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An Unusual Strain of Alfalfa Mosaic Virus Detected in *Crotalaria* L. Germplasm

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Abstract: Virus isolates from *Crotalaria* plants in germplasm regeneration plots in Griffin, GA were found to produce an unexpected wilting and necrosis on *Nicotiana benthamiana*. DAC-ELISA tests produced positive results for alfalfa mosaic virus (AlMV) and a mixture of viruses including cucumber mosaic, blackeye cowpea mosaic, peanut stunt and white clover mosaic viruses. After serial transfer of an isolate from *C. juncea* in *N. benthamiana* in the greenhouse, only AlMV could be detected by ELISA while the wilting and necrosis symptoms remained. In addition, RT-PCR tests produced a product of the expected size with the crotalaria isolate RNA as it did with a known isolate of AlMV. Sequencing of the 300 bp PCR-band indicated that there was 99% identity between the Crotalaria isolate and strain 425 of AlMV. These results show that this isolate from *Crotalaria* is AlMV and possibly a new strain of AlMV.

Key words: *Crotalaria*, viruses, RT-PCR, DAC-ELISA, germplasm

INTRODUCTION

Crotalaria L. is a legume consisting of more than 300 species have many uses throughout the world. Some are considered weeds, while others are used as cover crops, for fiber, weed or nematode control, green manure, or forage (Duke, 1983). Common names of *Crotalaria* include rattlepod and sunn hemp. There are 220 accessions of this genus in the USDA, ARS, National Plant Germplasm Collection and Griffin, Georgia is the site of the working collection. Accessions are planted in the field at Griffin periodically for seed regeneration. The plants in these regeneration plots are inspected for the presence of disease symptoms and diseased plants are tested further in host range studies and serologically. *Crotalaria* has been reported to be a host for a number of viruses. Viruses found in the routine tests are checked to be certain that no new viruses are introduced into the country. The URL site http://image.fs.uidaho.edu/vide/descro.html (Brunt *et al*., 1996) indicates that alfalfa mosaic (AlMV), blackeye cowpea mosaic (BlCMV), cucumber mosaic (CMV), peanut stunt (PSV) and white clover mosaic (WCMV) viruses will infect *Crotalaria*. These viruses were among those tested in our plots. The study to determine whether there were any new viruses in the germplasm being regenerated included host range studies, serological studies, testing via reverse transcription-polymerase chain reaction (RT-PCR) and sequencing of the expected band from the PCR are reported here. Results are presented to identify the unusual virus that was detected and to characterize this virus. The findings are discussed in light of previous reports of AlMV from other legumes.

MATERIALS AND METHODS

The host range study plant species included *Chenopodium amaranticolor* Coste and Reyn (Cam), *Nicotiana benthamiana* Domin. (Nb), *N. tobaccum* L. cv Burley 21 (Nt) and *Vigna unguiculata* subsp. *unguiculata cv.* Early Ramshorn *(Vu)*. These were planted in Metromix 300 (Sun Gro Horticultural Distribution, Inc., Bellevue, WA) in 10 cm pots in the green house.

Plants for the host range study and for isolate transfers were inoculated mechanically by rubbing carborundum dusted plants with an extract of leaf samples in 0.025 M phosphate buffer pH 7. The plants were observed daily for two to three weeks for the appearance of symptoms.

Serological tests were done by direct antigen coating-enzyme-linked immunosorbent assay (DAC-ELISA) (Gillaspie *et al*., 1995). Polyclonal rabbit antisera were supplied by and used at the following dilutions: CMV (1:5,000) and PSV (1:10,000), O.W. Barnett, Clemson University, Clemson, SC; BlCMV (1:20,000), Griffin, GA; AlMV (PVAS92; strain 425) (1:15,000) and WCMV (PVAS190) (1:20,000), American Type Culture Collection, Rockville, MD.

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For the RT-PCR testing, total RNA extracts of infected leaves were isolated by using the Qiagen miniprep kit (Qiagen Inc., Valencia, CA). Primers were designed by DesignerPCR software using sequences on Genebank. The forward primer is 5'-CGGTGCGAGATTCCTCTA-3' and the reverse is 5'-CAAAGGGCTACGGCATAG-3'. For first strand c-DNA synthesis, an RT mix (20 μL, consisting of 4 μL of 5X first strand RT buffer [Invitrogen, Carlsbad, CA], 2 μL each of 0.1 M dithiothreitol and 10 mM deoxynucleotide triphosphate [dNTP], 0.25 μL of SUPERSCRIPT RT RNase H$^-$ Reverse Transcriptase [Invitrogen], 0.2 μL RNasin RNase inhibitor [Promega Corp., Madison, WI], 9.35 μL nuclease-free water and 0.2 μL of the reverse primer [primers at 100 ρmol μL^{-1}]), was added to 2 μL of total RNA from test tissue. The reaction mix was then incubated at 37°C for 1 hr followed by treatment at 94°C for 2 min to inactivate the enzyme.

Viral cDNA was amplified in 25 μL of PCR mix containing 2.5 μL of 10X PCR buffer (Promega Corp.), 3.0 μL of 25 mM $MgCl_2$, 2.5 μL of 2 mM dNTP, 0.2 μL each of forward and reverse primers, 0.2 μL of *Taq* DNA polymerase (Promega), 13.4 μL of nuclease-free water and 3 μL of RT product as follows: 94°C, 2 min; 35 cycles of 94°C, 30 sec; 52°C, 30 sec; 72°C, 60 sec and one cycle of 72°C, 10 min. The amplification products were assessed by electrophoresis in a 1.5% agarose gel in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) and stained in ethidium bromide. The expected product size for AlMV was 300 bp.

The RT-PCR product was isolated by excising the band from an agarose gel and centrifuging the band through a Wizard minicolumn (Promega, Madison WI). This product (1 μL) was then subjected to another PCR procedure with the above protocol. The PCR product was then treated with 2 μL of Exonuclease 1 (10 U μL^{-1}) and 2 μL of Shrimp alkaline phosphatase (1 U μL^{-1}) (GE healthcare, Piscataway, NJ) and subsequently incubated at 37 and at 80°C for 15 min each. These enzymes digest single strand DNA and remove the 5' phosphate. The product was further treated with a Qiagen PCR cleanup column to ensure any remaining primers and dNTP's are removed. Then, 1 μL of the product was run on an agarose gel with a quantitative marker (Invitrogen) to determine concentration and thus prepare the sample for the sequencing reaction. Sequencing reactions were prepared by following instructions from the DTCS quick start sequencing kit (Beckman Coulter, Fullerton, CA). The sample was sequenced bidirectionally and PUC 18 was also sequenced as a positive control. Samples were injected and sequenced on a Beckman CEQ 8000 using the LFR-1 method. The sequence module of the software package CEQ 8000 Genetic Analysis System version 8.0.52 from Beckman was used to call the bases after the sequencing was performed. The forward and reverse strands were aligned using AlignIR version 2.0 (LI-COR, Lincoln, NE). This process was repeated with like results. These results were then compared to AlMV strain 425 and others via a BLAST search.

RESULTS AND DISCUSSION

The accessions of *Crotalaria* that showed virus symptoms during the examination times include two *C.* sp., four *C. juncea* and two *C. incana* lines. These lines originated from Brazil, India and Mexico or were donated from Myanmar and Peru. The outstanding host reaction when these samples were inoculated from *Crotalaria* plants directly to the greenhouse plants was the wilting and necrosis produced on Nb (Fig. 1C). If the inoculum is dilute enough, these Nb plants did survive enough that virus could be transferred from them. Strain 425 of AlMV produced a mild mosaic on Nb. The virus from *Crotalaria* also produced ringspots on Nt (Fig. 1A) unlike the mosaic and slight necrosis produced by strain 425 of AlMV. The Crotalaria isolate produced chlorotic local lesions on Cam (Fig. 1B) and necrotic lesions on Vu (Fig. 1D). The infection on Vu produced a systemic mosaic also. The DAC-ELISA results varied on the samples from the plants in the field with all plants producing a positive reaction to AlMV antiserum and reactions also to BlCMV, CMV, PSV and/or WCMV. In some cases, these additional viruses could be detected after several transfers on Nb. After transferring an isolate of the virus from *C. juncea* 20 times on Nb (extracts were diluted upto 100 X for these transfers to reduce the amount of wilting and necrosis), only AlMV could be detected via DAC-ELISA. At this time, healthy *Crotalaria* growing in flats in a growth chamber were inoculated from infected Nb plants. These *Crotalaria* plants became infected producing symptoms similar to those on the *Crotalaria* plants in the field. After several weeks, healthy host range plants were inoculated from the infected *Crotalaria*. The symptoms produced were those described above and shown in Fig. 1.

We suspect that this new virus is seedborne in *Crotalaria* since we have not detected the virus infecting any other plants in Georgia fields near the germplasm regeneration plots. Our attempts at detecting seed transmission with 200 seeds planted in flats in growth chambers has failed to produce the virus in any of these plants. WCMV was detected in two of the plants in these tests, but this seed transmission has previously been reported in Plant Viruses Online at URL http://image.fs.uidaho.edu/vide/ (Brunt *et al.*, 1996).

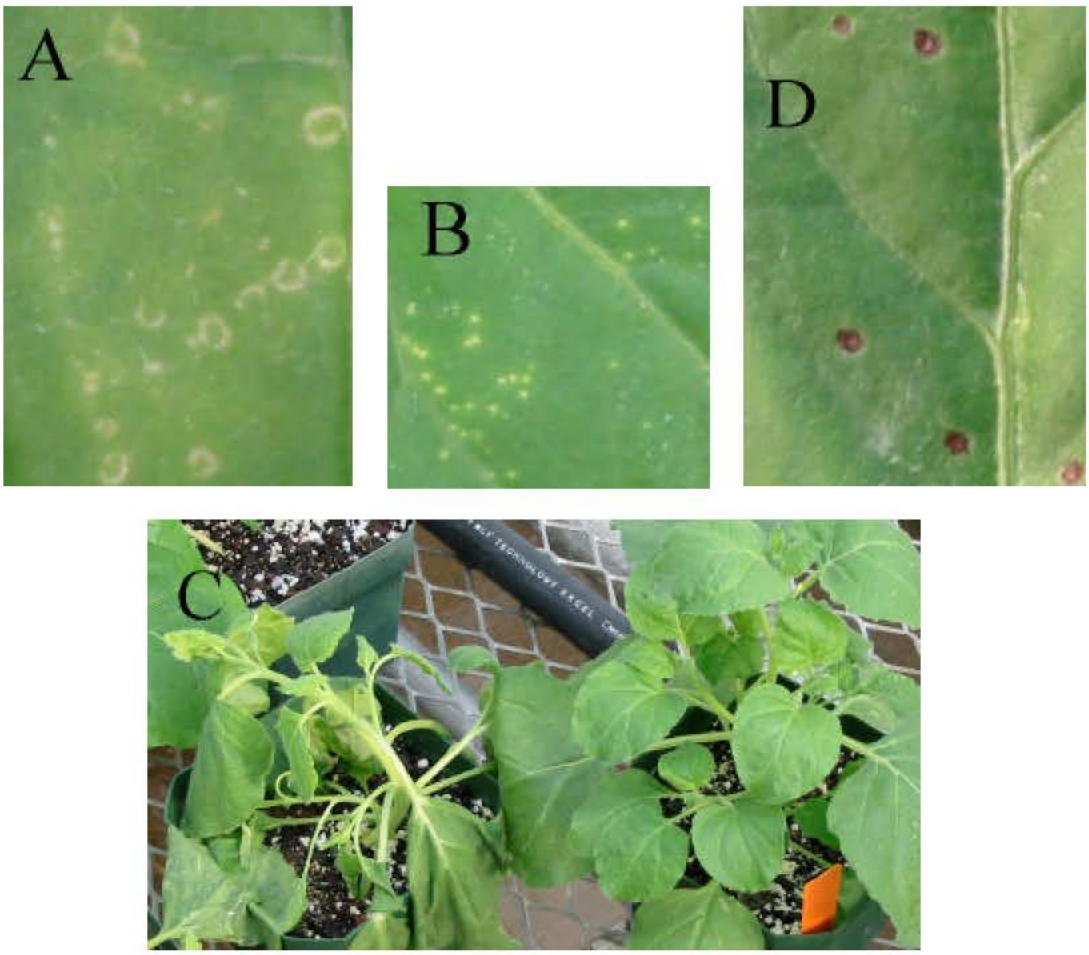

Fig. 1: Symptoms on plants infected with Crotalaria isolate of alfalfa mosaic virus from Crotalaria. A) infected *Nicotiana tabaccum* cv Burley 21, B) infected *Chenopodium amaranticolor*, C) *N. benthamiana* infected with Crot isolate on left with healthy plant on right and D) infected *Vigna unguiculata* cv Early Ramshorn

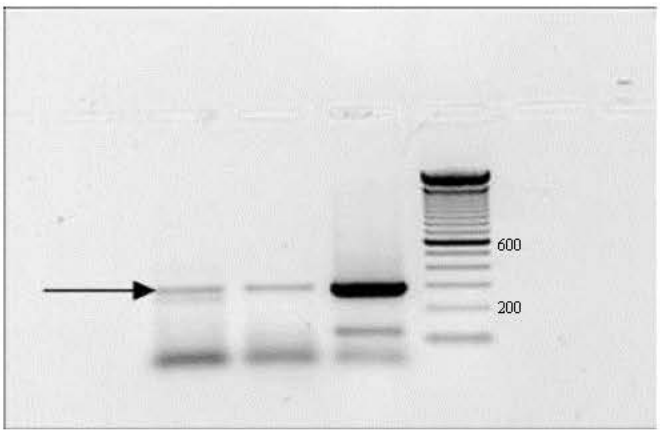

Fig. 2: RT-PCR products of Crotalaria isolate and 425 strain of alfalfa mosaic virus. From left to right: Lane 1 = Crot. isolate from *N. tabaccum* , lane 2 = Crot isolate from *N. benthamiana,* lane 3 = strain 425 from *N. tabaccum,* and lane 4 = 100 bp ladder

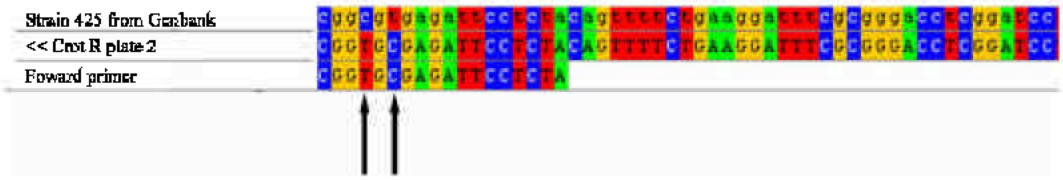

Fig. 3: DNA sequence matchups between the AlMV strain 425, the Crotalaria isolate of AlMV and the forward primer made for AlMV. The arrows denote the two points of difference. Note that there was point of difference at the area where the reverse primer attached (not shown)

Total RNA was extracted from infected Nb and Nt plants as well as Nt plants infected with AlMV (strain 425, Hagedorn and Hanson, 1963) and these RNAs were used in RT-PCR tests. The expected 300 bp band appeared with the AlMV and with the Crotalaria-virus isolate from both Nb and Nt (Fig. 2). BLAST search of the sequence of the band from the Crotalaria isolate gel showed a 99% identity with strain 425 and from 92-98% identity with nine other entries of AlMV from Genebank. The lack of identity between 425 and the Crot isolate occurred in the point of attachment of the forward primer (2 bp difference) and a single bp difference at; the point of attachment of the reverse primer (Fig. 3). This high identity along with the ELISA results indicate that the unknown virus is AlMV.

AlMV has been reported to infect members of genus *Crotalaria* (Brunt *et al*., 1996; Duke, 1983; Jaspars and Bos, 1980). Strains of this virus have been reported to infect Nb (Mayoral *et al*., 1998; Quacquarelli and Avgelis, 1975; Savino and Gallitelli, 1976), but only the first of these publications reports a somewhat similar symptom. Mayoral *et al*. (1998) reports an AlMV strain from alfalfa that causes chlorotic spots, wilting of the inoculated leaves and systemic mosaic of Nb. We saw no chlorotic spots and the wilting observed did progressed into total plant collapse when that inoculum was not diluted enough. However, these strains could be closely related, but we have no source of this strain to do direct comparisons.

In summary, 1) the production of wilting and necrosis symptoms on Nb, 2) the 300 bp product of RT-PCR with this isolate that produced by the known strain of AlMV, 3) the 99% identity of this 300 bp product with AlMV strain 425 and 4) the positive ELISA results with AlMV antiserum indicate that the isolate from *Crotalaria* is AlMV and probably a new strain of AlMV.

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