Iranian Journal of Virology 2014;8(1): 7-12

Original Article

Simultaneous Detection of *Arabis Mosaic Virus*, *Cherry Leafroll Virus* and *Cucumber Mosaic Virus* with Coamplification of Plant mRNA as Internal Control for Olive Certification Programs

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Abstract

Background and Aims: Certification programs of plant propagating materials rely on faster, cheaper and more importantly sensitive and reliable methods for detection of systemic pathogens as indicated in national and/or international health standards of plant propagating materials. Reverse transcription-polymerase chain reaction (RT-PCR) has been documented as an alternative assay for certification of plant propagating materials. RT-PCR has been shown to be necessary for olive certification due to the inefficiency of other methods in detecting viruses. The object of the present study was the optimization of a multiplex RT-PCR assay for simultaneous detection of *Arabis mosaic virus* (ArMV), *Cherry leafroll virus* (CLRV) and *Cucumber mosaic virus* (CMV) together with the plant mRNA as internal control for olive certification programs in Iran.

Materials and Methods: Total RNA was extracted from olive tissues infected by ArMV, CLRV and CMV as well as from healthy plants and subjected to cDNA synthesis by M-MuLV reverse transcriptase. Simplex, duplex and multiplex RT-PCR (s-, d-, and mRT-PCR) were optimized for amplification of target genes. Amplified fragments were further sequenced for evaluation of the accuracy of the assays.

Results: Genomic fragments of ArMV, CLRV, CMV and the plant internal control (*Nad 5* gene) were successfully amplified in all assays. The sequence information as well as application of the developed method on samples derived from different origins revealed the accuracy of all assays in olive certification schemes.

Conclusion: Results from the developed s-, d-, and mRT-PCR assays revealed RT-PCR as an excellent assay for olive certification. Moreover, coamplification of *Nad5* gene fragment suggested that was a robust marker for analyzing the accuracy of the developed RT-PCR as shown previously in other crops as well.

Keywords: Multiplex RT-PCR; *Nad*5; Virus detection; Olive

Introduction

Olive (*Olea europaea* L.) is an economically important crop with an estimated cultivated area of more than a hundred thousand hectare and its cultivation has made an important source of employment in both public and private sectors of Iran. Development and/or replacement of olive orchards demand saplings which normally produced by trained/untrained growers, that, in turn, necessitates meeting of certification schemes in regard to the Iranian national health

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standards for plant propagating materials (INHSPPM). On the basis of these standards, propagating materials of olive must be free from several pathogens including the viruses *Arabis mosaic virus* (ArMV), *Cherry leafroll virus* (CLRV) and *Cucumber mosaic virus* (CMV).

As members of Nepovirus (ArMV and CLRV) and Cucumovirus (CMV) genera, these viruses are transmited by nematode or vectors from Aphididae, but CLRV transmission by nematodes has not been reported in olive. Nepoviruses consist of two isometric particles containing RNA-1 and -2 and of Cucumoviruses three RNAs (1-3) are encapsidated in separate icosahedral particles.

Due to the nature of viruses, reliable, sensitive and cost effective methods are needed for their detection and characterization in certification of plant propagating materials. Several detection methods including biological indexing, serological assays and molecular characterization of a viral target gene as well as combination of different methods have been documented for virus detection in certification schemes. In addition to the advantages, any of these methods has considerable disadvantages for reliable certification. For instance, even though biological indexing is known as gold standard in detection of known and unknown viruses, the effect of viral concentration derived from resistant cultivars on disease appearance on indicator plants, environmental conditions on symptom development and diversity, time-consuming for some viruses for symptom appearance (ranging from months up to 2-3 years depending on virus-host interactions) and more importantly, the need for controlled conditions and expertise in diagnosis of symptoms that increases the cost of certification per sapling are some of disadvantages (4, 5, 12, 18). Indicator plants to assess virus infection in olive have not been reported (2).

Serological methods (e. g. different versions of Enzyme-linked immunosorbent assay) are routinely employed for virus detection in certification schemes. These methods allow specific, sensitive and simultaneous detection of viruses in single microplate, but complete relying on this method is still under questions for several reasons: First, when the target virus is in low concentration (i.e. derived from resistant cultivars) and the OD is equal to R [$\bar{x}$(average of negatives) +3sd)], conclusion on the virus presence in samples will not be quite clear. Second, antibodies for some viruses and virus-like agents are not commercially available. Third, some antibodies are isolate-specific and are unable to interact with the coat protein of other isolates of a given virus. The latter is well documented for Iranian isolates of *Citrus psorosis virus* (15). Serological tests are quite unreliable in detection of viruses in olive certification (2).

Molecular methods on the basis of polymerase chain reaction (PCR) amplification of nucleic acids enables greater sensitivity and reliability by analyzing the sequence data. Moreover, in the case of low concentration or uneven distribution of the target virus in asymptomatic tissues, co-application of external and internal primers (e. g. nested PCR) will increase the sensitivity of the assay. However, presence of inhibitors such as endogenous ribonucleases, polyphenolics and polysaccharides can drastically decrease the sensitivity of the assay as experienced in the case of pome and stone fruits (8, 19). Many of these problems could be circumvented by coamplification of an endemic mRNA such as *Rbcl* or *Nad*5 (13, 14, 18). Procedures that allow the multiple and simultaneous detection of plant viruses, viroids and bacteria have been developed and used routinely for certification of plant propagating materials in different plant taxons (3-5, 10, 11, 13, 14, 18). RT-PCR has been documented as one of the sensitive and reliable method for virus detection in olive certification schemes (2).

Due to the importance of optimization of reliable, sensitive, cost effective and fast method for certification of olive propagating materials in SPCRI, the present study was carried out with the objectives of development of simplex, duplex and multiplex RT-PCR (sRT-PCR, dRT-PCR and mRT-PCR) assays for simultaneous detection of ArMV, CLRV and CMV. Moreover the coamplification of plant *Nad*5 gene mRNA as a reliable candidate

for analyzing the RT-PCR accuracy was included in the assays.

Methods

Source of plant material and RNA extraction

Positive samples of ArMV, CLRV and CMV were purchased from Bioreba, Switzerland and used for RNA extraction and subsequent RT-PCR assays. Total RNA was extracted from these samples by phenol-chloroform precipitation as described previously (14).

Specific primers

Previously published and the newly designed primer pairs (table 1) for RT-PCR detection of ArMV, CLRV, CMV and the internal control *Nad*5 gene were used in this study. The previously reported primer pair for ArMV did not amplify the expected fragment of coat protein gene (9). In comparison to some of the published sequences for ArMV RNA2 (NC_006056, EF426853, D10086, X55460 and EU433920), six point mutations were found in reverse primer designed by Grieco et al. (2000). Using ArMV RNA2 accessions a new reverse primer was designed that showed higher match with forward primer to amplify a 427 bp fragment of ArMV RNA2. Moreover, new primers for CLRV RNA2 were designed on the basis of its published sequences on different host taxons [e. g. *Rheum* sp. (NC_015415), *Actinida chinensis* (KC937026), *Malus domestica* (KC937027), *Ribes rubrum* (KC937028), *Rubus ideaus* (KC937029), *Rumex obtusifolius* (KC937030), *Prunus avium* (JN104385) and *Vacciniu darrowii* (KC937031)]. Concerning to the primer positions within the published genome sequences of CLRV, CMV and *Nad* 5, these primer pairs were expected to amplify 331, 513, and 181 nucleotides, respectively. The new primers were designed using primer3 software (17).

First strand cDNA synthesis, simplex and duplex RT-PCR (s- and d-RT-PCR)

cDNA was synthesized using the M-MuLV reverse transcriptase (Invitrogen, Carlsbad, California, USA) following the manufacturer's instructions. In conventional sRT-PCR, 1 µl of each specific forward and reverse primers (10 pmol) of target viruses (table 1) were added separately to the PCR reaction mixture (25 µl) containing 2.5 µl PCR buffer (10X), 2.2 µl $MgCl_2$ (25 mM), 2 µl dNTP (10mM), 2 µl cDNA and 0.2 µl (1 unit) *Taq* DNA polymerase (Fermentas, GmbH, Germany). In the case of *Nad*5 gene, 0.5 µl of each primer was used. Polymerase chain reactions were done for 40 cycles using Biorad thermocycler (USA) following an initial denaturation of cDNA at 95°C for 5 minutes. The optimum temperatures for ArMV, CLRV, CMV and *Nad*5 gene were found at 62, 57, 62 and 60 degree celcius for 30s, respectively. Each cycle was consisted of a denaturation at 94°C for 30s, annealing temperatures as above, elongation at 72°C for 40s and a final extension at 72°C for 5 minutes. For dRT-PCR, the only modification was primer concentration (1.5 µl of each primer pair was used). The PCR reactions were analyzed by electrophoresis on 1.5% agarose gel buffered in TAE (0.04 M Tris-acetate, 1mM EDTA, pH 8.0) including GelRed™ and visualized by UV light.

Multiplex RT-PCR (mRT-PCR)

The mRT-PCR was optimized for the simultaneous detection of all four targets (ArMV, CLRV, CMV and *Nad*5) in one PCR reaction. The mRT-PCR reaction was performed in a total volume of 25 µl containing 0.6 µl of each of forward and reverse primers of ArMV and CMV and 0.3 µl of each of forward and reverse primers of CLRV and *Nad* 5, 2.5 µl PCR buffer (10X), 2.2 µl $MgCl_2$ (25 mM), 2 µl dNTP (10mM), 2 µl of cDNA and 0.2 µl (1 unit) *Taq* DNA polymerase (Fermentas, GmbH, Germany). Amplification was performed in 40 cycles in the Biorad thermocycler (USA). After an initial denaturation of cDNA at 95°C for 5 min., each cycle was consisted of a denaturation at 94°C for 30s, annealing temperatures at 60°C for 40s, elongation at 72°C for 40s and a final extension at 72°C for 5 minutes. The PCR products were analyzed by electrophoresis on 1.5% agarose gel buffered in TAE including GelRed™ and visualized by UV light.

Table 1. Primer pairs used for RT-PCR detection of ArMV, CLRV, CMV and the internal control *Nad 5* gene.

Virus/ *Nad* 5	Primers (5'-3')[a]	Primer Position[b]
ArMV	TTG GTT AGT GAA TGG AAC GG[c]	16200-16219
	CAA GCT ATC ATG TGG GCA AA[d]	16703-16684
CLRV	CGT GTA ACG GCA ACA GTG TTA[d]	1644-1664
	AGT CCG ACA CTC ATA CAA TAA GC[d]	1974-1952
CMV	TAA CCT CCC AGT TCT CAC CGT[e]	1488-1508
	CCA TCA CCT TAG CTT CCA TGT[e]	2000-1980
Nad 5	GAT GCT TCT TGG GGC TTC TTG TT[f]	968-987 and 1836-1838
	CTCCAG TCA CCA ACA TTG GCA TAA[f]	1995-1973

[a] The first and second primers are forward and reverse primers, respectively. [b]Primer positions for ArMV, CLRV , CMV and *Nad* 5 gene belong to the published genomic sequences NC_006056, JN104385, AB188235 and D37958, respectively. [c, d,e,f]primers designed by Grieco et al. (2000); this work; Grieco et al. (2000) and Menzel et al. (2002), respectively.

Sequencing

Amplicons of the expected sizes of ArMV, CLRV, CMV and *Nad*5 genes were excised from the agarose gel 1.5%, purified using the Gel Extraction Kit (QIAquick, Qiagen, Germany) and sequenced directly in MWG, Eurofins, Germany.

Results

cDNA synthesis and optimization of sRT-PCR and dRT-PCR

cDNA synthesis for s- and d-RT-PCR was performed using 1 µl of total RNA extracted from the infected tissues. For optimization of amplification of single and double targets in one PCR reaction, different combination of PCR reagents and especially primer concentrations were tested in several PCR reactions. The PCR reaction mixture and conditions mentioned previously, specifically amplified the expected fragment sizes of all targets studied and no amplification was seen in control cDNA (Fig. 1). As Shown in figure 1, monomorphic fragments of 513, 427 and 331 nucleotides were successfully amplified in samples containing CMV, ArMV and CLRV cDNA, respectively. In all cases, the application of mRNA primers of *Nad*5 gene resulted in amplification of a clear 181 bp fragment. Direct sequencing of the amplified fragments of CMV, ArMV, CLRV and *Nad*5 and their comparison with the reference sequences in the data bases (AB188235, NC_006056, JN104385 and D37958, respectively) revealed dependencies of sequences to these targets and further confirmed the accuracy of developed s- and d-RT-PCR.

Optimization of multiplex RT-PCR

cDNA synthesis for multiple amplification of all four targets in single PCR reaction was performed using 1 µl of RNA mixture containing ArMV, CLRV and CMV RNAs and 1 µl of random hexamer primer. Multiplex RT-PCRs for simultaneous amplification of viral and *Nad*5 genes were performed using different concentration of the PCR reagents and primer annealing temperatures. The amplification specificity and reliability were

compared with those amplified in s- and d-RT-PCR. Finally, the protocol shown in materials and methods successfully amplified all four targets at 60°C annealing temperature (Fig. 1, lane 1). The efficiency of the developed mRT-PCR was tested on at least 500 leaf and bark samples derived from olive trees in different geoghraphical positions (e. g. Khuzestan, Zanjan, Gilan and Tehran provinces).

Discussion

The present study describes the parallel detection of three economically important viral disease agents of olive (ArMV, CLRV and CMV), as indicated in the INHSPPM, using multiplex RT-PCR assay together with coamplification of host endemic gene (*Nad*5) mRNA. The accuracy and efficiency of the developed single, diplex and triplex RT-PCR was further rechecked in several samples infected with any of these viruses for certification purposes.

Several detection methods including biological and serological assays have already been developed for detection of viral diseases in propagating materials, but they are either labor-intensive, expensive, time consuming or tedious and even not applicable due to the reasons mentioned in introduction. Moreover, an internal control is lacking in serological methods and making ELISA unsuitable for routine pathogen testing if false negative or positive results occurred by inhibitors or contamination, in certification schemes. But, in spite of several inevitable but circumventable disadvantages, molecular detection of viral targets using ordinary RT-PCR has been widely used in certification programs. Simplex and/or multiplex RT-PCR assays for virus detection in olive certification schemes have been used widely, but in any of these reports coamplification of host internal gene (s) for evaluating the accuracy of different steps of the assay was not included (1, 4-7, 16, 20, 21). In the present study coamplification of *Nad*5 cDNA was included for checking the developed RT-PCR steps (i. e. RNA extraction, cDNA synthesis and PCR) and easily interpretation of results. The *Nad*5 primer pairs and specifically the forward primer that excellently designed based on the sequences of exones I and II of *Nad*5 genomic DNA (gDNA), spanning intron I (13), could be applied on cDNA derived from DNase-untreated RNA extracts and, in turn, decreases labourity of RNA extraction as well as contamination of cDNA with genomic DNA. This internal control has also been used in our previous studies on *Citrus* sp. certification schemes for detection of viruses and viroids (14).

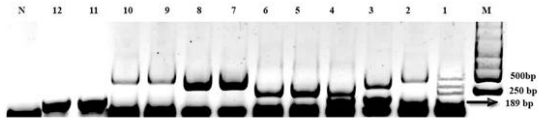

Fig. 1. Agarose gel electrophoresis of fragments belonging to exones I and II of mitochodorial *Nad*5 gene (lanes 11-12), CMV RNA2 (lanes 9-10), ArMV RNA2 (lanes 7-8) and CLRV RNA2 (lanes 5-6) amplified in sRT-PCR. dRT-PCR of CLRV-, ArMV- and CMV-*Nad*5 are shown in lanes 4, 3, and 2 respectively. Coamplification of all four targets is shown in lane 1. N indicates negative control and M is 1 kb molecular marker.

The application of inappropriate primers and/or the primers that designed based on the published sequences of virus isolates found in another regions/ continent is another source of problems for RT-PCR amplification of a certain target gene of a given virus in certification programs. The newly designed reverse primer for ArMV and specifically both reverse and forward primer for CLRV that designed on the basis of the published sequences for RNA 2 of this virus on different host taxons could easily be applied at least on Iranian isolates of CLRV on different hosts including olive, pome and stone fruit trees for certification purposes (the present work, data are not shown).

To our knowledge, developing of detection methods for olive certification schemes using a target-specific RT-PCR system together with amplification of plant mRNA as internal control is previously unreported event for olive certification. Specifically, the mRT-PCR

system reported in this study with new and robust primers for CLRV and ArMV should be considered as the new option for simultaneous detection of these pathogens.

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