Elicitation of Genes Encoding Enzymes of the Phenylpropanoid Pathway and Isoflavones in Soybean Seedlings by Pathogenic and Non-Pathogenic Fungi

Suha Jabaji, Philippe Seguin and Huilan Chen

Department of Plant Science, Macdonald Campus of McGill University, 21111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, H9X 3V9, Canada. Email: suha.jabaji@mcgill.ca

Abstract

Biocontrol properties of non-pathogenic *Rhizoctonia* isolates (np-R) lie in its ability to induce systemic resistance in plants. In this study we determined whether simultaneous inoculation of soybean seedlings with an np-R and infected with the plant pathogen *Rhizoctonia solani,* or single inoculation of each fungus has an impact on gene expression of key enzymes of the phenylpropanoid pathway, which are involved in plant defence and production of isoflavones. Temporal expression of 12 genes encoding enzymes in the phenylpropanoid pathway was done using QRT-PCR on seedlings inoculated with one of four treatments: i) np-R, ii) *R. solani*, iii) np-R and *R. solani*, and iv) untreated control. Compared to the control treatment, seedlings that were simultaneously np-R inoculated and infected with *R. solani* or seedlings that were only infected had significantly higher transcript levels of chalcone synthase coding for *CHS*1,3,4,5,6 and for *CHS*7, 8, chalcone reductase (*CHR*), isoflavone synthase coding for *IFS*2 and chalcone isomerase coding for *CHI*4. np-R caused a decrease of *CHI*4 compared to the other treatments. The remaining genes were minimally affected by np-R or *R. solani* treatment either singly or in combination. np-R caused an increase in total root isoflavone concentrations one week after treatment, but reduced root concentrations after two weeks, while *R. solani* increased total leave concentrations after two weeks. np-R was effective in controlling *R. solani.* Seedling dry weight, height, and nodule number one and three weeks after treatment were comparable to the control when np-R was applied with *R. solani*.

Media Summary

Genes encoding enzymes in the phenylpropanoid pathway in soybeans are altered by fungi.

Key Words

Quantitative gene expression, plant pathogens, *Rhizoctonia* species, defense genes, real-time QRT-PCR

Introduction

Protection of young seedlings of many crops against diseases caused by *Rhizoctonia solani* with non-pathogenic *Rhizoctonia* species (np-R) is well documented. Induced resistance is proposed as the plausible mechanism of np-BNR protection against *R. solani* in several crops including soybeans (Poromato et al. 1998). Pathogen attack and elicitors of biotic and non-biotic origins, cause massive changes in the pattern of RNA synthesis, and these include transcriptional activation of defense associated genes. We have shown that beans inoculated with np-R reduced *R. solani* infections, elicited a significant increase in peroxidases, glucanases and chitinases compared to the diseased and control plants (Xue et al. 1998), but did not increase gene expression of phenylalanine ammonia lyase (*PAL*), chalcone synthase (*CHS*) and glucanases (*GLUC*) (Wen et al. 2005). However, activation of all three transcripts was achieved in *Rhizoctonia* infected seedlings with significant 7-40 fold increases depending on the defense gene and tissue analyzed, relative to the control (Wen et al. 2005). The objectives of this study were to determine whether or not alteration in the temporal expression of soybean defense-related genes is a significant mechanism of disease suppression by np-R. Real-time QRT-PCR technology was used to quantify transcript levels of several genes in the phenylpropanoid pathway of soybean seedlings following infection with *R. solani* and compare them to levels estimated in tissues of np-R protected and infected or not with *R. solani*.

Methods

Biological materials: Two pre-germinated soybean seeds (AC Proteina) and three oat kernels colonized with the non-pathoegnic np-BNR 232-CG or non-colonized oat kernels (mock inoculum) were placed in one Cone-tainer™ containing autoclaved sand. The seeds and oat kernels were covered with 5 g of sand containing ground oat kernels previously colonized with the pathogen *R. solani* A76 or mock inoculum, depending on the treatment. Each Cone-tainer™ received Hoagland's solution. One ml of 1×10^9 CFU/ml *Rhizobium japonicum* 532C was added 5d after planting to each Cone-tainer™. *Treatments description:* 1. soybean seeds coated with water and inoculated with BNR and *R. solani* (B+R+), 2. soybean seeds coated with water and inoculated with BNR (B+R-), 3. Soybean seeds coated with water and inoculated with *R. solani* (B-R+), 4. soybean seeds coated with water (B-R- or control). The treatments were arranged as a RCBD with split-plot restriction and six replicates. Seedlings were harvested 7d, 14d and 21d post-sowing, and plant height, dry weight, photosynthesis and disease severity scores caused by *R. solani* were recorded.

Isoflavones extraction and quantification

Isoflavones were extracted from the upper part (leaves and stems) and lower part (roots) using the method of Seguin et al. (2004). Isoflavones were separated by HPLC and were detected at 254 nm. Purified isoflavones (daidzein, genistein; Sigma-Aldrich) were used as standards and concentrations were expressed on a DM basis. Concentrations of aglycones were summed to obtain total isoflavone concentration.

Gene expression

Primer Design: Based on soybean protein sequences or corresponding mRNA sequences published in Genbank primer pairs for *PAL*, *CHS*1,3,4,5,6, *CHS*6, *CHS*7,8, *CHI*1A, *CHI*1B, *CHI*2, *CHI*3, *CHI*4, *CHR*, *IFS*1 and *IFS*2 genes were designed using online software Primer 3 in biology workbench (http://workbench.sdsc.edu). The primers were evaluated by alignments with the original contigs from EST soybean bank and online standard nucleotide-nucleotide BLAST search (blastn in http://www.ncbi.nlm.nih.gov/BLAST/). For normalization purposes of quantification of target genes expression, the housekeeping genes (HKG) encoding actin was used. All amplifiable products were sequenced to ensure that the right sequence of the gene is amplified. *Isolation of RNA and Reverse Transcription and Quantitative PCR*: Total RNA was isolated using the RNeasy Plant Mini Kit and treated with RNase-free DNase I (Qiagen) and reverse transcribed to cDNA using QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's recommendations. *Quantitative reverse transcription (QRT)-PCR*: The reactions (20 µL) were performed for each of the target gene and for the HKG, actin using Mx3000P (Stratagene) and SYBR Green QPCR master mix. Each run included a negative control and a set of standard solutions prepared from the RT-PCR product.

Data analysis and statistics

Expression of the target genes was normalized against actin and relative transcript level ratios were calculated using equation 1 from Roche Applied Science: Ratio = $(E_{ref})^{CT\ ref}/(E_{target})^{CT\ target}$ where E_{ref} and E_{target} represent the PCR amplification efficiencies for the HKG and target gene, respectively, and which were calculated using equation 3 from Liu and Saint (2002): $E=(R_{n,A}/R_{n,B})^{1/(Ct,A-Ct,B)}$. Rn,B and Rn,A are two fluorescence levels taken during the exponential phase at two crossing points (Cts), Ct,B and Ct,A. Disease severity scores were subjected to Kurskal-Wallis test, using PROC NPAR1WAY in SAS. As the overall Kruskal-Wallis test was significant ($P<0.0001$), pairwise comparisons of treatments were done using Mann-Whitney tests. Other variables were tested for significance using PROC GLM in SAS. The experiment was repeated twice, and data from both trials were tested for homogeneity using the chi-square test. Where appropriate, the data were pooled and analyzed as 12 replicates. Data were tested for normality. Comparisons between means were made using least significant differences (LSD) at a 0.05 probability level when ANOVA indicated model and treatment significances.

Results

B+ and R+B+ seedlings had similar heights, weights, nodule numbers and photosynthesis as compared to control plants at all times except for plant height at 2-wks (Table 1). *R. solani* drastically decreased nodule number of 2 and 3-week old seedlings. Disease severity in 1 and 2-wk old seedlings was significantly decreased in infected seedlings treated with np-R as compared to those only infected.

Table 1. Effect of inoculation treatment on plant height, dry weight, disease score, nodule number and photosynthesis of soybean seedlings (n=6).

Treatment	Height (cm)	Dry Wt (g)	Disease Score	Nodule no.	Photosynthesis
		1-wk-old seedlings			
B+	5.65 A	0.13 A	0		
R+	3.19 B	0.16 A	4 A		
R+B+	4.65 AB	0.13 A	2 B		
Control	5.38 A	0.14 A	0		
		2 wk-old seedlings			
B+	11.7 AB	0.34 A	0	32 A	
R+	6.6 C	0.17 B	4 A	5 B	
R+B+	8.64 BC	0.28 AB	2 B	27 A	
Control	12.1 A	0.34 A	0	38 A	
		3-wk-old seedlings			
B+	16.8 A	0.54 AB	0	43 A	8.98 A
R+	7.73 B	0.24 B	3 A	8 B	6.40 A
R+B+	15.58 A	0.38 AB	3 A	31 A	6.98 A
Control	17.2 A	0.59 A	0	45 A	9.41 A

Means followed by different letters are significantly different ($P<0.05$).

Total root isoflavones increased significantly in np-R treated seedlings after one week compared to the other treatments. *R. solani* infected seedlings had significantly higher amounts of total leaves and stems isoflavones after two weeks. After three weeks, seedlings that were infected and treated with np-R showed substantially higher amounts of isoflavones in leaves and stems relative to that found in infected but not treated and non-infected np-R treated seedlings (Table 2).

Table 2. Effect of inoculation treatment on total isoflavones concentration (n=3).

Treatment	1-wk-old		2-wk-old		3-wk-old	
	Total isoflavones (µg/g dry wt)					
	L+S	Roots	L+S	Roots	L+S	Roots
B+	2322 A	4031 A	1506 B	4691 B	1375 B	6058 A
R+	3514 A	2286 B	2962 A	4628 B	1310 B	4124 A
B+R+	3936 A	2077 B	1729 B	5601 AB	2758 A	5684 A
Control	2924 A	1334 B	1610 B	6556 A	1895 AB	6843 A

Means followed by different letters are significantly different ($P<0.05$). L+S, Leaves and stems

Gene expression

The relative gene expression of 4 out of 12 genes or genes cluster was altered in seedlings that were simultaneously inoculated with np-R and infected with *R. solani*, or in seedlings that were only infected (Figure 1). Relative to the control, seedlings that were infected or simultaneously infected and treated with np-R had significantly higher transcript levels of *CHS*1,3,4,5,6, *CHS*7,8, *CHR,* and *IFS*2. Seedlings that were treated with np-R had similar genes expression levels as the control and were significantly lower compared to those that

were infected only. Irrespective of the treatment, the expression levels of the remaining genes remained similar to the control (data not shown).

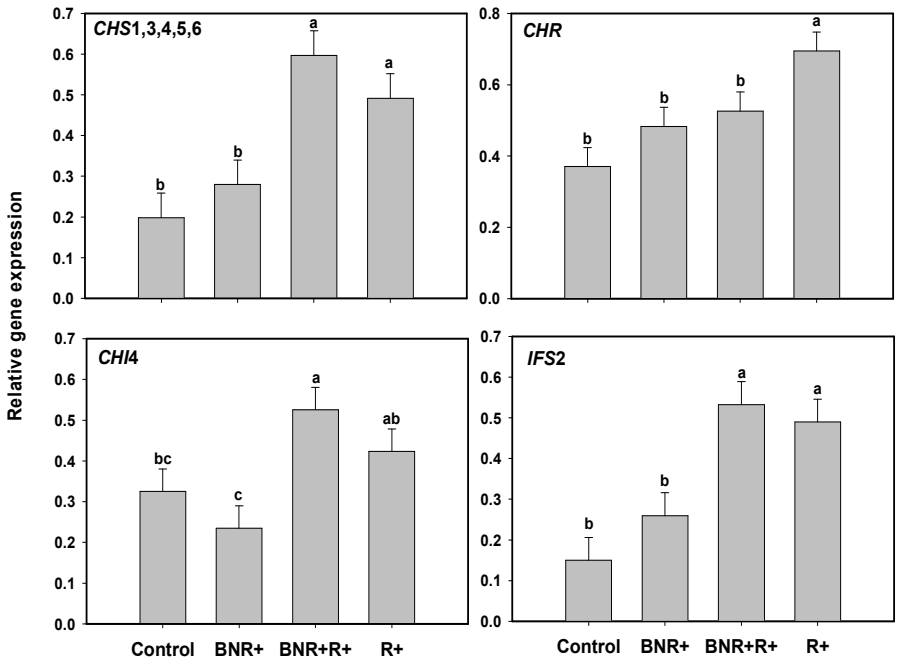

Figure 1. Relative transcript levels ratios of *CHS*, *CHR*, *CHI*, and *IFS* in soybean sprouts. Relative gene abundance represents number of target genes relative to actin.

Conclusions

These findings indicate that disease suppression by np-R isolate is not correlated to any of the genes encoding enzymes in the phenylpropanoid pathway. As previously reported for other pathogens of soybeans, infections cause substantial increase in gene transcripts.

References

Liu, W.H., and D.A. Saint. 2002. A new quantitative method of real time reverse transcription polymerase chain reaction assay based on simulation of polymerase chain reaction kinetics. Anal. Biochem. 302:52-59.

Poromarto, et al. 1998. Association of binucleate *Rhizoctonia* with soybean and mechanism of biocontrol of *Rhizoctonia solani*. Phytopathology 88:1056-1067.

Seguin et al. 2004. Isoflavone content of soybean cultivars grown in eastern Canada. J Sci. Food Agric. 84:1327-1332.

Wen, et al. 2005. Real-time quantitative RT-PCR of defense gene transcripts of *Rhizoctonia solani*-infected bean seedlings in response to inoculation with a non-pathogenic binucleate *Rhizoctonia* isolate. Phytopathology, 95:345-353.

Xue, et al. 1998. Systemic induction of peroxidases, 1,3-beta-glucanases, chitnases, and resistance in bean plants by binucleate *Rhizoctonia* species. Phytopathology 88: 359-365.