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ORIGINAL ARTICLE

Development of a SYBR Green-based RT-qPCR assay for the detection of Indian citrus ringspot virus

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Abstract

The Indian citrus ringspot virus (ICRSV) that causes ringspot disease, especially to 'Kinnow mandarin' hampers the sustainability of crop production. Presently, the disease is not amenable for control through host resistance or the introduction of chemicals, hence raising virus-free plants is one of the most effective approaches to manage the disease. Consequently, it is necessary to develop rapid, sensitive, specific, and early diagnostic methods for disease control. In the present study, newly designed primers targeting a 164 bp region of the ICRSV coat protein gene were used to develop and optimize a SYBR Green-based quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay, for the detection of ICRSV. The RT-qPCR assay was evaluated and confirmed using viral RNA extracted from ICRSV infected plants maintained in screen house as well as field samples. The standard curves displayed a dynamic linear range across eight log units of ICRSV-cRNA copy number ranging from 9.48.1 fmol (5.709×10^9) to 0.000948 amol (5.709×10^2), with detection limit of 5.709×10^2 copies per reaction using serial tenfold diluted in vitro transcribed viral cRNA. The developed RT-qPCR is very specific to ICRSV does not react to other citrus pathogens, and approximately 100-fold more sensitive than conventional RT-PCR. Thus, this assay will be useful in laboratories, KVKs, and nurseries for the citrus budwood certification program as well as in plant quarantine stations. To our knowledge, this is the first study of the successful detection of ICRSV by RT-qPCR.

Keywords Citrus · ICRSV · cRNA · RT-qPCR · SYBR green · Detection

Introduction

Citrus fruit is one of the most important horticulture crops cultivated in more than 150 countries under diverse climatic conditions (Meena et al. 2020; Kokane et al. 2020a). Citrus fruits are native to the tropical and subtropical areas of Southeast Asia, including India and China (Gmitter and Hu 1990). Citrus fruit is the third largest fruit industry in India after banana and mango, and it plays a vital role in the economy. It is used for the daily consumption as fresh fruit or as squash, cordial, jam, and marmalade, as well as many pharmaceutical uses. Citrus fruit production and productivity have been hampered by several diseases caused by viruses and virus-like pathogens (VLPs) in India. Among them, ringspot disease caused by Indian citrus ringspot virus (ICRSV) is one of the most detrimental diseases in 'Kinnow mandarin' (*Citrus reticulata*), a hybrid formed by the crossing of 'King' (*Citrus nobilis*) × 'Willow Leaf' (*Citrus deliciosa*). It is widely distributed in the north-western part of India (Ahlawat 1989; Sharma et al. 2004; Singh et al. 2006; Prabha and Baranwal 2011; Kokane et al. 2020b). ICRSV belongs to *Mandarivirus* genus within the family *Alfaflexiviridae* (Rustici et al. 2000; Adams et al. 2004). It is a flexuous rod-shaped virus (650 nm × 13 nm) containing a 7.5 kb ssRNA genome, excluding the 3′-poly (A) tail. It has six open reading frames (ORFs) along with 5′ and 3′ untranslated regions (UTRs) (Byadgi and Ahlawat 1995; Rustici et al. 2000; Adams et al. 2004).

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ICRSV has significant characteristic symptoms on 'Kinnow mandarin' and other citrus cultivars, namely viz., 'Malta', 'Mosambi', 'Kagzi limes', 'Pomelo', and 'Grapefruit'. However, the maximum incidence of the disease was recorded in Kinnow mandarin orchards (Byadgi and Ahlawat 1995; Kokane et al. 2020b). Plants infected with ICRSV exhibit mottling, conspicuous chlorotic rings, and irregular chlorotic patterns; these effects are most visible on mature leaves of the lower canopy as a key diagnostic symptom of ringspot disease (Thind et al. 2000; Rustici et al. 2002; Kokane et al. 2020b). While ICRSV is transmissible by graft, so far, there is no evidence of vectors or other modes of transmission (Pant and Ahalawt 1998; Singh et al. 2006).

At present, there is no effective management strategy available for ringspot disease; therefore, planting virus-free plants is the most effective way of disease prevention. The ICRSV is a quarantine pathogen and is restricted to India (Kokane et al. 2020b). Therefore, there is a need to develop rapid, highly sensitive, and early diagnostic techniques to prevent the spread of ringspot disease. Currently, several ICRSV diagnosis protocols have been developed. Biological indexing is a traditional method which is unpredictable and unreliable because symptom expression depends on several factors like environmental temperature, virus concentration in test sample, and whether it is a single or mixed infection (Thind et al. 1999). Other assays like, enzyme-linked immunosorbent assay (ELISA) is more popular for virus detection but less sensitive than nucleic acid-based detection techniques (Sharma et al. 2009). Electron microscopy requires an expensive instrument and a highly skilled person, and is also unable to discriminate between the similar particle viruses; on the other hand, reverse transcription polymerase chain reaction (RT-PCR) has been a routinely used technique (Rustici et al. 2002; Singh et al. 2006; Pant et al. 2018), but it is less sensitive than RT-qPCR. Kokane et al. (2020b) recently developed a robust, low-cost, single-step RT-LAMP assay for ICRSV detection, However, the LAMP assay is susceptible to aerosol contamination and may lead to a false-positive result (Karthik et al. 2014). To overcome this problem, it is necessary to develop early detection, highly sensitive, more valid, and reliable diagnostic method that is crucial for the prevention of virus transmission in the field (Chen et al. 2016).

Real-time PCR has been widely accepted due to its speed, sensitivity, reproducibility, and reduced risk of contamination. The amplification process is visualized by monitoring the emitted fluorescence signal and there is no need for post-PCR steps. Real-time PCR assays are usually carried out using a hydrolysis probe (Taqman) or a SYBR Green fluorescence dye. Compared to the Taqman probe, the real-time SYBR green assay is less expensive reporter system as the probe is not needed. It can also achieve an around similar detection performance and has been widely applied in the detection of a variety of plant viruses. SYBR Green binds to the minor groove of the double-stranded DNA and produces fluorescence, which is correlated with the quantity of amplified products present in the sample (Levin 2004).

The objective of this study is to develop a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay, based on coat protein (CP) gene-specific sequences, for the detection and quantification of ICRSV using SYBR Green. To the best of our knowledge, this is the first report of a successfully developed RT-qPCR assay to detect ICRSV in citrus plants.

Materials and methods

Sample collection and plant maintenance: eleven known positive ICRSV graft inoculated 'Kinnow mandarin' (2 year old) seedlings were maintained inside an insect-proof screenhouse at Indian Council of Agricultural Research–Central Citrus Research Institute (ICAR–CCRI), Nagpur, India (Fig. 1). Maintained samples were used for developing and optimizing a RT-qPCR assay. The developed RT-qPCR assay was validated by previously studied samples (Kokane et al. 2020b), which were collected from different regions of Punjab and Haryana, India during 2018–19. The specificity of the RT-qPCR assay was examined using other key citrus pathogens (two replicates of each) including viz. citrus tristeza virus (CTV) (Warghane et al. 2020), citrus yellow vein clearing virus (CYVCV) (Chen et al.2016), citrus yellow mosaic virus (CYMV) (Motghare et al. 2018), '*Candidatus* Liberibacter asiaticus' (*C*Las) (Kokane et al. 2020d), and citrus phytoplasma (Ghosh et al. 2019).

Sample processing and RNA extraction: for total RNA extraction, five to six symptomatic/infected leaves were taken from known positive graft inoculated plants as well as field-collected samples. The leaves were washed with tap water, wiped with 70% ethanol, and blot dried with tissue paper. The symptomatic conspicuous chlorotic ring portion on the mature leaf lamina was excised with scissor and ground with liquid nitrogen. Approximately 0.1 g of each ground sample was used for total RNA extraction using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), based on the manufacturer's guidelines. The extracted total RNA was purified using RNase-free DNase I (Ambion DNA-free kit, Invitrogen, USA) according to manufacturer's instructions to remove contaminating genomic DNA. The quality of total genomic RNA was analyzed on a 2% agarose gel. The concentration of total RNA extracted from each sample was measured using NanoDrop 2000 Spectrophotometer (Thermo Scientific). The RNA sample with the A260/A280 ratio within the range of 1.9–2.1 was used as a template in RT-qPCR.

Fig. 1 **A** Indian citrus ringspot virus (ICRSV) induced ring spot symptoms on 'Kinnow mandarin' with healthy leaf (extreme right). **B** Ring spot symptoms on 'Kinnow mandarin' trees. **C** Biological Indexing (ICRSV culture maintained in screen house)

Primer design: the primers for RT-qPCR were designed based on the conserved region of CP gene (GenBank Accession Number MN422617) of ICRSV using primer express software version 3.0 (Applied Biosystem, USA) (Table 1). The designed RT-qPCR primers were analyzed in silico for the specificity, stability, and cross-reactivity within primers using Oligo Analyzer 3.1 tool (http://eu.idtdna.com/calc/analyzer) to avoid false-positive results. In addition,

Table 1 Primer list used in assay standardization

Target gene	Assay	Primer code	Primer sequence (5′–3′)	Amplicon size (bp)
ICRSV coat protein	RT-qPCR (target)	ICRSV-RT-1F	GCTAACCCTAAACAGACCGAG	211
		ICRSV-RT-1R	GGCAGAAATCCCTAACCACTAG	
		ICRSV-RT-2F	TATACGCCATTGACCTCGCC	289
		ICRSV-RT-2R	GAAGCTGGCGAGAGGATACC	
		ICRSV-RT3-F	GCGCTTTTTGCGCTTACTAC	164
		ICRSV-RT3-R	TAGGGGCACATACAGGGAAG	
Plant cytochrome oxidase	RT-qPCR (internal control)	COX-F	F GTATGCCACGTCGCATTCCAGA	68
		COX-R	GCCAAAACTGCTAAGGGCATTC	
Citrus sinensis elongation factor 1alpha		EF-1α- F	CTCAAGCCTGGTATGGTGGT	185
		EF-1α -R	GGATCATCCTTCGAGTTGGA	
T7 Promoter containing ICRSV Coat protein	RT-PCR (cRNA)	T7-ICRSV-CP-F	TAATACGACTCACTATA**G**GGAGA ATGAGCTTTGACTACAC	988
		ICRSV-CP-3R	TCATTAGATGTTGAAAGGGGTC	
ICRSV Coat protein	RT-PCR	ICRSV-CP-3F	AGCTTTGACTACACACACC	978
		ICRSV-CP-3R	TCATTAGATGTTGAAAGGGGTC	
Nucleic acid binding region		NAB-F	TGGAACCTCATGATCAAAGCG	668
		NAB-R	TCATCTAGGGTCGAGGAGC	

the developed RT-qPCR assay was validated with conventional RT-PCR based on the coat protein gene (ICRSV-CP-3F/CP-3R) and the nucleic acid binding region (NAB-F/NAB-R).

Conventional RT-PCR: the total genomic RNA extracted from each sample was used for RT-PCR using the ICRSV coat protein gene (CP) specific primer set viz. ICRSV-CP-3F/ CP-3R. The RT-PCR was performed in two steps as described by Kokane et al. (2020b; c). Amplification was performed with the following conditions; initial denaturation at 94 °C for 2 min followed by 35 cycles of 0.30 s at 94 °C, 0.40 s at 58 °C, and 60 s at 72 °C, with a final extension period of 10 min at 72 °C. For further confirmation, RT-PCR was carried out with the NAB-F/NAB-R primers, as described by Kokane et al. (2020b) using the same ICRSV samples. Amplified products were separated on a 1.5% agarose gel and stained with 0.5 µg/ml ethidium bromide and visualized in an UV GelDoc system (G:Box, Syngene, USA).

Standardization of SYBR Green-based real-time RT-PCR assay: the RT-qPCR assay was performed in a StepOne real-time thermocycler (Applied Biosystem, USA) containing 48-well microtiter plates using the SYBR Green fluorophore. The three sets of primers specific to the CP gene were used for standardization of SYBR Green RT-qPCR assay. Among these primers, ICRSV-RT-3F/RT-3R provided a single amplicon with a sharp melt curve, and, therefore, used for all subsequent studies. The RT-qPCR was optimized for 100 nM, 150 nM, 200 nM, and 250 nM of each forward and reverse primers. The optimum concentration of the primer combination was then used for further amplification. For normalizing cycle-threshold (Ct) values, an initial set of primers based on the cox gene (Li et al. 2008; Ghosh et al. 2018a) was used as an internal control. Amplicons produced using primers based on the Cox gene provided multiple peaks as evidenced by the melt curve analysis. Subsequently, we used the sweet orange gene-specific elongation factor 1α for internal control primers (EF-1α-F/EF-1α-R) (Motghare et al. 2018). The amplification was carried out in 10 µl of reaction mixture including 1 × Go Taq qPCR master mix (Promega, Madison, USA), 200 nM of both reverse and forward primers for the target CP gene of ICRSV and 150 nM for internal control primers, and 1 µl of cDNA of each sample. The optimized thermal cycling conditions were as follows: an initial denaturation step at 95 ℃ for 2 min, 40 cycles of PCR amplification at 95 ℃ for 15 s, 60 ℃ for 1 min, followed by a post-melting curve analysis program run as a default parameter according to the instrument documentation. The data were analyzed using StepOne Software v2.1.

Synthesis of an in vitro ICRSV complementary RNA standard: to assess the sensitivity and generation of the standard curve for a qPCR assay, a ICRSV-cRNA standard was developed. The ICRSV-cRNA standard was synthesized using a forward primer containing T7 promoter sequence in the forward sites (T7-ICRSV-CP-F/ICRSV-CP-3R) based on CP gene of ICRSV as described by Kokane et al. (2020b) using the MEGAscript T7 Transcription Kit (Thermo Fisher Scientific, USA). Finally, the synthesized ICRSV-cRNA (Fig. 2) standard was purified, quantified and tenfold serial diluted with total RNA extracted from the healthy plant, and used to calculate a copy number according to the concentration and preparation of the standard curve.

Establishment of standard curve: tenfold serial dilutions of moles of cRNA standard ranging from 9.481 fmol, 948.1 amol, 94.81 amol, 9.481 amol, 0.9481 amol, 0.09481 amol, 0.009481 amol, 0.0009481 amol, and 0.00009481 amol were made from RNA extracted from healthy plants. To generate standard curves, the dilutions of each sample were tested in triplicate with non-template controls (NTC) as a negative control. The obtained mean cycle-threshold (Ct) values were plotted against the amount of cRNA copy number to construct the standard curve. The copy number was calculated using the following formula: Number of copies = (amount of cRNA in nanograms) × (Avogadro's number 6.0221×10^{23})/ length of amplicons in base pairs (bp) × 340 × 1 × 10^9 (Li et al. 2009). To determine the virus copy, the designed standard curve was used in unknown 500 ng RNA of each sample.

Sensitivity comparison of the SYBR green RT-qPCR with RT-PCR: the sensitivity of the SYBR Green real-time method developed in the present study was compared with conventional RT-PCR using the same set of primers (ICRSV-RT3-F/ICRSV-RT3-R). Three replicates of tenfold serial dilutions ICRSV-cRNA standards ranging from

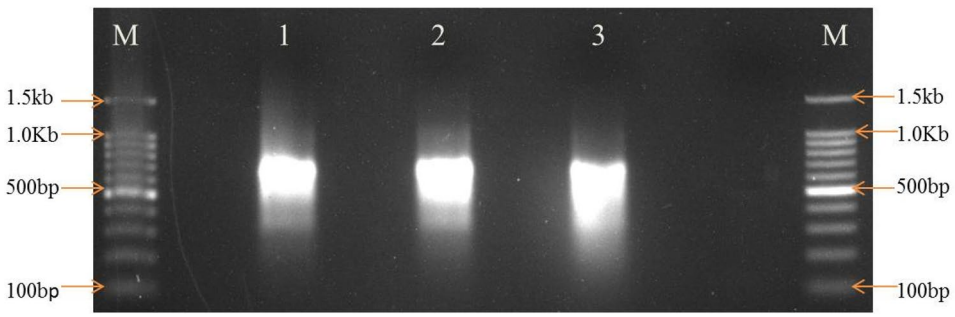

Fig. 2 Agarose gel electrophoresis of in vitro synthesized cRNA of ICRSV

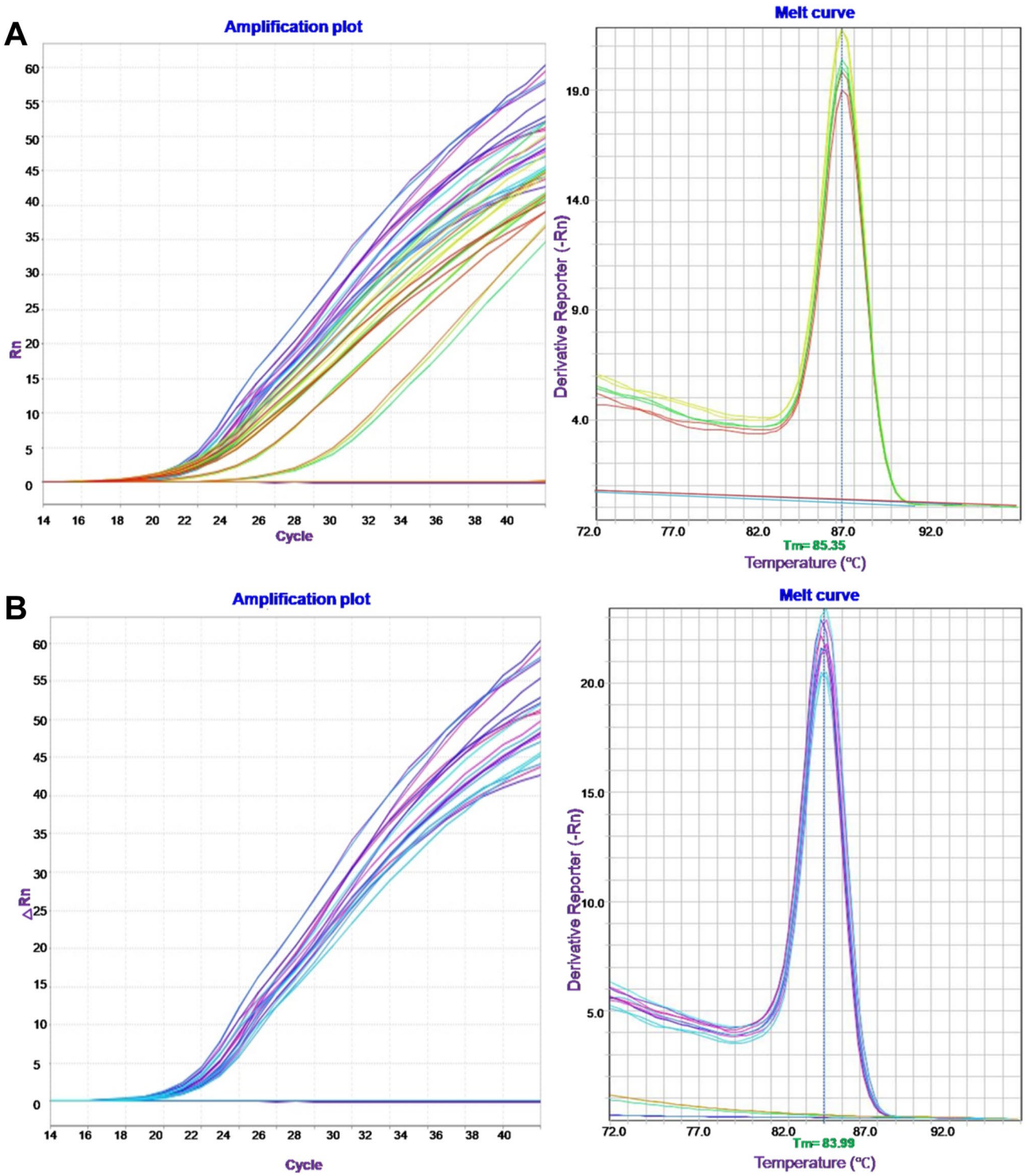

Fig. 3 Amplification plots and melting curves of representative samples: **A** ICRSV Coat Protein; **B** Internal control gene, EF-1α

5.709×10^{9} copies/µl to 5.709×10^{1}) copies/µl were used to perform RT-qPCR and RT-PCR.

Specificity and validation of RT-qPCR: the specificity of the RT-qPCR assay was assessed using a known positive sample of ICRSV, other major citrus pathogens, including CTV, CYVCV, CYMV, CLas, and citrus phytoplasma, as well as a healthy plant control. The total RNA/DNA extracted from the known infected citrus plants were used as a template to conduct the reaction under optimum conditions three times, as described earlier. In addition, validation

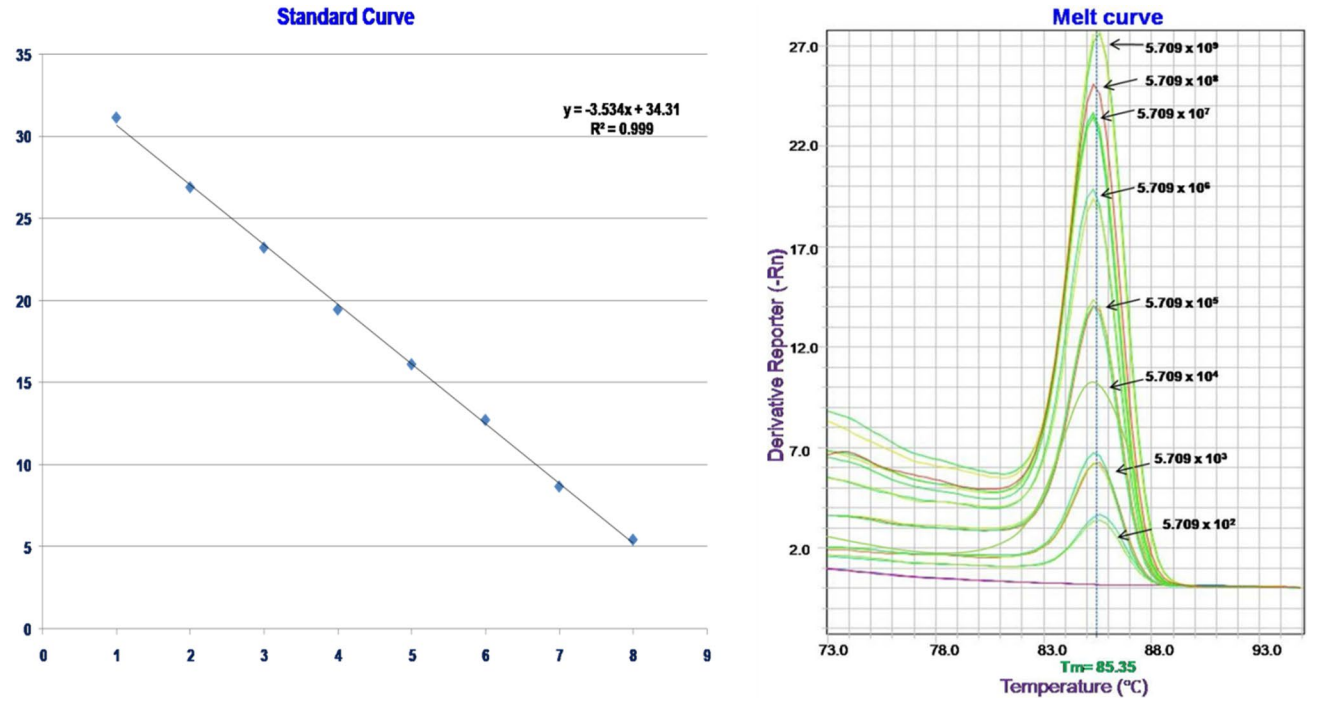

Fig. 4 Standard curve and melt curve specific to ICRSV obtained by a SYBR Green RT-qPCR assay using tenfold serial dilutions of moles of cRNA standard ranging from 9.481 fmol, 948.1 amol, 94.81 amol, 9.481 amol, 0.9481 amol, 0.09481 amol, 0.009481 amol, 0.0009481 amol, and 0.00009481 amol

of the RT-qPCR assay was carried out with field samples. For further confirmation, the RT-PCR was carried out using two sets of primers, and RT-PCR amplified products was analyzed on a 1.5% agarose gel.

Results

Detection of ICRSV by conventional RT-PCR: the ICRSV graft inoculated 11 'Kinnow mandarin' seedlings (Fig. 1) were re-tested by RT-PCR with CP and nucleic acid binding gene-specific primers. In all the ICRSV infected seedlings, successful amplification after RT-PCR with amplicons ~ 978 bp for CP gene and ~ 669 bp for nucleic acid binding gene were observed. To standardize the SYBR Green-based RT-qPCR assay, known ICRSV positive samples were used.

Standardization of SYBR Green-based RT-qPCR: three sets of primers specific to CP gene were used for optimization; among these, ICRSV-RT-3F/RT-3R primers provided single amplicons (~ 164 bp) along with a sharp melt curve peak at 85.35 °C (Fig. 3A). Amplification was not observed in the healthy citrus plant (negative control) and non-template control (NTC). The second, EF-1α gene-specific primer (EF-1α-F/ EF-1α-R) was used as an internal control, covering a fragment of ~ 185 bp. It provided the lowest Ct value (21.5 ± 3) for all the samples tested, and single amplicon with a melt curve peak at 83.99 °C (Fig. 3B).

Standard curve for ICRSV gene: to generate a standard curve, a known amount of tenfold diluted cRNA standard series ranging from 9.481 fmol (5.709×10^9) to 0.000948 amol (5.709×10^2) copies/µl and the respective Ct values obtained from (5.46 Ct) to (31.12 Ct) were plotted on amplification plot. The standard curve developed was highly specific and no primer dimer or non-specific amplification was observed in the analysis of the melting curve data (Fig. 4). A standard curve was generated by plotting the Ct values on the *Y*-axis and the number of virus copies (log 10 virus copies) on the *X*-axis. There was a linear correlation between the input amounts of the total RNA used as a template and the Ct values obtained during amplification with a regression coefficient (r^2) value of 0.999 [($y = -3.534x + 34.31$) (Fig. 4)].

Quantification of ICRSV in screen house inoculated and field-collected citrus plants: all the eleven ICRSV graft inoculated and 30 field samples were observed a positive result in the RT-qPCR assay. The Ct values obtained from ICRSV graft inoculated plants and the field sample of plants were determined from the standard curve to calculate the ICRSV copy number. However, ICRSV titer varied from plant to plant, ranging from 1.2×10^6 (16.25 Ct) to 4.9×10^3 (25.06 Ct) per 500 ng/µl of total RNA extracted in graft inoculated plants and varying in field-collected samples

Table 2 Detection and quantification of Indian citrus ringspot virus in biologically indexed plants along with field-collected citrus samples, and in samples of citrus trees known to be infected by other major citrus pathogens using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and reverse transcription polymerase chain reaction (RT-PCR)

Sr. No.	Sample code	Citrus cultivar	Scientific name	RT-qPCR		PCR with primer pairs	
				Ct values	Log 10 values of virus copy number/500 ng RNA	ICRSV-CP-3F/CP-3R	NAB-F/NAB-R
Biological indexing							
1	ICRSV-K-1	Kinnow	*C. reticulata*	16.25	5.11	+	+
2	ICRSV-K-2	Kinnow	*C. reticulata*	16.55	5.03	+	+
3	ICRSV-K-3	Kinnow	*C. reticulata*	17.12	4.86	+	+
4	ICRSV-M-4	Mosambi	*C. sinensis*	20.12	4.02	+	+
5	ICRSV-K-5	Kinnow	*C. reticulata*	18.56	4.46	+	+
6	ICRSV-K-6	Kinnow	*C. reticulata*	19.36	4.23	+	+
7	ICRSV-K-7	Kinnow	*C. reticulata*	20.35	3.95	+	+
8	ICRSV-M-8	Mosambi	*C. sinensis*	21.23	3.70	+	+
9	ICRSV-M-9	Mosambi	*C. sinensis*	19.63	4.15	+	+
10	ICRSV-M-10	Mosambi	*C. sinensis*	25.06	2.62	+	+
11	ICRSV-K-11	Kinnow	*C. reticulata*	21.65	3.58	+	+
Field collected citrus samples							
12	P-K-LUH-1	Kinnow	*C. reticulata*	22.12	3.45	+	+
13	P-K-LUH-2	Kinnow	*C. reticulata*	UD	UD	−	−
14	P-K-LUH-3	Kinnow	*C. reticulata*	18.12	4.58	+	+
15	P-D-LUH-4	Daisy	*C. reticulata*	17.39	4.79	+	+
16	P-K-LUH-5	Kinnow	*C. reticulata*	UD	−	−	−
17	P-G-LUH-6	Grapefruit	*C. paradisi*	16.64	5.00	+	+
18	P-G-LUH-7	Grapefruit	*C. paradisi*	17.62	4.72	+	+
19	P-P-LUH-8	Pomelo	*C. grandis*	UD	−	−	−
20	P-P-LUH-9	Pomelo	*C. grandis*	21.08	3.74	+	+
21	P-K-LUH-10	Kinnow	*C. reticulata*	UD	−	−	−
22	P-K-HSP-11	Kinnow	*C. reticulata*	22.12	3.45	+	+
23	P-K-HSP-12	Kinnow	*C. reticulata*	UD	−	−	−
24	P-K-HSP-13	Kinnow	*C. reticulata*	UD	−	−	−
25	P-K-HSP-14	Kinnow	*C. reticulata*	19.2	4.28	+	+
26	P-K-HSP-15	Kinnow	*C. reticulata*	18.33	4.52	+	+
27	P-K-ABH-16	Kinnow	*C. reticulata*	UD	−	−	−
28	P-K-ABH-18	Kinnow	*C. reticulata*	UD	−	−	−
29	P-K-ABH-19	Kinnow	*C. reticulata*	19.21	4.27	+	+
30	P-M-ABH-20	Malta	*C. sinensis*	17.32	4.81	+	+
31	P-M-ABH-21	Malta	*C. sinensis*	UD	−	−	−
32	P-K-ABH-22	Kinnow	*C. reticulata*	25.21	2.57	+	+
33	P-K-ABH-23	Kinnow	*C. reticulata*	UD	−	−	−
34	P-K-ABH-24	Kinnow	*C. reticulata*	23.78	2.98	+	+
35	P-K-ABH-25	Kinnow	*C. reticulata*	20.44	3.92	+	+
36	P-K-ABH-26	Kinnow	*C. reticulata*	19.65	4.15	+	+
37	P-K-ABH-27	Kinnow	*C. reticulata*	UD	−	−	−
38	P-K-ABH-28	Kinnow	*C. reticulata*	18.56	4.46	+	+
39	P-K-ABH-29	Kinnow	*C. reticulata*	20.87	3.80	+	+
40	P-K-ABH-30	Kinnow	*C. reticulata*	21.63	3.59	+	+
41	P-K-MUK-31	Kinnow	*C. reticulata*	UD	−	−	−
42	P-K-MUK-32	Kinnow	*C. reticulata*	17.39	4.79	+	+
43	P-K-MUK-33	Kinnow	*C. reticulata*	UD	−	−	−
44	P-K-MUK-34	Kinnow	*C. reticulata*	25.29	2.57	+	+

Table 2 (continued)

Sr. No.	Sample code	Citrus cultivar	Scientific name	RT-qPCR		PCR with primer pairs	
				Ct values	Log 10 values of virus copy number/500 ng RNA	ICRSV-CP-3F/CP-3R	NAB-F/NAB-R
45	P-K-MUK-35	Kinnow	*C. reticulata*	21.87	3.52	+	+
46	P-K-MUK-36	Kinnow	*C. reticulata*	UD	–	–	–
47	P-K-BTI-37	Kinnow	*C. reticulata*	20.56	3.89	+	+
48	P-K-BTI-38	Kinnow	*C. reticulata*	25.62	2.46	+	+
49	P-K-BTI-39	Kinnow	*C. reticulata*	22.66	3.30	+	+
50	P-K-BTI-40	Kinnow	*C. reticulata*	UD	–	–	–
51	P-K-FAZ-41	Kinnow	*C. reticulata*	24.61	2.74	+	+
52	P-K-FAZ-42	Kinnow	*C. reticulata*	21.69	3.57	+	+
53	P-K-FAZ-43	Kinnow	*C. reticulata*	UD	–	–	–
54	H-K-SIR-44	Kinnow	*C. reticulata*	25.63	2.46	+	+
55	H-K-SIR-45	Kinnow	*C. reticulata*	UD	–	–	–
56	H-K-SIR-46	Kinnow	*C. reticulata*	UD	–	–	–
57	H-K-SIR-47	Kinnow	*C. reticulata*	24.33	2.82	+	+
58	H-K-SIR-48	Kinnow	*C. reticulata*	30.21	1.16	+	+
59	H-K-SIR-49	Kinnow	*C. reticulata*	25.31	2.55	+	+
60	MH-M-1 (Healthy)	Mosambi	*C. sinensis*	UD	–	–	–
61	MH-M-2 (Healthy)	Mosambi	*C. sinensis*	UD	–	–	–
Other major citrus pathogens							
62	AL-1 (CTV)	Acid lime	*C. aurantifolia*	UD	–	–	–
63	M-1 (CTV)	Mosambi	*C. sinensis*	UD	–	–	–
64	K-1 (CYVCV)	Kinnow	*C. reticulata*	UD	–	–	–
65	K-2 (CYVCV)	Kinnow	*C. reticulata*	UD	–	–	–
66	M-1 (CYMV)	Mosambi	*C. sinensis*	UD	–	–	–
67	M-2 (CYMV)	Mosambi	*C. sinensis*	UD	–	–	–
68	AL-(*C*Las)	Acid lime	*C. aurantifolia*	UD	–	–	–
69	NM-1 (*C*Las)	Mandarin	*C. reticulata*	UD	–	–	–
70	AL-1 (citrus phytoplasma)	Acid lime	*C. aurantifolia*	UD	–	–	–
71	AL-2 (citrus phytoplasma)	Acid lime	*C. aurantifolia*	UD	–	–	–

UD undetermined

from 1×10^6 (16.64 Ct) to 4×10^2 (30.21 Ct) from 15 to 40 cycles (Table 2).

Sensitivity, specificity, and validation of SYBR Green-based RT-qPCR and RT-PCR assays: to determine the sensitivity, the same tenfold cRNA dilution series was used to compare the detection limit of RT-qPCR and conventional RT-PCR. Detection limits up to 0.000948 amol (5.709×10^2) copies/µl for RT-qPCR and up to 0.948 amol/µl (5.709×10^4) copies/µl for RT-PCR were observed (Fig. 5). The specificity of the assays was analyzed using other major pathogens infecting citrus in India extracted RNA from CTV, CYVCV and DNA from CYMV, *C*Las, and citrus phytoplasma, along with the RNA of healthy citrus plant. The reaction was performed in optimal conditions three times as described earlier, but no positive signal was obtained. Furthermore, the RT-qPCR assay was validated with 48 field samples, among which 30 field samples were observed as moderate to highly positive using RT-qPCR. Further authentication of the field samples as well as ICRSV positive mentioned samples were analyzed by RT-PCR using CP and nucleic acid binding gene-specific primers and amplified products ~ 978 bp (Fig. 6A) and ~ 669 bp (Fig. 6B), respectively, was observed.

Discussion

ICRSV is the endemic causal pathogen of citrus ringspot disease. The disease incidence was recorded mostly in 'Kinnow mandarin' orchards (83.8%) followed by 'sweet orange' (70%), and 'lime' (20%) that results in continuous reduction in plant vigor, flowering, and fruit yield (Byadgi and Ahlawat 1995; Prabha and Baranval 2011). Due to the unavailability of natural resistance to ICRSV in commercial citrus

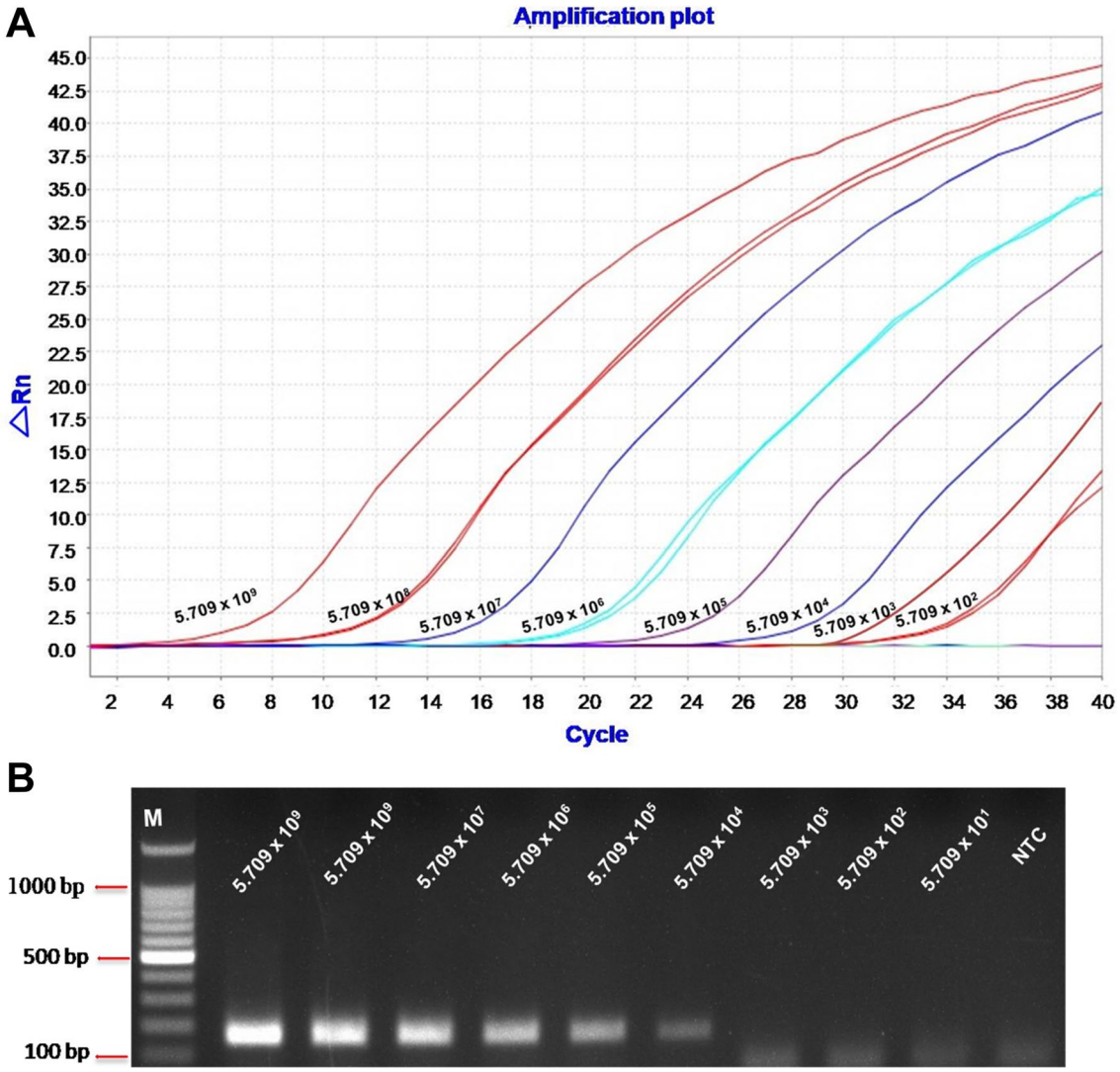

Fig. 5 Sensitivity of coat protein gene based developed assay: **A** Amplification plot of RT-qPCR assay using SYBR Green dye. **B** Reverse transcription polymerase chain reaction (RT-PCR). Lane M, 100 bp DNA Ladder; lane 2 to 9, indicate the cRNA concentration in RT-qPCR assay starting from 5.709×10^9 to subsequent tenfold dilution up to 5.709×10^1, respectively; and Lane NTC, non-template control

cultivars and/or chemical control measures over the ringspot disease, it has become a major threat to the Indian citrus industry. Moreover, the incomplete understanding of ICRSV and its pathogenesis makes it difficult to control the progression of ICRSV infection. ICRSV is a quarantined pathogen and so far, the disease remains limited to the north-western part of India. To restrict the spread of this quarantined pathogen from India to other major citrus producing countries through trade and germplasm exploration activities, there is a necessity to use quick, sensitive, and robust molecular diagnostic tools for early diagnosis of the virus.

In the present study, we have developed a SYBR Green-mediated ICRSV-RT-qPCR assay to detect and quantify ICRSV using CP gene-specific primers. The CP gene is unique to viruses and is, therefore, considered a suitable target for virus detection (Ghosh et al. 2020b). Currently, several techniques have been employed for the detection of plant viruses, including biological indexing (Ghosh et al. 2018b; Warghane et al. 2020; Kokane et al. 2020c), ELISA (Sharma et al. 2009; Shin et al. 2012), and RT-PCR (Rustici et al. 2002; Singh et al. 2006; Pant et al. 2018). More recently RT-LAMP (Kokane et al. 2020b), and Recombinase polymerase amplification-lateral flow assays (RPA-LFA) based CTV detection by Ghosh et al. (2020a) have been used. However, each method has its own limitation such as time-consumption, lack of robustness, less sensitivity, and carryover contamination. In an attempt to overcome these problems, the real-time PCR technique has been widely used

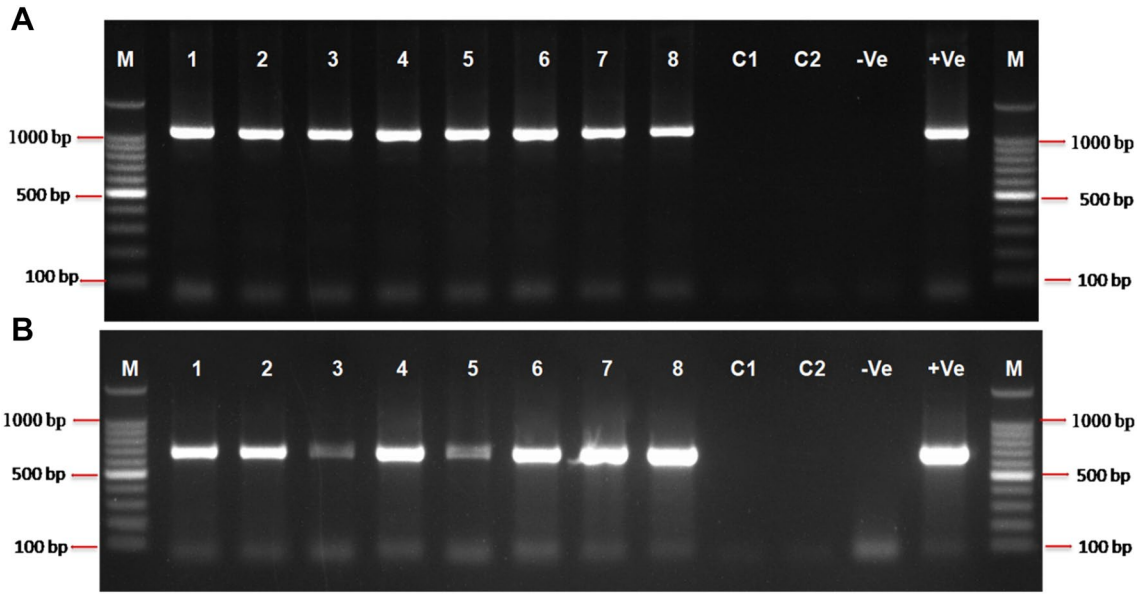

Fig. 6 Validation of RT-qPCR assay using RT-PCR of few representative field samples: **A** Amplified PCR product of ICRSV coat protein gene; **B** Amplified PCR product of ICRSV nucleic acid binding gene: Lane M, 100 bp Ladder; Lane 1–8, Field samples; Lane C1–C2, Healthy Plant; Lane −Ve, non-template control; and Lane + Ve, ICRSV positive control

by various researchers for the detection of citrus pathogens, including CYMV (Mothgare et al. 2018), CTV (Bertolini et al. 2008), CYVCV (Chen et al. 2016); '*C*Las (Ghosh et al. 2018a), *Xanthomonas axonopodis* (Cubero et al. 2001); citrus phytoplasma (Ito and Suzaki 2017) and *Guignardia citricarpa* (Van Gent-Pelzer et al. 2007).

In the present study, the SYBR Green-mediated ICRSV-RT-qPCR assay was developed using total genomic RNA extracted from known ICRSV infected plants maintained in screen houses and further validated with field samples. To specify the virus copy number, we used the in vitro synthesized cRNA standard to produce the standard curve of the tenfold serially diluted cRNA. Prior to this study, in vitro synthesized cRNA was utilized for generating standard curve and calculation of the virus copy number (Chen et al. 2016). SYBR Green dye binds non-specifically to any double-stranded DNA or primer dimer, and there is, therefore, need to design target gene-specific primers to get single amplicons and sharp melt curve peak analysis. To enhance the specificity of the RT-qPCR assay in this study, optimization of primer concentrations was crucial to prevent non-specific amplification of other irrelevant gene products. The outcomes are usually examined in terms of amplification plots combined with melting curves to discriminate non-specific amplification. The result suggests that the reaction was very efficient and there was minimum variation in PCR kinetics. The specificity of the RT-qPCR assay was analyzed using ICRSV isolates along with other major citrus pathogens present in India. It was observed that no specific amplification signals were observed in all reactions with. Furthermore, the specificity of the RT-qPCR primers was also, studied by in silico analysis through primer BLAST, and it was observed that the primers are specific to the target. Overall, the designed assay is very specific to detection of ICRSV and did not cross-react with any other major citrus pathogens. For the additional authentication, the developed assay was cross verified using conventional RT-PCR, which showed the target amplification of the ICRSV. A similar outcome was revealed by Motghare et al. (2018).

The RT-qPCR assay is a more powerful technique that usually provides a higher sensitivity than conventional PCR. Furthermore the post-PCR processing step is not required, which saves both time and reagents. The RT-qPCR was found to be up to 100 times more sensitive than conventional RT-PCR. Similar sensitivity was observed by other researchers (Ruiz-Ruiz et al. 2007). In conclusion, SYBR Green-based RT-qPCR assay is the most powerful, reliable, sensitive, and reproducible assay for early detection of ICRSV. Other benefits of SYBR Green are the relative simplicity and the reduction of operating costs as compared to probe-based chemistry which might be more suitable for use in laboratories. The developed RT-qPCR assay will be useful for the citrus budwood certification program to produce virus-free planting material in nurseries, small laboratories, Krishi Vigyan Kendras (KVKs), and other agriculture centers in India. Additionally, it will be useful at quarantine centers to prevent the spread of plant viruses from India to other countries.

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Author contributions DK, AK, KL, and PM conceived and designed the experiments. AK, SK, and MG performed the experiments and analyzed the data. DK and AK wrote and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and material The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

Consent for publication I, the undersigned, give my consent for the publication of identifiable details, which can include photograph(s) and details within the text ("Material") to be published in the 3 Biotech. Therefore, anyone can read material published in the Journal.

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