Analysis of Double-Stranded RNAs from Cherry Trees with Stem Pitting in California

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ABSTRACT

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Five distinct dsRNA species were recovered from Bing sweet cherry (*Prunus avium* (L.) L.) trees with stem pitting symptoms. A 4.7-kilobase pair (kbp) dsRNA was isolated from mahaleb rootstock (*P. mahaleb* L.); an unrelated 4.7-kbp dsRNA, always co-purified with a 1.3-kbp dsRNA, and a 9-kbp dsRNA were from Bing cherry. In addition, an 8.5-kbp dsRNA found in diseased Shirofugen flowering cherry and in Bing cherry was identified as sour cherry green ring mottle virus (CGRMV). The larger, 8.5- and 9.0-kbp dsRNA species were graft-transmissible, while the smaller ones were non-transmissible and appeared cryptic in nature. Reverse transcription-polymerase chain reaction (RT-PCR) assays were developed for each dsRNA species by cloning and sequencing cDNA synthesized from the dsRNA templates. When several diseased collections were assayed by RT-PCR, approximately 14% reacted positively with primers for the 9.0-kbp dsRNA or CGRMV. Although CGRMV and the 9.0-kbp dsRNA caused wood-marking symptoms in graft-inoculated Mazzard (*P. avium*) seedling trees, no xylem or canopy symptoms developed in grafted Bing cherry. The causal agent or agents of cherry stem pitting have not been identified.

In the 1960s, an outbreak of *Prunus* stem-pitting disease occurred in peach orchards in Maryland, New Jersey, and Pennsylvania (3,15,16,23). The causal agent was identified as tomato ringspot virus (TmRSV; 22). Also, in Canada, a stem pitting of cherry was reported and associated with tomato bushy stunt virus (TBSV) infections (8). In California, Mircetich and co-workers (17) graft-transmitted another infectious agent from sweet cherry trees with stem-pitting (CSP) symptoms, where diseased trees were free of TmRSV and cherry leafroll virus (CLRV; 10,21). Thus, the search for other agent or agents causing CSP was warranted.

Initial symptoms of CSP include premature defoliation from the lower portion of affected trees and severe pitting in the woody cylinder of Bing scion and the sweet cherry rootstock, Mazzard (also *P. avium* (L.) L.). Wood-marking symptoms are much less conspicuous on the Mahaleb rootstock (*P. mahaleb* L.). With chronic

infections, Bing trees on either rootstock exhibit sparse canopies, unmarketable fruit, limb dieback, and eventually tree death (24). The disease is widespread in California and may also occur in Oregon and Washington (1,14). Disease incidence in California orchards ranges from a few infections to 44%, and several orchards were replanted due to rapid tree decline often associated with CSP (24).

More than 90% of plant viruses have single- or double-stranded RNA genomes (6,12,26). During the replication of single stranded-RNA viruses, a complementary strand of viral RNA is synthesized. An annealed double-stranded form (dsRNA) can be isolated by phenol extraction. Morris and Dodds (18) developed methods for the isolation and analysis of viral dsRNAs in diseased plant tissues. Their protocols have been used extensively to diagnose and characterize plant viral infections (7,25) and cryptic viruses (4,5).

We have searched for dsRNAs in CSP-affected Bing cherry trees in an attempt to determine the etiology of the disorder. We have isolated and partially characterized four dsRNAs from diseased sweet and flowering cherry trees. Complementary DNAs corresponding to these dsRNAs were cloned and partially sequenced in order to identify oligonucleotide primers which could be used in reverse transcription-polymerase chain reaction (RT-PCR) assays. We report here the results of our investigations.

MATERIALS AND METHODS

Bing cherry trees with and without stem-pitting symptoms were sampled from 10 commercial orchards (minimum of 10 samples per orchard) and budwood source trees at two nurseries (five samples each) located in the Stockton-Lodi or Modesto areas in California. Sampling of the trees was done during the summer months, usually following an irrigation, because canopy symptoms on diseased trees were evident and for easy removal of trunk bark to view wood markings. Several collections of shoots, leaves, pedicles, and roots were made over a 2-year period. Also, collections from two Shirofugen flowering cherry (*P. serrulata* Lindl.) trees in different orchards in Davis, California were made. Both of these tree sources exhibited bark necrosis and stem-pitting symptoms of the scion and stem pitting on the Mazzard understocks. The two sources were graft-inoculated on Kwanzan flowering cherry (*P. serrulata*), which later developed symptoms resembling those described for sour cherry green ring mottle virus (CGRMV; 13). Therefore, virus cultures from Prosser, Washington (CGRMV-WA) and from Michigan (CGRMV-MI) were used as positive controls in RT-PCR. All infected tissues were destructively sampled, and were not propagated in California.

Tissue extracts of dsRNA were prepared (see below) from each collection and tested by RT-PCR. Also, dsRNAs of cucumber mosaic virus were used as molecular size markers in polyacrylamide gel electrophoresis assays.

dsRNA isolation. The dsRNAs used for cDNA cloning were purified from several collections of sweet or flowering cherry or Mahaleb rootstock, as previously described (18,25). Leaf, pedicle, or root tissues (6 g) were frozen in liquid nitrogen, then ground to a fine powder in a mortar and pestle and extracted in a mixture of 14 ml 1× STE (0.05 M Tris, pH 6.8, 0.1 M NaCl, and 1 mM EDTA), 2 ml 10% sodium dodecyl sulfate (SDS), 1 ml 2% bentonite, and 18 ml 2× STE-saturated phenol. The entire mixture was shaken for 30 min and centrifuged for 15 min at 8,000 rpm in a Beckman SS34 rotor. The supernatant was adjusted to 16.5% ethanol and passed through a cellulose (Whatman, CF-11) column that bound the dsRNA. After an extensive wash with 1× STE buffer at

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16.5% ethanol, the dsRNA was eluted from the column with 1× STE and precipitated with one-tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol. The dsRNA was resuspended in 50 µl water and half of the preparation was electrophoresed through a 6% polyacrylamide gel. The dsRNA was stained with ethidium bromide (1 µg/ml) and visualized by UV light. Purified preparations of dsRNAs were used to produce cDNA, which was cloned and sequenced.

The dsRNA templates used in RT-PCR detection assays were isolated from field collections by a small-scale purification procedure (27). Eight leaf discs (100 mg) per sample were processed in a 1.5-ml microfuge tube. The tube and contents were immersed in liquid nitrogen and the frozen tissue ground to a fine powder with a flame sealed-pipette tip (1,000-µl size) serving as a pestle. The powdered tissue was suspended in 600 µl 1× STE, 80 µl 10% SDS, and 1 ml 2× STE-saturated phenol. The mixture was vortexed and centrifuged for 5 min at maximum speed (14,000 rpm) in an Eppendorf microfuge. The upper aqueous phase was removed and adjusted to a final concentration of 16.5% ethanol. Approximately 0.1 mg CF-11 was added, mixed by vortexing, and the CF-11 pelleted by centrifugation. The buffer was discarded. The CF-11 pellet was dispersed in 1 ml 1× STE at 16.5% ethanol and repelleted by centrifugation. This process was repeated three times. After the third wash, the bound dsRNA was eluted from the CF-11 with 200 µl 1× STE. The elution-repelling step was repeated a second time and the aqueous phase combined; total 400 µl. The dsRNA was precipitated as above, then collected by centrifugation, air-dried, resuspended in 15 µl distilled water, and used as templates for RT-PCR amplification.

cDNA cloning and sequencing. Purified dsRNAs were cloned following a pro-

cedure described by Hillman et al. (9) with some modifications. All reagents were purchased from Gibco BRL (Gaithersburg, MD) unless otherwise stated. Approximately 1 µg dsRNA was denatured using 40 mM methyl mercury hydroxide (CH₃HgOH) instead of dimethyl sulfoxide (DMSO). Synthesis of complimentary DNA was done with a cDNA synthesis kit. The resulting double-stranded cDNA was tailed with oligo d(C), annealed to oligo d(G) tailed pUC 9 (Pharmacia Biotechnology, Inc., Uppsala, Sweden), and transformed *Escherichia coli* strain DH5α following the heat shock procedure provided by the manufacturer. White, ampicillin-resistant colonies were screened using a ³²P-labeled cDNA probe made from the respective dsRNA templates. Hybridizing cDNA clones were characterized by restriction analysis and Southern blot hybridization. Both ends of selected cDNA clones were sequenced using M13/pUC forward and reverse universal primers and a double-stranded DNA cycle sequencing kit following manufacturer's recommendation.

RT-PCR assay. Primer pairs were designed based on the sequence information obtained. In the reverse transcription reaction, 2 µl of a dsRNA preparation was mixed with 0.75 µl of 10 µM per primer and 6.2 µl water. The mixture was overlaid with a drop of mineral oil, incubated 8 min at 95°C to denature the dsRNA, and chilled in an ice bath. Reagents from commercial stock solutions were added to final concentrations of 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM MgCl₂; 5 mM per deoxynucleotide triphosphate; 5 U/µl RNase inhibitor; and 5 U/µl MMLV-reverse transcriptase; final volume was 15 µl. The mixture was incubated for 30 min at 42°C followed by 5 min at 95°C to denature the RNA/cDNA hybrid, then chilled in ice. For use as template, 5 µl of this cDNA mixture

was added to 25 µl of a PCR reaction mixture containing a final concentration of 10 mM Tris-HCl, pH8.3; 50 mM KCl; 200 µM per deoxynucleotide triphosphate; 2.75 mM MgCl₂; 0.15 µM per primer; and 1.5 U Taq DNA polymerase (5 U/µl stock solution, Perkin-Elmer Inc., Branchburg, NJ). The PCR reaction was performed in a Perkin-Elmer DNA Thermal Cycler Model 480, which was programmed for 3 min at 95°C, followed by 35 alternating cycles of 1 min at 94°C, 1 min for annealing at specific temperatures per primer pairs (Table 1), and 1 min at 72°C accompanied by a final cycle for 10 min at 72°C. Fifteen µl of the PCR reaction was electrophoresed through a 1% agarose gel, stained with ethidium bromide, and the products visualized with UV illumination.

Graft-transmission test. Collections of bud wood or root tissues from CSP-affected cherry trees (all had tested positive previously by RT-PCR for at least one of the dsRNA species) were graft-inoculated on healthy indicator trees of Bing scion/Mahaleb rootstock and Shirofugen flowering cherry/Mazzard rootstock. In addition, two viruses from Shirofugen flowering cherry (later identified as CGRMV and designated CGRMV-F and -N; see below) were transmitted to more trees of Shirofugen flowering cherry, and CGRMV-F was further tested on several seedling trees of Mazzard cherry. The graft-inoculation was accomplished by placing three bud chips beneath the bark flap and wrapping with budding rubber or, in a process called bottle-grafting, by inserting the wedge-shaped end of mature limbs (approximately 25 cm in length) into an angled cut (up to 2.5 cm deep) made in a similar-size limb on the indicator tree. The cambial margins of the inoculum unit and indicator limb were placed in direct contact before binding with adhesive tape. The bottom end of the inoculum unit was

Table 1. cDNA clones, oligonucleotide primers, annealing temperature, and the size of the reverse-transcription-polymerase chain reaction (PCR) products in kilobases (kb) for each of the five dsRNA isolates

dsRNA isolate	cDNA clone	Primer	Primer sequence	Temperature (°C)	PCR product (kb)
R	pR18	R18f	5'GAGCATAGGATGAAGCCA3'	50	0.6
		R18r	5'CTCTGCTCTACAGACATGTC3'	50	0.6
	pR60	R60f	5'TTCCTCACCACGAGGCACCT3'	59	0.6
		R60r	5'TCAGGCGTCGTCGTTAG3'	59	0.6
P	pP22	P22f	5'TCAAGGATAATGCGCGCAAC3'	55	0.2
		P22r	5'GTGTAGTAATGGCCGCTCT3'	55	0.2
	pP33	P33f	5'GAGAGTAGGCTTGGCGCGTG3'	61	0.2
		P33r	5'GAAGCTTGCTTCAAGGGATGA3'	61	0.2
S	pS7	S7f	5'ACCAAGAGTAGCTGCCTTGCT3'	59	0.7
		S7r	5'CGTGGACTTATTTCGATATGACTT3'	59	0.7
	pS22	S22f	5'GCTTCTAATGCTTCAGTCACAG3'	59	1.0
		S22r	5'GACATCCAGACACCCCTTATC3'	59	1.0
F	pF39	F39f	5'TGCAGTCTAGCTCCGATCAATT3'	59	0.35
		F39r	5'ACCTTCTCGTTAGCTCATTACA3'	59	0.35
	pF401	F401f	5'ATGAGTTGAGGGGTTGGGCT3'	59	1.1
		F401r	5'ACCACGCCAGCAAGATTGT3'	59	1.1
N	pN5	N5f	5'AGACTGTCAATCCGATGCTCG3'	59	1.2
		N5r	5'GAGCCATAGTGTGGTTGAGCT3'	59	1.2
	pN34	N34f	5'ACCCAGTCTGATGTGATTGCCT3'	61	1.2
		N34r	5'GCTCATTACATCAACAGTTGCAC3'	61	1.2

then immersed in water held in plastic bottles taped to the tree. In addition to bud chip and bottle-grafts, root bark grafts were made onto the Mahaleb rootstocks of Bing cherry trees. All grafted trees (minimum of two indicator trees per collection) were observed for disease symptoms for 3 years and assayed by RT-PCR. Ungrafted trees served as healthy controls. The indicator trees ranged from 2 to 4 years old and were planted in an orchard site maintained by the Department of Plant Pathology, University of California, Davis.

Sap-transmission assays. Collections of flower, pedicel, and root tissues were obtained from trees of Bing cherry growing on Mahaleb rootstock and exhibiting symptoms of CSP. Tissue (1 g) was triturated in 10 ml of 0.1 M sodium phosphate buffer, pH 7.4, containing 3% nicotine, and gently rubbed onto carborundum-dusted leaves of *Nicotiana tabacum* L. cvs. Xanthi n, Xanthi nc, Turkish, and Havana 425; *N. benthamiana* Domin.; *N. glutinosa* L.; *N. clevelandii* Gray, *N. occidentalis* Wheeler No. 1 and 37B; *Chenopodium amaranticolor* Coste & Reyn.; *C. quinoa* Willd.; *Cucumis sativus* L. cv. National Pickling; *Lycopersicon esculentum* Mill.; *Gomphrena globosa* L.; and *Cucurbita maxima* Dcne. cvs. Buttercup and Butter-nut. Three plants of each species were used per collection and an equal number of control plants were similarly rubbed with extraction buffer. All plants were maintained for 2 months in a white-washed greenhouse equipped with two evaporative coolers and observed for symptoms.

RESULTS

Specific primers used in RT-PCR comprised two cDNA clones selected for each dsRNA species present in Mahaleb-source R, Bing-sources P and S, and Shirofugen-sources F and N. Both ends of the cloned inserts were sequenced (27) and oligonucleotide primers were designed based on the sequence information (Table 1). These primers were used in RT-PCR assays to amplify the corresponding region of the dsRNAs isolated from plant samples.

Each primer pair specifically amplified a portion of the homologous dsRNA template. However, two universal primer pairs derived from the Shirofugen source trees F (F401f,r; Table 1) and N (N34f,r) were cross-reactive and amplified products in RT-PCR using heterologous dsRNA templates (Table 2). Also, strain-specific primers of CGRMV-F (F39f,r) and -N (N5f,r) amplified only the homologous dsRNA preparations. Both universal primer pairs and the specific primer pair F39 reacted with CGRMV-WA and CGRMV-MI and closely related to CGRMV-F. The specific primer pair of CGRMV-N did not react with dsRNA templates of CGRMV-F (Table 2), CGRMV-WA, or CGRMV-MI (*data not shown*).

During a 2-year period, a total of 150 CSP-affected trees from the 10 commercial

orchards and 10 healthy appearing trees from two nurseries were tested by RT-PCR using all of the primers developed in this study. The 4.7-kilobase pair (kbp) dsRNA-R was detected in 3.3% of the root samples, but not in the Bing scions of the same trees. The doublet (4.7/1.3 kbp) dsRNAs of P were detected in 67.3% of the scions collected from nine commercial orchards and five trees sampled at one nursery. Our attempts to graft-transmit dsRNAs-R and -P failed; grafted trees continued to appear healthy and they tested negative by RT-PCR.

The largest dsRNA (designated S; approximately 9 kbp) was recovered from a single CSP-affected Bing cherry orchard, and two slightly smaller dsRNAs (designated CGRMV-F and CGRMV-N; approximately 8.5 kbp) from two stem-pitted Shirofugen flowering cherry trees. In collections made of commercial orchard trees, the incidence of dsRNAs S, CGRMV-F, and CGRMV-N were 6.7, 4.7, and 2.7%, respectively. In grafted Bing/Mahaleb indicator trees, there were no canopy symptoms or internal wood markings after 3 years of incubation. However, on grafted Shirofugen flowering cherry trees, infections associated with presence of dsRNA-S produced slightly raised "rash-like" markings in sucker growths arising from the Mazzard understock. With CGRMV-F infections, symptoms of bark necrosis, stem pitting, leaf stunting, and twisting were observed. Also, Mazzard trees grafted with CGRMV-F developed stem-pitting symptoms after 2 years. The CGRMV-N inoculum also induced bark necrosis and stem pitting as well as leaf vein necrosis, leaf scorch, and defoliation in Shirofugen flowering cherry. In Bing cherry scions, RT-PCR tests of the grafted trees confirmed that dsRNA-S, CGRMV-F, and CGRMV-N were infectious, but did not induce symptoms.

Similarly, all herbaceous host test plants remained free of symptoms.

DISCUSSION

We found several unique dsRNAs in our study of the etiology of CSP. cDNAs of these dsRNAs were prepared, sequenced,

and oligonucleotide primers synthesized for use in RT-PCR assays. A total of four dsRNA species were isolated from either CSP-diseased or apparently healthy Bing cherry. Also, two related, but biologically and molecularly distinct, strains of CGRMV were recovered from diseased Shirofugen flowering cherry trees.

In collections from nursery scionwood trees and diseased cherry orchards, tissue extracts assayed by RT-PCR demonstrated that all Bing cherry trees sampled at one nursery and in nine commercial orchards were positive for the 4.7/1.3-kbp dsRNAs. Incidence of the Mahaleb 4.7-kbp dsRNA was low. These unique dsRNA species were not infectious. The noninfectious doublet dsRNAs always appeared together and were detected in CSP-affected and in healthy appearing Bing trees. Also, Bing cherry trees from one nursery and a commercial orchard had assayed free of these dsRNA species using graft-indexing and RT-PCR test procedures. The doublet dsRNAs shared some properties in common with those described for cryptic viruses (5,12).

Since the Mahaleb 4.7-kbp dsRNA was similar to the genome size of TBSV (11), a comparison between both entities was made. However, the Mahaleb dsRNA species was not related to TBSV in biological and molecular assays reported elsewhere (27).

The largest dsRNA species (approximately 9 kbp in size, originally found in a CSP-affected tree in orchard S) was only detected in 10 diseased collections from the same orchard. Although latent infections (detected by RT-PCR) occurred in graft-inoculated Bing/Mahaleb trees, the same inoculum source grafted onto Shirofugen flowering cherry was positive by RT-PCR assays and caused wood marking in the Mazzard understock.

The 8.5-kbp dsRNAs of CGRMV-F and -N were isolated from different trees of Shirofugen flowering cherry with stem-pitting symptoms. These dsRNAs were later shown to be related, but distinct, strains of CGRMV by the symptoms caused in woody indicator hosts and by molecular genetic analyses reported else-

Table 2. Specificity and results (percentage of positive examples) of a reverse-transcription-polymerase chain reaction (PCR) survey of 150 sweet cherry trees using oligonucleotide primers specific for five dsRNAs

dsRNA isolate (% trees surveyed)	Primer designation	PCR amplification of each dsRNA				
		R	P	S	F	N
R (3.3)	R18f, r	+	—	—	—	—
	R60f, r	+	—	—	—	—
P (67.3)	P22f, r	—	+	—	—	—
	P33f, r	—	+	—	—	—
S (6.7)	S7f, r	—	—	+	—	—
	S22f, r	—	—	+	—	—
F (4.7)	F39f, r	—	—	—	+	—
	F401f, r	—	—	—	+	+
N (2.7)	N5f, r	—	—	—	—	+
	N34f, r	—	—	—	+	+

where (27). Historically, CGRMV infections in sweet cherry trees were latent and caused no obvious symptoms (2,19,20). With CGRMV-F, however, stem pitting symptoms were found in graft-inoculated mazzard trees and observed on the Mazzard understocks on both of the original diseased Shirofugen trees (J. K. Uyemoto, *unpublished*), suggestive of a causal relationship.

In summary, CSP appears to be a disease complex for which the primary causal agent or agents remains unknown. Because of the difficulties in graft-transmitting the agent or agents, the etiology of this disease remains unclear, and other biotic and abiotic factors such as DNA viruses, viroids, and the use of additional indicator tree species need to be considered in future studies of CSP.

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