Alstroemeria-Infecting Cucumber Mosaic Virus Isolates Contain Additional Sequences in the RNA 3 Segment

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Abstract

The coat protein (CP) genes and flanking regions of three *alstroemeria*-infecting cucumber mosaic virus isolates (CMV-ALS), denoted ALS-LBO, ALS-IPO, and ALS-NAK, were cloned and their nucleotide sequence determined and compared at both nucleic acid and deduced protein level with the published sequences of CMV RNA 3. The sequences of these isolates showed more than 95% nucleotide and peptide sequence homology to each other and to other members of subgroup II CMV. Strikingly, an additional sequence of 218 nt was found in the central region of the 3'-non-translated region (NTR) of RNA 3 of two out of three isolates. The additional sequence appeared to have arisen from RNAs 1 or 2. A subgroup-specific DIG-labeled probe has been developed from this additional sequence and applied to detect subgroup II CMV strains in dot blot hybridization and to differentiate the *Alstroemeria* isolates containing additional sequences in Northern blot hybridization.

INTRODUCTION

Alstroemeria has become an important ornamental crop during the past decade in the Netherlands and all over the world (van Zaayen, 1995). Several viruses infecting alstroemeria have been described, including alstroemeria mosaic potyvirus (AlMV), alstroemeria carla virus (AlCV) (Phillips et al., 1986), *alstroemeria* streak potyvirus (AlSV) (Wang et al., 1992; van Zaayen et al., 1994), tomato spotted wilt tospovirus (TSWV) (Hakkaart and Versluijs, 1985). Also cucumber mosaic cucumovirus (CMV) (Hakkaart, 1986) has been reported in *Alstroemeria*. However, very little was reported on the interaction between CMV and alstroemeria plants because of its sporadic occurrence. Since CMV in alstroemeria usually was reported together with other viruses (van Zaayen, 1995), the symptoms caused by CMV-infection were not clear and not described until 1996. Bellardi and Bertaccini (1996) reported that CMV-infected alstroemeria showed necrosis on the leaves of *Alstroemeria* cv. Red Sunset and malformed flowers on *Alstroemeria* cv. Rossella. In addition, chlorotic mosaic on the leaves and stunting also were observed in *Alstroemeria* Red Sunset. The combination of CMV with other alstroemeria viruses can induce different symptoms (necrosis. red stripes, yellowing) which could depend upon environmental conditions, alstroemeria cultivars and growth stage when infection occurred (Bellardi and Bertaccini, 1997). Seed transmission of CMV in alstroemeria was reported as well (Bellardi and Bertaccini, 1997). Antisera against alstroemeria CMV strains have been developed and have been applied to routine inspection.

Cucumber mosaic virus (CMV) is the type species of the genus *Cucumovirus* in the family of *Bromoviridae* (Rybicki, 1995) and consists of isometric particles with a diameter of about 28 nm. Its genome consists of three functional single-stranded RNA molecules designated as RNA 1, 2, and 3, which are required for infection. RNA 1 and 2 encode components of the viral RNA-dependent RNA polymerase (Nitta et al., 1988), while RNA 3 encodes the movement protein (MP) (Suzuki et al., 1991) and coat protein (CP) (Schwinghamer and Symons, 1977). The CP is translated from the subgenomic RNA 4 which is encoded by the 3'-half of the RNA 3 (Gould and Symons, 1982). A small

overlapping gene (2b), encoded by RNA 2, was discovered and is most likely expressed through subgenomic RNA (Ding et al., 1994). The product of the 2b gene is involved in the virulence of the virus, possibly by suppression of gene silencing (Brigneti et al., 1998). The complete nucleotide sequences of the genomic RNAs of several CMV strains have been reported and have been classified into two major subgroups I and II (Anderson et al., 1995; Palukaitis et al, 1992; Quemada et al., 1989). Moreover, a further grouping of subgroup I, designated as IA and IB, has been proposed based on the nucleotide sequences of 5' NTRs of RNA 3 (Palukaitis and Zaitlin, 1997; Roossinck et al. 1999). CMV is one of the most widespread plant viruses in the world and has a host range of over 1,000 species of plants (Palukaitis et al, 1992). Also *alstroemeria* has been reported a host of CMV (Hakkaart, 1986).

In a previous survey on CMV strains infecting ornamental crops, we characterized 13 CMV strains of ornamental crop origins on the basis of nucleotide sequence of RNA 4 and flanking regions (Chen et al., *manuscript in preparation*). The sequence data showed that an additional sequence of 218 nucleotides, with similarity to RNA 1 or 2 of the homologous virus, resided in the central region of 3' NTR of the RNA 3 of the *alstroemeria*-infecting CMV isolate (ALS-LBO) (Chen et al, 1999). To assure that the occurrence of the additional sequence in ALS-LBO is not incidental, two other sources of *alstroemeria* CMV (ALS-IPO and ALS-NAK) were used to confirm the results of our previous studies. In this study, we report the cDNA sequences of the CP gene and its flanking regions of three *alstroemeria*-infecting CMV isolates obtained from different sources and compare these sequences at nucleotide and protein level. Moreover, the additional sequence is used as a template for the production of a specific probe to distinguish alstroemeria CMV isolates in Northern analysis.

MATERIALS AND METHODS

Virus Sources and Plant Materials

The first isolate of *alstroemeria*-infecting CMV (ALS-LBO) was obtained from the Bulb Research Center (LBO-LNV), Lisse, The Netherlands. The second , ALS-IPO, was a stock isolate (code: N-11) of the Department of Virology, Plant Research International (PRI), Wageningen, The Netherlands. The third source (ALS-NAK) was obtained from the Inspection Service for Floriculture and Arboriculture (NAKB), Den Haag, The Netherlands. ALS-LBO and ALS-IPO were originally maintained in *Nicotiana clevelandii* and as dried material in *Cucumis sativa*, respectively, whereas ALS-NAK was isolated from a CMV-infected *alstroemeria* plant. All tested isolates were mechanically inoculated to *Chenopodium quinoa* and the local lesions were mechanically inoculated to *N. benthamiana* for maintenance. *Alstroemeria* plants (clone VV024-6) (Lin et al, 1998) were kindly offered by Dr. M. de Jeu of Laboratory of Plant Breeding, Department of Plant Sciences, Wageningen University and were used for pathogenicity tests.

Virion and RNA Purification and Total RNA Extraction

All three isolates were propagated on *N. tabacum* cv. White Burley *or N. benthamiana* for virion purification which were carried out as described by Lot et al. (1972). Viral RNA was isolated from the purified virions by the method of Palukaitis and Symons (1980). Total RNA was extracted from leaf tissue of inoculated *N. benthamiana* with the method of Seal and Coates (1998).

RT-PCR

The degenerated oligonucleotide primer pair for RT-PCR to amplify CMV coat protein gene and flanking regions was designed according to some of the determined sequences of CMV RNA 3. BamHI sites were added for cloning purposes. The sequence of the forward primer (CMVCP-1) is 5'- CCCCGGATCCACATCAYAGTTTTRAGATTCAATTC-3' and that of the reverse primer (CMVCP-5) is 5'-CCCGGATCCTGGTCTCCTT-3'. The predicted length of the

amplified DNA fragment is 1111 to 1122 bp for subgroup II CMV strains. The forward and reverse primers, denoted as ALSEXT-F and ALSEXT-R, to amplify the additional fragment of 218 nucleotides were 5'-CGGGTATCGCCTGTGG-3' and 5'-CAAGGGTACCTCGACAACCC-3', respectively. Total RNA of infected plants was used to synthesize first strand cDNA using MMLV-reverse transcriptase with primer CMVCP-5. PCR amplifications of cDNA were carried under the following conditions: denaturation for 3 min at 94ºC and then 35 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 52ºC, and extension for 1 min at 72ºC. The final extension was a 7 min incubation at 72ºC. PCR products were analyzed by 1% agarose gel electrophoresis and the DNA bands were visualized by ethidium bromide staining and UV transilluminator.

cDNA Cloning and Sequencing

PCR products of the expected size were eluted from agarose gel (Concert Matrix, GibcoBRL) and precipitated with ethanol. Purified DNA was ligated to the pGEM-T vector (Promega) transformed to *E. coli*. The plasmids were denoted as pLBOCP51, pIPOCP51, pNAKCP51 for the gene and flanking regions of ALS-LBO, ALS-IPO, ALS-NAK, respectively, and pALS218 for the additional sequence. The cloned sequences of all three tested CMV isolates were compared with that of subgroup I and subgroup II CMV strains, whose sequences were available in the literatures and in GenBank, i.e. Fny-CMV (Owen et al., 1990; D10538) and NT9-CMV (Hsu et al., 1995; D28780) for subgroup I CMV, and Q-CMV (Davies and Symons, 1988; J02059) and Trk7-CMV (Salanki et al., 1994; L15336).

Preparation of Digoxigenin-Labeled RNA Probe

RNA probes were prepared from the plasmid DNA of pALS218 using SP6 or T7 RNA polymerase. One µg of the plasmid DNA was put in a 25 µl T7 or SP6 RNA transcription mixture containing 1x DIG RNA labeling mixture, 1x T7/SP6 RNA polymerase buffer, 10mM DTT, 0.5 µl RNasin and 1.5 µl T7 or SP6 RNA polymerase (GibcoBRL), and incubated at 37ºC for I hr. Transcribed probe RNAs were ethanol precipitated and dissolved in 50 µl distilled water. DIG-labeled RNA probes were checked by electrophoresis in 1% agarose gel.

Dot-Blot and Northern Hybridization

For dot-blot, total RNA extract from healthy or virus-infected leaf tissue were diluted with 20x SSC to final concentration of 0.2 µg/µl and 1 µg spotted onto a nylon membrane and baked at 80ºC for 2 hr. For Northern blot, purified viral RNA or extracted total RNA were separated in 0.8% agarose gel in 1x methyl mercuric hydroxide buffer system, transferred to nylon membrane by capillary force and baked at 80ºC for 2 hr. Hybridization was carried out as in Webster and Barker (1998), by using DIG-labeled RNA probe and Goat-anti-DIG-AP conjugated complex both in a dilution of 1:2500.

RESULTS

Pathogenicity Tests

Using mechanical inoculation, all CMV-ALS isolates were capable of systemically infecting *N. benthamiana* and *N. tabacum* cv. White Burley. *Chenopodium quinoa* could be infected locally only. *Alstroemeria* plants (clone VV024-6) could also be inoculated by all three isolates, however, the infection rates of ALS-LBO and ALS-NAK was much higher than that of ALS-IPO, with an average infection rate of 60% for ALS-LBO and ALS-NAK, but only 5% for ALS-IPO. All isolates infected tobacco in an infection rate of 100%.

RT-PCR and Sequence Analysis

The designed degenerated primers, CMVCP-5 and CMVCP-1, amplified an

expected single DNA band of about 1,100 bp for ALS-IPO; however, a DNA band with a length of about 1,300 bp was produced from ALS-LBO and ALS-NAK (Fig. 1). These products were cloned and their sequences determined. Sequencing data showed the precise length of 1,118 nt of the amplified ALS-IPO cDNA and 1,333 nt for both ALS-LBO and ALS-NAK. This size difference between the 3 isolates appeared to be caused by an additional sequence of 218 nucleotides in the 3' NTR (see Fig. 2). This additional sequence made the length of RNAs 3 and 4 in both isolates longer than that of ALS-IPO the isolate (Fig. 3). Flanking the inserted sequence, a pair of repeated sequences of 16 nucleotides (5'-TGTCGAGGTACCCTTG - 3'), were observed in ALS-LBO and ALS-NAK. In fact, this 16-nt sequence is a congenital sequence and resides directly upstream of the 3' terminal tRNA-like structure of all CMV strains.

The partial RNA 3 sequences of both ALS-LBO and ALS-IPO will be available in the EMBL database with accession number of AJ131622 and AJ672587, respectively. ALS-NAK has not been submitted as yet.

Comparison of Nucleotide and Deduced Amino Acid Sequences

The nucleotide sequences of the 3'-halves of RNA 3 and the putative amino acid sequence of the CP ORFs of the three *alstroemeria* CMV isolates were compared with those of some strains in both subgroups of CMV, i.e. Fny (D10538), NT9 (D28780), Q (J02059) and Trk7 (L15336). The homology at the nucleotide level are more than 97% between tested isolates and subgroup II CMV strains, whereas only 70 to 75% homology between tested isolates and subgroup I strains was observed (Table 1). The nucleotide sequences of RNA3 are identical between ALS-LBO and ALS-NAK and, apart from the 218-nt additional sequence, share 99% homology with that of ALS-IPO. The differences are 7 nt in 5'-NTR of the CP ORF and 3 nt in the CP ORF itself. The sequence of the 218-nt inserted fragment, which was found only in the RNAs 3 and 4 of ALS-LBO and ALS-NAK, is highly homologous to the sequences of RNAs 1 and 2 (more than 83% homology) but neither to RNAs 3 nor 4 (less than 70% homology) of subgroup II CMVs. Also no homology was observed to any segment of subgroup I CMV isolates or related viruses (Table 2).

Dot-Blot and Northern Blot Hybridization

DIG-labeled RNA probes developed from the 218-nt additional sequence of ALS-LBO were used in dot-blot hybridization assays to assess specificity. This probe specifically hybridized with total RNA extracted from alstroemeria and tobacco that were infected with subgroup II CMV strains including the alstroemeria CMV isolates. None of the subgroup I CMV isolates cross reacted to the probe (Fig. 4). *Alstroemeria* CMV isolates that contain additional sequences, i.e. ALS-LBO and ALS-NAK, could be distinguished from other subgroup II CMV strains in Northern hybridization by their deviant hybridization pattern (Fig. 5).

DISCUSSION

RT-PCR can be used to detect plant viruses rapidly and sensitively. Indeed, the RT-PCR method has proven to be more sensitive than ELISA and dot-blot hybridization (Hu et al., 1995). Also cucumber mosaic virus (CMV) can be effectively detected with different sets of degenerate primers (Choi, et al. 1999; Hu et al, 1995; Singh et al., 1995). The set of primers used in this study amplified the CP ORF and flanking regions of CMV isolates from ornamental crops successfully. In addition, it allows screening for additional sequences observed in alstroemeria-infecting CMV isolates.

Strains of cucumber mosaic virus can be classified into two major subgroups (I and II) on the basis of host range, serological relationships, peptide mapping, and nucleic acid hybridization (Palukaitis et al., 1992). Strains in the same subgroup share 91-100% homology in nucleotide and putative amino acid sequences, while only 76-84% homology can be observed between strains of different subgroups (Owen et al., 1990; Quemada et al., 1989). Based on the nucleotide sequence of the 3'-half of RNA 3 and deduced amino

acid sequences of the coat protein, three studied isolates infecting *alstroemeria* plants in the Netherlands were classified as subgroup II CMV and can be distinguished as two different isolates based on the sequence of 3' NTR of RNA 3.

A nonradioactive RNA probe, derived from the additional sequence residing in RNA 3 of ALS-LBO, is highly specific to subgroup II CMV strains (Chen et al., 1999). In combination with the degenerated primer set and specific probe developed in this study, the detection and subgroup differentiation of CMV strains can be achieved. Moreover, the probe can be efficiently used for differentiating isolates of CMV in *alstroemeria* when applied in Northern hybridization.

To date, the occurrence of RNA recombination has been established in several members of the *Bromoviridae* family, Brome mosaic virus (Bujarski and Kaesberg, 1986) and Cowpea chlorotic mosaic virus (Allison et al., 1990). The additional sequence in the 3' NTR of RNAs 3 and 4 of ALS-LBO and ALS–NAK share high homology with RNAs 1 and 2 of homologous virus and subgroup II CMV strains, suggesting a RNA recombination event occurred between RNA 3 and RNA 2 or RNA 1.

Due to its limited incidence in the crop, cucumber mosaic virus infecting *alstroemeria* has not been studied as intensive as CMV occurring in other crops (van Zaayen, 1995). The routine indexing of CMV in *alstroemeria* plants is carried out mainly by serological methods, which are sensitive enough to detect CMV infection in *alstroemeria* (Maat, 1980). However, serological methods can not distinguish CMV-ALS isolates that contain additional sequences outside the CP ORF. This might be one of the reasons that CMV isolates containing additional sequences in the 3' NTR have not been reported previously, neither in *alstroemeria* nor in other crops. Consequently, recombination events may be much more common among plant-infecting viruses, but are simply not detected due to the common use of serological methods rather than RNA-based detection systems.

The biological relevance of the additional sequences to CMV in *alstroemeria* requires further elucidation. For TMV (Leathers et al., 1993) and BMV RNA 3 (Gallie and Kobayashi, 1994) it is described that the 3'-NTR, not only the structure but also the primary sequence, enhances the translation rate. The pseudoknots located just upstream of the tRNA-like structure of TMV and BMV RNA 3 are necessary for RNA replication and participate in replication and/or translation (Leathers et al. 1993; Lahser et al., 1993). The insertion of an additional sequence in the RNA 3 of ALS-LBO and ALS-NAK alters the primary sequence and structure of their 3'-NTR and make it different from those of common CMV strains. An altered 3'-NTR may subsequently change the biological function of the RNA resulting in increased infectivity or stability in the host. Mechanisms causing the recombination, the role of the 16-nt repeated sequences flanking the additional sequence and the biological effects of the additional sequence are unclear at present and warrant further detailed study.

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Tables

Table 1. Sequence homology (%) of 3'-half RNA 3 and CP peptide between tested isolates and some CMV strains

Nucleotide sequence

	LBO	NAK	IPO	Q	Trk7	Fny	NT9
ALS-LBO		100	99.5	98.2	97.5	70.6	73.0
ALS-NAK	100		99.5	98.2	97.5	70.6	73.0
ALS-IPO	98.2	98.2		98.6	97.8	72.2	75.2
Q-CMV	96.8	96.8	98.6		97.8	72.7	75.4
Trk7-CMV	94.5	94.5	96.3	97.7		71.4	74.6
Fny-CMV	85.3	85.3	87.2	88.5	86.7		92.9
NT9-CMV	84.9	84.9	86.7	88.0	86.2	99.5	

Peptide Sequence of CP

Table 2. Sequence homology (%) between 218-nt additional sequence and genomic RNAs of some virus strains of *Bromoviridae*

	RNA 1	RNA 2	RNA 3	Accession numbers *
ALS-LBO	84.9	94.5	100	AJ276584, AJ276585, AJ131622
ALS-NAK	84.9	94.5	100	AJ304404, AJ304395, AJ304398
ALS-IPO	83	89.5	69.6	AJ304405, AJ276586, AJ276587
Q-CMV	83.8	84.3	69.2	X02733, X00985, J02059
Trk7-CMV	83.0	88.1	70.0	AJ007933, AJ007934, L15336
Fny-CMV	56.3	60.7	59.0	D00356, D00355, D10538
NT9-CMV	55.4	62.4	60.3	D28778, D28779, D28780
PSV	58.2	54.0	57.3	U33145, U83332, U31366
TAV	68.2	65.9	64.7	D10044, D10663, D01015
AlMV	36.2	42.7	40.1	L00163, K02702, X00819
BMV	44.2	39.0	44.5	X02380, X1678, V00099
TSV	43.1	41.3	39.0	U80934, U75538, X00435

*: the accession numbers are in the order of RNA 1, RNA 2, and RNA 3, respectively.

Figures

Fig. 1. Amplified DNA fragments obtained by the RT-PCR with degenerate primers, CMVCP-1 and CMVCP-5, from total RNA of tobacco leaves infected with alstroemeria CMV isolates, ALS-LBO, ALS-NAK, and ALS-IPO. Marker is lambda DNA digested with restriction enzyme, PstI.

Fig. 2. Schematic diagram of 3'-non-translated region (3'-NTR) of RNA 3 of alstroemeria-infecting cucumber mosaic virus isolates. An additional sequence of 218-nt (hatched box), which is flanked by a repeated sequence of 16-nt (solid box), resides in the central region of 3'-NTR of ALS-LBO and ALS-NAK but not in ALS-IPO. Dotted box indicates the coat protein open reading frame (CP ORF) and the numbers indicate the length in nucleotides.

Fig. 3. RNA pattern of CMV strains in 0.8% agarose gel. The additional sequence which resides in the 3'-half of RNA 3 made the RNAs 3 and 4 of ALS-LBO and ALS-NAK longer than those of ALS-IPO and CMV-LILY.

Fig. 4. Specificity of DIG-labeled RNA probe reacted with total RNA extracted from plant tissue infected by subgroup II CMV strains but not with that from subgroup I CMV-infected plants and healthy control. Dots 1- 4 are RNA extracted from alstroemeria leaves and dots 5 - 12 are from *Nicotiana benthamiana*. 1: healthy control, 2:ALS-IPO, 3: ALS-LBO, 4: ALS-NAK, 5: ALS-IPO, 6: ALS-LBO. 7:ALS-NAK, 8: healthy control, 9:CMV-CRY, 10: CMV-S, 11:CMV-LILY, 12:CMV-GPP. CMV-CRY and CMV-S are subgroup II strains, while CMV-LILY and CMV-GPP are subgroup I strains.

Fig. 5. Specificity of a DIG-labeled RNA probe developed from the additional sequence of 218-nt resides in the RNA 3 of LAS-LBO isolate, in Northern hybridization. The probe reacted with RNA of subgroup II CMV but not with subgroup I CMV (lily strain). Moreover, it reacted specifically with RNAs 1, 2, 3, and 4 of ALS-LBO and ALS-NAK but only RNAs 1 and 2 of ALS-IPO.

Figures: see next pages)

Fig. 1.

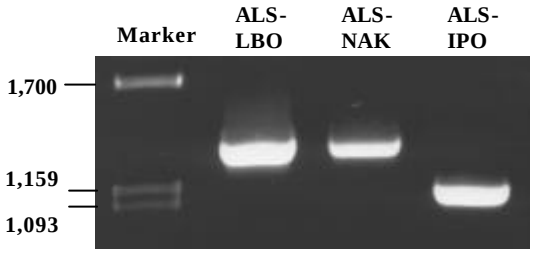

Fig. 2.

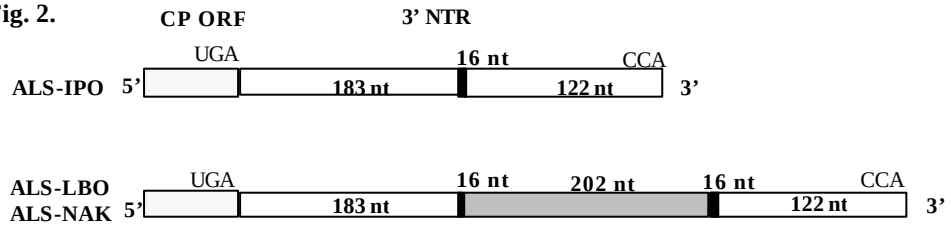

Fig. 3.

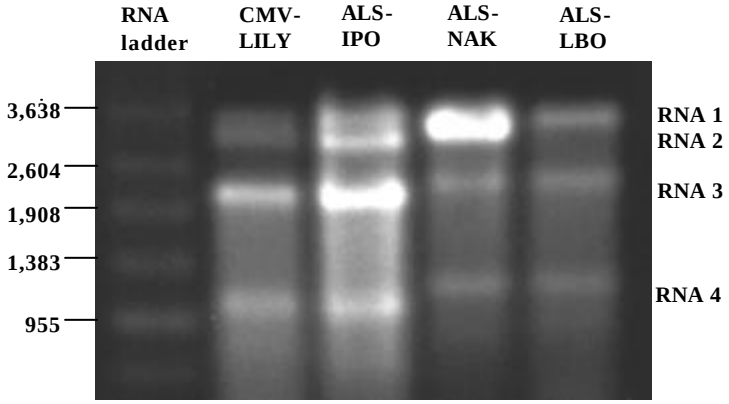

Fig. 4.

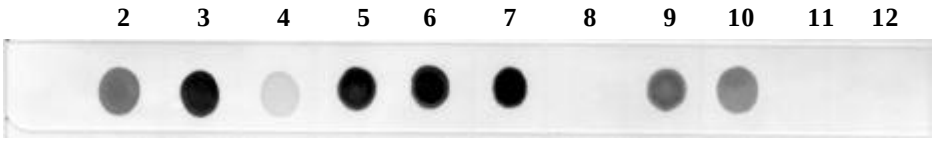

Fig. 5.