Association of a Badnavirus with Citrus Mosaic Disease in India

Y. S. Ahlawat and **R. P. Pant,** Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi-110012, India; **B. E. L. Lockhart,** Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108; and **M. Srivastava, N. K. Chakraborty,** and **A. Varma,** Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi-110012, India

ABSTRACT

Ahlawat, Y. S., Pant, R. P., Lockhart, B. E. L., Srivastava, M., Chakraborty, N. K., and Varma, A. 1996. Association of a badnavirus with citrus mosaic disease in India. Plant Dis. 80:590-592.

A previously unreported badnavirus was found to be associated with a mosaic disease occurring commonly in orchard trees and nursery plants of citrus in India. The virus had 30 × 150 nm nonenveloped bacilliform particles typical of badnaviruses, and was found by immunosorbent electron microscopy to be related serologically to sugarcane bacilliform and to eight other badnaviruses. Upon PCR amplification using degenerate oligonucleotide primers based on conserved badnavirus genomic sequences, the viral genomic nucleic acid yielded a product similar in size to that obtained with other badnaviruses which have been shown to contain dsDNA genomes. The virus, named citrus mosaic badnavirus (CMBV), was graft- and dodder-transmitted to 13 of 14 citrus species and cultivars, and was transmitted by mechanical inoculation from symptomatic citrus to healthy *Citrus decumana*.

Additional keywords: citrus badnavirus, citrus mosaic virus

A viruslike disorder of citrus, referred to as citrus mosaic, occurs commonly in India, especially in sweet orange (*Citrus sinensis* (L.) Osbeck) and pummelo (*Citrus grandis* (L.) Osbeck) (3,20). The incidence of mosaic disease ranges from 10 to 70% in different Satgudi sweet orange orchards in the Hindupur region of Andhra Pradesh. Pummelo is not grown commercially in India, and no data are available on incidence of mosaic in this cultivar. The losses caused by the mosaic disease were apparent in Satgudi sweet orange orchards in Andhra Pradesh and Karnataka because several orchards with trees 4 to 10 years old were abandoned since they were no longer productive. The reduction in fruit yield was 77% in trees 10 years old, and fruit from affected trees had 10% less juice and ascorbic acid (20). The disease has been recorded in up to 46% of trees in some commercial nurseries at Kodur in Andhra Pradesh. In 1992, foliar symptoms consisting of bright yellow mosaic and vein-banding were observed on a 10-year-old tree of pummelo on rough lemon (*Citrus limon* (L.) Burm.) rootstock in Bangalore. Of 10 pummelo trees in this orchard, only one was found infected. Leaf samples and budwood were collected from this tree to investigate whether the disease was similar to the mosaic disease of citrus observed elsewhere in India. The causal agent of the disease was graft transmitted, and bacilliform virions were observed in both field samples and samples from inoculated glasshouse plants (2). During 1993, a similar disease was recorded in three 8-year-old trees of pummelo at the experimental orchard of the Indian Agricultural Research Institute, New Delhi. A study was initiated to establish the role of the bacilliform virus in the etiology of citrus mosaic.

Corresponding author: B. E. L. Lockhart
E-mail: anna@puccini.crl.umn.edu

Accepted for publication 7 February 1996.

Publication no. D-1996-0306-05R

MATERIALS AND METHODS

Virus isolate. The citrus mosaic virus isolate used in these studies was obtained from a naturally infected pummelo tree in Bangalore and was transmitted by wedge-grafting to 6-month-old pummelo seedlings. The virus culture was multiplied in pummelo seedlings by graft inoculation and maintained in an insect-proof greenhouse.

Transmission. Dodder transmission tests were conducted using *Cuscuta reflexa,* as described previously (4). Insect transmission was attempted using aphids (*Myzus persicae* (Sulzer) and *Aphis gossypii* (Glover)) and mealybugs (*Planococcus citri* (Risso)). Tests were done for both persistent and nonpersistent modes of transmission. Mealybugs were maintained on healthy grapefruit (*Citrus paradisi* Macf.) and were fed on the virus source plant for 7 days. They were then transferred in groups of 20 to each of 10 healthy pummelo seedlings. The mealybugs were then transferred serially at 24-h intervals to two fresh lots of healthy pummelo seedlings. Mechanical transmission was done from the original source of virus. Inoculum was prepared from young, fully expanded symptomatic leaves of pummelo. The tissue was powdered in liquid nitrogen and suspended in 600 mM phosphate buffer, pH 7.0. Leaves of test plants were immediately inoculated using Carborundum as an abrasive. The test plants used in these experiments were 3-month-old seedlings of pummelo, grapefruit, Satgudi sweet orange, Rangpur lime (*Citrus limonia* Osbeck), and *Citrus decumana* (L.) L., a native of India. Ten plants of each test species were inoculated and maintained in the glasshouse for observation.

Host range. During these studies, available citrus species were inoculated by wedge-grafting from the original mosaic-infected source plant. Five 1-year-old seedlings of each test species were inoculated.

Electron microscopy (EM) and immunosorbent electron microscopy. Leaf samples were examined by EM using either the leaf-dip method or partially purified preparations. The latter were prepared from 5-g leaf tissue samples, which were powdered in liquid nitrogen and then extracted with 18 ml of 200 mM phosphate buffer, pH 6.0, containing 10 mM EDTA, 0.5% (wt/vol) Na_2SO_3, 3% (wt/vol) polyethylene glycol (PEG, av. mol. wt. 8,000), and 2% (wt/vol) polyvinylpyrrolidone (PVP, av. mol. wt. 40,000). The extract was filtered through cheesecloth and centrifuged at 12,000 × *g* (max) for 10 min, and the pellet was discarded. Triton X-100 was added to the supernatant to a final concentration of 2% (vol/vol). After mixing by inversion for 30 s, the mixture (17 ml) was layered over a 5-ml cushion of 30% (wt/vol) sucrose in 100 mM phosphate buffer, pH 7.2, and centrifuged for 1 h at 30,000 rpm (109,000 × *g* max.) in a Beckman type 50.2 Ti rotor. Each pellet was resuspended in 100 µl of 10 mM phosphate and 150 mM NaCl, pH 7.2. The suspension was clarified by shaking briefly with 50 µl of chloroform, followed by microfuging for 6 min at 12,400 × *g* (max). The upper aqueous phase was removed and constituted the partially purified preparation. Trapping and decoration of particles in ISEM tests were done as

described previously (16). Antisera against the following badnaviruses were used in ISEM tests: banana streak virus (BSV) Morocco isolate (13), BSV Rwanda isolate (17), BSV Trinidad Mysore isolate (17), sugarcane bacilliform virus (ScBV) (15), Kalanchoë top-spotting virus (KTSV) (7), Dioscorea bacilliform virus (DBV) (8,10), cacao swollen shoot virus (CSSV) (1), and Commelina yellow mottle virus (CoYMV) (14,18). Antiserum against DBV was provided by A. A. Brunt, Horticulture Research International, Littlehampton, U.K.; and CSSV antiserum was provided by H. L. Vetten, Institut für Viruskrankheiten der Pflanzen, Braunschweig, Germany. EM and ISEM preparations were negatively stained using 2% sodium phosphotungstate, pH 7.0, (PTA) or 2% aqueous uranyl acetate (UA), both containing bacitracin at 100 µg/ml.

Viral DNA extraction and amplification by polymerase chain reaction (PCR). Genomic nucleic acid was extracted from partially purified preparations of CMBV as described previously (14). This included predigestion of the samples with DNase and RNase prior to extraction of virion nucleic acid to eliminate nonencapsidated nucleic acids, including host plant DNA. Nucleic acid extracted from virions was used as template for PCR amplification employing two degenerate oligonucleotide primers based on consensus sequences located in the reverse transcriptase and RNase H domains of ORF III of the badnavirus genome (17). The nucleotide sequences of these two primers, designated Badna 2 and Mys 3′, were, respectively, TAY ATH GAY GAY ATH YT and CCC CAT RCA NCC RTC NGT YTC. Products generated by PCR amplification were analyzed by electrophoresis in 1.5% agarose gels in Tris-acetate-EDTA (TAE) and stained with ethidium bromide. Lambda DNA digested with *Hin*dIII was used as molecular weight markers.

Table 1. Reaction of various *Citrus* species and cultivars to graft inoculation with citrus mosaic badnavirus (CMBV)

Species	Plants infected of 5 inoc.	Symptoms[a]
C. limonia	5	CS, VB, MM
C. volkameriana	5	YS, YB, YM
C. jambhiri	4	VF, M, YS
C. sinensis cvs.		
Satgudi	3	M, YR
Mosambi	5	M, YR
Chini	5	CS, YM
C. reticulata		
cv. Nagpur	3	VB
C. limettiodes	1	MM
C. aurantifolia	0	...
C. grandis	5	YM, SL
C. paradisi		
cv. Duncan	2	VF, YM
C. medica	1	VY
C. aurantium	5	CS, YS, YM
C. mitis	5	YR, SL

[a] CS = chlorotic spot, VB = vein banding, MM = mild mosaic, YS = yellow spots, YB = yellow blotches, YM = yellow mosaic, VF = vein flecking, M = mosaic, YR = yellow rings, SL = smalling of leaves, VY = vein yellowing.

RESULTS

Transmission. The virus associated with citrus mosaic disease was transmitted from infected pummelo to 13 of 14 citrus species and cultivars by wedge-grafting and by dodder (Table 1). No symptoms were observed in plants inoculated by aphids or mealybug 6 months after inoculation, and no virus was detected in these plants by EM examination. Except for one out of 10 *C. decumana* plants inoculated, none of the plant species inoculated by sap inoculation developed any symptoms. The infected *C. decumana* developed typical mosaic symptoms 90 days postinoculation and was shown by EM examination to contain bacilliform particles.

Symptoms. The characteristic symptoms of the disease in field-infected sweet orange and pummelo trees consisted of bright yellow mottling of the leaves and yellow flecking along the veins (Fig. 1A and B). Greenhouse-inoculated pummelo plants developed typical mosaic symptoms similar to those observed on naturally infected plants. Symptoms that were produced in various *Citrus* spp. upon graft inoculation are given in Table 1 and in Figure 1C to F. Except for *C. aurantifolia*, graft-inoculated plants of all other citrus species tested developed varying degrees of symptoms within 70 days postinoculation. Indexing by EM confirmed that all symptomatic test plants contained bacilliform virions that were not detectable in healthy, asymptomatic plants.

PCR amplification of CMBV genomic nucleic acid. A region of the genomic nucleic acid extracted from CMBV virions was successfully amplified by PCR using the badnavirus-specific oligonucleotide primer pair Badna 2 and Mys 3′, which is known to prime amplification of all mealybug-transmitted badnaviruses (17). A badnavirus-specific PCR product, approximately 450 bp in size, was obtained when genomic dsDNA of CoYMV and BSV, and genomic nucleic acid of CMBV, were used as template (Fig. 2). No PCR product was obtained from an extract of healthy citrus prepared in a manner identical to that used for CMBV.

Electron microscopy. Typical bacilliform virions measuring approximately 130 × 30 nm were observed in negatively stained leaf-dip and in partially purified preparations of samples both from field-infected pummelo trees and from inoculated greenhouse test plants. No virions were observed in similar preparations from uninoculated control plants.

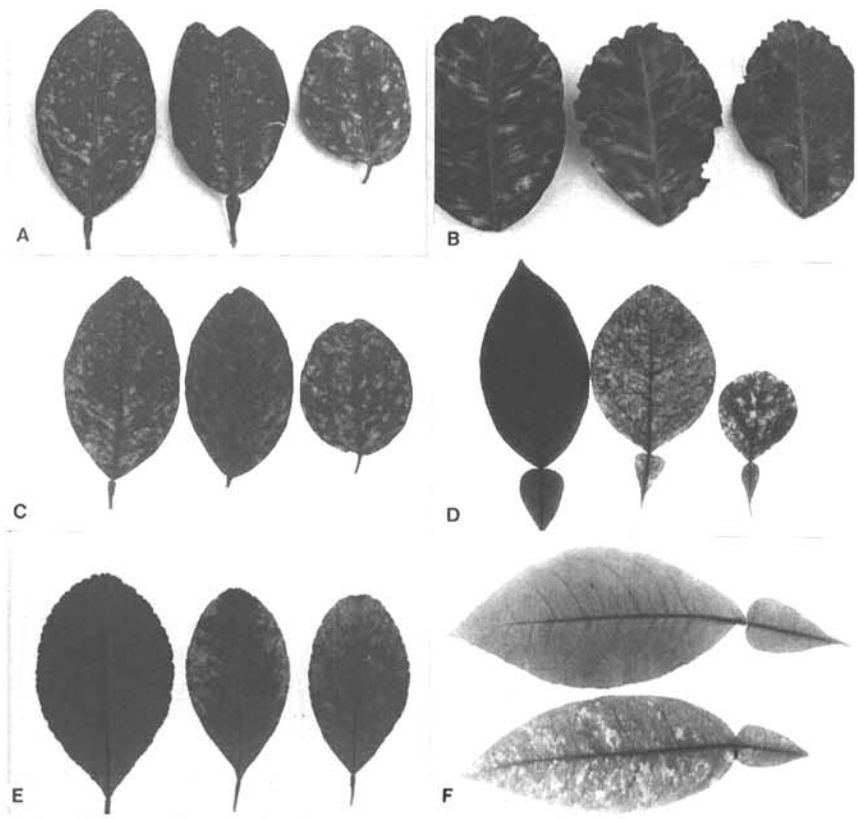

Fig. 1. Foliar symptoms associated with citrus mosaic badnavirus (CMBV) infection in various *Citrus* species. **(A)** Sweet orange, field symptoms. **(B)** Pummelo, field symptoms. **(C)** Sweet orange, inoculated greenhouse plant. **(D)** Pummelo, inoculated greenhouse plant. **(E)** Lemon, inoculated greenhouse plant. **(F)** Sour orange, inoculated greenhouse plant.

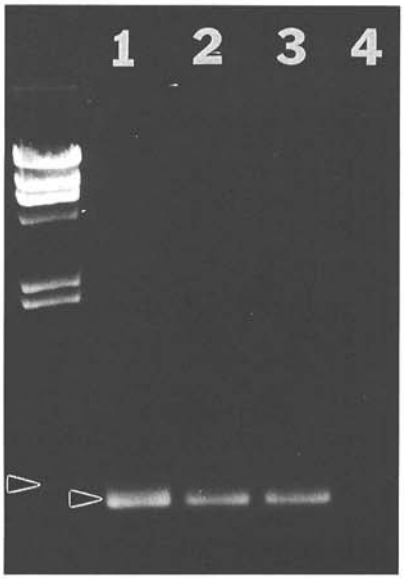

Fig. 2. Analysis by agarose gel electrophoresis of a badnavirus-specific 450-bp product generated by polymerase chain reaction (PCR) amplification using the degenerate badnavirus oligonucleotide primers Badna 2 and Mys 3′ and genomic nucleic acid from three badnaviruses as template. Lane 1, Commelina yellow mottle virus (CoYMV) genomic DNA; lane 2, banana streak virus (BSV) genomic DNA; lane 3, citrus mosaic badnavirus (CMBV) genomic nucleic acid. Lane 4, extract of healthy citrus prepared in a manner identical to that used for sample in lane 3. Arrows indicate 564-bp Lambda *Hin*dIII marker and badnavirus-specific PCR products, respectively. Electrophoresis was done in 1.5% agarose gels in Tris-acetate-EDTA (TAE) at 45 volts for 75 min, and the gel was stained with ethidium bromide.

Fig. 3. Virions of citrus mosaic badnavirus (CMBV) trapped by antiserum to sugarcane bacilliform virus (ScBV) and stained with 2% sodium phosphotungstate, pH 6.5. Scale bar = 100 nm.

Serology. Virions of CMBV were successfully trapped and decorated with antisera to BSV, CoYMV, DBV, KTSV, CSSV, and ScBV in ISEM tests. The greatest number of CMBV particles was trapped by antiserum to ScBV (Fig. 3).

DISCUSSION

Mosaic diseases in citrus have been reported from India (3,5) and Japan (11). Isometric particles were reported to be associated with Japanese citrus mosaic (21), but no viruslike particles have been previously associated with Indian citrus mosaic. No serological relationship between the pathogens of these two diseases could be established using antiserum against Japanese citrus mosaic (Y. S. Ahlawat, *unpublished*). The association of bacilliform virions with Indian citrus mosaic further distinguishes the latter disease from the one reported from Japan. A bacilliform virus has been reported to be associated with citrus leprosis disease in Brazil (12). The bacilliform virions associated with citrus leprosis, like those of the morphologically similar orchid fleck virus (6), occur in the nucleus (6,12), unlike those of badnaviruses, which occur only in the cytoplasm (8,9,18,19).

Citrus mosaic disease is widely distributed in India and is of great economic importance to the citrus industry. The presence of the disease in commercial nurseries suggests inadvertent spread of the disease through contaminated budwood. CMBV, as indicated in Table 1, is capable of infecting the major commercial citrus cultivars and rootstocks used in India. The disease is transmitted mechanically to *C. decumana*, a native of India. This will help in separating the virus from mixed infections, which are quite common in orchard trees.

Reaction of CMBV in ISEM tests with antisera to a range of badnaviruses, and PCR-mediated amplification of CMBV genomic nucleic acid with badnavirus-specific oligonucleotide primers, confirm that CMBV is a member of the genus *Badnavirus*. This is the first report of a badnavirus infection in citrus.

ACKNOWLEDGMENTS

Published as paper 22,253 of the contribution series of the Minnesota Agricultural Experiment Station based on research conducted under Project 22-79H, supported in part by funds from USAID PSTC Grant AID/HRW-5600-G-00-2017-0.

LITERATURE CITED

1. Adomako, D., Lesemann, D.-E., and Paul, H. L. 1983. Improved methods for the purification and detection of cacao swollen shoot virus. Ann. Appl. Biol. 103:109-116.
2. Ahlawat, Y. S., Chakraborty, N. K., Jagadishchandra, K, Srivastava, M., and Varma, A. 1993. Association of a rhabdovirus with yellow vein mosaic a new disease of citrus. Pages 455-457 in: Proc. Conf. IOCV, 12th. IOCV, Riverside, California.
3. Ahlawat, Y. S., Chenulu, V. V., Vishwanath, S. M., and Pandey, P. K. 1984. Studies on a mosaic disease of citrus in India. Curr. Sci. 54:873-874.
4. Ahlawat, Y. S., and Dhingra, K. L. 1973. Dodder transmission of some temperate fruit viruses in Simla hills. Indian Phytopathol. 26:748-749.
5. Dakshinamurti, V., and Reddy, G. S. 1975. Mosaic—a transmissible disorder of sweet oranges. Indian Phytopathol. 28:398-399.
6. Doi, Y., Chang, M. U., and Yora, K. 1977. Orchid fleck virus. CMI/AAB Descriptions of Plant Viruses No. 183. Commonw. Mycol. Inst./Assoc. Appl. Biol., Kew, Surrey, U.K.
7. Ferji, Z. 1988. Role of a small bacilliform virus in the etiology of top-spotting disease of *Kalanchoë blossfeldiana*, and some properties of the virus. M.S. thesis. University of Minnesota, St. Paul.
8. Harrison, B. D., and Roberts, I. M. 1973. Association of virus-like particles with internal brown spot of yam (*Dioscorea alata*). Trop. Agric. (Trin.) 50:335-340.
9. Hearon, S. S., and Locke, J. C. 1984. Graft, pollen and seed transmission of an agent associated with top spotting in *Kalanchoë blossfeldiana*. Plant Dis. 68:347-350.
10. Hughes, J. d'A. 1986. Viruses of the Araceae and *Dioscorea* species: Their isolation, characterization and detection. Ph.D. thesis. University of Reading, U.K.
11. Ishigai, T., and Jinno, M. 1958. On citrus mosaic. Ann. Phytopathol. Soc. Jpn. 23:29.
12. Kitajima, E. W., Muller, G. W., Costa, A. S., and Yuki, W. 1972. Short rod like particles associated with citrus leprosis. Virology 50:254-258.
13. Lockhart, B. E. L. 1986. Purification and serology of a bacilliform virus associated with banana streak disease. Phytopathology 76:995-999.
14. Lockhart, B. E. L. 1990. Evidence for a double-stranded circular DNA genome in a second group of plant viruses. Phytopathology 80:127-131.
15. Lockhart, B. E. L., and Autrey, L. J. C. 1988. Occurrence in sugarcane of a bacilliform virus related serologically to banana streak virus. Plant Dis. 72:230-233.
16. Lockhart, B. E. L., Autrey, L. J. C., and Comstock, J. C. 1992. Partial purification and serology of sugarcane mild mosaic virus, a mealybug-transmitted closterolike virus. Phytopathology 82:691-695.
17. Lockhart, B. E. L., and Olszewski, N. E. 1993. Serological and genomic heterogeneity of banana streak badnavirus: Implications for virus detection in *Musa* germplasm. Pages 102-113 in: Breeding Banana and Plantain for Resistance to Diseases and Pests. J. Ganry, ed. CIRAD/INIBAP, Montpellier, France.
18. Migliori, A., and Lastra, R. 1978. Étude de virus presents chez *Commelina diffusa* Burm. en Guadeloupe. Ann. Phytopathol. 10:467-477.
19. Milne, R. G., Masenga, V., Accotto, G., Scapin, I., and D'Agliano, G. 1985. Un virus bacilliforme associato alla maculatura gialla della *Yucca elephantipes*. Inftore. Fitopatol. 35:43-44.
20. Reddy, G. S., and Murti, V. D. 1985. Citrus diseases and their control. Indian Council of Agricultural Research Publication, New Delhi.
21. Tanaka, H., and Imada, J. 1976. Purification of viruses of citrus mosaic and navel orange infectious mottling. Proc. Int. Org. Citrus Virol. 7:116-118.