Real-Time Quantitative RT-PCR of Defense-Associated Gene Transcripts of *Rhizoctonia solani*-Infected Bean Seedlings in Response to Inoculation with a Nonpathogenic Binucleate *Rhizoctonia* Isolate

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Accepted for publication 3 December 2004.

ABSTRACT

Wen, K., Seguin, P., St.-Arnaud, M., and Jabaji-Hare, S. 2005. Real-time quantitative RT-PCR of defense-associated gene transcripts of *Rhizoctonia solani*-infected bean seedlings in response to inoculation with a nonpathogenic binucleate *Rhizoctonia* isolate. *Phytopathology* 95:345-353.

Certain isolates of nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) are effective biocontrol agents against seedling root rot and damping-off. Inoculation of bean seed with np-BNR strain 232-CG at sowing reduced disease symptoms in bean (*Phaseolus vulgaris*) seedlings caused by *R. solani*. Molecular analyses of the spatial expression of three defense-associated genes were carried out using real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) assays. This method allowed accurate quantitative evaluation of transcript levels of *pG101* encoding for 1,3- β -D-glucanase, *gPAL1* encoding for phenylalanine ammonia lyase, and *CHS17* encoding for chalcone synthase in

1- and 2-week-old bean seedlings that were inoculated simultaneously with np-BNR and infected with *R. solani*, and in seedlings that were singly inoculated with either fungi or not inoculated. In the seedlings that were infected with *R. solani* only, results revealed that, following infection, activation of all defense-associated gene transcripts was achieved with significant increases ranging from 7- to 40-fold greater than the control, depending on the defense gene and tissue analyzed. Seedlings that were treated with np-BNR and infected with *R. solani* had expression similar to those that were treated with np-BNR only, but the levels were significantly down-regulated compared with those that were infected with *R. solani* only. These findings indicate that disease suppression by np-BNR isolate is not correlated to *pG101*, *gPAL1*, and *CHS17* gene activation.

Additional keywords: biological control, induced systemic resistance, plant defense response.

Several studies have demonstrated protection of young seedlings of many crops against damping-off and crown and root rot diseases caused by *Pythium* Pringsh. spp. and *Rhizoctonia solani* Kühn with nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) belonging to the anastomosis groups AG-A, AG-B(a), AG-B(o), AG-G, AG-I, AG-K, and AG-P (2,4,20,21,38,43). Induced resistance has been proposed as the plausible mechanism of np-BNR protection against *R. solani* in poinsettia cuttings (21), corn (29), soybean (31), and bean (22,46) in greenhouse studies. A biochemical study by Xue et al. (46) demonstrated that inoculation of the hypocotyl of etiolated bean seedlings (*Phaseolus vulgaris* L.) with the np-BNR isolate 232-CG (AG-G) elicited induced systemic resistance in the roots, crown, and cotyledons to later challenges with either the root rot pathogen *R. solani* (AG-4) or the foliar pathogen *Colletotrichum lindemuthianum* (Sacc. & Magnus) Lams.-Scrib. In addition, the np-BNR elicited a significant and systemic increase in total peroxidases, 1,3- β -glucanases, and chitinases compared with the diseased and control plants, and this increase was negatively correlated with disease incidence.

One type of induced resistance, called systemic acquired resistance (SAR), has been extensively studied and characterized (34). SAR is regulated by endogenous amounts of the systemic signal molecule, salicylic acid (SA) (34), and is effective against a broad spectrum of pathogens (19). It is an active process dependent on RNA and protein synthesis (34,41). Pathogen attack and elicitors

of biotic and nonbiotic origins cause massive changes in the pattern of RNA synthesis, and these include transcriptional activation of defense-associated genes that encode cell wall hydroxyproline-rich-glycoproteins (HRGP), production of phytoalexins, and synthesis of pathogenesis-related (PR) proteins (11,16,42).

Determining how np-BNR isolates affect defense-associated gene expression in bean is crucial to understanding this mechanism of bioprotection and, further, to lead to a more efficient use of these biocontrol agents. The recent development of real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) assays has allowed the accurate expression profiling of RNA transcripts, and has become the most useful method for characterizing gene expression in human research (3). Real-time QRT-PCR technology relies on the ability to progressively monitor fluorescence emitted from specific double-stranded DNA binding dye such as SYBR green I (25) or fluorophore-labeled probes that hybridize to target sequences during the exponential phase of the PCR reaction such that quantification is accomplished (28). Few reports have been published on the application of real-time QRT-PCR in plants, to profile the spatio-temporal expressions of hormones in strawberry (1), and to estimate gene copy number in transgenic maize and soybean (26,39). This technology clearly has not yet been completely exploited in plant-microbe interaction studies. One study, by McMaugh and Lyon (28), reported on the application of real-time QRT-PCR to analyze the expression of class II chitinase in Bermudagrass following infection with the root pathogen *Ophiostoma narmari*.

The main objective of this study was to determine whether or not alteration in the spatial expression of plant defense-associated genes is a significant mechanism of disease suppression by np-

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DOI: 10.1094/PHYTO-95-0345

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BNR. Our goal was to apply real-time QRT-PCR technology to quantify transcript levels of defense-associated genes coding for 1,3- β -D-glucanases (GLUC), phenylalanine ammonia lyase (PAL), and chalcone synthases (CHS) in roots, stems, and leaves of 1- and 2-week-old bean seedlings following infection with *R. solani*, and to compare them to levels estimated in tissues of np-BNR protected and infected or not infected with *R. solani*.

MATERIALS AND METHODS

Fungal cultures and inoculum preparation. A highly pathogenic isolate of *R. solani* AG-4 (A76) and np-BNR isolate 232-CG were cultured on potato dextrose agar plates and grown at 24°C in the dark in an incubator for 1 week. The inocula for *R. solani* and np-BNR were prepared by inoculating 50 g of sterile oat kernels in 250-ml Erlenmeyer flasks with three plugs and incubated at 24°C for 3 weeks according to the method of Cardoso and Echandi (4). Colonized oat kernels were stored at 4°C until further use. Sterile oat kernels were used as mock inoculum for the control treatment.

Plant material and inoculation. Bean seed (*Phaseolus vulgaris*) cv. Contender UT15 (Stokes Seeds Ltd., St. Catharines, ON, Canada) were surface sterilized in 30% H₂O₂ for 5 min, rinsed with sterile distilled water, and then pre-germinated on filter papers for 48 h at 24°C in the dark in an incubator. Cone-tainers (Stuewe and Sons, Corvallis, OR) of 164-ml capacity were used to grow the plants. Prior to use, the Cone-tainers were surface sterilized for 45 min in 2.5% (wt/vol) NaOCl and rinsed with distilled water. A glass marble was inserted in each one to plug the drain hole, followed by the addition of 16 ml of washed and autoclaved 65-mm gravel and then 100 ml of sand previously pasteurized for 1 h at 70°C. Two pregerminated bean seeds and three oat kernels colonized with np-BNR 232-CG or non-colonized oat kernels (mock inoculum) were placed on the top of the sand depending on the treatment. The seed and oat kernels were covered with 30 ml of pasteurized sand containing ground oat kernels (0.5 g/liter) previously colonized with *R. solani* A76 or mock inoculum, depending on the treatment. There were four treatments: (i) uninoculated control receiving mock inoculum (–B–R), (ii) inoculated with np-BNR isolate (+B–R), (iii) infected with *R. solani* isolate (–B+R), and (iv) inoculated with both np-BNR and *R. solani* isolates (+B+R). The treatments were arranged in a randomized complete block design with split-plot restriction and three replicates. The harvesting weeks (1 and 2 weeks after seeding) were randomized among the main plots whereas the four inoculation treatments (–B–R, +B–R, –B+R, and +B+R) were randomized among subplots. The entire experiment was repeated twice.

Each Cone-tainer received 15 ml of sterile water at planting and then 10 ml of sterile water each day until emergence of seedling. The sterile water then was replaced once every 3 days with 15 ml of 50% Hoagland's solution containing 0.5 M K₂SO₄, 1 M

MgSO₄, 0.05 M Ca(H₂PO₄), 0.01 M CaSO₄, FeEDTA at 0.134 g/100 ml, and micronutrient solution at 1 ml/liter consisting of 2.86 g of H₃BO₃, 1.81 g of MnCl₂ · 4H₂O, 0.22 g of ZnSO₄ · 7H₂O, 0.08 g of CuSO₄ · 5H₂O, and 0.02 g of H₂MoO₄ · H₂O in 1 liter of H₂O. Plants were grown in growth cabinets maintained at 23 and 20°C day and night, respectively, with 80% relative humidity. Lighting was maintained for 16 h/day generating a photon flux of 210 μ mol m^{–2} (PAR) at the surface of the Cone-tainer.

Evaluation of disease. At harvesting (1 and 2 weeks after seeding), the plants were removed from the Cone-tainers and the root system was washed with tap water. The severity of hypocotyl rot caused by *R. solani* was evaluated visually for each plant using a disease severity scale ranging from 0 to 5 on the basis of necrotic lesion development (4), where 0 = no lesions on hypocotyls, 1 = lesions $\leq$ 2.5 mm long, 2 = lesions 2.5 to 5.0 mm long, 3 = lesions $\geq$ 5.0 mm long, 4 = lesions girdling the seedling, and 5 = seedling is damped-off. Disease severity in each replicate was determined by averaging the sum of individual scores of two seedlings per Cone-tainer.

Primer design. All primers used in this study (Table 1) were designed using the software DNAMAN (version 4.13) based on bean mRNA sequences deposited in GenBank. The specificity of the primers was checked by alignments with the original GenBank sequences using the standard nucleotide-nucleotide BLAST (blastn; provided online by NCBI). Because each primer set is located on the same exon of the gene, they can amplify genomic DNA as well as cDNA. Therefore, DNase treatment was carried out to ensure that RNA samples were DNA free. Conventional RT-PCR and PCR assays followed by gel electrophoresis were performed to verify the amplification of bean cDNA and genomic DNA using the designed primers. Melting point analysis was performed at the end of the real-time RT-PCR to confirm the amplification of a unique product for each gene (Fig. 1A).

RNA extraction and RT. After scoring the plants (at 1 and 2 weeks old) for disease severity, the plants were separated into roots, stems, and leaves and flash-frozen with liquid nitrogen and stored at –80°C. The entire explant tissue of each treatment was ground into fine powder in liquid nitrogen using a mortar and pestle. Total RNA from 1- and 2-week-old plants was extracted from 100 mg of ground tissue using the RNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) and DNase treated by the RNase-Free DNase Set (Qiagen), according to the manufacturer's recommendations. Contamination of residual genomic DNA in all RNA samples was verified by conventional PCR amplification on total RNA using the designed primers listed in Table 1. RNA samples containing DNA were further DNase treated until no PCR amplification of RNA samples was obtained. Prior to retro-transcription experiments, the concentration and integrity of RNA were verified by an optical density at 260 nm (OD₂₆₀)/OD₂₈₀ absorption ratio using Beckman DU640 spectrophotometer and electrophoresis on formaldehyde agarose gel according to the manufacturer's recommendations. Total RNA was quantified

TABLE 1. Primers designed and used in real-time reverse transcription-polymerase chain reaction (RT-PCR) for amplifying defense-associated genes of *Phaseolus vulgaris*^a

Target gene	Primer name	Primer sequence (5'–3')	PCR product size (bp)	Gene location (accession no.)	Reference
<i>pG101</i>	PvglucF	GCTGTAAGGGCTCAAGGCCTC	427	357–783 (X53129)	9
	PvglucR	CCAAGTACACAGTGCCTTGTC	...	...	...
<i>gPAL1</i>	PvpalF	AAGCCATGTCCAAAGTGCTG	240	798–1037 (M11939)	6
	PvpalR	GAGTTCTCCGTTGCCACCT	...	...	...
<i>CHS17</i>	PvchsF	CAAACCTCTGGGACTTCGGC	180	492–671 (X06411)	35
	PvchsR	CTGGGTGTCACTTGGCCC	...	...	...
<i>Actin homolog</i>	PvactinF	CACCGAGGCACCGCTTAATC	126	177–302 (AB067722)	(K. Takahara, S. Hamada, H. Matsui, and H. Ito, unpublished data)
	PvactinR	CGGCCACTAGCGTAAAGGGAA	...	...	...

^a *pG101* = 1,3- β -D-glucanase; *gPAL1* = phenylalanine ammonia lyase; *CHS17* = chalcone synthase; and actin homolog.

Fig. 1. Example of analysis of specificity of real-time quantitative reverse transcription-polymerase chain reaction products amplified for the following defense-associated genes: **A and B**, *pG101*; **C and D**, *gPAL1*; **E and F**, *CHS17*; and **G and H**, actin, using the designed primers listed in Table 1. **A, C, E, and G**, Melting peak profiles. **A**, *pG101* transcripts; **C**, *gPAL1* transcripts; **E**, *actin* transcripts. Symbols: ● = cDNA from leaves, ○ = cDNA from stems, ▼ = cDNA from roots, ▽ = negative control, and ■ = RNA sample. **B, D, F, and H**, Corresponding agarose gel (1.0%) electrophoresis. Lanes: L = 100-bp DNA ladder; lanes 1 to 3 = amplification of cDNA from 62.5 µg of RNA of bean leaves, stems, and roots respectively; lane 4 = negative control; and lane 5 = amplification of 17 ng of bean RNA template.

assuming that OD₂₆₀ of 1.0 approximates RNA at 40 µg/ml. An amount of 0.5 µg of total RNA was reverse transcribed into 20 µl of cDNA using the QIAGEN Omniscript RT Kit according to the manufacturer's recommendation.

cDNA amplification of defense-associated genes by conventional RT-PCR. Conventional RT-PCR assays were performed on cDNA templates, prepared from total RNA that was extracted from bean tissues, using the designed primers listed in Table 1. cDNA samples were diluted 100-fold with autoclaved Elga pure water in order to minimize the suppression of PCR by RT solution; whereas, with diluted samples showing low amplification, original concentrations were used. Amplification was performed in a 25-µl reaction mixture containing 2.5 µl of 10× PCR buffer, 2.5 µl of 8 mM (2 mM each) dNTP, 2.5 µl of 15 mM MgCl₂, 2.5 µl of 2 µM of each primer, 10.4 µl of PCR water, 0.1 µl of 5 µl/µl *Taq* polymerase (Invitrogen Life Technologies, Burlington, ON, Canada), and 2 µl of cDNA. The amplification conditions for *pG101* cDNA were 94°C for 3 min for denaturing, followed by 30 cycles of amplification at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min. Following amplification, a 10-min extension at 72°C was added at the end of the run. The amplification conditions for *gPAL1*, *CHS17*, and the internal control gene, actin, were as follows: 94°C for 2 min, followed by 30 cycles amplification at 94°C for 1 min, 64°C for 1 min, and 72°C for 2 min. An extension step similar to that for *pG101* was performed at the end the run. In all RT-PCR runs, appropriate negative controls containing no cDNA template were subjected to the same procedure in order to exclude or detect possible contamination or carryover.

The RT-PCR products for all target genes were purified using QIAquick PCR Purification Kit and quantified using Beckman DU640 spectrophotometer. Depending on the gene, serial dilutions of purified RT-PCR products in the range of concentration from 10¹ to 10⁹ copies and over 4 to 5 orders of magnitude were prepared to generate standard curves for real-time QRT-PCR assays.

Gene expression analysis by real-time QRT-PCR. Real-time QRT-PCR was performed for each of the defense-associated genes and for actin on cDNA templates prepared from total RNA that was extracted from bean tissues using the LightCycler (Roche, Montreal, QC, Canada) and SYBR Green PCR master mix (Qiagen) according to the manufacturer's recommendations (Qiagen). A set of standard solutions prepared from RT-PCR products was included in each run. Reactions were prepared in capillaries using the following concentrations: 10 µl of SYBR Green master mix, 5 µl of PCR water, 2 µl of each primer set (Table 1), and 1 µl of cDNA or standard solution as template. The amplification conditions for *pG101* cDNA were 95°C for 15 min to activate the hot-start recombinant *Taq* DNA polymerase, followed by 55 cycles of amplification at 94°C for 15 s, 65°C for 20 s, 72°C for 35 s, and 79°C for 5 s to avoid dimer formation. Following amplification, a melting curve program (70 to 95°C with a

heating rate of 0.1°C/s) and finally a cooling step to 40°C were added. The amplification conditions for *gPAL1*, *CHS17*, and actin cDNA were as follows: 95°C for 25 min, followed by 45 cycles amplification at 94°C for 15 s, 64°C for 20 s, and 72°C for 6 s. Similar conditions for melting curve and cooling step as those for *pG101* were conducted.

The Fit Points Method was performed using the LightCycler software 3.3 for the analysis of the standard curve. The crossing points (CPs) were determined at a fixed fluorescence threshold of 0.2. A linear relationship was obtained for each run by plotting the threshold cycle numbers (CTs) against the logarithm of cDNA copies of a set of standard solutions. The equation of the line that best fits the data was determined by minimizing error for regression analysis. The *R*² value was calculated to estimate the accuracy of the real-time RT-PCR as a quantification method. The slope of the standard curve was used to calculate the efficiency of the real-time QRT-PCR according to the formula $E = 10^{(-1/\text{slope})}$. The fold changes of defense-associated gene transcripts in different treatments versus control (i.e., mock treatment) were normalized using the CT and efficiency obtained for the actin amplification run on the same cDNA templates using the formula established by Pfaffl (30) and modified by McMaugh and Lyon (28): fold change = $[(E_{\text{gene}})^{\Delta\text{CT}_{\text{gene}}(\text{control} - \text{sample})}] / [(E_{\text{actin}})^{\Delta\text{CT}_{\text{actin}}(\text{control} - \text{sample})}]$, where *E*_{gene} is the real-time RT-PCR efficiency of a defense gene transcript, *E*_{actin} is the real-time RT-PCR efficiency of the housekeeping gene actin transcript, $\Delta\text{CT}_{\text{gene}}$ is the CT deviation of control – sample of the target gene transcripts, and $\Delta\text{CT}_{\text{actin}}$ = CT deviation of control – treatment of actin transcripts.

Statistics. Disease severity scores were subjected to the Kurskal-Wallis test, a nonparametric ranking procedure, using PROC NPAR1WAY in SAS (36). Because the overall Kruskal-Wallis test was significant (*P* < 0.0001), pairwise comparisons of treatments were done using Mann-Whitney tests. The fold changes of defense-associated gene transcripts were tested for significant difference between harvesting time (1- and 2-week-old) and inoculation treatments using two-way analysis of variance (ANOVA). The data generated from repeated trials were tested for homogeneity using the χ^2 test (12). Where appropriate, the data were pooled and analyzed as six blocks. Data were tested for normality and were transformed using logX+1 wherever necessary. Statistical analyses of fold change of defense gene transcripts were done using PROC GLM in SAS (36). Comparisons between means were made using least significant differences at a 0.05 probability when ANOVA indicated model and treatment significances.

RESULTS

Effect of np-BNR on stem rot of bean. Bean seedlings inoculated with np-BNR (+B–R) like mock-inoculated plants (–B–R) showed no symptoms regardless of the harvesting period (Table 2). Seedlings protected with isolate 232-CG and infected with *R. solani* (+B+R) had significantly (*P* < 0.05) lower disease severity compared with those that were infected with *R. solani* but nonprotected (–B+R).

Quantification of defense-associated gene transcripts in bean. The choice of the defense-associated genes studied was dictated by the fact that all of them have been reported to be activated in bean tissues upon elicitation, wounding, or infection (Table 1). Each primer set, designed to amplify a particular defense gene or the housekeeping gene actin, had successfully amplified a unique putative product of similar size when cDNA (Fig. 1B, D, F, and H) and DNA (data not shown) were used as template in conventional RT-PCR runs. However, before initiating real-time QRT-PCR experiments, all total plant RNA samples were treated with RNase-free DNase prior to reverse transcription and tested for the presence of DNA by PCR amplification assays using the designed primer pairs. No signal or PCR products were

TABLE 2. Effect of nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) inoculation on disease severity caused by *Rhizoctonia solani*

Treatment description and designation	Disease scale ^a	
	Week 1	Week 2
Mock inoculated (–B–R)	0 c	0 c
Plants inoculated with np-BNR 232-CG only (+B–R)	0 c	0 c
Plants infected with <i>R. solani</i> only (–B+R)	5 a	5 a
Plants inoculated with np-BNR 232-CG and infected with <i>R. solani</i> (+B+R)	2 b	2 b

^a Disease severity scale for *R. solani* ranged from 0 = no lesions on hypocotyls, 1 = lesions ≤2.5 mm long, 2 = lesions 2.5 to 5.0 mm long, 3 = lesions ≥5.0 mm long, 4 = lesions girdling plant, and 5 = plant damped-off. Values represent median of six replicates. Values in a column with different letters are significantly different (*P* < 0.05) (Mann-Whitney tests).

detected when RNA was used as template (Fig. 1B, D, F, and H). A LightCycler melting curve analysis was performed for specificity of quantitative RT-PCR products for each primer set which resulted in single products with the following specific melting temperature: *pG101*, $81.87 \pm 0.84^\circ\text{C}$; *gPAL1*, $79.49 \pm 0.58^\circ\text{C}$; *CHS17*, $82.83 \pm 1.33^\circ\text{C}$; and actin, $79.74 \pm 1.18^\circ\text{C}$ (Fig. 1A, C, E, and G).

Standard curves and sensitivity of real-time QRT-PCR. In this study, standard curves for each of the four genes were constructed using different concentrations of amplified cDNA products that ranged over four- to fivefold magnitude. A linear regression relation between logarithm of known gene cDNA copy number and real-time CTs over the range of cDNA concentrations was established (Fig. 2). For all of the four genes studied, the square regression correlation coefficient (R^2) of detection ranged between 0.9901 and 1.0000 (Fig. 2).

Depending on the gene, titers as low as 10^1 of template molecules of target transcripts could be reproducibly detected using SYBR Green Dye1. Amplification plots were highly reproducible between triplicate samples of the same treatment (Fig. 3A to D), and fluorescence data from negative controls con-

taining no templates always remained well below the threshold level.

Validation of reference gene for normalization. The amount of the housekeeping gene actin did not show significant change between treatments ($P > 0.05$) (Fig. 3D). Thus, the relative expression ratio of defense-associated genes in the treated plants versus the control (-B-R) was estimated by normalizing against actin (Fig. 4).

Spatial expression of defense-associated gene transcript levels. There were no significant differences ($P > 0.05$) in temporal expression of defense-related gene transcripts between the two harvesting times (i.e., 1 and 2 weeks). Therefore, we present the results only for the first harvesting time point. There was a systemic and spatial expression of transcript levels of *pG101*, *gPAL1*, and *CHS17* genes in bean seedlings that were either infected with *R. solani*, protected with np-BNR, or both; although, in general, transcript levels were lower in roots than in leaves and stems (Fig. 4). Compared with the mock treatment, the highest defense gene expression was achieved in *R. solani*-infected stems for *CHS17* (41-fold) and *pG101* (26-fold) (Fig. 4C and A), followed by *gPAL1* (8-fold) in leaves of the same treatment (Fig.

Fig. 2. Example of real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) efficiencies for the defense-associated genes *pG101*, *gPAL1*, *CHS17*, and bean actin. Depending on the gene, purified RT-PCR products were diluted serially in the range of concentration from 10^1 to 10^5 copies and over four to five orders of magnitude to be amplified in QRT-PCR assay. The crossing points (CPs) were determined at a fixed fluorescence threshold of 0.2. A linear relationship was obtained for each run by plotting the threshold cycle number (Ct) against the logarithm of the known copies of starting template in the dilution series. The equation of the line that best fits the data was determined by minimizing error for regression analysis. The R^2 value was calculated to estimate the accuracy of the real-time RT-PCR as a quantification method. The slope of the standard curve was used to calculate the efficiency of the real-time QRT-PCR according to the formula $E = 10^{(-1/\text{slope})}$. A, *pG101*; B, *gPAL1*; C, *CHS17*; and D, actin homolog transcripts.

4B). Protected and infected seedlings (+B+R) showed a significant down-regulation ($P < 0.05$) of transcript levels in all tissues for *pG101* and in leaves and stems for *gPAL1* and *CHS17* genes compared with those that are infected and not protected (–B+R). This decrease ranged between two- and fourfold depending on the tissue and the gene analyzed (Fig. 4). Regardless of the tissue analyzed, transcript levels of *pG101*, *gPAL1*, and *CHS17* in np-BNR-protected and *R. solani*-infected (+B+R) bean seedlings were not significantly different from those estimated in protected but noninfected seedlings (+B–R). Of interest, the relative expression ratio of *gPAL1* and *CHS17* genes in protected but noninfected root tissue (+B–R) was <1 , lower than the background expression value estimated in mock treatments (Fig. 4B and C).

DISCUSSION

Our results indicate that inoculation of bean seed at planting with np-BNR isolate 232-CG significantly protected bean seedlings from infection with the pathogen *R. solani*. Similar protection by the same np-BNR isolate and by different isolates belonging to several anastomosis groups was achieved on potato (10), poinsettia (21), snap bean (4), cucumber (7,43), corn (29), creeping bentgrass (2), cabbage (33), and several nursery plants (17). The protection efficiency of np-BNR isolates has been reported to vary, depending on the np-BNR isolate used (10,43), inoculation time (29,43), inoculum type (5), and environmental factors (2,33). Pre-inoculation of plants with np-BNR isolates 1 to 3 days earlier to subsequent challenge with *R. solani* has been reported to be effective in controlling disease (2,18,29). In this

study, we have achieved 60% reduction in *Rhizoctonia* stem rot by simultaneous inoculation of bean plants with np-BNR 232-CG and *R. solani* at seeding. These results indicate that np-BNR 232-CG played an important role in preventing the onset of the disease and in reducing the rate of disease expansion.

To the best of our knowledge, we are the first to perform real-time QRT-PCR assays to quantify the levels of spatial alteration of defense-related gene transcripts during interaction of bean plants with a nonpathogenic fungus in the presence of a pathogen. Several factors that may affect the reliability of real-time QRT-PCR were carefully controlled in conducting our experiments. (i) Specific primer sets were designed that amplified unique single products that were confirmed by melting curve and gel analyses. Only the primers amplifying unique bands and revealing single melting peaks were chosen for use in this study. (ii) DNase treatment on all RNA samples was a must because the reference gene and all the defense genes used in this study contained only one exon, and the designed primers in this case could not avoid amplifying genomic DNA. (iii) Constructing linearity standard curves with coefficient correlations (r^2) ranging from 0.99 to 1.00 led to accurate calculation of the amplification efficiency that was used in calculation of the relative level of gene transcripts. (iv) It was statistically validated that the expression levels of the house-keeping gene actin remained constant ($P > 0.05$) with treatment. This was essential because it has been reported that actin varies considerably and that it cannot be used reliably as an internal control (15,37,40). (v) A proper mathematical model (28,30) that accounts for both differences in amplification efficiency and threshold cycle numbers was chosen. The results obtained with

Fig. 3. Kinetics of fluorescence signal versus cycle numbers measured during amplification of the defense-associated genes *pG101*, *gPAL1*, *CHS17*, and the house-keeping gene bean actin cDNA subjected to four inoculation treatments. **A**, *pG101* cDNA transcripts; **B**, *gPAL1* cDNA transcripts; **C**, *CHS17* cDNA transcripts; and **D**, actin transcripts. Symbols: ○ = +B–R, ● = –B+R, ▽ = +B+R, ▼ = –B–R, and ■ = negative control.

this formula were identical to those calculated by the Roche Light Cycler Relative quantification software (32).

The significant increase in the spatial expression of transcript levels of *pG101*, *gPAL1*, and *CHS17* in bean seedlings due to *R. solani* infection is a strong indication that *R. solani* stimulated a systemic accumulation of GLUC, a PR protein (27), and production of CHS and PAL, two key enzymes of the phenylpropanoid pathway that lead to phytoalexin accumulation (6,35). In addition, the results of this study clearly showed that the expression of mRNA did not remain restricted to the vicinity of *R. solani* infection sites (crowns and hypocotyls), but spread rapidly to non-protected and noninfected tissues (i.e., the cotyledons and leaves). This phenomenon implies, therefore, the existence of a signal that spreads systematically from the hypocotyls to the rest of the plant. Many studies have indicated that SA, a natural phenolic compound, is an important signaling factor in the induction of SAR (23,24,34). In a previous histochemical study conducted by Jabaji-Hare et al. (22), *R. solani* successfully invaded the bean cortical tissue, causing cell wall degradation. It is very likely that cell wall fragments might have served as elicitors to produce a signal in infected plants, such as SA leading to substantially higher expression levels of the defense gene encoding GLUC, PAL, and CHS.

Of interest, inoculation of bean seed with np-BNR (+B-R) did not elicit a significant defense response as demonstrated by levels of gene expression similar to that of the noninoculated control. However, the presence of np-BNR significantly affected the plant's defense responses only during a pathogenic infection (+B+R) by significantly down-regulating the expression of the defense genes at the mRNA levels relative to those estimated in infected seedlings (-B+R). These findings indicate that protection of bean seedlings with np-BNR against *R. solani* is not the result of the activation of *pG101*, *gPAL1*, and *CHS17* genes 1 or 2 weeks after inoculation. Instead, protection could be associated with a stronger cell wall fortification resulting from the activation of defense genes encoding structural proteins such as HRGP (11) or the deposition of phenols, suberin, and pectic substances in np-BNR-protected epidermal and cortical cells. This ultimately would strengthen and alter the host's cell wall, providing a barrier for the invading hyphae of *R. solani* (22). Alternatively, the down-regulation also could be viewed as a weaker elicitation because of a lower development of the pathogen in protected plants that may be mediated by any other possible mechanism involved in disease suppression.

Another plausible possibility is that the activation of the plant's defense response occurred earlier than 1 week. We previously have provided evidence that substantial increases in enzymatic activities of peroxidases and PR proteins (46), as well as in total mRNA transcripts encoding PR proteins and PAL (45) in etiolated bean seedlings 48 h after inoculation with np-BNR, were associated with reduction in disease severity caused by *R. solani*. The discrepancy in the results of these studies could be explained by the fact that np-BNR stimulated an earlier (hours after inoculation) but transient systemic activation of some defense genes during the establishment of np-BNR colonization on bean plants (earlier studies) followed by down-regulation at later stages (this study). The early but transient activation of defense genes may have been sufficient to provide bean plants protection against *R. solani*.

Down-regulation or suppression of the plant's defense encoding genes in response to other nonpathogenic interactions is not a new phenomenon. During the arbuscular mycorrhizal (AM) symbiotic interaction, the expression of *MD17*, a cDNA encoding a basic chitinase, was suppressed during the establishment and colonization of the AM fungus *Glomus intraradices* in tobacco roots (8); whereas, during dual inoculation of bean and alfalfa with *G. intraradices* and *R. solani*, mRNA transcripts encoding key enzymes in the phenylpropanoid pathway (13,14) or encoding

glucanases (44) were down-regulated to levels below those observed in infected plant tissues. Taken together, the results of the current study along with previous studies clearly strengthen the concept that plants suppress or down-regulate the defense response elicited by pathogenic fungi during root and stem colonization by AM fungi and np-BNR, respectively. Further research is needed to understand the mechanism implicated in down-regulation during plant-pathogen-np-BNR interaction. In AM-plant-pathogen interactions, it has been proposed that suppressor molecules originating from AM fungi can suppress the plant's defense response for facilitating establishment of AM colonization, and this suppression overcomes pathogen elicitation (13). Whether similar suppressor molecules exist during np-BNR colonization and can overcome the pathogen elicitation needs to be further investigated.

Fig. 4. Effect of inoculation with nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) and infection with *Rhizoctonia solani* on transcript levels of the defense-associated genes **A**, *pG101*, **B**, *gPAL1*, and **C**, *CHS17*. Transcript levels were estimated using real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR). The crossing points were determined at a fixed fluorescence threshold of 0.2. Fold change was calculated from the real-time QRT-PCR efficiencies and the threshold cycle (Ct) deviation of an inoculation treatment versus control. The fold change represents the means of three replicates. Significance was determined among treatments using least significant difference (LSD) ($P < 0.05$). +B-R = plants inoculated with np-BNR only; -B+R = plants infected with *R. solani* only, and +B+R = plants inoculated with np-BNR and infected with *R. solani*. The fold change ratio of control (-B-R) was always 1 and was not included in the statistical analyses.

In summary, the present study provides evidence that QRT-PCR is a powerful technique in quantification of defense-associated genes transcript levels, especially with low transcript levels. Inoculation of bean seed at sowing with a nonpathogenic isolate of binucleate *Rhizoctonia* sp. (np-BNR) reduced disease symptoms caused by *R. solani*. However, this protection seems to be unrelated to *pG101*, *gPLA1*, and *CHS17* activation elicited by np-BNR. For better understanding of the mechanisms responsible for bioprotection of nonpathogenic fungi, analysis of several defense-associated genes, earlier sampling times (hours after inoculation), characterizing signals, and study of competition for infection sites need to be further investigated.

ACKNOWLEDGMENTS

This work was supported by a research grant from the Fond pour la Formation des Chercheurs et l'Aide à la Recherche (FCAR-IRDA) grant to S. H. Jabaji-Hare (leader), C. Hamel, M. St-Arnaud, and R. Hogue; and partly by an Natural Sciences and Engineering Research Council of Canada (NSERC) grant to S. H. Jabaji-Hare. We thank G. Taylor for his technical help.

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