Eur J Plant Pathol
DOI 10.1007/s10658-015-0599-6

TaqMan real-time PCR assay for the detection and quantification of *Sclerospora graminicola*, the causal agent of pearl millet downy mildew

Bandamaravuri Kishore Babu · Rajan Sharma

Accepted: 20 January 2015

Abstract Early detection and quantification of *Sclerospora graminicola*, causal agent of pearl millet downy mildew, from seed, plant and infested soil could help in initiating preventive measures to control the spread of the disease. Two sensitive assays, SgK PCR (conventional PCR) and SgTqK q-PCR (real-time PCR), were developed for the detection of *S. graminicola*. Both assays were targeted on the 28S region of the nuclear large subunit (nuLSU) of the rDNA cluster. In conventional PCR, the species-specific primers (SgK F/R) amplified a 436 bp product in *S. graminicola*. TaqMan real-time PCR specifically amplified an 86 bp fragment in diverse DNA samples of *S. graminicola* obtained from infected seed, root, leaves and infested soil. Both assays did not amplify or produce any fluorescent signal from DNA obtained from other test microbes or from healthy pearl millet leaves. The absolute quantity of target molecules (28S) in the DNA sample obtained from *S. graminicola* sporangia (6×10^8/ml) was estimated at 25 pg/μl and the copy number was calculated as 2.69×10^8 molecules/μl. The copy number of target molecules in a diploid nucleus (2n) of *S. graminicola* was estimated to be ~27 molecules/nucleus. This assay can be used as a rapid and efficient detection tool for the identification and diagnosis of *S. graminicola*, and will be helpful in ensuring safe exchange of *S. graminicola*-free pearl millet germplasm across borders.

Keywords q-PCR · Species-specific primers · Downy mildew · Obligate pathogen · *Sclerospora graminicola*

Introduction

Downy mildew caused by *Sclerospora graminicola* (Sacc.) Schroet is a widespread disease of pearl millet (*Pennisetum glaucum* (L.) R. Br). The disease is severe in the arid and semi-arid tropics of Asia and Africa, where pearl millet provides food security for millions of people (Thakur 2008). Grain yield losses of 10–60 % due to *S. graminicola* have been reported in various countries of Asia and Africa (Singh et al. 1993). In India, 9.8 million hectares of pearl millet is cultivated with an annual production of 7.01 million tons (Khairwal 2008). Downy mildew epidemics caused substantial yield losses in F_1 hybrids in India during 1970–76, when grain yield losses as high as 60–70 % were recorded (Singh et al. 1993).

The primary infection of pearl millet seedlings is caused by resting oospores present in the soil and by sporangia that are produced on the lower surface of diseased leaves. The sporangia release zoospores, which disperse to cause secondary infection on plants in the neighbouring fields and generally cause systemic infection early in crop growth (Thakur et al. 2011). Early detection of the pathogen in the soil and/or plant

B. K. Babu · R. Sharma (✉)
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, 502324 Hyderabad, Telangana, India
e-mail: r.sharma@cgiar.org

Published online: 06 February 2015

material at an early stage of crop growth might thus permit the use of preventive measures to halt the spread of the pathogen (Blanco and Ristaino 2011).

Development of molecular diagnostics for plant pathogens may help pathologists to monitor the global spread of pathogens through infected plants, propagation materials and postharvest products. Moreover, the quantitative estimation of pathogen populations is essential, not only to predict disease outbreaks, but also for plant quarantine measures (Tooley et al. 2006, 2010). Also, early detection of a pathogen in plant materials and in field-soil is crucial for framing disease management strategies. Conventional methods of identifying pathogens have relied on symptoms and morphological characters which are time consuming to monitor and mostly suited for pathogens that can be cultured on artificial media. Detection is more difficult in the case of obligate pathogens such as downy mildews (Göker et al. 2007), powdery mildews (Braun et al. 2002), rusts (Leonard and Szabo 2005) and smuts (Bauer et al. 2001). A large number of species within the peronosporaceae are obligate biotrophs which cannot be cultured (Göker et al. 2007). Detection of these obligate biotrophs is difficult prior to the expression of disease symptoms on infected plants, and by that time the pathogen has spread and caused severe damage to the crop. Furthermore *S. graminicola* is considered an important quarantine pest which requires strict monitoring for safe movement of germplasm (Thakur et al. 2010). However, effective techniques for the rapid detection of *S. graminicola* are not available. Therefore, development of PCR-based methods are a good option for the identification and early detection of *S. graminicola*.

Real-time PCR (q-PCR) based assays have the advantages of speed, accuracy and sensitivity over other detection techniques (Gachon et al. 2004; Mumford et al. 2006; Schaad and Frederick 2002). In addition, q-PCR has been used to quantify the pathogen in different samples without the need to culture it (Mumford et al. 2006; Vandemark and Ariss 2007). q-PCR is often developed to target conserved region of DNA, including ribosomal DNA (Blanco and Ristaino 2011; Hayden et al. 2006), elongation factor-1α (Mogens et al. 2009), histone 3 (Mesapogu et al. 2011), beta-tubulin (Tooley et al. 2010) and topoisomerase-II genes (Yadav et al. 2011), or organism-specific regions such as sequence characterized amplified regions (SCARs) (Babu et al. 2011), and/or toxin-producing genes (Sarlin et al. 2006). The internal transcribed spacer (ITS) region and the nuclear large subunit (nuLSU) of the rDNA cluster have been widely used for the detection and identification of phytopathogenic fungi as they display species-specific variations and exists as multiple copies in the fungal genome (Braun et al. 2002; Bridge and Spooner 2001; Viaud et al. 2000). Although the LSU region has been used to study phylogenetic relationships of *S. graminicola* (Göker et al. 2007; Riethmüller et al. 2002), there is no report using the region as a tool for molecular detection of this pathogen. The present study describes the development of a rapid and sensitive q-PCR assay for the detection of *S. graminicola*.

Materials and methods

S. graminicola isolates

S. graminicola isolates used in this study were obtained from a pathogen collection being maintained at ICRISAT, Patancheru, Hyderabad, Telangana, India. Ten isolates of *S. graminicola* collected from different geographical locations in India were selected (Table 1). Other fungal and bacterial cultures used as test microbes were obtained from different laboratories.

Sample preparation and DNA extraction

DNA extraction was done using fresh sporangia (Sharma et al. 2010; Babu et al. 2007). The fresh sporangia were collected in 10 ml sterile distilled water (SDW), centrifuged at 3000 rpm for 5 min at 4 °C, discarding the supernatant. Approximately 200 mg of sporangia was added to a 1.5 ml eppendorf tube, crushed with a micro-pestle, mixed with 300 ml lysis buffer (50 mM Tris–HCl, pH 7.8, 50 mM Na_2-EDTA, 3 % SDS) (Babu et al. 2007) and incubated at 65 °C for 1 h. Subsequently, the total genomic DNA was extracted and purified by phenol-chloroform extraction and precipitated with ethanol. Genomic DNA was isolated from bacteria, actinomycetes and other fungal species used in this study using protocols described earlier (Babu et al. 2007, 2011). DNA was extracted from infected and healthy pearl millet leaves and from seed obtained from partially infected (green ear) panicles. About 1 g of each seed and leaf was crushed in liquid N_2 separately followed by thawing in lysis buffer, which was incubated at 65 °C for 1 h. DNA extraction and purification was

carried out as described above. The pure DNA samples were stored at −20 °C.

PCR amplification and sequencing of a partial 28S region of *S. graminicola*

A partial 28S region of *S. graminicola* isolate Sg 445 was amplified using LROR (5′-ACCCGCTGAACTTA AGC-3′) and LR5 (5′-TCCTGAGGGAAACTTCG-3′) eukaryotic primers (Pinnoi et al. 2007). The PCR amplified product was gel purified and subjected to direct sequencing using an ABI automated DNA Sequencer with an ABI Big Dye termination cycle sequencing ready reaction kit (Applied Biosystems, Foster City, California, USA) following the manufacturer's protocol.

Sequence analysis and design of specific primers

Multiple sequence alignment was performed between the partial 28S sequence of *S. graminicola* and closely related oomycetes and fungal species (retrieved from GenBank) using molecular evolutionary genetic analysis software (MEGA 5.1) (Tamura et al. 2011). The aligned sequences were visually checked for the hallmark regions, which were conserved and specific to *S. graminicola*. The hallmark regions were used to design species-specific primers using Primer3 online software (Steve and Helen 2000). Two primer sets named SgK PCR (SgK F/R) and SgTqK q-PCR (SgTqK F/R) were designed for conventional PCR and q-PCR, respectively (Table 2). For the TaqMan q-PCR detection, one internal minor grove binding (MGB) oligonucleotide probe SgTqK P (24 mer) was designed to match the conserved region between the SgTqK F and SgTqK R primers. All the primers were evaluated separately for theoretical specificity, G+C %, 3′-self complementarities, hair pin loop formation and self-dimerization. The primers and dual labelled (quencher and reporter) probe were custom synthesized (Invitrogen Bioservices, Whitefield, Bangalore, India).

Validation of primers by conventional PCR

Specificity of the primers SgK F/R was validated using conventional PCR. Individual PCR amplifications were done with DNA obtained from *S. graminicola* isolates and other test microbes, downy mildew infected plant material and soil samples (Tables 1 and 3). PCR was performed by mixing 2 μl of 10×PCR buffer, 0.2 mM dNTPs, 5 pmol of each primer, 0.4 U of *Taq* DNA polymerase, and 1 μl template DNA; SDW was added to make a final volume of 20 μl. The PCR reaction had initial denaturation at 95 °C for 1 min followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 60 °C for 30 s, extension at 72 °C for 45 s, and a final extension step of 72 °C for 10 min.

To confirm the sequence similarity among *S. graminicola* isolates, partial 28S segments from 10 different isolates were amplified with species-specific primers (SgK F/R) (Table 1). The PCR products (436 bp) were sequenced as described above and multiple sequence alignment was performed among all 10 sequences obtained from the *S. graminicola* isolates. For SgK PCR validation, individual PCR assays were conducted in which the genomic DNA of *S. graminicola* was used as a positive control and DNA from the other test microbes used as unknown targets. All the PCR assays were analyzed in duplicate reactions and repeated to confirm the results.

SgTqK real-time PCR

q-PCR assays were performed using a model 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA) using forward (SgTqK F) and reverse (SgTqK R) primers and a TaqMan probe SgTqK P (Table 2). All reactions were performed in 96 well plates in a total reaction volume of 20 μl. The reaction mixture consisted of 1 μl template DNA, 1X TaqMan© universal master mix (Applied Biosystems, Branchburg, New Jersey, USA), 0.6 μl of each forward and reverse primer (100 pmol/μl), 0.2 μl probe (100 pmol/μl); and nuclease-free water was added to make a final volume of 20 μl. The q-PCR thermal cycling conditions were an initial soak for 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. All the PCR assays had three replicates and were repeated to confirm the results.

Sensitivity and standard curve analysis of SgTqK q-PCR

A standard curve was constructed based on the cycle threshold (C_T) values from a 10-fold dilution series (10^{2}–10^{-5} ng/μl) with three replicates for each concentration of DNA. Gel-purified SgK PCR product (436 bp) generated from *S. graminicola* genomic

Table 1 Microorganisms tested in conventional PCR and q-PCR detection assays

Name	Origin/source	Conventional PCR/q-PCR results[a]	GenBank accession
Sclerospora graminicola, Sg 019*	ICRISAT, Patancheru, Telangana, India	+	JX569258
S. graminicola, Sg 334*	Bhiwani, Haryana, India	+	JX569259
S. graminicola, Sg 384*	Barmer, Rajasthan, India	+	JX569260
S. graminicola, Sg 457*	Jaipur, Rajasthan, India	+	JX569261
S. graminicola, Sg 520*	Bhiwani, Haryana, India	+	JX569262
S. graminicola, Sg 526*	Jodhpur, Rajasthan, India	+	JX569263
S. graminicola, Sg 530*	Aurangabad, Maharashtra, India	+	JX569264
S. graminicola, Sg 542*	Aurangabad, Maharashtra, India	+	JX569265
S. graminicola, Sg 561*	IARI, New Delhi, India	+	JX569266
S. graminicola, Sg 445	Banaskantha, Gujarat, India	+/+	JX569267
Fusarium oxysporum	ICRISAT, Patancheru, Telangana, India	−/−	–
Fusarium thapsinum	ICRISAT, Patancheru, Telangana, India	−/−	–
Fusarium proliferatum	ICRISAT, Patancheru, Telangana, India	−/−	–
Curvularia lunata	ICRISAT, Patancheru, Telangana, India	−/−	–
Macrophomina phaseolina	ICRISAT, Patancheru, Telangana, India	−/−	–
Colletotrichum graminicola	ICRISAT, Patancheru, Telangana, India	−/−	–
Pyricularia grisea	ICRISAT, Patancheru, Telangana, India	−/−	–
Alternaria alternata	ICRISAT, Patancheru, Telangana, India	−/−	–
Aspergillus flavus	ICRISAT, Patancheru, Telangana, India	−/−	–
Sclerotium rolfsii	ICRISAT, Patancheru, Telangana, India	−/−	–
Phytophthora sp. (castor)	Directorate of Oilseeds Research, Hyderabad, Telangana, India	−/−	–
Phytophthora sp. (sunflower)	Directorate of Oilseeds Research, Hyderabad, Telangana, India	−/−	–
Puccinia substriata var. *indica*	ICRISAT, Patancheru, Telangana, India	−/−	–
Peronosclerospora sorghi (sorghum isolate)	ICRISAT, Patancheru, Telangana, India	−/−	–
Peronosclerospora sorghi (maize isolate)	ICRISAT, Patancheru, Telangana, India	−/−	–
Escherichia coli, JM101strain	…	−/−	–
Mesorhizobium ciceri	ICRISAT, Patancheru, Telangana, India	−/−	–
Streptomyces sp. MMA32	ICRISAT, Patancheru, Telangana, India	−/−	–
Pseudomonas sp. SRI360	ICRISAT, Patancheru, Telangana, India	−/−	–
Metarhizium anisopliae	ICRISAT, Patancheru, Telangana, India	−/−	–
Acinetobacter sp. SRI 305	ICRISAT, Patancheru, Telangana, India	−/−	–
Streptomyces sp. KAI 27	ICRISAT, Patancheru, Telangana, India	−/−	–

[a] −= no amplification, +=amplification

*Isolate used only for conventional PCR

DNA was used as a target for the standard. The standard DNA concentration (100 ng/μl) was adjusted using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA) and a standard curve was plotted with serially diluted (1:10) standard DNA of up to eight orders of magnitude. The sensitivity or minimum detection limit of the assay was estimated so as to quantify and detect the lowest amount of target DNA, when the cycle threshold was attained with up to 40 cycles. All the individual SgTqK q-PCR assays were repeated to confirm the results.

Table 2 Species-specific oligonucleotides developed for conventional PCR (SgK PCR) and quantitative real-time PCR (SgTqK q-PCR) based assays for the detection of *Sclerospora graminicola*

Name and sequence (5′−3′)	Assay developed
SgK F−CGGCGAATGTGTGCGTGCG (19 mer) SgK R−GCCCTTCACGTTAATTACGCGTCC (24 mer)	SgK PCR (conventional PCR)
SgTqK F−CGGCGAATGTGTGCGTGCG (19 mer) SgTqK R−ACAGGGCACCAGCAAGGAAGC (21 mer) SgTqK P−6-FAM-GCGGCTTTTTGGCTGCGCTTGGTA-MGBNFQ (24 mer)	SgTqK q-PCR (q-PCR)

FAM- 6-carboxy-fluoroscein, *MGBNFQ*, minor groove binder non-fluorescent quencher

SgTqK q-PCR assay validation

The species specific q-PCR assay was evaluated for *S. graminicola* sporangia (asexual spores) and oospores (sexual spores).

Assay for sporangia

Sporangia were obtained from isolate Sg 445 as described above. The sporangia were suspended in PBT (Phosphate buffer+1 % Tween 20) and the concentration adjusted to 6×10^8 sporangia/ml using a haemocytometer and the DNA was isolated as described above.

Assay for oospores

For oospore formation, mixed sporangial inoculum (1:1) of two self-sterile isolates of different mating types, Sg 445 (*Mat*-1) and Sg 019 (*Mat*-2), was sprayed on 48 h old seedlings of the susceptible pearl millet genotype 7042S. The seedlings were incubated at 20 °C at>90 % relative humidity (RH) for 20 h, and susequently transferred to an isolation chamber in a greenhouse for disease development. Infected leaves were allowed to mature for oospores formation. Dried leaf samples infested with oospores were collected, powdered and the oospores were counted (Pushpavathi et al. 2006). The powdered leaf sample (10 g) was mixed with 100 g fine sterilized soil (25 % moisture) and incubated for 35 days. After incubation, 1 g soil (oospore infested soil) was collected for DNA extraction. Both the oospores infested soil and the dry leaf samples (1 g each) were crushed in liquid N_2 followed by thawing in lysis buffer and incubation at 65 °C for 1 h. DNA extraction and purification was carried out as described above.

Target/template description

The q-PCR assays were run for the DNA samples obtained from sporangia, oospores (infected dry leaves), test microbes, infected plant material, oospore infested soil and healthy leaves. The sporangial DNA of

Table 3 Evaluation of species-specific PCR and q-PCR assays

Source of DNA sample	SgK PCR[a]	SgTqK q-PCR assay	
		Mean of C_T value[d]	DNA conc. (ng/µl)/ Copy no. of target molecules/µl
Sporangial suspension[b]	+	21.2	$0.025/2.69\times10^8$
Downy mildew infected plant/field samples			
Seed from infected panicles	−	35	$0.00001/1.02\times10^5$
Infected leaves	+	25.2	$0.007/7.13\times10^7$
Infected stems and roots	−	32	$0.0002/2.04\times10^6$
Oospore infested soil	−	33	$0.007/7.13\times10^7$
Dry leaf sample[c]	−	29.5	$0.002/2.04\times10^7$

[a] −= no amplification, += amplification

[b] 6×10^8 sporangia/ml from isolate Sg 445

[c] 1×10^2 oospores/cm^2

[d] SD of C_T=1

S. graminicola (isolate Sg 445) was used as positive control; while DNA from healthy leaves of pearl millet line 7042S was used as negative control. All PCR reactions were replicated three times alongside controls of the DNA standards under the same PCR conditions employed for the standard curve. All the PCR assays were repeated to confirm the results.

Data analysis

The data obtained from different q-PCR assays were analyzed using Detection 1.2 software (Applied Biosystems, Foster City, California, USA). The mean of the concentration of target DNA in each sample was measured in two separate q-PCR runs with three replicates. In order to determine the cycle threshold value (C_T) a base line was drawn from the amplification plot to normalize the fluorescence emission intensity of the reporter dye (Rn) with reference to the fluorescence emission intensity of the passive reference dye. The mean C_T value of each run was calculated and the standard deviation was estimated. The absolute quantification of target DNA in a given sample was estimated through the standard curve. The copy number of 28S target molecules in the DNA samples was calculated from the DNA concentration obtained in comparison to the standard curve using the equation described by Linde et al. (2009) or using the online DNA copy number calculator (http://endmemo.com/bio/dnacopynum.php).

Results

28S amplification, sequence analysis and design of specific oligonucleotides

A partial 28S segment (1101 bp) was amplified from the *S. graminicola* isolate Sg 445 using LROR and LR5 primers. Multiple sequence alignment of the 28S region of isolate Sg 445 with other related oomycetes and fungal species revealed several unique regions that are highly conserved in *S. graminicola*. These hallmark regions were used for the development of species-specific primers. For the conventional PCR assay, two specific oligonucleotide primers (SgK F and SgK R) were designed to amplify a 436 bp product (Table 2). For q-PCR detection and quantification, a set of species-specific primers (SgTqK F/R) was designed to amplify an 86 bp product and a TaqMan probe SgTqK P (24 mer) was designed to bind adjacent to the SgTqK F primer on the forward strand (Table 2).

Specificity of the SgK PCR assay

With the conventional PCR, the species-specific primers (SgK F/R) amplified a 436 bp product in all the *S. graminicola* isolates tested (Fig. 1). The sequences of the PCR products of ten different *S. graminicola* isolates showed>99 % sequence identity (data not shown). All the sequences obtained in this study were submitted to the NCBI GenBank database (Table 1). In the SgK PCR validation assays, the primers specifically amplified a 436 bp product from the DNA samples obtained from the downy mildew infected pearl millet leaves and from sporangia of *S. graminicola* (positive control). However, no PCR of DNA from the test microbes, infected stems/roots, soil/seed samples or from the healthy pearl millet samples resulted in any amplification (Tables 1 and 3).

Standard curve analysis and sensitivity of SgTqK q-PCR

Under optimized conditions, the q-PCR assay showed an exponential increase in fluorescence representing amplification of a PCR product. A standard curve was established over eight orders of magnitude using standard DNA at an initial concentration of 100 ng/μl (Fig. 2a), and a corresponding copy number of 28S target molecules calculated as 1.13×10^{12} molecules/μl. An amplification plot was drawn between delta Rn (ΔRn) and cycle number; a threshold base line was drawn for the normalized reporter dye (Rn) fluorescence signal at ΔRn 0.148 (Fig. 2b). The lowest detection limit of the assay was 10 fg/μl, obtained at a C_T value of 35.0 (Table 3). The standard curve provided a linear range and high correlations between C_T values and DNA quantity (R^2=0.997).

Detection and quantification assays

In the quantification assay, the target 28S molecule concentration in *S. graminicola* DNA obtained from

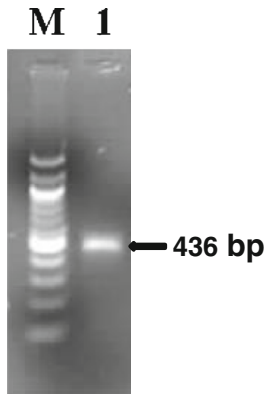

Fig. 1 Validation of the SgK PCR assay: PCR amplification of *S. graminicola* (isolate Sg 445) showing the 436 bp product in lane 1. M=100 bp DNA ladder

the sporangial solution (6×10^8 sporangia/ml) was estimated to be 25 pg/µl using the standard curve (Table 3). The absolute quantity of 28S (86 bp) copy number was calculated as 2.69×10^8 molecules in 25 pg/µl of target DNA. Serial dilutions of sporangial DNA provided a linear range of the standard curve between C_T value and copy number. The minimum detection limit and/or minimum copies of target molecules (28S) in sporangial DNA was achieved at 26.9 molecules/µl (Fig. 3). The specificity of both assays (SgTqK q-PCR and SgK PCR) was demonstrated by no fluorescent signal or DNA amplification in any of the test microbes (Table 1) or in the negative control (healthy pearl millet DNA). Exponential amplification of the fluorescent signal was obtained only from samples of downy mildew-infected pearl millet (leaf, root, stem and seed material), and from oospore samples with C_T values between 21 and 35 (Table 3).

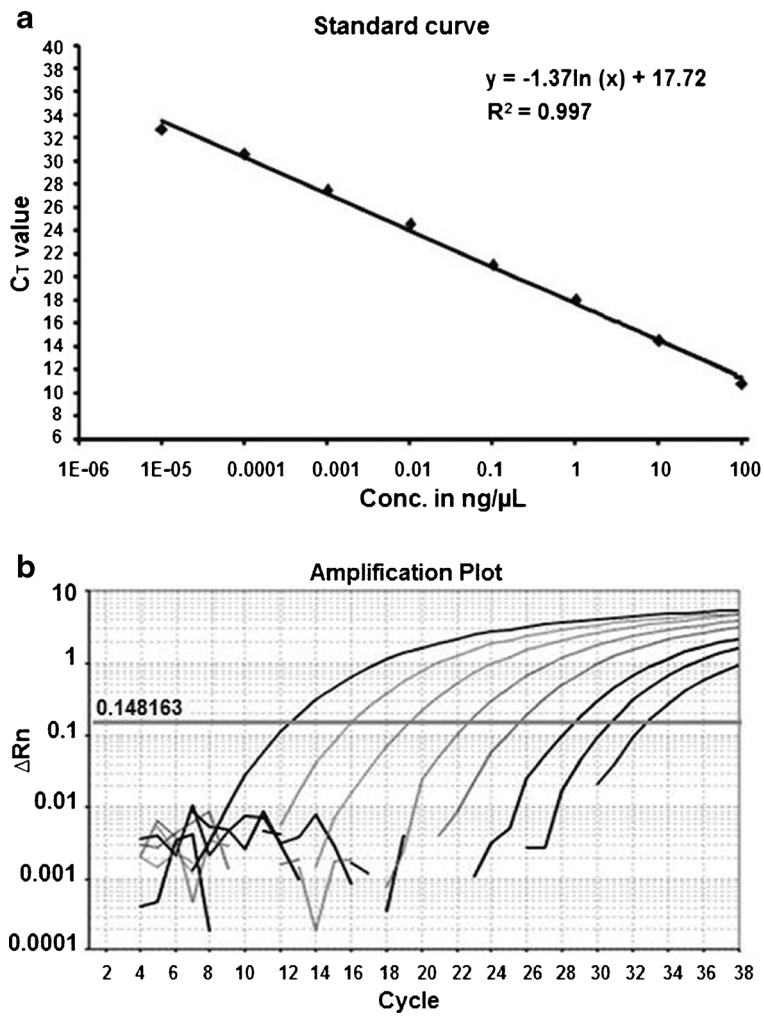

Fig. 2 Standard curve analysis: **a**. the standard curve derived from the 10–fold serially diluted standard DNA obtained from gel-purified SgK PCR product of *S. graminicola* isolate Sg 445. **b**. the amplification plot representing the exponential amplification of target molecules in the serially diluted standard DNA of *S. graminicola*. Base line at 0.148 shows the normalized fluorescence emission intensity of the reporter dye (Rn) with reference to the passive dye

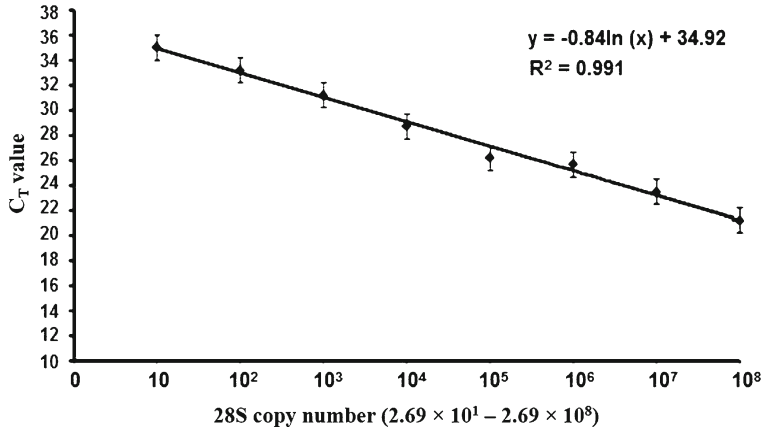

Fig. 3 Standard curve derived from sporangial DNA (25 pg/μl) over eight magnitudes of dilution; the least possible copy number of the target molecule detectable was calculated as 26.9 molecules/μl

Discussion

Detection of pathogens within a plant or in the rhizosphere, and elimination of infected plants or planting material before the development of visible symptoms is an important step towards prevention of disease epidemics. A rapid, sensitive and specific assay for in vitro detection and quantification of *S. graminicola* in diverse samples was developed. Since, *S. graminicola* is an obligate parasite of pearl millet, whole plant DNA from infected and healthy plant material was included in the study. The rDNA cluster including ITS and nuLSU regions have been targeted to develop species-specific probes and primers for detection of culturable (Guzmán-Franco et al. 2008; Raidl et al. 2005; Viaud et al. 2000) and non-culturable obligate (Blanco and Ristaino 2011; Crouch and Szabo 2011; Ioos et al. 2012) oomycetes and fungal pathogens. Similarly, the present study used the 28S region of nuLSU in the rDNA cluster to develop species-specific primers for the detection of *S. graminicola*. *S. graminicola* isolates collected from different pearl millet growing areas exhibited 28S rDNA sequence similarity and inter-species variability compared with other related species. Thus, two sets of oligonucleotide primers were designed, one for conventional PCR (SgK PCR) and one for q-PCR (SgTqK q-PCR). There are several reports that rDNA-based detection and quantification assays are more sensitive than those based on other regions such as the β-tubulin, Cox-I & II and EF-1α genes etc. (Yan et al. 2008; Malvick and Impullitti 2007; Dombrowski et al. 2006; Tooley et al. 2010). In this study, conventional PCR and q-PCR assays could detect the pathogen at the lowest tested DNA concentration of 7 pg/μl and 10 fg/μl, respectively (Table 3). Though the primers showed a high degree of specificity towards *S. graminicola* in conventional PCR assays, due to limitations of template concentration and size of the amplicon, the PCR assay was confined to the identification of *S. graminicola* from pure DNA samples.

For rDNA based phylogenetic studies, specific primers can be used to avoid amplification of host DNA. Thus, to circumvent template contamination by DNA of the host plant and/or other plant microflora, the universal primers have been replaced with specific or semi-specific primers such as species-specific (Babu et al. 2007) and genus/group specific (Chemidlin et al. 2011; Gardes and Bruns 1993; Vilgalys and Gonzalez 1990) ones designed from the rDNA cluster. We focused on development of a q-PCR assay suitable for the detection of *S. graminicola* in diverse samples including leaves, seed and oospores infested soil. The conserved/species-specific nature of the target region was assessed by sequence alignment of the 436 bp target region of ten different isolates of *S. graminicola*. The specificity of q-PCR assay is dependent on the binding affinity of the primers and probe, and the size of the target region/product (Bustin 2000). Our results demonstrated the effectiveness of the q-PCR assay developed by amplifying a short fragment (86 bp) of the target region. In a similar study, a q-PCR detection assay for *P. halstedii* targeted a 94 bp fragment (Ioos et al. 2012).

For fungal pathogens, earlier reports of q-PCR based assays demonstrated absolute quantification of copy number of a target DNA with respect to hyphal/mycelial biomass (Parlade et al. 2007; Raidl et al. 2005). However, in the case of *S. graminicola*, which is an obligate parasite, the copy number of the 28S

molecules was estimated in relation to the diploid nucleus of sexual/asexual spores. The absolute quantification of target molecules in the DNA obtained from sporangia (6×10^8/ml) was estimated to be 25 pg/μl, and the copy number was calculated as 2.69×10^8 molecules/μl. The lowest possible copy number of target molecules quantified at the lowest dilution on the standard curve was 26.9 molecules/μl. Therefore, it is presumed that approximately 27 molecules of 28S could be present in a diploid nucleus (2n) of *S. graminicola*. Although whole genome sequencing is the only possible way to know absolute copy number of the 28S molecule per genome, several researchers have estimated ITS region copy numbers for different fungi (whose genome has not been sequenced) using q-PCR (Linde et al. 2009; Parlade et al. 2007; Raidl et al. 2005).

In this study, the q-PCR assay of pearl millet seed samples estimated the absolute quantity of target 28S region of nuLSU as 1.02×10^5 molecules/μl. These results indicated the presence of pathogen hyphae or pathogen propagules on the seed coat or inside the seed. Although, earlier reports have indicated the possibility of *S. graminicola* being externally seed borne, experimental evidence of the viability of the pathogen has not been demonstrated (Thakur et al. 2010). Since detection of pathogen DNA in seed samples indicate the presence of the pathogen either on the seed coat or inside the seed tissue, this kind of material is worthy of quarantine restrictions (Ioos et al. 2012). Thus, the q-PCR assays developed in this study can be utilized for the quick detection of early infection of the downy mildew pathogen in field samples, and for monitoring the safe exchange of mildew-free pearl millet germplasm across boundaries.

Acknowledgments We thank Dr Harikishan Sudini, Groundnut Pathology, ICRISAT; Dr S Gopalakrishnan, Biocontrol Lab, ICRI SAT and Dr R D Prasad, Directorate of Oilseeds Research, India for providing microbial cultures. We are also thankful to Dr Rajeev Varshney and Dr Mahender Thudi, Applied Genomic Lab, ICRI SAT for providing constructive suggestions during this study.

References

Babu, B. K., Srivastava, A. K., Saxena, A. K., & Arora, D. K. (2007). Identification and detection of *Macrophomina phaseolina* by using species-specific oligonucleotide primers and probe. *Mycologia, 99*, 733–739.

Babu, B. K., Mesapogu, S., Sharma, A., Reddy, S. S., & Arora, D. K. (2011). Quantitative Real-Time PCR assay for rapid detection of plant and human pathogenic *Macrophomina phaseolina* from field and environmental samples. *Mycologia, 103*, 466–473.

Bauer, R., Begerow, D., Oberwinkler, F., Piepenbring, M., & Berbee, M. L. (2001). Ustilaginomycetes. In D. J. McLaughlin, E. G. McLaughlin, & P. A. Lemke (Eds.), *The mycota 7 part B-systematics and evolution* (pp. 57–83). Berlin: Springer.

Blanco, M. M., & Ristaino, J. B. (2011). Detection and quantification of *Peronospora tabacina* using a real-time polymerase chain reaction assay. *Plant Disease, 95*, 673–682.

Braun, U., Cook, R. T. A., Inman, A. J., & Shin, H. D. (2002). The taxonomy of the powdery mildew fungi. In R. R. Bélanger, W. R. Bushnell, A. L. Dik, & T. L. W. Carver (Eds.), *The powdery mildews: a comprehensive treatise* (pp. 13–55). St. Paul: APS Press.

Bridge, P., & Spooner, B. (2001). Soil fungi: diversity and detection. *Plant and Soil, 232*, 147–154.

Bustin, S. (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology, 25*, 169–193.

Chemidlin, P. N., Christen, R., Dequiedt, S., Mougel, C., Lelie'vre, M., Jolivet, C., Shahbazkia, H. R., Guillou, L., Arrouays, D., & Ranjard, L. (2011). Validation and application of a PCR primer set to quantify fungal communities in the soil environment by real-time quantitative PCR. *PLoS ONE, 6*(9), e24166.

Crouch, J. A., & Szabo, L. J. (2011). Real-time PCR detection and discrimination of the southern and common corn rust pathogens *Puccinia polysora* and *P. sorghi. Plant Disease, 95*, 624–632.

Dombrowski, J. E., Baldwin, J. C., Azevedo, M. D., & Banowetz, G. M. (2006). A sensitive PCR-based assay to detect *Neotyphodium* fungi in seed and plant tissue of tall fescue and ryegrass species. *Crop Science, 46*, 1064–1070.

Gachon, C., Mingam, A., & Charrier, B. (2004). Real-time PCR: what relevance to plant studies? *Journal of Experimental Botany, 55*, 1445–1454.

Gardes, M., & Bruns, T. D. (1993). ITS primers with enhanced specificity for basidiomycetes−application to the identification of mycorrhizae and rusts. *Molecular Ecology, 2*, 113–118.

Göker, V. H., Riethmüller, A., & Oberwinkler, F. (2007). How do obligate parasites evolve? A multi-gene phylogenetic analysis of downy mildews. *Fungal Genetics and Biology, 44*, 105–122.

Guzmán-Franco, A. W., Atkins, S. D., Alderson, P. G., & Pell, J. K. (2008). Development of species-specific diagnostic primers for *Zoophthora radicans* and *Pandora blunckii*; two co-occurring fungal pathogens of the diamondback moth, *Plutella xylostella*. *Mycological Research, 112*, 1227–1240.

Hayden, K., Ivors, K., Wilkinson, C., & Garbelotto, M. (2006). TaqMan chemistry for *Phytophthora ramorum* detection and quantification, with a comparison of diagnostic methods. *Phytopathology, 96*, 846–854.

Ioos, R., Fourrier, C., Wilson, V., Webb, K., Schereffer, J. L., & Tourvieille de Labrouhe, D. (2012). An optimized duplex real-time PCR tool for sensitive detection of the quarantine

oomycete *Plasmopara halstedii* in sunflower seeds. *Phytopathology, 102*, 908–917.

Khairwal, I. S. (2008). *Pearl millet Coordinator's review report.* Mandor, Jodhpur: Indian Council of Agricultural Research.

Leonard, K. J., & Szabo, L. J. (2005). Stem rust of small grains and grasses caused by *Puccinia graminis. Molecular Plant Pathology, 6*, 99–111.

Linde, S., Alexander, I. J., & Anderson, I. C. (2009). Spatial distribution of sporocarps of stipitate hydnoid fungi and their below ground mycelium. *FEMS Microbiology Ecology, 69*, 344–352.

Malvick, D. K., & Impullitti, A. E. (2007). Detection and quantification of *Phialophora gregata* in soybean and soil samples with a quantititative, real-time PCR assay. *Plant Disease, 91*, 736–742.

Mesapogu, S., Babu, B. K., Bakshi, A., Reddy, S. S., & Saxena, S. (2011). Rapid detection and quantification of *Fusarium udum* in soil and plant samples using real-time PCR. *Journal of Plant Pathology & Microbiology, 2*, 1–7.

Mogens, N., Skaidre, S., Linda, K. N., Irene, L., Niels, H. S., & Annemarie, F. (2009). Real-time PCR for quantification of eleven individual *Fusarium* species in cereals. *Journal of Microbiological Methods, 76*, 234–240.

Mumford, R., Boonham, N., Tomlinson, J., & Barker, I. (2006). Advances in molecular phytodiagnostics–new solutions for old problems. *European Journal of Plant Pathology, 116*, 1–19.

Parlade, J., Hortal, S., Pera, J., & Galipienso, L. (2007). Quantitative detection of *Lactarius deliciosus* extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and inter-specific competition. *Journal of Biotechnology, 128*, 14–23.

Pinnoi, A., Jeewon, R., Sakayaroj, J., Hyde, K. D., & Gareth Jones, E. B. (2007). *Berkleasmium crunisia* sp. nov. and its phylogenetic affinities to the pleosporales based on 18S and 28S rDNA sequence analyses. *Mycologia, 99*, 378–384.

Pushpavathi, B., Thakur, R. P., & Rao, C. K. (2006). Fertility and mating type frequency in Indian isolates of *Sclerospora graminicola*, the downy mildew pathogen of pearl millet. *Plant Disease, 90*, 211–214.

Raidl, S., Bonfigli, R., & Agerer, R. (2005). Calibration of quantitative real-time TaqMan PCR by correlation with hyphal biomass and ITS copies in mycelia of *Piloderma croceum. Plant Biology, 7*, 713–717.

Riethmüller, A., Voglmayr, H., Göker, M., Weiß, M., & Oberwinkler, F. (2002). Phylogenetic relationships of the downy mildews (Peronosporales) and related groups based on nuclear large subunit ribosomal DNA sequences. *Mycologia, 94*, 834–849.

Sarlin, T., Yli-Mattila, T., Jestoi, M., Rizzo, A., Paavanen-Huhtala, S., & Haikara, A. (2006). Real-time PCR for quantification of toxigenic *Fusarium* species in barley and malt. *European Journal of Plant Pathology, 114*, 371–380.

Schaad, N. W., & Frederick, R. D. (2002). Real-time PCR and its application for rapid plant disease diagnostics. *Canadian Journal of Plant Pathology, 24*, 250–258.

Sharma, R., Rao, V. P., Varshney, R. K., Prasanth, V. P., Kannan, S., & Thakur, R. P. (2010). Characterization of pathogenic and molecular diversity in *Sclerospora graminicola*, the causal agent of pearl millet downy mildew. *Archives of Phytopathology and Plant Protection, 43*, 538–551.

Singh, S. D., King, S. B., & Werder, J. (1993). Downy mildew disease of pearl millet. Information Bulletin No. 37. International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502324, Andhra Pradesh, India.

Steve, R., & Helen, J. S. (2000). Primer3 on the WWW for general users and for biologist programmers. In S. Krawetz & S. Misener (Eds.), *Bioinformatics methods and protocols: methods in molecular biology* (pp. 365–386). Totowa, New Jersey: Humana Press.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution, 28*, 2731–2739.

Thakur, R. P. (2008). Pearl millet. In S. Lodha, M. Ritu, & B. S. Rathore (Eds.), *Disease management in arid land crops* (pp. 21–41). Jodhpur, India: Scientific Publishers.

Thakur, R. P., Gunjotikar, G. A., & Rao, V. P. (2010). Safe Movement of ICRISAT's Seed Crops Germplasm. Information Bulletin No. 81. International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502324, Andhra Pradesh, India.

Thakur, R. P., Sharma, R., & Rao, V. P. (2011). Screening Techniques for Pearl Millet Diseases. Information Bulletin No. 89. International Crops Research Institute for the Semi-Arid Tropics, Patancheru 502 324, Andhra Pradesh, India.

Tooley, P. W., Martin, F. N., Carras, M. M., & Frederick, R. D. (2006). Real-time fluorescent polymerase chain reaction detection of *Phytophthora ramorum* and *Phytophthora pseudosyringae* using mitochondrial gene regions. *Phytopathology, 96*, 336–345.

Tooley, P. W., Carras, M. M., Sechler, A., & Rajasab, A. H. (2010). Real-time PCR detection of sorghum ergot pathogens *Claviceps africana*, *Claviceps sorghi* and *Claviceps sorghicola. Journal of Phytopathology, 158*, 698–704.

Vandemark, G. J., & Ariss, J. J. (2007). Examining interactions between legumes and *Aphanomyces euteiches* with real-time PCR. *Australasian Plant Pathology, 36*, 102–108.

Viaud, M., Pasquier, A., & Brygoo, Y. (2000). Diversity of soil fungi studied by PCR-RFLP of ITS. *Mycological Research, 104*, 1027–1032.

Vilgalys, R., & Gonzalez, D. (1990). Organization of ribosomal DNA in the basidiomycete *Thanatephorus praticola. Current Genetics, 18*, 277–280.

Yadav, M. K., Babu, B. K., Saxena, A. K., Singh, B. P., Singh, K., & Arora, D. K. (2011). Real-time PCR assay based on topoisomerase-II gene for detection of *Fusarium udum. Mycopathologia, 171*, 373–381.

Yan, L., Zhang, C., Ding, L., & Ma, Z. (2008). Development of a real-time PCR assay for the detection of *Cladosporium fulvum* in tomato leaves. *Journal of Applied Microbiology, 104*, 1417–1424.