Simultaneous Detection of Mixed Infection of *Onion yellow dwarf virus* and an *Allexivirus* in RT-PCR for Ensuring Virus Free Onion Bulbs

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Abstract Reduced seed production in onion is associated with *Onion yellow dwarf virus* (OYDV), a filamentous *Potyvirus*. Onion is also infected with other filamentous virus particles suspected to be *Allexivirus*. RT-PCR was used to detect mixed infection of both the viruses in leaves and bulbs. A duplex RT-PCR was developed, which simultaneously detected the presence of these two viruses in winter (Rabi) onion bulb. In summer (Kharif) onion bulbs only *Allexivirus* was detected. The absence of OYDV in summer crop is discussed. The sequencing of RT-PCR amplified products confirmed the identity of OYDV and *Allexivirus*, the latter showing closer identity to *Garlic virus C* (GVC)/Garlic mite-borne mosaic virus. This makes the first detection of an *Allexivirus* in onion crop in India. The duplex RT-PCR to detect these viruses (OYDV and *Allexivirus*) would be an improvement for indexing of viruses in onion bulbs for seed production.

Keywords *Allexivirus* · Leaf · *Onion yellow dwarf virus* · Onion bulb · Virus indexing

Introduction

Onion is a major bulbous crop among vegetables and is of global importance [11]. *Onion yellow dwarf virus* (OYDV) causing onion yellow dwarf disease is most common virus

in onion. The yield of bulb and seed can be greatly reduced in virus infected onion plants [3]. Disease incidence has been reported up to 92% in Argentina [6] and up to 95% in USA [13]. In India OYDV occurs in epiphytotic proportion and was implicated in the reduced onion seed production [1]. Additionally an unidentified flexuous virus with particle size of ~900 nm was also observed in electron microscopy and was suspected to be particles of mite-borne filamentous virus which belongs to *Allexivirus* [10]. *Allexiviruses* can cause significant yield reduction in garlic (14–32%) [4] and might affect onion crop in mixed infection with OYDV. A tospovirus (*Irish yellow spot virus*) which produces typical symptoms of diamond-shaped or near-diamond shaped lesions has been previously reported in onion from western part of India [14] but the same was not observed in our experimental onion field.

Typical symptom of yellow stripe, diffuse chlorotic spots, twisting of leaves and mosaic generally caused by OYDV were observed up to 70% on the leaves of Rabi onion crop during survey of experimental fields at Indian Agricultural Research Institute, Pusa, New Delhi in the month of January 2007 and 2008. Kharif onion on the other hand was apparently symptom less. In a previous study presence of OYDV and *Allexivirus* particles in onion was observed in immunosorbent electron-microscopy [10]. Although, RT-PCR has been developed to detect OYDV in onion leaves [2], in the present study duplex RT-PCR detection of OYDV and *Allexivirus* was developed to detect the mixed infection of OYDV and *Allexivirus* in bulb tissues and used to determine the presence of these two viruses in Rabi and Kharif onion bulbs so that virus free onion bulbs can be ensured for seed production. Specific identification of both viruses was done by sequencing of amplified product and comparing the nucleotide sequences from the database.

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Materials and Methods

Leaves from one accession line Pusa Red (PR) showing yellow striping; dwarfing and mosaic symptoms were collected and tested for the presence of OYDV and *Allexivirus* by standard RT-PCR. At the end of the season, bulbs from the five cultivars of Rabi onion viz., Pusa Red (PR), Pusa Madhavi (PM), Early Grannow (EG), Pusa White Flat (PWF), and Pusa White Round (PWR) showing typical symptoms of mosaic and yellowing and four cultivars from Kharif onion viz., selection 100-02-1, selection-157, selection-397 and H44 without any apparent symptoms were obtained from the experimental field of the Division of Vegetable Science, IARI, New Delhi. Five bulbs were grown in the glasshouse. Remaining bulbs (5–20) were stored at 4°C. Healthy plants were obtained by growing onion seeds in glasshouse under insect free conditions.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 50 mg tissues of leaves and bulb of infected onion and healthy onion leaves using RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. RNA from each sample was eluted in 40 µl of RNase free water.

For the detection of allexiviruses previously published primers Allexi1 and Allexi2 were used [8]. The primer pair produced an amplicon of ~200 bp. For the detection of OYDV three sets of primers were synthesized from the conserved regions of large nuclear inclusion protein (Nib), coat protein (CP) and 3' untranslated region (3'UTR). Sequences used for designing primers were available in NCBI GenBank. BLAST programme in NCBI GenBank was used on the primer sequences to confirm their specificity. The details of primers and their location on the viral

genome are given in the Table 1. The three sets of primers viz., primer set 1, primer set 2 and primer set 3 producing amplicons of ~1.1 kb, ~500 bp and ~350 bp, respectively (Table 1) were initially evaluated for OYDV detection in the infected leaves of PR cultivar. The primer pair giving best amplification was used for detection of OYDV in leaf and bulb tissues of onion cultivars.

c-DNA Synthesis

For RT-PCR first strand of cDNA was synthesized separately for OYDV and *Allexivirus* using 4 µl of total RNA and a reverse transcription (RT) mixture containing the reverse primer of OYDV or allexivirus at a concentration of 0.2 µM, 20 U M-MuLV reverse transcriptase (Fermetas, Lithuania), 4 µl of 5× reaction buffer and 0.3 mM dNTPs. The total reaction mixture of 20 µl was incubated at 42°C for 45 min. The enzyme was inactivated by heating at 70°C for 10 min.

Polymerase Chain Reaction

Based on annealing temperature (Table 1) for primer pairs of OYDV and *Allexivirus*, RT-PCR was performed in Bio-Rad MyCycler. PCR was performed using a reaction mix containing 5 µl of RT reaction mix, 5 U of Taq DNA polymerase (Promega), 5 µl of 10× reaction buffer, 1.5 mM MgCl₂, primer pairs at a concentration of 0.2 µM and 0.2 mM dNTPs. The temperature profile consisted of a denaturation step at 94°C for 5 min, then 30 cycles of 45 s at 94°C, 20 s at annealing temperature and 1 min at 72°C and one final extension step at 72°C for 10 min.

10 µl of amplified products were separated by electrophoresis in a 1.2% agarose gel containing ethidium bromide at a concentration of 0.5 µg ml⁻¹ and photographed under UV illumination with an imaging system (Biorad XR documentation system). Rest of the product was purified

Table 1 Primer sets used for detection of OYDV and *Allexivirus* in the different tissues of onion

Set no.	Primers, their location on genome and sequences	T _m (°C)	Annealing temperature (°C)	References	Position on viral genome
01.	OYDV.VKB1F-5'ATAGCAGAAACAGCTCTTA3'	53	52	[2]	9442 (Nib) ^a
	OYDV.VKB 2R-5'GTCTCYGTAATTCACGC3'	56			10539 (3'UTR) ^a
02.	OYDV.VKB 2F-5'GGAAATTGGACTATGATGGA3'	56.3	52	Designed in this study	9913 (coat protein) ^a
	OYDV.VKB 2R-5'GTGTTKACGTTACCATC3'	53.5			10247 (coat protein) ^a
03.	OYDV.VKB3F-5'ATAGCAGAAACAGCTCTTA3'	53	52	[2]	9442 (Nib) ^a
	OYDV.VKB 3R 5'TCCATCATAGTCCAATTTCC3'	56		Designed in this study	9932 (coat protein) ^a
04.	Allexi 1 F 5'CYGCTAAGCTATATGCTGAARGG3'	60	56	[8]	(NABP)
	Allexi 2 R 5'TGTTTCAARGTAAGTTTATAGYAATATCAACA3'	62			(3'UTR)

^a OYDV nucleotide sequence No. as per GenBank: NC_005029

Table 2 Detection of OYDV and *Allexivirus* in field samples of different selection lines of onion using standardized duplex RT-PCR

Selection lines/ cultivars	Range of symptoms	Total no of samples tested	No. of samples positive for OYDV	No. of samples positive for <i>Allexivirus</i>
<i>Rabi onion</i>				
Pusa Red (PR)	Curling, yellowing, mosaic, stunting	20	20	17
Pusa Madhavi (PM)	Mosaic, stunting	5	5	5
Early Grannow (EG)	Yellowing, mosaic, stunting	5	5	5
Pusa White Flat (PWF)	Mosaic, stunting	4	4	4
Pusa White Round (PWR)	Mosaic	4	4	4
<i>Kharif onion</i>				
Selection 100-02-1	No symptom	4	0	4
Selection -157	No symptom	4	0	4
Selection- 397	No symptom	3	0	3
H44	No symptom	4	0	4

using PCR Purification kit (Qiagen). The purified PCR product was sequenced at Chromous Biotech, Bangalore, India. BLAST programme from NCBI was used to determine the identity of PCR product sequences.

On the basis of results obtained from evaluation of primer pairs, primer set 3 producing an amplicon of ~500 bp was used for further detection of OYDV in standard and duplex RT-PCR. The primer pair Alexi 1 and Alexi 2 initially used for standard RT-PCR for *Allexivirus* was used in duplex PCR. RNA was extracted from leaves and bulb tissues of each five accessions of Rabi onion. Minimum of three random samples from each accession lines were used for standard RT-PCR detection of OYDV and *Allexivirus*.

For standardization of the duplex PCR, cDNAs of OYDV and allexiviruses were prepared simultaneously in a single-tube reaction using 4 µl of total RNA from PR onion bulbs. The RT mixture contained reverse primer for OYDV and *Allexivirus* at a concentration of 0.2 µM and other components were same as described for standard cDNA preparation. The PCR mixture contained 5 µl of RT reaction mixture, 5 U of Taq DNA polymerase (Promega, USA), 5 µl of 10× reaction buffer, primers as used for detection in the previous section were used at a concentration of 0.2 µM for both OYDV and *Allexivirus*. Optimized higher concentration of dNTPs (0.4 mM) and MgCl₂ (2.5 mM) were used in the PCR mixture. To determine the appropriate annealing temperature for the duplex PCR, a gradient PCR was set up with annealing temperatures of 52/54/56°C for 20 s. The denaturation and other reaction steps were the same as described for standard PCR. The duplex RT-PCR was validated by determining the presence of OYDV and allexiviruses in twenty bulb samples of PR accessions and 3–5 samples of other accessions from Rabi and Kharif onion (Table 2). As a control, standard RT-PCR was performed for each of two viruses associated with PR accession of

onion. Healthy onion bulbs were used as negative control. Standard RT-PCR was done to reconfirm the negative results. All the experiments were repeated twice.

Results

RT-PCR for OYDV with Primer set 1 produced multiple amplification bands including the expected size of 1 Kb. Primer set 2 and 3 produced very specific single amplified product of ~350 and ~500 bp, respectively (result not shown). OYDV could be detected in leaves and bulbs of all the five cultivars using the primer set 3 (Fig. 1a, b). Similarly *Allexivirus* was also detected both in bulb and leaf tissues but it could be observed in bulb tissues of 4 cultivars compared to only leaf tissues of two cultivars (Fig. 2a, b). No amplification was observed in healthy onion leaves (result not shown).

Sequencing of purified PCR product revealed that OYDV fragment was 490 bp long and BLAST analysis of the nucleotide sequence showed that it has maximum sequence identity of 93% with NIB and CP region of another OYDV sequence from Japan (Acc. No. AB000843). The sequence of PCR products specific to allexiviruses was 179 bp long. With BLAST analysis it was found that the nucleotide

Fig. 1 Detection of OYDV using primer set 3 producing amplicons of ~500 bp **a** in leaves, **b** in bulbs; lane M 100 bp ladder, lanes 1–5 PR, PM, EG, PWF, and PWR accessions

Fig. 2 Detection of an *Allexivirus* in **a** in leaves, **b** in bulbs; lane *M* 100 bp ladder, lanes 1–5 PR, PM, EG, PWF, PWR accessions

Fig. 3 **a** Simultaneous detection of OYDV and allexiviruses in bulbs of Rabi onion. Lane *M* 100 bp marker, lane 1 only OYDV by standard RT-PCR, lane 2 only *Allexivirus* by standard RT-PCR, lanes 3–13 amplification of OYDV and *Allexivirus* by Duplex RT-PCR in different Rabi onion accessions, lanes 3,4 PR, lanes 5, 6 PM, lanes 7, 8 EG, lanes 9, 10 PWF, lanes 11, 12 PWR, lane 13 healthy control. **b** Simultaneous detection of OYDV and allexiviruses in bulbs of Kharif onion. Lane *M* 100 bp marker, lane 1 only OYDV by standard RT-PCR; lane 2 only *Allexivirus* by standard RT-PCR, lanes 3, 12 amplification of OYDV and *Allexivirus* by Duplex RT-PCR in different Kharif onion accessions, lane 3 PR (Positive control), lanes 4, 5 selection 100-02-1, lanes 6, 7 selection-157, lanes 8, 9 selection-397, lanes 10, 11 H44, lane 12 healthy control

sequence had maximum sequence identity of 79% with *Garlic virus C* (Acc. No. AB010302) and Garlic mite-borne mosaic virus (D49443) in the nucleic acid binding protein (NB) region and 3' untranslated region (3'UTR). It showed identity of 33–68.9% with other allexiviruses.

OYDV and *Allexivirus* could be detected simultaneously by the duplex PCR (Fig. 3a, b), and both viruses were amplified optimally at annealing temperature of 54°C (result not shown). The duplex RT-PCR successfully detected the presence of OYDV and an *Allexivirus* in the bulbs of the five selected accessions of Rabi onion (Fig. 3a). Only *Allexivirus* and no OYDV was detected in the bulbs of Kharif onion cultivars (Fig. 3b; Table 2). There was no amplification in RNA from healthy onion tissue.

Discussion

OYDV and *Allexivirus* are two common flexuous viruses which infect onion in Delhi region. Although ELISA has

often been employed for virus diagnosis in garlic and onion [7, 15], RT-PCR is a more efficient and sensitive method of virus detection [8, 16]. Standard RT-PCR has been used to detect OYDV [2, 8, 19] and allexiviruses [8]. In a previous study we had demonstrated duplex RT-PCR for simultaneous detection of OYDV and SLV in garlic [12]. Here we have developed a duplex RT-PCR for determining the mixed infection of OYDV and allexiviruses in onion bulbs so that it can be used for raising a virus free onion seed crop. It is often necessary to optimize the reaction parameters like concentrations of primers, MgCl₂, dNTPs, and reaction conditions like annealing temperature while performing multiplex RT-PCR [5, 17]. In the present study optimization was done for primer specificity and their annealing temperature and determination of appropriate tissues for RNA isolation and RT-PCR detection of viruses infecting onion. Generally within a certain fragment length, e.g., 100–1000 bp, the shorter the targeted fragment, the more efficient the amplification [9, 18] and our studies also demonstrated that primer sets 2 and 3 producing specific ~350 bp and ~500 bp amplicons provided better and more specific amplification of OYDV than the primer set 1 producing non specific amplifications including the targeted amplicon of ~1.1 bp.

OYDV and *Allexivirus* are transmitted by aphid and mite vector, respectively, and infected onion plants can serve as reservoir of these viruses for other economically important *Allium* crops. It is interesting to observe that duplex PCR protocol detected the presence of both OYDV and *Allexivirus* in all the selection lines of Rabi onion. However, only *Allexivirus* was detected in Kharif onion. The absence of OYDV in Kharif onion may be explained by the fact that aphid population is very low during Kharif season (February to September) and thereby it might escape transmission of OYDV by aphids. The sequencing of PCR products of *Allexivirus* indicated that it was closely related with *Garlic virus C*/Garlic mite-borne mosaic virus. This confirms the previous observation of presence of an unidentified flexuous virus particle in OYDV infected onion leaves in electron microscope in Delhi [10]. A recent report has indicated the presence of *Garlic virus C* in onion from Iran [15].

Development of a duplex PCR for detection of OYDV and *Allexivirus* is important tool for epidemiological and management studies and our study indicated that most of the onion accessions grown in Delhi region are affected by these two viruses. Therefore, this study will be highly useful as an improved indexing protocol in screening of onion bulbs used for seed production so that the virus free bulbs can be used for the attainable production of seeds.

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