived from line 03-009 supported this conclusion. These results offer a valuable model for CMV resistance in spinach and will enhance future breeding programs.

Introduction

Cucumber mosaic virus (CMV) has a wide range of hosts, infecting over 1000 plant species and causing significant economic losses in various crop [1]. CMV is a member of the genus *Cucumovirus* in the family *Bromoviridae*. It is a positive-sense RNA plant virus with a genome consisting of three single-stranded RNA molecules, designated RNA1, RNA2 and RNA3; RNA4, a subgenomic RNA that encodes the coat protein, is generated from RNA3 during replication [2]. Spinach (*Spinacia oleracea* L.) is a common food crop that is affected by 14 naturally-occurring viral diseases, of which CMV, often referred to as “Spinach Blight”, is historically the most significant [3, 4]. Blight-resistant spinach varieties have long been cultivated, but while producers generally use host genetic resistance to manage CMV infection [5, 6], the specific genetic regulatory mechanisms involved in host-virus interactions are not yet fully understood.

Effector-triggered immunity (ETI) is part of the plant immune system. It is activated by R genes in response to attacks from pathogens [7]. ETI can lead to a strong defense reaction known as the hypersensitive response (HR), which is characterized by localized cell death around the infection site [8, 9]. The HR is effective in suppressing pathogen infection and provides long-lasting protection. Extreme resistance (ER) is a type of antiviral resistance mediated by R genes that suppresses virus accumulation effectively in initially infected cells without triggering programmed cell death [10]. The *Arabidopsis thaliana*, ecotype C24 is resistant to the yellow strain of CMV (CMV-Y) and displays the HR following

inoculation. In C24, a single dominant allele of the gene *RCY1*, which encodes a CC-NBS-LRR-type protein, is responsible for conferring resistance [11, 12]. Similar R genes that confer CMV resistance have not yet been identified or isolated from other plants.

In this study, the resistance of various spinach germplasm accessions and lines to CMV-Y was determined to establish a model system for understanding the genetic and molecular basis of CMV resistance. In addition to characterizing resistance phenotypically, we conducted genetic analyses of progeny from the resistant, susceptible, and segregating lines identified during the study.

Materials and Methods

Plant material

The spinach lines investigated consisted of three United States Department of Agriculture (USDA) germplasm accessions (PI 699741, PI 699746, and PI 699758) derived from “blight-resistant” cultivars, three monoecious lines (03-336, 03-258, and 03-263), a near-isogenic line (NIL-M), and a dioecious line (03-009) (Table 1). All lines other than NIL-M were bred by the Tohoku Seed Co., LTD (Utsunomiya, Japan). NIL-M was produced by introgression of the monoecious gene from line 03-336 into the 03-009 genetic background [13, 14]. CMV resistance was also investigated in five populations (#10, #11, #13, #14, and #16) of sib-crossed progeny derived from line 03-009 (Table 2). These populations were produced by crossing pairs of male and female plants selected at random from line 03-009 without prior knowledge of their susceptibility to CMV. Finally, F_1 and F_2 populations derived from the cross NIL-M $\times$ 03-336 were analyzed for CMV resistance (Table 2). The observed segregating ratio of resistant and susceptible traits was statistically tested using the Chi-square test, with a significance level $\alpha = 0.05$.

All plants were grown in BioTRON growth chambers (Nippon Medical & Chemical Instruments, Osaka, Japan) at 20°C with an 8-h photoperiod.

Table 1
Germplasm accessions and lines used in this study.

Germplasm accession / line ID	Name	Acquisition source	Origin
PI 699741	Blight-resistant savoy	USDA	New York, USA
PI 699746	Virginia blight-resistant	USDA	Pennsylvania, USA
PI 699758	Blight-resistant savoy	USDA	Wyoming, USA
03-336	-	Tohoku Seed Co., LTD.	Japan
03-009	-	Tohoku Seed Co., LTD.	Japan
03-258	-	Tohoku Seed Co., LTD.	Japan
03-263	-	Tohoku Seed Co., LTD.	Japan
NIL-M	-	Yamano et al. [12]	Japan

Table 2
Responses of spinach germplasm accessions and lines to inoculation with CMV-Y.

Plant population	Number of plants		χ^2 (3: 1)	P	χ^2 (1: 1)	P
	Resistant (Asymptomatic)	Susceptible (Symptomatic)				
PI 699741	9	0	n.a.	n.a.	n.a.	n.a.
PI 699746	10	0	n.a.	n.a.	n.a.	n.a.
PI 699758	5	0	n.a.	n.a.	n.a.	n.a.
03-258	0	8	n.a.	n.a.	n.a.	n.a.
03-263	0	8	n.a.	n.a.	n.a.	n.a.
03-336	0	107	n.a.	n.a.	n.a.	n.a.
NIL-M	156	0	n.a.	n.a.	n.a.	n.a.
03-009	32	32	n.a.	n.a.	0	1
03-009-derived sib- crossed progeny #10	65	25	0.37	0.54	n.a.	n.a.
03-009-derived sib- crossed progeny #11	0	34	n.a.	n.a.	n.a.	n.a.
03-009-derived sib- crossed progeny #13	0	32	n.a.	n.a.	n.a.	n.a.
03-009-derived sib- crossed progeny #14	14	16	n.a.	n.a.	0.13	0.72
03-009-derived sib- crossed progeny #16	72	21	0.29	0.59	n.a.	n.a.
03-336 × NIL-M F ₁	12	0	n.a.	n.a.	n.a.	n.a.
03-336 × NIL-M F ₂	139	41	0.47	0.49	n.a.	n.a.
n.a., not applicable						

CMV inoculation

The yellow strain of CMV (CMV-Y) was used in this study [15–17]. *Nicotiana benthamiana* leaves harboring CMV-Y were ground in 0.1 M phosphate buffer to produce leaf sap. Leaves of 4-week-old spinach plants at the 5–6 leaf stage were dusted with carborundum powder (600 mesh) (Nacalai Tesque, INC., Japan) and rub-inoculated with leaf sap. Plants mock-inoculated with phosphate buffer were used as negative controls.

RT-PCR analysis of CMV infections

Total cellular RNA was extracted from leaf tissues using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). cDNA was synthesized by reverse transcription using GoScript™ Reverse Transcription Mix and random primers (Promega, Madison, USA). To confirm CMV infection in plants, a 285-bp cDNA fragment, reverse transcribed from CMV RNA3 [17], was amplified using GoTaq® DNA Polymerase (Promega, Madison, USA) and the primers 5'-TCCTCCCTCCGATCTCTGTGG-3' and 5'-TGGTCTCCTTTTGGAGGCC-3', followed by 2% agarose gel electrophoresis. The spinach *GADPH* (GenBank ID, NM_001426410) cDNA was amplified using the primers 5'-TGTGTGCGACATCCCCCTCGTGTCAG-3' and 5'-CCACCCGTTGGCTGTAACCCCACTCAT-3' as a positive control in the RT-PCR analysis.

Results & Discussion

CMV-Y inoculation of spinach lines and germplasm accessions

The response to CMV-Y was tested in 5–20 individual spinach plants from three germplasm accessions and five lines (Tables 1 and 2). Plants from lines 03-336, 03-258, and 03-263 exhibited symptoms of infection. Inoculated leaves developed chlorotic spots (known as infection spots) at 6–8 days post-inoculation (dpi), and the chlorosis spread gradually across the leaves; Fig. 1a–c shows representative images from 03-336. By 8 dpi, the top leaf of susceptible plants became yellowish and chlorotic from its base (Fig. 1a, b). Furthermore, CMV-Y was detected in the top leaves of symptomatic plants at 5 and 10 dpi (Fig. 1d). These results suggested that lines 03-336, 03-258, and 03-263 were susceptible to CMV-Y and a systemic infection had been established. By contrast, the three germplasm accessions and NIL-M neither exhibited symptoms of CMV-Y infection nor developed the lesions typical of the HR, although they showed minor physical damage caused by rub-inoculation (Table 2; Fig. 1a–c). Line 03-009 contained both susceptible and resistant plants (Fig. 1c). RT-PCR analysis did not detect cDNA from CMV-Y in NIL-M (Fig. 1d) or the germplasm accessions following inoculation, indicating these lines were resistant to CMV-Y infection. The HR was not observed in the germplasm accessions, NIL-M, or 03-009; instead, although further verification is required for certainty, resistance in these lines appeared to result from ER.

To establish a model for studying CMV resistance in spinach, line 03-336 and NIL-M were selected as representative susceptible and resistant lines. Unlike the germplasm accessions, these lines result from many generations of self-fertilization and thus each is a homogeneous population suitable for genetic study. Examination of over 100 plants from each line confirmed that 03-336 and NIL-M were true-breeding for CMV susceptibility and resistance, respectively (Table 2). Line 03-009, a dioecious line produced by sib-crossing over many generations that contains both susceptible and resistant individuals, was also subjected to further analysis. Examination of 64 plants from line 03-009 revealed they segregated for susceptibility and resistance in a 1:1 ratio (Table 2). This line will be particularly useful in

identifying the gene(s) responsible for resistance since it showed clear segregation for the trait but has high genetic homogeneity.

Genetic characterization of CMV resistance in lines NIL-M and 03–009

The F_1 and F_2 progeny resulting from a cross between the CMV-resistant line NIL-M and the CMV-susceptible line 03-336 were examined to determine the genetic nature of CMV resistance in spinach. Following CMV-Y inoculation, 12 F_1 plants showed no lesions or other symptoms of infection, although some exhibited physical damage from rub-inoculation, and no CMV-Y was detected (Table 2 and Fig. 1e–f). This suggested that dominant factors were responsible for CMV resistance in spinach.

Next, 180 F_2 progeny were inoculated with CMV-Y and their resistance determined. This F_2 population contained 41 symptomatic and 139 asymptomatic plants (Table 2; Fig. 1g). To confirm that the presence and absence of symptoms judged by visual observation properly represented susceptibility and resistance, the top leaves of randomly selected individual F_2 plants, as well as the parental lines (NIL-M and 03-336), were sampled at 14 dpi and subjected to an RT-PCR assay designed to amplify a fragment of cDNA produced from viral RNA3. This fragment was detected in line 03-336 (the susceptible parent) and in symptomatic F_2 plants, but not in NIL-M (the resistant parent) or in asymptomatic F_2 plants (Fig. 1h). Such a result indicated that F_2 plants with symptoms were susceptible, and developed systemic infections with CMV-Y, while F_2 plants without symptoms were resistant. The numbers observed in the F_2 population (139 resistant and 41 susceptible plants) were consistent with a segregation ratio of 3:1, which would be expected if a dominant allele at a single locus controlled resistance (Table 2). Given that NIL-M was derived from a cross between lines 03-336 and 03–009 [14], it is reasonable to assume that the resistance shown by NIL-M originated in dioecious line 03–009, which was observed to segregate into resistant and susceptible individuals (Table 2).

To confirm whether resistance to CMV in line 03–009 was also regulated by a single factor, the responses to CMV-Y inoculation were monitored in five sib-crossed progeny populations (#10, #11, #13, #14, and #16) derived from 03–009 (Table 2). Populations #11 and #13 consisted only of susceptible plants, thus these populations were likely to result from crosses between two homozygous parents. By contrast, ~ 90 individual plants from populations #10 and #16 inoculated with CMV-Y segregated into resistant and susceptible individuals in a 3:1 ratio. This result suggested that populations #10 and #16 each resulted from a cross between two heterozygous parents, lending further support to the assumption that resistance was determined by a single locus. Finally, of 30 plants from population #14 inoculated with CMV-Y, 16 displayed susceptibility and the other 14 showed resistance, which was consistent with a 1:1 segregation ratio (Table 2). This result can again be explained by assuming that a single pair of dominant and recessive alleles determined resistance, and would be expected if one parent was homozygous and the other heterozygous. Taken together, these results indicated that CMV resistance in NIL-M and 03–009 shared a common origin and was controlled by a single gene. Hereafter, we will refer to this resistance gene as *SRCm1*.

Efficient identification of CMV resistance genes in spinach requires access to genomic sequence information and well-characterized plant materials. Recently, high quality pseudomolecules representing the six chromosomes of line 03–009 have become available. They have a total length of 879.2 Mb and a 97.0% BUSCO completeness score. The genetic background of NIL-M is nearly identical to that of line 03–009. Furthermore, whole genome sequencing reads (DRX401202) are also available for line 03-336 [14]. Currently, a genetic mapping analysis of the lines NIL-M, 03–009, and 03-336 is in progress to identify the CMV resistance gene, *SRCm1*. As resistant plants did not exhibit HR, unlike *Arabidopsis* ecotype C24, these three lines offer insight into a novel mechanism of CMV resistance. Such knowledge will improve the design of spinach breeding programs aimed at developing resistance against CMV.

Limitations

Because spinach virus diseases are usually not accompanied by severe, distinct disease symptoms, there have not been many studies worldwide that have targeted virus disease resistance factors for spinach breeding. Despite the mild symptoms, it is crucial to breed for virus disease resistance to enhance spinach quality. Seed companies often protect spinach varieties and breeding lines with valuable genotypes, such as disease resistance. As a result, there are not many opportunities to obtain useful information on CMV resistance factors from past papers and commercially available spinach seeds. Therefore, it may be up to our own future efforts to determine how practical the *SRCm1* gene, we have discovered here, is for breeding.

Abbreviations

CMV
cucumber mosaic virus
CMV-Y
yellow strain of cucumber mosaic virus
HR
hypersensitive resistance
ER
extreme resistance

Declarations

Ethics approval and consent to participate

Not applicable for this study.

Consent for publication

Not applicable.

Author Contribution

Y.W. collected the data and prepared the initial draft of the manuscript; H.H. acquired funding and designed the conception of the work; C.M. acquired funding, designed the conception of the work, and reviewed the manuscript; Y.O. acquired funding, designed the conception of the work, and reviewed the manuscript. All authors read and approved the final manuscript.

Acknowledgement

The authors thank Tohoku Seed Co. Ltd. (Utsunomiya, Tochigi, Japan) very much for providing the spinach breeding lines used in this study. We appreciate the technical assistance provided by Mrs. H. Yokomoto.

Data Availability

Data is provided within the manuscript.

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Figures

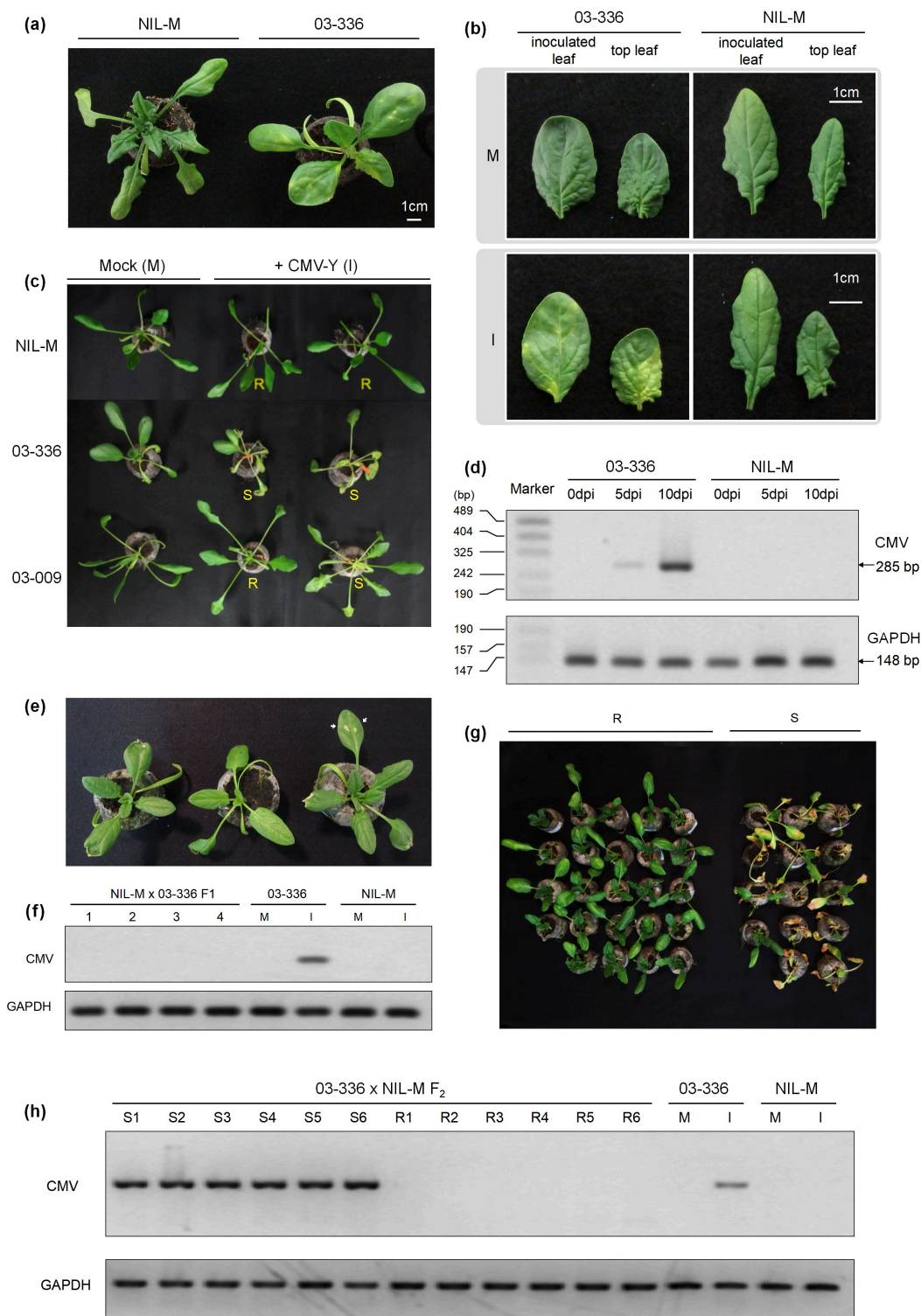


Figure 1

Figure 1

Representative images of plants inoculated with CMV-Y and of RT-PCR analyses to detect CMV-Y infections.

(a-b) Plants from NIL-M and line 03-336 at 8 days post-inoculation (dpi). (c) Plants from NIL-M, line 03-336, and line 03-009 at 15 dpi. (d) RT-PCR analysis of CMV-Y infections in NIL-M and line 03-336. (e) F₁

plants from line 03-336 and NIL-M at 11 dpi. Arrows indicate physical damage caused by rub-inoculation. (f) RT-PCR analysis of CMV-Y infections in F₁ plants. (g) F₂ progeny derived from plants shown in (e) at 14 dpi. (h) RT-PCR analysis of CMV-Y infections in F₂ progeny derived from plants shown in (e). *GAPDH* was amplified as a positive control in RT-PCR analyses (d, f, h). R: resistant (asymptomatic); S: susceptible (symptomatic); M: mock-inoculation control; I: inoculated.