SHORT COMMUNICATION

Molecular detection and characterization of a divergent isolate of *Plantago asiatica* mosaic virus in *Plantago asiatica*

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Abstract Four samples of *Plantago asiatica* showing mottle or mottled yellowing symptoms were collected and three of them were confirmed to be infected with *Plantago asiatica* mosaic virus (PIAMV) by Illumina HiSeq 2500 paired-end RNA sequencing. Consequently, the complete genomic sequence of a Korean isolate of PIAMV (PIAMV isolate Gunwi) from *P. asiatica* was determined. The nucleotide sequence of PIAMV-Gunwi was most closely related to a Russian isolate of PIAMV (PIAMV-Ru) collected from *P. asiatica* but highly dissimilar (~23 %) to other isolates of PIAMV from other plant species. Pairwise comparisons revealed that the complete replicase protein and coat protein of PIAMV-Gunwi share 85.15–91.20 and 84.54–90.82 % amino acid sequence identities, respectively, to the corresponding proteins of other PIAMV isolates. To our knowledge, this is the first report of the natural infection of *P. asiatica* by PIAMV in Korea, and

the divergent PIAMV isolate Gunwi expands our understanding of the epidemiology of PIAMV.

Keywords PIAMV · Divergent isolate · *Plantago asiatica* · Next-generation sequencing

Plantago asiatica mosaic virus (PIAMV; genus *Potexvirus*, family *Alphaflexiviridae*, order *Tymovirales*) was initially identified from *P. asiatica*, a member of the family *Plantaginaceae*, and the complete genomic sequence of PIAMV was determined about two decades later [10]. To date, the complete genomic sequences of 17 PIAMV isolates are available in the GenBank database. Although the first-reported isolate was characterized from *P. asiatica* [10], the other isolates of PIAMV have been identified in *Lilium* spp., *Nandina domestica*, and *Primula sieboldii* [3, 5] except for one PIAMV isolate from *P. asiatica* in this work. The majority of these have been identified in *Lilium* spp. samples in recent years, and PIAMV has become an economically important pathogenic virus in this species [2, 4, 5, 8, 9]. In Korea, six complete genomic sequences of PIAMV isolates sampled from *Lilium* spp. have been reported recently. In this study, we detected PIAMV in *P. asiatica* samples using high-throughput RNA sequencing and compared the genomic sequences of the PIAMV isolate and known PIAMV isolates.

In April 2014, four samples of *P. asiatica* showing mottle or mottled yellowing symptoms were collected from an apple orchard located in Gunwi County, Korea. To identify the causal agent(s) of the symptoms, the plants were analysed using paired-end RNA sequencing. The total RNAs extracted from the symptomatic leaves of each sample with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) were pooled and the ribosomal

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RNAs were eliminated with the Ribo-ZeroTM rRNA Removal Kit (Plant Leaf) (Epicentre, Madison, WI, USA). Illumina HiSeq 2500 paired-end RNA sequencing by the Theragen Bio Institute (Suwon, Korea) and data analysis with SeqGenesis (Daejeon, Korea) were conducted as described in previous studies [6, 7]. One contig, representing nearly complete genomic sequence of PIAMV, with 79–87 % nucleotide sequence identity to previously sequenced PIAMV isolates, was obtained.

To confirm the contig sequence derived with RNA sequencing, five primer sets were designed to amplify almost the entire genome, except both ends (Supplementary Table S1). The total RNA was extracted from each sample with TRI Reagent (Molecular Research Center), and the cDNA was synthesized with a random N25 primer and RevertAid Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA), for use as the template in a PCR performed with AccuPower ProFi Taq PCR PreMix (Bio-ner, Daejeon, Korea). The PCR results confirmed that three of the four *P. asiatica* samples were infected with PIAMV (data not shown). One sample showing mottled yellowing symptoms was then used to obtain the complete genomic sequence of a Korean isolate of PIAMV from *P. asiatica*. The amplified PCR products were cloned into the RBC T&A Cloning Vector (RBC Bioscience, Taipei, Taiwan) for Sanger sequencing by Macrogen (Daejeon, Korea). To determine both ends of the PIAMV genome, rapid amplification of cDNA ends (RACE) was conducted using the 5'/3' RACE Systems (Invitrogen, Carlsbad, CA, USA) and three additional primers designed based on the contig sequence and the sequences obtained with Sanger sequencing (Supplementary Table S1). We sequenced at least three clones per PCR reaction, and there was no any significant variation between clones. The sequences of seven fragments, comprising overlapping PCR products covering the entire PIAMV genome, were assembled using the DNAMAN 5.0 program (Lynnon Biosoft, Quebec, Canada).

The complete genomic sequence of the PIAMV isolate Gunwi was determined and deposited in GenBank (Accession no. KU697313). Its genome is composed of 6130 nucleotides (nt), excluding the 3'-terminal polyadenosine tail, and the 5' and 3' untranslated regions (UTRs) are 85 and 125 nt in length, respectively. A National Center for Biotechnology Information (NCBI) BLASTN search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) showed that the entire nucleotide sequence of PIAMV-Gunwi shares the greatest identity with a Russian PIAMV isolate (PIAMV-Ru) sampled from *P. asiatica* (GenBank accession no. Z21647; 87 % identity). A phylogenetic tree and pairwise comparison, based on the complete genomic sequence of PIAMV-Gunwi and all existing complete genomic sequences of other PIAMV isolates, were constructed with

Fig. 1 Phylogenetic tree (a) and pairwise comparison (b) based on a complete genomic nucleotide sequence alignment of *P. asiatica* mosaic virus (PIAMV) isolate Gunwi (GenBank accession no. KU697313) and other PIAMV isolates. The phylogenetic tree and pairwise comparison were constructed with CLC Main Workbench 6.1.1 (CLC Bio, Cambridge, MA, USA), and the lengths of the branches and percentage bootstrap frequencies are marked. *Tulip virus X* (TVX) was used as the outgroup

CLC Main Workbench 6.1.1 (CLC Bio, Cambridge, MA, USA) and showed that the nucleotide sequence of PIAMV-Gunwi is most closely related to PIAMV-Ru but has a high level of nucleotide sequence dissimilarity to other isolates (~23 %) (Fig. 1). On the other hand, Korean isolates of PIAMV, which originate from *Lilium* spp., share more than 98 % nucleotide sequence identity with that of the PIAMV-Concador from Hungary but they are distantly related to PIAMV-Gunwi (Fig. 1).

Five putative open reading frames (ORFs) were predicted using the DNAMAN 5.0 program (Lynnon Biosoft): ORF1 (nt 86–4249), ORF2 (nt 4251–4943), ORF3 (nt 4909–5241), ORF4 (nt 5045–5410), and ORF5 (nt 5382–6005), (Supplementary Fig. S1). The protein families database (Pfam; <http://pfam.xfam.org/>; [1]) identified the conserved domains of PIAMV-Gunwi, encoding a methyltransferase, RNA helicase, RNA-dependent RNA polymerase, movement protein, and coat protein (Supplementary Fig. S1). The highest amino acid sequence identities of the individual ORFs of PIAMV-Gunwi shared with those of other PIAMV isolates were identified with the NCBI Protein BLAST program (BLASTP), and the results were 91 % (query coverage 100 %) for ORF1, 92 % (query coverage 100 %) for ORF2, 93 % (query coverage 100 %) for ORF3, 81 % (query coverage 100 %) for ORF4, and 91 % (query coverage 100 %) for ORF5. All the amino acid sequences that were most similar to those of PIAMV-Gunwi were derived from PIAMV-Ru. To understand the molecular relationships of these PIAMV isolates, phylogenetic trees were constructed based on amino acid sequence alignments of the complete replicase proteins (ORF1) or the complete coat proteins (CP; ORF5) of PIAMV-Gunwi and other isolates (Supplementary Fig. S2). Like the results of the NCBI BLASTP database search, the phylogenetic trees showed that PIAMV-Gunwi is most closely related to PIAMV-Ru, the only other isolate sampled from *P. asiatica* (Supplementary Fig. S2). The pairwise comparisons revealed that the complete replicase protein and CP of PIAMV-Gunwi share 85.15–91.20 and 84.54–90.82 % amino acid sequence identities, respectively, to the corresponding proteins of other PIAMV isolates. Although six Japanese PIAMV isolates sampled from *L. maximowiczii* Regel were more closely related to a Japanese PIAMV isolate from *P. sieboldii* than to any other

(b)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
AB360791_PIAMV-Li2	1																	
AB360794_PIAMV-Li5	2	99.79																
AB360795_PIAMV-Li6	3	99.67	99.79															
AB360790_PIAMV-Li1	4	99.93	99.85															
AB360792_PIAMV-Li3	5	99.90	99.79	99.67														
AB360793_PIAMV-Li4	6	99.49	99.61	99.49	99.56	99.49												
AB360796_PIAMV-Pr	7	91.45	91.49	91.36	91.46	91.43	91.31											
LN794199_PIAMV-Concador	8	85.12	85.15	85.04	85.09	85.02	84.94	85.74										
KT717325_PIAMV-kr	9	84.87	84.91	84.79	84.84	84.78	84.66	85.51	99.52									
KU159089_PIAMV-Ko-JJ_2-1	10	84.94	84.97	84.86	84.91	84.84	84.76	85.59	99.67									
KU159091_PIAMV-Ko-JJ_2-2	11	85.00	85.04	84.92	84.97	84.91	84.82	85.64	99.57	99.49	99.67							
KU159092_PIAMV-Ko-JJ_3-1	12	84.97	85.00	84.89	84.94	84.87	84.79	85.59	99.56	99.62	99.52							
KU159090_PIAMV-Ko-JJ_2-4	13	84.99	85.02	84.91	84.96	84.89	84.81	85.64	99.62	99.52	99.72	99.57						
KU159093_PIAMV-Ko-YS	14	85.10	85.14	85.02	85.07	85.00	84.92	85.74	98.95	98.85	98.87	98.90						
KU697313_PIAMV-Gunwi	15	78.42	78.50	78.42	78.40	78.39	78.24	78.68	78.36	78.21	78.34	78.23	78.34	78.39				
Z21647_PIAMV-Ru	16	78.03	78.08	77.98	78.03	78.00	77.88	78.44	78.10	77.92	78.10	77.98	78.08	78.18	86.83			
AY800279_PIAMV-Na	17	77.65	77.68	77.59	77.57	77.54	77.41	76.88	76.85	76.90	76.90	76.85	76.90	76.95	77.61	77.38		
AB066288_TVX	18	68.72	68.78	68.73	68.72	68.67	68.52	68.31	68.08	68.02	68.10	68.03	68.10	68.29	68.91	68.21	67.07	

PIAMV isolate from *Lilium* spp., the genetic variability of the PIAMV isolates generally reflected their different host plants.

The complete genome sequence of PIAMV-Gunwi is composed of 6130 nt. This is very similar to the 6128 nt of PIAMV-Ru from *P. asiatica* but distinct from the 6101 nt of PIAMV-kr (KT717325) from lily, 6102 nt of six lily and one primrose isolates (AB360790-AB360796) from Japan, one lily isolate from Hungary (LN794199) and five lily isolates (KU159089-KU159093) from Korea, 6099 nt of one lily isolate from Netherlands (assembled sequence of KF601695 and KF471012), and the 6066 nt of the *Nandina* isolate (AY800279). As the differences may be related to host specificity, we searched for insertion and deletion in nucleotide sequence of PIAMV-Gunwi compared to all other complete genomic sequences. Six insertion and deletion regions were detected in PIAMV-Gunwi compared to lily and primrose isolates. One nucleotide deletion (PIAMV-Gunwi nt position between 50 and 51) in 5' UTR and eight nucleotide insertion (PIAMV-Gunwi nt positions 6089–6095 and 6130; the same with the *Nandina* isolate in nt position 6130) in 3' UTR were detected. In the coding region, nucleotide insertions were detected in three regions of ORF1, PIAMV-Gunwi nt positions 1425–1439 (not the *Nandina* isolate), 1630–1633, and 1695–1696. The *Nandina* isolate has some additional nucleotide deletion regions. Further studies will be required to determine whether these differences are related to host specificity.

In this study, the detection and molecular characterization of the genome of a divergent PIAMV isolate, Gunwi, showed that it is distantly related to the sequences of all previously described PIAMV isolates. To our knowledge, this is the first report of the natural infection of *P. asiatica* by PIAMV in Korea, and the isolate Gunwi expands our understanding of the epidemiology of PIAMV.

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