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Identification of differentially expressed genes from *Fusarium oxysporum* f. sp *cubense* and *Trichoderma asperellum* (prr2) interaction in the susceptible banana cultivar Grand Naine

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Abstract: Identification of defense-related genes in the host is one of the most essential steps in understanding disease resistance mechanisms in plants. In this study, a suppression subtractive hybridization (SSH) library was constructed to study differential gene expression in banana plants mediated through a Fusarium wilt pathogen (*Fusarium oxysporum* f.sp *cubense* – *Foc*) and its interaction with the *Foc* effective biocontrol agent *Trichoderma asperellum* (prr2). Here cDNAs from the roots of banana cv. Grand Naine infected by *Foc* were used as the driver and cDNAs from *Foc* + *T. asperellum* inoculated banana plants as the tester population. After hybridization and cloning, an EST library of 300 nonredundant clones was obtained. Based on sequence analysis and a homology search in the NCBI database, the clones were assigned to different functional categories. The expression patterns of six selected defense-related genes, namely *endochitinase*, *polyubiquitin*, *calmodulin binding protein*, *pleotropic drug resistant gene*, *isoflavone reductase*, and *mannose binding lectin*, were analyzed through quantitative real-time PCR in *Foc* alone inoculated and *Foc* + *T. asperellum* inoculated banana plants. It was observed that the expression of these genes during initial progression of disease was higher in *Foc* + *T. asperellum* inoculated plants as compared to *Foc* alone inoculated plants. Our results constitute a step toward a better understanding of the role of mycoparasitic *T. asperellum* in plant defense during its interaction with *Foc* in the susceptible banana cultivar Grand Naine.

Key words: *Foc*–biocontrol interaction, *Fusarium oxysporum* f. sp. *cubense*, *Musa* defense genes, suppression subtractive hybridization

1. Introduction

Fusarium wilt of banana is a serious disease threat throughout the world (Vuylsteke, 2000). The disease is caused by systemic vascular infection by *Fusarium oxysporum* f.sp. *cubense* (*Foc*) (Snyder and Hanson, 1940), which results in disruption of water translocation to the shoot (Duniway, 1971; Beckman and Roberts, 1995). This disruption leads to typical wilt symptoms of foliage drooping, chlorosis, and eventually necrosis, as the leaves collapse around the pseudostem. As the disease progresses, the crown and pseudostem also collapse, resulting in plant death (Rishbeth, 1955).

Options for the control of Fusarium wilt are limited by ineffectual chemical control and the lack of commercially suitable resistant cultivars (Shi et al., 1991; Smith et al., 2006). Under these circumstances, use of antagonistic microbes, which protect and promote plant growth by colonizing and multiplying within the plant and in the rhizosphere, could be a potential alternative approach for the management of Fusarium wilt of banana. Several soil antagonists such as *Pseudomonas fluorescens* (Saravanan et al., 2003), *Burkholderia cepacia* (Pan et al., 1997), *Trichoderma* spp. (Beckman and Roberts, 1995; Nel et al., 2006), and *Streptomyces* sp. (Getha et al., 2005) have been used to control Fusarium wilt disease. Among the effective microbes, *Trichoderma* fungi as biocontrol agents have been exploited and reported against several soil-borne phytopathogenic fungi, invertebrates, and bacteria (Papavizas et al., 1982; John et al., 2010).

Although biocontrol agents have been shown to control *Foc* infection, knowledge of the molecular mechanisms of plant responses to *Foc*–biocontrol interaction helps in formulating better strategies to develop resistant varieties. Endophyte-induced defenses in plants are expressed through structural and biochemical mechanisms. Structural mechanisms include the reinforcement of plant cell walls by deposition of newly formed molecules of callose, lignin, and phenolic compounds (He et al., 2002; Jeun et al., 2004). Other physical mechanisms of resistance include the occlusion of colonized vessels by gels, gums, and tyloses (Gordon and Martyn, 1997; Olivian and Alabouvette, 1999), which are formed by the plant to prevent further ingress of the invading organisms (Schmelzer, 2001). Major biochemical changes

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following endophyte colonization include accumulation of secondary metabolites such as phytoalexins (Kuć and Rush, 1985; Baldridge et al., 1998), and production of pathogen related (PR) proteins such as β-1,3-glucanases, chitinases, and peroxidases (Duijff et al., 1998; Xue at al., 1998; Yedidia et al., 1999; Bargabus et al., 2004). These enzymes are reported to release elicitors for phytoalexin synthesis (Keen and Yoshikawa, 1983).

In the present study, efforts were made to understand the molecular mechanisms due to *Foc*-biocontrol agent (*T. asperellum* (prr2)) interactions in a *Foc* susceptible banana cultivar, Grand Naine, using suppression subtractive hybridization (SSH). This technique suppresses the amplification of common transcripts and enriches the differentially expressed transcripts. It has been successfully employed to isolate differentially expressed genes from *Musa* and its pathogen interaction such as *Foc* (Van Den Berg et al., 2007), *Mycosphaerella fijiensis* (Portal et al., 2011), and *Pratylenchus coffeae* (Backiyarani et al., 2014). We identified differentially expressed genes and particularly focused on a series of genes to understand the molecular basis of the defense mechanism in *Musa* due to *Foc* + *T. asperellum* interaction.

2. Materials and methods

2.1. Plant materials and RNA isolation

The banana cultivar Grand Naine (AAA) grown in mud pots containing sterile sand and red soil (1:1) was inoculated with 30 g of *Foc* (1×10^8 cfu/g) grown in sand maize medium. One week after *Foc* inoculation, 30 g of Fusarium wilt suppressive biocontrol agent *Trichoderma asperellum* (prr2) (1×10^8 cfu/g) mass produced in rice chaffy grain was applied for combating the infestation of Fusarium wilt pathogen (*Foc* race 1 – VCG 0124 and *T. asperellum* (prr2)) inoculums were obtained from the culture collection - pathology lab, NRC for Banana, India). Total RNA (nucleo spin RNA plant kit, Macherey-Nagel, Germany) was isolated from the roots of *Foc* alone inoculated control (driver) and *Foc* + *T. asperellum* inoculated (tester) banana plants. From these total RNA and mRNA were purified using a nucleotrap mRNA kit (Macherey-Nagel) and cDNA was synthesized from mRNA by reverse transcription with oligo (dT) primers according to the manufacturer's protocol (Promega, USA).

2.2. SSH library construction

Suppression subtractive hybridization was performed using a PCR-Select cDNA subtraction kit (Clontech, USA). Forward and reverse subtraction libraries were constructed using cDNA samples of driver versus tester treatments. The subtracted cDNAs were subjected to two rounds of PCR to normalize and enrich cDNA populations. The PCR products were cloned using an InsTAclone PCR cloning kit and transformed into One Shot OmniMAX 2TIR competent cells (Invitrogen) in LB medium with IPTG and X-Gal for screening recombinants. A single white colony was picked and grown overnight at 37 °C. Glycerol stocks (15% conc.) were prepared and all the clones were stored at –80 °C until further use.

2.3. DNA sequencing and initial processing

Plasmids of positive clones were isolated using a GenElute plasmid kit (Sigma, USA), and validated by colony PCR using nested primers and finally sequenced with M13 primers. The resultant sequences were trimmed and end clipped using a Codoncode aligner initially. Later, vector sequences still present were removed by VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen) and poly A tails and adapter sequences were eliminated by Trimest (http://emboss.bioinformatics.nl/cgi-bin/emboss/trimest). Sequences were assembled by CAP3 (http://pbil.univ-lyon1.fr/cap3.php) and annotated by Blast2GO (http://www.blast2go.com).

2.4. Validation of defense genes identified by real-time PCR

Total RNA from roots of *Foc* alone inoculated and *Foc* + biocontrol (*T. asperellum* (prr2)) inoculated banana plants cv. Grand Naine were isolated at 1, 3, 5, and 7 days after inoculation (DPI). The mRNA was purified using nucleotrap mRNA kit (Macherey-Nagel, Germany). First strand cDNA was synthesized from mRNA by reverse transcription with oligo (dT) primers according to the manufacturer's protocol (Promega). The first strand cDNA was diluted (1:10) with water and was used as template for PCR. RT-PCR primer sets were designed from the selected representative unigenes by using Primer3 software (as shown in Table 1).

Musa 25S rRNA was used as endogenous control. Quantitative real-time PCR (qRT-PCR) was carried out in a StepOnePlus real-time PCR machine (Applied Biosystems, USA) with three biological replicates and normalized with 25S rRNA. A 10-µL reaction contained 10 µL of power SYBR qPCR master mix (Applied Biosystems), 2 µL of forward and reverse primer (10 µM), 1 µL of cDNA template (1 ng), and 2 µL of PCR-grade water (Sigma). The PCR cycling profile consisted of denaturation at 94 °C for 1 min and annealing at 60 °C for 1 min for 35 cycles. Melting curve amplification was also kept for the SYBR green assay to examine the amplification specificity. At the same time, the amplification efficiency of primer pairs was determined by a standard curve derived from 5 serial dilutions of cDNA and the comparative (Ct) method was used to determine the expression level of analyzed genes.

3. Results

3.1. Construction of subtracted library

A subtractive cDNA library was constructed from the cDNAs of *Foc* inoculated root samples (driver) and *Foc* +

Table 1. Primers used for quantitative RT-PCR analysis for genes enriched by SSH in the banana cultivar Grand Naine inoculated with *Foc* and challenged with *T. asperellum* (prr2).

GenBank accession	Gene	Forward primer	Reverse primer
KT967972	*Mannose binding lectin*	5'GCACACAACACGATGAACG-3'	5'CCGTCTTCCCGTAGTAGGTG -3'
KT967977	*Calmodulin binding protein*	5'GGCGATGAACATGTGAACAG-3'	5'CCATAGCAGCGTTCCAGTTT -3'
KT967976	*Polyubiquitin*	5'GGCAGGTGTCGTGACAGTAG-3'	5'CTTTGCTGGCAAGCAGCTC -3'
KT967973	*Pleotropic drug resistance*	5'TGCAAAGAGGAAAGCAAACA-3'	5'ACGGTCTAGTCGCCTCACAG -3'
KT967974	*Isoflavone reductase*	5'TCCGGAGGAAGAAGTCTTGA-3'	5'CGTCAACGGTGGTGTACTTG -3'
KT967975	*Endochitinase*	5'CTGCCATGATGTGATTACCG-3'	5'AACGGGCTCTGGTTATAGCA -3'
AF399949	*Musa25S*	5'ACATTGTCAGGTGGGGAGTT-3'	5'CCTTTTGTTCCACACGAGATT-3'

T. asperellum inoculated root samples (tester) of banana cv. Grand Naine. The subtraction method overcomes the problem of differences in mRNA abundance through normalization, thereby enriching the less abundant genes. The subtraction efficiency was confirmed by evaluating the expression of 28S rRNA between subtracted and unsubtracted cDNAs. The amplification of 28S rRNA could be detected in agarose gel only at 28 PCR cycles in subtracted sample, whereas in the unsubtracted sample the amplification was observed even at 18 PCR cycles. The reduction in expression level suggested that the subtraction procedure was successful. A total of 500 positive clones were randomly picked from the SSH library for plasmid isolations. All the plasmids were digested with EcoRI to eliminate false positive clones. Based on the digestion, it was observed that nearly 300 clones had the inserts and these clones alone were sequenced. Out of 300 clones sequenced, 258 readable sequences were obtained. All the readable sequences were assembled through CAP3 analysis, which resulted in the construction of a unigene set of 109 ESTs. Among them, 87 were singletons and the remaining sequences were grouped into 22 contiguous sequences (contigs).

3.2. Annotation of differentially expressed cDNA sequences

Gene ontology analysis was carried out for all the unigenes using the Blast2GO software. The properly assembled contigs and singletons were annotated using different tools (Blastn and BlastX) and databases (InterProScan, KEGG) provided in the Blast2GO software. The sequences were classified into 3 categories: biological process, molecular function, and cellular component (as shown in Figure 1). The biological process category was subdivided into 11 groups: metabolic process (9 genes), response to stimulus (7 genes), cellular process (12 genes), biological regulation (6 genes), single-organism process (5 genes), localization (7 genes), immune system process (3 genes), developmental process (3 genes), signaling (4 genes), biogenesis (3 genes), and reproduction (1 gene). The molecular function category was subdivided into 5 groups containing 10 genes and in this more than 50% of the transcripts are related to binding and catalytic activity followed by the transcription factor activity category and others are grouped in the antioxidant and transporter activities. The cellular component category was subdivided into seven groups containing 29 genes and, among these, transcripts related to the cell group were present at high percentages followed by transcripts of organelle, membrane, membrane-enclosed lumen, symplast, cell junction, and extracellular region. The remaining 10 ESTs were found to hit with no functional characterization and included in hypothetical and predicted proteins. The similarity search for all these ESTs with the existing sequences in GenBank using BlastX resulted in 88 hits and among these only 24 sequences that had significant matches (E value $<10^{-3}$) were categorized into five groups, namely, defense/resistance, signal transduction, transcription, protein synthesis, and metabolism (as shown in Table 2). Out of these 24 genes, six defense-related genes, namely *banana lectin-methyl-alpha-mannose complex* (*mannose binding lectin*), *calmodulin binding protein*, *pleotropic drug resistance gene*, *endochitinase*, *isoflavone reductase*, and *poly ubiquitin* were selected for further studies by RT-PCR.

3.3. RT-PCR analysis of differentially expressed genes

The validation for the six defense-related genes performed by RT-PCR in cv. Grand Naine indicated that all these genes were expressed in the root tissues of both *Foc* alone and *Foc* + *T. asperellum* inoculated banana plants. However, the expression level of all these genes was consistently higher in *Foc* + *T. asperellum* inoculated plants as compared to *Foc* alone inoculated plants (as shown in Figure 2). With regard to time of expression of these defense related genes, the transcript level of *endochitinase*, *mannose binding lectin*, and *polyubiquitin* genes reached the maximum at 5 DPI, whereas the *isoflavone reductase* and *pleotropic drug resistance gene* reached maximum level (10-fold) at 7 DPI and *calmodulin binding protein* at 3 DPI in *Foc* + *T. asperellum* inoculated plants compared to *Foc*

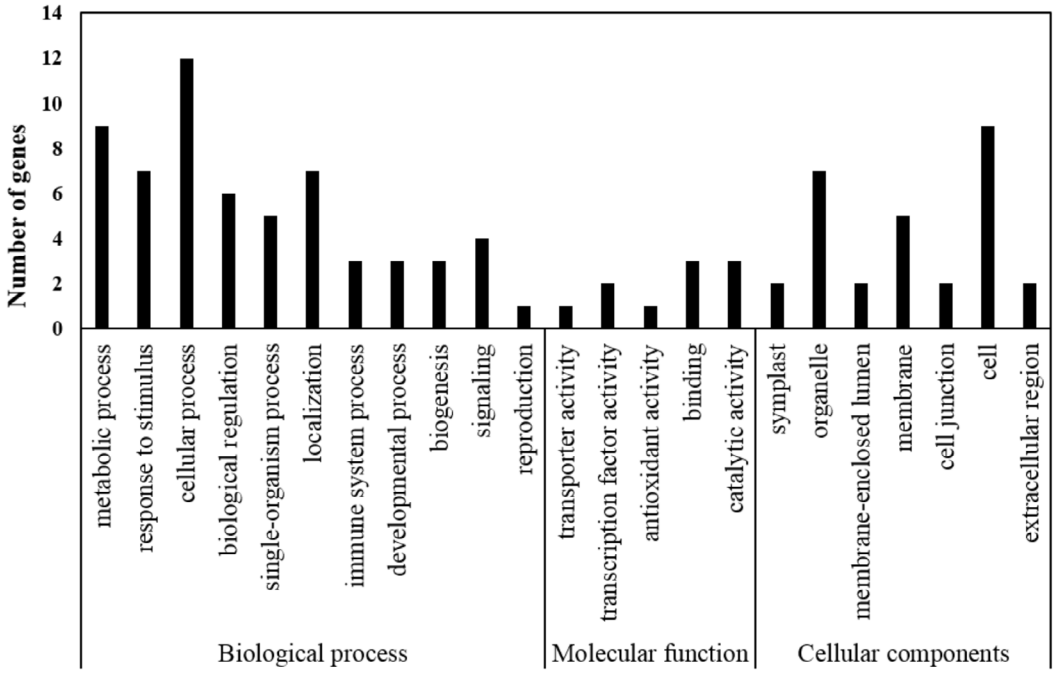

Figure 1. Gene ontology (GO) annotations of unigenes obtained from suppression subtractive hybridization in the banana cultivar Grand Naine inoculated with *Foc* and challenged with *T. asperellum* (tester) as against *Foc* alone inoculated driver genes.

alone inoculated plants. Altogether this expression study showed that the defense-related gene mechanism peaked between 3 DPI and 7 DPI in *Foc* + *T. asperellum* inoculated banana plants.

4. Discussion

In the present study, we aimed to construct a SSH library to study the transcriptional diversity during *Foc*–biocontrol interaction in the *Foc* susceptible cultivar Grand Naine (AAA). Suppression subtractive hybridization is a powerful technique that allows the rapid and cost effective investigation of gene expression at different stages of development and produces a library of differentially expressed genes (Diatchenko et al., 1996; Lukyanov et al., 2007). The high level of enrichment of rare transcripts has been achieved by the inclusion of a normalization step in the subtraction procedure (Diatchenko et al., 1996). In our study, 109 differentially expressed unigenes were obtained during *Foc* + *T. asperellum* interaction in the susceptible banana cultivar Grand Naine through SSH technique. Functional annotation of SSH derived ESTs showed that nearly 91% (99 out of 109 genes) of the ESTs hit with known biological function categories. However, our goal was to mainly focus on the genes that are involved in defense response and to characterize the resistance pathways to *Foc* in banana. In general, our analysis revealed that the upregulated ESTs are involved in *Foc* recognition, signal transduction, protection mechanism, and hypersensitive response (HR). The remaining 9.1% (10 out of 109 genes) of ESTs with unknown function suggested that the responses to *Foc* + *T. asperellum* interaction are rather complex and multigenic.

The SSH results were validated using quantitative RT-PCR with six defense specific representative genes including *chitinase* and *mannose glucose lectin*. Our study clearly demonstrated the difference between *Foc* alone inoculated and *Foc* + *T. asperellum* inoculated susceptible cultivars in transcript diversity with respect to timing and the level of gene expression in response to *Foc* infection. The degree of resistance depended not only upon qualitative differences in the activated defense genes, but also on differences in the timing and magnitude of their expression (Khraiwesh et al., 2012). For example, the chitinase gene was upregulated in both *Foc* alone and *Foc* + *T. asperellum* inoculated plants, but the level of chitinase expression in *Foc* + *T. asperellum* was abundant and several fold (up to 8-fold) higher than the *Foc* alone inoculated plants at 5 and 7 DPI. As a mechanism for perceiving chitin, plants developed chitinases to release the active polymers from the cell walls of invading fungi, thereby triggering defense responses. Thus, the mycotrophic activity of *Trichoderma* chitinases can also release chito oligosaccharides and indirectly contribute to the induction of this defense mechanism (Hermosa et al., 2012). The chitinases of mycoparasitic species, e.g., *Trichoderma asperellum*, are involved in the antagonistic ability of plant pathogenic fungi especially

Table 2. List of selected ESTs obtained by suppression subtractive hybridization derived from *Foc* + *T. asperellum* interaction in *Foc* susceptible cultivar Grand Naine.

Clone no.	Size bp	Sequence homology	Accession no.	E-value	Sequence identity
Defense/Resistance					
Contig3	948	*NPR1-like protein*	AHF20179	1.95E-06	98%
Contig7	459	*Pleiotropic drug resistance protein*	XP_004506879	1.75E-26	80%
Contig6	434	*Peroxidase*	DQ317315	1.45E-12	97%
Contig5	451	*Horcolin*	Q5U9T2	2.43E-06	63%
C18	354	*Banana Lectin- Methyl-Alpha-Mannose Complex*	1X1V_A	1.27E-14	78%
C38	446	*Isoflavone reductase*	XP_004136926	1.63E-30	92%
C312	1202	*Catalase*	Q59296	1.93E-10	76%
C82	463	*Lipoxygenase*	FM164378.1	6.21E-43	89%
C146	627	*Endochitinase*	AGC39032	3.45E-17	90%
Signal transduction					
Contig12	1268	*Ubiquitin2*	AFW66446	1.55E-59	100%
Contig18	387	*Serine threonine-protein kinase*	XP_009412046	3.44E-13	94%
C538	755	*Fiber polyubiquitin*	AGZ15412	3.22E-18	82%
C348	835	*Polyubiquitin*	CAD27944	6.66E-28	83%
C911	235	*Proline rich protein*	AB182103.1	2.00E-03	76%
C10	1092	*Choline transporter-like protein*	XP_007648439	8.77E-04	85%
C31	1182	*Glycosyl transferase*	WP_002166579	4.27E-10	80%
Transcription					
Contig9	823	*Bzip transcription factor*	Q949G3	1.78E-14	77%
C243	388	*Transcription factor*	AM293617.1	3.10E-09	100%
C1138	407	*Calmodulin-binding transcription activator*	Q9FY74	1.04E-13	71%
Protein synthesis					
C421	398	*Ribosomal protein S11*	D85128.1	2.00E-137	91%
C911	235	*Proline rich protein*	AB182103.1	2.00E-03	96%
Metabolism					
C121	559	*Glucose-6-phosphate-1-dehydrogenase*	AM497810.1	5.00E-12	100%
C52	326	*Phosphoglycerate kinase*	AM497801.1	1.83E-07	86%
C316	676	*Ethylene signal transcription factor*	AB266320.1	2.50E-08	76%

against *Rhizoctonia solani*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* (Woo et al., 1999; Sharma et al., 2011). A similar kind of expression profile was observed for a pleiotropic drug resistance (PDR) gene in which the *Foc* + *T. asperellum* inoculated plants recorded a significant increase (up to 10-fold) in expression level at 7 DPI as compared to the *Foc* alone inoculated plants. PDR genes are group of transporter genes belonging to the ATP-binding cassette (ABC) family and play important roles in detoxification processes, preventing water loss, transport of phytohormones, and secondary metabolites (Stukkens et al., 2005).

In addition to preformed physical and chemical barriers, plants have an immune system that is able to detect motifs or domains with conserved structural traits typical of entire classes of microbes but not present in their host, namely the pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs, respectively). *Trichoderma* strains produce a variety of MAMPs, which to date are those most widely described among plant-

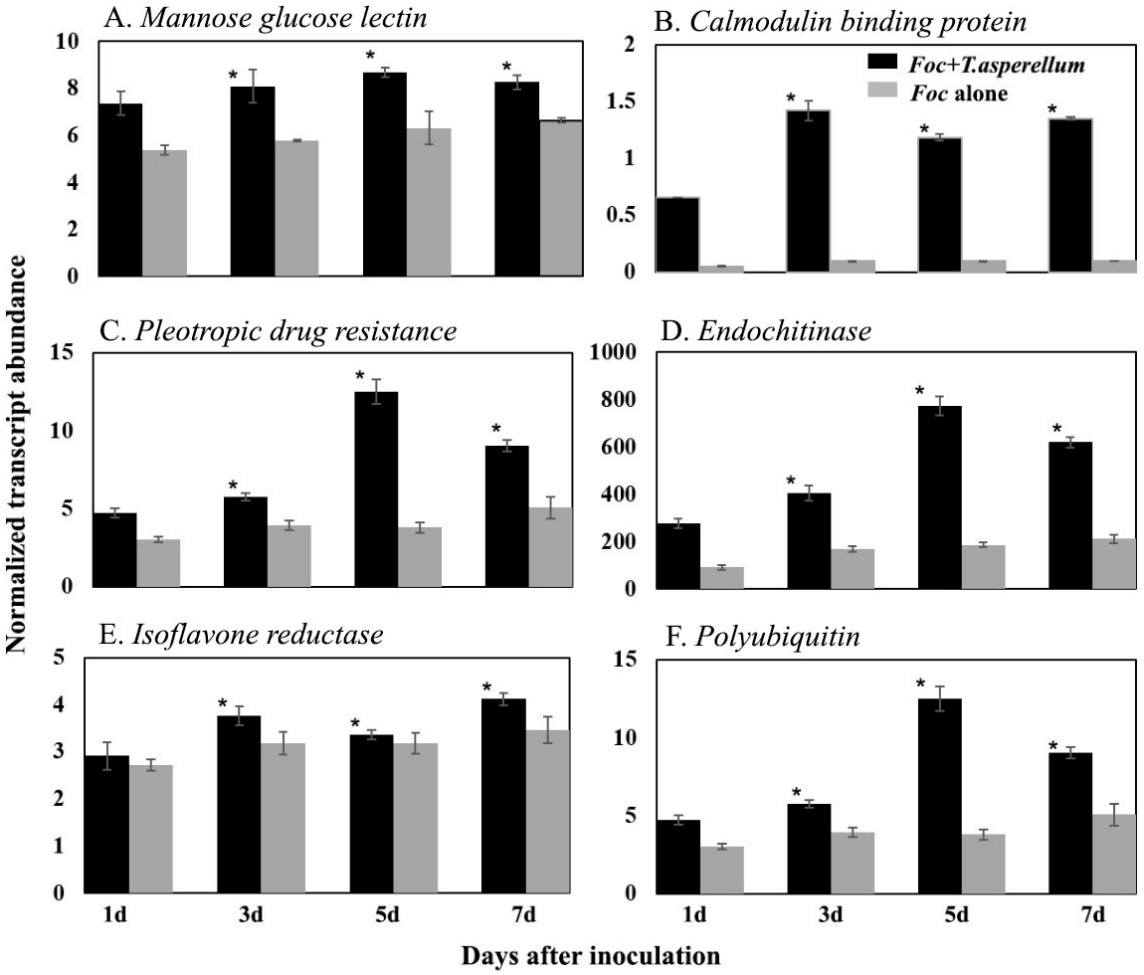

Figure 2. Relative quantification of defense-related genes in roots of the banana cultivar Grand Naine inoculated with *Foc* and challenged with *T. asperellum* (prr2). Gene expression quantified by RT-PCR after 1, 3, 5 and 7 DPI on *Mannose binding lectin* (A), *Calmodulin binding protein* (B), *Pleotropic drug resistance* (C), *Endochitinase* (D), *Isoflavone reductase* (E), *Polyubiquitin* (F). Gene expression is shown as PCR/25S rRNA product. Data were normalized with endogenous control. All experiments were performed with 3 biological replicates (n = 3). Designation of treatments is as follows: Black bars, *Foc* + *T. asperellum* inoculated plants; Gray bars, *Foc* alone inoculated plants. Means ± SE, n =3. *Tukey's test was used for proof of significance ($P < 0.05$) compared with corresponding *Foc* + *T. asperellum* or *Foc* alone inoculated treatments.

beneficial fungi (Beckman and Roberts, 1995). Certain secondary metabolites produced by *Trichoderma* exert an antimicrobial effect at high doses but act as MAMPs and as auxin-like compounds at low concentrations. At 1 ppm concentration, 6-pentyl-a-pyrone, harzianolide, and harzianopyridone activate plant defense mechanisms and regulate plant growth in pea, tomato, and canola (Kuć and Rush, 1985), suggesting that plant defense mechanisms and their developmental responses to *Trichoderma* share common components. Similarly, lignans and isoflavonoids play important roles in plant defense. Their constitutive deposition significantly helps confer durability, longevity, and resistance to plants against pathogens (Gang et al., 1999). Similarly, plant mannose-binding lectins (MBLs) are also crucial for plant defense signaling during pathogen attack by recognizing specific carbohydrates on pathogen surfaces (Hwang and Hwang, 2011). The abundance of MBLs and *isoflavone reductase* gene upregulation in *Foc* + *T. asperellum* inoculated plants as compared to *Foc* alone inoculated plants observed in the present investigation supports the hypothesis that *T. asperellum* facilitates pathogen recognition and plant defense.

Both plant and animal cells elevate their cytosolic-free calcium level in response to a variety of external stimuli, including pathogen attack. *Calmodulin binding protein* (CaM) regulates transient influx of Ca^{2+}, constituting an early event in the signaling cascades that trigger plant defense responses (Kim et al., 2001). The most

prevalent calcium-binding motif is the EF-hand motif, which generally occurs in intramolecular EF-hand pairs. The role of CaM was evidenced by its overexpression during the colonization of cacao seedlings by endophytic isolates of *Trichoderma* species (Bailey et al., 2006). In the present study, upregulation of CaM was observed up to 7 DPI in *Foc* + *T. asperellum* inoculated plants, which indicated that Ca^{2+} signals play critical roles in regulating plant defense responses against pathogens. The intricate molecular mechanisms that govern plant immune responses engage a high degree of proteomic plasticity to which posttranslational protein modification through ubiquitination contributes crucially (Marino et al., 2012). In our experiment, *polyubiquitinin* was clearly upregulated in *Foc* + *T. asperellum* as early as 1 DPI and the expression continued to increase up to 5 DPI. Modification of pathogen proteins by the covalent attachment of ubiquitin sentences the protein to proteolysis or other facets such as relocalization or endocytosis (Ikeda and Dikic, 2008).

The role of 6 defense genes evaluated in this study by RT-PCR analysis has provided an insight into defense-related gene expression in banana mediated through *Foc* + *T. asperellum* interaction. The role of these genes in mycoparasitism must be studied in more detail, because it might involve the participation of a wide range of other molecular functions not described in this work.

To conclude, our results constitute a step towards a better understanding of the molecular role of mycoparasitic *T. asperellum* during its interaction with *Foc* in a susceptible banana cultivar and how it potentiates plant defenses to wilt pathogen. However, future studies aimed at the functional characterization of genes reported here will help better to define pathways involved in *T. asperellum*'s interaction with *Foc*. A better understanding of the expression profiles of these genes could enhance *T. asperellum* performance, either by predicting the regulation of the genes involved in the mycoparasitism or by improving their use in biotechnology processes such as transgenic expression in plants.

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