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A survey of viruses of *Alstroemeria* in the UK and the characterisation of carlaviruses infecting *Alstroemeria*

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Abstract

Alstroemeria samples collected in the UK were tested for a range of viruses using ELISA. Alstroemeria mosaic virus (AlMV), alstroemeria carlavirus (AlCV), lily symptomless virus (LSV), cucumber mosaic virus (CMV) and tobacco rattle virus (TRV) were detected either singly or in combination in 67.5% of 203 samples. AlCV and LSV isolates from *Alstroemeria* and lily were studied and characterised serologically using existing antisera, and by PCR, using primers to an 11 kDa open reading frame (ORF) unique to carlaviruses and to the coat protein gene of LSV. Sequences of isolates of AlCV and LSV from the coat protein gene were 94–99% similar and were 99% similar in the 11 kDa ORF, supporting the view that these are strains of the same virus.

Introduction

Alstroemeria cut flower production is rapidly increasing in the EU and worldwide. *Alstroemeria* plants can be raised from seed, but for many years the commercial crop has been multiplied vegetatively by splitting the rhizomes of mature plants or by micropropagation. Reproduction through seed is now usually used only in breeding. Viruses infecting *Alstroemeria* have been described in Denmark (Rønde Kristensen et al., 1962), UK (Brunt and Phillips, 1981), the Netherlands (Hakkaart and Versluijs, 1985; Van der Vlugt and Bouwen, 1997) and Italy (Bellardi and Bertaccini, 1991). They include alstroemeria carlavirus (AlCV), alstroemeria mosaic potyvirus (AlMV), cucumber mosaic virus (CMV), tomato spotted wilt (TSWV) and impatiens necrotic spot (INSV) tospoviruses and tobacco rattle tobravirus (TRV) (Van Zaayen, 1995). Alstroemeria streak potyvirus (AlSV) has also been reported to infect *Alstroemeria* (Van Zaayen et al., 1994), but recently AlMV and AlSV have been reported to be strains of the same virus (Van der Vlugt and Bouwen, 1999) with the name AlMV having precedence. However, these are not identical and some antisera are capable of distinguishing those isolates previously called AlSV from other AlMV strains and in this work such isolates will be identified as the AlSV strain of AlMV.

In previous reports, AlCV isolated from *Alstroemeria* had a close serological relationship to lily symptomless virus (LSV) and carnation latent virus (CarLV) and was considered to be a host-adapted strain of LSV (Derks et al., 1982; 1983; Phillips and Brunt, 1986). The only known natural hosts of AlCV are from the *Liliaceae*, and include *Alstroemeria*, *Lilium longiflorum* and *Tulipa gesneriana* which are all infected systemically. Symptoms are leaf curling and striping in cool conditions. AlCV is transmitted by *Myzus persicae* in a non-persistent manner and by mechanical inoculation. It is probably distributed worldwide. AlCV is also closely related to, but distinct from, chrysanthemum B, potato M, potato S, carnation latent and passiflora latent carlaviruses (Brunt et al., 1986).

The genomes of carlaviruses are arranged (from 5′ to 3′) as a replicase gene, a triple gene block (25 kDa, 12 kDa and 7 kDa), coat protein gene and 11 kDa

protein gene. There is extensive similarity at the amino acid level between the equivalent proteins of potex- and carlaviruses. However, relative to potexviruses the carlaviruses have an additional open reading frame (ORF) downstream from the coat protein at the 3′ terminus. This ORF potentially encodes a protein of approximately 11 kDa in size and contains domains that are highly conserved between all carlaviruses. The location of this ORF between the coat protein and the poly(A) tail differentiates carlaviruses from potex- and all other plant RNA viruses. Polymerase chain reaction (PCR) amplification of this ORF has been used as a diagnostic test for carlaviruses (Badge et al., 1996). In this study, several isolates of AlCV from *Alstroemeria* and one of LSV from lily were characterised using serological and molecular techniques to try to provide definitive evidence that AlCV and LSV are strains of the same virus.

Materials and methods

Plant material and virus isolates

Leaves from *Alstroemeria* plants with possible virus symptoms were collected during surveys of UK *Alstroemeria* nurseries. For each sample, records were taken of location, cultivar or species and disease severity. Leaf samples were stored in insulated cool bags and later refrigerated at 4–5 °C before ELISA tests. Cultures of AlCV, AlMV (including one previously identified as AlSV), CMV, LSV and TRV were maintained for comparison. *Alstroemeria* plants that were subsequently found to be infected by AlCV or LSV were dug up and potted up in glasshouses at HRI for future work. Similarly, some infected plants were received from project partners in the Netherlands and Germany.

ELISA

ELISA tests were performed using polyclonal antibodies against the AlSV strain of AlMV (IPO-DLO), AlCV (IPO-DLO), AlMV (HRI), CMV (IPO-DLO), LSV (HRI) and TRV (HRI). Similar tests were used to detect AlCV and LSV in *Alstroemeria* samples collected in surveys carried out by all the partners in the EU-funded project. The double direct antibody sandwich (DAS) test (Clark and Adams, 1977) was used with Nunc-Immuno I (A/S Nunc, Denmark) plates. Coating globulins (at 1 µg/ml) and virus specific antibodies conjugated with alkaline phosphatase (1/1000) were used to detect the trapped virus antigen. Absorbance values ($A_{405\,nm}$) were measured with a Titertek Multiskan MCC/340 reader after 60 min. Tests were considered to be positive if the absorbances were a least twice that of the mean of the uninfected control sap. More detailed ELISA tests were performed using the antibodies to AlCV and LSV. Samples of sap from three selected isolates of carlavirus from *Alstroemeria* (HRI-12, HRI-A92 and HRI-A95) and LSV were tested at three sap dilutions (1/10, 1/100 and 1/1000) with antibodies to AlCV (IPO-DLO) and LSV (HRI).

Polymerase Chain Reaction (PCR)

One set of primers was used to amplify a 130 bp fragment from part of the start of the 11 kDa protein to the poly(A) tail (T-NOT1: 5′-TTT TTT TTT TTT TTT CGC CGG CGC TTA AC-3′ and Carla-Uni: 5′-GGA GTA ACY GAG GTG ATA CC-3′) (Badge et al., 1996). A second set of primers (LSV F: 5′-AAC TCT CAC TTG TTA CCG CAA-3′ and LSV R: 5′-CTA TGG TAG TTC GTC GCT TCA-3′) was designed at HRI, using the published sequence of LSV (accession number D43801; Takamatsu et al., 1994) to amplify a 930 bp fragment from the the coat protein gene.

Immuno-capture PCR was used in a one-step reaction (Barbara et al., 1995) with either set of primers and with AlCV antiserum to trap virus particles. Microfuge tubes (Treff Lab) were coated with AlCV immunoglobulin, 4 ng ml^{-1}, in carbonate buffer, pH 9.6, for either 3–4 h at 30 °C or overnight at 4 °C. After washing three times with PBS-Tween (PBS with 0.5 ml/l Tween 20), leaf samples were homogenised at a suitable dilution (usually 0.1 g to 5 ml) in PBS-Tween, added to the coated tubes and incubated as above. After washing again three times with PBS-Tween, tubes were washed once briefly with water and the RT/PCR reactants added (200 µM dNTPs, 1.5 mM magnesium chloride, 3 µl ml^{-1} Triton X-100, 1 × PCR reaction buffer, 1 U *Taq* polymerase, 0.25 U AMV reverse transcriptase (both Gibco/BRL, Life Technologies, UK), 10 pmoles each primer and 2 µl PBS-Tween in a final volume of 25 µl). After overlaying with oil, tubes and contents were heated according to the following: 42 °C/45 min; 92 °C/2 min; 54 °C (Carla-uni primers) or 60 °C (LSV primers)/1 min; 72 °C/2 min; 92 °C/1 min for 35 cycles; 54 or 60 °C/1 min; 72 °C/5 min. PCR products were analysed by electrophoresis in an agarose gel

(usually 15 g l^{-1}; BioRad 162-0126), stained with ethidium bromide (0.5 μg ml^{-1}) and photographed in UV light.

The 11 kDa ORF and coat protein PCR fragments from five isolates of carlavirus from *Alstroemeria* were cloned into pMos*Blue* T-vector (Amersham) and sequenced in both directions using a commercial ABI sequencing service (Sequiserve, Germany). Sequences were compared with published sequences for LSV and another unrelated carlavirus, potato virus S (PVS) (Foster and Mills, 1992).

Results and discussion

Virus surveys

A total of 203 *Alstroemeria* samples were collected from glasshouses and polythene houses of *Alstroemeria* growers in the UK and from an outdoors field trial. Samples were taken from a wide range of *Alstroemeria* varieties, mostly produced by vegetative propagation but some were raised from seed. *Alstroemeria* varieties of different ages were sampled and tested by ELISA using virus-specific antibodies. Samples from 137 plants (67.5%) were virus infected (Table 1). The majority of samples in which virus was detected were infected by at least one potyvirus, with AlMV (including the AlSV strain) being the most common (Table 1).

Samples were infrequently infected separately by AlCV, CMV, LSV and TRV (6.5%) or two of these in combination (5.4%). More commonly samples were infected with a mixture of just a potyvirus (33.5%) and at least one virus other than a potyvirus (22.2%) (Table 1). Some samples were infected with one or more carlaviruses only (8.4%). Symptom expression varied. Sometimes infected plants were symptomless but more usually there was a mild leaf streak; a yellow or chlorotic streak was often associated with CMV infection. Infection by CMV alone occurred only in two plants of *Alstroemeria pulchella* that were raised from seed.

ELISA

In the more detailed ELISA tests performed using the antibodies to AlCV and LSV, both reacted with all samples supporting the suggestion that the two are closely related even if not the same virus. However, there was a higher absorbance value in most reactions with the AlCV antiserum, possibly due to a higher antibody titre.

Table 1. Summary of viruses identified by ELISA in *Alstroemeria* samples collected in surveys in the UK

Viruses	Number of samples	%
AlMV only	54	26.6
AlSV[1] only	14	6.9
AlCV only	6	3.0
LSV only	3	1.5
CMV only	2	1.0
TRV only	2	1.0
AlMV and AlCV	15	7.4
AlMV and LSV	2	1.0
AlMV and CMV	1	0.5
AlMV and TRV	10	4.9
AlCV and LSV	8	3.9
LSV and TRV	1	0.5
CMV and TRV	2	1.0
AlMV, AlCV and LSV	12	5.9
AlMV, CMV and TRV	4	2.0
AlMV, AlCV, CMV, LSV and TRV	1	0.5
Total no. of infected plants	137	67.5
Total not infected	66	32.5
Totals for incidence of each individual virus, singly or in combination		
AlMV	99	48.8
AlSV[1]	14	6.9
AlCV	42	20.7
LSV	27	13.3
CMV	10	4.9
TRV	20	9.9

Alstroemeria carlavirus (AlCV), alstroemeria mosaic potyvirus (AlMV), [1]alstroemeria streak potyvirus strains of AlMV (AlSV), cucumber mosaic cucumovirus (CMV), lily symptomless virus (LSV) and tobacco rattle tobravirus (TRV).

Differentiation of carlaviruses by PCR and sequencing

When ALCV-infected samples were tested by PCR, the Carla-Uni primers produced DNA fragments of approximately. 140 bp for all AlCV and LSV isolates tested. This corresponds with the predicted size of fragment, largely from the putative 11 kDa protein that is unique to carlaviruses. The DNA fragments from four isolates of AlCV (HRI-2, HRI-3, HRI-A95 and HRI-F) and two of LSV (LSV-7 and LSV-21) were cloned and sequenced and compared with the published sequence

Table 2. Multiple alignment of % identity predicted in the amino acid sequence of the coat protein gene from alstroemeria carlavirus (AlCV) isolates

	PVS	LSV	HRI-A95	HRI-23	HRI-F	HRI-12	HRI-13
PVS	—	54	56	55	56	53	55
LSV	—	—	96	95	94	96	96
HRI-A95	—	—	—	96	96	96	96
HRI-23	—	—	—	—	94	95	94
HRI-F	—	—	—	—	—	94	94
HRI-12	—	—	—	—	—	—	99
HRI-13	—	—	—	—	—	—	—

All isolates are Alstroemeria carlavirus (AlCV) except lily symptomless virus (LSV) and potato virus S (PVS).

for LSV. There was 99% nucleotide identity between the isolates sequenced and with LSV (data not presented). This corresponded to 100% identity of the predicted amino acid sequence between AlCV isolates and LSV.

Using the LSV primers in PCR, DNA fragments of approximately 930 bp were obtained from several isolates of AlCV. The DNA fragments from five isolates (HRI-12, HRI-13, HRI-23, HRI-A95 and HRI-F) were cloned and sequenced and compared with the published sequence for LSV. There was high nucleotide and amino acid sequence similarity of the coat protein genes within the AlCV isolates (94–99% identity of the amino acid sequence) and between these isolates and the published sequence for LSV (94–96% identity of the amino acid sequence) (Table 2). This is in contrast to only 53–56% identity with another carlavirus, PVS. The results indicate that AlCV and LSV are strains of the same virus, and explain the ELISA results in this study and support conclusions of earlier reports, based on biological and serological properties, that AlCV is a host-adapted strain of LSV (Derks et al., 1982). The very high amino acid homology found between isolates from different geographical sources may indicate a common origin in propagation material many years ago.

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