Detection and molecular characterization of the *Iris severe mosaic virus*-Ir isolate from Iran

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Abstract: Iris belongs to the Iridaceae family. It is one of the most important pharmaceutical and ornamental plants in the world. To assess the potyvirus incidence in natural resources of iris plants in Iran, Antigen Coated-Plate ELISA (ACP-ELISA) was performed on 490 symptomatic rhizomatous iris leaf samples, which detected the potyvirus in 36.7% of the samples. Genomic 3' end of one mechanically non-transmitted potyvirus isolate, comprising a 3' untranslated region (390 bp) and C-terminus of the coat protein (CP) gene (459 bp), was amplified by reverse transcription polymerase chain reaction (RT-PCR), which was ligated into pTG19-T vector. The nucleotide sequence of amplicons was compared with related sequences, using Blastn software available at NCBI GenBank, and showed the highest similarity with *Iris severe mosaic virus* (ISMV) isolates. The nucleotide and deduced amino acid sequence of the CP C-terminus region was more than 83% identical with other ISMV isolates, therefore this isolate was designated as ISMV-Ir. This new ISMV isolate is closely related to the Chinese ISMV-PHz in phylogenetic analysis, based on the partial nucleotide and deduced amino acid sequence of the CP region. This is the first report of ISMV occurrence on *Iris* spp. in Iran.

Key words: *Iris* spp., ISMV-Ir, partial coat protein, phylogeny, 3'-UTR

Introduction

Irises have been cultivated and used for centuries, not only as ornamentals but also as a source of perfume, medicine, and for decoration (Köhlein 1987). The rhizomatous iris plants belong to one of the important groups of irises. The rhizomatous Iris plants are used in the medical field as a pharmaceutical plant, in the garden as ornamentals, and produced commercially as cut flowers in the greenhouse (De Munk and Schipper 1993). Some potyviruses, such as *Iris mild mosaic virus* (IMMV), *Iris severe mosaic virus* (ISMV), *Bean yellow mosaic virus* (BYMV), *Turnip mosaic virus* (TuMV), and *Ornithogalum mosaic virus* (OrMV), have been previously detected on infected Iris plants around the world (Inouye and Mitsuhata 1978; Asjes 1979; Brunt and Phillips 1980; Derks *et al*. 1980; Hammond *et al*. 1985). All the above-mentioned viruses are transmitted by aphids in a non-persistent manner, and also transmitted by mechanical sap inoculation onto diagnostic host plants (Harrison and Robinson 1988; Atreya *et al*. 1991; Van der Vlugt 1994). *Tobacco ring spot nepovirus* (TRSV), *Tobacco mosaic tobamovirus* (TMV), and *Tobacco rattle tobravirus* (TRV) are other viruses naturally infecting *Iris* spp. (Asjes 1979).

ISMV is a member of the genus *Potyvirus,* family Potyviridae. This virus has filamentous particles (750 × 12 nm), transmitted especially by *Macrosiphum euphorbiae* and *Myzus persicae* (Brierley and McWhorter 1936; Asjes 1979). A severe mosaic symptom was described by Brierley and McWhorter (1936) as occuring in the bulbous and rhizomatous Iris, and in *Crocus vernus* by Brunt *et al*. (1988). The virus was also partially characterised by several researchers (Brunt and Phillips 1980; Alper *et al*. 1984; Derks 1985; Derks and Hollinger 1986). ISMV produces inclusions such as cylindrical inclusions. Morphological analysis of these inclusions indicated that they belonged to type II according to the classification of Edwardson, with a pinwheel-like configuration in the cross section and parallel lines in the longitudinal section (Edwardson *et al*. 1984; Yan *et al*. 2010). ISMV causes conspicuous chlorotic stripes and/or mosaic patterns in the leaves, and breaking in the flowers of sensitive cultivars of bulbous and rhizomatous *Iris* spp. and *C. vernus* (Van der Vlugt 1994), resulting in considerable damage to iris plants. Three strains of the virus have been recognised by the severity of the symptoms they induce on *Iris domestica* [*Belamcanda chinensis* (L.) DC] (Brunt *et al*. 1988). This virus can infect in combination with other viruses such as IMMV or *Cucumber mosaic virus* (CMV). When infection occurs in combination with other viruses, the symptoms are mild or severe mosaic on the leaves, consisting of pale-green and yellowish-green stripes and wider bands in irregular patterns extending upward from below the soil level (Asjes 1979; Van der Vlugt 1994).

Exportation of ornamental bulbous and rhizomatous plants such as iris, narcissus, and lilium adds a significant contribution to the economy of many countries including

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the Netherlands, Israel, and Germany. It should also be noted, that Iran is one of the most important countries famous as "the land of tulip and iris flowers" and has a great potential for cultivation of various wild ornamental and cutting species. Recently, the total cultivation area of the cut flower plants has been reduced, and may be affected by diseases such as viral ones. The objective of this survey was to partially characterise the potyvirus infecting iris plants in Iran. This potyvirus is named the Iris potyvirus isolate.

Materials and Methods

Sampling and plant materials

During the 2012–2013 time period, a total of 490 individual rhizomatous iris (*Iris* spp.) plants with symptoms including mosaic, yellowing or stunting, were collected from greenhouses and natural sites located in the Tehran, Isfahan, Alborz, and Markazi provinces of Iran (Table 1).

ACP-ELISA

Collected symptomatic samples were investigated for the presence of *Potyvirus* genus by Antigen Coated-Plate ELISA (ACP-ELISA) (Joisson *et al*. 1992) using DSMZ--AS-0573/1 broad-spectrum monoclonal antibody against potyviruses (1 : 1,000) and RAM-AP conjugate (1 : 500). To detect positive reactions, p-nitrophenyl phosphate substrate (1 mg · ml^{-1}) was used. The absorbance of each well was measured at 405 nm (OD_{405}) by ELISA-reader (Beckman AD340). Samples were considered positive only if the absorbance value was more than three times that of the negative control. Such samples were subsequently used in biological and molecular assays.

Mechanical inoculation and laboratory host range studies

Crude sap (1 g of fresh leaves ground in 10 ml of 0.01 M sodium phosphate buffer, pH 7, containing active charcoal) from symptomatic iris plant leaf extract was mechanically inoculated on test plants species including *Gomphrena globosa* L., *Chenopodium amaranticolor* L., *C. quinoa* L., *C. album* L., *Cucurbita pepo* L., *Cucumis sativus* L., *Capsicum frutescens* L., *Phaseolus vulgaris* L. cv. Red Kidney, *P. vulgaris* cv. Bountiful, *P. vulgaris, Vigna unguiculata* (L.) Walp., *Nicotiana tabacum* L. cv. White Burley, *N. tabacum* cv. Sumsun, *N. glutinosa* L., *N. clevelandii* L., *N. debneyi, N. benthamiana* (A. Gray), *N. rustica* L., *Datura stramonium* L., *D. metel* (A. Gray), *Vicia faba* L., and *Lactuca sativa* L. The plants were pre-dusted with carborundum (600 mesh) and then kept in a glasshouse for symptoms development. All inoculated plants were assayed by ACP-ELISA, 20 days post-inoculation (dpi), and assayed by reverse transcription polymerase chain reaction (RT-PCR) as described below.

RT-PCR

An extraction of the total RNA was taken from a potyvirus-infected Iris plant using RNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacture's protocol. The extract was then used in an RT-PCR protocol to amplify 3′ end of iris infecting potyvirus genome using a combination of *Potyvirus* genus specific forward (Pot) and Potyviridae family specific reverse (M4T) primers (Table 2). Effective cDNA synthesis was performed in 20 µl of reaction mixture containing 5 µl RNA, 4 µl PCR buffer 5×, 10 mM dNTPs, 100 mM DTT, 40 U RNase inhibitor enzymes (Fermentase) and 100 pM M4T reverse primer, and sterile distilled water. The samples were incubated at 72°C for 3 min, immediately placed on ice, and 200 U MMLV reverse transcriptase (Fermentase) was added to each reaction. The reaction was incubated at 42°C for an hour in a thermocycler (Eppendorf, Autorisierter, Germany), then reverse transcription was inactived by heating at 72°C. Five µl of the RT reactions were used for PCR containing 5 µl cDNA, 14 µl H_2O, 2.5 µl PCR buffer 10×, 10 mM dNTPs, 50 mM $MgCl_2$, 5 U SmarTaq polymerase, 100 pM of each M4T reverse and Pot forward primers.

The cycling parameters were the initial denaturation at 95°C for 3 min, followed by 35 cycles at 94°C for 1 min, 53°C for 90 s and 72°C for 90 s, and a final extension step at 72°C for 10 min. An analysis of the PCR products was done by electrophoresis on 1% agarose gel with 0.5× TBE. Finally, the gel was stained with 1 µg · ml^{-1} of ethidium bromide solution for 20 min and photographed with gel documentation (White/Ultraviolet transiluminotor-UVP-UK) (Feinberg and Vogelstein 1983).

Table 1. Location and the number of symptomatic and potyvirus infected *Iris* spp. samples based on ACP-ELISA

Geographical location	Symptomatic samples No.	Potyvirus infected No.	Potyvirus infection rate [%]
Alborz	60	26	43.3
Tehran	120	45	37.5
Isfahan	250	82	32.8
Markazi	60	27	45.0
Total	490	180	36.7

Table 2. Characteristics of primer pair used in this study to amplify 3′ genomic end of iris infecting potyvirus

Primer	Sequence	Tm [°C]	Reference
M4T (reverse)	5′-GTT TTC CCA GTC ACG ACT(15)-3′	64.0	Hasiów-Jaroszewska *et al*. 2013
Pot (forward)	5′-TGA GGA TCC TGG TG(CT) AT(ACT) GA(AG) AA(CT) GG-3′	64.5	Langeveld *et al*. 1991

Cloning, sequencing and phylogenetic analysis

Following electrophoresis of the PCR product on agarose gel, the amplified fragment of the viral genome in the predicted size was excised from agarose gel, cleaned with a QIAquick gel extraction kit (QIAGEN Inc.), and ligated into a T-cloning vector (pTG19-T Promega, Madison, Wisconsin, USA) using T4 DNA ligase (Fermentase). Ligated pTG19-T plasmids were transformed into *Escherichia coli* strain DH5α by using heat shock. Recombinant clones were selected on Luria broth (LB) agar containing 100 μg · · ml^{-1} ampicillin. Recombinant plasmids were extracted by standard alkaline lysis protocol (Sambrook *et al*. 1989). Insertion of amplified DNA fragment in the expected size was verified by digestion reaction using *Bam*HI. Recombinant plasmids were subjected to nucleotide sequencing in both directions using M13 and T7 universal primer pair, which was done by Bioneer Inc. (South Korea). The contigs were assembled based on forward and reverse nucleotide sequence of amplicon using DNA STAR Lasergene (SEQMAN and EDITSEQ, version 10). The obtained nucleotide sequences were compared with related sequences based on the Blastn analysis software available at NCBI GenBank. Nucleotide sequence of Ir isolate genomic 3′ end was deposited in the NCBI GenBank database with the accession number (KJ878662). Nucleotide and deduced amino acid sequence multiple alignments of amplified fragment against sequence data of related potyviruses (Table 3) were performed by MEGA 6 (Tamura *et al*. 2013). Phylogenetic analysis was carried out using the maximum likelihood method and bootstrap analysis of 1,000 replications. *Barley yellow mosaic virus* (*Bymovirus*, Potyviridae) was used as an outgroup in this analysis.

Results and Discussion

Serological assays and laboratory host range of the virus

Antigen-coated plate enzyme-linked immunosorbent assay results showed that 32.8–45% of symptomatic rhizomatous iris plants collected from four different provinces of Iran, were infected with a potyvirus, maximally in the Markazi and minimally in the Isfahan provinces (Table 1). Infected plants mainly showed mosaic, stunting and conspicuous chlorotic stripes on leaves with different severity, compared to healthy plants (Fig. 1). Following mechanical inoculation of indicator plants with potyvirus infected symptomatic Iris leaf extract, no symptoms developed on the plants. The virus-free status of inoculated test plants was confirmed by ACP-ELISA at 20 dpi.

RT-PCR, sequencing and phylogenetic analysis

Reverse transcription-polymerase chain reaction (RT--PCR) assays were performed using the Pot/M4T primer pair and resulted in amplification of a fragment with 917 bp in length, with no amplification in healthy iris plants. Nucleotide sequence of Iris potyvirus isolate amplified fragment was determined as 917 bp, comprising the PolyA signal, 3′ untranslated region (3′-UTR, 390 bp) and the partial coat protein (CP) gene (459 bp). The nucleotide sequence comparison of the amplicon showed the highest homology with ISMV isolates, as 84–86%, using NCBI Blastn tool. Based on multiple alignments of partial nucleotide and amino acid sequence data of this potyvirus isolate coat protein with available sequence data of related potyviruses by DNAMAN version software package7 (Lynnon, Biosoft, Quebec, Canada), the highest identity was seen as 86.6 and 87.1%, with Chinese ISMV-PHz isolate, respectively, therefore, the designation was ISMV-Ir (Table 4). The maximum likelihood phylogenetic trees, based on nucleotide and deduced amino acid sequence alignment of carboxyl terminus of CP, were similar in topology and showed an ISMV separated clade including the Ir isolate (Fig. 2).

Despite the occurrence and vegetative transmission importance of viral infections in iris plants, there has been little research conducted on ISMV throughout the world. This means that there should be further investigations done on ISMV, and determination of complete genome nucleotide sequence of more isolates should follow. Irises are perennial plants growing from bulbs or creeping

Table 3. Properties of isolates used in phylogenetic analysis in this study

Virus isolate	Origin	Host	Accession number
ISMV-Ir	Iran	*Iris* spp.	KJ878662
ISMV-Indian	India	*Iris* x *hollandica*	AJ549755
ISMV-Netherland	Netherlands	*Crocus vernus* (L.) Hill	X75939
ISMV-Korean (ISMV-K)	Korea	*Iris* spp.	AF034839
ISMV-Chinese (ISMV-PHz)	China	*Iris ensata* Thunb.	FJ481099
Onion yellow dwarf virus (OYDV)	Japan	*Allium wakegi* Araki	AB000472
Onion yellow dwarf virus (OYDV)	India (Dehli)	*A. sativum* L.	DQ519034
Ornithogalum mosaic virus (OrMV)	Japan (Akita)	*Ornithogalum dubium* Houtt.	AB091836
Ornithogalum mosaic virus (OrMV)	Japan	*O. thyrsoides* Jacq.	AB079649
Iris mild mosaic virus (IMMV)	New Zealand	*Iris* x *hollandica* cv. Wedgewood	DQ436918
Iris mild mosaic virus (IMMV)	New Zealand	*Iris* x *hollandica* cv. Wedgewood	DQ436919
Barley yellow mosaic virus (BaYMV)	China	*Hordeum vulgare* L.	NC-002990

ISMV – *Iris severe mosaic virus*

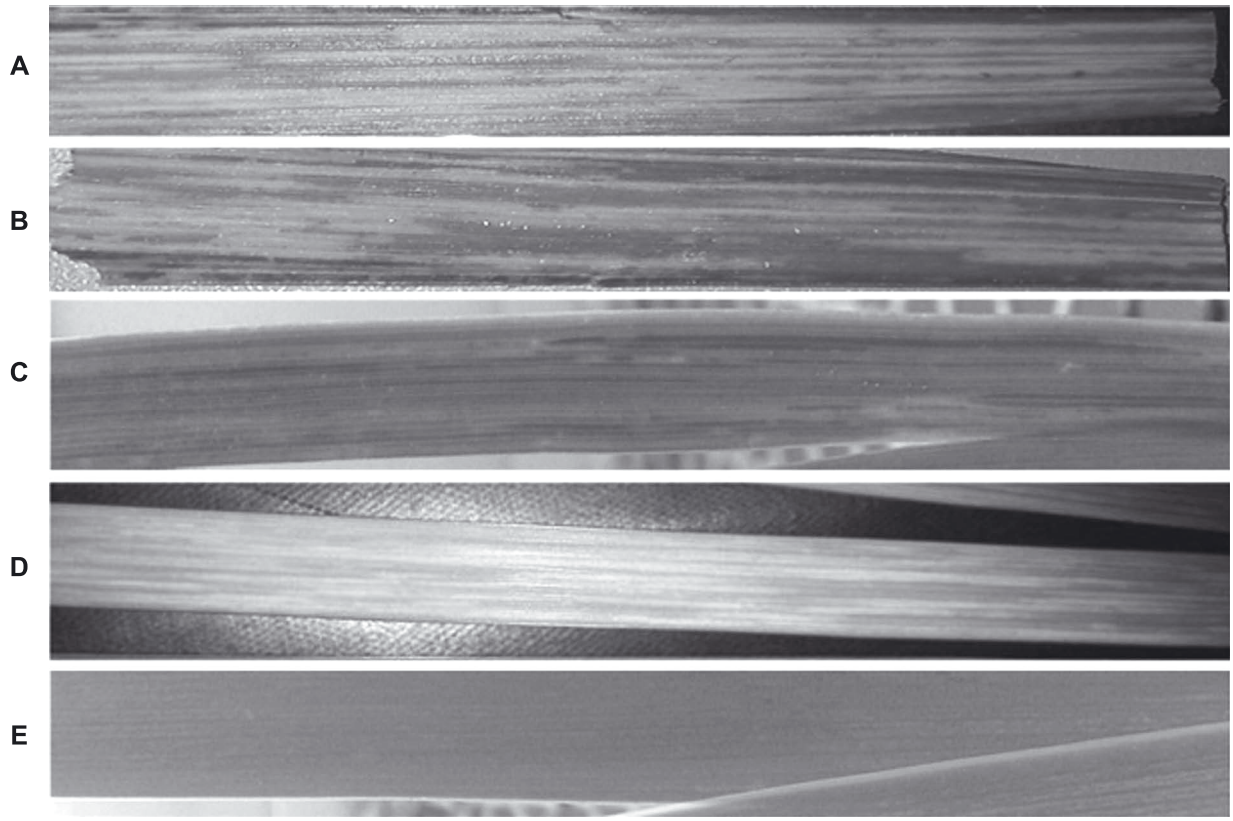

Fig. 1. Disease symptoms caused by ISMV on *Iris* spp.: A–D – mosaic and chlorotic stripe symptoms of different severity; E – healthy iris plant

Table 4. Comparison between sequences (% identity) of 3′ end ISMV-Ir genome and the most closely related potyviruses by observed divergence method using DNAMAN version7

*Virus	Accession No.	Partial CP [%]		3′-UTR [%]
		(nt)	(aa)	
ISMV-Indian	AJ549755	83.8	84.1	–
ISMV-Netherland	X75939	84.9	85.2	64.8
ISMV-K	AF034839	84.9	85.2	–
ISMV-PHz	FJ481099	86.6	87.1	64.2
OYDV	AB000472	66.6	65.7	37.7
OYDV	DQ519034	68.5	68.4	30.8
OrMV	AB091836	63.6	62.9	37.1
OrMV	AB079649	65.2	64.6	–
IMMV	DQ436918	59.5	59.3	42.1
IMMV	DQ436919	60.8	60.7	44.7
BaYMV	NC-002990	38.6	39.0	27.7

CP – coat protein; (nt) – nucleotide; (aa) – amino acid
*explanations – see table 3

rhizomes, so these vegetative propagation organs are an important and critical source of the virus, as described before by Van der Vlugt (1994). In this study, infection of the rhizomatous type of Iris plants with potyviruses was proved. In our surveys, 36.7% of plant samples showing mosaic symptoms were positively reacted with *Potyvirus* genus specific antibody in ACP-ELISA (Table 1). Pakdasht, in the Tehran province, is one of the major distributive centres of ornamentals, cut flowers, and vegetative propagation organs in Iran. Symptomatic iris plants collected from this location showed a high infection rate with potyvirus(es). Biological and molecular aspects of a potyvirus isolate, originating from an individual Pakdasht infected iris, was further studied. 849 bp fragment of genomic 3′ end of this isolate (excluding polyA tail), showed the highest identity (84–86%) with ISMV isolates sequences. While, 3′-UTR of the genome maximally showed only 64.8% identity with the Netherland isolate of ISMV; its CP carboxyl terminal was > 83% identical with other ISMV isolates. According to the genetic spe-

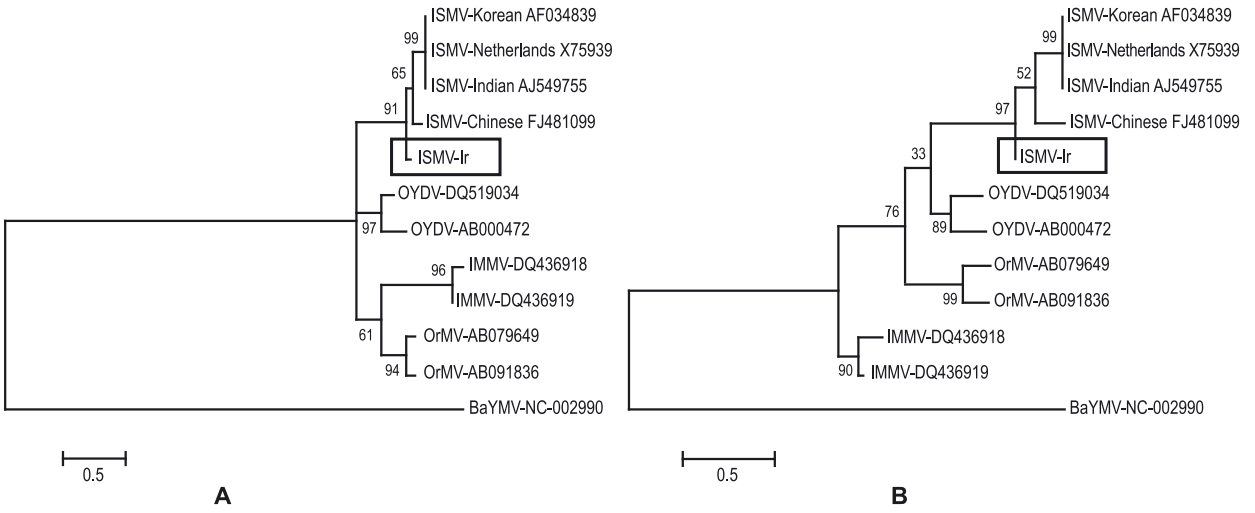

Fig. 2. Phylogenetic trees of different virus species which belong to Potyviridae based on partial coat protein sequence: A – 459 nucleotide; B – deduced amino acid sequence of the carboxyl terminus region of the coat protein alignments of ISMV-Ir and other related sequences using the maximum likelihood method and MEGA6 with 1,000 bootstrap replicates. Bootstrap values in percentages are shown at the branch point. BaYMV was defined as outgroup

cies demarcation criteria in the Potyviridae family, isolates with more than 76% and 82% identity in nucleotide and amino acid of the coat protein gene, belong to a single species (Adams *et al*. 2005). For this reason, the Iris potyvirus in this investigation was characterised as an Iranian isolate of ISMV and designated as ISMV-Ir. Phylogenetic analysis of partial CP nucleotide and deduced amino acid sequence showed that ISMV-Ir is closely related to the Chinese ISMV-PHz isolate, which demonstrates their probable common origin. To our knowledge, it is the first report of ISMV occurrence in Iran. We found that ISMV-Ir was not able to infect the number of mechanically inoculated test plant species, which was similar to the results of previous research carried out by Barnett *et al*. (1971), Brunt *et al*. (1980, 1988), Derks and Hollinger (1986), Van der Vlugt *et al.* (1994). Only a couple of tested plant species (*C. vernus* and *I. domestica)* are diagnostically susceptible to ISMV. For our study, these plant species were not available to be inoculated with ISMV-Ir, and other tested plant species did not show any symptoms following mechanical inoculation – even after a long period of time.

On the other hand, potyviruses are one of the most commonly encountered viruses in *Crocus* spp. (Miglino *et al*. 2005). The presence of potyvirus latent infections in *C. sativus* (Russo *et al.* 1979) and *C. cartwrightianus*, and probably progenitor species of saffron (Caiola and Canini 2010), demonstrate an ancient infection of *Crocus* spp. with potyviruses which resulted in the selection of mild virus strains (Caiola and Faoro 2011). Four tentative consensus (TC) sequences from symptomless saffron stigma cDNA library with potyviral sequence similarity were identified by D'Agostino *et al.* (2007). Transmission and distribution of ISMV is not only a serious threat on iris plants but also on the other natural host plants such as *C. vernus* and even *C. sativus*. Iran now accounts for approximately 90% of the world's production of saffron, which explains the fact that further studies need to be performed about the natural distribution of ISMV on other host plants and ISMV transmission by aphids, in Iran.

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