European Journal of Plant Pathology **103:** 303–311, 1997.

Identification of *Piper* yellow mottle virus, a mealybug-transmitted badnavirus infecting *Piper* spp. in Southeast Asia

B.E.L. Lockhart[1], Kittisak Kiratiya-Angul[2], P. Jones[3], Lily Eng[4], Padmini De Silva[5], N.E. Olszewski[6], Nikki Lockhart[6], Nuanchan Deema[2] and J. Sangalang[7]

[1]*Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA (Fax: 1-612-625-9728)*
[2]*Division of Plant Pathology and Microbiology, Department of Agriculture, Bangkhen, Bangkok 10900, Thailand*
[3]*Department of Crop and Disease Management, IACR-Rothamsted, Harpenden, Hertfordshire, AL5 2JQ, UK*
[4]*Department of Agriculture Research Centre, Semongok, PO Box 977, 93720 Kuching, Sarawak, Malaysia*
[5]*Department of Export Agriculture, Research Station, Matale, Sri Lanka;* [6]*Department of Plant Biology, University of Minnesota, St. Paul, MN 55108, USA;* [7]*Department of Horticulture, University of the Philippines Los Banos, College, Laguna 4031, Philippines*

Accepted 2 December 1996

Key words: Piper badnavirus, black pepper virus, betelvine virus, mealybug transmission, badnaviruses

Abstract

A previously undescribed badnavirus was found to be a causal agent of a disease of black pepper (*Piper nigrum*) in Malaysia, the Philippines, Sri Lanka and Thailand, and was also associated with a disease of betelvine (*P. betle*) in Thailand. Disease symptoms included chlorotic mottling, chlorosis, vein-clearing, leaf distortion, reduced plant vigor and poor fruit set. The virus, named *Piper* yellow mottle virus (PYMV), had non-enveloped bacilliform virions averaging 30 × 125 nm in size and containing a double-stranded DNA genome. An isolate of PYMV from Thailand was transmitted by mechanical inoculation and by the citrus mealybug, *Planococcus citri*, from infected *P. nigrum* and *P. betle* to healthy *P. nigrum* seedlings, which developed symptoms similar to those observed in naturally-infected plants. A serological relationship between PYMV and isolates of banana streak (BSV) and sugarcane bacilliform (ScBV) viruses, but not six other badnaviruses, was detected by immunosorbent electron microscopy (ISEM). Genomic PYMV sequences were amplified by polymerase chain reaction (PCR) using badnavirus-specific oligonucleotide primers, and sequence analysis comparisons of the putative reverse transcriptase (RT) domain showed PYMV to be closely related to other mealybug-transmitted badnaviruses. Black pepper infected with PYMV sometimes contained one or more isometric virus-like particles, and PYMV may therefore be only one component of a virus complex infecting black pepper in Southeast Asia.

Introduction

Black pepper, prepared from the seeds of *Piper nigrum* L., is an important item of international commerce, and the leaves of the betelvine, *P. betle* L., are widely used as a masticatory in countries of southeast Asia and the Indian subcontinent. These areas are also the principal producers and/or exporters of these two commodities. Virus-like disorders of *P. nigrum* have been reported from several countries in Southeast Asia (Barat, 1952; Holliday, 1959; Prakasam et al., 1990), but the causal agents were not identified. More recently, three virus or virus-like diseases of betelvine have been described from India (Dubey, 1987; Singh and Rao, 1988; Johri et al., 1990). In two of these cases the presumed causal agents were described as flexuous 750 nm rods (Johri et al., 1990) and rigid 350 nm rods (Singh and Rao, 1988), respectively.

In 1990 a virus-like disorder of black pepper and betelvine was observed in Bangkok, Thailand. Symptoms consisted of vein-clearing, leaf distortion and interveinal chlorotic mottle (Figures 1A and B). Non-enveloped bacilliform virus-like particles resembling those of badnaviruses (Lockhart and Olszewski,

1994) were observed by electron microscopy (EM) in partially purified extracts of symptomatic, but not of asymptomatic black pepper and betelvine plants. This report describes the etiology of the disease, which was named *Piper* yellow mottle, and the nature, mode of transmission and distribution of the causal agent, which was named *Piper* yellow mottle virus (PYMV).

Materials and methods

Virus source

The isolate of PYMV used in most of the studies described herein was obtained from infected *P. nigrum*, cultivar unknown, in Bangkok, Thailand. The virus was transmitted by mechanical inoculation (see below) to seedlings of *P. nigrum* cv. Krabi and maintained in this cultivar by vegetative propagation.

Virus purification

PYMV was purified from symptomatic leaves of several cvs. of *P. nigrum* using a modification of the method described for other badnaviruses (Lockhart, 1986; Lockhart, 1990, Lockhart and Autrey, 1988). Fresh leaf tissue was powdered in a mortar with liquid nitrogen and the powder extracted 3:1 (vol:wt) with 250 mM Tris-HCl, pH 7.4 containing 1% (w/v) Na_2SO_3 and 0.5% (v/v) 2-mercaptoethanol. The extract was filtered through cheesecloth and clarified by centrifugation at 18,000 *g* (max.) for 15 min. Triton X-100 was added to the supernatant to a final concentration of 2% (v/v), the mixture was shaken for 1 min, layered over 6 ml of 30% (w/v) sucrose in extraction buffer, and centrifuged for 45 min at 148,000 *g* (max.) (35,000 rpm in a Beckman Type 50.2Ti rotor). The resulting pellets were resuspended overnight in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4, and clarified by shaking with one-half volume of chloroform and centrifuging at 18,000 *g* (max) for 10 min. The aqueous phase constituted a partially purified virus suspension. Further purification was achieved in pre-formed Cs_2SO_4-sucrose gradients (Gumpf et al., 1981) for 6 h at 128,000 *g* (max) in a Beckman SW 28.1 rotor. Fractions of 250 μl were collected from the top of the gradient columns using a gradient fractionator (ISCO, Lincoln, NE) and Fluorinert FC 40 (3M Company, St. Paul, MN) as chase solution. Virus-containing fractions were identified by EM.

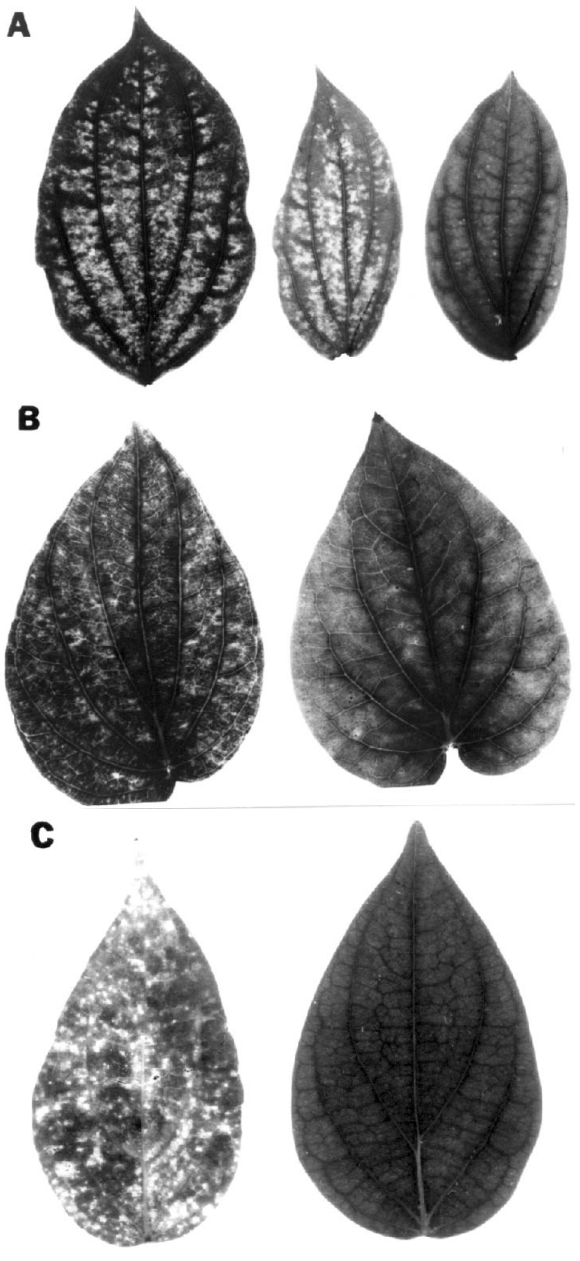

Figure 1. Symptoms associated with *Piper* yellow mottle virus infection in naturally- and experimentally-infected *Piper* spp. (A) Foliar symptoms in *Piper nigrum* naturally infected with *Piper* yellow mottle virus (Philippine isolate) and containing no other virus-like particles. Symptoms vary from severe chlorotic mottling (left) to interveinal chlorosis (right). (B) Left: Chlorotic mottle symptoms in *Piper betle* (cv. unknown, Thailand) Right: Healthy leaf. (C) Left: Systemic yellow mottle symptoms produced in *Piper nigrum* 'Krabi' following mechanical inoculation with partially purified *Piper* yellow mottle virus (Thailand *P. nigrum* isolate). Right: Healthy leaf.

Distribution of PYMV infection in black pepper

Occurrence of PYMV in several black pepper-producing areas of southeast Asia was determined by EM examination of partially-purified leaf tissue extracts. Leaf samples showing chlorotic mottle, chlorosis, leaf deformation or other virus-like symptoms were collected in Sarawak (Republic of Malaysia), Sri Lanka, Thailand and the Philippines. Leaf samples were collected from both commercial plantings and *P. nigrum* germplasm collections.

For EM examination, partially purified extracts were prepared as described above from 8-10 g leaf samples. The pellet from the first cycle of ultracentrifugation was resuspended in 150 μl of 50 mM Tris-HCl, pH 7.4, clarified by shaking with one-half volume of chloroform, and centrifuged at 20,000 *g* for 5 min. The pellet was discarded and the supernatant was negatively stained for EM examination as described below.

Mechanical inoculation

Carborundum-dusted leaves of test plants were mechanically inoculated using partially purified suspensions of PYMV. No inoculations were done using crude sap because previous experience with other badnaviruses suggested that failure of mechanical inoculation using crude sap inoculum was probably due to low virus concentration in such extracts (Lockhart and Autrey, 1988; Lockhart and Ferji, 1988). Furthermore, the use of partially purified preparations allowed verification by EM examination that the inoculum contained PYMV particles and was free of other virus-like particles. Inoculated test plants were kept in the greenhouse at 25–30 °C and observed for up to 2 years after inoculation. Both inoculated leaves and new growth were assayed by EM and ISEM examination for presence of badnavirus particles in partially purified extracts.

Mealybug transmission

Mealybug transmission tests were done using the citrus mealybug, *Planococcus citri* (Risso) raised on virus-free *Commelina diffusa* Burm. grown from seed. Early-instar mealybugs were given a 24-h acquisition access feed on symptomatic PYMV-infected *P. nigrum* and *P. betle* collected in Thailand. Eight to ten insects were then transferred to each of 6 healthy test seedlings of *P. nigrum* cv. Krabi at the 4-leaf stage. Because of the low germination rate of black pepper seeds, larger numbers of healthy test plants could not be used. After an inoculation access period of 24 h the plants were sprayed with insecticide.

Electron microscopy

Partially purified or purified virus preparations were negatively stained with 2% sodium phosphotungstate, pH 7.0 (PTA) or 4% aqueous uranyl acetate (UA). Ultrathin leaf tissue sections were prepared as described previously (Lockhart et al., 1985). Electron microscope magnification was calibrated using the lattice spacing of stained catalase crystals (Wrigley, 1968). Electron microscopy of DNA extracted from purified virions was done as described previously (Lockhart, 1990).

Serological tests

A polyclonal antiserum against PYMV was prepared by immunizing rabbits with partially-purified virus emulsified in Freund's adjuvant and administered intramuscularly. Four injections were given over a period of 35 days and blood samples were collected starting at 45 days after the first injection. Serological tests were done by ISEM as described previously (Lockhart and Autrey, 1988), using partially-purified virus preparations.

Antisera to banana streak virus (BSV) (Lockhart, 1986), *Commelina* yellow mottle virus (CoYMV) (Lockhart, 1990), Kalanchoë top-spotting virus (KTSV) (Lockhart and Ferji, 1988) were used. A polyclonal antiserum against a mixture of serologically distinct isolates of sugarcane bacilliform virus (ScBV) was prepared in rabbits by intramuscular injection of purified virus emulsified in Hunter's Titermax (Sigma Chemical Company, St. Louis, MO, USA) and Freund's incomplete adjuvants. Antisera to *Dioscorea* bacilliform virus (DBV) (Harrison and Roberts, 1973), cacao swollen shoot virus (CSSV) (Adomako et al., 1983) and rice tungro bacilliform virus (RTBV) (Omura et al., 1983) were provided by A.A. Brunt, Littlehampton, U.K., J. Vetten, Braunschweig, Germany, and H. Hibino, IRRI, Philippines, respectively.

Characterization of the PYMV genome

Nucleic acid was extracted from PYMV virions banded on preformed Cs_2SO_4-sucrose gradients as described above. Gradient fractions were examined by EM to

verify presence of PYMV virions and/or other virus-like particles. PYMV-containing fractions not contaminated by host membrane debris were pooled and dialyzed overnight against two changes of 25 mM Tris-HCl, pH 7.4, pelleted by ultracentrifugation as described above, and the pellet resuspended in 200 μl of 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 (TN buffer). The virus suspension was incubated at 37 °C for 30 min in a reaction mixture containing 6 mM $MgCl_2$, 4 units of ribonuclease-free deoxyribonuclease (RQ DNase, Promega Corporation, Madison, WI, USA) and 25 μg/ml ribonuclease (RNase A) (Sigma Chemical Company, St. Louis, MO, USA). Sodium ethylenediamine tetraacetate (Na-EDTA, pH 8.0) was then added to a final concentration of 10 mM and the mixture was digested at 37 °C for 30 min with 250 μg/ml proteinase K in the presence of 1% (w/v) sodium dodecyl sulfate (SDS). The mixture was extracted once with TN-equilibrated phenol containing 0.1% (w/v) 8-hydroxyquinoline and 0.2% (v/v) 2-mercaptoethanol, and once with chloroform:isoamyl alcohol (24:1 v/v). The nucleic acid was precipitated from the aqueous phase by addition of 2.5 vol absolute ethanol in the presence of 300 mM sodium acetate, pH 5.5. The precipitate was collected by centrifugation, rinsed with 70% ethanol, dried under vacuum, and resuspended in 100 μl of TN buffer. Electrophoretic analysis of nucleic acid was done as described for CoYMV (Lockhart, 1990) in 0.8% agarose gels in 90 mM Tris-borate, 2mM EDTA (TBE), except that SDS was not added to the TBE electrophoresis buffer. Treatment of extracted viral nucleic acid with RNase, DNase and S1 nuclease was done as described previously (Lockhart, 1990). Genomic nucleic acid extracted from purified tobacco mosaic virus (TMV), brome mosaic virus (BMV), maize chlorotic mottle virus (MCMV) and bacteriophage M13 were used as single-stranded RNA and DNA controls, respectively.

Amplification of PYMV DNA by PCR

Genomic nucleic acid extracted from PYMV (Thailand isolate) purified as described above was used as template for PCR-mediated amplification using badnavirus-specific degenerate oligonucleotide primers based on consensus sequences in the reverse transcriptase (RT), RNase H and $tRNA^{MET}$-binding domains of the badnavirus genome (Medberry et al., 1991). These primers were designated BADNA 2 (TAY ATH GAY GAY ATH YT), BADNA 3 (ATH ATH ATH GAR ACY GAY), BADNA T (MYM WNG CTC TGA TAC CA) and MYS 3′ (CCC CAT RCA NCC RTC NGT YTC), respectively (Lockhart and Olszewski, 1993; Ahlawat et al., 1996). BADNA 2 and BADNA 3 are forward primers located in the RT and RNase H domains, respectively. BADNA T and MYS 3′ are reverse primers located in the $tRNA^{MET}$-binding and RNase H domains, respectively. Products generated by PCR amplification were analyzed by agarose gel electrophoresis as described previously (Ahlawat et al., 1996). The primers contained restriction enzyme recognition sequences to facilitate ligation of the PCR product into the cloning vector.

Molecular cloning and nucleotide sequence of the putative RT domain of the PYMV genome

The PCR product obtained from PYMV genomic DNA using the degenerate oligonucleotide primer pair BADNA T + BADNA 2 was digested with *Sma* I and *Not* I and ligated into the cloning vector pBluescript kS (Stratagene Cloning Systems, La Jolla, CA, USA). Sequencing of cloned PCR products and the generation of trees depicting molecular relationships using the neighbour-joining method (Nei, 1987) to analyze distance matrices based on the nucleotide sequence of a portion of the conserved RT domain was done as described previously (Bouhida et al., 1993).

Results

Yellow mottle disease and PYMV incidence

Disease symptoms including mild to severe chlorotic mottling, vein-clearing, interveinal chlorosis, reduction in leaf size and leaf puckering and deformation (Figure 1A) were observed in black pepper samples from Sarawak (Malaysia), Sri Lanka, the Philippines and Thailand. Chlorotic mottle symptoms without leaf deformation were also observed on betelvine in Thailand (Figure 1B). In all cases, disease symptoms were associated with PYMV infection. However, PYMV-infected black pepper from Malaysia and Sri Lanka frequently contained isometric virus-like particles 30 nm and 50 nm in diameter. Similar isometric virus-like particles were not observed in PYMV-infected black pepper from Thailand and the Philippines.

Three double-stranded RNA (dsRNA) species were detected in sucrose density gradient fractions containing the 30 nm virus-like particles (data not shown). No information was obtained regarding the possible nature

of the 50 nm virus-like particles. Several PYMV-infected black pepper clones also contained flexuous rod-shaped particles. These particles were similar in appearance to proteinaceous rod-shaped structures (Figure 2B), 15–17 nm in width, of widely varying length and having rounded ends, which are associated with badnavirus infections in several other plant species including banana (Lockhart, unpublished), citrus (Ahlawat et al., 1991), *Commelina* (Ayala-Navarette, 1993) and Schefflera (Lockhart and Olszewski, 1996). A purified preparation of these rod-shaped structures associated with PYMV infection in a *P. nigrum* cv. from Sarawak had a UV adsorbtion spectrum characteristic of proteins, and contained no nucleic acid detectable by ethidium bromide staining (Sharp et al., 1973) (Data not shown).

Mechanical transmission of PYMV and symptom expression

The Thailand isolate of PYMV, verified by EM examination to be free of other virus-like particles, was transmitted by mechanical inoculation to 3 of 5 healthy seedlings of the black pepper cv. Krabi. Systemic chlorotic mottle and vein-clearing symptoms (Figure 1C), similar to those observed in the original virus source plants, first appeared at 4–5 weeks after inoculation. The presence of badnavirus particles (Figure 2A) in symptomatic leaves of inoculated test plants was verified by EM and ISEM. No other virus-like particles were observed by EM in partially-purified extracts from these plants, which were observed for two years, during which symptoms including leaf reduction and deformation appeared. Severe symptoms developed sporadically on flushes of new growth, while other leaves showed milder symptoms or were symptomless. The Thailand PYMV black pepper isolate was not transmitted by mechanical inoculation to *Piper colubrinum, P. betle* (cv. unknown, Logee's Greenhouses, Danielson, CT), *Peperomia argyreia* E. Morr., *P. caperata* Yunck., *P. obtusifolia* (L.) A. Dietr., *Chenopodium quinoa* Willd. or *Nicotiana benthamiana* Domin. No local or systemic symptoms appeared in indicator plants, and no virions were detected by EM or ISEM examination of partially purified extracts of these plants.

Mealybug transmission of PYMV

The Thailand isolates of PYMV from both *P. nigrum* and *P. betle* were transmitted by *Planococcus citri*

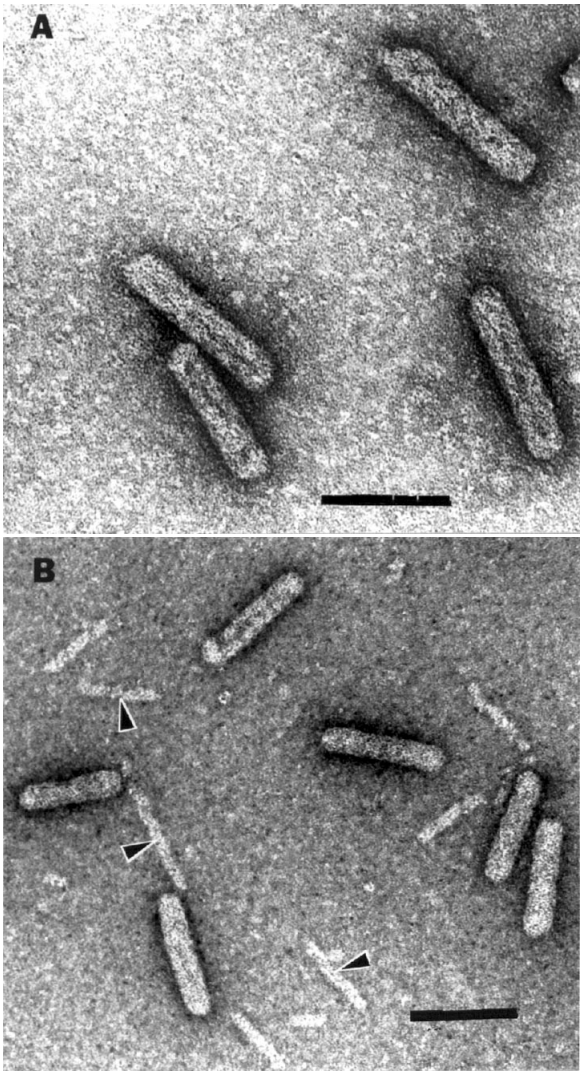

Figure 2. Appearance of *Piper* yellow mottle virus virions and associated elongated proteinaceous structures in negatively-stained EM preparations. (A) Virions of *Piper* yellow mottle virus (Thailand *P. nigrum* isolate); purified preparation stained with 4% aqueous uranyl acetate. Scale bar equals 100 nm. (B) Partially purified preparation of *Piper* yellow mottle virus (Sarawak isolate) showing virions and rod-shaped proteinaceous structures (indicated by arrowheads). Scale bar equals 100 nm.

from naturally infected *P. nigrum* and *P. betle*, respectively, to healthy seedlings of *P. nigrum* cv. Krabi. In both cases 3 of 3 test plants developed vein-clearing and chlorotic mottle symptoms at 5–8 weeks after inoculation by viruliferous mealybugs. The symptoms were similar to those described above (Figure 1C) for mechanically-inoculated plants, and the presence of

badnavirus particles was verified by EM examination of partially-purified extracts. Neither of the two Thailand isolates of PYMV was transmitted by *P. citri* to the *P. betle* cv. mentioned above, *P. colubrinum, P. argyreia, P. caperata, P. obtusifolia* or *N. benthamiana*. No symptoms occurred in plants observed for up to 18 months after inoculation by viruliferous mealybugs, and no particles were detected by EM or ISEM in partially purified extracts of either inoculated leaves or new growth.

Electron microscopy

One hundred and fifty virions of PYMV (Thailand isolate) were measured. The virions were 28–32 nm in width and had a modal length of 125 nm, with 90% of the particles falling in the 115–135 nm range. In ultrathin sections of infected black pepper, virions of PYMV were observed in the cytoplasm but not in the nucleus of infected cells. The particles were randomly arranged and were observed in several tissue types including epidermis, mesophyll and phloem.

Serology

In ISEM tests the antiserum prepared against PYMV trapped virions of PYMV isolates from the Philippines, Sawawak, Sri Lanka and Thailand, and one isolate of BSV, but did not trap particles of CoYMV, KTSV and an isolate of ScBV. In reciprocal tests PYMV particles were trapped by the antiserum prepared against a mixture of ScBV isolates, but not by antisera to BSV, CoYMV, CSSV, DBV, KTSV or RTBV.

Characterization of the PYMV genome

The electrophoretic migration pattern of nucleic acid extracted from virions of the Thailand isolate of PYMV was similar to that of CoYMV (Lockhart, 1990), but there were differences in the number and relative position of bands observed (Figure 3). PYMV nucleic acid was resistant to RNase but was completely degraded by DNase (Figure 3). Under similar conditions ssRNA's of TMV, BMV and MCMV were completely degraded by RNase but not affected by DNase (data not shown). PYMV DNA was also not degraded by single-strand-specific S1 nuclease, which completely digested M13 ssDNA (Figure 4). However, while digestion with S1 nuclease gave rise, in the case of CoYMV, to two smaller genomic fragments resulting from cleavage at the two discontinuities in the circular genome

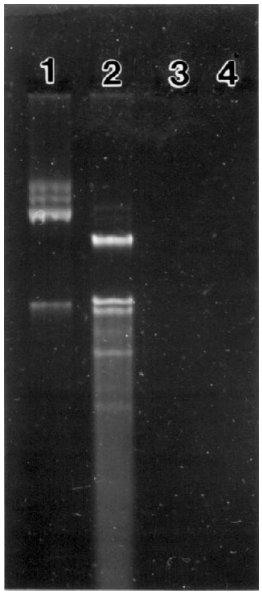

Figure 3. Electrophoretic migration pattern of nucleic acid extracted from purified virions of *Piper* yellow mottle virus (Thailand *P. nigrum* isolate). Lane 1: *Commelina* yellow mottle virus DNA, purified by isopycnic banding in CsCl and treated with RNase. Lane 2: *Piper* yellow mottle virus nucleic acid, treated with RNase. Lanes 3 and 4: *Commelina* yellow mottle virus DNA and *Piper* yellow mottle virus nucleic acid, respectively, treated with RNase-free DNase. Enzyme treatments and electrophoresis conditions are as described in the text.

(Medberry et al., 1991), no change occurred in the electrophoretic pattern of PYMV treated with S1 nuclease under the same conditions. The reason for this difference is not yet clear, as it would be reasonable to assume that the PYMV genome, like that of other pararetroviruses (Medberry et al., 1991), would have one or more discontinuities. EM examination of PYMV DNA (Figure 5A–D) revealed that while circular, convoluted-circular and linear forms could be observed, a large proportion of the molecules occurred as tightly coiled rod-like structures (Figure 5D) rather than the open-circular or linear forms reported for CoYMV (Lockhart, 1990).

Amplification of PYMV genomic DNA by PCR using badnavirus-specific degenerate primers

Regions of the PYMV genome were amplified by PCR using the badnavirus-specific primer pairs BADNA T + BADNA 2, BADNA T + BADNA 3, and BADNA 2 + MYS 3′. These oligonucleotide sequences have been shown to prime the amplification of a wide range of badnaviruses (Lockhart and Olszewski, 1993). The

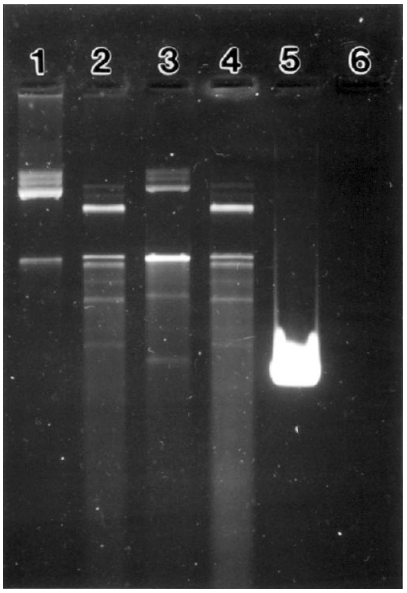

Figure 4. Effect of S1 nuclease digestion on *Commelina* yellow mottle virus and *Piper* yellow mottle virus genomic DNA. Lane 1: *Commelina* yellow mottle virus DNA, untreated; Lane 2: *Piper* yellow mottle virus DNA untreated. Lane 3: *Commelina* yellow mottle virus DNA, digested with S1 nuclease as described in text; Lane 4: *Piper* yellow mottle virus DNA, digested with S1 nuclease as described in text. Lane 5: Bacteriophage M13 ssDNA, untreated. Lane 6: M13 ssDNA digested with S1 nuclease as described in text.

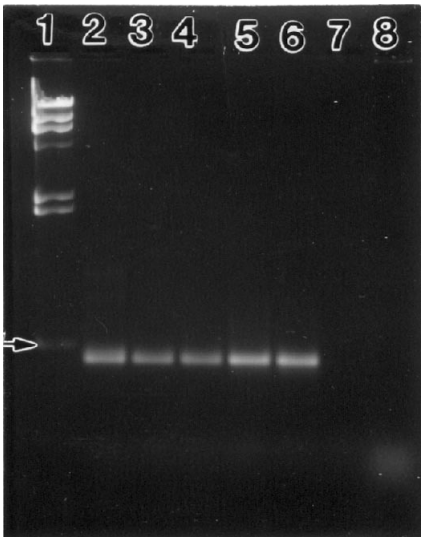

Figure 6. Analysis by agarose gel electrophoresis of a badnavirus-specific 450 bp product generated by PCR amplification using the degenerate, oligonucleotide primer pair BADNA 2 and MYS 3' and genomic nucleic acid from the following five badnaviruses as template. Lane 2: *Commelina* yellow mottle virus. Lane 3: Banana streak virus. Lane 4: Sugarcane bacilliform virus. Lane 5: Citrus mosaic badnavirus. Lane 6: *Piper* yellow mottle virus. Lanes 7 and 8: Partially-purified extracts from healthy banana and black pepper, respectively. Lambda *Hind* III markers are in Lane 1, with the arrow indicating the position of the 564 bp fragment. Electrophoresis conditions are described in the text.

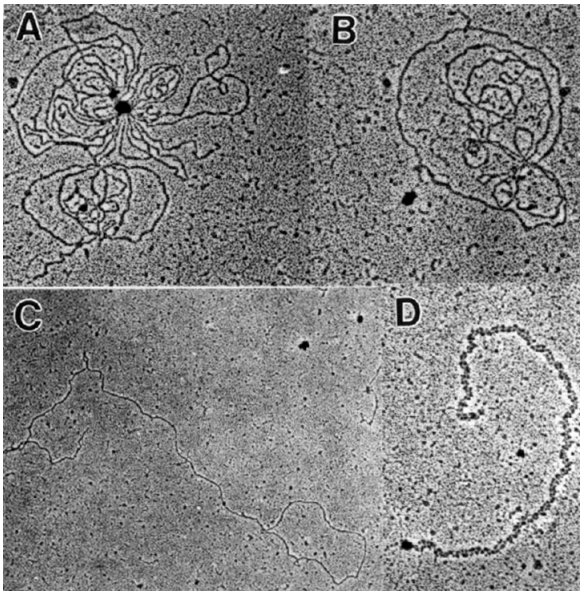

Figure 5. Configurations of *Piper* yellow mottle virus DNA observed by electron microscopy. (A and B) Convoluted circular forms (54,000X). (C) Linear form (30,000X). (D) Tightly coiled rod-like form (39,000X).

450 bp PCR product obtained with the primer pair BADNA 2 + MYS 3′ is shown in Figure 6 together with products of similar size generated by other badnavirus genomic templates.

Molecular relationship between PYMV and other plant pararetroviruses

Based on nucleotide sequence comparisons, a cloned PCR-generated segment of the putative RT-encoding region of PYMV ORF III was found to be more closely related to the mealybug-transmitted badnaviruses BSV, CoYMV and ScBV than to RTBV, a leafhopper-transmitted badnavirus (Omura et al., 1983), and more distantly related to the caulimoviruses cauliflower mosaic (CaMV), carnation etched-ring (CERV), figwort mosaic (FMV) and soybean chlorotic mottle (SoyCMV) viruses (Figure 7).

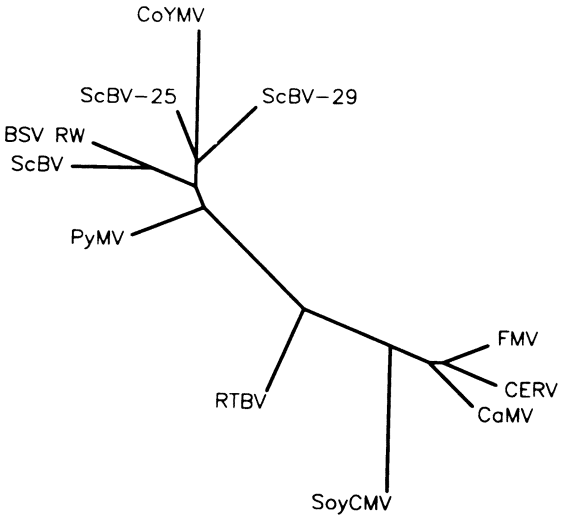

Figure 7. Tree illustrating the relationship of *Piper* yellow mottle virus to other plant pararetroviruses based on sequence comparisons of a portion of the putative reverse transcriptase-encoding region of the respective genomes. Branch lengths represent the extent of dissimilarity between the various viruses. The tree also illustrates genomic heterogeneity within sugarcane bacilliform virus. Preliminary evidence (data not shown) suggests that a similar genomic heterogeneity occurs in *Piper* yellow mottle virus.

Discussion

The data presented above on particle morphology, genome composition, serological and molecular affinities, and mealybug transmissibility support the conclusion that PYMV is a previously undescribed member of the *badnavirus* genus (Lockhart and Olszewski, 1994). *Piper* yellow mottle virus resembles the majority of badnaviruses in having a restricted host range, but differs from other badnaviruses in the unusual configuration of a large proportion of its genomic DNA molecules observed by EM (Figure 5D) and a relative resistance of its extracted genomic DNA to S1 nuclease digestion (Figure 4). The rod-like configuration and resistance to S1 nuclease of some PYMV DNA molecules suggest that these may represent covalently-closed, supercoiled molecules. However, there is no published evidence that supercoiled genomic DNA is encapsidated within virions of plant pararetroviruses, and no experiments (e.g. centrifugation in CsCl-ethidium bromide density gradients) were done to determine whether such configurations of DNA existed in the preparations used in the experiments described above. It therefore remains to be determined whether these observed properties of PYMV DNA represent intrinsic characteristics of the encapsidated DNA or artefacts resulting from the method of DNA extraction, or to other factors such as nuclease-inhibiting contaminants co-purified with the viral DNA.

The Thailand isolate of PYMV was demonstrated to be transmitted by mechanical inoculation and by the citrus mealybug, *Planococcus citri*, and to induce yellow mottle symptoms in black pepper. Symptomatic black pepper from Malaysia and Sri Lanka which contained one or more isometric virus-like particles were always infected with PYMV, and no symptomatic plants free of PYMV infection were found. It was therefore concluded that PYMV by itself could induce yellow mottle symptoms in black pepper, and that the other unidentified virus-like particles could possibly play a role in symptom development, but that this remains to be demonstrated. In particle size and association with three dsRNA species, the 30 nm particle found in black pepper in Sarawak and Sri Lanka resembles several cryptic plant viruses (Francki et al., 1985).

While this is the first published report of the identification and partial characterization a virus from black pepper, previous reports (Barat, 1952; Holliday, 1959) described symptoms essentially similar to those described above, and evidence was also cited for the probable role of a mealybug vector in disease transmission (Barat, 1952; Holliday, 1957). Together, these data support the conclusion that PYMV and possibly other unidentified viruses have been one of several factors in a disease complex limiting black pepper production and contributing to black pepper plantation decline in southeast Asia (Barat, 1952; Holliday, 1959). There has also been a recent report of a virus-like disorder of black pepper in India (Prakasam et al., 1990) and symptoms similar to those described above for PYMV infection were observed previously in India and Indonesia (Holliday, 1959). This suggests that PYMV may occur in black pepper in Southeast Asian countries other than those mentioned above. Because black pepper cultivars are clonally propagated, it would be important to establish the identity, epidemiology, distribution and potential economic effect of PYMV and other possible viral pathogens of *P. nigrum*, and to develop methods for producing and certifying virus-free planting material.

This report also describes the occurrence of PYMV in betelvine. The properties of PYMV are distinct from those reported for two viruses infecting *P. betle* in India (Singh and Rao, 1988; Johri et al., 1990), and PYMV therefore appears to be the third virus identified in betelvine.

Acknowledgement

Published as paper No. 20,523 of the contribution series of the Minnesota Agricultural Experiment Station based on research conducted under Project 22-79H.

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