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# COMPARISON OF CHERRY GREEN RING MOTTLE VIRUS STRAINS USING RT-PCR AND COAT PROTEIN SEQUENCE PHYLOGENY

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## SUMMARY

Assays for Cherry green ring mottle virus (CGRMV) were developed and used to compare different sources from North American and Mediterranean countries by differential reverse transcription-polymerase chain reaction (RT-PCR) amplification and phylogenetic analysis of coat protein sequences. DsRNAs of approximately 8.5 kb in size were purified from all diseased samples. Universal and specific primer pairs derived from sequences of two Californian strains of CGRMV were used in RT-PCR assays. Parsimony analyses using coat protein nucleotide sequences separated the 12 CGRMV sources into three major phylogenetic clades.

*Key words*: Cherry, green ring mottle viruses, Foveovirus, RT-PCR, molecular cloning.

## INTRODUCTION

Cherry green ring mottle virus (CGRMV) occurs throughout North America infecting several *Prunus* species (Rasmussen *et al*., 1951; Parker *et al*., 1976). The virus causes epinasty of Kwanzan and Shirofugen flowering cherry (*Prunus serrulata* Lindl.) (Milbrath, 1966) and severe fruit necrosis of sour cherry (Parker *et al*., 1953; Milbrath, 1966; Parker *et al*., 1976). Although CGRMV from different geographic areas were compared on indicator hosts, no biological differences were previously reported. Recently, we identified two biologically and molecularly distinct strains of CGRMV in California on Shirofugen flowering cherry and by cloning and sequencing their dsRNAs (Zhang *et al*., 1998b).

Viral coat proteins are presumed to have evolved more rapidly than proteins involved in replication and expression of virus genomes. For this reason, the coat protein genes have been used in phylogenetic comparisons (Zimmern, 1988; Koonin and Gorbalenya, 1989). Phylogenetic analyses have indicated that coat protein genes of several filamentous viruses represented separate ancestors (Dolja *et al*., 1991) and that coat protein gene sequences were highly conserved within these groups (Magome *et al*., 1997). In our study, several CGRMV sources were compared by RT-PCR and phylogenetic analyses of the complete coat protein genes. The results are reported herein.

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## MATERIALS AND METHODS

Virus isolates, CGRMV-F and -N from California were maintained in Fay Elberta peach [*P. persica* (L.) Batsch]. Additional virus sources were CGRMV-Wp1 and -Wp2 from Prosser, Washington, and CGRMV-Ww from Wenatchee, Washington (provided by G. Mink); CGRMV-MI from Michigan (provided by D. Ramsdell); CGRMV-BC from British Columbia, Canada (provided by D. Thompson); and putative sources from Italy, CGRMV-Ita1, -Ita5, -Ita6 and -Ita7, and from Lebanon, CGRMV-Leb. These diseased sources were either destructively sampled or autoclaved and discarded. The dsRNA preparations served as templates and were prepared as previously described (Zhang *et al*., 1998b). Also, extracts were prepared by the small-scale procedure for RT-PCR assays (Zhang *et al*., 1998c).

The sets of primers designed by Zhang *et al.* (1998a, 1998b), which are reprinted in Table 1, were used in RT-PCR.

The cDNA synthesis and RT-PCR reaction conditions were the same as described previously (Zhang *et al*., 1998b), except that the annealing temperature was lowered from 59°C to 55°C and the total number of cycles reduced to 25. The RT-PCR amplified DNA was ligated to the TA cloning vector pCRII (Clontech, Palo Alto) and cloned in INV F One-Shot *E. coli* competent cells following manufacture's protocol. Both strands of the cloned CGRMV coat protein gene were sequenced by the dideoxy chain termination method (Sanger *et al*., 1977) using an automated nucleotide sequencer at the University of California Sequencing Facility, Davis.

**Table 1.** PCR primers used for amplification from CGRMV.

| Primer | Nucleotide sequence (5'-3') | Location (nt position) | Product |
|---|---|---|---|
| F401h | ATGAGTTGAGGGGTTGGGTCT | Replicase (5450-5470) | 916 nt |
| F401c | ACCACGCCCAGCAAGATTTGT | Triple gene (6365-6345) | |
| N34h | GCTCATTACATCAACAGTTGCAC | Triple gene (6495-6517) | 1181 nt |
| N34c | ACCCAGTCTGATGTGATTGCCT | Coat protein (7675-7654) | |
| F39h | ACCTTCTCGTTAGCTCATTCACA | Replicase (4313-4335) | 380 nt |
| F39c | TGCAGTCTAGCTCCGATCAATT | Replicase (4692-4671) | |
| N5h | GAGCCATAGTGTGGTTGAGCT | Replicase (3765-3785) | 1086 nt |
| N5c | AGACTGTCAATCCGATGCTCG | Replicase (4892-4872) | |
| NCPh | TGGATCCATGGCTGATGAAGAATTTGA | Upstream CP (7367-7393) | 821 nt |
| NCPc | AGGATCCTCATTCATCACCACCAATTT | Downstream CP (8187-8161) | |

h: homologous; c: complementary.

The putative coat protein sequences obtained were aligned using the Pileup program of the UW-GCG software system (Genetic Computer Group, Madison). The aligned sequences were analyzed as previously described (Zhang *et al.*, 1998a). The coat protein sequence of apple stem pitting virus (ASPV; Jelkmann, 1994) was used as an out group.

The cDNA synthesis reagents were from Gibco BRL (Gaithersburg), and PCR reagents from Perkin-Elmer Inc. (Branchburg).

## RESULTS

All 12 CGRMV sources yielded dsRNAs of approximately 8.5 kb in size (data not shown). No dsRNAs were observed in similarly prepared extracts from healthy tissues. Some minor bands did appear in the gels, but their origins and relationships to CGRMV dsRNA were not determined.

Both sets of universal primers amplified the expected size products with all 12 CGRMV sources. In total, the specific primers F39h and F39c reacted with homologous CGRMV-F and other CGRMV sources, except CGRMV-N (Table 2). With the specific primers N5h and N5c, these reacted with three sources, *i.e.* homologous CGRMV-N and two Mediterranean sources, CGRMV-Ita6 and – Leb. The latter two sources had also reacted with the specific primers F39h and N39c.

The phylogram generated from PAUP using nucleotide sequence comparisons of the coat protein for all 12 CGRMV cultures yielded three major clades (Fig. 1). CGRMV-Ita6 and –MI were grouped in clade I, while CGRMV-N and –BC were in clade III. All others were grouped together in clade II.

**Table 2.** RT-PCR amplification of dsRNAs of 12 CGRMV sources using various universal and specific primers CGRMV sources.

| Primer pairs | N | F | Wp1,Wp2, Ww, MI, BC | Ita1, 5, 7 | Ita6, Leb |
|---|---|---|---|---|---|
| F401h, F401c, N34h, N34c[1] | + | + | + | + | + |
| F39h, F39c[2] | – | + | + | + | + |
| N5h, N5c[2] | + | – | – | – | + |
| NCPh, NCPc[3] | + | + | + | + | + |

[1] Universal primers: pair F from CGRMV-F; pair N from CGRMV-N.
[2] Specific primers F and N series from CGRMV-F and -N, respectively.
[3] Primer pair for coat protein gene derived from CGRMV-N.
h: homologous; c: complementary.

RT-PCR assays with coat protein primers and small-scale tissue preparations also yielded the expected size product.

Sequence comparisons of the protein gene using the Gap program showed that percent identities of the nucleotide and amino acid levels were, in general, very high among the 12 CGRMV isolates (94 to 100% in Table 3). With isolates CGRMV-Ita6 and -Leb (these reacted with both specific primers), four nucleotide sequences per virus source were compared and they showed identities of 98 to 99% for each virus (data not shown).

**Table 3.** Comparisons of the percent identities (numbers in bold type) and similarities among 268 amino acids in the coat proteins of twelve different CGRMV strains.

| Phylogenetic group | | I | | II | | | | | | | | III | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Strains | Ita6 | MI | Ita1 | Ita7 | Ita5 | Ww | Wp1 | F | Wp2 | Leb | BC | N |
| I | Ita6 | – | 97 | 97 | 97 | 96 | 96 | 97 | 97 | 97 | 97 | 97 | 97 |
| I | MI | 96 | – | 97 | 97 | 97 | 95 | 97 | 97 | 97 | 96 | 97 | 97 |
| II | Ita1 | 94 | 95 | – | 100 | 99 | 99 | 100 | 100 | 100 | 99 | 99 | 99 |
| II | Ita7 | 94 | 95 | 100 | – | 99 | 99 | 100 | 100 | 100 | 99 | 99 | 99 |
| II | Ita5 | 94 | 96 | 99 | 99 | – | 98 | 99 | 99 | 99 | 98 | 99 | 98 |
| II | Ww | 94 | 94 | 98 | 98 | 97 | – | 98 | 98 | 98 | 97 | 98 | 97 |
| II | Wp1 | 95 | 96 | 99 | 99 | 98 | 98 | – | 100 | 100 | 99 | 100 | 99 |
| II | F | 95 | 96 | 99 | 99 | 98 | 98 | 99 | – | 100 | 99 | 100 | 99 |
| II | Wp2 | 96 | 96 | 99 | 99 | 99 | 98 | 100 | 100 | – | 99 | 100 | 99 |
| II | Leb | 95 | 96 | 98 | 98 | 97 | 97 | 99 | 99 | 99 | – | 99 | 98 |
| III | BC | 95 | 96 | 97 | 97 | 97 | 97 | 98 | 98 | 99 | 98 | – | 99 |
| III | N | 96 | 97 | 97 | 97 | 97 | 96 | 98 | 98 | 98 | 97 | 98 | – |

**DISCUSSION**

Our RT-PCR results confirm the occurrence of CGRMV in the Mediterranean region, which was previously identified by biological assays (Savino *et al.*, 1997).

With two specific primer pairs capable of distinguishing between CGRMV-F (primers F39h and F39c) and CGRMV-N (primers N5h and N5c), 11 virus sources produced the expected product size with the specific primers for CGRMV-F. However, the Italian (CGRMV-Ita6) and Lebanese (CGRMV-Leb) sources also reacted with specific primers for CGRMV-N, suggesting that these sources may be representatives of mixed virus infections or examples of putative recombinants of CGRMV-N and -F.

Phylogenetic analyses of coat protein genes have been used to determine the relationships of different strains of potyviruses (Wang *et al.*, 1994), luteoviruses (Vincent *et al.*, 1990), and ilarviruses (Guo *et al.*, 1995; Scott *et al.*, 1998). Comparisons of CGRMV coat protein genes showed that at the nucleotide level, the virus strains CGRMV-MI and -Ww differed by as many as 89 nucleotides. However, these nucleotide changes resulted in as few as 15 amino acid changes. Because of this, the overall impact of such changes may be minimal.

Based on phylogenetic analyses of coat protein sequences, the 12 virus strains were clustered into three clades. Interestingly, the two Californian strains appeared in separate clades and, concomitantly, induced strain specific symptoms in Shirofugen flowering cherry (Zhang *et al.*, 1998b). The apparent wide geographic dispersion of

coat protein variants underscore the possible consequence from unrestricted world trade of propagative materials and that no clear-cut clusters of variants, indicative of specific geographic origins can be identified.

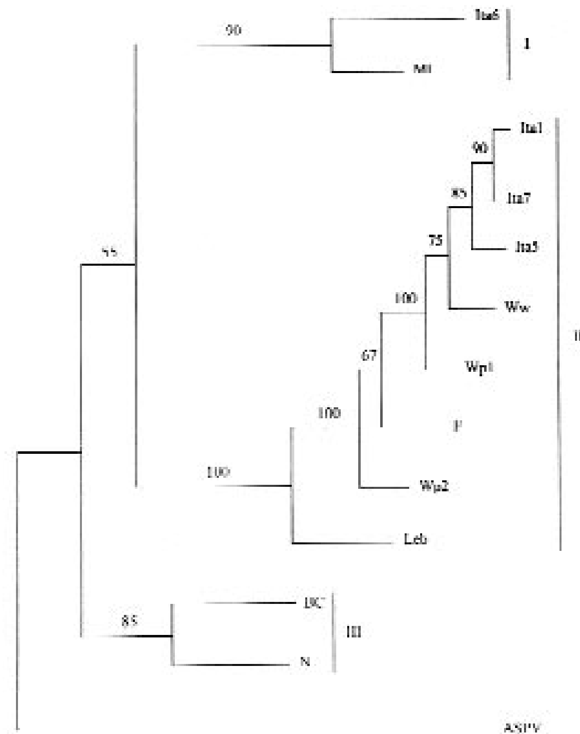


**Fig. 1.** A phylogram based on nucleotide sequences of coat protein genes from 12 CGRMV sources. Strains CGRMV-F and –N were from California; CGRMV-Wp1, -Wp2, and –Ww from Prosser and Wenatchee, Washington; CGRMV-MI, Michigan; CGRMV-BC, British Columbia, Canada; CGRMV-Ita1, -Ita5, -Ita6, and -Ita7, Italy; and CGRMV-Leb, Lebanon. The numbers above the branch lines are bootstrap confidence value. The nucleotide sequence of apple stem pitting virus (ASPV) coat protein was used as the out group.

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