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SHORT COMMUNICATION

Occurrence of endogenous Piper yellow mottle virus in black pepper

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Abstract Some badnaviruses are known to occur as endogenous viruses integrated into their host genome. In the present study, Piper yellow mottle virus (PYMoV), a badnavirus infecting black pepper was shown to occur as endogenous virus based on the PCR, reverse transcription (RT)-PCR, ELISA and Southern hybridization tests. Black pepper plants that tested positive in PCR for PYMoV gave negative reaction in RT-PCR indicating that they harbour endogenous PYMoV (ePYMoV) sequences. The RT-PCR (−ve) plants tested negative in ELISA and also in PCR using outword primers to amplify the full circular genome. Further, the presence of ePYMoV sequences in the black pepper genome was confirmed by Southern hybridization analysis using cloned PYMoV genomic fragments as probes. Among different open reading frames (ORFs) of the virus, ORF 3 was more frequently integrated. This is the first report of occurrence of ePYMoV sequences in black pepper genome.

Keywords Piper yellow mottle virus (PYMoV) · Endogenous PYMoV (ePYMoV) · Black pepper · PCR · RT-PCR · Southern hybridization

Introduction

Piper yellow mottle virus (PYMoV), a member of the genus *Badnavirus*, Family *Caulimoviridae*, is known to infect black pepper in Indonesia, Brazil, Malaysia, Thailand, Philippines, Sri Lanka and India [2, 12]. The virus induces chlorotic mottling, vein clearing, leaf distortion, reduced plant vigour and poor fruit set in affected black pepper plants. In addition to black pepper, PYMoV is reported to infect many '*Piper*' species including betelvine and Indian long pepper. PYMoV is transmitted primarily through vegetative means (stem cuttings) and seeds while secondary spread in the field occur through various species of mealybug vectors. It is a bacilliform shaped virus containing a circular covalently closed double stranded DNA genome of about 7.5 kb [2, 8, 12]. Complete genome sequence of four isolates of PYMoV is currently available [3, 7].

Badnaviruses are pararetroviruses that replicate through an RNA intermediate. Even though the replication cycle of pararetroviruses do not have a mandatory integration step, some badnaviruses and other pararetroviruses are known to integrate their genome into the genome of their hosts [2, 9]. These integrated sequences are referred as endogenous plant pararetroviruses (EPRVs). EPRVs are known in banana, citrus, fig, grape, kalanchoe, pineapple, taro and yam [5, 6, 11, 15, 20]. The EPRVs present in the plant genomes can be either dead sequences, which are incapable of giving episomal infection or can be functional and can trigger a virus infection under certain abiotic stress conditions. So far only three endogenous badnaviruses present in banana are known to give rise to infective episomal viruses under abiotic stress conditions [9, 10, 13]. In the present study, we report for the first time the occurrence of endogenous PYMoV (ePYMoV) in black pepper.

Plant materials used for this study include leaf tissues from PYMoV infected black pepper plants of variety Panniyur 1, IISR-Thevam and Sreekara maintained in the green house facility at the ICAR-Indian Institute of Spices Research, Kozhikode. The plants were identified by PCR

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using three different sets of primers corresponding to different open reading frames (ORFs) of the PYMoV genome. The PCR positive plants were further tested for the presence of viral transcripts by total RNA extraction followed by RT-PCR using the same set of primers. By analyzing the results of PCR and RT-PCR, tested plants were categorised as: (1) PCR (+) and RT-PCR (+) plants and (2) PCR (+) and RT-PCR (−) plants. These plants were then subjected to ELISA and PCR using outward primers (Forward 5′ TATGCCAAGGTAAGCCCAAC 3′; Reverse 5′ TCCAGCATTGCGACTAAGTG 3′) to amplify full circular genome of the virus. Southern hybridization was done using standard protocol [14] with total DNA isolated as described previously [1]. Thirty µg of sample DNA was restricted using *Eco*RI, *Sac*I, *Bam*HI, *Hind*III, *Kpn*I or *Xba*I (New England Biolabs, UK) and size fractionated in a 0.7% agarose gel by electrophoresis, and subsequently transferred to a nylon membrane membrane (Amersham Hybond^{TM}-N^{+}, GE Healthcare, UK), by capillary method. Probe preparation, hybridization, and detection were performed using 'DIG High Prime DNA Labeling and Detection Starter Kit II' (Roche Applied Science, IN, USA) following the manufacturer's instructions. The DNA probe was prepared by PCR amplification of different regions of PYMoV (Table 1). Amplified DNA fragment was isolated after gel fractionation, purified, and labeled with digoxigenin (DIG) using the random primed labeling method as specified in the kit. Hybridization procedures and incubation with chemiluminescence substrate CSPD were carried out in accordance with the manufacturer's instructions. The tracks of chemiluminescence were captured, developed, and fixed in X-ray film as per standard procedures.

Five plants each from black pepper varieties Panniyur 1, IISR-Thevam and Sreekara were tested for PYMoV by DNA PCR and RT-PCR. In DNA-PCR all plants tested positive for PYMoV for all or at least one set of primers (not shown). When total RNA from these plants were tested by RT-PCR using PYMoV specific primers, all except two plants of Panniyur 1 gave positive reaction. A plant with episomal and endogenous form of badnavirus may test positive in DNA-PCR due to sequence similirities in the episomal and endogenous forms [2]. As badnaviruses replicate via RNA intermediate, the virus transcripts are formed only in plants infected with episomal form of badnaviruses while plants with only endogenous badnaviruses donot produce viral transcripts, hence would test negative in RT-PCR. Thus two plants that tested negative in RT-PCR indicate absence of PYMoV transcripts and the occurrence of ePYMoV in black pepper. The RT-PCR (−ve) plants when subjected to ELISA using polyclonal antiserum to PYMoV showed negative reaction. Similarly, PCR performed using outword primers also did not give expected product of 7.5 kb indicating the absence of circular full genome of the virus in RT-PCR (−ve) plants (not shown).

The above results were further confirmed through Southern hybridization using probes representing different genomic regions of PYMoV. *Eco*RI has a single restriction site in the PYMoV genome; hence if a plant is infected with only episomal form of PYMoV, single hybridized band at 7.5 kb is expected in the Southern hybridization. *Eco*R1 digests probed with PYMoV 187,188 and PYMoV 36,107 probes (Table 1) in the present study showed multiple bands ranging from 250 bp to 20 kb indicating the occurrence of ePYMoV in Panniyur 1 variety of black pepper though banding pattern differered from plant to plant (Fig. 1a, b). The PCR (+) and RT-PCR (+) Panniyur 1 plants showed multiple bands including the 7.5 kb band indicating that they may have both PYMoV and ePYMoV. The two PCR (+) and RT-PCR (−) plants also showed multiple hybridized bands ranging from about 500 bp to 20 kb including a faint hybridization at the expected size for episomal form of PYMoV in one plant (Fig. 1a, b). The same plant DNA restricted with *Xba*I (which do not have any restriction site in the PYMoV genome) and probed with PYMoV 36,107 and PYMoV 157,163 probes (Table 1) also hybridized to multiple DNA fragments ranging from 750 bp to 20 kbp (Fig. 1c, d) though the size and number of bands varied. Similar kinds of multiple bands were also observed when *Sac*I, *Bam*HI, *Hind*III and *Kpn*I restricted DNA were hybridized with different PYMoV specific probes confirming the preence of

Table 1 List of probes representing different regions of PYMoV genome used for Southern hybridization. PYMoV genome cloned in a plasmid vector was used as template for amplification

Probe	Primers used for amplification (F/R)	Region	Size of probe (bp)
PYMoV 36,107	TATGCCAAGGTAAGCCCAAC (F) CAGCTGGTCTTGATAATAG (R)	ORF 3 & 4	899
PYMoV 187,188	GAGTACCAACAGGTGATGA(F) GTGCTTCCTCTTCTCAATC (R)	ORF 3	539
PYMoV 157,163	GAATGGTGTGAACTGGAAATG (F) AATAACGCATCAATATGCTTAAGG (R)	ORF 3	1025
PYMoV 252,253	GAGAGATCAATCGAGGATTG (F) CAACCTTGGCTATCATCAAC (R)	ORF 1	379
PYMoV 254,255	TTTGTCAAGCCAAGAGACCAC (F) TTGAGTGATTTGGTCCTCCAC (R)	ORF 2	352

ORF open reading frame

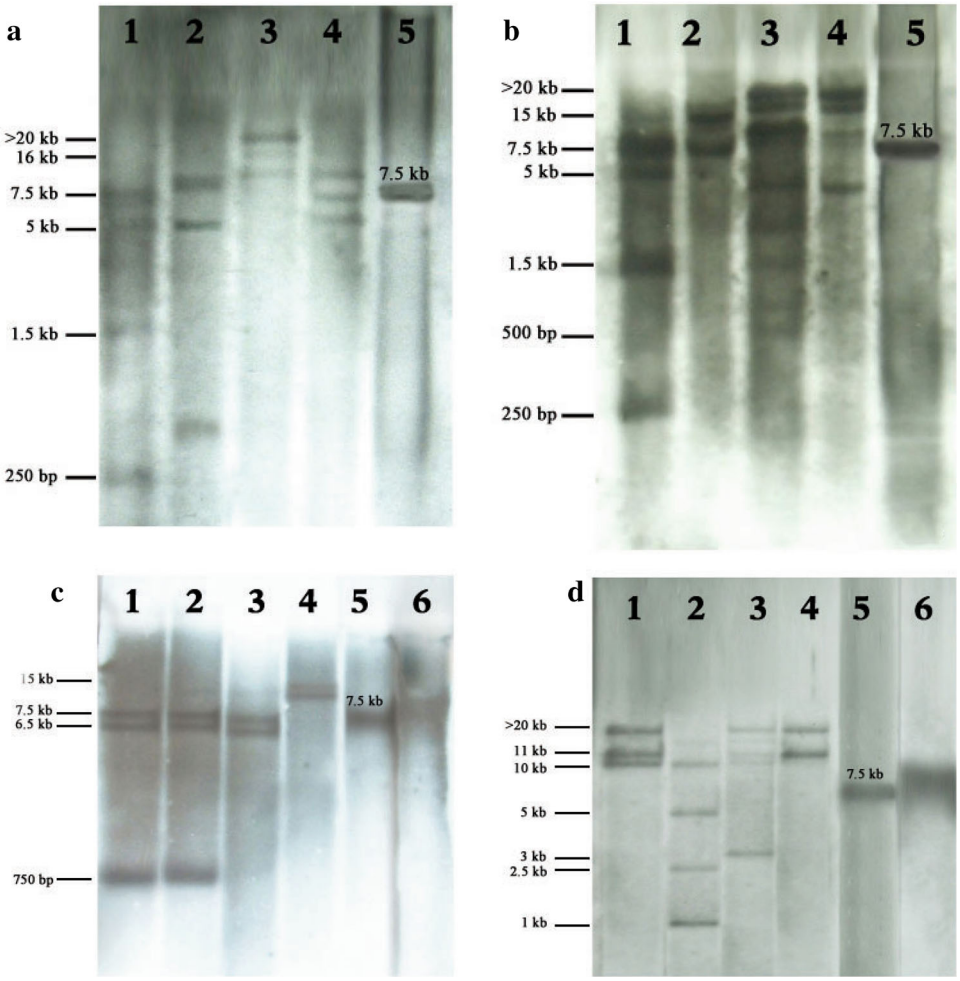

Fig. 1 Southern hybridization analysis of *Eco*RI (**a** & **b**) and *Xba*I (**c** & **d**) restricted Panniyur 1 black pepper plants using PYMoV 187,188 probe (**a**), PYMoV 36,107 probe (**b** & **c**) and PYMoV 157,163 probe (d). Lane 1 & 2: PCR (+) and RT-PCR (+) plant, Lane 3 & 4: PCR (+) and RT-PCR (−) plant and lane 5: Positive control (Linearized PYMoVclone containing ORF 3 and 4 region in a plasmid vector with a total size of 7.5 Kb). Lane 6: Unrestricted total DNA

ePYMoV in Panniyur 1 plants (not shown). The unrestricted plant DNA of the plant showed a diffused band at about 8.5 kb region indicating the presence of episomal form of PYMoV (Fig. 1c, d).

In order to test the presence of ePYMoV in other varieties of black pepper (Sreekara and IISR-Thevam) *Eco*RI digested plant DNA from these plants were hybridized using the probe PYMoV 36,107. Result showed a single hybridized fragment (4 kb) in the IISR-Thevam and Sreekara (not shown). The above results confirm integration of ORF 3, ORF 4 and intergenic region (IR) of PYMoV in all tested varieties of black pepper genome. In order to test the integration of ORF 1 and ORF 2 regions, *Eco*RI DNA digests of black pepper *var.* Sreekara, IISR-Thevam and Panniyur 1 were probed with corresponding probes (PYMoV 252,253 and PYMoV 254,255; Table 1). The linearized PYMoV full genome clone in a plasmid (total size, 10 kb) was used as a positive control (Fig. 2a, b). The probes hybridized to multiple DNA fragments ranging from 2 to 20 kb in the variety Panniyur 1 while in the Sreekara, a single band at 4 kb and in the IISR-Thevam a single band at 9 kb were observed (Fig. 2). No hybridization was seen with ORF 2 probe in the case of var. IISR Thevam indicating non-integratin of this region (Fig. 2b). Overall the results clearly showed that among different ORFs, ORF 3 is more frequently integrated than other ORFs.

Occurrence of endogenous badnaviral sequences in banana [6], *Dracaena* [16], yam [15, 18], fig [11], kalanchoe [20] and taro [19] were reported based on the PCR and Southern hybridization tests. In the present study we used PCR, RT-PCR, PCR with outword primers and Southern hybridization to show the occurrence of ePYMoV in black pepper. Among three varieties tested for PYMoV integration in the present study, Panniyur 1 is a hybrid while Sreekara and IISR-Thevam are selctions. The more number of hybridized DNA fragments observed in the digests of Panniyur 1 plants suggest integration of multiple copies of PYMoV genomic fragments. This could be due to hybrid nature of Panniyur 1 as similar results were reported in interspecific hybrids of other crops such as banana [9]. The low number of hybridized bands observed in the varieties, Sreekara and IISR-Thevam indicate that these varieties may have only a portion of the PYMoV genome

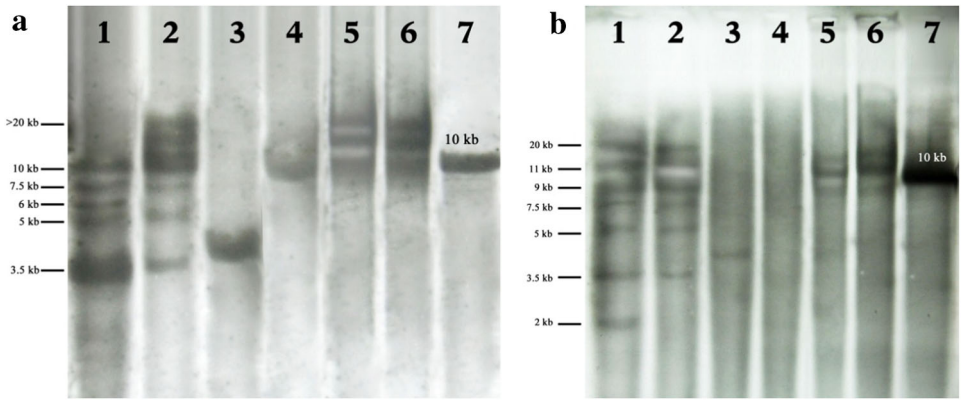

Fig. 2 Southern hybridization analysis of *Eco*RI restricted Panniyur 1, Sreekara and IISR-Thevam plants using PYMoV 252,253 probe (**a**) and PYMoV 254,255 probe (**b**). Lane 1, 3–4: *Eco*RI digested [PCR (+) and RT-PCR (+)] Panniyur 1, Sreekara, and IISR-Thevam DNA samples respectively. Lane 2: *Eco*RI digested [PCR (+) and RT-PCR (−)] Panniyur 1; lane 5–6: unrestricted DNA and lane 7: Positive control (Linearized PYMoV full genome cloned in a plasmid vector with a total size of 10 kb)

integrated. Overall, it was observed that frequency of integration of ORF 3 region was more compared to other regions. This kind of variation in the region integrated and its copy number was reported in eDBV (endogenous Dioscorea bacilliform virus) in yam [15, 18] and eDMV (endogenous Dahlia mosaic virus) in dahlia (*Dahlia variabilis*) [4]. In eDBV, integration frequency of ORF 3 region especially 3′ end of ORF 3 was much more than other regions [18]. At species and variety level, changes in copy number or activation and inactivation of endogenous virus elements happen due to the activity of retrotransposons or the inerspecific hybridization events [9, 10].

Recently Geering et al. [5] described a new genus in the family, Caulimoviridae that occur only in endogenous form called '*Florendovirus*', members of which have colonized the genome of large diversity of flowering plants including apple, citrus, cocoa, grape, cassva, rice, potato, maize, papaya, soybean, tomato etc. Endogenization takes place by illegitimate recombination into host genomes, and their presence is not necessarily associated with infection [9]. Except for the three endogenous badnaviruses reported in banana, rest of the endogenous badnaviruses reported so far in different plant species are not known to give rise to episomal infections as only a small portions of the viral genome are integrated [2, 9]. PYMoV is known to infect all varieties and cultivars of black pepper and other related *Piper* species. A detailed study may be needed to see the presence and extent of ePYMoV in different cultivars of black pepper and related species. Asymptomatic nature of PYMoV infected black pepper plants is common. Symptoms would re-appear in such plants when they are subjected to abiotic stress such as temperature and nutrient [17]. Whether this kind of symptom expression is due to increased viral replication or activation of ePYMoV into infective PYMoV is not known. Studies based on immuno capture (IC)-PCR and rolling circle amplification (RCA) in combination with PCR, RT-PCR and Southern hybridization are needed to identify and differentiate plants with endogenous and episomal PYMoV. Once these methods are developed, plants with only ePYMoV may be subjected to different abiotic stresses to see whether they can give rise to episomal infections or not.

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