DOI : 10.5958/0974-0112.2017.00110.4

Indian J. Hort. 74(4), December 2017: 574-578

Occurrence of *Plantago asiatica mosaic virus* infecting oriental lily in India

S. Rajamanickam*, S. Rageshwari, P. Renukadevi and S. Nakkeeran

Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore 641 003

ABSTRACT

Lilium is a major cut flower grown under protected cultivation in Tamil Nadu and Bengaluru provinces by importing lilium bulbs from European countries. The oriental lilium grown under protected cultivation in Nilgiris provinces expressed the symptom of viral infection. The symptoms in Oriental lily include chlorotic and necrotic streaks on leaves and stunting of the infected plants. Leaf samples expressing the characteristic symptoms of virus infection were indexed using DAC-ELISA and reverse transcription polymerase chain reaction (RT-PCR). The coat protein (CP) gene of PlAMV was amplified with an amplicon size of 722 bp and sequence analysis confirmed the viruses as *Plantago asiatica mosaic virus* (PlAMV) with 99 to 100% nucleotide and 99.5 to 100% amino acid homology with other PlAMV isolates. Comparison of multiple sequence alignment analyses confirmed the close relationship between PlAMV and *Tulip virus* × (TVX), which had 70% nucleotide sequence identity. Phylogenetic analysis of the nucleotide confirms that our PlAMV isolates formed a single subgroup with other PlAMV isolates. The result provides important clues about spread of the virus and to the best of our knowledge it is the first detailed study of PlAMV infecting lily in India.

Key words: Lily, PlAMV, Occurrence, CP gene analysis.

INTRODUCTION

Lilies (*Lilium* sp.) belongs to the family *Liliaceae*, is a commercial high value cut flower crop cultivated in Nilgris province of South India under protected cultivation. The genus Lilium includes 294 genera with 4500 species with three commercially important divisions of lily including Easter lily (*Lilium longiflorum*), Asiatic and Oriental hybrids. In India, Asiatic and Oriental lilies are commonly grown in hilly areas and under temperate condition (Sharma *et al*., 15). Owing to the commercial value, lilium is grown throughout the year in Nilgris province of India (Hemamoorthy and Prakasam, 6). The *Cucumber mosaic virus* (CMV), *Lily symptomless virus* (LSV), *Strawberry latent ringspot virus* (SLRSV) and *Lily mottle virus* (LMoV) are the major viruses infecting lily in India (Sharma *et al*., 16). Apart from these viruses, *Prunus necrotic ringspot virus* (PNRSV) (Han and Liu, 2007), *Tobacco mosaic virus* (TMV), *Lily virus* × (LVX), *Tobacco rattle virus* (TRV), *Lily mild mosaic virus* (LMV), *Tomato ringspot virus* (ToRSV), *Narcissus mosaic virus* (NMV) and *Arabis mosaic virus* (ArMV) (Lee *et al*., 11; Asje, 1; Komatsu *et al*., 8) have also been reported in lily worldwide. The CMV, LSV, SLRSV predominate in India, whereas PlAMV has not been experienced. An unusual symptom including chlorotic and necrotic streaks was observed on the leaves in oriental lily in Nilgris province of Tamil Nadu during October 2015. However, it was not associated with infection by any of major viruses detected through serological reaction with tospovirus, potyviruses and ilarvirus specific antibody. Hence, the oriental lilium infection was suspected as *Plantago asiatica mosaic virus* (PlAMV). The *Plantago asiatica mosaic virus* (PlAMV) pertains to the genus *Potexvirus*, family *Flexiviridae*, a mechanically transmitted virus with unknown vectors (Komatsu *et al*., 8). The occurrence of PlAMV in oriental hybrid lilies has been reported in Southern Italy, Netherlands, South Korea, and USA (Parrella *et al*., 12; Hammond *et al*., 4). Though the occurrence of PlAMV has been reported from many countries, reports on molecular characterization of PlAMV in India are not known. However in the present investigation, we report the natural occurrence of PlAMV in lilum and its molecular properties and phylogenetic relationship.

MATERIALS AND METHODS

The lilium varieties grown under protected cultivation were observed for the presence of PlAMV symptoms in field at Nilgris district of Tamil Nadu, India. The characteristic symptoms were observed and described. The PlAMV infected plant samples collected from lilium was subjected to direct antigen coating-ELISA (DAC-ELISA) with tospovirus, potyviruses and ilarvirus specific antibody as per the procedure described by Hobbs *et al*. (7). The polystyrene plates were coated with 200 µl of plant extract ground in 0.05 M carbonate buffer pH 9.6 @ 1:10 dilution (1.59 g = Na_2CO_3; 2.93 g = $NaHCO_3$;

*Corresponding author's E-mail: rajamanickam.path@gmail.com

dissolved in 1 l deionized water) incubated at 37°C for 1.30 h. The plates were washed three times in PBS Tween (2.89 g of Na_2HPO_4; 0.4 g of KH_2PO_4; 0.4 g of KCl; 16.0 g of NaCl; dissolved in 2 l deionized water; pH 7.4 and add 0.5 ml/ l of Teen 20®) with an interval of 3 min. for each washing. The polyclonal antibody diluted in antibody buffer to a dilution of 1:1000 were added at the rate of 200 µl per well separately. Then the plates were incubated at 37°C for 3 h and washed with PBS-Tween with an interval of 3 min. for each washing. Universal conjugate was diluted in antibody buffer to a dilution of 1:2000 and then added to each well. The plate was incubated at 37°C for 3 h and washed in PBS-Tween. Substrate buffer containing 0.5 mg/ ml of PNPP (p-nitrophenyl phosphate pH 9.8) was then dispensed to each well @ 200 µl/ well and incubated at room temperature under dark for 20-30 min. Light orange to yellow colour development indicated a weak to strong positive reaction and the results were quantitatively recorded in an ELISA reader at 405 nm (Biotek EL × 800).

The total RNA was extracted from 100 mg lilium leaves using RNeasy plant extraction kit (Qiagen Inc., Chatsworth, CA, USA) according to the manufacturers' protocol and resuspended in 50 µl nuclease free water. For cDNA synthesis of PlAMV, 1 µg total isolated RNA (200 ng/ µl) was annealed with 0.3 µM downstream primers (PlAMV CPR - 5'AAACGGTAAAATACACACCGGG 3') at 70°C for 10 min. To the transcription mixture, various reaction components were added [(RNase inhibitor 1 µl (20 U); dNTPs 2 µl (10 mM); 4 µl 5´ reverse transcriptase buffer containing Tris-HCl 250 mM, pH 8.3 at 25°C, KCl 250 mM, $MgCl_2$ 20 mM, 1 µl DTT 50 mM)]. The reaction mixture was incubated at 37°C for 10 min., 40 U M-MuLV reverse transcriptase was added and the mixture was re-incubated at 37°C for 60 min. The reaction was stopped by heating the mixture at 70°C for 10 min.

PlAMV:cDNA product (5 µl) was added to 50 µl of PCR reaction mixture containing 0.20 mM each of dNTPs, 0.25 µM of each primer (PlAMV CPF-5' CAAGACATTC TCCACCATGGCACTC 3' and PlAMV CPR - 5'AAACGGTAAAATACACACCGGG 3', 5 µl 10X *Taq* polymerase buffer, 2.0 mM $MgCl_2$ and 2 U *Taq* DNA polymerase. The RT-PCR was performed in Eppendorf Mastercycler Gradient ES with the following thermal programme: initial denaturation at 94°C for 2 min., followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. and final extension of 72°C for 10 min. The PCR product was analyzed on a 1.2% agarose gel, stained with ethidium bromide and viewed under transilluminator.

The amplicon of coat protein gene was purified using QIAGEN gel extraction kit (Qiagen Inc., Chatsworth, CA, USA) and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) following the manufacturer's instructions and transformed into *Escherichia coli* DH5α by following standard molecular biology procedures. Plasmid DNA was isolated from the potential recombinant clones using Wizard plus DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol and the positive clones were identified by restriction digestion analysis using *EcoRI* enzyme. The three independent clones were sequenced (Chromos Biotech Pvt. Ltd., Bengaluru) from both orientations for each fragment separately. The sequences were then edited using the BIOEDIT Software (Hall, 3). Sequence similarity search of the GenBank database was done using the Basic Local Alignment Search Tool (BLAST) program.

The sequence variability of PlAMV was analysed with the nucleotide and amino acid sequences of CP genes compared with sequences of the previously reported isolates available in GenBank. The amino acid sequences of the PlAMV coat protein gene was translated from the consensus nucleotide sequences using the EMBOSS Transeq program (Rice *et al*., 13). Both the nucleotide and amino acid sequences were then aligned with selected sequences of *Potexvirus* along with selected sequences of PlAMV using the CLUSTAL W program (Larkin *et al*., 10). Phylogenetic analysis of CP sequence was done on MEGA 5.1 (Tamura *et al*., 16) and trees were created using the Neighbour-joining method (Saitou and Nei, 14). The robustness of the trees was determined by bootstrap using 1,000 replicates. *Lily virus* × (LVX) was used as a reference out group member of the genus potexvirus for rooting the phylogenetic tree.

RESULTS AND DISCUSSION

Viruses are the major constraints in lily cultivation under protected condition that often decrease the yield and quality of flowers. Characterization of PlAMV infecting lily in Tamil Nadu provides knowledge on better understanding the occurrence and genetic composition. The oriental lily grown under protected cultivation expressed the characteristic symptoms of PlAMV on leaves, which showed severe chlorotic and necrotic symptoms. The severely infected plants were stunted. Symptom development of PlAMV starts with the end of the vegetative growth stage with brown coloured veins on the bottom side of a leaf along with chlorotic streaks and turns necrotic. In severe stage of infection brown-coloured and necrotic symptoms were noticed on the top side of the leaves (Fig. 1). Immunological assays have been developed and successfully used for a number of years for the

Fig. 1. Symptoms of PlAMV on lilium with chlorotic and necrotic streaks on the leaves.

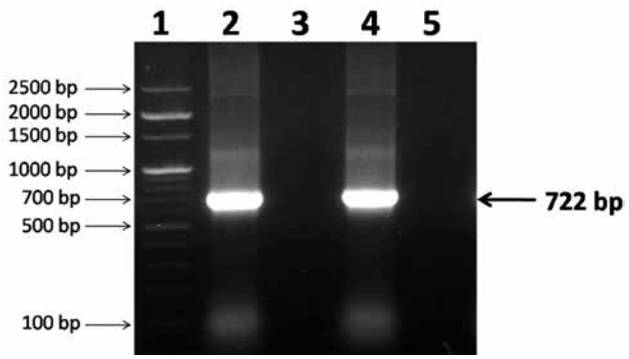

Fig. 2. RT-PCR amplification of PlAMV coat protein gene from naturally infected leaves of lilium. Lane 1:100 bp ladder; Lanes 2 & 4: Amplified DNA fragment from infected samples; Lane 3 & 5: Healthy samples (control).

detection of plant viruses. Bulbs of the infected plants were collected and the leaf samples were tested for the presence of tospovirus, potyviruses and ilarvirus by DAC-ELISA with specific antibody and conjugate (provided by ICRISAT, Hyderabad, Telangana, India). All the samples tested were negative to tospovirus, potyvirus and ilarvirus group antiserum, respectively. This could help in understanding that, virus infecting lily is different from previously reported lilium viruses. The PCR assay has been used as a tool for identification of unreported viruses from lily, since PCR has been shown to be effective in rapid and sensitive detection of many plant viruses (Kwon *et al*., 9). Parrella *et al*. (12) has reported severe necrotic streaking in midstem leaves caused by PlAMV on plants of lily hybrids (*Lilium* sp., Liliaceae) in several greenhouses of Campania region of Southern Italy. Hammond *et al*. (4) reported the infection of PlAMV in Asiatic and Oriental lilies in the United States.

RNA isolated from infected sample was subjected to RT-PCR assay using self designed primers corresponding to coat protein gene based on alignment of KM205357 with all PlAMV sequences available in GenBank. RT-PCR using PlAMV CPF/ PlAMV CPR yielded an amplicon size of 722 bp from infected leaves, while amplification was not observed with total RNA extracted from healthy plants (Fig. 2). The amplified fragments were separated on agarose gel and cloned into pGEM-T easy vector. The three independent clones were sequenced (Chromos Biotech Pvt. Ltd., Bengaluru) from both the orientations for each fragment separately. The nucleotide sequence analysis using the NCBI BLAST confirmed the association of *Plantago asiatica mosaic virus* in lilium. The CP gene sequence of PlAMV virus lilum isolate TN-1 from Ooty and TN-2 from Devashola of Nilgris district were submitted in NCBI GenBank database (Acc. No. KU845394 and KX130954). The CP gene of PlAMV isolate was compared with corresponding gene from known PlAMV isolates and other genus of potexvirus at the nucleotide levels. The sequence analysis revealed the high homologies between the PlAMV isolates, including Ko-JJ-2-2 isolate from South Korea (KU159091), CES5 isolate from Italy (LN827658), kr isolate from South Korea (KT717325) and Concador isolate from Hungary (LN794199), respectively. They showed the highest nucleotide sequence identity of 100 per cent with CES5 isolate from Italy (LN827658), kr isolate from South Korea (KT717325) and Concador isolate from Hungary (LN794199). Similarly, sequence had 99 per cent similarity with Sorbonne isolate from the Netherlands (KF471012), LIL6 isolate from Italy (LN651194) and SEG2 isolate from Italy (LN827660). Analysis of the 239 deduced amino acid sequence of coat protein gene revealed that our isolates had 99.5 to 100% homology with other strains of the same virus (Table 1). Phylogenetic analysis of nucleotide sequences of CP gene supported a single cluster PlAMV, indication the absence of geographical variation. The combined analysis of species representing the *Potexvirus* revealed a distinct group of PlAMV, which was closely related to *Tulip virus* × (TVX) (Fig. 3). Similarly, Hammond *et al*. (2015) amplified the sequence of PlAMV using RT-PCR from *C. quinoa* and *N. benthamiana*, which yielded 1.3 kb product and the consensus sequence (KM205357) had 98.7% nucleotide identity to a Dutch isolate of PlAMV (PlAMV, KF471012). Similarly, Parrella *et al*. (12) demonstrated that,

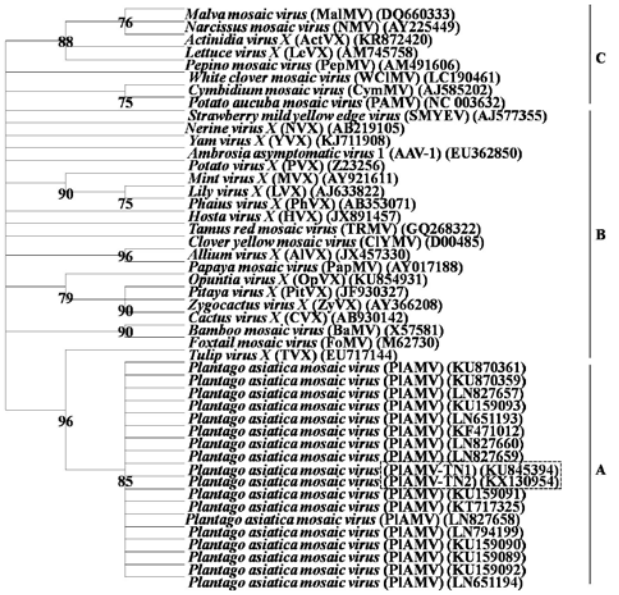

Fig. 3. Neighbor-joining phylogenetic tree based on the nucleotide sequences of the coat protein gene of PlAMV (KU845394 and KX130954) and *Lily virus* × (LVX) is defined as an out-group. Numbers above each branch are the neighbor-joining bootstrap scores given as percentage of 1,000 replicates. Bootstrap scores lower than 70% are not shown. Group B and C contain potexviruses, while group A contains members of representative species of PlAMV within potexviruses collected from NCBI database.

the amplified coat protein gene with an amplicon size of approximately 1.0 kb encompassing the complete ORF had the highest nucleotide sequence identity with the Dutch isolate Sorbonne of PlAMV (KF471012), ranging from 99.4 to 99.8%. This clearly indicate that Indian isolates of PlAMV infecting lily showed a high genetic stability that may be due to these isolates having evolved from the same parental source in lily, which is propagated vegetatively through bulbs and bulblets. The combined analysis of species representing the *Potexvirus* revealed a distinct group of PlAMV, which was closely related to TVX as reported previously by Fajolu *et al*. (2). He illustrated that, the TVX and PlAMV are sister group in the genera potexvirus based on nucleotide percentage identity of CP from TVX. Based on the sequence similarity of PlAMV isolates TN-1 and TN-2 detected from India, closely mimics the identity of CES5 isolate from Italy or Sorbonne isolate from the Netherlands, indicating that the virus might have been introduced through the import of bulbs from either Netherlands or Italy. This is the first report of PlAMV in *Lilium* spp. in India, since, PlAMV infection of lilies have not been reported earlier.

Table 1. Nucleotide (nt) and amino acid (aa) identities of the coat protein gene of *Plantago asiatica mosaic virus* (PlAMV) lilium strain (KU845394 and KX130954) with corresponding sequences of selected strains of PlAMV and *Lily virus* × (LVX) is defined as an out-group.

Accn. No.	Strain	Country	Percentage (%) identity	
			nt	aa
KU159091	PlAMV	South Korea	100.00	100.00
KT717325	PlAMV	South Korea	100.00	100.00
LN827658	PlAMV	*Lilium* sp.	100.00	100.00
LN794199	PlAMV	Hungary	100.00	100.00
KU159090	PlAMV	South Korea	99.80	100.00
KU159089	PlAMV	South Korea	99.80	100.00
KU159093	PlAMV	South Korea	99.80	100.00
KU159092	PlAMV	South Korea	99.80	100.00
LN651194	PlAMV	South Korea	99.80	99.50
LN827657	PlAMV	Italy	99.80	100.00
LN651193	PlAMV	Italy	99.60	100.00
LN827660	PlAMV	Italy	99.60	99.50
LN827659	PlAMV	Italy	99.60	99.50
KF471012	PlAMV	Netherlands	99.60	99.50
KU870361	PlAMV	Netherlands	99.50	100.00
KU870359	PlAMV	Netherlands	99.50	100.00
AJ633822	LVX	Italy	46.10	36.00

ACKNOWLEDGEMENT

The authors acknowledge the Tamil Nadu Agricultural University, Coimbatore for support of facilities.

REFERENCES

1. Asjes, C.J. 2000. Control of aphid-borne lily symptomless virus and lily mottle virus in *Lilium* in the Netherlands. *Virus Res*. **71**: 23-32.

2. Fajolu, O.L., Wen, R.H., Windham, A.S., Windham, M.T., Moulton, J.K. and Hajimorad, M.R. 2009. Genetic variability and phylogenetic analysis of *Hosta virus X*. *Arch. Virol*. **154**: 1909-16.

3. Hall, T.A. 1999. Bioedit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/ NT. *Nucleic Acids Symp. Series*, **41**: 95-98.

4. Hammond, J., Bampi, D. and Reinsel, M.D. 2015. First report of *Plantago asiatica mosaic*

virus in imported Asiatic and Oriental lilies (*Lilium* hybrids) in the United States. *Plant Dis.* **99**: 292.

5. Han, L.J. and Liu, W.H. 2007. Studies on *Prunus* necrotic ringspot virus (PNRSV) occurring on lily. *Agric. Sci. China*, **6**: 1201-08.

6. HemaMoorthy, T. and Prakasam, V. 2013. First report of *Penicillium expansum* causing bulb rot of *Lilium* in India. *American-Eurasian J. Agric. Env. Sci*. **13**: 293-95.

7. Hobbs, H.A., Reddy, D.V.R., Rajeshwari, R. and Reddy, A.S. 1987. Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. *Plant Dis.* **71**: 747-49.

8. Komatsu, K., Yamaji, Y., Ozeki, J., Hashimoto, M., Kagiwada, S., Takahashi, S. and Namba, S. 2008. Nucleotide sequence analysis of seven Japanese isolates of *Plantago asiatica mosaic virus* (PlAMV): A unique potexvirus with significantly high genomic and biological variability within the species. *Arch. Virol*. **153**: 193-98.

9. Kwon, J.Y., Ryu, K.H. and Choi, S.H. 2013. Reverse transcription polymerase chain reaction-based system for simultaneous detection of multiple lily-infecting viruses. *Plant Pathol. J*. **29**: 338-43.

10. Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J. and Higgins, D.G. 2007. Clustal W and Clustal × version 2.0. *Bioinformatics*, **23**: 2947-48.

11. Lee, K.H., Choi, H.S., Choi, G.S. and Kim, J.S. 1996. Virus diseases of lilies in Korea. *Acta Hort*. **414**: 195-201.

12. Parrella, G., Pasualini, A., Greco, B. and Nappo, A.G. 2015. *Plantago asiatica mosaic virus* found in protected crops of lily hybrids in southern Italy. *Plant Dis.* **99**: 1289.

13. Rice, P., Longden, I. and Bleasby, A., 2000. EMBOSS: The European molecular biology open software suite. *Trends Genet*. **16**: 276-77.

14. Saitou, N. and Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*. **4**: 406-25.

15. Sharma, A., Mahinghara, B.K., Singh, A.K., Kulshrestha, S., Raikhy, G., Singh, L., Verma, N., Hallan, V., Ram, R. and Zaidi, A.A. 2015. Identification, detection and frequency of lily viruses in Northern India. *Scientia Hort.* **106**: 213-27.

16. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*. **28**: 2731-39.

Received : February, 2017; Revised : September, 2017; Accepted : November, 2017