Detection of Alfalfa Mosaic Virus in Five Alfalfa Cultivar Seeds in Riyadh K.S.A Using RAPD-PCR Technique

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Abstract: Random Amplification of Polymorphic DNA (RAPD-PCR) technique, was used for detection of alfalfa seed born mosiac virus (ASBMV) in seed homogenates of five common Saudi varieties of alfalfa *Medicago sativa*, two resistant varieties (Kaf 101 and Iraqui), two very sensitive varieties (Serveer and Qasimy) and local variety (Aquarious). Eleven virus specific primers used to amplify DNA fragments. Specific bands of PCR products observed at the position were corresponding to the expected size of DNA amplification product. The results indicated that primers could permit the specific detection of ASBMV and RAPD-PCR technique are efficient to distinguish between alfalfa varieties seeds in their infection by alfalfa mosaic virus. The technique is useful for screening of seed lots before cultivation and for palnt quarantine for the presence of seed-borne viruses.
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1. Introduction

Alfalfa mosaic virus (AMV) is the type member of the genus *Alfamovirus* in the *Bromoviridae* family of plant viruses. AMV has 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 (Jaspars and Bos, 1980). AMV can cause various mosaics, mottles, and malformations in alfalfa (*Medicago sativa*), yellowing of leaves in pea (*Pisum sativum*), calico and tuber necrosis in potato (*Solanum tuberosum*), and various symptoms in tobacco (*Nicotiana tabacum*) (Howard *et al*., 1994). Sixteen species of aphids, including *Myzus persicae,* can transmit AMV in a nonpersistent manner (Jeffries, 1998). AMV also can be transmitted through potato pollen and true seed (Valkonen, *et al*., 1992). Based on the differential reaction on one or two indicator species, AMV isolates have been classified into many strains or variants (Jaspars and Bos, 1980).

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 (Bol,1999). Viral movement protein (MP) is translated directly from RNA 3 (Erny *et al*., 1992). The viral coat protein (CP) gene located downstream of the MP gene in RNA 3 is translated via a subgenomic RNA, RNA 4 (Tenllado and Bol, 2000). All RNAs have a cap structure at the 5′ end and a t-RNA-like structure at the 3′ end (de Graaff *et al*., 1995). Nucleotide sequences of some strains have been determined and the genome structures and functions of most of the genes identified have been revealed (*Van Rossum et al., 1997; Parrella et al*., 2000)

Enzyme-linked immunosorbent assay (ELISA), nucleic acid hybridization, and bioassay using *Phaseolus vulgaris* and *Chenopodium amaranticolor* or *C. quinoa* have been evaluated (Ahoonmanesh *et al*., 1990, Hajimorad and Francki, 1988) for the diagnosis of AMV. Transmission electron microscopy (TEM) is also a useful tool for the identification of an *Alfamovirus* based on the shape of viral particles. Reverse-transcription polymerase chain reaction (RT-PCR), developed for screening potato samples for the presence of several viruses, has many advantages over ELISA and bioassay. Sequences of many AMV strains have been determined and are available in public databases (Xu, and De Boer, 2004, Xu et *al*., 2005). The recent outbreaks of AMV in Saudi Arabia renewed the interest for this virus as a threat to vegetables production. Little is known on AMV strains from Saudi Arabia and how they correlate with those already described elsewhere and characterized in more detail. Information on the variability of this virus can supply basic knowledge for studying the evolution of resident population and can assist in determining useful criteria for selecting alfalfa cultivars for AMV resistance.

The purpose of this reseach is to use Random Amplification of Polymorphic DNA(RAPD-PCR) to distinguish between seeds of susceptible and resistant alfalfa cultivars in their infection by alfalfa mosaic virus.

2. Materials and methods

2.1. Alfalfa varieties

Seeds of five common Saudi varieties of alfalfa *Medicago sativa* were used in this study. Two resistant varieties (Kaf 101 and Iraqui), two very sensitive varieties (Serveer and Qasimy) and local variety (Aquarious).

2.2.Isolation of Genomic DNA from plant tissue

Seeds of alfalfa were grinding into fine powder using morter and pestle. 40 mg of the powder was transfered to 1.5 ml microcentrifuge tube. 600 ul of Nuclei Lysis solution was added to each tube and vortex for 1-3 seconds to wet the sample then incubated at 65 ^{o}C for 15 min. 3 ul of RNase solution was added to the cell lysate and the sample mixed by inverting the tube 2-5 times. The resultant mixtures were incubated at 37^{o}C for 15 minutes then allow cooling for 5 min. at room temperature. 200 ul of protein precipitation solution was added and vortex vigorously at high speed for 20 seconds then centrifuged for 3 mintues at 13,000-16,000 xg. The resultant supernatant containing DNA was carefully transfared to a clean 1.5 ml microcentrifuge tube containing 600 ul of isopropanol, the solution was then mixed gently by inversion until thread-like strands of DNA form a visible mass. Resultant solution was centrifuged at 13,000-16,000 xg for 1 minute at room temperature. Supernatant was decanted and the resulting pellets were resuspended in 600 ul of 70% ethanol and the tube inverted gently several times to wash the DNA, then aspirate the ethanol carfully using sequencing pipette tip. The tubes were inverted onto clean absorbent papers and air-dried the pellet for 15 minutes. DNA solution then rehydrated at 65^{o}C for 1 hour and DNA stored at 2-8 ^{o}C untill further use.

2.3. Random Amplification of Polymorphic DNA (RAPD-PCR) amplification

Virus detection was carried out at Agricultural Genetic Engeinnering Research Institut (**AGERI**) by RAPD-PCR according to the procedure given (Alibrahem, 1990) with minor modifications. The amplification reaction was carried out in 25 ul reaction volume containing 1 X PCR buffer, 1.5 mM Mg Cl_2, 0.2 mM d NTPs, 1 uM primer (Table 1), 1U tag DNA polmerase and 25 ng template DNA.

2.4. Theromocyling Profile and detection of the PCR products

PCR amplification was performed in a Perkin-Elmer/ Gene Amp^{R} PCR system 9700 (PE applied biosystems) programmed to fulfill 40 cycles after an initial denaturantion cycle for 5 min at 94 ^{o}C. Each cycle consisted of a denaturation step at 94 ^{o}C for 1 min, an annealing step at 36 ^{o}C for 1 min, and an elongation step at 72 ^{o}C for 1.5 min. The primer extension segment was extended to 7 min at 72 ^{o}C in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 ug/ml) in 1 X TBE buffer at 95 volts. PCR products were visualized on UV light and photographed using a Polaroid camera. Amplified products were visually examined and the presence or absence of each size class was scored as 1 or 0, respectively.

Table (1); Sequence of primers assayed in RAPD-PCR.

Primer*	Sequence (5'-3')	Primer	Sequence (5'-3')
OPA-03	AGTCAGCCAC	OPB-02	TGATCCCTGC
OPA-10	GTGATCGCAG	OPB-03	CATCCCCCTG
OPA-09	GGGTAACGCC	OPB-04	GGACTGGAGT
OPP-06	GTGGGCTGAC	OPB-06	TGCTCTGCCC
OPC-12	TGTCATCCCC	OPB-17	AGGGAACGAG
		OPB-20	GGACCCTTAC

Primer designed polymerase chain reaction based on coat protein gene sequence of alfalfa mosaic virus.

3.Results .

The Random Amplification of Polymorphic DNA- PCR (RAPD-PCR) method was used to investigate the dynamic of alfalfa seed-borne mosaic virus (ASBMV) in seed homogenate of five common Saudi varieties of alfalfa *Medicago sativa* two are resistant varieties (Kaf 101 and Iraqui), two very sensitive varieties (Serveer and Qasimy) and a local variety(Aquarious). Eleven virus specific primers were used to amplify DNA fragments. Specific bands of PCR products were observed at the position corresponding to the expected size of DNA amplification product (Fig.1)

Amplification of DNA using different primers.

1) Primer OPA-10.

A band observed at 560bp by using the virus specific primer OPA-10 in Iraqui resistant variety which is absent in the samples of other tested varieties. Comparing the two resistant varieties, two bands were observed at 1500 and 560 bp in Iraqui while it is missing in Kaf 101 variety. Comparing the two susceptible varieties, five bands were observe in both. Moreover, a band was observed at 1500bp in Serveer variety and not exist in Qasimy samples. In the meantime two bands were observed in local variety samples (Aquarious) at 1070 and 870 bp while they are absent in the samples of other varieties. Those bands existed in the samples of alfalfa varieties are corresponding to the expected size of DNA amplification products for alfalfa mosaic virus.

2) Primer OPB-02.

Seven bands observed in the samples of the two resistant varieties. Comparing the two susceptibl, four bands observed in bothvarieties. Meanwhile two bands one at 900 bp observed in Serveer variety and was absent in Qasimy and the other band at 950 bp

observed only in Qasimy. Furthermore, two bands observed at 2000 and 460 bp in all inspected varieties except for the local variety.

3) Primer OPB-03.

For resistant varieties seven bands were observed in both. Suceptible varieties varied in their bands, whereas four bands observed in Qasimy and were absent in Serveer variety (at 950, 870, 190 and 180 bp). Moreove, a band at 600bp observed in Serveer and not present in Qasimy. Comparing the five varieties a band at 870 bp observed in all varieties except Serveer and another band at 600 bp in all varieties except Qasimy. Furthermore, a band was observed at 190 bp in the susceptible variety, Qasimy, while it was absent in the other four varieties.

4) Primer OPB-17.

Three bands observed at 650, 450 and 230bp in the resistant variety Kaf 101 and the other varieties except Iraqui variety. Comparing susceptible varieties, two bands were observed at 900 and 600 bp in Serveer and were absent in Qasimy.

5) Primer OPB-20

For resistant varieties four bands were observed in Kaf 101 at 600, 400, 300 and 280 bp and were absent in the Iraqui. Comparing the five varieties, three bands observed at 400, 300 and 280 bp in all varieties except Iraqui. Susceptible varieties shared five bands except a band at 600 bp observed in Serveer and absent in Qasimy. Furthermore, all the bands were observed in the local variety while some of the bands were absent in resistant and susceptible variety.

6) Primer OPA-03

Comparing resistant varieties eleven bands were observed in both and varied in one band at 740 bp observed in Iraqui variety and was absent in Kaf 101 or in Suceptible and local. Meanwhile, ten bands observed in both susceptible and local varieties.

7) Primer OPA-09

Five bands observed in resistant or susceptible varieties. The susceptible varieties varied from resistant ones in the presence of one band at 1700 bp and absent in resistant ones. Meanwhile a band at 1250 bp was obseved in resistant varieties but not in susceptible ones. Furthermore, five bands observed in both susceptible and local varieties.

8) Primer OPB-04

For resistant and susceptible varieties, seven bands observed and a band at 980 bp observed only in samples of susceptible varieties. Moreover, another band at 260 bp was present only in resistant varieties. Furthermore, a band observed in the local variety at 300 bp and was not present in the other varieties.

9) Primer OPB-06

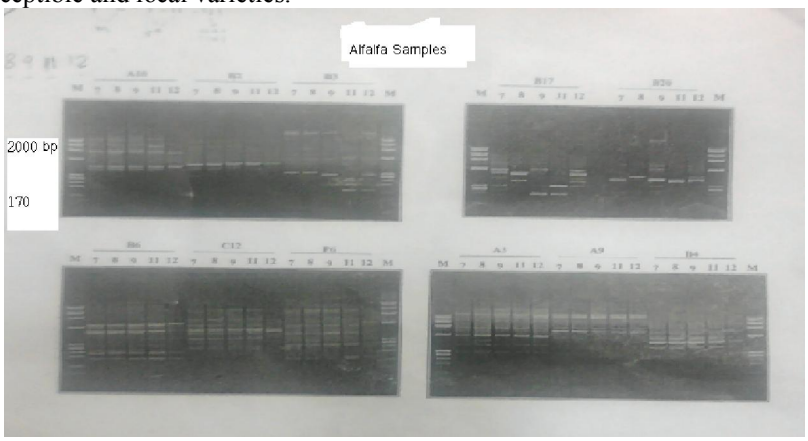
A band observed in the local variety at 660 bp and was not present in the other varieties. A band of resistant variety Kaf 101 observed at 310 bp and absent in the other varieties. Susceptible varieties are silimilar in their bands except a band at 310 bp observed in Kaf 101 only. Meanwhile, susceptible and resistant varieties, varied in presence of three bands at 270, 500 and 650 bp compared with the local one.

10) Primer OPC-12

All varieties are analogous in the presence of bands except a band at 560 bp observes only in the susceptible variety Serveer.

11) Primer OPP-06.

For resistant varieties all observed bands were in Kaf 101 and Iraqui except two band at 680 and 280 bp observed only in Iraqui. Meanwhile two bands observed in local variety at 610 and 270 bp and absent in both resistant and susceptible varieties. Further, three bands at 1070, 870 and 600 bp were absent in local variety compared with resistant and susceptible varieties.



Figure(1):DNA products amplified in PCR from seeds of different varieties of alfalfa susceptible and resistance to mosiac virus.

*Primers (A10, B2, B3, B17, B20, B6, C12, P6, A3, A9 and B4), M (markers)

* Alfalfa cultivars, Resistant No.(7) Kaf 101 and (8) Iraqui; Susceptibe (9, Serveer and 11, Qasimy) Local (12, Aquarious).

The percent of similiarity between the five varieties of alfalfa reached 89.4% between the resistant varieties, Kaf 101 and Iraqui. While it was 88.9% between the susceptible varieties, Serveer and Qasimy. For the local variety, Aquarious the similarity reduced to 77.7% and 69.6% compared with resistant varieties, Kaf 101 and Iraqui. While it was 78.4 and 81.1 % compared with susceptible varieties, Serveer and Qasimy, respectively. (Table 2).

Table (2) Similarity matrix

Variety	Kaf 101	Iraqui	Serveer	Qasimy	Aqarious
Kaf 101	1.00	-			
Iraqui	0.89	1.00			
Serveer	0.90	0.83	1.00		
Qasimy	0.83	0.79	0.89	1.00	
Aquarious	0.78	0.70	0.78	0.81	1.00

4. Discussion

RAPD-PCR have potential application for detecting AMV in bulk samples in which the incidence of virus infection might be low. There always has been a need for rapid procedures to detect virus infection in seeds. This technique appears to be useful. Analysis of amplified sequences offers a simple and accurate means of grouping AMV isolates and could prove useful for the analysis of AMV dynamics in natural populations. From the previous results it is obvious that primers are extremly important for RAPD-PCR detection of AMW virus. The results indicated that primers could permit the specific detection of AMW. This agreed with results that the RT-PCR with degenrate primers is an efficient method for virus detection (Routh *et al*. 1998). Menzel *et al*., 2003, reported that multiplex RT-PCR-ELISA technique could be a reliable and suitable tool for indexing apple plants for ACLSV, ASGV, ASPV and ApMV and may replace presently used less advantageous techniques. Also, Niimi *et al*., 2003, showed that RT-PCR is more sensitive method than ELISA to determine the presence of viruses in Lilium plants of various species. (Xu and Nie, 2006), reported that, RT-PCR followed by restriction fragment length polymorphism analysis may be a useful approach for screening potato samples on a large scale for the presence of AMV. The results show that this molecular technique is more reliable for the detection of the above mentioned alfalfa viruse in seeds of alfalfa, thereby helping to reduce cost and time during the certification of plant material. RAPD-PCR might be widely used to detect this virus in other plant species, since the degenrated primers used designed according to various strains of virus in different palnts. OPB-04 and OPA-03 are good for detecting and distinguish the presence of AMV in resistant and susceptible varieties of different plants.

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