Real-Time Quantitative RT-PCR Assay of Gene Expression in Plant Roots during Fungal Pathogenesis

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ABSTRACT

Real-time quantitative RT-PCR is becoming the preferred method for high-sensitivity, rapid-throughput RNA transcript quantification. However, due to the significant developmental costs of dedicated fluorogenic probes, a real-time assay that is simple to establish, comparatively inexpensive, and readily adaptable would be advantageous for the detailed analysis of large sets of expressed sequences. We have devised a flexible real-time quantitative RT-PCR assay that employs a nonspecific DNA binding dye for product detection and uses a relative quantification formula to account for differences in PCR amplification efficiency between the target and reference products. The latter permits the use of an exogenous reference transcript and therefore avoids the normal requirement for the construction of a recombinant RNA reference transcript or extensive characterization of housekeeping gene expression. In an investigation of class II chitinase expression in two varieties of Bermuda grass (*Cynodon spp.*), following infection with the fungal root pathogen *Ophiostoma narmari*, this assay identified 16- and 28-fold peaks in gene expression at 24 and 96 h after inoculation, respectively.

INTRODUCTION

Spring dead spot of Bermuda grass (*Cynodon spp.*), caused by the fungal root pathogen *Ophiostoma narmari*, is the most important disease complex identified in turf grasses in Australia and the United States. The characterization and manipulation of host plant resistance is a possible strategy for effective disease control. Most plants possess pathogenesis-related proteins, such as chitinase and glu-

canase, which are encoded by genes that are rapidly induced following pathogen infection. Chitinase degrades chitin (N-acetylglucosamine)_n, a major structural polymer of the fungal cell wall, and these enzymes have been shown to halt the growth of some fungi in vitro (1,2). Moreover, the expression of class II chitinase transgenes has increased the resistance of wheat to fungal pathogens (3,4). Investigation of the expression of class II chitinase mRNA in the roots of Bermuda grass varieties during fungal infection may indicate the importance of such genes in host plant resistance toward *O. narmari*.

In recent years, quantitative RT-PCR has become the method of choice for high-sensitivity, rapid-throughput quantification of mRNA. Following reverse transcription, PCR amplification products can be detected by fluorescent emission from sequence-specific, fluorogenic oligonucleotides such as TaqMan® (Applied Biosystems, Foster City, CA, USA) (5), molecular beacons (6) or dual fluorescence resonance energy transfer (FRET) probes (7), or from non-specific dsDNA binding dyes (8). Instruments that can detect a range of fluorescence chemistries instantaneously (in real time) have added to the utility of the technique and provide an abundance of options for any experiment (9,10).

A real-time quantitative RT-PCR assay that is easy to establish and highly adaptable yet economical to operate would be invaluable for projects with limited budgets or when large sets of expressed sequence tags (ESTs) or microarray data require further analysis. Low-cost assays inevitably use dye-based detection and are restricted to rel-

ative quantification against an endogenous (housekeeping) gene or an exogenous reference transcript. However, housekeeping genes need to be identified and have their expression confirmed as invariant under broad experimental conditions before they can be validly used as reference transcripts.

Here we report the development of a convenient and flexible real-time quantitative RT-PCR assay that uses an exogenously derived reference transcript and SYBR® green I detection. This is combined with a relative quantification formula that accounts for differences in PCR amplification efficiency between the target and reference products (11). The assay was found to be suitable for the analysis of variable chitinase transcript levels in *Cynodon spp.* roots during fungal pathogenesis.

Figure 1. Design of the quantitative RT-PCR assay. The RNA of the reference transcript (Kan 1.2) is added before RNA extraction. The pooled RNA is treated with DNase I and then reverse transcribed. Quantitative reactions are conducted in triplicate. Paired tubes receive equivalent cDNA from either the reference (1) or target (2) primer pairs. To control for DNA contamination, a third tube without cDNA template also receives the target primers (3).

MATERIALS AND METHODS

Four-week-old, axenically propagated Bermuda grass (*Cynodon* spp.) plants were transferred into liquid hormone- and sucrose-free Murashige and Skoog media and incubated in constant high-light conditions with rotary shaking at 100 rpm (12). *O. narmari* inoculum was a homogenate of eight-week-old potato dextrose agar (PDA) culture plates. Propagule numbers were estimated by an adaptation of the most probable number method, in which the serial dilutions were cultured on PDA plates (13). Plant roots were infected by the addition of approximately 1000 *O. narmari* propagules to each culture vessel. Uninfected plants received a water control, and root tissue was harvested at 0, 12, 24, 48, or 96 h after infection.

The RNA was isolated using the RNeasy® plant RNA extraction kit (Qiagen, Clifton Hill, Victoria, Aus-

tralia) with elution for 10 min in 50 µL RNase-free water. Root tissue (100 mg) was ground into a fine powder in liquid nitrogen and dispersed into extraction buffer. Approximately 2.5×10^5 copies of Kanamycin 1.2 kb Control RNA (Kan 1.2; Promega, Madison, WI, USA) were then added to each extraction. After elution, 40 U RNaseOUT™ RNase inhibitor (Invitrogen, Carlsbad, CA, USA) were added to each sample. RNA integrity was assessed by examining the rRNA bands after electrophoresis on a standard agarose gel. Contaminating DNA was then removed using DNA-free™ (Ambion, Austin, TX, USA) with 4 U DNase I per sample and incubation for 1 h.

Reverse transcription was performed in a total volume of 20 µL. Total RNA amounts were not normalized between reactions. Total RNA solution (12 µL) was combined with 500 ng oligo(dT)₁₈ primer (1 µL), heated at 70°C

for 10 min, and cooled on ice. Each reaction tube then received 4 µL of 5× first-strand buffer (Invitrogen), 2 µL 0.1 M DTT, 1 µL 10 mM of dNTPs, and 100 U SuperScript™ II RNase H-reverse transcriptase (Invitrogen) as a master mixture. Control tubes (RT-) for each sample received a mixture with no enzyme. The reactions were incubated at 25°C for 10 min (primer annealing) and at 50°C for 50 min (cDNA synthesis). The cDNA solutions were then treated with 1 U RNase H (Fermentas, Vilnius, Lithuania) for 30 min at 37°C, and cDNA solutions and controls were diluted 10-fold to a final volume of 200 µL with sterile Milli-Q® water (Millipore, Bedford, MA, USA).

Quantitative PCR was performed in a 32-well Rotor-Gene™ centrifugal real-time thermal cycler (Corbett Research, Mortlake, NSW, Australia). Each quantitative PCR contained 2.5 mM MgCl₂, 1× reaction buffer (20 mM Tris-HCl, pH

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8.4, 50 mM KCl), 200 nM of each dNTP, 5 µL of template (RT/RT-), 0.1× SYBR green I (Molecular Probes, Eugene, OR, USA), 0.5 U of recombinant *Taq* DNA polymerase (Invitrogen), and 200 nM of each primer for either target or reference primer pair in a total reaction volume of 25 µL. CHTIIa (target) PCR primers were sense 5'-ACCAGG-CCCAACCTCGAT-3' and antisense 5'-CGGATTGAATCTACACATTGACA-3'. Kan 1.2 (reference) PCR primers were sense 5'-GCCATTCTCACCGG-ATTCAGTCGTC-3' and antisense 5'-AGCCGCCGTCCCGTCAAGTCAG-3' (Promega). Both primer pairs were optimized for equivalent annealing temperatures. The thermal profile for quantitative PCR was 94°C for 3 min, and then 94°C for 15 s, 60°C for 1 min, 15 s at 80°C (fluorescence measurement) repeated for 50 cycles, followed by a melting step in which the temperature was raised in one-degree increments from 50°C to 99°C with a 5-s hold at each degree.

The identity of each RT-PCR product was confirmed initially by direct sequencing and subsequently from the observation of a single melt peak in the respective reactions. Every quantitative PCR run contained "no template" controls for each primer pair. The 134-bp CHTIIa and 323-bp Kan 1.2 products were cloned into pBluescript® SK⁺ (Stratagene, La Jolla, CA, USA), and pCHTIIa and pKan323 were serially diluted over a five-log range from 10⁵ to 10¹ molecules. PCRs were per-

formed with the points in the two dilution series as templates. The threshold was set at 0.15 fluorescent units, and the threshold cycle (C_t) values were plotted against the log of starting template concentration (5). The slope *m* of the line *y* = *mx* + *b* is related to the amplification efficiency by the formula *E* = 10^[-1/*m*] (14). The fold change in CHTIIa expression during fungal infection was calculated from the C_t values and the PCR amplification efficiencies using the formula (11):

$$\text{fold change} = \frac{(E_{\text{target}})^{\Delta C_{t\text{target}}(\text{control} - \text{infected})}}{(E_{\text{ref}})^{\Delta C_{t\text{ref}}(\text{control} - \text{infected})}} \quad [\text{Eq. 1}]$$

RESULTS

The process of sampling limited stocks of axenically propagated plant tissue at multiple timepoints, combined with the relatively low yields of RNA harvested from infected Bermuda grass roots (data not shown), required the development of a highly sensitive method for the detection of chitinase gene transcripts. Figure 1 shows the components of the two-step quantitative RT-PCR assay system. To provide an internal reference for each sample, a heterologous kanamycin transcript (Kan 1.2 RNA) was added during the RNA extraction, co-transcribed with the target transcript in the reverse transcription reaction, and PCR amplified separately to the target.

A partial class II chitinase gene clone was obtained by the amplification of *Cynodon* spp. genomic DNA with degenerate primers designed from conserved plant chitinase sequences, and PCR walking was used to extend the 3' sequences of this clone to create a chitinase gene contig that was designated CHTIIa (data not shown). The specific PCR primers designed from this contig encompass 42 bp of the CHTIIa open reading frame together with 92 bp of the 3' untranslated region and amplify a single 134-bp CHTIIa product from the genomic DNA of the two Bermuda grass varieties, "Greenlees Park" (GLP) and "Tifgreen" (TG).

For both the CHTIIa target and Kan 1.2 reference products, Pearson correlation coefficients confirmed the relationship between starting template concentration and C_t value (Figure 2). PCR amplification efficiencies were 1.90 for the CHTIIa product and 1.97 for the Kan 1.2 reference product. To test the low level of variability in the assay, the Kan 1.2 reference was amplified from 15 different cDNA backgrounds in a total of 87 replicates and gave a mean C_t value of 19.88 cycles with a variation of 3.2%.

Root tissue from the Bermuda grass varieties GLP and TG was harvested at 0, 12, 24, 48, and 96 h, following inoculation with propagules of the *O. narmari* pathogen. Fold changes in the levels of CHTIIa gene transcripts were determined using the quantitative RT-PCR assay (Figure 3). The expression

Figure 2. Amplification efficiencies of CHTIIa and Kan 1.2 products. Threshold cycle (C_t) for the CHTIIa (■) and Kan 1.2 (●) products plotted against log₁₀ of the known number of starting template molecules in the dilution series. The line equations and Pearson correlation coefficients (R²) are given.

Figure 3. Bermuda grass chitinase mRNA expression during fungal pathogenesis. Fold change in the expression of CHTIIa mRNA in the two *Cynodon* spp. varieties, GLP and TG, at 0, 12, 24, 48 and 96 h after inoculation with *O. narmari* propagules.

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of CHTIIa in TG was shown to peak 16-fold after only 24 h, whereas CHTIIa expression in GLP achieved a larger but delayed increase of 28-fold after 96 h.

DISCUSSION

The aim of this work was to develop a cost-effective, real-time quantitative RT-PCR assay suitable for the analysis of mRNA levels in *Cynodon* roots during microbial pathogenesis. Our data show that the resultant assay can accurately detect changes in transcript levels in small samples of Bermuda grass tissue at progressive stages of fungal infection. The quantitative RT-PCR assay was sensitive, highly reproducible, and comparatively inexpensive to set up and operate.

The devised assay structure has several advantages. The use of an exogenous reference transcript avoids the need to construct a recombinant RNA reference or to confirm the expression of housekeeping genes as invariant under experimental conditions. Such a reference transcript is adjustable toward the range of the target and is not subject to temporal variation. The addition of the reference RNA to the nucleic acid extraction limits error to single mass and volume measurements and controls for the yield and quality of RNA. Thus, the problem of normalizing sample RNA of low concentration between reactions is eliminated. In addition, the reference transcript used here is commercially available, thus avoiding in vitro transcription and purification. We suggest that reverse transcriptase inhibition would influence all of the transcripts within a reaction equally and that differences in reverse-transcription efficiency between any two transcripts due to sequence composition would be at a fixed ratio.

It should be noted that the use of a heterologous reference transcript would not normally provide for matched PCR amplification efficiencies between the reference and target products. However, this requirement can be eliminated by the use of the relative quantification formula, which neatly accounts for any difference in the amplification efficiency between the two products (11). As a result, the assay

can be easily adapted for the analysis of alternative or additional target genes using the same reference transcript. The flexibility of the assay is further enhanced by the SYBR green I detection of the amplification products.

The Corbett Research Rotor-Gene PCR machine used in these experiments is centrifugal, and the PCR tubes spin through a single air mass. The low level of variation in the C_t values for the reference transcripts shows that the PCR conditions are very uniform and that the external amplification of the reference transcript in paired tubes containing equivalent cDNA templates closely approximates the ideal of an internal RNA reference.

The application of this quantitative PCR assay to the study of fungal pathogenesis in Bermuda grass has indicated a potential role for CHTIIa in host plant defense. However, despite the observed differences in chitinase expression, the chosen cultivars have not been found to differ significantly in their physiological responses to disease (data not shown). Future work might analyze the expression of CHTIIa and other chitinase II genes within a broader selection of *Cynodon* varieties that exhibit different degrees of disease resistance. The flexibility of the quantitative RT-PCR assay readily lends itself to such expansion. In a more general sense, the assay would be ideal for conducting detailed analyses of microarray data and EST expression in which large sets of genes require investigation and the cost of manifold gene-specific fluorogenic probes would be prohibitive.

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