Etiology and Host Range of a *Closterovirus* Associated with Plum Bark Necrosis-Stem Pitting Disease

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ABSTRACT

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Diseased plum (*Prunus salicina*) cv. Black Beaut trees developed stem gumming, severe bark necrosis, and stem pitting symptoms on the woody cylinder of the main trunk and scaffold branches. The sucker shoots of the peach (*Prunus persica*) cv. Nemaguard understocks exhibited oak-leaf patterns, but lacked the wood or bark markings. Other susceptible hosts included almond (*Prunus dulcis*), sweet (*Prunus avium*) and Japanese flowering (*Prunus serrulata*) cherries, and several plum (*Prunus salicina*) and prune (*Prunus domestica*) varieties. A purified preparation containing high molecular size dsRNAs was obtained initially from diseased cherry (*P. avium* × *Prunus pseudocerasus*) cv. Colt tissues. Healthy preparations were devoid of similar sized dsRNAs. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays with degenerate oligonucleotide *Closterovirus* primers, designed from the HSP70 gene, were used to amplify two DNA fragments of 0.67 and 0.56 kbp. The larger cDNA product was cloned, sequenced (AF195501), and compared with other viral sequences. Depending on the number of nucleotides used in the comparisons, identities ranged from 77% for *Grapevine leafroll associated virus* to 3 to 44% for *Little cherry virus*-1. Specific primers from the 0.67 kbp cDNA sequence were designed and used in subsequent RT-PCR assays. The associated 0.67 kbp HSP70 amplicon of *Plum bark necrosis-stem pitting associated virus* was detected in all graft-inoculated *Prunus* species and varieties except prune (*P. domestica* var. French Improved).

Plum bark necrosis-stem pitting (PBNSP) disease was first observed in 1986 affecting plum (*Prunus salicina* Lindl.) cv. Black Beaut trees in two orchards in Dinuba, CA (8). All of the Black Beaut plum trees had been whip-grafted with a single cultivar Wickson plum scion per tree to serve as a pollinizer, and all Wickson grafts failed. A year later, the same trees were grafted a second time with another source of Wickson scion wood, which successfully callused and grew. Meanwhile, scaffold branches with the failed grafts began to exude copious amounts of dark-colored gum and developed necrotic bark and stem pitting symptoms. Disease incidence approached 100%, and both sites were eventually replanted. We hypothesized the first collection of Wickson scions was diseased and the second healthy.

Subsequent graft-transmission tests performed at University of California at Davis (UCD) proved the infectious nature of PBNSP (8). In the present study, an associ-

ated virus, and the putative causal agent, was identified as a *Closterovirus* and designated as *Plum bark necrosis stem-pitting associated virus* (PBNSPaV). The host range, symptoms induced, and development of a reverse transcriptase-polymerase chain reaction (RT-PCR) assay for the associated *Closterovirus* are described.

MATERIALS AND METHODS

Inoculum source. The virus source was a Black Beaut plum tree maintained at UCD that was graft-inoculated with tissue from diseased Black Beaut plum trees taken in a commercial orchard (8). In the second year postinoculation, the trunks and scaffold branches of grafted trees developed gummosis, bark necrosis, and stem pitting symptoms. In addition, sucker growth arising from peach (*Prunus persica* (L.) Batsch) cv. Nemaguard understocks showed chlorotic oak-leaf patterns. No wood marks were observed on the main peach trunks. Plum bark and peach bud tissues (i.e., root sucker growth) were used as inocula for graft-inoculations and as positive controls in RT-PCR assays. The inoculum source tree had previously tested negative for *Prunus necrotic ringspot virus* and *Prune dwarf virus* by enzyme-linked immunosorbent assay and flowering cherry (*Prunus serrulata* Lindl.) cv. Shirofugen indexings, viruses commonly found in *Prunus* species.

Plum cultivars and *Prunus* species. Five trees each of nine *P. salicina* and *P.*

domestica L. plum cultivars—Angeleno, Black Amber, Black Beaut, Casselman, French Improved, Friar, Shiro, Stanley, and Laroda—were propagated (by a commercial propagator) on Citation rootstock (peach × plum hybrid). In spring 1997, the trees were transplanted to the field at UCD and spaced 45 cm apart and 90 cm between sets of five trees per cultivar. Choice of plum cultivar was based, in part, on the relative popularity and acreage planted in California. Later that fall, two or three trees per cultivar were graft-inoculated, each with three peach budchips. Non-grafted trees served as healthy controls. In September 2000, all plum trees were removed, trunks autoclaved, and bark tissue removed to expose the woody cylinder.

In the greenhouse, dormant seedlings of cherry (*Prunus tomentosa* Thunb.) cv. Nanking, cherry (*P. avium* (L.) L.) cv. Mazzard, and peach cv. GF 305 were potted during January 1998. Later in March, three diseased peach budchips or plum bark patches per tree were graft-inoculated on three to five plants per species. All potted plants were transplanted in the field at UCD in April 1998. One to three trees per species were used as healthy controls.

In the orchard, two or three mature trees each of peach cv. Fay Elberta, cherry cv. Bing, almond (*P. dulcis* (Mill.) D.A. Webb) cv. Mission, and cherry (*P. avium* × *P. pseudocerasus*) cv. Colt supporting scions of flowering cherry cv. Shirofugen were inoculated with 15 peach budchips each. One healthy tree per species was included as a control.

cDNA cloning and sequencing. Purified dsRNAs were prepared as described (9) from leaves removed from inoculated-diseased and noninoculated-healthy trees of Colt cherry, Shirofugen flowering cherry, and peach suckers and bark tissues of Black Beaut plum trees. These preparations were used as templates in RT-PCR where cDNAs were amplified using the *Closterovirus* HSP70 degenerate primers (7). The products were resolved by electrophoresis in 1.5% agarose gel and size estimates determined with a 1-kb DNA Ladder standard (Life Biotechnologies Inc., Rockville, MD). The PCR products were extracted and purified from the agarose gels using a commercial Bio-Spin DNA purification column (BIORAD Inc., Hercules, CA) and cloned with TOPO TA Cloning kit (Invitrogen Inc., Carlsbad, CA). The cloning plasmid was pCR 2.1-

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Fig. 1. Bark necrosis symptoms on *Plum bark necrosis-stem pitting associated virus*-infected Black Beaut plum tree.

TOPO, and the resulting recombinant DNA plasmids were purified with the QIAprep spin miniprep kit (Qiagen Inc., Valencia, CA). Recombinant plasmids were selected, and those containing inserts were identified by restriction enzyme analysis (4).

The clones with the 0.67 fragments of PCR-amplified DNAs were sequenced at the DNA Sequencing Facility, UCD. Specific primers for the associated *Closterovirus* were then designed from the sequence information. The RT-PCR products were separated by electrophoresis through 1.5% agarose gels using TAE buffer as per Zhang et al. (10).

Sample preparation and RT-PCR assays. A rapid assay method developed for grapevine viruses (3) was used to prepare petiole and bark tissues of diseased and healthy Black Beaut plum trees.

In May and in September 1999 (2 years postinoculation), all graft-inoculated and healthy tree species were assayed by RT-PCR. Approximately 0.5 g of bark tissues (located immediately beneath the budchip/bark patch inocula) and leaf petioles arising nearest the inoculation sites were sampled. On some trees, leaves with virus-like symptoms along with bark samples were also assayed. All extracts were made at a tissue-to-grinding buffer ratio of 0.2 g:5.0 ml.

RESULTS

Among field-grafted orchard trees, 85 to 100% of the inoculum peach budchips callused after a 30-day incubation period. In greenhouse inoculations, 33% of all peach budchips and 22 and 83% of plum bark patches survived on Mazzard and Nanking cherry trees, respectively. In the field, all grafted Nanking transplants died in the second growing season due to unknown causes, while all control trees survived. On GF 305 peach, 83% of the plum bark patches and all peach budchips were united with the host plant and these trees survived in the nursery.

Fig. 2. Profile of dsRNAs purified from symptomatic leaves of Colt cherry trees infected with *Plum bark necrosis-stem pitting associated virus* (PBNSPaV). Lane 1, molecular size marker of *Cucumber mosaic virus* dsRNAs; lane 2, dsRNA profile of healthy Colt cherry leaves; lane 3, dsRNA profile of PBNSPaV-infected Colt leaves; lane 4, dsRNA profile of PBNSPaV-infected Colt roots; and lane 5, 1-kb DNA marker (Life Biotechnologies). Electrophoresis was done in 6% polyacrylamide gel.

After budbreak and incubation for 6 months, both inoculated Colt cherry trees developed leaves with chlorotic ring spots. On GF 305 peach, Casselman, Laroda, and Shiro plum cultivars, leaf symptoms of chlorotic ring spots, oak-leaf and line-pattern were observed 12 months postinoculation. Similarly, during the second full growing season postinoculation, bark gummosis, necrosis, and stem pitting symptoms began to develop on trunks of Angeleno, Black Beaut, and Friar trees. In the third year postinoculations, bark and stem symptoms intensified (Fig. 1). Concomitantly, leaf symptoms were absent on these cultivars.

Hosts with latent infections included Fay Elberta peach, Shirofugen flowering cherry, Mission almond, Bing and Mazzard cherry, and Black Amber and Stanley plum. French Improved plum was refractory to infection by PBNSPaV. All healthy controls were normal in appearance.

Purified nucleic acid preparations from diseased leaf and bark tissues of two cherry species, plum, and peach contained three distinct dsRNA species, estimated at 17, 16, and 2.1 kbp in size (Fig. 2), typical of monopartite members of the *Closteroviridae*. Similar bands were absent in preparations of healthy tissues. Molecular size markers used in the gels were *Cucumber*

mosaic virus (CMV) dsRNAs and the 1-kb DNA ladder standard.

Due to conserved sequences among *Closteroviruses*, use of the HSP70 degenerate primers resulted in amplification of two fragments (0.67 and 0.56 kbp). Only the larger cDNA fragment was cloned and sequenced (GenBank accession number: AF195501) and compared with sequences in the GenBank. The percentages of HSP70 nucleotide sequence identities were 44% with *Little cherry virus*-1 (LChV-1); 50 to 51% with *Grapevine leafroll associated virus*-4 and -5 (GLRaV-4, -5); 59% with *Citrus tristeza virus*; and 76 to 77% with *Beet yellows virus* and GLRaV-3.

From our sequence data, three pairs of primers were designed and tested in all combinations. One forward primer and all three reverse primers yielded products of 414, 484, and 541 bp (Fig. 3). Sequences of the primer pair producing the largest product were: forward primer-PBNSP-3f, 5'-CTT CGG CAC TAC TTT CAG CAG T-3', and reverse primer-PBNSP-2r, 5'-AGT CGC ACC ACC AGT CTT CT-3'. This pair of primers was used for further testing and detection of PBNSPaV in the different test plants. Analyses with these primers revealed that with petiole tissues collected in spring and summer, the grape extraction protocol consistently yielded

Fig. 3. An agarose gel electrophoresis profile from reverse transcriptase–polymerase chain reaction (RT-PCR) amplification of *Plum bark necrosis-stem pitting associated virus* (PBNSPaV) dsRNA isolated from diseased leaves of a Colt cherry tree. Lane 1 contains the 1-kb DNA ladder (Life Bio-technologies). Lanes 2 to 4 represent combinations of different primers used for PCR amplification: lane 2, PBNSPaV-3f and -1r; lane 3, 3f and 3r; lane 4, 3f and 2r. Lane 5 is healthy control using 3f and 2r primers. Primer combination of 3f-2r was selected for routine virus assays.

larger amounts of the expected product compared with the small-scale extraction method (data not shown).

All *Prunus* hosts assayed in the study tested positive for PBNSPaV by RT-PCR assays, except French Improved plum, which tested negative in two assays, and Nanking cherry trees that died prior to performing the assay.

DISCUSSION

Several *Prunus* cultivars, including Colt cherry, GF 305 peach, and Casselman, Laroda, and Shiro plums, produced symptomatic leaves in the presence of PBNSPaV. In contrast, infections in Angeleno, Black Beaut, and Friar plums incited only bark and wood marking symptoms. Susceptible, latent hosts of PBNSPaV included Fay Elberta peach, Shirofugen flowering cherry, Mazzard, and Bing cher-

ries, Mission almond, and the plum cultivars Black Amber and Stanley. We failed to infect French Improved plum and possibly Nanking cherry. All other grafted trees tested positive for PBNSPaV by RT-PCR assays. Noninoculated controls remained healthy and were negative by RT-PCR.

To date, only three *Closteroviruses* have been reported in *Prunus* species, and all were recovered from little cherry diseased (LChD) trees in Germany (2,6). Comparisons of limited sequence data for LChV-2 by Rott and Jelkmann (6) with another LChV characterized in North America (1) showed the viruses to be similar. Biologically, both viruses are vectored by the apple mealybug (*Phenacoccus aceris* (Signoret)) (5). In contrast, the vector for LChV-1 is unknown and distinct from the apple mealybug vectored viruses. Lastly, a third distinct *Closterovirus* was found in an

LChD source tree that tested negative with LChV-1 and -2 primers (6).

A tentative identification of the putative PBNSPaV agent was made when the banding pattern of the dsRNA species showed similarity to *Closteroviruses*. This was confirmed when cDNA fragments were amplified in RT-PCR assays using the HSP70-degenerate primers. Definitive proof of a causal relationship, however, must await completion of Koch's postulates.

The incidence and distribution of PBNSPaV in the various *Prunus* orchards in California is unknown. However, virus incidence is likely low. This is because Shiro plum is used as a standard *Prunus* virus indicator in the California Stone Fruit and Nut Tree Registration and Certification program and would easily detect PBNSPaV. Also, development of RT-PCR assay should prove useful for rapid detection and identification of the virus.

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