

Cloning of the 5'-end and Amplification of Full-Length cDNA of Genomic RNA of *Lily symptomless virus*

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This paper describes the cloning and sequence analysis of the 5'-terminal region and full-length cDNA production of genomic RNA of *Lily symptomless virus* (LSV), a species of the genus *Carlavirus*. A single DNA band about 600 bp harboring the 5'-end of genomic RNA of the virus was successfully amplified by reverse transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE), and was cloned for nucleotide sequence determination. Sequence analysis of selected RACE cDNA clones revealed that the LSV 5' non-translated region consists of 67 nucleotides long of AT rich stretch followed GC rich from the 5'-end. To produce full-length cDNA products for the viral genomic RNA, a set of LSV-specific primers could be designed based on the obtained sequence in this study and the known sequences of 3'-terminal region for the virus. Full-length cDNA copies of LSV, an 8.4 kb long, were directly amplified by the long-template RT-PCR technique from the purified viral genomic RNA samples. This full-length cDNA copies were analyzed by restriction mapping. The molecules produced in this study can be useful for the production of *in vitro* infectious cDNA clone, as well as, for the completion of genomic RNA sequence and genome structure for the virus.

Keywords : *Carlavirus*, 5'-end sequence, full-length cDNA, *Lily symptomless virus*, RACE.

Several viruses such as *Cucumber mosaic virus* (CMV), *Lily latent virus*, *Lily mottle virus*, *Lily symptomless virus* (LSV), *Strawberry latent ringspot virus*, and *Tulip breaking virus* infecting native and cultivated lilies have been reported worldwide (Ahn et al., 1999; Allen, 1972; Beijersbergen et al., 1980; Cohen et al., 1995; Derks, 1995; Kim et al., 1995; Ryu et al., 2000; Ryu et al., 2002; Yamaji et al., 2001). LSV, a species of the genus *Carlavirus*, is the most prevalent virus infecting lily plants and is widespread throughout the world where lily plants are grown (Allen, 1972; Derks, 1995; Kim et al., 1995). LSV-infected leaves

usually shows very mild mosaic or no visible symptom in lily plants infected singly. However, LSV frequently co-infects with CMV in cultivated lily cultivars and this mixed infection results in severe mosaic with dwarf pattern, which threatens the yield and production of lily commercially.

The members of the genus *Carlavirus* are filamentous virus particles, 610-690 nm long and 12-13 nm in diameter, which are transmitted by aphids in a non-persistent manner or by whitefly (Cavileer et al., 1994; Foster et al., 1990). The size of the carlaviral genomes ranges from 7.4 to 8.5 kb. These are composed of monopartite, single-stranded, plus sense RNA molecules, and have a poly (A) tract at their 3' terminus and cap structure or a monophosphate at their 5' terminus (Cavileer et al., 1994; Foster et al., 1990; Zavriev et al., 1991). *Carlavirus* genomic RNA is encapsidated by a single type of coat protein with Mr of 31 to 34 kDa (Ahn et al., 1999; Cavileer et al., 1994; Foster et al., 1990). The triple gene block (TGB), known to function for the cell-to-cell movement in the *Potexvirus* genus, also found in carlaviruses genomes (Ahn et al., 1999; Cavileer et al., 1994; Foster et al., 1990; Turner et al., 1993; Zavriev et al., 1991).

The partial or complete nucleotide sequences of several members of the *Carlavirus*, namely, *Aconitum latent virus* (AcLV), *Blueberry scorch virus* (BISV), *Carnation latent virus*, *Chrysanthemum virus B*, *Helenium virus S*, *Hop latent virus* (HpLV), *Garlic latent virus* (GarLV), LSV, *Poplar mosaic virus*, *Potato virus M* (PVM), *Potato virus S*, and *Scorch latent virus* have been reported previously (Cavileer et al., 1994; Foster et al., 1990; Fuji et al., 2002; Hataya et al., 2000; Henderson et al., 1992; Levay and Zavriev, 1991; Mackenzie et al., 1989; Meehan and Mills, 1991; Memelink et al., 1990; Turner et al., 1993; Zavriev et al., 1991). To date, the partial nucleotide sequences of four strains of LSV (2 Korean, 1 Japanese and 1 Dutch isolates) have been published (Ahn et al., 1999; Memelink et al., 1990; Takamatsu et al., 1994) and the sequences determined so far covered about 2.4 kb long from the 3'-terminal region. This indicates that there is no report of the sequences of 5'-end and RNA dependent RNA polymerase gene for the LSV and this block the relationship and molecular taxonomy between the virus and other carla-

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viruses.

The objectives of this study were to clone the cDNA of the 5'-terminal region for the LSV for production of full-length cDNA copies by the RT-PCR and rapid amplification of cDNA ends (RACE) techniques, and to compare the 5' non-translated region (NTR) between the virus and other known carlaviruses.

Materials and Methods

Sources of plant and virus, purification of virus, and extraction of viral RNA. Lily (*Lilium longiflorum* cv. Georgia) plants infected with LSV and showing mild mosaic leaf symptom were maintained in the light/temperature-controlled glasshouse and used as source of virus. The virus, denoted as LSV-KR, was isolated and purified by extraction and clarification with chloroform, followed by differential centrifugation method (Ahn et al., 1999). Total genomic RNA of the virus was extracted from purified virus particles by the method of Ryu et al. (1995).

cDNA cloning of the 5'-end region of LSV by the rapid amplification of cDNA ends (RACE). First strand cDNA was synthesized from 20 µg of purified viral genomic RNA using the cDNA synthesis kit (GIBCO BRL) with a LSV-specific RACE primer, LS-RACE (5'-GAA TTC GTT GCC ATA TCT TAT CTT-3') according to the manufacturer's instructions. RACE was performed with the SMART RACE cDNA amplification kit (Clontech, USA) with the LS-RACE and 5'-CDS (Clontech, USA) primers in a thermal cycler (i-cycler, Bio-Rad, USA). PCR cycling consisted of 20 seconds at 94°C, 40 seconds at 54°C, and 60 seconds at 72°C, followed by 3 minutes at 94°C. Final extension was done for 10 minutes at 72°C. The 5'-RACE products were electrophoresed on an agarose gel and DNA was eluted from the pieces of agarose gel by the GeneClean kit (Bio101) according to the manufacturer's instructions. Purified DNAs were cloned into the pGEM-T-Easy plasmid vector (Promega Corp., USA). Ligated plasmids were transformed into competent *Escherichia coli* (strain JM109) cells. Recombinant plasmids were selected and purified for nucleotide sequence determination.

Nucleotide sequence determination. The RACE cDNA clones were obtained and purified for nucleotide sequence determination. Nucleotide sequence of each clone was determined in both directions by the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Model 377 automatic DNA sequencer (ABI). Nucleotide sequences were analyzed using the DNASTAR software package (Madison, WI).

Sequence comparison and phylogenetic tree analysis. Nucleotide sequence alignment and phylogenetic analysis of the 5' NTR of LSV and other members of the genus *Carlavirus* were performed to address the molecular taxonomic status of the carlaviruses. Sequences of members of the genus *Carlavirus* for analysis were retrieved from the GenBank database under the following accession numbers: AcLV, AB051848; LSV (used in this study); BiScV, L25658; GarLV, AJ292226; HpLV, AB032469; and PVM, X53026. Pairwise sequence comparisons and multiple-

alignment were done with a multiple sequence editor program in the DNAMAN package (version 5.1: Lynnon Biosoft, Canada) with a gap open penalty of 10 and a gap extension penalty of 5.

Results and Discussion

cDNA cloning of the 5'-terminal region of the virus and sequence determination. The cDNA product of the 5'-terminal region of the genomic RNA of a Korean isolate of *Lily symptomless virus* (LSV-KR) was produced by the RACE and RT-PCR techniques. Nucleotide sequences of N-terminal and parts of replicase gene of LSV could be obtained from cDNA clones (our unpublished results) and LSV-specific primer for 5'-RACE was designed. The 5'-RACE RT-PCR products about 600 bp were successfully amplified and the major band could be detected in the agarose gel electrophoresis (Fig. 1).

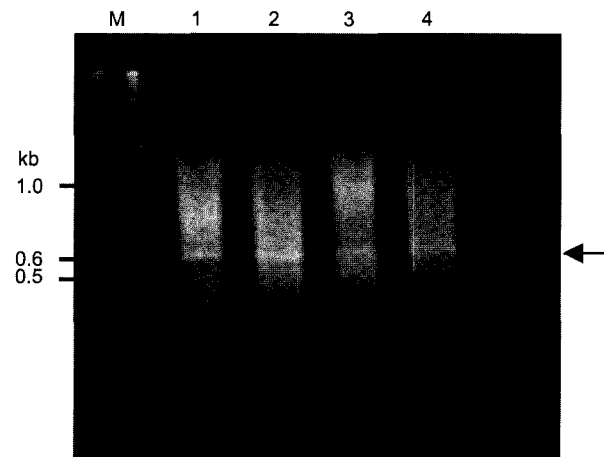


Fig. 1. Agarose gel electrophoresis of RT-PCR products of RACE procedure for 5'-end of LSV. Lane M, 1kb plus DNA ladder; Lanes 1-4, RT-PCR products of 5'-RACE for LSV.

The products were column-purified from the gel and cloned into the vector for determination of nucleotide sequence. In total, three independent RACE cDNA clones were obtained and used for sequence determination. A contig master sequence was generated from the aligned nucleotide sequences of the three independent 5'-RACE clones and it contains the 5'-end sequences (Fig. 2A). The 5'-NTR was 67 nucleotides long preceding a viral RNA-dependent RNA polymerase (RdRp) gene. This sequence contains ATG start codon (68th-70th nucleotides) for viral RdRp and 111 amino acid residues were, the N-terminal part of LSV RdRp as predicted by ORF Finder program search from the GenBank server generated (Fig. 2A). When 111 amino acid residues of the putative LSV RdRp were analyzed in the BLAST search, the sequence matched well with the carlaviral RdRp, which ranged from 53% identity

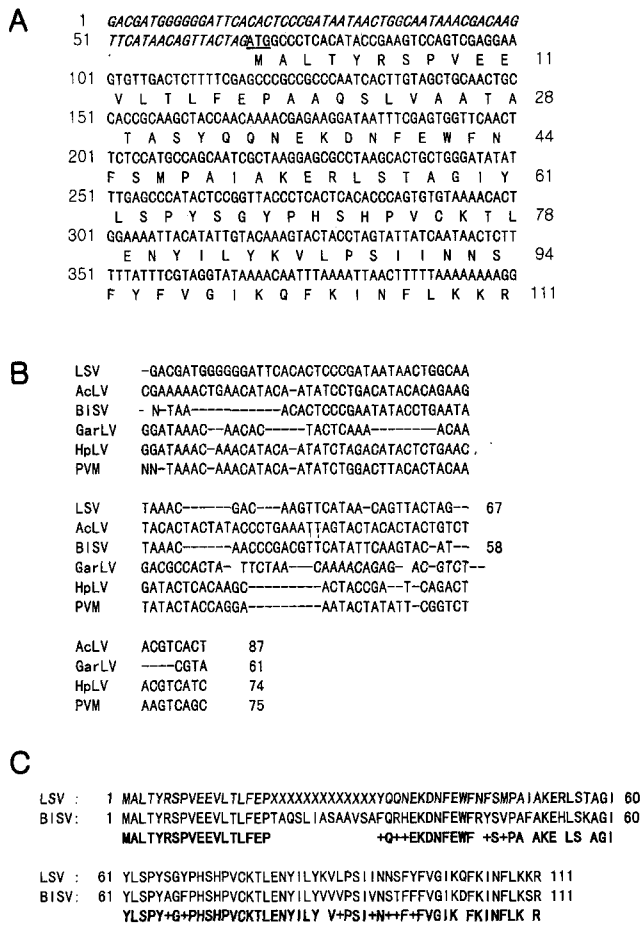


Fig. 2. Nucleotide sequences of the 5'-NTR and N-terminal regions of RdRp of LSV and their deduced amino acid residues (A); Multiple alignment of 5'-NTR between LSV and 5 other carlaviruses (B); and Alignment of N-terminal regions of RdRp of LSV with BISV generated by the BLAST search analysis (C).

to GarLV to 71% identity to BISV (Fig. 2C). This suggests that the cDNA clones obtained in this study contained 5'-end part of genomic RNA of the virus.

Sequence comparison and phylogenetic analysis. The 5'-NTR of LSV showed 37.1% (with GarLV) to 60.4% (with BISV) nucleotide sequence identities with that of other carlaviruses. Interestingly, LSV has AT rich stretch followed GC rich from the 5'-end (Fig. 2A, B). The 5'-NTR was highly divergent and the total nucleotides numbers, 58-87

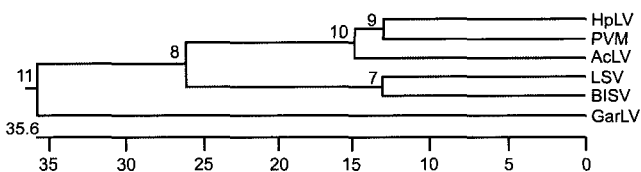


Fig. 3. Phylogenetic tree analysis of 5'-NTR of LSV with the corresponding region of other 5 members of carlaviruses.

Table 1. Percentage sequence similarities of nucleotide sequence of 5'-NTR of LSV with other carlaviruses

	AcLV	LSV	BISV	GarLV	HpLV	PVM
AcLV	—					
LSV	38.6	—				
BISV	46.4	60.4	—			
GarLV	38.3	37.1	36.8	—		
HpLV	66.2	43.8	44.9	40.0	—	
PVM	64.8	46.7	41.7	32.3	69.0	—

nucleotides long, was variable for each virus species in the same genus. The phylogenetic tree analysis of the 5'-NTR comparison indicates that LSV is closest to BISV (Fig. 3). This relationship matched well with the comparison and phylogenetic tree analyses of the viral coat protein and TGB genes as reported previously (Ahn et al., 1999).

Production of full-length cDNA product. To amplify full-length cDNA products for the viral genomic RNA, a set of LSV-specific primers could be designed based on the obtained sequence in this study and the known sequences of 3'-terminal region for the virus (Ahn et al., 1999; Memelink et al., 1990). Full-length cDNA copies of LSV, an 8.4 kb long, were directly amplified by the long-template RT-PCR technique (Yoon et al., 2002) from the purified viral genomic RNA samples (Fig. 4, lane 1). Complete viral RdRp gene of 5.9 kb long with the LSV-specific primers by the same procedure (Fig. 4, lanes 2, 3) could be produced. This full-length cDNA copies were analyzed by restriction mapping (Fig. 5), and this information can be useful for the

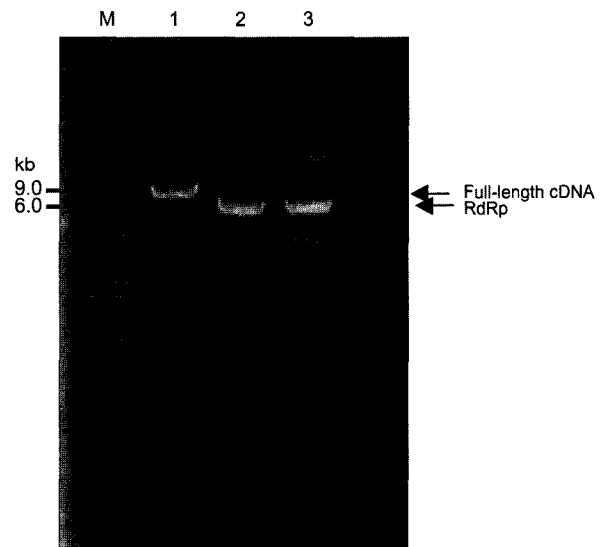


Fig. 4. Full-length cDNA products of genomic RNA for LSV by long-template RT-PCR with a set of LSV-specific primers. Lane M, 1 kb DNA ladder; Lane 1, full-length cDNA of entire genomic RNA of LSV; Lanes 2, 3, full-length cDNA copies of LSV RdRp.

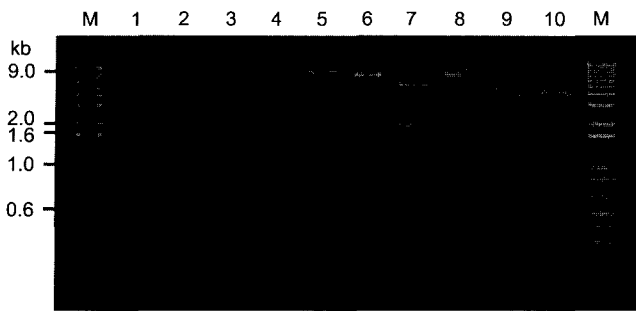


Fig. 5. RFLP analysis of full-length cDNA product of LSV with 10 restriction endonucleases. Lane M, 1 kb DNA ladder; Lane 1, *ApaI*; Lane 2, *BamHI*; Lane 3, *BglII*; Lane 4, *EcoRI*; Lane 5, *EcoRV*; Lane 6, *KpnI*; Lane 7, *MluI*; Lane 8, *SalI*; Lane 9, *XbaI*; Lane 10, *XhoI*.

comparison of other LSV strains by RFLP, as well as, for the generation of sub-clones for determination of nucleotide sequence. This is the first report of the 5' terminal regions and N-terminal sequences of RdRp of LSV. The molecules produced in this study can be useful for the production of *in vitro* infectious cDNA clone (Yoon et al., 2002) and development of virus-resistant transgenic lily plants, as well as, for the completion of genomic RNA sequence and genome structure for the virus. We are interested in the molecular taxonomy of members of the genus *Carlavirus*, and work is now underway on the completion of the genomic sequencing of LSV.

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