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Commentary

Comparison of Relative RT-PCR and Northern Blot Analyses to Measure Expression of β-1,3-Glucanase in *Nicotiana benthamiana* Infected With *Colltotrichum destructivum*

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Abstract. Although northern blot analysis is effective for quantifying gene expression, reverse transcription–polymerase chain reaction (RT-PCR) is much more sensitive. Obtaining quantitative RT-PCR results, however, can be challenging. Relative RT-PCR uses standard PCR techniques but permits the comparison of transcript quantities between samples by coamplifying the gene of interest with a housekeeping gene that acts as an internal control. To analyze the expression of a plant gene encoding a pathogenesis-related protein, such as β-1,3-glucanase, a translation elongation factor 1α (EF-1α) gene was selected as an internal control. Northern blot analysis demonstrated constitutive expression of the plant EF-1α gene following infection of *Nicotiana benthamiana* by *Colletotrichum destructivum*. Primers for the gene of interest and internal control were compatible, and 35 cycles of amplification gave reproducible relative RT-PCR results for β-1,3-glucanase gene expression. A high correlation was observed between the relative quantification of β-1,3-glucanase gene expression as determined by northern blot and relative RT-PCR analyses, demonstrating the validity of relative RT-PCR with a plant EF-1α gene as a control.

Key words: β-1,3-glucanase, *Colletotrichum*, *Nicotiana*, northern blot, relative RT-PCR, translation elongation factor 1α

Abbreviations: EF-1α, translation elongation factor 1α; hpi, hours postinoculation; PR2, pathogenesis-related protein 2; RT-PCR, reverse transcription–polymerase chain reaction.

Introduction

Gene expression levels are commonly determined using northern blot analysis. However, this technique is time-consuming and requires a large quantity of RNA (Chelly and Kahn, 1994). RT-PCR converts RNA into first strand cDNA, which is then used as a template for PCR. RT-PCR is more rapid and sensitive and can be more specific than northern blot analysis, but quantification can be difficult because many sources of variation exist, including template concentration and amplification efficiency. One approach to obtaining quantitative RT-PCR results is

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relative RT-PCR, which coamplifies the gene of interest with an internal control and quantifies the gene of interest relative to the internal control (Gause and Adamovicz, 1995). For relative RT-PCR to be accurate, specific PCR conditions and an appropriate internal control must be determined. For quantification, the reaction must be analyzed in the linear range of amplification before the products reach the PCR plateau for either the gene of interest or internal control. Therefore, the number of starting target molecules and amplification efficiency of both the target and control must be comparable.

A reliable internal control should show minimal change, whereas a gene of interest may change greatly over the course of an experiment. Housekeeping genes are often selected because they encode proteins essential to cell viability and therefore are assumed to be expressed similarly in different cell types. However, expression of some commonly used internal controls, such as actin, glyceraldehyde-3-phosphate dehydrogenase, and cyclophilin, has been found to vary in different tissues and can be affected by experimental treatments (Stürzenbaum and Kille, 2001). Plant-microbe interactions also present challenges for finding appropriate internal controls. For example, lupine cyclophilin transcripts accumulated following infection by *Bradyrhizobium* (Nuc et al., 2001), and actin transcripts of round-leaved mallow increased following infection by *Colletotrichum* (Jin et al., 1999). In addition, ribosomal subunits are not suitable controls for diseased plants because they can be highly homologous between plants and pathogens, and the ratio of plant to pathogen RNA will decrease as the infection progresses (Mahe et al., 1992).

One housekeeping gene commonly used as a control is EF-1α, which catalyzes the first step of protein synthesis by binding the aminoacyl-tRNA to the ribosome aminoacyl site (Stürzenbaum and Kille, 2001). Mahe et al. (1992) selected a bean EF-1α gene to normalize the expression of a chitinase gene of bean after infection by *C. lindemuthianum*.

In this study, we have evaluated the effectiveness of relative RT-PCR for observing relative gene expression patterns in *N. benthamiana* infected by *C. destructivum* using a *N. benthamiana* EF-1α gene, *NbEF-1α*, as a constitutive control. The expression of *NbEF-1α* was compared to that of the *N. benthamiana* basic PR2 gene, *NbPR2*. *NbPR2* encodes a β-1,3-glucanase, which degrades glucans found in fungal cell walls and has been induced in plants following attack by fungal pathogens, including *Phytophthora, Alternaria, Peronospora,* and *Colletotrichum* species (Leubner-Metzger and Meins, 1999). Both northern blot and relative RT-PCR analyses were used to observe the expression pattern of *NbPR2* after infection, and the suitability of an EF-1α gene for relative quantification was assessed.

Materials and Methods

Biological materials

Cultivation of the host and pathogen and the method of inoculation were done according to Shen et al. (2001). RNA extraction was performed according to Chen et al. (2000).

Northern hybridization

Ten micrograms of RNA was separated on a 1% formaldehyde agarose gel (0.4 g agarose, 34.8 mL diethyl pyrocarbonate [DEPC]–treated water, 1.2 mL 37% formaldehyde, 4 mL 10 X MOPS buffer [pH 7] [200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA]) in 1 X MOPS buffer and transferred to a positively charged microporous (0.45-µm pores) nylon 66 membrane (Roche Diagnostics, Laval, PQ). Hybridization was performed, and probes were produced as described in the DIG System Users Guide (Roche Diagnostics). The *NbEF-1*α probe was produced with primers Tobef2A (5'-CATCCATCTTGTTACAGC-3') and Tobef2S (5'-CAAGTATGCCTGGGTGCT-3') and the amplification conditions described below. The *NbPR2* probe was produced using the primers described below. The relative intensity of the bands was determined by scanning the northern blot or PCR gel and analyzing the band intensity using the NIH Image program (Scion Corporation, Frederick, MD). Relative quantification was done by comparing intensities of the *NbPR2* and *NbEF-1*α bands (Mahe et al., 1992).

Relative RT-PCR

RNA was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Burlington, ON) as recommended by the manufacturer and stored at -20°C until needed. PCR reactions were performed in 15-µL reactions with the following:

- 1 µL of cDNA from *N. benthamiana* infected with *C. destructivum*
- 0.04 U/µL *Tth* polymerase (Interscience, Markham, ON)
- 1 X *Tth* polymerase buffer
- 2 mM dNTPs
- 5 mM of $MgCl_2$
- 1 µM of primers TobefS (5'-CTCCAAGGCTAGGTATGATG-3') and TobefA (5'-CTTCGTGGTTGCATCTCAAC-3') to amplify *NbEF-1*α
- 0.5 µM of primers Pr2S (5'-CATCACAGGGTTCGTTTAGGA-3') and Pr2A (5'-GGGTTCTTGTTGTTCTCATCA-3') to amplify *NbPR2*

Amplification was as follows: 94°C for 3 min; 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 10 min. RNA samples were tested for the presence of genomic DNA by using extracted RNA directly as a PCR template, prior to cDNA synthesis, under the same PCR conditions. cDNA PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). RT-PCR products were separated on 1.4% agarose gels, scanned, and saved in tagged information file format (tiff) files for quantification using the NIH Image program (Scion Corporation). The optical density was determined for both *NbPR2* and *NbEF-1*α, and a ratio of *NbPR2* band intensity to *NbEF-1*α band intensity was calculated.

Results

To verify the amount of RNA loaded for northern blot analysis, total RNA was quantified by image analysis of the ethidium bromide–stained RNA gel prior to blotting (Figure 1A). Degradation of total RNA was clearly visible as the disease

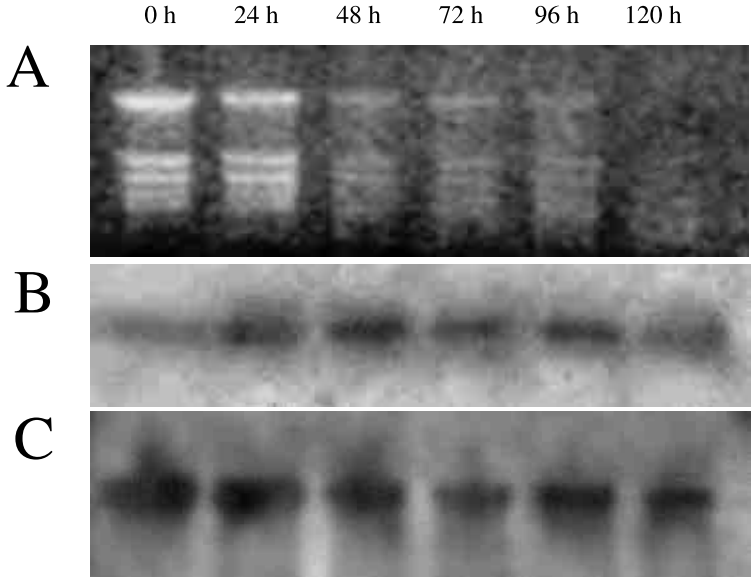

Figure 1. Northern blot analysis of *NbPR2* and *NbEF-1α* expression. (A) Total RNA used for northern blotting was from *N. benthamiana* infected with *C. destructivum* before inoculation (0 hpi) and at 24, 48, 72, 96, and 120 hpi. (B) Northern blot probed with *NbPR2*. (C) Northern blot probed with *NbEF-1α*.

progressed, particularly by 120 hpi (Figure 1A), but the hybridization bands for *NbPR2* (Figure 1B) and *NBEF-1α* (Figure 1C) remained clear and distinct from 0-120 hpi. Northern blot analysis of *NbPR2* expression (Figure 1) normalized for the amount of RNA loaded showed increasing levels of transcripts throughout the infection by *C. destructivum* (Figure 2). From the pattern of expression, a faster rate of increase appears to have occurred after 48 hpi (Figure 2A). In contrast, *NbEF-1α* expression (Figure 1C) remained constant throughout the infection (Figure 2A).

A different pattern of *NbPR2* expression was observed when *NbPR2* expression was quantified relative to *NbEF-1α* (Figure 2B). The rate of increase in *NbPR2* expression slowed in the latter stages of infection. Linear regression analysis of *NbPR2* expression relative to *NbEF-1α* expression against hpi produced an R^2 value of 0.927 (P<.001).

Coamplification of *NbPR2* and *NbEF-1α* showed that the level of *NbPR2* expression increased during the infection (Figure 3A). After 48 hpi, the expression level increased more slowly (Figure 3B). Linear regression analysis of *NbPR2* expression against hpi produced an R^2 value of 0.917 (P<.002).

The *NbPR2* expression patterns quantified by means of northern blot analysis and relative RT-PCR were very similar (Figure 4A). However, from 0-48 hpi, *NbPR2* expression levels were slightly lower, as measured by means of northern blot analysis. A strong correlation was observed between *NbPR2* expression using relative RT-PCR and northern blot analyses with an R^2 value of 0.919 (P<.002) (Figure 4B). Because both northern blot and RT-PCR analyses were corrected for

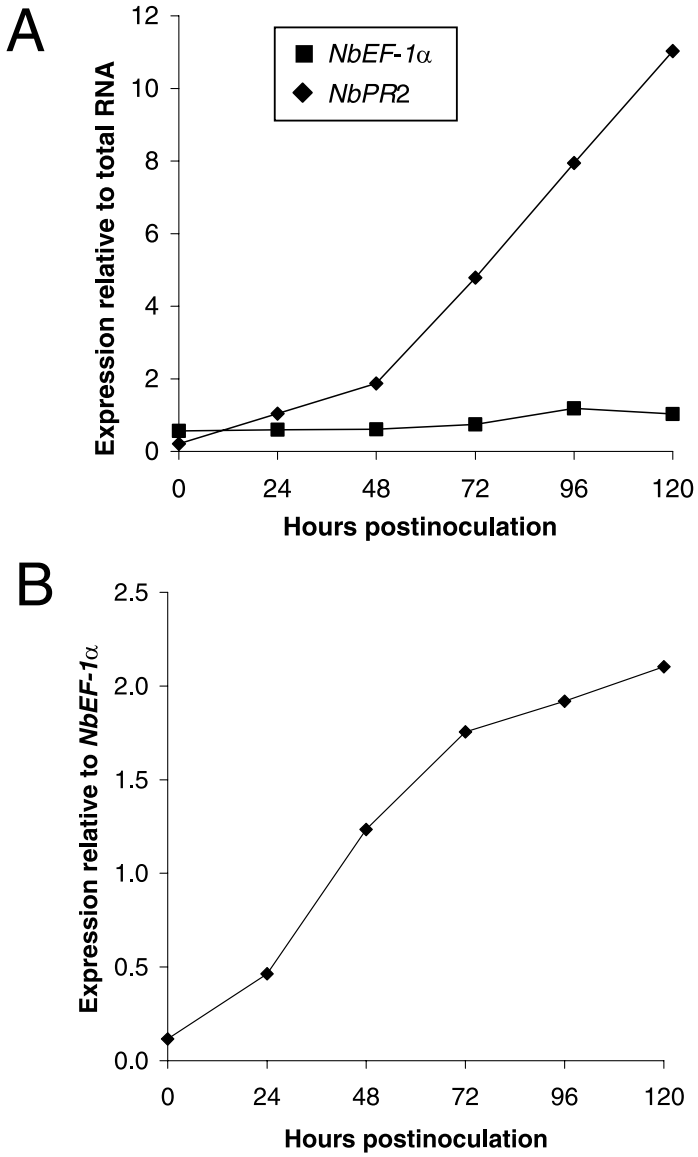

Figure 2. Quantification of *NbPR2* and *NbEF-1α* expression based on northern blot analysis. (A) Expression was calculated from the optical density of the hybridization bands of *NbPR2* and *NbEF-1α* and then normalized for the amount of RNA loaded per lane by dividing by the optical density of total RNA. (B) Quantification of *NbPR2* expression relative to that of *NbEF-1α*.

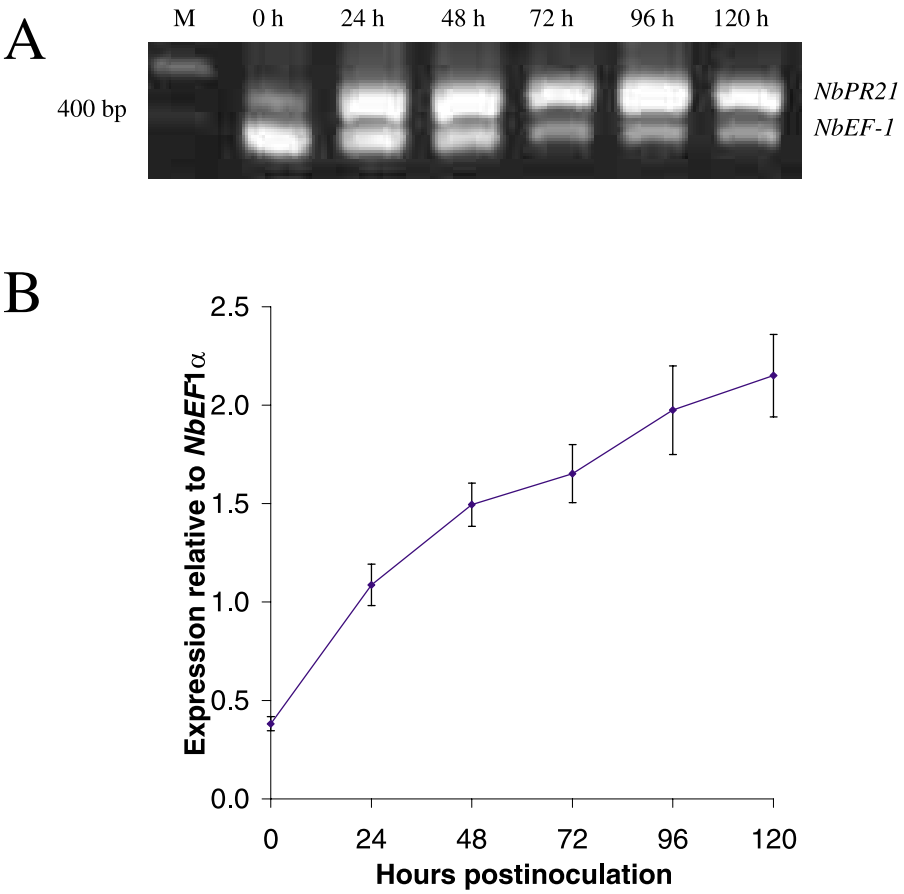

Figure 3. Relative RT-PCR analysis of *NbPR2* expression relative to that of *NbEF-1α*. (A) Relative RT-PCR gel of *NbPR2* (440 bp) and *NbEF-1α* (370 bp) before inoculation (0 hpi) and at 24, 48, 72, 96, and 120 hpi. (B) *NbPR2* expression relative to that of *NbEF-1α* Standard error bars represent the variation of 3 relative RT-PCR experiments. Lane M, 100-bp ladder.

background, the positive intercept supports the view that, on the basis of the threshold of detection, RT-PCR is more sensitive than northern blot.

Discussion

Although quantification of RNA transcript levels can be performed using northern blot analysis, relative RT-PCR has many advantages, such as being able to detect lower levels of gene expression. Results from northern blot and relative RT-PCR analyses of *NbPR2* expression were similar, thereby validating the use of relative RT-PCR with an EF-*1α* internal control for measuring host gene expression during infection. The inclusion of primers to amplify an EF-*1α* gene fragment in each sample helped to account for variation in template concentration and amplification efficiency.

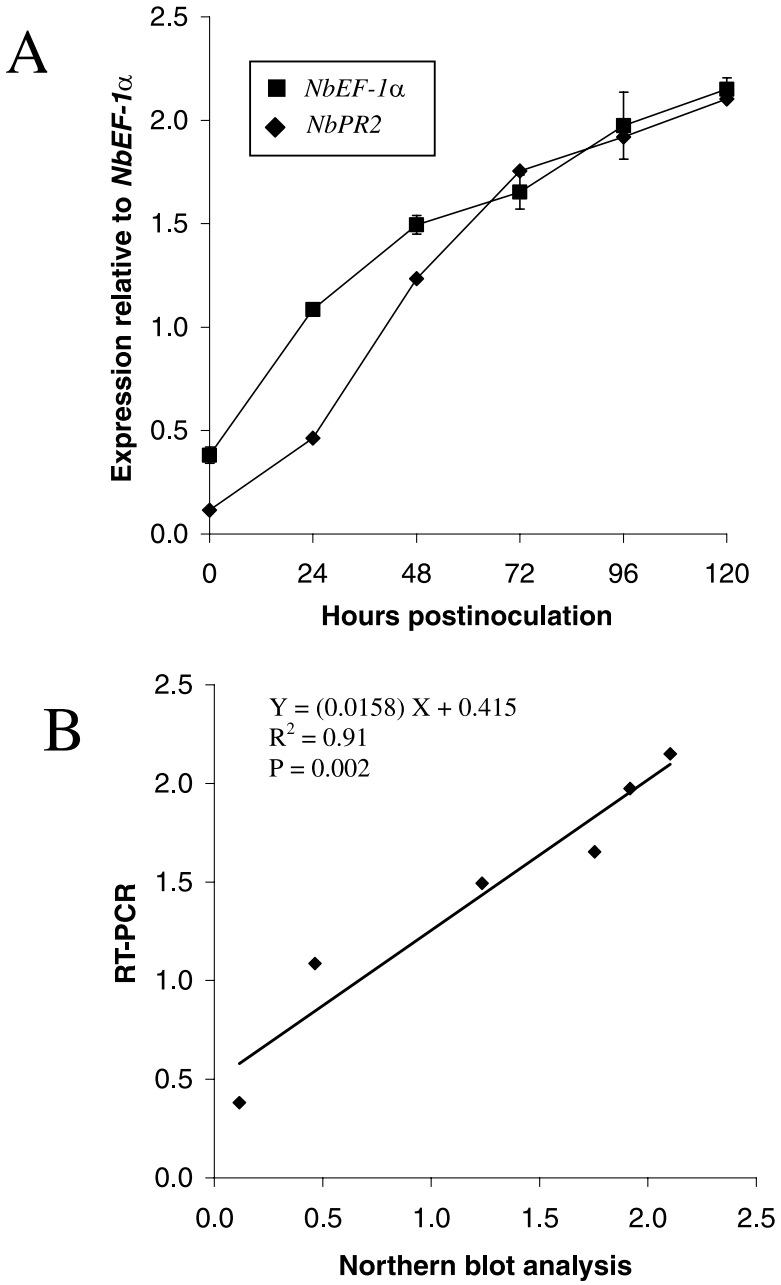

Figure 4. Comparison of *NbPR2* and *NbEF-1*α expression determined by northern blot and relative RT-PCR analyses. (A*)* Relative expression of *NbPR2* before inoculation (0 hpi) and at 24, 48, 72, 96, and 120 hpi as determined by northern blot and relative RT-PCR analyses. (B) Correlation between *NbPR2* and *NbEF-1*α expression as determined by relative RT-PCR and northern blot analyses.

Several internal controls have been used in relative RT-PCR. For example, glyceraldehyde-3-phosphate dehydrogenase was selected as an internal control for relative RT-PCR of cytokine gene expression in dogs (Markus et al., 2002), whereas the 18S rRNA was found to be a superior control to glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes for measuring gene expression in human melanoma cells (Goidin et al., 2001). For relative RT-PCR of plant genes, the 18S rRNA was used as an internal control to measure nutrient transporter gene expression in *Medicago truncatula* roots colonized by an arbuscular mycorrhizal fungus (Burleigh, 2001) and phosphoenolpyruvate carboxylase expression in developing and germinating *Triticum aestivum* seeds (Gonzalez et al., 2002). However, Burleigh (2001) recognized that 18S rRNA sequences are highly conserved; therefore, the internal control was amplifying both plant and fungal 18S rRNA from the fungal infected roots, leading to an underestimation of plant gene expression.

For *Phaseolus vulgaris* infected by *C. lindemuthianum,* Mahe et al. (1992) suggested using EF-*1*α as an internal control because the similarity between fungal and plant EF-*1*α genes is much lower than between 2 plant EF-*1*α genes. Using northern blot analysis, expression of the bean EF-*1*α gene was shown to be constitutive in mock-inoculated leaves and only declined late in the infection. When the EF-*1*α expression level was used to normalize expression levels of a pathogenesis-related protein gene for chitinase, chitinase expression increased 48-fold in diseased leaves rather than 17-fold as had been determined without the EF-*1*α as an internal control (Mahe et al., 1992).

For *N. benthamiana* infected with *C. destructivum*, northern blot analysis revealed relatively little change in *NbEF-1*α expression during infection. Determination of *NbPR2* expression relative to *NbEF-1*α expression using both northern blot and relative RT-PCR analyses revealed that the rate of *NbPR2* expression increased throughout the infection but was induced more rapidly in the early phase. Without normalization with *NbEF-1*α, however, one might have assumed that *NbPR2* expression increased relatively less rapidly early in the infection. The infection by *C. destructivum* occurs by intracellular hemibiotrophy, where the infection is initially biotrophic and then converts to a necrotrophic phase at approximately 72 hpi (Shen et al., 2001). The expression pattern of the basic PR2 tobacco gene has been extensively studied and compared to the expression patterns of other genes following infection (Brederode et al., 1991; Guo et al., 2000; Vögeli-Lange et al., 1994). Basic PR2 gene expression is induced by ethylene (Leubner-Metzger and Meins, 1999), and a peak in ethylene production from 24-48 hpi in the biotrophic phase of infection of *N. benthamiana* by *C. destructivum* (Chen, 2002) may be the reason for the faster rate of induction of *NbPR2* during the biotrophic phase of infection. *NbPR2* encodes a β-1,3-glucanase that degrades fungal cell walls, directly damaging them and releasing fungal cell wall materials, which can act as elicitors to induce defense reactions (Leubner-Metzger and Meins, 1999).

This work demonstrates that EF-*1*α is not only an adequate control for northern blot analysis but also for relative RT-PCR, where factors such as transcript abundance and amplification efficiency are important. It can be a challenge to create conditions in which the internal control mRNA is not too abundant

compared to a rare mRNA of interest. To be quantitative, RT-PCR must be analyzed in the linear range of amplification for both the gene of interest and internal control before reaction components become limiting. When target transcripts are abundant, PCR amplification may plateau after only 20 cycles; therefore, relative RT-PCR conditions must be determined to ensure that both bands are still accumulating at the end of the PCR. In this study, 35 cycles were used for RT-PCR analysis, which is usually considered to be too many cycles for quantification. However, our results show that the results of RT-PCR analysis with 35 cycles closely resemble those of northern blot analysis, indicating a relatively low template amount or low amplification efficiency from diseased leaves. The number of cycles to reach saturation greatly depends on the amplification efficiency. For example, a 10% reduction in the relative efficiency of amplification results in more than 95% reduction in the amount of PCR product (Diaco, 1995). The expression of other genes from diseased leaves of *N. benthamiana* and *N. tabacum* that have been quantified by coamplification with *NbEF-1*α using 35 cycles include glutathione S-transferase, pathogenesis-related protein 1a, and 1-aminocyclopropane-1-carboxylic acid oxidase genes (Goodwin PH, unpublished); the level of *NbEF-1*α amplification was always below saturation, indicating that the conditions described here are more widely applicable.

Relative RT-PCR requires less RNA, takes less time to complete, is less expensive than northern blot analysis, and only requires standard PCR techniques. The correlation analysis reported in this work also supports the contention that RT-PCR has greater threshold sensitivity than northern blot analysis. Relative RT-PCR and northern blot analyses gave similar results, suggesting that relative RT-PCR using a host EF-*1*α gene as an internal control is effective for determining gene expression in fungal infected plants.

Acknowledgments

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