cDNA cloning and molecular characterization of cherry green ring mottle virus

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The complete nucleotide sequence of the cherry green ring mottle virus (CGRMV) genome was determined to be 8372 nt excluding a 3' poly(A) tail. Based on computer analysis and sequence comparison, five open reading frames (ORFs) were identified on the virion strand encoding: a putative RNA-dependent RNA polymerase, a triple gene block and a coat protein. Two other ORFs with \( M \) values over 10000 and internal to the helicase and coat protein genes, but of unknown function, were also identified. Sequence and genome structure comparisons with other filamentous viruses indicated that CGRMV is most similar to apple stem pitting virus, some carlaviruses and potexviruses. However, it is different from members of any of these virus groups in regard to sequence homology and genome organization. A chimeric fusion coat protein was expressed in E. coli and antibodies specific for the CGRMV coat protein were raised in rabbits. The antibody was used in Western blot analyses to detect the CGRMV coat protein in infected cherry tissue.

Introduction

Cherry green ring mottle virus (CGRMV) was first reported on Montmorency sour cherry (Prunus cerasus L.) trees in 1937 in Michigan. CGRMV infects several Prunus species, including sweet cherry (P. avium L.), sour cherry (P. cerasus L.), oriental flowering cherry (P. serrulata L.), peach [P. persica (L.) Batsch] and apricot (P. armeniaca L.) in fruit-growing regions throughout North America and Europe (Parker et al., 1976). On Montmorency sour cherry, CGRMV produces yellow mottling and ring-like bands on leaves, and misshapen, bitter and unmarketable fruit. Sweet cherry, peach and apricot are symptomless hosts of CGRMV. Indicator hosts of CGRMV include Kwanzan and Shirofugen flowering cherry trees (P. serrulata L.) in which CGRMV produces vein necrosis and epinasty of leaves 2–3 months after graft-inoculation. A Michigan strain of CGRMV was isolated and characterized by Zagula et al. (1989). The purified virus particles ranged from 1000–2000 nm in length and 5–6 nm in diameter, with a buoyant density of 1.24–1.25 g/cm³, an ssRNA of 2.5 \( \times \) \( 10^6 \) Da and coat protein of 25 kDa. There were also fibrillar inclusions in cells of CGRMV-infected plants. Based on these findings, it was suggested that CGRMV was possibly a closterovirus (Zagula et al., 1989).

During a search for the agent that causes cherry stem pitting disease, we found a virus disease of Shirofugen flowering cherry that was also detected in some commercial sweet cherry trees in California (Zhang, 1996). The symptoms induced on graft-inoculated trees of Kwanzan flowering cherry closely resembled those described for green ring mottle disease (Milbrath, 1960). RT–PCR assays that were developed for this virus also amplified products from reference strains of CGRMV from Michigan, Washington, British Columbia, Canada and Italy (Zhang, 1996). Based on these results, it was concluded that this virus was a strain of CGRMV.

The closterovirus group is a collection of plant viruses whose taxonomy is still evolving. Some members in one of the two original closterovirus subgroups that possessed smaller size genomes were subsequently reclassified into new groups on the basis of additional molecular genetic information. Examples include apple stem grooving virus (ASGV) reclassified as a capillovirus (Yoshikawa et al., 1992; Francki et al., 1991) and apple chlorotic leaf spot virus (ACLSV) reclassified as a trichovirus (Martell et al., 1994; Sato et al., 1993; German et al., 1992). Due in part to these recent changes in virus taxonomy and because the original classification of CGRMV was based on limited morphological and cytopathological information, we cloned and characterized the full-length CGRMV genome in order to better assess its taxonomic status. Here, we report the molecular characterization of CGRMV and
its relationship to other filamentous plant viruses on the basis of its genome sequence and organization and phylogenetic analysis of its RNA-dependent RNA polymerase and coat protein genes.

Methods

■ Virus source and host range. The CGRMV isolate used in this study was from a diseased Shirofugen flowering cherry found in Davis, CA and designated the ‘N’ strain. The virus was maintained in peach trees following its graft-transmission with bud chips from the original Shirofugen tree.

For sap inoculations onto herbaceous plants to determine the herbaceous host range of the virus, leaf tissues of CGRMV-N infected Shirofugen were triturated in 0.1 M sodium phosphate buffer, pH 7.4, and used as inoculum to sub-inoculate carbonbund dusted leaves of Nicotiana tabacum L. cv. Xanthi n. Xanthi nc, Turkish and Hannah 425, N. benthamiana Domini, N. glutinosa, N. clevelandii, N. occidentalis Wheeler ‘No.1’ and ‘37B’, Chenopodium quinoa Willd., C. amaranticolor Coste & Reyn., Cucumis sativus L. cv. National Pickling, Lycopersicon esculentum Mill., Gomphrena globosa L., Cucurbita maxima cv. Buttercup and Buttercut squash. Three plants of each species were inoculated. Test plants and mock-inoculated controls were observed for viral symptoms for up to 8 weeks in the greenhouse.

On woody plants, diseased or healthy budchips of Shirofugen flowering cherry were grafted onto one to three trees of Bing cherry, Fay Elberta peach, Nanking cherry (P. pomentosa Thunb.), Nonpareil almond (P. dulcis Mill.) Webb and Shirofugen flowering cherry. Two years after inoculation test trees and healthy controls were assayed for presence of CGRMV by dsRNA analysis (Valverde, 1990) and RT–PCR analysis using primers specific for CGRMV-N (see below).

■ cDNA cloning of the CGRMV genome. dsRNA was isolated from infected peach leaves as described by Valverde (1990) and used as template for cDNA cloning. Various strategies were used at different stages of the cloning process. Initial cDNA cloning was performed using random primers following the procedure described by Hillman et al. (1989), using the ‘SuperScript plasmid system for cDNA synthesis and plasmid cloning kit’ (Gibco BRL). The major portion of the CGRMV genome was cloned by using viral specific oligonucleotide primers NR1 (5′-GCGGCCGCTTACCTTAG-3′) and NR4 (5′-GCGGCCGCGTCTGATGTGATTGCCT-3′), which contains a NotI site (underlined) at the 5′ end, and the ‘SuperScript plasmid system for cDNA synthesis and plasmid cloning kit’. The 5′ and 3′ end sequences were cloned using the ‘CapFinder PCR cDNA library construction kit’ (Clontech) in combination with viral specific primers SSKTF1, 5′-ATCCCTATCATCACCACAAATTT-3′. The PCR products were ligated into the EcoRI site of the pRSET fusion protein expression vector (Invitrogen). The fusion protein was expressed in E. coli (strain DE3::BL21), purified from bacterial lysates using a NTA + agarose column (Qiagen Inc., Valencia, CA), and electrophoresed on a 13% denaturing SDS–PAGE gel. The fusion protein band was excised and electroeluted from the gel matrix using an ‘Electro-elutor’ (Bio-Rad). Approximately 0.5 mg of the protein was used to immunize New Zealand White rabbits for antibody production. Antisera were collected and the IgG purified from the serum using a Protein A–Sepharose (Pharmacia) chromatography column.

Virions of CGRMV were partially purified from 20 g of infected cherry leaf tissue using a part of the procedure of Zagula et al. (1989) and analysed by SDS–PAGE. Electrophoresed proteins were transferred to a nitrocellulose membrane using a ‘Mini trans-blot electrophoretic transfer cell’ (Bio-Rad) following the manufacturer’s protocol. The nitrocellulose membrane was probed with IgG (5 μg/ml) from the CGRMV coat protein fusion antibody as described by Harlow & Lane (1988).

■ Sequence comparison and phylogenetic analysis. The genome sequence and ORF organization of CGRMV were compared to the following viruses: apple stem pitting virus (ASPV, D21829; Jelkmann, 1994), potato X potexvirus (PVX, D00344; Huisman et al., 1988), lily X potexvirus (LVX, X15342; Memelink et al., 1990), lily symptomless carlavirus (LSV, D43801; Takamatsu et al., 1994), potato virus S carlavirus (PVS, D00461; MacKenzie et al., 1989) and potato M carlavirus (PVM, X53062; Zavriev et al., 1991).

Phylogenetic analysis was performed to determine the relationship of CGRMV with other filamentous plant viruses. A sequence of about 300 amino acids surrounding the two core sequences (GxxxCxxTxxNT/S and GDD) which are near the C terminus of the RNA-dependent RNA polymerase was selected for the comparison. The following viruses were included in the analysis: potato Y potyvirus (PVY, M94591; Thole et al., 1993), tobacco etch potyvirus (TEV, M15239; Allison et al., 1986), beet yellows closterovirus (BYV, X73474; Agranovsky et al., 1994), citrus tristeza closterovirus (CTV, U16304; Karasev et al., 1995), papaya mosaic potexvirus (PMV, D13957; Sit et al., 1989), PVX, white clover mosaic potexvirus (WCVM, X16636; Beck et al., 1990), blueberry scorch carlavirus (BSV, L25658; Cavileer et al., 1994), PVMS, PVAM, ASPV, apple chlorotic leaf spot trichovirus (ACLTS, D41996; Sato et al., 1993) and apple stem grooving carlavirus (ASCV, D14995; Yoshikawa et al., 1992). Tobacco mosaic tobamovirus (TMV, D13438; Ikeda et al., 1993) sequence was used as the outgroup. The sequences were aligned using the ‘Plepu’ program of GCG with a gap weight of 2 and gap length weight of 1. The sequence alignment was then analysed by PAUP (Phylogenetic Analysis Using Parsimony), version 3.1.1 (Illinois Natural History Survey, Champaign, Ill.). A phylogram was generated by performing bootstrap analysis of 100 replicates with 10 random additions of each replicate. Branch swapping was carried out using the tree bisection and reconnection method. Phylogenetically uninformative bases were not included in this analysis.

Amino acid sequences of the following viral coat proteins were also compared using the same program: BSV, PVX, PVAM, ASPV, WCVM, PMV, PVX and CGRMV. ACLSV sequence was used as the outgroup. Coat protein sequences were analysed as described above except using a gap weight of 3 and gap length weight of 1.

Results and Discussion

Host range

CGRMV-N was successfully graft-transmitted to Shirofugen flowering cherry, Bing sweet cherry, Nanking cherry and
Fay Elberta peach, but not to almond, as determined by RT–PCR detection assays. On inoculated Shirofugen flowering cherry, CGRMV-N caused epinasty and necrosis of leaves, necrotic bark tissues, pitting of the woody cylinder and dieback of limbs. However, CGRMV-infected sweet cherry and Nanking cherry trees were symptomless, results which agree with previous research (Zagula et al., 1989). Because our early attempts to purify CGRMV viroids directly from woody plant hosts were unsuccessful, it was only possible to isolate and clone the CGRMV genome using dsRNA isolated from infected plant tissue. Because dsRNA titres were highest in infected peach, this host was used as the dsRNA source plant for this study.

**Nucleotide sequence of CGRMV genome**

Multiple, overlapping cDNA clones were sequenced in both orientations to determine the complete nucleotide sequence of the CGRMV genome. The CGRMV genome was determined to be 8372 nt, excluding the 3’ poly(A) tail. The nucleotide sequence of CGRMV was submitted to GenBank under accession number AF017780.

**Coding regions**

An open reading frame search, using the frames program of GCG in all three reading frames of both the positive and complementary strands of the CGRMV genome, revealed seven significant ORFs on the positive strand and no significant ORFs on the negative strand of the CGRMV-N genome.

ORF1 (nucleotides 103–6174) potentially encodes a poly-peptide of 2023 amino acids with a calculated Mr of 230,000. Database searches showed that this protein has significant amino acid sequence similarities to the RNA-dependent RNA polymerases of ASPV (61–0%) (Jelkmann, 1994), some carlaviruses (57–4% with PVM) and some potexviruses (50–7% with PVX). The conserved sequence motifs GxGxxGKS/T and GDD, which reside within the core of the viral RNA-dependent RNA polymerase, are located at nucleotide positions 5668–5694 and 5764–5772 in the CGRMV genome. A methyltransferase domain, a papain-like proteinase domain and an NTP-binding/helicase domain were also identified on ORF1 by sequence homology. For these reasons, it is likely that ORF1 encodes an RNA-dependent RNA polymerase.

ORF2 (nucleotides 6174–6842) potentially encodes a poly-peptide of 223 amino acids with a calculated Mr of 24,700. This sequence has significant similarities to the NTP-binding helicase of ASPV (65–8%), potexviruses (54–5% with PVX) and carlaviruses (50–7% with PVM), and the NTP-binding helicase motif GxGxxGKS/T was identified at positions 6255–6278 in the CGRMV-N genome. Therefore, ORF2 may be an NTP-binding helicase gene; however, the activity of the ORF2 protein was not determined experimentally.

ORF3 (nucleotides 6843–7184) potentially encodes a poly-peptide of 114 amino acids with a calculated Mr of 12,400. This sequence has significant similarities to the membrane protein of carlaviruses (60–7% with PVM), potexviruses (58–7% with PVX) and ASPV (58–0%). Therefore, ORF3 is tentatively classified as a membrane protein gene.

ORF4 (nucleotides 7120–7323) potentially encodes a poly-peptide of 67 amino acids with a calculated Mr of 7,000. This sequence has extensive similarity to polypeptides of potexviruses (Huismann et al., 1988), carlaviruses (Zavriev et al., 1991) and ASPV (Jelkmann, 1994) which comprise part of the triple gene block.

ORFs 2, 3 and 4 constitute a triple gene block whose organization is conserved among potexviruses and carlaviruses (Rupasov et al., 1989). A triple gene block has also been identified in ASPV (Jelkmann, 1994), barley stripe mosaic Hordeivirus (Gustafson & Armour, 1986), beet necrotic yellow vein Furovirus (Bouzoubaa et al., 1986) and Nicotiana velutina mosaic virus (Randles & Rohde, 1990). These three partially overlapping ORFs, which encode a putative NTP-binding helicase (Skryabin et al., 1988; Gorbalenya et al., 1988) and two membrane-bound proteins (Morozov et al., 1990), are thought to be involved in cell-to-cell movement of the viruses (Huismann et al., 1988; Skryabin et al., 1988; Petty & Jackson, 1990). It is thought that the reason these genes overlap is because they can be coordinately translated either through the expression of multi-cis-tronic mRNAs (Morozov et al., 1990) or through the production of fusion proteins by translational frameshifting (Prüfer et al., 1992).
ORF5 (nucleotide 7374–8180) potentially encodes a polypeptide of 269 amino acids with a calculated Mr of 29700. Database searches showed that this protein has significant amino acid sequence similarities to coat proteins of ASPV (51.7%), carlaviruses (48.7% with PVM) and potexviruses (47.6% with PVX). Also, the antibody produced against the chimeric fusion protein expressed from ORF5 detected a protein in CGRMV-infected plants whose size would correspond to the predicted size of the viral coat protein (see below). Therefore, ORF5 is likely the CGRMV coat protein gene.

ORF2a (nucleotide 6202–6573) and ORF5a (nucleotide 7381–7872), which are located within ORF2 and ORF5 respectively, potentially encode polypeptides of 124 and 164 amino acids with calculated Mr values of 14000 and 18300. No sequence similarity was found between the proteins potentially encoded by these ORFs and any other known protein in GenBank. It is not known if these ORFs are functionally expressed in planta.

Noncoding regions at the 5′ and 3′ ends of the CGRMV genome

The 5′ end noncoding region consists of 102 nucleotides, a portion of which is conserved among potexviruses and carlaviruses (Huisman et al., 1988; Zavriev et al., 1991), suggesting CGRMV is related to these viruses. The 3′ noncoding region consists of 132 nucleotides excluding the poly(A) tail. A polyadenylation signal, AAUAAA, is located at position 8293–8298. A hexanucleotide motif (5′ ACUUAA 3′), which is conserved among potexviruses and carlaviruses (Skryabin et al., 1988; Sit et al., 1990; Bancroft et al., 1991), is located just downstream from the polyadenylation signal at position 8299–8304. This latter motif has been suggested to play a functional role in the synthesis of viral RNAs (White et al., 1992).

Serological characterization of CGRMV coat protein

The putative CGRMV coat protein gene (ORF5) was expressed in E. coli and antisera were produced against the fusion proteins in rabbits. In Western blots, two proteins (Mr values 30000 and 23000) were detected in the CGRMV-infected cherry leaf sample which were not detected in protein preparations of healthy cherry (Fig. 1). The larger protein is most likely the CGRMV coat protein because the fusion coat protein (Mr 33000) contained a leader sequence with an Mr of 3000. The smaller protein may be a breakdown product of the coat protein or possibly another viral component. The detection of the CGRMV coat protein in infected plant tissue
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Fig. 3. Phylograms of (A) a portion of the putative RNA-dependent RNA polymerase region showing the relationship of CGRMV to other filamentous plant viruses, which was generated by PAUP analysis of an aligned region of about 300 amino acids around the two core sequences of the replicase; (B) relationship of CGRMV with ASPV and selected members of potexvirus and carlaviruses groups generated by PAUP analysis of an alignment of coat protein amino acid sequences. Arrow denotes position of CGRMV. The numbers above the branches indicate the bootstrap confidence values.

using antibodies made to fusion protein expressed in E. coli provides further evidence that the dsRNA used as the cloning template was indeed associated with CGRMV.

Genome comparison and phylogenetic analysis of CGRMV

As shown in Fig. 2, the CGRMV genome organization has extensive similarities to ASPV, potexviruses (PVX, LVX) and carlaviruses (LSV, PVS and PVM). It has a 3' poly(A) tail, a similar number and organization of ORFs, and a triple gene block as reported for these viruses (except for LVX). Without considering ORF2a and ORF5a, whose function or expression is unknown, the genome organization of CGRMV is most similar to ASPV; however, CGRMV has a smaller RNA-dependent RNA polymerase and a smaller coat protein.

Fig. 3 shows the phylogenetic relationship between CGRMV and other filamentous viruses based on analysis of a portion of the putative RNA-dependent RNA polymerase (Fig. 3A) and the full-length coat protein genes (except for ASPV which was truncated to the size of carlavirus coat protein) (Fig. 3B). The members of each virus group clustered together, as expected. Phylogenetic analysis of both genes shows that CGRMV is most similar to ASPV, while CGRMV was most similar to carlaviruses by analysis of the replicase gene and most similar to potexviruses by analysis of the coat protein gene.

Although its precise taxonomic classification remains uncertain, these molecular analyses clearly show that CGRMV is not a clodterovirus as previously suggested (Zagula et al., 1989). The overall genomic organization and properties of specific CGRMV genes have attributes in common with ASPV, potexviruses and carlaviruses. Although it is likely that
CGRMV would be in a group with ASPV and grapevine rupestris stem pitting associated virus (Y.-P. Zhang, unpublished data), the CGRMV genome organization and physical properties of some of its genes appear to be unique among known plant viruses.

References


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