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Full Length Article

Molecular Characterization of Two *Alfalfa Mosaic Virus* Isolates Infecting Potato Crop in Central Region of Saudi Arabia

Mohammed A. Al-Saleh[1], Mahmoud A. Amer[1,2*], Ibrahim M. Al-Shahwan[1], Omer A. Abdalla[1] and M.T. Shakeel[1]
[1]*Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, Kingdom of Saudi Arabia*
[2]*Viruses and Phytoplasma Research Department, Plant Pathology Res. Institute, Agricultural Research Center, Egypt*
*For correspondence: mamaamery@yahoo.com

Abstract

During the autumn growing season 2012, thirty-two leaf samples of potato (*Solanum tuberosum*, L. cv. Herms) plants showing mottling symptoms suspected to be due to viral infection were collected from Hail and Wadi aldawasser regions in Saudi Arabia. These leaf samples were tested serologically using DAS ELISA against *Alfalfa Mosaic Virus* (*AMV*). Twenty-three out of the thirty-two samples were positive for *AMV*. To determine host range, plant sap was extracted from two diseased potato samples representing the two regions and different healthy plants were mechanically inoculated. The disease symptoms induced by the two selected isolates were divided into three groups: systemic and calico symptoms, local chlorotic lesions, and non-symptomatic ones. Total RNA was extracted from different plants showing positive reaction to *AMV*. RT-PCR was performed using *AMV* coat protein specific primers. Approximately 700-bp RT-PCR products were amplified, and these PCR products were sequenced in both directions. Nucleotide sequencing analysis showed that these two Saudi Arabian *AMV* isolates [*AMV*-PSA-Ha (for the Hail isolate) and *AMV*-PSA-Wd (for the Wadi Al Dawasser isolate] had a 96.5% identity. Sequence comparison showed that these two isolates of *AMV* shared 93.2% to 99.7% sequence similarity with the twenty-eight reported isolates of *AMV* obtained from GenBank. The cDNA probe was prepared and hybridized with RNA extraction from symptomatic potato plants. No hybridization was shown with RNA extracts from asymptomatic plants. This is the first report on the genetic variability of *AMV* isolates infecting potato crop in Saudi Arabia. © 2014 Friends Science Publishers

Keywords: Potato; *AMV*; ELISA; RT-PCR; CP sequence

Introduction

Potato (*Solanum tuberosum* L.) is a very important staple crop in many parts of the world due to its nutritional values. It is used for human consumption, animal feed, as well as a source of starch, carbohydrates, alcohol, and protein (Al-Saikhan, 2000). In Saudi Arabia, potato is considered to be one of the most important and potential vegetable crops. It is grown during spring and autumn growing seasons. Seed tubers of different potato cultivars are usually imported from France, Holland, and USA for planting the spring season, whereas the autumn season is planted from seed tubers propagated locally from the imported tubers harvested from the previous spring season. Potato acreage has increased in some areas and this expansion is mainly due to the government policy of diverting food production and reducing wheat acreage. Potato is grown in different regions in Saudi Arabia, and the total area reached 15,312 ha with a production that reached up to 444,138 tons in 2010 (Anonymous, 2010). Many viruses, viroids and phytoplasmas, have been reported to infect potato. More than forty different viruses or virus like-diseases have been reported as pathogenic to potato (Ross, 1986; Salazar, 1996; Al-Shahwan *et al*., 1998). These plant viruses are responsible for severe diseases in potato plants, resulting in severe losses in potato production (Aranda and Maule, 1998). In Saudi Arabia, twelve potato viruses have been detected (Al-Shahwan *et al*., 1998). Out of these 12 viruses, *Alfalfa Mosaic Virus* (*AMV*) was found to be the most important and widely distributed one, infecting potato plants in several locations and was considered as one of the most prevalent viruses in the country (Al-Shahwan *et al*., 1998; Al-Shahwan, 2003). The main objective of this study was to molecularly study the genetic variability of *AMV* isolates recovered from potato plants from two different regions in Saudi Arabia and assess the DNA homology between the Saudi *AMV* isolates and other *AMV* strains reported elsewhere and available in GenBank database.

Materials and Methods

Source of Virus Isolates, Serological and Biological Identification

In December, 2012, thirty-two symptomatic and asymptomatic leaves of potato plants of cv. Hermes growing under field conditions which exhibited leaf distortion and mottling symptoms suspected to be virus

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infections (Fig. 1) were collected from Hail (North of the Riyadh region) and Wadi Aldawasser (South of the Riyadh region), Saudi Arabia. These samples were tested serologically using Direct ELISA against *AMV* (Agdia Co., France). One sample from each region (Hail and Wadi aldawasser) was chosen for further plant host range characterization. The sap was extracted and ground separately in phosphate buffer (1:3 w/v, 0.1 M, pH 7.2), passed through a double layer of cheesecloth and mechanically inoculated onto carborandoum (600 mesh)-dusted leaves by gently rubbing the freshly prepared inoculums (Xu and Nie, 2006). The selected plant species belonged to different plant families including *Chenopodium amaranticolor* Cost and Reyn, *C. quinoa*, *Vigna unguiculata*, *Pisum sativum*, *Vicia faba*, *Nicotiana tabacum*, *N. benthamiana*, *Capsicum annum*, *Cucumis sativus*, *N. rustica* and *S. tuberosum*. For biological purification, the single local lesion technique was carried out according to Kahn and Monroe, 1963 using *C. amaranticolor* as a local lesion host. The resulting local lesions were singly back inoculated onto potato plants, whereas *N. tabacum* was used as propagative host for the *AMV* isolates (Fig. 2). Five seedlings of each host plant were inoculated and observed daily for symptom development. A number of healthy seedlings of the same species and age were inoculated with buffer to serve as a control. The inoculated plants were maintained in an insect-proof greenhouse with natural illumination and temperatures of 25–32°C. The symptoms expressed on each species were described and recorded. The experiment was repeated twice.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted from four out of twenty-three samples which tested positive for *AMV* by ELISA (two samples from each region). These samples and one uninfected potato sample, served as a negative control were extracted using the Isolate Plant RNA Mini Kit (Bioline). The oligonucleotide primers designed according to (Xu and Nie, 2006) was as follows, the upstream primer *AMV*-coat-F: 5'-GTG GTG GGA AAG CTG GTA AA-3'] and downstream primer *AMV*-coat-R: 5'-CAC CCA GTG GAG GTC AGC ATT-3' were used. RT-PCR was performed using a thermal cycler (Eppendorf, Germany) according to Xu and Nie, 2006; Al-Saleh and Amer, 2013. DNA was visualized and photographed using DNA documentation gel analysis (IN GENIUS, Syngene Bio Imaging, UK). HyperLadder IV DNA marker (Bioline) was used to determine the size of RT-PCR amplified cDNA products.

Probe Preparation and Dot Blot Hybridization

The viral fragment was labeled according to the Digoxigenin Non-radioactive Labeling System (Roche, Boehringer Mannheim, Germany). Total clarified sap preparations from infected plant materials were diluted ten-fold with 6X SSC buffer, heated to 95ºC for 10 min, and chilled on ice. A volume of 5 µL of total RNA extracted from each of the twenty-three symptomatic and healthy samples that were collected were prepared and directly applied to nitrocellulose membranes according to established methods (Podleckis *et al*., 1993; Pallas *et al*., 1998). Prehybridization, hybridization, and colorimetric detection with a single digoxigenin labeled probe were carried out using a hybridization oven (Amersham Biosciences, Piscataway, NJ, USA) following the protocol recommended by Boehringer Mannheim. The results were documented by photography.

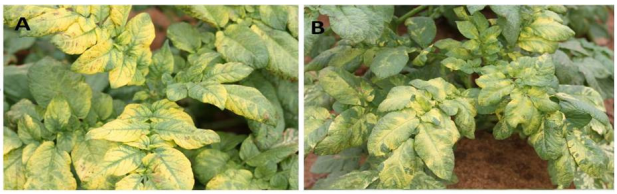

Fig. 1: Natural infection of AMV on *Solanum tuberosum* L. cv. Herms. A: Calico symptoms on potato collected from the Wadi aldawasser; and B: from the Hail region

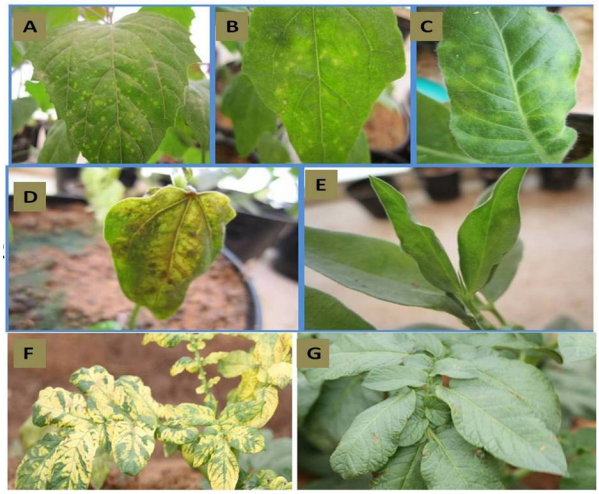

Fig. 2: Reaction of select plant species to mechanical inoculation with the two AMV isolates collected from the Hail and Wadi aldawasser regions. A and B: chlorotic local lesions on inoculated *Ch. ammaranticolor* and *Ch. quinoa*. C, D and E: Systemic mosaic on *N. tabaccum*, *V. unguiculata*, and *V. faba.* F: calico symptoms on *S. tuberosum*; and G: Uninoculated *S. tuberosum* as a negative control

Nucleotide Sequence and Phylogenetic Analyses

Virus-specific RT-PCR products from two out of the four samples tested positive for *AMV* and were designated as *AMV*-PSA-Ha (for the Hail isolate) and *AMV*-PSA-Wd (for the Wadi Al Dawasser isolate). The RT-PCR products were sequenced using two directional sequencing with the *AMV*-

coat-F and *AMV*-coat-R primer specific for *AMV*-CP (Xu and Nie, 2006) using an Applied Biosystem AB3730xI DNA analyzer (Life Technologies, USA). The partial nucleotide sequence of the CP gene was subjected to a Blast N search for comparison with published *AMV*-CP gene sequences retrieved from GenBank. The multiple sequence alignment and phylogenetic relationships for the *AMV* isolates available in GenBank (Table 1) were analyzed and reconstructed using Lasergene DNASTAR, V5-05 Software.

Results

Serological and Biological Identification

Twenty-three out of thirty-two tested samples were shown to be positive to *AMV* by ELISA. Two isolates (*AMV*-PSA-Ha and *AMV*-PSA-Wd) were selected and inoculated mechanically onto various plants to determine host range. The induced symptoms were divided into three groups. The first group included the variable systemic symptoms exhibited by *V. unguiculata, N. tabacum*, and *V. faba.* Calico symptoms were induced on inoculated *S. tuberosum* plants. The second group included chlorotic local lesions exhibited by *Ch. quinoa* and *Ch. amaranticolor*. The third group included the other plants that were not infected by these isolates (*P. sativum*, *N. benthamiana*, *C. annum*, *C. sativus*, and *N. rustica*).

RT-PCR

Figure (3) show electrophoresis analysis of the RT-PCR products of *AMV*-CP gene generated from leaf samples from symptomatic potato plants collected from Hail (lanes 2,3) and Wadi aldawasser (lanea 4,5) regions, respectively. No fragments were amplified from the RNA extracted from healthy plants (lane 1).

Dot Blot Hybridization Assay

Dot blot hybridization was used to detect the virus in infected potato plant tissues. Fig. 4 shows a moderate reaction (blue signal) resulting from dot blot hybridization of the DIG labeled probe with nucleic acids extracts from all twenty-three potato samples collected from Hail (Row A: 1-8 and Row B: 1-5) and Wadi aldawasser regions (Row B: 6-8 and Row C: 2-8). However, hybridization was not observed between the probe and total nucleic acids from uninfected potato leaf tissue (Row C: 1).

Nucleotide Sequence and Phylogenetic Analyses

Sequence comparisons showed that the percentage of similarity between *AMV*-PSA-Ha and twenty-eight reported isolates of *AMV* obtained from GenBank ranged from 96.5 to 99.7%, while the identity ranged from 93.2% -97.4%

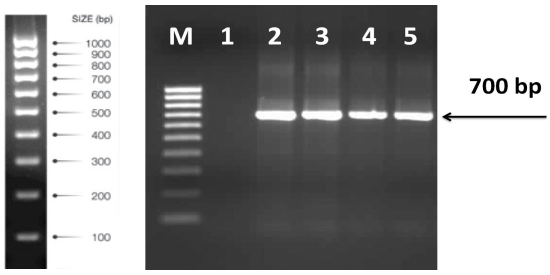

Fig. 3: Gel electrophoresis of RT-PCR amplification of a 700 bp fragment from the AMV-CP gene using the AMVcoat-F/AMVcoat-R primer pair. Lane M represents HyperLadder IV DNA marker (Bioline). Lanes 2 and 3 show samples from infected potato plants collected from Hail. Lanes 4 and 5 show leaf samples from infected potato plants collected from the Wadi aldawasser region, and Lane 1 shows healthy potato as a negative control

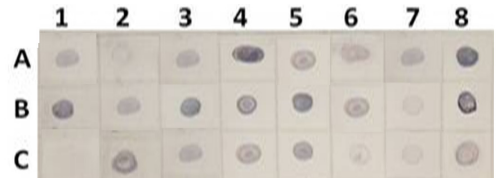

Fig. 4: Dot blot hybridization of the AMV DIG-cDNA probe with the total RNA extracted from the infected and uninfected potato samples. Row A: 1-8 and Row B: 1-5 represent samples collected from the Hail region, and Row B: 6-8 and Row C: 2-8 represent samples collected from the Wadi aldawasser region. No hybridization reaction was observed with uninfected potato samples (Row C: 1)

with the *AMV*-PSA-Wd isolate. The *AMV*-PSA-Ha isolate was closely related (99.7%) to *AMV*-Saj, Saudi Arabia (KC434083) and Aq, Australia (JX112758) isolates, also high similarity (99.5%) was shares with Hu (JX112759) in Australia and Brazil (FJ858265) isolates isolated from alfalfa. Also this isolate was shares the high sequence identity (99.1%) with N20 (HM807304), S40 (HM807306), W1(HM807307), isolates in Australia, one isolate from China (JQ281522) isolated from alfalfa and Ca175 isolate (DQ314750) in Canada which isolated from potato. *AMV*-PSA-Ha shares the lowest sequence homology (94.1%) with the *AMV*-WD isolate (KC434084) which was isolated from alfalfa in Wadi aldawasser region, Saudi Arabia

The *AMV*-PSA-Wd isolate shares the highest sequence homology (97.4%) with the *AMV*-Wd isolate (KC434084) isolated from alfalfa grown in Wadi Aldawasser region, Saudi Arabia. The high similarity (96.2 to 96.3%) was shares with the Chinese (JQ281522) Brazilian (FJ858265), Australian isolates of N20 (HM807304), S40 (HM807306) and W1 (HM807307) isolated from alfalfa. The *AMV*-PSA-Wd isolate shares the lowest sequence homology (93.2%) with a Serbian isolate (FJ527748) isolated from alfalfa (Fig. 5 and Table 1).

The partial nucleotide sequences containing the CP genes of the *AMV*-Saudi Arabian isolates were deposited in the GenBank under the accession number no. KC569796 and KC569797, for the *AMV*-PSA-Wd and *AMV*-PSA-Ha isolates, respectively.

Discussion

Potato plants are infected by many viruses under field conditions (Salazar, 1996; Al-Shahwan *et al*., 1998). Such viruses have been shown to cause great economic losses and are considered to be the major limiting factors of potato production. Among such viral diseases *AMV* was found to be widely distributed on potato plants. *AMV* is the type member of the genus *Alfamovirus* in the *Bromoviridae* family of plant viruses. *AMV* is a world-wide distributed virus (Jasper and Bos, 1980) with a very wide host range. This virus can naturally infect many herbaceous and some woody plant hosts, and causes diseases of many economically important crops including the families Solanaceae and Leguminosae (Hiruki and Miczynski, 1987), however *AMV* was considered as economically less important when compared to potato viruses (Salazar, 1996), but was considered as one of the most prevalent viruses in the Saudi Arabia (Al-Shahwan *et al.*, 1998; Al-Shahwan, 2003), and also in other countries (Jung *et al*., 2000; Xu and Nie, 2006; Pourrahim *et al*., 2007; El-Helaly *et al*., 2012). In this study, *AMV* was mechanically inoculated onto different host plants, and the results obtained in this report were compatible with the findings of other reports (Xu and Nie, 2006; Baldo *et al*., 2010; Parrella and Acanfora, 2010).

In this study, host range studies and serological reactions with *AMV* antisera showed that the two isolates isolated from potato were of the *Alfamoviruses*, previously reported to be pathogenic on several crop species (Paliwal, 1982; Valkonen *et al*., 1992). The close relationship between the two isolates was confirmed based on biological assays, serological reactions, and molecular techniques including RT-PCR, molocular hybridization and sequencing.

The size of the major RT-PCR product from *AMV*-infected tissue was identical to that of the 700 bp CP gene of *AMV*; however, the specific primer pair did not amplify viral cDNA from extracts of uninfected potato plants. The primers for detection of *AMV* (*AMV* coat-F and *AMV* coat-R were specific for *AMV* RNA (Xu and Nie, 2006). PCR and RT-PCR assays (Saiki *et al*., 1988) which involve the enzymatic amplification of a DNA fragment defined by two oligonucleotide primers have been used to diagnose a number of plant viruses belonging to several different groups (Henson and French, 1993). These method has been successfully utilized to detect viruses from infected potato tubers and leaves (Lopez-Moya and Loez-Abella, 1992; Singh and Singh, 1995).

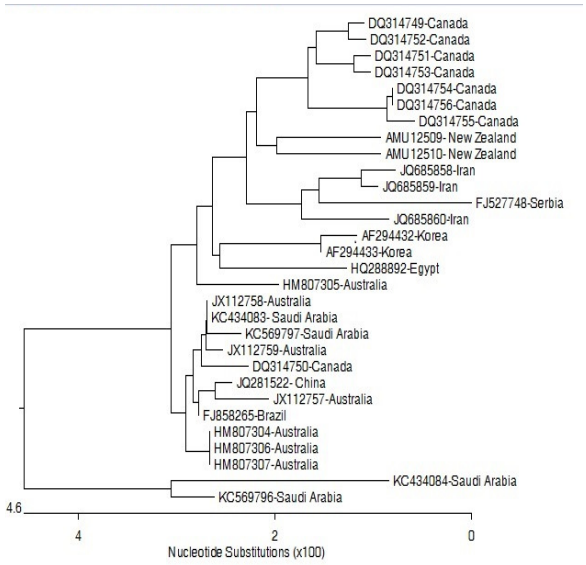

Fig. 5: A phylogenetic relationships of the CP gene of the two AMV Saudi Arabian isolates isolated from potato and twenty-eight AMV isolates obtained from GenBank (Table 1) using Lasergene DNASTAR, V5-05

Table 1: Nucleotide identity percentage of the CP gene sequences of the two AMV Saudi Arabian isolates isolated from potato with those of AMV isolates originating in different countries

AMV GenBank Accession Number	Country	Isolate/ Strain	Organic Host	Percentage of Identity AMV-PSA-Ha	AMV-PSA-Wd
AF294432	Korea	KR1	*S. tuberosum*	96.8%	94.4%
AF294433	Korea	KR2	*S. tuberosum*	97.1%	94.7%
AMU12509	New Zealand	NZ1	*M. sativa*	96.1%	93.2%
AMU12510	New Zealand	NZ2	M. sativa	96.1%	93.2%
DQ314749	Canada	Ca375	*S. tuberosum*	96.1%	93.7%
DQ314750	Canada	Ca175	*S. tuberosum*	96.3%	95.1%
DQ314751	Canada	Ca399	*S. tuberosum*	96.1%	93.5%
DQ314752	Canada	Ca400	*S. tuberosum*	96.1%	93.7%
DQ314753	Canada	Ca401	*S. tuberosum*	96.1%	93.5%
DQ314754	Canada	Ca508	*S. tuberosum*	95.8%	93.2%
DQ314755	Canada	Ca518	*S. tuberosum*	95.4%	93.0%
DQ314756	Canada	Ca616	*S. tuberosum*	95.8%	93.2%
FJ527748	Serbia	95-08	*M. sativa*	96.1%	92.9%
FJ858265	Brazil	-	*M. sativa*	98.6%	95.4%
HM807304	Australia	N20	*M. sativa*	98.3%	95.4%
HM807305	Australia	S30	*M. sativa*	96.8%	94.0%
HM807306	Australia	S40	*M. sativa*	98.3%	95.4%
HM807307	Australia	W1	*M. sativa*	98.3%	95.4%
JX112757	Australia	EW	*M. sativa*	98.0%	94.7%
JX112758	Australia	Aq	*M. sativa*	98.8%	95.6%
JX112759	Australia	HU	*M. sativa*	98.6%	95.4%
JQ685858	Iran	Ke.Sh.Al	*M. sativa*	96.3%	94.2%
JQ685859	Iran	Ke.Si.Al	*M. sativa*	96.4%	94.4%
JQ685860	Iran	Ke.Ba.Po	*S. tuberosum*	96.4%	94.1%
HQ288892	Egypt	-	*S. tuberosum*	96.1%	93.5%
JQ281522	China	-	*M. sativa*	98.1%	95.4%
KC434083	Saudi Arabia	Saj	*M. sativa*	99.7%	96.4%
KC434084	Saudi Arabia	Wd	*M. sativa*	94.0%	97.4%
KC569796	Saudi Arabia	PSA-Wd	*S. tuberosum*	96.5%	100%
KC569797	Saudi Arabia	PSA-Ha	*S. tuberosum*	100%	96.5%

Dot blot hybridization show that the DIG-labeled cDNA probe is sensitive enough to allow for detection of *AMV* in infected potato plants. Moreover, the hybridization was more sensitive than ELISA as more samples tested positive for *AMV* infection. For routine testing of large numbers of samples, the dot blot hybridization assays are more convenient. Dot blot hybridization assays have been used widely to detect *AMV* and other viruses and viroids in infected plants (Peiró *et al*., 2012).

Sequencing analysis of both Saudi Arabian *AMV* isolates showed slight differences in the nucleotide sequences of their CP genes. Sequence comparison revealed that the two Saudi Arabian isolates of *AMV* isolated from potato plants shared 93.2% to 99.7% sequence similarity with the twenty-eight reported isolates of *AMV* obtained from GenBank. The occurrence of *AMV* isolates that have nucleotide sequences similar to the Saudi isolates isolated from potato plants may suggest the common origin of distantly distributed isolates, which can be attributed to the widespread tuber and mechanical transmission of the two virus isolate.

This study confirmed that the *AMV* is still threatening the potato crop production in Saudi Arabia. DNA analysis showed that there was high genetic similarity of the CP gene of the collected isolates indicating that the virus may spread through potato tubers among the two geographic distant potato-growing regions in the Kingdom. Also, as long as the virus was isolated from alfalfa, further studies are needed to investigate the presence of this virus in weeds and other crops, in order to study the epidemic of the virus in the Kingdom.

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