



Development of a qPCR Strategy to Select Bean Genes Involved in Plant Defense Response and Regulated by the *Trichoderma velutinum* – *Rhizoctonia solani* Interaction

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OPEN ACCESS

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Specialty section:

This article was submitted to
Crop Science and Horticulture,
a section of the journal
Frontiers in Plant Science

Received: 30 May 2016

Accepted: 12 May 2016

Published: 04 August 2016

Citation:

Mayo S, Cominelli E, Sparvoli F,
González-López O,
Rodríguez-González A, Gutiérrez S
and Casquero PA (2016)
Development of a qPCR Strategy
to Select Bean Genes Involved
in Plant Defense Response
and Regulated by the *Trichoderma*
velutinum – *Rhizoctonia solani*
Interaction. *Front. Plant Sci.* 7:1109.
doi: 10.3389/fpls.2016.01109

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Bean production is affected by a wide diversity of fungal pathogens, among them *Rhizoctonia solani* is one of the most important. A strategy to control bean infectious diseases, mainly those caused by fungi, is based on the use of biocontrol agents (BCAs) that can reduce the negative effects of plant pathogens and also can promote positive responses in the plant. *Trichoderma* is a fungal genus that is able to induce the expression of genes involved in plant defense response and also to promote plant growth, root development and nutrient uptake. In this article, a strategy that combines *in silico* analysis and real time PCR to detect additional bean defense-related genes, regulated by the presence of *Trichoderma velutinum* and/or *R. solani* has been applied. Based in this strategy, from the 48 bean genes initially analyzed, 14 were selected, and only *WRKY33*, *CH5b* and *hGS* showed an up-regulatory response in the presence of *T. velutinum*. The other genes were or not affected (*OSM34*) or down-regulated by the presence of this fungus. *R. solani* infection resulted in a down-regulation of most of the genes analyzed, except *PR1*, *OSM34* and *CNGC2* that were not affected, and the presence of both, *T. velutinum* and *R. solani*, up-regulates *hGS* and down-regulates all the other genes analyzed, except *CH5b* which was not significantly affected. As conclusion, the strategy described in the present work has been shown to be effective to detect genes involved in plant defense, which respond to the presence of a BCA or to a pathogen and also to the presence of both. The selected genes show significant homology with previously described plant defense genes and they are expressed in bean leaves of plants treated with *T. velutinum* and/or infected with *R. solani*.

Keywords: biotic stress, systemic acquired resistance, induced systemic resistance, hypersensitive response, defense genes, biocontrol agent, *Phaseolus vulgaris*

INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is the most important food legume crop worldwide. Bean production is often affected by biotic and abiotic factors (Guerrero-González et al., 2011) by microorganisms, humidity, temperature... that are detected as signals for the activation of plant response mechanisms. This crop is affected by a wide diversity of fungal pathogens (*Sclerotinia* spp., *Fusarium* spp., *Phytophthora* spp., *Botrytis* spp.,...) among them *Rhizoctonia solani* JG Kühn [Teleomorph: *Thanatephorus cucumeris* (AB Frank) Donk] has a remarkable importance as responsible of important economic losses in this crop (Valenciano et al., 2006). *R. solani* is a necrotrophic pathogen responsible for the root and hypocotyl diseases. Plant infection occurs through wounds or by the direct action of the fungal mycelium, which tears the cuticle and penetrates the epidermis (Guerrero-González et al., 2011).

As a strategy to control bean infectious diseases, mainly those caused by fungi, the use of biocontrol agents (BCA) can reduce the negative effects of plant pathogens and they also can promote positive responses in the plant (Shoresh et al., 2010). The genera *Trichoderma*, *Gliocladium*, *Rhizobium*, *Pseudomonas*, are beneficial organisms that have shown good efficiency as BCAs against pathogenic microorganisms. *Trichoderma* (Teleomorph: *Hypocrea*) is a fungal genus that is found in the soil, and it is a secondary fast growing opportunistic invasive. In addition, *Trichoderma* biocontrol strains are able to induce the expression of genes involved in defense response and also to promote plant growth, root development, and nutrient uptake (Hermosa et al., 2012).

The relationships established between plant and microorganisms are very diverse. When a plant is exposed to a pathogenic microorganism, the production of molecules associated to salicylic acid is increased, being this a systemic acquired resistance (SAR) response. The response of plants against non-pathogenic microorganisms is different, resulting in activation of signaling cascades that are dependent on jasmonic acid and ethylene, such as hydroperoxide lyase, peroxidase, and phenylalanine ammonia lyase, all of which belong to an induced systemic resistance (ISR) response (Druzhinina et al., 2011). Other responses result in a rapid cell death in infected tissues, then plants activate the hypersensitive response that involves the accumulation of salicylic acid, reactive oxygen species and an increased the influx of Ca^{2+} (Guerrero-González et al., 2011).

In the tripartite interaction of bean plants with the pathogen *R. solani* and a biocontrol *Trichoderma* species, several changes are produced in the plant, such as the increase in phenolic acid and lignin, accumulation of phytoalexins (Guerrero-González et al., 2011), and down- or up-regulation of defense-related genes expression (Mayo et al., 2015). Different categories of defense-related genes whose expression is modulated by biotic stresses have been described in bean plant interacting with pathogen and non-pathogenic microorganisms (Mayo et al., 2015).

Our hypothesis is that the combination of real time PCR with “*in silico*” analysis is a valid strategy to identify bean defense-related genes regulated by BCAs and/or plant

pathogens. The aim is develop a systematic strategy to detect bean defense-related genes regulated by the presence of *Trichoderma velutinum* and/or *R. solani*. Finally, the procedure has been validated by the analysis of expression of the selected genes in the presence or absence of these two fungi.

MATERIALS AND METHODS

Trichoderma and *Rhizoctonia solani* Isolates and Culture Collections

Trichoderma velutinum T028, was collected from the bean traditional production area (Protected Geographical Indication, PGI), called “Alubia La Bañeza - León” (EC Reg. n.256/2010 published on March 26th, 2010, OJEU L880/17), from a High Quality variety of beans (Figure 1) without any genetic manipulation. It was isolated from soil plot bean in the Astorga region (León, Spain). This isolate gave percentages of inhibition greater than 60% in membrane assays and 40% in direct confrontation assays with *R. solani*, and that was able to sporulate on potato-dextrose-agar (PDA) medium.

Rhizoctonia solani R43 was isolated from bean plants of the same PGI and selected based on its high virulence. The isolated strains were stored in the collection “Pathogens and Antagonists of the Laboratory Diagnosis of Pests and Diseases” (PALDPD, University of León, León, Spain).

Isolates were inoculated on PDA (Becton Dickinson, Germany) medium and grown at 25°C in the dark for 1 week. After this incubation time *T. velutinum* T028 was exposed to light in order to induce the spore’s formation.

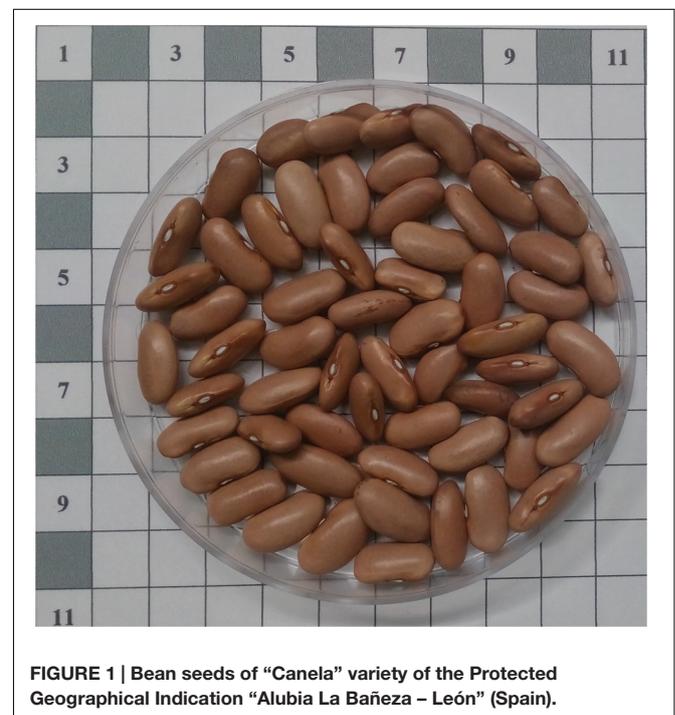


FIGURE 1 | Bean seeds of “Canela” variety of the Protected Geographical Indication “Alubia La Bañeza – León” (Spain).

Plant Materials and Growth Conditions

Sixty bean seeds (Canela landrace, PGI “Alubia de la Bañeza – León,” **Figure 1**) per treatment were germinated and cultured in presence or absence of the fungi in four conditions according to the procedure previously described by Mayo et al. (2015): (i) *T. velutinum* (T028) isolate plus *R. solani* (R43) (RT028); (ii) *T. velutinum* isolate (T028) without pathogen (C = control) (CT028); (iii) control (without *T. velutinum*) with *R. solani* (RC) and (iv) control without fungi (CC). The culture was carried out in climatic chamber and growth conditions were performed as previously described (Mayo et al., 2015). Six bean leaves from 45 day-old plants of each treatment were randomly collected and stored at -80°C until use.

RNA Extraction and Purification

The procedures for RNA extraction were performed as described previously (Reid et al., 2006). Bean leaves were lyophilized and were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was mixed with 20 ml of extraction buffer/g of sample (extraction buffer: 0.1% SDS, 100 mM LiCl, 10 mM EDTA, 100 mM Tris-HCl, pH9) pre-warmed at 65°C , and 20 ml/g of phenol-chloroform-isoamyl alcohol 25:24:1 (Sigma-Aldrich, St. Louis, MO, USA). Then, the mixtures, in eppendorf tubes, were centrifuged at 13,000 rpm for 10 min at 4°C . The aqueous layer was transferred to a new tube. This step was repeated twice. Nucleic acids were precipitated with 1 volume of LiCl 4 M, mixed and kept overnight at 4°C . Tubes were then centrifuged at 13,000 rpm for 30 min at 4°C , and the resulting pellets were washed with ice cold ethanol 70%-DEPC, centrifuged again at 13,000 rpm for 10 min at 4°C and air dried. Finally, the pellets were dissolved in 50–200 μl H_2O -DEPC and stored at -20°C until use.

RNA concentrations and its purity were estimated from the A260/280 absorbance ratio with a NanoDrop (Thermo Scientific, Wilmington, DE, USA), considering the ideal absorbance ratio ($1.8 \leq \text{A260}/280 \leq 2.0$) and 1% agarose gel was run to visualize the integrity of the RNA.

cDNA Synthesis

Approximately 5 μg of RNA were treated with DNase using the TURBO DNaseTM Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer instructions. cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied-Biosystems, Foster City, CA, USA) according to the manufacturer's manual.

qPCR Conditions and Analysis

qPCR reactions were performed with 7300 System (Applied Biosystems, Foster City, CA, USA) using SYBR[®] Green. Each reaction was performed in 20 μl containing 10 μl of 2 X Power SYBR Green PCR Master Mix (Life Technologies), 0.2–0.3 μM primers and cDNA samples diluted 1:20. Each qPCR reaction was performed in triplicate. Reactions were run using the cycling parameter described previously (Reid et al., 2006) and the qPCR data were analyzed by the $2^{-\Delta\Delta\text{Ct}}$ method (Pfaffl, 2001). In order to analyze the qPCR data, *Act11* gene was used as housekeeping

to determine the relative expression level of the other genes analyzed in this work (Borges et al., 2012). *T. velutinum* T028 strain was selected as reference strain in this study based on its positive effects on bean phenotype with and without *R. solani* infection (data no published). For the determination of qPCR efficiency of each primer pairs, a standard curve was performed using the following cDNA dilutions: 1:4, 1:16, 1:64; 1:256 and 1:1024. Every measurement was made in triplicate. The corresponding qPCR efficiencies (E) were calculated for every primer pair with the software 7300 System SDS software (Applied Biosystems, Foster City, CA, USA) according to the equation $E = (10^{-1/\text{slope}} - 1) \times 100$ (Rutledge and Stewart, 2008).

The significance of the differences between the gene expressions levels were compared by the Student's *t*-test using SAS (SAS Institute Inc., 2004, Cary, NC, USA).

RESULTS

Selection of Putative Bean Defense-Related Genes

Following an exhaustive and systematic analysis, summarized in the **Figure 2**, several bean genes were selected for their expression analysis in leaves from bean plants grown in interaction with *T. velutinum* and infected or not with *Rhizoctonia solani*. Thus, as result of the search in the literature, 48 genes were firstly found, showing stress and/or defense response (**Table 1**). Only those genes that resulted to be expressed in *P. vulgaris* leaves, based on transcriptomic data reported in the Phytozome database¹, were considered for qPCR expression analysis in leaves. The genes for which we confirmed expression in leaves were considered for further analyses.

As result, from the 48 genes selected for their involvement in bean stress and/or defense responses, only 19 were selected which showed a detectable level of expression in bean leaves.

The selected genes can be included in nine different groups (**Table 2**): (i) involved in the regulation of the balance between necrotrophic and biotrophic pathogen responses: *WRKY33* (WRKY transcription factor) (NM129404.3) (Bakshi and Oelmüller, 2014); (ii) pathogenesis related genes: *PR1* (pathogenesis related 1) (HO864272) (Guerrero-González et al., 2011), *PR2* (β 1-3 endoglucanase) (HO864270) (Guerrero-González et al., 2011), *PR3* (chitinase class I) (TC18606) (Pereira et al., 2014), *PR4* (pathogenesis related 4) (HO864354) (Guerrero-González et al., 2011), *PR16a* (germin.like protein 8) (CB540239) (Borges et al., 2012), *IPER* (basic peroxidase) (AF007211) (Upchurch and Ramirez, 2010), *PPO* (polyphenol oxidase) (EF158428) (Upchurch and Ramirez, 2010); (iii) related with the ethylene signaling pathway: *ERF1* (ethylene-responsive transcription factor 1) (AF076277) (Lorenzo et al., 2003), *ERF5* (ethylene-responsive transcription factor 5) (At5g47230) (Moffat et al., 2012), and *CH5b* (endochitinase precursor) (FE897014.1) (Vellicce et al., 2006); (iv) involved in phytoalexin

¹<http://phytozome.jgi.doe.gov/pz/portal.html>

biosynthesis: *PAL1* (phenylalanine and histidine ammonia-lyase) (KF279696) (Kim and Hwang, 2014); (v) related in osmotin biosynthesis: *OSM34* (osmitin-like protein) (At4g11650) (Sharma et al., 2013); (vi) involved in Ca^{2+} signaling: *CNGC2* (cyclic nucleotide-gated ion channel 2) (CB542582) (Borges et al., 2012); (vii) needed for antimicrobials and oxylipins (defense signaling molecules): *HPL* (hydroperoxide lyase) (AW733791) (Upchurch and Ramirez, 2010), *Lox2* (lipoxygenase 2) (D13949) (Upchurch and Ramirez, 2010), *Lox7* (lipoxygenase 2) (Upchurch and Ramirez, 2010); (viii) *GSTa* (2,4-D inducible glutathione S-transferase) (HO864392) (Guerrero-González et al., 2011); and (ix) *hGS* (homogluthathione synthetase) (HO864377) both related with oxidative stress (Guerrero-González et al., 2011).

However, only 14 genes were selected to the study of the expression genes because *PR16a*, *IPER*, *PPO*, *Lox2*, and *Lox7*, showing negative qPCR results, were finally discarded.

Selection of a *Trichoderma* Strain to Validate the Gene Selection Strategy

Trichoderma velutinum T028 was the selected isolate, based on its positive effect on bean growth. Thus, plants inoculated with this strain showed a significant increase in dry weight of both aerial parts and root system, including when *R. solani* was present in the substrate (Figure 3). Thus, when bean plants were treated with *T. velutinum* T028 they increased respect to control plants (CC) 4.75% their diameter of hypocotyl, 10.75% their length of root system, 4.27 and 5.51% in dry weight of aerial parts and root system, respectively. When plants were infected with *R. solani*, the action of *T. velutinum* T028 caused an increased respect to the control plant with the pathogen (RC) of the diameter

of hypocotyl in 8.76, 21.15% in the length of root system, and 11.05 and 3.43% in dry weight of aerial parts and root system respectively.

Based on these results, this isolate was used for further studies. In addition, this is the first report in which the effects of this strain on bean phenotype and plant gene regulation are studied.

Effect of *R. solani* Infection on Expression of the Selected Genes. Validation of the Procedure Used to Select Bean Genes Involved in Defense Responses (Strategy Validation I)

A significant down-regulation of expression of *PR2*, *PR3*, *PR4*, *ERF1*, *ERF5*, *PAL1*, *HPL*, and *GTSa* genes with ratios of expression ranging from 0.149 fold for *PAL1* and 0.763 fold for *PR3* was observed in bean plants grown in the presence of *R. solani* (RC) compared to control plants (CC). Conversely, expression of *PR1*, *OSM34*, *CNGC2*, and *hGS* genes was up-regulated, but with non-statistically significant differences with a ratios between 1.289 and 1.193 for *PR1* and *hGS*, respectively (Figure 4).

Effect of *Trichoderma* on Expression of the Selected Genes (Strategy Validation II)

Trichoderma treatment also down-regulates expression of most of the bean defense-related genes, but at a lower level than *R. solani*. Thus, when *T. velutinum* T028 was in the substrate (CT028), *PR2*, *PR3*, *PR4*, *ERF1*, *ERF5*, *PAL1*, *CNGC2*, *HPL*, and *GSTa*

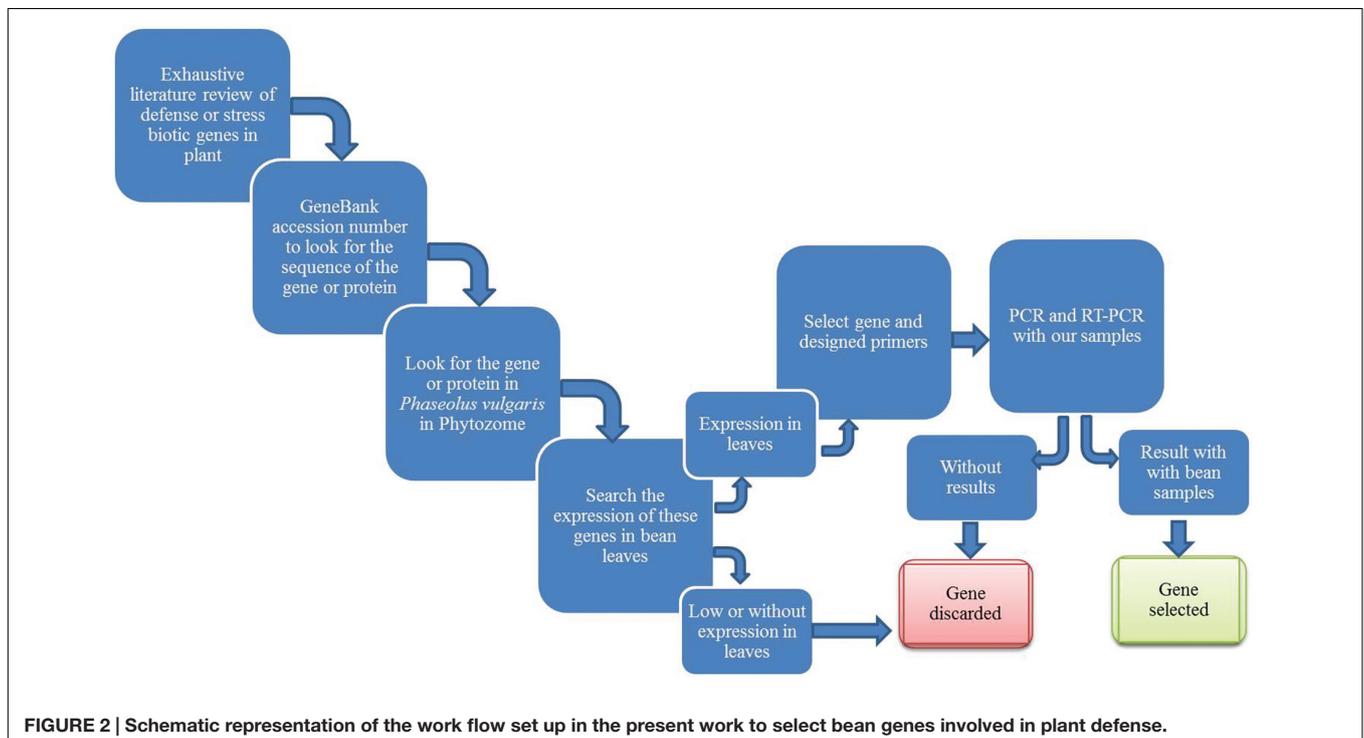


TABLE 1 | Genes selected for stress and/or defense response and their empirical expression in *Phaseolus vulgaris* leaves.

Id	Gene	Accession number	Functional annotation	NCBI Phytozome
Pereira et al., 2014				
1	<i>Chit</i>	AY357300.2	Chitinase	
2	<i>Glu1</i>	DQ093563.1	β -1,3-glucanase	
3	<i>Pod3</i>	AF485265.1	Peroxidase	
4	<i>PR3</i>	TC18606	Chitinase class I	Phvul.009G116600
5	<i>Lox1</i>	U76687.2	Lipoxygenase	
Upchurch and Ramirez, 2010				
6	<i>PPO</i>	EF158428	Polyphenol oxidase	
7	<i>PR10</i>	AJ289155	Stress-induced ribonuclease-like protein	
8	<i>PR12</i>	BU964598	Defensin precursor	
9	<i>MMP2</i>	AY057902	Matrix metalloproteinase 2	
10	<i>CHS</i>	X53958	Chalcone synthase	
11	<i>AOS</i>	DQ288260	Allene oxide synthase	
12	<i>HPL</i>	AW733791	Hydroperoxide lyase	Phvul.005G116800
13	<i>LOX2</i>	D13949	Lipoxygenase 2	Phvul.005G156700
14	<i>LOX7</i>	U36191	Lipoxygenase 2	Phvul.005G156900
15	<i>IPER</i>	AF007211	Basic peroxidase	Phvul.009G215000
Borges et al., 2012				
16	<i>PR16a</i>	CB540239	Germin-like protein 8	Phvul.010G129900
17	<i>PGla</i>	CB542106	Polygalacturonase-inhibitor-like protein	
18	<i>MAPKK</i>	CB543156	MEK map kinase kinase	
19	<i>PROF</i>	CB543496	Profilin	
20	<i>CNGC2</i>	CB542582	Cyclin nucleotide-gated ion channel 2	Phvul.008G036200
Guerrero-González et al., 2011				
21	<i>PR1</i>	HO864272	Pathogenesis related protein 1	Phvul.003G109100
22	<i>PR2</i>	HO864270	Pathogenesis related protein 2	Phvul.003G109200
23	<i>PR4</i>	HO864354	Pathogenesis related protein 4	Phvul.006G102300
24	<i>PR10</i>	HO864271	Pathogenesis related protein 10)	
25	<i>LTP2</i>	HO864366	Lipid-transfer protein 2	
26	<i>SIP</i>	HO864290	Syringolide-induced protein B13-1-9	
27	<i>DAAP</i>	HO864358	Defense associated acid phosphatase	
28	<i>CHI</i>	HO864289	Chalcone isomerase	
29	<i>hGS</i>	HO864377	Homogluthathione synthetase	Phvul.006G094500
30	<i>aDO1</i>	HO864351	Alpha- dioxygenase 1	
31	<i>CPRD14</i>	HO864341	CPRD14 protein	
32	<i>OPR5</i>	HO864304	12-oxophytodienoic acid 10, 11-reductase	
33	<i>GST22</i>	HO864275	Glutathione S-transferase 22	
34	<i>CPRD8</i>	HO864396	CPRD8 protein	
35	<i>UDPGT</i>	HO864301	UDP-glucosyl transferase 72E1	
36	<i>ERD15</i>	HO864375	ERD15 protein	
37	<i>GTSa</i>	HO864392	2,4-D inducible glutathione S-transferase	Phvul.002G241400
38	<i>GST15</i>	HO864369	Glutathione S-transferase 15	
Gallou et al., 2009				
39	<i>GST1</i>	J03679	Glutathione-S-transferase 1	
Lehtonen et al., 2008				
40	<i>TSI-1</i>	BQ121547	TSI-1 protein	
41	<i>Lip</i>	BQ112158	Lipase-like protein	
42	<i>Amintransf2</i>	BQ517030	Aminotransferase 2	Phvul.006G029100
Bakshi and Oelmüller, 2014				
43	<i>WRKY33</i>	NM129404.3	WRKY transcription factors	Phvul.008G090300
Vellicce et al., 2006				
44	<i>CH5b</i>	FE897014.1	Endochitinase precursor	Phvul.009G116500
Lorenzo et al., 2003				

(Continued)

TABLE 1 | Continued

Id	Gene	Accession number	Functional annotation	NCBI Phytozome
45	<i>ERF1</i>	AF076277	Ethylene-Responsive Transcription Factor 1	Phvul.007G127800
Moffat et al., 2012				
46	<i>ERF5</i>	At5g47230	Ethylene-Responsive Transcription Factor 5	Phvul.002G055700
Kim and Hwang, 2014				
47	<i>PAL1</i>	KF279696	Phenylalanine and histidine ammonia-lyase	Phvul.001G177800
Sharma et al., 2013				
48	<i>OSM34</i>	At4g11650	Osmotin-like protein	Phvul.002G155500

were significantly down-regulated with expression ratios ranging from 0.168 for *PR4* to 0.754 for *ERF1*. However, *WRKY 33*, *CH5b*, and *hGS* were up-regulated when compared with the levels of expression in control plants, with relative expression levels between 2.462 for *CH5b* and 1.576 for *hGS* (Figure 5). *OSM34* was slightly but not significantly up-regulated.

Effect of Interaction of *T. velutinum* and *R. solani* on Expression of the Selected Genes (Strategy Validation III)

When *T. velutinum* T028 and *R. solani* (RT028) were in the substrate, the genes *WRKY33*, *PR2*, *PR3*, *PR4*, *ERF1*, *ERF5*, *PAL1*, *OSM34*, *HPL* and *GSTa* were significantly down-regulated with values between 0.179 for *PAL1* and 0.631 for *WRKY33*. In the case of *PR1* and *CNGC2*, they were also down-regulated but not significantly respect to control plant (C). Conversely, *hGS* was up-regulated with a significant ratio of 1.589 respect to control plants, while *CH5b* was not significantly up-regulated with a ratio of 1.613 (Figure 6).

DISCUSSION

Plants have developed some defensive strategies to perceive pathogen attack and to translate this perception into an appropriate adaptive response. During attack, plants are able to enhance their resistance (induced, acquired, hypersensitive) (Lodha and Basak, 2012). Contact with pathogenic and non-pathogenic microorganisms triggers two mechanisms: (i) SAR that is usually triggered by local infections, it provides long-term systemic resistance to pathogen attack and requires the involvement of the signal molecule salicylic acid (Durrant and Dong, 2004), and (ii) ISR that is known to result from colonization of roots by certain non-pathogenic microorganisms and is dependent on components of the jasmonic acid and ethylene signaling pathways (Shoresh et al., 2010). Then, the combination of both types of induced resistance response can protect the plant against pathogens and can even result in additive level of induced protection against pathogens through both the jasmonic acid/ethylene and salicylic acid pathways (Verhagen et al., 2006).

In the present work we developed a strategy to select genes involved in bean defense response, which would belong to those pathways, but also genes that can contribute to plant defense by other mechanisms. In this sense several previous works have

described genes involved in bean defense response (Guerrero-González et al., 2011; Mayo et al., 2015). However, in the present work, by a systematic approach, 48 genes were initially considered, and 14 finally selected, which match with the criteria set up in this work: (i) they showed significant homology with previously described plant defense genes, and (ii) were expressed in bean leaves of plants treated with *Trichoderma* and/or infected with *R. solani*.

The expression of *P. vulgaris* defense-related genes was analyzed in leaves, although the interaction with *Trichoderma* and/or *R. solani* is initially produced at the root level, to determine if the signals generated in roots as result of this interaction are able to systematically stimulate the bean defense along long distance from roots to the leaves. The isolate *T. velutinum* T028 was selected following a similar strategy to that previously described (Mayo et al., 2015), and based on its positive effect on bean growth. In this work, to select a *Trichoderma* isolate, the results of the *in vitro* membrane assays and direct confrontation assays against *R. solani* were analyzed. Isolate *Trichoderma* T019 was then selected, showing a percentage of inhibition higher than 40% in the membrane assays, and/or 20% in the direct confrontation assays. This isolate also showed the best positive effects on plant phenotype among all the analyzed isolates.

WRKY transcription factors have been involved in the regulation of plant defense gene expression (Rushton and Somssich, 1998; Singh et al., 2002). Thus, *WRKY33* has a role in biotic stress defense, where it regulates the balance between necrotrophic and biotrophic pathogen responses (Lippok et al., 2007; Pandey and Somssich, 2009; Birkenbihl et al., 2012). Previous studies have pointed out the involvement of *Arabidopsis* *WRKY* transcription factors in regulating the expression of *PR* genes by direct binding (Chen et al., 2002; Kim et al., 2006). A rapid pathogen-induced *WRKY33* expression did not require salicylic acid signaling but a downregulation of this gene involved a direct activation of jasmonic acid (Bakshi and Oelmüller, 2014). In the present case, when bean plants were in contact *T. velutinum* T028 without pathogen, the *WRKY33* gene expression was significantly up-regulated while the *PR* genes expression (*PR2*, *PR3* and *PR4*) was significantly down-regulated compared to expression levels in plants without *Trichoderma* treatment. In the present work, when *R. solani* was added to the substrate, expression of *WRKY33* was significantly down-regulated in plants with *Trichoderma* inoculation, while *PR2*, *PR3* and *PR4* were down-regulated. In the study by Mayo et al. (2015),

TABLE 2 | Common bean sequences used for primer design for RT-PCR analysis.

Gene	Functional annotation	NCBI Phytozome	Forward/Reverse	Efficiency Reference
Reference genes				
<i>Act11</i>	Actin-11	Phvul.008G011000	TGCATACGTTGGTGATGAGG AGCCTTGGGGTTAAGAGGAG	1.084
<i>Ukn1</i>	Unknown	Phvul.011G023200	ATTCCCATCATGCAGCAAAG AGATCCCTCCAGGTCAATCC	0.937
Balance between necrotrophic and biotrophic pathogen responses				
<i>WRKY33</i>	WRKY transcription factors	Phvul.008G090300	TTTCACAGGACAGGTTCCAGC CCTTTGACAGAAATGACTGAAGGA	0.938
Pathogenesis related genes				
<i>PR1</i>	Pathogenesis Related 1	Phvul.003G109100	TGGTCCTAACGGAGGATCAC TGGCTTTTCCAGCTTTGAGT	1.094 Mayo et al., 2015
<i>PR2</i>	Beta 1-3 Endoglucanase	Phvul.003G109200	GTGAAGGACGCCGATAACAT ACTGAGTTTGGGGTTCGATTG	1.048 Mayo et al., 2015
<i>PR3</i>	Chitinase class I	Phvul.009G116600	TGGAGTTGGTTATGGCAACAA ATTCTGATGGGATGGCAGTGT	1.034
<i>PR4</i>	Pathogenesis-related 4	Phvul.006G102300	CGCAGTGAGTGCATATTGCT TGTTTGTCCCTCAAGCAC	0.922 Mayo et al., 2015
<i>PR16a</i>	Germin-like protein 8	Phvul.010G129900	GGCAGTCTCATGTTATGGTTT GCATGCTCAAGTCTCAACACAT	–
<i>IPER</i>	Peroxidase precursor	Phvul.009G215000	GGCAAGCATTATATGGTTGAAA GATGGCAACATCCATCACTTTA	–
<i>PPO</i>	Polyphenol oxidase	Phvul.008G073200	GAAGACGATGATTTGCTGGTTA AAGAAACATTTTCTTTGTGAAA	–
Ethylene signaling pathway				
<i>ERF1</i>	Ethylene-Responsive Transcription Factor 1	Phvul.007G127800	CGCTCTCAAGAGGAAACTCC TGAATCAGAAGGAGGGAAT	0.937
<i>ERF5</i>	Ethylene-Responsive Transcription Factor 5	Phvul.002G055700	GGCTCCAAGTGGATTGAGAAC TCAGAATCAGATAACTACAAGCACAA	0.932
<i>CH5b</i>	Endochitinase precursor	Phvul.009G116500	CAGCCAAAGGCTTCTACACC TTGTTTCGTGAGACGTTTGC	0.883 Mayo et al., 2015
Phytoalexins biosynthesis				
<i>PAL1</i>	Phenylalanine and histidine ammonia-lyase	Phvul.001G177800	TGAGAGAGGAGTTGGGCACT TTCCACTCTCCAAGGCATTCC	1.034
Osmotin biosynthesis				
<i>OSM34</i>	Osmotin-like protein	Phvul.002G155500	GAACGGAGGGTGTCAAAAATC CGTAGTGGTCCACAAGTTCTC	0.927
Involved in Ca²⁺ signaling				
<i>CNGC2</i>	Cyclic nucleotide-gated ion channel 2	Phvul.008G036200	ATTC AATTTGCTTGGAGACGTT ACAGTTTTATTGAAGGCCAGGA	0.98
Antimicrobials and oxylipins (defense signaling molecules)				
<i>HPL</i>	Hydroperoxide lyase	Phvul.005G116800	TCAAGGCTACATTTGTATTTCCA TGGTGACATTTCTTAGTAGCAA	0.984
<i>Lox2</i>	Lipoxygenase 2	Phvul.005G156700	ATGCAAGGCTAAAGAGATCCAA ATGGTGACAGGAGCTAAACACA	–
<i>Lox7</i>	Lipoxygenase 2	Phvul.005G156900	GAAGGCTTGACTTTTCAGAGGAA AACACACGAGAAGATTCAACCA	–
Oxidative stress				
<i>GSTa</i>	2,4-D inducible glutathione S-transferase	Phvul.002G241400	AGGGAGTCACACTGGCTATGTT ATGTGCCATTTGCATTTTAGTG	1.013
<i>hGS</i>	Homogluthathione synthetase	Phvul.006G094500	GTGGCTATATGGTGCGTACAAA GAAACAAGAATGCATCTCCTCA	1.023
<i>Amintransf2</i>	Aminotransferase 2	Phvul.006G029100	TTCTTCTTTTCTGCTCTTTCAA AGATGACAAGATGCAATGATTTTT	–

(–) Genes that empirically showing expression but showing negative qPCR results.

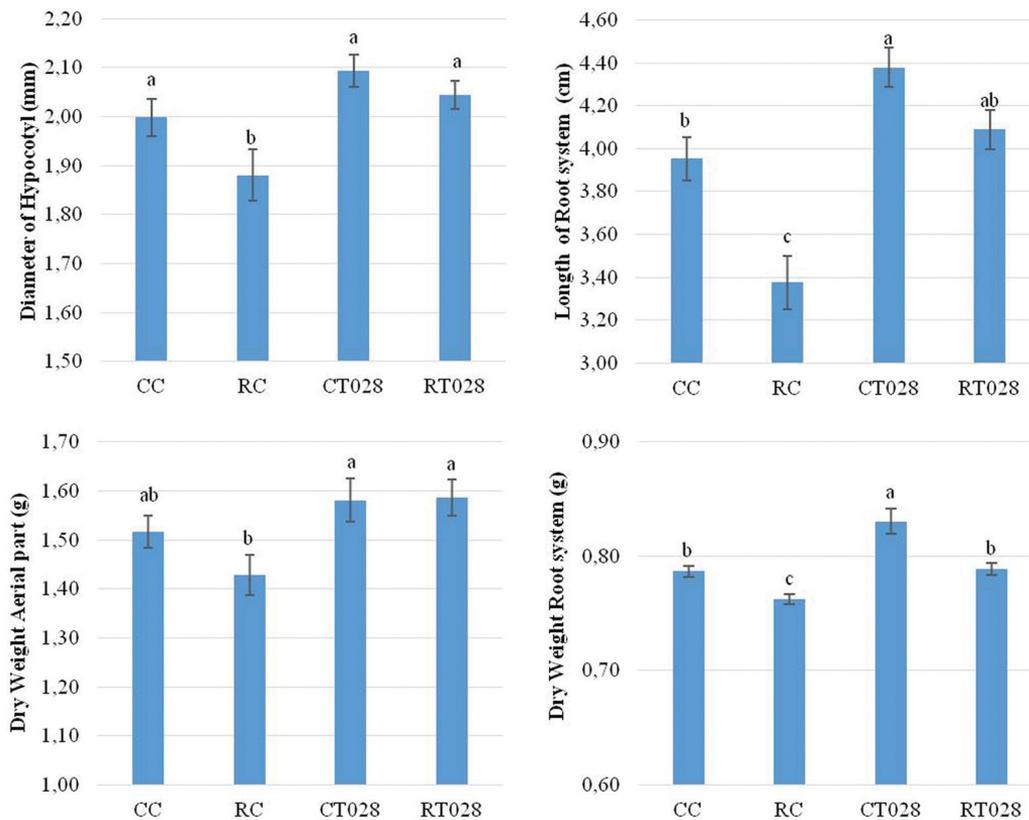


FIGURE 3 | Evaluation of the diameter of the hypocotyl (above left), length of root system (above right) dry weight of the aerial part (below left) and root system (below right) of bean plants grown during 45 days after sowing. [*Trichoderma velutinum* T028 without pathogen (CT028), *T. velutinum* T028 with *Rhizoctonia solani* (RT028), *R. solani* control (RC) and control without fungus (CC)]. Differences statistically significant respect to control plants ($p < 0.05$) are indicated with different letters.

the expression of *PR1*, *PR2*, *PR3*, and *PR4* was down-regulated when beans were inoculated with *R. solani*.

WRKY33 is also involved in the regulation of the expression of genes modulated by components of the ethylene signaling pathway. In this work, expression of the *ERF1* and *ERF5* reached similar significant values either with or without *Trichoderma* and or *R. solani* in the substrate. This result contrasts with previous reports showing that *ERF5* was up-regulated and *WRKY33* was down-regulated in *Arabidopsis* infected with *Alternaria brassicicola* (Son et al., 2012). *WRKY33* would act as a repressor of *ERF1* and *ERF5* expression. Thus, when the expression of *WRKY33* is increased, expression of *ERF1* and *ERF5* is down-regulated.

CH5b encodes an endochitinase precursor and it is also related with the ethylene signaling pathway. In previous works, it has been shown that, when this gene was over-expressed the *R. solani* symptoms were reduced in crops like *Nicotiana tabacum* and *Brassica napus* (Broglie et al., 1991). However, in this study, when bean plants were in contact with *R. solani*, the expression of this gene was down-regulated but not significantly, while treatment of these infected plants with *T. velutinum* resulted in its significant up-regulation. These results are in agreement with previous data,

showing that the pathogen represses its expression, and the presence of *Trichoderma* induced it (Mayo et al., 2015).

PAL plays an important role in plant defense; it is involved in the biosynthesis of salicylic acid, which is related to plant systemic resistance (Nugroho et al., 2002; Chaman et al., 2003). *PAL* gene expression is also regulated in response to pathogen infection. In this work, the presence of *T. velutinum* and *R. solani* in the soil resulted in a significant down-regulation of this gene compared with control plants.

Osmotins have plant protective effects against pathogen infection (Narasimhan et al., 2009). In this study, when *T. velutinum* or *R. solani* were present in the soil, the expression of *OSM34* was not significantly up-regulated respect to control plants, but when both fungi were in the soil at the same time, *OSM34* was slightly but significantly down-regulated.

The *CNGC* genes can be related to early plant defense responses due to changes in ion flux, including H^+ and Ca^{2+} influx and K^+ and Cl^- efflux (Atkinson et al., 1996). The up-regulation of *CNGC2* can confirm the importance of ion channels for the plant resistance response (Borges et al., 2012). In this work, this gene was up-regulated when *R. solani* was present in the soil not significant. Conversely, *CNGC2* was down-regulated

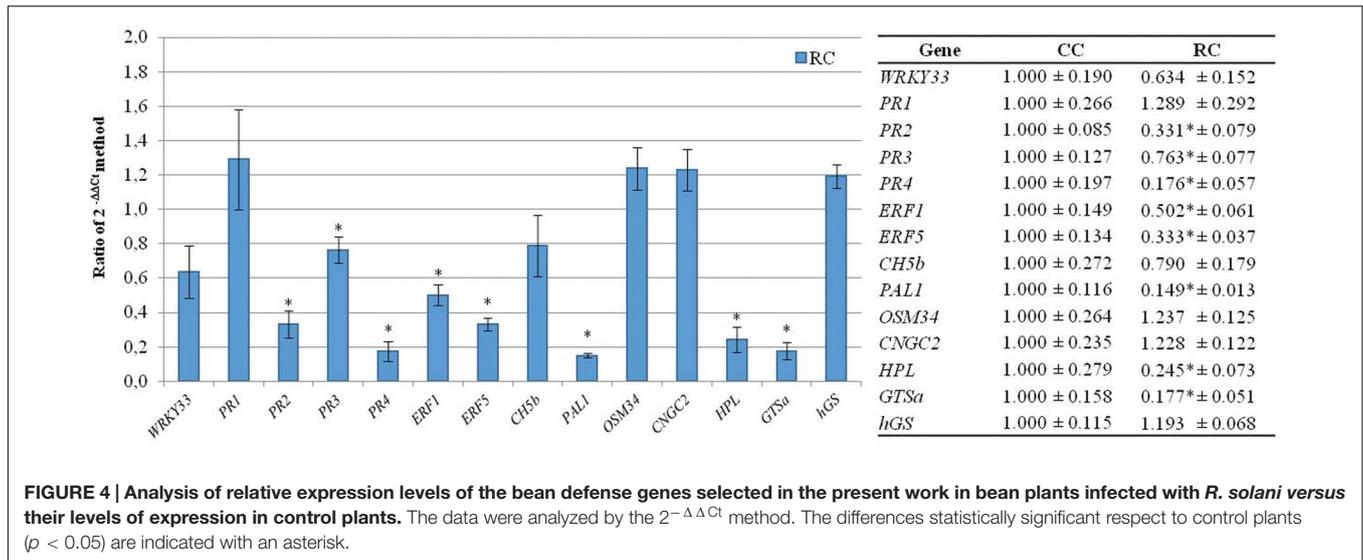


FIGURE 4 | Analysis of relative expression levels of the bean defense genes selected in the present work in bean plants infected with *R. solani* versus their levels of expression in control plants. The data were analyzed by the $2^{-\Delta\Delta C_t}$ method. The differences statistically significant respect to control plants ($p < 0.05$) are indicated with an asterisk.

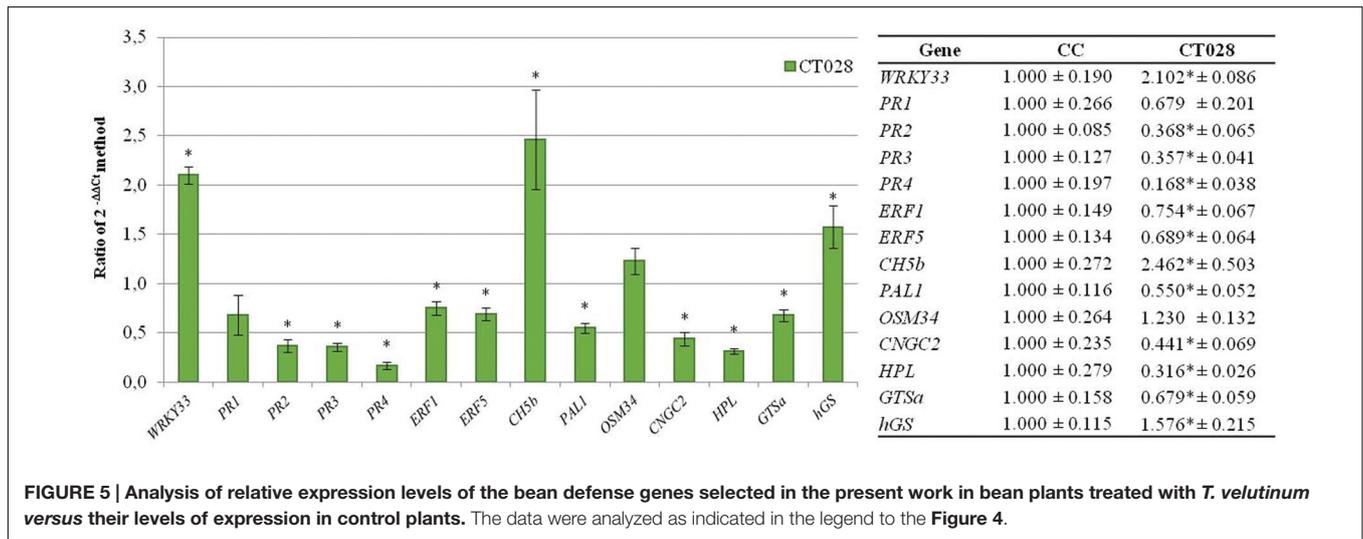


FIGURE 5 | Analysis of relative expression levels of the bean defense genes selected in the present work in bean plants treated with *T. velutinum* versus their levels of expression in control plants. The data were analyzed as indicated in the legend to the Figure 4.

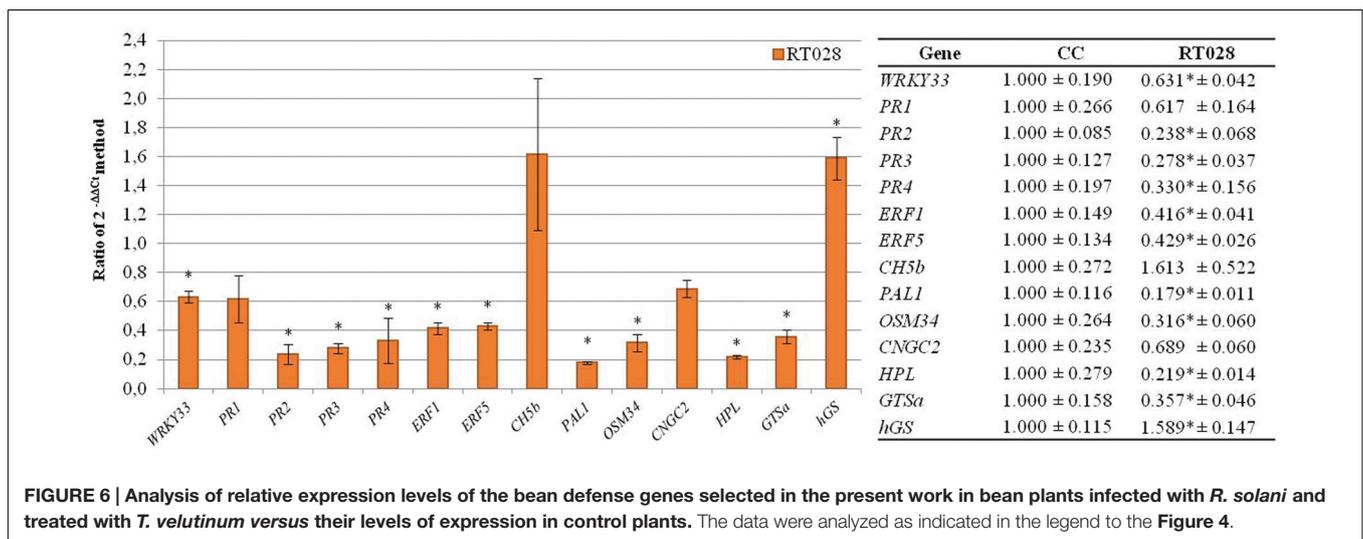


FIGURE 6 | Analysis of relative expression levels of the bean defense genes selected in the present work in bean plants infected with *R. solani* and treated with *T. velutinum* versus their levels of expression in control plants. The data were analyzed as indicated in the legend to the Figure 4.

in plants treated with *T. velutinum*. Then, the pathogen would induce an activation of hypersensitive defense mechanisms.

Hydroperoxide lyase (*HPL*) is involved in the production of antimicrobial and defense signaling oxylipins (Noordermeer et al., 2001; Huang et al., 2010). In this study, the presence of *T. velutinum* and *R. solani*, resulted in a down-regulation of this gene expression respect to control plants. In previous works, when tomato plants were in contact with *Botrytis cinerea*, *HPL* expression increased 24 h after gray mold infection, but after that time the expression of this gene decreased gradually (Wan et al., 2013). In the present case, after 45 days in contact with the fungus *T. velutinum* and/or *R. solani*, its expression was down-regulated, indicating that the plant identifies *Trichoderma* and *Rhizoctonia* as two invader organisms, and some of the mechanisms activated against the presence of both are similar, independently of the final response specifically activated in the plant by each one.

GSTa (2,4-D inducible glutathione S-transferase) expression also responds to pathogen attack (Mauch and Dudler, 1993) and can be induced by molecules such as salicylic acid, methyl jasmonate, abscisic acid and H₂O₂ (Dixon et al., 2002; Moons, 2005). In *Gossypium arboreum*, *GST* provides resistance to fungal pathogens and oxidative stress (Barthelson et al., 2010). *GST* expression was up-regulated during fungal infection in barley, *Arabidopsis*, and cotton (Dowd et al., 2004; Durrant and Dong, 2004; Lu et al., 2005). However, in banana *GST* was down-regulated following *Fusarium oxysporum f. sp. cubense* infection (Wang et al., 2013), which is in agreement with the present case, where the expression of *GSTa* was down-regulated when *T. velutinum* and/or *R. solani* were present in the soil.

hGS encodes a homogluthathione synthetase that is involved in response to oxidative stress. There is not much information about the behavior of this gene in the plant. In the present study, when bean plants were in contact with *T. velutinum* and/or *R. solani*, expression of this gene was significantly up-regulated compared to control plants. In other studies, treatment of *Medicago truncatula* plants with compounds that release nitric oxide, a key signaling molecule in plants, induced expression of *GST* but not *hGS* in roots (Innocenti et al., 2007). Similarly, common bean plants treated with H₂O₂ showed up-regulation of *hGS* in nodules, whereas treatments with cadmium, sodium chloride, or jasmonic acid had no effect (Loscos et al., 2008).

CONCLUSION

From 48 genes initially analyzed, 14 bean genes were selected in the present work and only *WRKY33*, *CH5b* and *hGS* showed

an up-regulatory response in the presence of *T. velutinum*, the other genes were or not affected (*OSM34*) or down-regulated by the presence of this fungus. *R. solani* infection resulted in a down-regulation of most of the genes analyzed, except *PR1*, *OSM34* and *CNGC2* that were not affected, and the presence of both, *T. velutinum* and *R. solani*, up-regulates *hGS* and down-regulates all the other genes analyzed, except *CH5b* which was not significantly affected.

As conclusion, the strategy described in the present work has been shown to be effective to detect genes involved in plant defense, which respond to the presence of a BCA or to a pathogen and also to the presence of both. The selected genes showed significant homology with described plant defense genes and they are expressed in bean leaves of plants treated with *T. velutinum* and/or infected with *R. solani*. The proposed strategy will be very useful in studies about the interaction of bean with pathogens and biocontrol fungi.

AUTHOR CONTRIBUTIONS

PC and SG conceived the research. SM, OG-L, and AR-G designed the research. SM, OG-L, and AR-G conducted the experiments. SM, PC, and SG analyzed data. SM, PC, SG, EC, and FS interpreted the data. SM, PC, SG, EC, and FS wrote the manuscript. All authors were agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors critically revised the manuscript. All authors approved the final version to be published.

ACKNOWLEDGMENTS

The grant awarded to Sara Mayo Prieto (FPU12/00677) by the Ministry of Education, Culture and Sport (Spain) according the Resolution of April 25, 2012 (BOE of 10 May), as amended by Resolution of May 18, 2012 (BOE of 22 May), and by Resolution of October 31, 2012 (BOE of 12 November) and granted in the Resolution of December 20, 2012. Ministry of Economy and Competitiveness for National project “Farnesol as self-regulatory molecule in *Trichoderma*. Tyrosol and farnesol signaling in *Trichoderma*-bean interaction” (AGL2012-40041-C02-02). Junta de Castilla y León, Consejería de Educación for the project “Effect of terpenes and physiologically related compounds produced by *Trichoderma parareesei* in the development of common bean (*Phaseolus vulgaris* L.) and in defensive responses of this plant” (LE228U14).

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The reviewer EVG declared a past co-authorship with one of the authors SG to the handling Editor, who ensured that the process met the standards of a fair and objective review.

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