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Molecular evidence for association of *Tomato leaf curl New Delhi virus* with leaf curl disease of papaya (*Carica papaya* L.) in India

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Abstract. Association of *Tomato leaf curl New Delhi virus* with leaf curl disease of papaya (*Carica papaya* L.) was detected by polymerase chain reaction using begomovirus-specific primers and confirmed by highest sequence similarities and close phylogenetic relationships.

Papaya (*Carica papaya* L.) is cultivated commercially throughout the world's tropical and subtropical regions for its edible fruits. Papain, an enzyme prepared from dried latex of immature fruits, is used for meat tenderising, food processing and in the leather industry (Singh 2006). The vasculature latex of papaya has medicinal value and is used to treat ulcers, dissolve membranes in diphtheria, and reduce swelling, fever and adhesions after surgery (Singh *et al.* 1983). The limiting factor of papaya cultivation is its susceptibility to ring spot, leaf curl, mosaic and distortion diseases. Among them leaf curl disease caused by *Begomovirus* species is one of the most serious threats to papaya cultivation in most of the papaya growing countries (Singh 2006). Various RNA viruses have been reported on papaya worldwide, viz. *Papaya ring spot virus* (*Potyvirus*), *Papaya mosaic virus* (*Potexvirus*), *Tomato spotted wilt virus* (*Tospovirus*), *Papaya apical necrosis virus* (*Rhabdovirus*), *Tobacco ring spot virus* (*Nepovirus*), *Tobacco streak virus* (*Ilarvirus*), *Tobacco rattle virus* (*Tobravirus*) and *Cucumber mosaic virus* (*Cucumovirus*) (Singh 2006). However, a limited number of DNA viruses are known to be associated with the leaf curl disease of papaya: *Papaya leaf curl virus* (Nadeem *et al*. 1997; Singh 2006), *Papaya leaf curl China virus* and *Papaya leaf curl Guangdong virus* (Wang *et al*. 2004), and *Papaya leaf curl Taiwan virus* (Chang *et al*. 2003). These viruses are members of

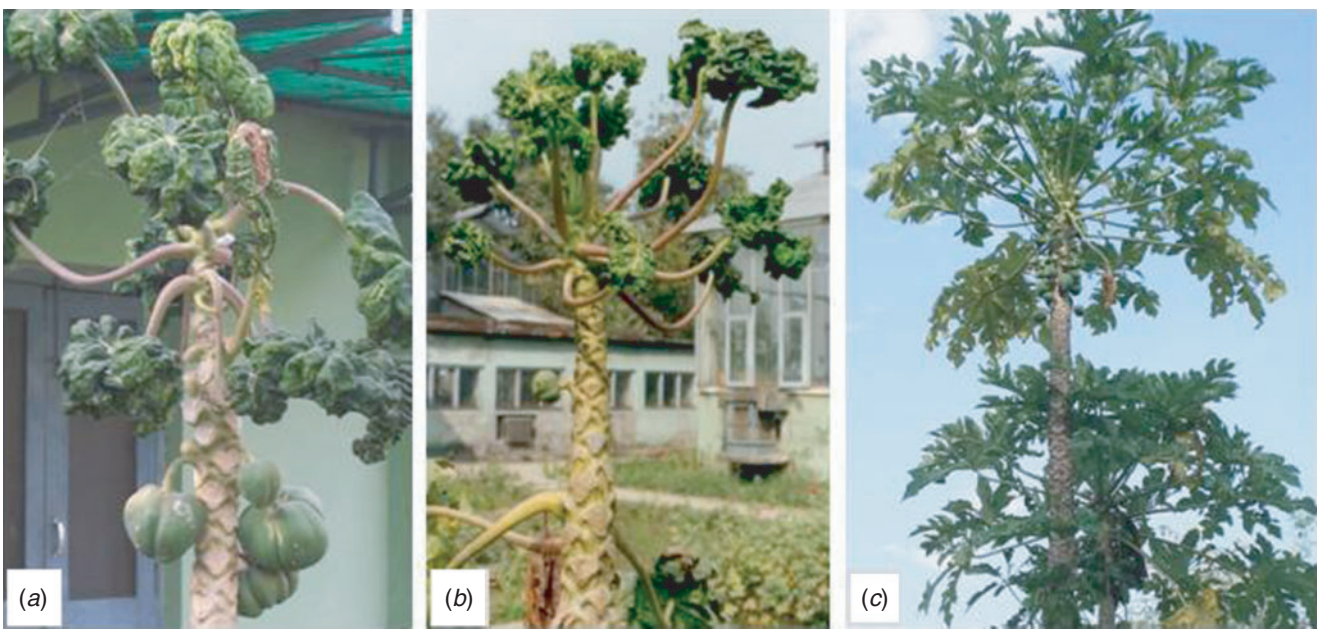

Fig. 1. Naturally infected *Carica papaya* plants exhibiting (*a*, *b*) severe downward leaf curl, swelling of veins, twisting and reduction of petioles, and distorted fruits, compared with (*c*) healthy plants.

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the *Begomovirus* group of the family *Geminiviridae.* The family comprises viruses with circular single stranded DNA genomes encapsidated in geminate quasi-isometric virion particles of ~20–30 nm in size which are transmitted through whitefly (*Bemisia tabaci*) (Harrison 1985).

A typical leaf curl disease was observed on papaya grown in and around Lucknow (India) during November 2006. Naturally infected papaya plants showed severe downward leaf curling, swelling of veins, twisting and reduction of petioles, and stunted growth of the whole plant which bore a few small, distorted fruit (Fig. 1*a*, *b*) compared with the healthy one (Fig. 1*c*). The whitefly population was also noticed in the papaya growing area, therefore, transmission of the disease was attempted through whiteflies (*B. tabaci*) in an insect proof glasshouse using an acquisition access period (AAP) of 18 h and inoculation access period (IAP) of 24 h. The disease was successfully transmitted by *B. tabaci* from naturally infected papaya to healthy seedlings of papaya, tobacco, tomato and chilli and all the inoculated test species displayed typical leaf curl symptoms indicating the association of a whitefly transmissible infectious agent, possibly a begomovirus, with the leaf curl disease of papaya.

For molecular detection, the total DNA was extracted from 100 mg leaf tissues of naturally infected and healthy papaya samples collected from six locations by a method described earlier (Dellaporta *et al*. 1983) and subjected to polymerase chain reaction (PCR). The PCR was performed using begomovirus genus specific degenerate primers (Wyatt and Brown 1996) and begomovirus coat protein (CP) region

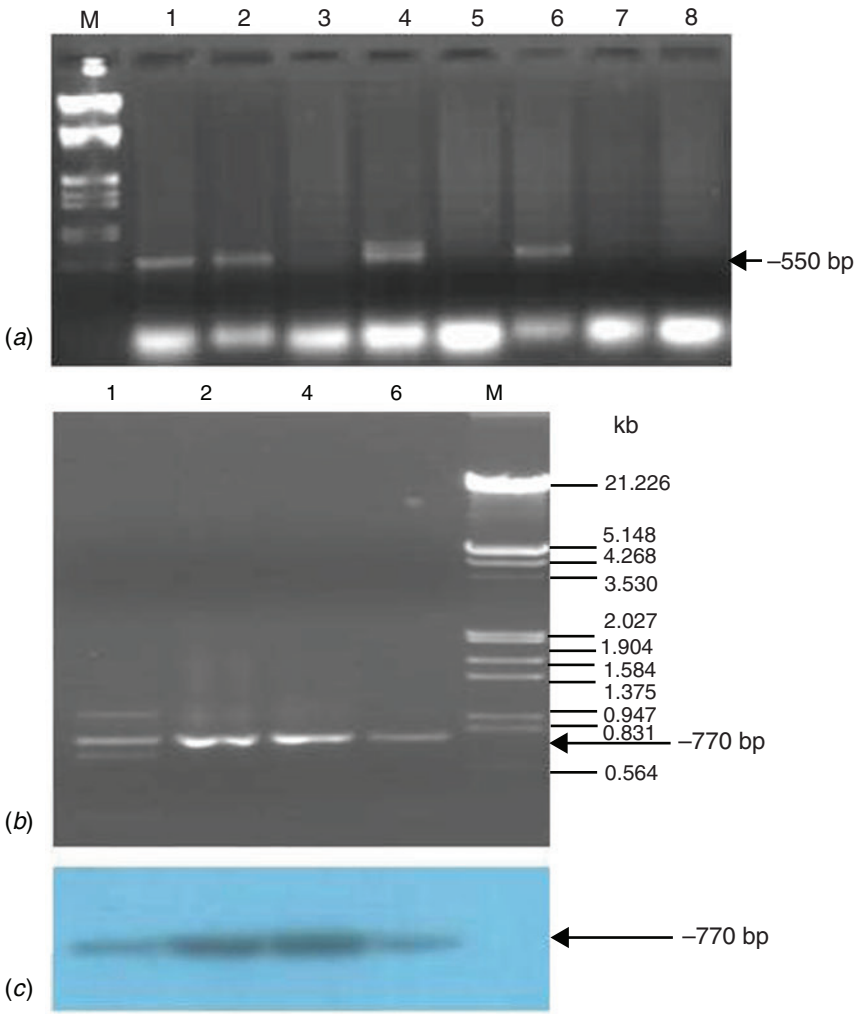

Fig. 2. Molecular evidence of a begomovirus present in symptomatic papaya. (*a*) Agarose gel electrophoresis of PCR products obtained by PCR using begomovirus genus specific degenerate primers (Wyatt and Brown 1996) from infected and healthy papaya leaf samples (lanes 1–8) showing a ~550 bp amplicon in 4 of 6 infected samples (lanes 1, 2, 4 and 6) but not from 2 of 2 healthy samples (lanes 7 and 8). M is Lambda DNA digested with *Eco*RI/*Hin*dIII (Genei Pvt. Ltd, Bangalore, India). (*b*) PCR amplicons of ~770 bp obtained by ToLCNDV-CP specific primers (Singh 2005) in 4 of 4 samples that were positive in (*a*) (lanes 1, 2, 4 and 6). (*c*) Southern blot hybridisation with a probe generated from the CP clone of ToLCNDV showing strong signals of hybridisation with all amplicons from (*b*) (lanes 1, 2, 4 and 6).

specific primers (Singh 2005) in a 50 µL reaction mixture containing: template DNA (100 ng), dNTPs (10 mmol each), primers (each 25 pmol), *Pfu* DNA polymerase (1.5 U), assay buffer (10×) in an automated thermal cycler (MJ Research, USA). The PCR conditions were: initial denaturation at 94°C for 5 min followed by 30 cycles of PCR of 1 min at 94°C, 30 s at 52°C (for Wyatt and Brown 1996 primers) or 47°C (for Singh 2005 primers) and extension at 72°C for 1.5 min, and a final extension of 5 min at 72°C. The PCR products were analysed by electrophoresis in 1.2% agarose gels. As expected, a band of ~550 bp in 4 of 6 symptomatic samples was successfully amplified by the Wyatt and Brown (1996) primers; however, no such amplicon was obtained in 2 of 2 healthy samples collected from the same location (Fig. 2*a*). The Singh (2005) primers were then used for PCR on DNA that had previously tested positive by PCR using the Wyatt and Brown (1996) primers and these all gave ~770 bp band (Fig. 2*b*). To confirm the authenticity of the ~770 bp amplicons, the gel was blotted onto nylon membrane and was allowed to hybridise with a probe of a CP clone of *Tomato leaf curl New Delhi virus* (DQ431846) which showed strong signals of hybridisation with the probe (Fig. 2*c*), indicating that PCR amplicons originated from the CP region of the begomovirus. Presence of begomovirus was also confirmed by PCR by begomovirus CP-specific primers in test plants that had been infected through transmission by whitefly.

The ~770 bp amplicons obtained from three leaf samples were cloned into the pGEM-T easy vector system-1 (Promega Life Corporation, USA). One clone of each sample was sequenced in both orientations using SP6 and T7 primers (Bangalore Genei Pvt. Ltd, India). The consensus sequence data of three identical sequences, producing a complete CP gene sequence of 771 nucleotides, encoding 256 amino acid residues, was deposited in the GenBank database (Accession EF194275). Basic Local Alignment Search Tool (BLAST) analysis of the papaya isolate (EF194275) revealed highest 95–97% nucleotide sequence identities with isolates of *Tomato leaf curl New Delhi virus* (ToLCNDV, AY691902, AY691899, AM286433, AY939926 and EU366163).

The Genomatix DiAlign2 program (Morgenstern 1998) was used to align the new begomovirus nucleotide and amino acid sequences (EF194275) with selected begomoviruses from a diverse range of host species including tomato, luffa, cotton, hibiscus, bottle gourd, and chilli (Table 1). EF194275 showed maximum 93–97% identities (at both nucleotide and amino acid levels) with ToLCNDV isolates. The identities of the new isolate with *Papaya leaf curl virus* (PLCV) isolates PLCV-PD, PLCV-AD, PLCV-HD, PLCV-Luc were low: 76–81% and 91–92% at nucleotide and amino acid levels, respectively (Table 1).

On the basis of the positive whitefly transmission test, PCR amplification of fragments of the expected size (~550 bp with begomovirus genus specific degenerate primers or ~770 bp with begomovirus CP specific primers), highest (96%) sequence identities with isolates of ToLCNDV and in accordance with the latest report of the International Committee on Taxonomy of Viruses (ICTV, Fauquet *et al*. 2008), the virus associated with leaf curl disease of papaya was identified as an isolate of ToLCNDV and designated as ToLCNDV-Papaya.

Table 1. Percentage identities in the coat protein gene region of the virus isolate from *Carica papaya* (EF194275) at nucleotide and amino acid levels with various begomoviruses, prepared using the Genomatix DiAlign program

ToLCNDV, *Tomato leaf curl New Delhi virus*; ToLCKV, *Tomato leaf curl Karnataka virus*; ToLCGV, *Tomato leaf curl Gujrat virus*; ToLCBV, *Tomato leaf curl Bangalore virus*; PLCV, *Papaya leaf curl virus*

Accession number	Abbreviation	Natural host	Location	Identity (%)	
				Nucleotide	Amino acid
AY691902	ToLCNDV	Tomato	Varanasi, India	97	93
AY691899	ToLCNDV	Tomato	Coimbatore, India	95	96
AM286434	ToLCNDV	Pumpkin	New Delhi, India	96	97
AY939926	ToLCNDV	Luffa	Northern India	95	97
DQ272541	ToLCNDV	Potato	New Delhi, India	94	96
DQ272540	ToLCNDV	Bottle gourd	India	94	96
EF063145	ToLCNDV	Cotton	India	94	96
EF123060	ToLCNDV	Hibiscus	Lucknow, India	94	96
DQ116880	ToLCNDV	Chilli	Pakistan	93	96
DQ116885	ToLCNDV	Tomato	Pakistan	93	96
NC_003897	ToLCKV	Tomato	Banglore, India	80	93
AY754812	ToLCKV	Tomato	India	79	93
AY190290	ToLCGV	Tomato	Varanasi, India	74	78
AF449999	ToLCGV	Tomato	Varanasi, India	73	78
AY428770	ToLCBV	Tomato	Bangalore, India	74	87
AF165098	ToLCBV	Tomato	Bangalore, India	73	85
DQ376036	PLCV-PD	Papaya	India	81	92
DQ376039	PLCV- PD	Papaya	Gujarat, India	77	92
DQ989326	PLCV-AD	Papaya	New Delhi, India	77	91
DQ376037	PLCV-AD	Papaya	India	77	92
NC_004147	PLCV-Luc	Papaya	Lucknow, India	77	91
DQ376038	PLCV-HD	Papaya	Gujarat, India	76	91
AJ436992	PLCV	Cotton	Pakistan	77	92

Papaya leaf curl disease has been reported from India by Thomas and Krishanaswamy (1939) and by Nariani (1956). The disease was known to be transmitted by the whitefly (*B. tabaci*) in a persistent manner and a begomovirus was detected from papaya by nucleic acid hybridisation tests (Saxena *et al*. 1998*a*, 1998*b*). *Papaya leaf curl virus* (PLCV) has been reported from Pakistan (Nadeem *et al*. 1997), Taiwan (Chang *et al*. 2003), China (Wang *et al*. 2004; Zhang *et al*. 2005; Huang and Zhou 2006) and India (Singh 2006). To the best of our knowledge this is a first report on association of ToLCNDV with leaf curl disease of papaya in India.

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