GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Quantitative detection of the rice false smut pathogen *Ustilaginoidea virens* by real-time PCR

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Genet. Mol. Res. 12 (4): 6433-6441 (2013)
Received April 25, 2013
Accepted September 15, 2013
Published December 10, 2013
DOI http://dx.doi.org/10.4238/2013.December.10.4

ABSTRACT. Rice false smut (RFS) is an important rice disease that is caused by the pathogen *Ustilaginoidea virens*. In this study, we developed a real-time polymerase chain reaction (PCR) assay to detect *U. virens* and to estimate the level of disease. The genomic DNAs of *U. virens* and rice were extracted together from the rice samples. Real-time PCR assays were performed and compared to conventional nested-PCR assays. The real-time PCR assay presented a consistent linearity of the standard curve (R^2 = 0.9999). The detection limit could be as low as 40 fg *U. virens* DNA with a rice genomic DNA background on using the real-time PCR assay, which

showed significantly higher sensitivity than the conventional nested-PCR assay. We conclude that the real-time PCR quantitative assay is a useful tool for detecting *U. virens* and for early defense and control of RFS.

Key words: Rice false smut; Real-time PCR; Detection; Quantification; *Ustilaginoidea virens*

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for half of the world's population, especially in Asian countries. Rice false smut (RFS), which is caused by *Ustilaginoidea viren*s (Cke.) Tak., is one of the most common and serious diseases in rice-growing areas of the world (Ou, 1972; Tanaka and Tanaka, 2008). The disease was first reported in Tirunelveli District of Tamil Nadu State of India in 1878 (Ou, 1972). The RFS disease symptoms appear on the spikelets at maturity. The diseased spikelets, the so-called green balls, are covered with powdery dark-green chlamydospores (Ou, 1972). Outbreaks of the disease could lead to yield loss and reduced grain quality (Lu et al., 2009). In addition, ustiloxins derived from false smut balls are toxic to animals and are a potential source of food contamination (Koiso et al., 1994).

RFS had been considered to be a minor disease because of its infrequent occurrence. However, in recent years, it has become a serious grain disease because of the apparent change in climate, widespread cultivation of high nitrogen fertilizer-responsive varieties, and excessive application of nitrogen. In China, RFS has become a serious fungal disease on rice since the 1980s because *japonica* rice varieties are widely cultivated, most of which are very susceptible to RFS and are high nitrogen fertilizer-responsive cultivars (Ji and Zheng, 1995). There have been many reports of severe outbreaks of RFS since 2001 in many rice-growing provinces of China, such as Liaoning, Hubei, Sichuan, and Anhui; the yield loss ranged from 20 to 50% in different areas and varied with rice varieties. In 2005, the occurrence area of RFS disease was approximately 330,000 ha and a third of the panicles in Sichuan were affected (Lu et al., 2009).

Isolation on nutrient-rich media and morphological examinations are the conventional detection and identification methods for *U. virens* (Zhou et al., 2003). However, these procedures are time consuming and nonspecific. Conventional polymerase chain reaction (PCR) and nested-PCR techniques can qualitatively detect *U. virens*. Because of the use of two pairs of amplification primers, the nested-PCR assay has higher sensitivity and specificity than the conventional single-round PCR assay (Zhou et al., 2003). However, it is difficult to quantify the amount of DNA by the conventional PCR or nested-PCR assay. It is necessary to quantify the amount of pathogen DNA when quantitative data about pathogen severity are required. Further, gel electrophoresis, which is the normal tool for analyzing conventional PCR and nested-PCR results, could lead to the detection of contamination or false-positive or false-negative results (Harrison et al., 2002). In contrast, the widely used real-time PCR method has better quantitative performance, greater sensitivity and greater accuracy (Maciel et al., 2011). Real-time PCR can monitor the progression of DNA amplification after each cycle through fluorescence probes (Ginzinger, 2002). In addition, the chances of contamination are obviously reduced compared to that in conventional PCR because amplification and detection occur in a single sealed tube (Nejat et al., 2010). The TaqMan probe was widely used in real-time PCR because it had better sensitivity and reliability than SYBR Green I, a commonly used fluores-

cent DNA-binding dye (Nejat et al., 2010). By using the TaqMan probe to detect the ribosomal DNA (rDNA) of *U. virens*, a linear correlation between cycle threshold (C_T) values and the number of false smut balls in the soil was revealed (Ashizawa et al., 2010). Therefore, real-time PCR can be used to detect and quantify *U. virens*. However, little information is available about the relationship between the amount of *U. virens* DNA and the level of RFS disease.

For rapid and early detection of *U. virens* and quantification the *U. virens* DNA level, a quantitative detection method is urgently needed. Therefore, the aim of this study was to develop a sensitive and reproducible real-time PCR assay to detect, quantify the *U. virens*, and evaluate the level of disease in rice.

MATERIAL AND METHODS

Isolation and culture of *U. virens* and rice plants

An isolate (Uv-A12) of *U. virens* was obtained from a false smut ball produced on a floret of rice that was planted in a paddy field in Hefei, Anhui Province, China. Preparation of the chlamydospore suspension, hyphal culture, and storage were performed according to the method reported by Ashizawa et al. (2010).

To obtain artificially infected rice plants, a conidial suspension (1 x 10^6 conidia/mL) was injected into the flag leaf sheath of a highly susceptible rice cultivar, Wanjing97 (*Oryza sativa* cv. Wanjing97), 10 days before heading at the booting stage. From the time of injection to the maturation of rice seeds, rice spikelets were harvested every 10 days. To obtain rice plants in the field, Wanjing97 seedlings were planted in Jinzhai, Anhui Province (RFS outbreak area); Hefei, Anhui Province (RFS epidemic area); and Sanya, Hainan Province (RFS non-epidemic area). All the seeds were harvested and mixed as the detection samples.

DNA extraction

For *U. virens* or rice DNA extraction, 50 mg frozen mycelia samples of *U. virens* or 50 mg rice spikelet powder of every sample were placed in 1.5-mL microcentrifuge tubes. The DNA extraction procedure has been described in a previous report (Li et al., 2013). Briefly, the sample was suspended in 300 µL extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0; 1 M NaCl; 1.25% sodium dodecyl sulfate; and 1% 2-mercaptoethanol) and vigorously vortexed for 1 min. Next, 150 µL 5 M potassium acetate was added to the tube, and the tube was vigorously vortexed again for 1 min. The suspension was centrifuged for 10 min at 12,000 *g*. Then, 300 µL supernatant was transferred to a new tube with 200 µL isopropanol. The suspension was centrifuged for 10 min at 12,000 *g*. The supernatant was discarded, and the DNA was washed with 600 µL 70% ethanol and air dried on a clean bench. The DNA was dissolved in 150 µL 0.1X TE buffer (1 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0). The DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific,Wilmington, DE, USA) according to the manufacturer protocol.

Real-time PCR primers and probe design

The primers and fluorochrome-labeled probe were designed using the Primer Express

3.0 software (Life Technologies, Carlsbad, CA, USA) based on the 2852-bp rDNA sequences of *U. virens* (GenBank accession No. AB105954). This rDNA region, which is a unique region in the *U. virens* genome and is not present in any of the other organisms, allowed for the design of specific primers and a probe for real-time PCR (Ashizawa et al., 2010). The primers and probe were synthesized (Life Technologies), and their sequences were shown in Table 1. The probe was labeled at the 5' terminus with fluorescein (FAM) and near the 3' terminus with a dark quencher dye (TAMRA). The primer pair amplified a product that was 84 bp in length.

Table 1. Oligonucleotide primers and TaqMan probe sequence used for real-time PCR detection.

Primer or probe	Sequence (5' to 3')
RFS-F	GCGAGAGGATGCTACAATGGA
RFS-R	AATCCGCAGCCAAGCCCCTACG
Probe	GCATACGGGGAAGGTTCAGA

Standard curves and real-time PCR conditions

To obtain the real-time PCR detection limits for *U. virens* DNA, a standard curve based on the threshold cycle (C_T) for a dilution series of pure *U. virens* DNA (1, 0.5, 1 x 10^{-1}, 1 x 10^{-2}, 1 x 10^{-3}, and 1 x 10^{-4} ng/µL, diluted with 0.1X TE buffer) was constructed and triplicate real-time PCR was performed. In order to quantitatively detect *U. virens* DNA in the actual rice samples, another standard curve based on the C_T for the same dilution series of *U. virens* DNA (pure *U. virens* DNA diluted with 20 ng/µL negative-control rice spikelet DNA solution) was also constructed. The negative control rice samples were planted in the sterile culture room.

Real-time PCR was performed on an Applied Biosystems 7500 Real Time PCR System (Life Technologies). The amplification was carried out in a total volume of 25 µL, which contained 500 nM each primer, 200 nM probe, 12.5 µL ABGene Absolute QPCR Rox Mix (Thermo Scientific), 5 µL DNA template (different dilution samples), and nuclease-free water. The amplification was carried out using the following program: 5 min at 95°C for pre-denaturation and then 50 cycles of 10 s at 95°C and 30 s at 60°C. The C_T values were automatically calculated with the 7500 software version 2.0.5 (Life Technologies). The standard curves were obtained by plotting the C_T value versus the logarithm of the concentration of each dilution of *U. virens* DNA.

Quantitative detection of different samples by real-time PCR

DNA from different artificially infected rice plants or field rice plants were extracted and analyzed by real-time PCR using the primer pair and probe. The DNA concentration of each rice sample was diluted to 20 ng/µL with 0.1X TE buffer. The quantity of *U. virens* DNA in a rice sample was calculated using the standard curve. For comparative purposes, the DNA samples were also analyzed by a nested-PCR assay according to the method of Zhou et al. (2003).

Comparison of the real-time PCR assay and the pathogenicity test

In order to compare the results of the real-time PCR assay and the pathogenicity test

of *U. virens*, artificially infected rice plants were also infected by the dilution series of the conidial suspension (10^5, 10^6, 10^7, and 10^8 conidia/mL). At least 10 panicles were inoculated for each treatment. Fifty days after the artificial injection, the number of smut balls per panicle was counted. Rice panicle samples from all treatments were harvested and mixed. Then, DNA was extracted and analyzed by real-time PCR by using the primer pair and probe.

RESULTS

DNA extraction

The DNA quality is critical for reproducible results in the real-time PCR assay (Cankar et al., 2006). *U. virens* and rice have a different cell structure. Different DNA extraction methods were used in many studies (Ashizawa et al., 2010; Nejat et al., 2010). However, different DNA extraction methods would influence the quantitative results of the real-time PCR (Tilburg et al., 2010). In this study, *U. virens* and rice genomic DNA were extracted individually or together by the same extraction method. Typically, DNA samples have an A_{260}/A_{280} ratio >1.8 and A_{260}/A_{230} ratio >2.0 (Table 2), which suggests that the DNA quality is sufficient to further examination. The A_{260}/A_{280} ratio ranged from 1.92 to 2.06, and the A_{260}/A_{230} ratio ranged from 2.03 to 2.14 (Table 2). These results indicated that the DNA had little contamination of proteins, polysaccharides, and polyphenols. These contaminants, especially polyphenols, would inhibit the enzymatic activity of the DNA polymerase and affect the PCR amplification efficiency (Li et al., 2013).

Table 2. Concentrations, purities of DNA of *Ustilaginoidea virens* and the representative rice samples DNA.

Samples	DNA concentration (ng/μL)	A_{260}/A_{280}	A_{260}/A_{230}
U. virens	39.08 ± 7.51	1.95 ± 0.06	2.04 ± 0.07
Rice-T_0	83.08 ± 9.13	2.06 ± 0.05	2.14 ± 0.05
Rice-T_1	77.55 ± 8.43	1.99 ± 0.03	2.09 ± 0.05
Rice-T_2	75.32 ± 6.33	1.95 ± 0.06	2.05 ± 0.04
Rice-T_3	71.80 ± 5.22	1.98 ± 0.04	2.08 ± 0.08
Rice-T_4	65.28 ± 7.86	1.92 ± 0.07	2.03 ± 0.06

Data are reported as means ± SD, N = 8; Rice-T_0 = rice samples sampled before artificial injection; Rice-T_1 = rice samples sampled after artificial injection 10 days; Rice-T_2 = rice samples sampled after artificial injection 20 days; Rice-T_3 = rice samples sampled after artificial injection 30 days; Rice-T_4 = rice samples sampled after artificial injection 40 days.

Standard curves for real-time PCR

In order to quantitatively detect *U. virens* DNA in the actual rice samples, the standard curves were generated using *U. virens* DNA concentrations that ranged from 0.1 pg/μL to 1 ng/μL by 2 different dilution methods. The real-time PCR detection limit for *U. virens* DNA was 0.1 pg/μL (1 x 10^{-4} ng/μL) (Figure 1). The curves of the 2 different dilution methods are described by y = -3.3692x + 19.583 (*y* is the C_T, and *x* is the logarithm of the starting *U. virens* DNA concentration) and y = -3.2341x + 20.515. The R^2 value (coefficient of determination) was 1 for the samples diluted with 0.1X TE buffer and 0.9999 for the samples diluted with a negative-control rice spikelet DNA solution. The *U. virens* DNA concentration and C_T were highly correlated for the primer pair and probe.

Standard curves have been constructed in other studies by using pure pathogen DNA or recombinant plasmid DNA diluted with water (Ashizawa et al., 2010; Nejat et al., 2010). Because the components of the practical rice samples of DNA are different from those of the pure pathogen DNA or recombinant plasmid DNA samples, using these artificial standard curves to quantify the amount of DNA in the actual samples might result in deviations between the real-time PCR assay results and the actual amount. We observed that the negative-control rice spikelet DNA dilution method provided a higher C_T value than the 0.1X TE dilution method at the same *U. virens* DNA concentration (Figure 1), which suggested that PCR replication was affected by the other components that were in the practical samples. Therefore, the results would be more accurate using a standard curve based on DNA samples that were diluted with rice genomic DNA.

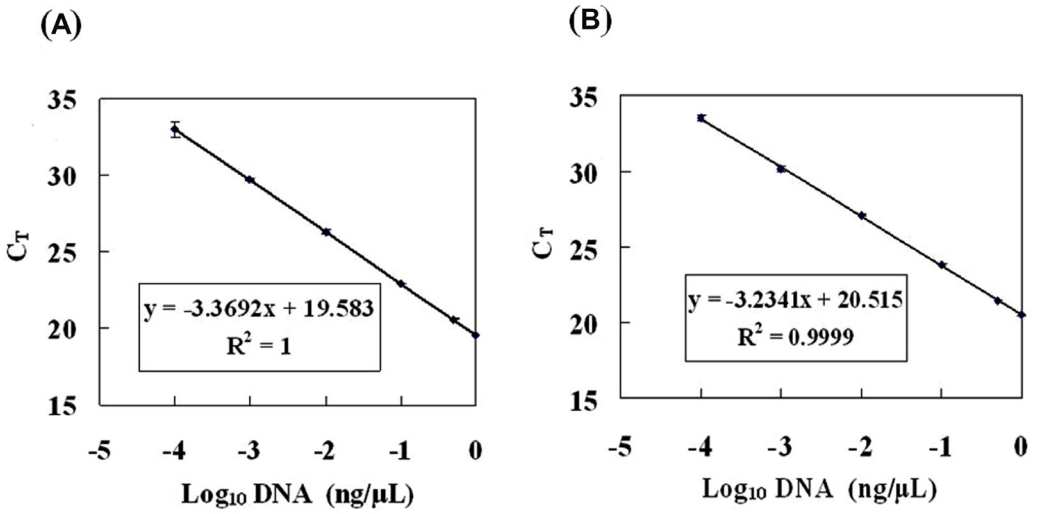

Figure 1. Standard curve for *Ustilaginoidea virens* DNA in real-time PCR assay. (**A**). Pure *U. virens* DNA diluted by 0.1X TE buffer. (**B**). Pure *U. virens* DNA diluted by 20 ng/μL negative control rice spikelets DNA solution. (Each point represents the mean of three replicates. Error bars represent the standard deviation.).

Quantitative of *U. virens* by real-time PCR

The mean C_T and quantity of *U. virens* DNA from different artificially infected rice plants or field rice plants are shown in Table 3. The C_T values obtained from different artificially infected rice plants or field rice plants ranged from 26.76 to 34.79, whereas the negative control had C_T values above 38 (Table 3). In the real-time PCR and nested-PCR assays, the detection limits of *U. virens* DNA were 40 fg (3.85×10^{-5} ng/μL) and 850 fg (8.53×10^{-4} ng/μL), respectively (Table 3). Overall, the real-time PCR assay showed significantly higher sensitivity for detecting the *U. virens* DNA than the conventional nested-PCR assay.

Comparison of the real-time PCR assay and the pathogenicity test

The mean C_T values and smut balls per panicle from the rice plants infected by dif-

ferent concentrations of a conidia suspension are shown in Table 4. The concentrations of the conidia suspension ranged from 10^5 to 10^8 conidia/mL, the relevant C_T values ranged from 27.48 to 23.62, and the average number of smut balls per panicle ranged from 2.5 to 8.6 (Table 4). In other words, the *U. virens* DNA concentration obtained from the real-time PCR assay was consistent with the average number of smut balls per panicle.

Table 3. Real-time PCR and nested-PCR results for the representative samples.

Samples	Replicates	Mean C_T	Nested-PCR	*U. virens* DNA concentration (ng/μL)*
Rice-T_0	3	37.34	-	-
Rice-T_1	3	34.79	-	3.85×10^{-5}
Rice-T_2	3	32.55	-	1.90×10^{-4}
Rice-T_3	3	30.44	+	8.53×10^{-4}
Rice-T_4	3	28.21	+	4.18×10^{-3}
Rice-SY	3	37.55	-	-
Rice-HF	3	29.43	+	1.75×10^{-3}
Rice-JZ	3	26.76	+	1.17×10^{-2}
Positive control	3	20.51	+	1.0
Negative control	3	38.62	-	-

The symbol (+) means where the product was detected, and the symbol (-) means no product detected. *DNA concentration calculated from the standard curve (Figure 1B) and is based on the average of 3 independent estimates for each replicate. Rice-T_0 = rice samples sampled before artificial injection; Rice-T_1 = rice samples sampled after artificial injection 10 days; Rice-T_2 = rice samples sampled after artificial injection 20 days; Rice-T_3 = rice samples sampled after artificial injection 30 days; Rice-T_4 = rice samples sampled after artificial injection 40 days; Rice-SY = rice samples sampled from Sanya of Hainan province (RFS non-epidemic area); Rice-HF = rice samples sampled from Hefei of Anhui Province (RFS epidemic area); Rice-JZ = rice samples sampled from Jinzhai of Anhui Province (RFS outbreak area); Positive control = pure *U. virens* DNA diluted by 20 ng/μL negative control rice spikelets DNA; Negative control = negative control rice spikelets DNA.

Table 4. Comparison of real-time PCR assay and pathogenicity test of *Ustilaginoidea virens*.

Concentration of conidia suspension	Mean C_T	*U. virens* DNA concentration (ng/μL)*	Average number of smut balls per panicle
10^5 conidia/mL	27.48	7.02×10^{-3}	2.5
10^6 conidia/mL	26.29	1.64×10^{-2}	3.2
10^7 conidia/mL	25.05	3.96×10^{-2}	5.3
10^8 conidia/mL	23.62	1.10×10^{-1}	8.6

*DNA concentration calculated from the standard curve (Figure 1B) and is based on the average of 3 independent estimates for each replicate.

DISCUSSION

The reliability of real-time PCR could be influenced by many factors, such as DNA quality, primers, and the probe (Bustin et al., 2009). Different DNA extraction methods could influence the DNA quality, which could affect PCR amplification (Tilburg et al., 2010). In order to obtain reproducible results and eliminate the influence of the DNA extraction method (Cankar et al., 2006), we used the same extraction protocol for DNA isolation from *U. virens* and rice samples. The standard curve of the DNA dilutions showed linearity ($R^2 = 0.9999$) in the real-time PCR assay; this result indicated that the quality of the DNA was suitable for the real-time PCR assay. The primer pair and probe in this study showed better detection results than the universal probe from the universal probe library (data not shown) (Ashizawa et al.,

2010). Moreover, the primers that amplified short DNA fragments showed better results than those that amplified long DNA fragments in the real-time PCR assay (Tilburg et al., 2010). The primer pair in this study amplified a product that was 84 bp in length. Taken together, the PCR system that we established is reliable and sensitive.

Relative and absolute quantification are widely used strategies to estimate DNA concentrations from C_T values. Absolute quantification requires the construction of a standard curve, which involves the amplification of known amounts of target DNA. Plasmid DNA is often used to construct a standard curve because of its high quality and purity. For absolute quantification, the assumption that plasmid DNA and target genomic DNA amplify with the same efficiency must be made (Sivaganesan et al., 2008). In fact, many factors such as DNA stability, complexity, and secondary structure could significantly affect the amplification (Sivaganesan et al., 2008). Therefore, using the standard curve constructed with plasmid DNA would affect the quantification target DNA template that is complex genomic DNA instead of plasmid DNA. Moreover, a standard curve of genomic DNA isolated from actual rice samples is better fit to quantify the DNA amount than a standard curve of DNA isolated from pure cultures (Martín et al., 2006). In this study, *U. virens* DNA was diluted using a solution of the negative control rice spikelets DNA to construct the standard curve. It is fit to quantify the amount of *U. virens* DNA in the actual samples.

The conventional nested-PCR assay has been used to detect *U. virens* in rice (Zhou et al., 2003). The nested-PCR assay is less sensitive than the real-time PCR assay and cannot be used to quantify the amount of target DNA; therefore, it can only be used to indicate the presence or absence of the target DNA. The real-time PCR assay has the advantage of calculating the absolute quantity of starting target DNA within 2 h. The real-time PCR assay has been employed for universal phytopathogen detection based on the 16S ribosomal RNA (rRNA) gene and 23S rRNA gene (Nejat et al., 2010). Ashizawa et al. (2010) developed a real-time PCR assay to clarify the relationship between the false smut occurrence on rice and the quantification of *U. virens* from soil in Japan. As a result, the real-time PCR technology provides new opportunities to detect and study *U. virens* and other plant phytopathogens.

In conclusion, the method for quantifying *U. virens* DNA in this study is more sensitive and reproducible than the conventional nested-PCR assay, in both artificially and naturally infected rice samples. The *U. virens* DNA concentration obtained from the real-time PCR assay was consistent with the average number of smut balls per panicle. The real-time PCR assay will be a useful tool for rapidly diagnosing and monitoring *U. virens*. Additionally, it would be beneficial with respect to breeding the RFS-resistant rice variety.

ACKNOWLEDGMENTS

Research supported by the National Key Technology R&D Program of China (grant #2012BAD07B01), Youth Foundation of President of the Anhui Agricultural Academy of Sciences (#13B0101, #13B0102, #13B0113, #13B0114), and Creative Team Foundation of the Anhui Agricultural Academy of Sciences (#13C0101).

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