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Research article

MOLECULAR IDENTIFICATION OF ALFALFA MOSAIC VIRUS ISOLATED FROM NATURALLY INFECTED ALFALFA (*MEDICAGO SATIVA*) CROP IN SAUDI ARABIA.

Jehan S. Al-Abrahim

Department of Biology, Faculty of Science, Princess Nora bent Abdul-Rahman University, Postal Code 11474, Riyadh, Saudi Arabia.
For correspondence: farhati@hotmail.com

ABSTRSACT: Alfalfa mosaic alfamovirus (AMV) causing a disease in Alfalfa (*Medicago sativa)* crop in Saudi Arabia has been isolated and identified on the basis of determination of an experimental host range, symptom expression, morphological properties and a positive immunological test of the virus isolates. The primers designed on the basis of published sequences were applied for amplification of AMV RNA fragments in reverse transcription-polymerase chain reaction (RT-PCR) using host plants infected with AMV. The detection of AMV in Alfalfa was confirmed by RT-PCR technique. For virus detection, a pair of primers, AMV-F and AMV-R, specific to the AMV coat protein (CP) gene, was designed based on the nucleotide sequence alignment of known AMV strains. Assessments showed that RT-PCR using this primer set was specific and sensitive for detecting AMV and that the molecular technique is more reliable for the detection of Alfalfa virus in Alfalfa leaves. The sequencing and alignment of the RT-PCR amplified fragments has been stored in Gen-Bank AMV strain (accession no. KF487082), which was confirmed by the sequence analysis of their (CP) gene. Further studies for better understanding into AMV spreading over the crops, the effect on the physiological performance of the plant and eventually the effect on the crop product quality are urgently needed.
Key words: Alfalfa, Alfalfa mosaic virus, identification, ELISA, RT-PCR**.**

INTRODUCTION

In Saudi Arabia, Alfalfa (*Medicago sativa* L.) is considered of great economic value, and accounts for about 30% of crop production with a very high yield potential compared with that of other forage crops. It is also an integral component of many crop rotations because of its ability to fix nitrogen, improve soil structure, and control weeds in subsequent crops [1]. In terms of cultivated area and production, Alfalfa (*Medicago sativa* L.) is the major and most important forage crop in Saudi Arabia. Forage which are either directly or indirectly consumed by cattle and poultries, play important roles in animal production. Medics and clovers including berseem clover (*Trifolium alexandrinum* L.) and Alfalfa (*Medicago sativa* L.) have constituted the backbone of forage crops production [2].
Under field conditions, Alfalfa plants are subjected to infection with many viruses which have been shown to cause great economic losses. Among such viruses Alfalfa mosaic virus was found to be widely distributed in Alfalfa plants. *Alfalfa mosaic virus* (AMV) is the type member of the genus *Alfamovirus* in the *Bromoviridae* family of plant viruses with a very wide host range. This virus can naturally infect many herbaceous and some woody plant hosts (150 species in 22 families) and is transmissible to over 430 species of 51 dicotyledonous families [3]. In nature, AMV is preserved and transmitted with the seed of Alfalfa, pepper and weeds (*Datura stramonium* L., *Solanum nigrum* L., *Chenopodium quinoa* Willd. and possibly *Melilotus* sp.) It has been found naturally infecting potato (*S. tuberosum* L.) and Alfalfa (*Medicago sativa* L.) and the symptoms may be covered. In many species, bright yellow mottle or mosaic is common. Severe necrosis may also occur. Spread from Alfalfa to the surrounding crops via aphids is common. At least 15 aphid species are known to transmit the virus in the stylet-borne or non-persistent manner. Aphids can transmit AMV after having fed on the infected plants for only one second [4]. The virus is transmitted by pollen and grafting and not transmitted by contact among the plants [5].

The genome of AMV consists of three single-stranded RNA molecules of plus-sense polarity, conventionally numbered RNA 1 to 3 in order of decreasing size [6]. Many viruses possess RNA genomes, and those with single-stranded plus-sense RNA genomes represent an important sub-group that includes many pathogenic plant, animal, and human viruses [7]. [8] reported the first presence of AMV in three different regions in Saudi Arabia (Asser, Gasim and Najran) and of its spread in 16 locations belonging to the Riyadh region in Saudi Arabia.

ELISA and transcription polymerase chain reaction (RT-PCR) are currently used for the diagnosis of AMV, methods developed for screening of potato, tomato samples and Alfalfa seeds for the presence of several viruses. The RT-PCR technique has many advantages over ELISA and bioassay [9,10,11,12].

The current work was initiated to investigate the disease in Alfalfa crop caused by AMV, to isolate and identify the causal agent and to establish some properties (host range, symptoms, morphology of virions) of this virus. Moreover, our interest was to evaluate the reliability of RT-PCR techniques in detection of AMV isolate.

MATERIALS AND METHODS

Alfalfa leaf samples collection

Leaf samples were collected in 2011 from plants showing bright yellow blotching symptoms on Alfalfa leaflets from Dirab experimental farm, Riyadh, Saudi Arabia and were confirmed to be positive for AMV by initial ELISA and RT-PCR tests. The diagnostic study of the pathogen was done at Plant Virus Laboratory in the Department of Botany (Ekarda, Syria) using standard methods for mechanically transmitted viruses. Study of the host range was determined after which ELISA test was well performed to detect AMV in infected plants. Serological confirmation of virus presence in test samples of the experimentally infected plants was conducted using the commercially-prepared polyclonal antiserum to AMV (DSMZ Plant Virus Collection, Germany). Enzyme-substrate reactions were measured at wave length of 405 nm using an ELISA reader (Labsystem Multiskan RC). The ELISA readings were banked on buffer controls, and a sample was considered positive when its A_{405} nm value exceeded three times the mean value of the extracts from negative control (non-infected plants).

Detection of AMV by reverse transcription-polymerase chain reaction

For detection of AMV by reverse transcription-polymerase chain reaction (RT-PCR), infected leaf tissues of Alfalfa plants were used. Total RNA extraction was carried out according to the instruction of the Quick Prep total RNA extraction kit for the direct isolation of total RNA from most eukaryotic tissues or cells (Amersham Pharmacia Biotech, UK). Frozen tissue samples (25 – 50 mg) of experimentally-infected test plant tissues were ground in liquid nitrogen and transferred to 1.5 ml microfuge tubes. Exactly 150 µl of the extraction buffer was poured into the tube and 3 µl of 14.3 M β-mercapto ethanol was added. The solution was mixed thoroughly to obtain a homogeneous suspension. Thereafter, 350 µl of lithium chloride (LiCl) solution was added to the homogenized samples. The homogenization was continued by mixing the components. The tubes containing the suspension were placed on ice, then 500 µl of caesium-tri-fluor-acetate (CsTFA) was dispensed into the homogenized samples and mixed well. The tubes were placed on ice for 10 min and later spinned for 15 min at 14000 r.p.m. The RNA formed a pellet at the bottom of the micro-tubes. The proteins form a coat at the top of the tubes and DNA remains in the liquid phase. The protein coat and the liquid phase were carefully removed. Subsequently, RNA pellets were washed with three "kit" components: 75 µl extraction buffer, 175 µl LiCl solution and CsTFA solution. The tubes were vortexed several times to wash the RNA pellet. The samples were spinned in a micro-centrifuge at 14 000 r.p.m for 5 min. The supernatants were discarded without disturbing the pellets. One ml of 70% ethanol was added to the samples and incubated at –20 °C for 2 hours. The samples were spinned in a micro-centrifuge at 14 000 r.p.m for 5 min. The pellet was air-dried for 10 – 15 min keeping the tubes on ice. DEPC‑treated (Diethyl Pyrocarbonate) water containing 1% of RNAs inhibitor was added to the RNA pellets. The pellet was broken by pulse vortexing 5 – 10 times. The samples were incubated on ice for 15 – 30 min. For the first strand cDNA synthesis, to the denaturated RNA solution a mixture containing 5x reaction buffer, 1% of RNAse inhibitor, 10 mM deoxynucleoside triphosphate (dNTP) mixture and 10 Units of RevertAidTM M-MuLV reverse transcriptase (MBI Fermentas, Vilnius, Lithuania) were added. The first strand cDNA synthesis was carried out at 37°C for 60 min and 70°C for 10 min. DNA amplification was performed in 55 µl reaction mixtures containing each of the four dNTP at a concentration of 200 µM of each primer at a concentration of 0.4 µM, 10 × PCR buffer, 25 mM $MgCl_2$ and 0.25 U of recombinant *Taq*polymerase (MBI Fermentas). PCR was carried out for 40 cycles using the following parameters: 1 min at 94°C (4 min for the first cycle), 2 min at 52°C and primers extension for 2 min (10 min in the final cycle) at 72°C. The resulting PCRs products were analyzed by electrophoresis in 5% polyacrylamide gel stained with ethidium bromide, and DNA bands were visualized using a UV transilluminator. The primer pairs designed on basis of published sequences successfully amplified cDNA templates in RT-PCR.

Table 1: Primer pairs designed for reverse-transcription polymerase chain reaction based on coat protein gene sequence of Alfalfa mosaic virus (AMV) at 58°C

Primer pair	Primer sequence	Locationa	Product size (bp)
AMV1-F	5′-CCATCATGAGTTCTTCACAAAAG-3′	1,188–1,210	351
AMV1-R	5′-TCGTCACGTCATCAGTGAGAC-3′	1,518–1,538	

RESULTS

Naturally infected Alfalfa plants with AMV generally exhibited bright yellow mottle symptoms in Alfalfa leaves. Later, the leaf yellowing was accompanied by plant stunting and leaflet deformation. For accurate determination of the experimental host range and symptoms of the causal agent, five leaf samples showing these symptoms were collected from Alfalfa plants. The isolates produced similar symptoms in inoculated test plant species.

ELISA test

The data obtained from ELISA analysis revealed that the leaf samples were positive for AMV. However, all samples were confirmed to be positive for AMV by all applied tests, including ELISA, after inoculation onto Alfalfa plants on which they all produced typical symptoms in plants which confirm the dependability of the virus.

RT-PCR tests

The molecular technique, RT-PCR was employed for confirmation of AMV detection in Alfalfa, and to facilitate identification of Saudi isolates of AMV from Alfalfa plants. Primer pairs (AMV1-F/ AMV1-R) which have been designed in this study were specific for AMV RNAs and did not show amplicons from Alfalfa leaves infected with different kinds of viruses.

Sequence analysis

Total RNAs extracted from Alfalfa leaves were confirmed to be positive for AMV by ELISA and RT-PCR, which flank the entire CP gene of AMV, resulting in PCR amplicons of (310 – 603 bp). The nucleotide sequence of the CP gene of AMV isolates from Saudi Alfalfa leaves was determined. AMV isolate was closely related (> 97%) in the amino acid sequences of their coat protein gene to another known AMV strains (Table 2, Fig. 1). The complete CP gene sequence of the Saudi Alfalfa isolates of AMV (Jehan 1) was deposited in NCBI Gen Bank (accession no. KF487082).

Table 2: Relationship of the Saudi Alfalfa mosaic virus (AMV) isolates and other known AMV strains based on the alignment of the nucleotide sequence of the coat protein gene.

AMV	Percentage %	Citation
tr_B8YEK0	99%	[13]
tr_L0BXF9	99%	[14]
tr_Q7T448	98%	[15]
tr_I6PBM6	98%	[16]
tr_G4U4J2	97%	[17]
tr_L0BVS1	97%	[14]

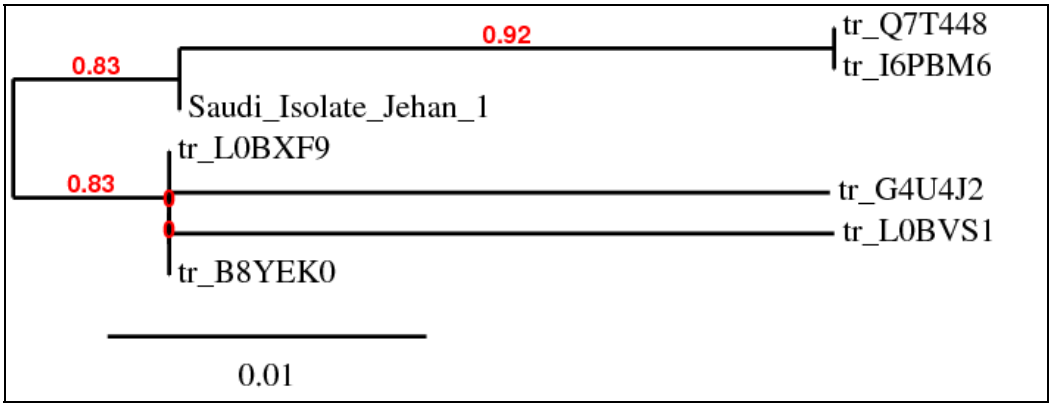

Figure 1: Phylogenetic dendrogram showing the relationship of the Saudi Alfalfa mosaic virus (AMV) isolates and other known AMV strains based on alignment of the the nucleotide sequence of the coat protein gene. The bar indicates the relative evolution distance.

The dendrogram demonstrated in Figure 1 obviously showed that the Saudi AMV Alfalfa isolate (Jehan 1) characterized in this study was closely related to AMV strains from Iran [14], USA [15], Serbia [16] and Italy [17] which showed that AMV isolates formed a strain group which is more distantly related to the group of Saudi AMV isolate based on the sequence analysis of their CP gene (Table 2). Further study revealed that the sequence analysis of the CP gene of AMV from Korea and Argentina (Q9DYE1 and T2BRB3, respectively) had 100% similarity with Saudi isolate, Jehan 1 [18,19].

Moreover, the results of biological, morphological, serological and molecular analysis of this virus disease agent confirmed that it is an AMV isolated from Alfalfa plants. Based on the results obtained from our current experiments and the existing data in the literature, it might be reported that these virus isolates could be attributed to AMV from the genus *Alfamovirus*. This is the first report of AMV infection detected in a field-grown Alfalfa crop in Saudi Arabia.

DISCUSSION

The present study was conducted to provide information on the response of field-grown Alfalfa crop to AMV infection. In phytopathology, real-time PCR has recently become an exceptionally useful and satisfactory tool for studying various causal agents of plant diseases [11,12]. In this study, AMV was detected in Alfalfa samples collected from an experimental farm in Dirab, Riyadh, Saudi Arabia. AMV isolates were characterized by ELISA, RT-PCR and sequence analysis of the CP gene. Data from all these tests positively identified the virus as AMV. Interestingly, data generated from sequence analysis of the nucleotides showed that this isolate was closely related to other known AMV and there were only slight differences in their nucleotide sequences in their CP gene. The dendrogram depicted in Figure 2 showed that the Saudi AMV characterized in this study was closely related to AMV strains from USA, Serbia, Iran and Italy. Furthermore some strains isolated from Korea and Argentina showed 100% relationship with Saudi isolate. This could be attributed to the fact that it might be a different strain because only partial CP gene was detected for Saudi Isolate. Recently, it has been well demonstrated that RT-PCR is a more sensitive method than ELISA test to determine the occurrence of viruses in Alfalfa seeds, paper and in Lilium plants of various species [11,12,21]. It has also been reported that RT-PCR may be a useful approach for detection of the presence of AMV for screening potato samples on a large scale [9,22].

CONCLUSIONS

Alfalfa mosaic alfamovirus (AMV) has been isolated, detected and identified in Alfalfa plants in Saudi Arabia. The identity of the detected virus disease agent with AMV has been confirmed by the methods based on test plant reaction, the morphology of virions, ELISA and RT-PCR which might be considered as faster method, but also most significantly, a more sensitive method for the detection and quantification of Alfalfa mosaic viruses directly from Alfalfa plants compared with the present assays, such as ELISA methods. However, this technology requires the use of special instruments and reagents which are relatively expensive at the present time. Furthermore, their genetic relationships with other known AMV strains were recognized.

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