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Plant endophytic *Pseudomonas putida* BP25 induces expression of defense genes in black pepper roots: Deciphering through suppression subtractive hybridization analysis

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ABSTRACT

Black pepper associated endophytic *Pseudomonas putida* BP25 displayed broad spectrum antagonistic activity against a wide range of plant pathogens. The molecular responses of black pepper upon endophytic colonization by *P. putida* BP25 was studied using a PCR based suppression subtractive hybridization (SSH) method. The transcripts induced by endophytic *P. putida* BP25 in black pepper included pathogenesis related proteins such as **PR-4 and PR-1**; reactive oxygen species (ROS) scavenging **catalase and metallothionein**; stress induced **glutathione S-transferase**; **5-enolpyruvylshikimate-3-phosphate synthase** involved in phenylpropanoid metabolism. Differential expression of transcripts was validated by quantitative real time PCR (qPCR) for selected ESTs. This information helps in understanding the ability of an endophytic strain *P. putida* BP25 in inducing a wide array of defence responses in black pepper roots.

1. Introduction

Plant associated beneficial bacteria is known to suppress plant diseases either directly through microbial antagonism or indirectly by induction of systemic resistance (ISR) in plants [1,2]. ISR confers on the plant an enhanced defensive capacity [3]. Such mechanisms include reinforcement of plant cell walls, production of antimicrobial phytoalexins as well as an enhanced defensive capacity against pathogen colonization [4,5]. Fluorescent pseudomonads are known to trigger ISR by their lipopolysaccharides, flagella and siderophores [6]. Phytohormones such as ethylene (ET) and jasmonic acid (JA) play a pivotal role in modulating ISR in plants [7,8]. In *Arabidopsis thaliana*, ISR triggered by root colonizing strains of *Pseudomonas fluorescens* was shown to be ET and JA dependent but salicylic acid (SA) independent [9–11]. ISR in tobacco plants triggered by specific strains of *P. fluorescens* 89B-61, *Bacillus pumilus* and *Serratia marscescens* involved SA signaling [12]. In our recent studies, triggering of SA signaling associated genes by the endophytic *P. putida* BP25 was clearly demonstrated in the model plant, *A. thaliana* [13]. It appears that the endophytic bacteria mediated ISR is different from that of root colonizing rhizobacteria. Additionally molecular cross-talk among various signaling pathways is also reported [14–16].

Foot rot disease caused by *Phytophthora capsici* and slow decline disease caused by *Radopholus similis* are the two major diseases in black pepper (*Piper nigrum* L.) which cause severe economic losses. *P. putida* BP25, a potential biocontrol agent, was isolated as an endophyte from black pepper which exhibited excellent colonization in black pepper roots and also inhibited a broad range of pathogens such as *P. capsici, Pythium myriotylum, Gibberella moniliformis, Athelia rolfsii, Rhizoctonia solani, Colletotrichum gloeosporioides* and plant parasitic nematode, *R. similis* through its volatile organic compounds [17,18]. Interestingly *P. putida* BP25 induced several defense related genes in *A. thaliana* and triggered SA signaling that restricted its own population in a feedback loop [13].

The endophytic *P. putida* BP25 suppressed the oomycete pathogen, *P. capsici*, on black pepper cut shoots. Understanding the mechanisms of disease suppression by *P. putida* BP25 is important for deployment of bacterial bioinoculants for successful

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management of this disease. In this study, molecular responses triggered in black pepper upon colonization by *P. putida* BP25 was deciphered using suppression subtractive hybridization (SSH) method. This is a powerful technique that enables to specifically clone ESTs that are differentially expressed in different mRNA populations [19]. The technique is used in various plant species to isolate transcripts involved in responses to biotic and abiotic stresses [20–25]. Therefore, the objectives of this study were to understand the pattern of differential gene expression in black pepper roots upon *P. putida* BP25 colonization by constructing a cDNA library by SSH and to validate the expression of selected transcripts through quantitative real time PCR (qPCR).

2. Materials and methods

2.1. Bacterial strain and plant growth conditions

Spontaneous rifamycin resistant laboratory mutant of *P. putida* designated as PpBP25 was used for plant inoculation. PpBP25 was cultured in Luria Bertani agar (LBA, Himedia) at 37 °C for 24 h. Bacterial cells were pelleted at 8000 × g for 5 min and washed pellets were used for inoculating the plants after resuspending in sterile distilled water. The roots of two-leaf stage black pepper rooted cuttings (cv. Sreekara) were treated with bacterial cell suspension (~ × 10^8 cfu ml^{-1}), and plants treated with sterile distilled water served as control. The cuttings were planted in sterile potting mixture and maintained under greenhouse conditions at 28–30 °C with 60–80% humidity.

2.2. RNA extraction and mRNA purification

In order to get a wide range of induced transcripts, bacterized and non-bacterized roots of plantlets were sampled at different intervals such as 1 day, 2 days, 3 days, 5 days and 7 days (two plants/time points). Roots were flash frozen, ground in liquid nitrogen and total RNA was extracted from treated and control samples for each time point using TRI reagent (Sigma Chemicals, USA). RNA samples corresponding to each treatment (PpBP25 inoculated and control plants) were pooled separately to obtain two independent RNA pools. Poly A + RNA was purified from 400 μg of total RNA using mRNA Purification Kit (MN, Germany) following manufacturer's instructions. The quality of mRNA was checked on 1% agarose gels and its quantity as well as purity was checked spectrophotometrically using Biophotometer (Eppendorf, Germany).

2.3. Construction of a SSH cDNA library

The SSH library enriched with differentially expressed genes was generated using the PCR-Select cDNA subtraction kit (Clontech Laboratories, USA) following the manufacturer's instructions. Briefly, cDNAs were separately synthesized from 1 μg of mRNA of each PpBP25 inoculated (tester) and control (driver) plants and digested with *Rsa* I to obtain short and blunt ended fragments. *Rsa* I digested tester cDNA was divided in to two subpopulations; and was ligated to adaptor 1 and adaptor 2R, respectively. Each adaptor ligated tester population was then hybridized separately with an excess of driver cDNA and then the reactions were mixed together for a second subtractive hybridization with driver cDNA. Two rounds of PCR amplification were performed to enrich differentially expressed sequences. Primary PCR amplification was carried out in 25 μl final volume using PCR primer 1 provided in the kit. Amplification conditions consisted of 1 min of denaturation at 94 °C, followed by 27 cycles of 20 s at 94 °C, 30 s at 66 °C and 1.5 min at 72 °C, with a final extension step of 5 min at 72 °C. Secondary PCR was carried out using nested PCR primers with amplification conditions; 1 min of denaturation at 94 °C, followed by 20 cycles of 10 s at 94 °C, 30 s at 68 °C and 1.5 min at 72 °C, with a final extension step of 5 min at 72 °C. Enriched subtracted cDNAs obtained were ligated in the pTZ57R/T (Thermo Scientific, USA) and cloned into *Escherichia coli* DH5α cells using InsTAclone PCR Cloning Kit (Thermo Scientific, USA). Positive transformants were selected based on white/blue colour selection on LB agar with ampicillin, X-gal and IPTG and confirmed by colony PCR using M13 primers.

2.4. Sequencing and analysis of the cloned ESTs

Plasmids of 100 bacterial clones of the resulting SSH library were eventually isolated and sequencing was done using M13 universal primer (SciGenome Labs Pvt. Ltd., Cochin). The sequences of the clones were checked for vector contamination using VecScreen (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) and trimmed off the vector and adaptors sequences. Low quality and short (<100 bp) sequences were excluded from the analysis. Computational annotation of ESTs from the black pepper- PpBP25 interaction was performed using 'Blast2GO' version 3.3.5. Homologies were checked in the NCBI protein database by running blastx with the E-value set to $1.0e^{-1}$. Blast2GO software was used to obtain Gene Ontology (GO) information from retrieved database matches and annotation of all sequences was performed. InterProScan analysis was applied to find functional motifs using the specific tool in the Blast2GO software with the default parameters. Enzyme mapping of annotated sequences was retrieved by direct GO and used to query the Kyoto Encyclopaedia of Genes and Genomes (KEGG) to define the main metabolic pathways involved.

2.5. Expression validation by quantitative Real-Time PCR

Quantitative Real-Time PCR (qPCR) experiments were carried out to validate the induction of selected ESTs presumed to have functional roles. Five ESTs such as pathogenesis related protein (PR-4), catalase, glutathione S-transferase, metallothionein and WRKY transcription factor 40 were selected for qPCR validation. Specific primer pairs for each sequence were designed using the primerQuest tool (https://eu.idtdna.com/Primerquest/Home/Index). Total RNA was isolated from roots (both treated and control) sampled at different time points; 1 d, 2 d, 3 d, 5 d and 7 days after post inoculation. cDNA was synthesized from 2 μg of DNase treated total RNA using reverse transcriptase (Fermentas, USA). qPCR experiments were carried out in a final volume of 20 μl containing 10 ng of RNA equivalent cDNA, 0.5 μM primers and 1× QuantiFast SYBR Green mastermix (Qiagen, Germany). The following thermal cycling profile was used for qPCR reactions: 94 °C for 5 min hold, 35 cycles of 94 °C for 30 s, 60 °C for 30 s. Melting curves of qPCR products were assessed from 62 °C to 99 °C to confirm the amplification of single PCR products. qPCR analyses were finally performed using Rotorgene Q Real-Time PCR System (Qiagen, Germany). Relative fold differences were calculated based on the comparative Ct method using actin as an internal standard. The sequences of actin primers (ACT-FP: 5′-ACATCCGCTGGAAGGTGC-3'; ACT-RP: 5′-TCTGTATGGTAACATTGTGCTC-3′) were as described previously by Alex et al. [26]. To determine relative fold differences for each sample in each experiment, the Ct values for the genes were normalized to the Ct value for the reference gene actin and were calculated using the formula $2^{-\Delta\Delta Ct}$ [27].

3. Results

3.1. Generation of cDNA library by SSH

To construct SSH library enriched with differentially expressed genes in black pepper, good quality RNA isolated from bacterized and uninoculated roots was used. The enriched subtracted cDNA was analyzed on gel and appeared as a smear ranging from 300 bp-1500 bp. Sequencing was done for selected clones of different insert sizes based on colony PCR using M13 primers (Supplementary Fig. 1). A total of 100 clones were sequenced to generate a forward subtracted cDNA library representing genes up-regulated in black pepper upon endophytic colonization by PpBP25. Computational analysis of sequences showed a number of ESTs with homology to genes of known functions. Sequence length varied from 154 to 621 bp with an average length of 426 bp. Querying with Blastx allowed hits for 61 sequences (Table 1).

3.2. Functional classification and identification of differentially expressed genes in library

Annotation of putative transcripts obtained through Blast2GO analysis, showed that many genes were induced in black pepper roots upon colonization by PpBP25. Some of the transcripts are represented by more than one independent clone encoding different parts of the same coding DNA sequences: Bet v1 and metallothionein (10 clones each); PR-4, PR-1, glutathione S-transferase and heat shock protein (3 clones each); and catalase and dnaJ (2 clones each). Majority of the ESTs represented coding DNA sequences (CDS) belonging to the genomes of *Nelumbo nucifera, Populus trichocarpa, Camellia sinensis, Cicer arietinum* and *Brassica rapa* indicating the lack of genetic information for black pepper (Fig. 1). Analysis of ESTs showed that many defence related genes are induced in roots upon PpBP25 colonization (Table 2). The ESTs were deposited in NCBI under GenBank accession numbers KX305935 to KX305954 and are furnished in Table 2. Analysis of the EST set by the Blast2GO software allowed the annotation of expressed sequences according to the terms of the three main GO vocabularies, i.e. 'biological process', 'molecular function' and 'cellular component'. GO data revealed up-regulation of 33% transcripts belonging to biological process, 30% to cellular component and 51% to molecular function. Functional categorization of the expressed sequences is shown in Fig. 2. The most represented classes of ESTs in terms of 'biological processes' are signal transduction and response to stress. With respect to the GO term 'cellular component' the most represented class included nucleus and cytosolic part. Different categories such as binding, oxidoreductase activity, transferase activity and enzyme regulator activity could be identified under the main GO vocabulary term 'molecular function'. The distribution of GO terms of different ESTs is shown in Table 3.

InterProScan analysis was done to find out functional motifs in the transcripts. The most represented domains identified were START like domain and Bet v 1 allergen (Fig. 3). Enzyme code mapping was done to find the upregulation of enzymes representing different classes, oxidoreductase, transferases and lyases. The KEGG database was queried to assign sequences to specific metabolic and/or biosynthetic pathways. KEGG maps analysis showed that proteins related to different pathways were upregulated. ESTs such as 5-enolpyruvylshikimate 3-phosphate synthase, glutathione S-transferase, catalase and inositol oxygenase were found to be involved in different metabolic pathways (Table 4).

3.3. Validation of selected transcripts by quantitative Real-Time PCR

Validation using qPCR analyses of expression of transcripts from the subtracted cDNA library revealed significant up-regulation of catalase, WRKY transcription factor 40, pathogenesis related protein (PR-4), glutathione S-transferase and metallothionein. Specific primers used for qPCR analysis is furnished in Supplementary Table 1. The PCR products were analyzed using gel electrophoresis for single PCR bands (Supplementary Fig. 2). Melt curves of q-RT PCR products also showed the amplification of single PCR products (Supplementary Fig. 3). The gene expression pattern along time: ie., at 1, 2, 3, 5 and 7 dpi of PpBP25 in black pepper roots was analyzed (Supplementary Fig. 4). All the transcripts analyzed showed a general increase in the expression in bacterized roots of black pepper. The PR-4 protein showed up-regulation with fold change of 128.4, 40.3, 89.5 and 115.7 at 1, 2, 3 and 5 dpi, respectively. The expression level of the catalase showed up-regulation with an estimated fold change of 23.9 at 1 dpi and 15.3 at 2 dpi as compared to mock. The expression of WRKY transcription factor was found to be maximum at 3 dpi (2.4) as well as the expression of glutathione S-transferase was found to be maximum at 5 dpi (3.0). Metallothionein was found to be up regulated at 1 dpi (2.34) and 2 dpi (2.40) (Table 5, Fig. 4).

4. Discussion

Endophytic bacteria are reported to promote plant growth and to reduce/prevent the deleterious effects of several plant pathogens [28]. Plant growth promotion is exerted through direct growth promotion effects and also indirectly through the production of antimicrobial metabolites or through ISR [28–30]. ISR is known to play an important role in potentiating plant health by reinforcing the physical and mechanical strength of the plant cell wall as well as changing the physiological and biochemical reaction of the host, leading to the synthesis of defence chemicals against the challenge pathogen [31–33]. The interaction between many beneficial bacteria with its host plants is well studied and induction of resistance is reported in several crops against a broad spectrum of pathogens [34,35] and/or abiotic stresses [36]. Black pepper associated PpBP25 exhibited antagonistic activity against *P. capsici* and *R. similis* in black pepper [17,37] and broad spectrum activity against several pathogens through its volatile organic compounds [18]. The genetic responses upon colonization of PpBP25 was investigated in *A. thaliana* using microarray and found that the bacterium induced several genes involved in defense and salicylic acid signaling. It is suggested that the PpBP25 colonization triggered expression of defense genes in regulating its own population in a feedback loop [13]. It is important to understand the underlying genetic responses in black pepper. Previously, transcriptional analysis of maize plants colonized with *P. putida* KT2440 revealed more transcriptional changes in roots compared to leaves [38]. In this study, a forward SSH library of differentially expressed transcripts was generated which aided in understanding the defense responses in black pepper roots upon colonization by PpBP25.

Blast2GO analysis of differentially expressed transcripts allowed hits for 61 sequences among which many of them were potentially involved in plant defense/stress responses. Functional annotation of these revealed up-regulation of genes involved in metabolism, transcription, signal transduction, response to stress, binding, oxidoreductase activity etc. in PpBP25 colonized black pepper roots.

A few studies have deciphered local responses exerted by

Table 1
Pseudomonas putida BP25 induced expressed sequence tags (ESTs) in black pepper roots identified by Blast2GO analysis.

Sequence name	Sequence description	Sequence length	Blast E-value min	Blast Top hit species
BPC1	Pleiotropic drug resistance 7	376	1.40E-42	*Elaeis guineensis*
BPC11	Type 2 metallothionein	424	3.70E-16	*Ilex paraguariensis*
BPC24	WRKY transcription factor 40	475	1.40E-11	*Coffea canephora*
BPC27	Major pollen allergen Bet v 1-D H	458	2.80E-41	*Populus trichocarpa*
BPC33	26S protease regulatory subunit 7-like	431	1.80E-22	*Populus trichocarpa*
BPC36	Type 2 metallothionein	423	3.40E-17	*Camellia sinensis*
BPC46	Pathogenesis-related 1-like	621	1.18E-50	*Nelumbo nucifera*
BPC49	Elongation factor 1-alpha	479	3.50E-82	*Cicer arietinum*
BPC51	Heat shock protein 70	555	3.00E-84	*Ziziphus jujube*
BPC60	Glutathione S-transferase F13-like	385	1.00E-21	*Nelumbo nucifera*
BPC61	Type 2 metallothionein	427	4.20E-17	*Ilex paraguariensis*
BPC65	Type 2 metallothionein	423	3.40E-17	*Camellia sinensis*
BPC69	Catalase partial	476	7.10E-68	*Manihot esculenta*
BPC72	Pathogenesis-related PR-4-like	335	7.80E-20	*Brassica rapa*
BPC77	High mobility group B 1-like	506	1.10E-42	*Nelumbo nucifera*
BPC79	Metallothionein type 2	413	6.30E-18	*Pyrus x bretschneideri*
BPC80	Heat shock protein 70	459	7.00E-91	*Ziziphus jujube*
BPC85	Major pollen allergen Bet v 1-D H	558	9.50E-52	*Nelumbo nucifera*
BPC157	Pathogenesis-related PR-4-like	438	1.00E-55	*Brassica rapa*
BPC166	Major pollen allergen Bet v 1-D H	494	2.00E-46	*Populus trichocarpa*
BPC171	Glutathione S-transferase F13-like	391	1.90E-20	*Nelumbo nucifera*
BPC179	Cell number regulator 8	532	7.90E-59	*Prunus avium*
BPC191	Uncharacterized mitochondrial protein	292	6.30E-44	*Nicotiana tabacum*
BPC220	Major pollen allergen Bet v 1-D H	598	6.40E-50	*Nelumbo nucifera*
BPC224	Ubiquitin	342	7.80E-26	*Nicotiana benthamiana*
BPC229	Major pollen allergen Bet v 1-D H	564	8.70E-52	*Nelumbo nucifera*
BPC244	uncharacterized protein	514	3.30E-02	*Sesamum indicum*
BPC258	5-enolpyruvylshikimate-3-phosphate synthase	526	2.40E-100	*Elaeis guineensis*
BPC269	Type 2 metallothionein	456	4.80E-17	*Camellia sinensis*
BPC273	Pathogenesis-related 1-like	295	3.50E-16	*Populus trichocarpa*
BPC283	Type 2 metallothionein	425	4.20E-17	*Ilex paraguariensis*
BPC285	Major pollen allergen Bet v 1-D H	448	1.60E-37	*Nelumbo nucifera*
BPC298	Inositol oxygenase	296	9.90E-55	*Nelumbo nucifera*
BPC301	Histone	365	4.88E-32	*Glycine soja*
BPC302	Pathogenesis-related PR-4-like	415	3.30E-49	*Brassica rapa*
BPC304	Major pollen allergen Bet v 1-D H	588	1.20E-51	*Nelumbo nucifera*
BPC308	High mobility group B 1-like	529	4.20E-43	*Nelumbo nucifera*
BPC310	Heat shock protein 70	378	1.70E-38	*Eriobotrya japonica*
BPC314	Glutathione S-transferase F13-like	384	1.90E-15	*Nelumbo nucifera*
BPC316	Major pollen allergen Bet v 1-D H	519	2.70E-48	*Populus trichocarpa*
BPC321	Catalase partial	509	3.90E-67	*Ananas comosus*
BPC323	dnaJ homolog	393	5.60E-33	*Gossypium raimondii*
BPC324	Elongation factor 1-alpha	375	5.70E-51	*Cicer arietinum*
BPC326	Major pollen allergen Bet v 1-D H	477	1.60E-41	*Populus trichocarpa*
BPC328	Type 2 metallothionein	440	4.10E-17	*Camellia sinensis*
BPC335	Major pollen allergen Bet v 1-D H	458	2.80E-41	*Populus trichocarpa*
BPC337	Plasma membrane intrinsic protein 1,4 isoform 1	425	1.40E-66	*Pyrus x bretschneideri*
BPC338	NADPH–cytochrome P450 reductase-like	484	2.90E-36	*Oryza sativa Indica Group*
BPC341	Proline transporter 2	477	2.20E-21	*Musa acuminata* subsp. *malaccensis*
BPC344	Pathogenesis-related 1-like	276	8.80E-17	*Populus trichocarpa*
BPC348	Actin-depolymerizing factor 7-like	304	1.50E-32	*Setaria italica*
BPC353	Elongation factor 1-alpha	476	2.40E-81	*Cicer arietinum*
BPC354	Superoxide dismutase [Cu-Zn]	542	2.60E-94	*Phoenix dactylifera*
BPC358	60S ribosomal L5	587	6.10E-100	*Musa acuminata* subsp. *malaccensis*
BPC368	Hypothetical protein	518	1.40E-48	*Morus notabilis*
BPC370	Casein kinase II subunit alpha like	394	5.94E-49	*Coffea canephora*
BPC372	Elongation factor 1-alpha	505	3.20E-79	*Cicer arietinum*
BPC373	Metallothionein type 2	478	1.10E-16	*Pyrus x bretschneideri*
BPC387	Type 2 metallothionein	475	6.70E-17	*Camellia sinensis*
BPC389	dnaJ homolog	424	3.20E-32	*Gossypium raimondii*
BPC422	(RS)-norcoclaurine 6-*O*-methyltransferase-like	325	7.60E-25	*Jatropha curcas*

endophytic bacteria previously. Verhagen et al. [39] reported substantial changes in the expression of 97 genes including genes involved in cell rescue and defence, metabolism, transcription, cellular communication and signal transduction, particularly those involved in ethylene signaling in *Arabidopsis* roots challenged with *P. fluorescens* WCS417r using an *Arabidopsis* GeneChip microarray. In another study, Sanchez et al. [40] found that 58 genes including genes involved in signal transduction and transcription were upregulated in roots of the legume species *Medicago truncatula* in response to colonization by the growth promoting strain *P. fluorescens* C7R12. The genetic responses induced in olive roots upon colonization by the biocontrol endophytic bacterium *P. fluorescens* PICF7 were also elucidated and induction of several defense related genes were confirmed [41]. In our study, several defense/stress related transcripts such as pathogenesis related (PR) proteins, reactive oxygen species (ROS) scavenging transcripts,

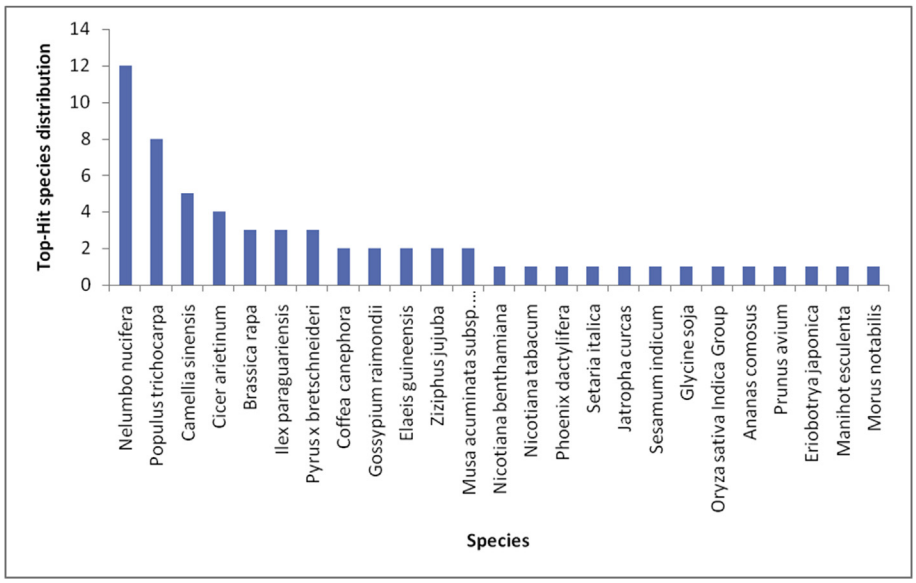

Fig. 1. Distribution of hits of the complete assembly obtained against sequences available for different plant species.

Table 2
Functional details of defense related ESTs induced in black pepper roots upon colonization by *Pseudomonas putida* BP25.

Gene name	GenBank accession No.	Gene function	Remarks
5-enolpyruvylshikimate-3-phosphate synthase	KX305940	3-phosphoshikimate 1-carboxyvinyltransferase activity, chorismate biosynthetic process, tryptophan biosynthetic process, tyrosine biosynthetic process, L-phenylalanine biosynthetic process	Involved in phenyl alanine, tyrosine and tryptophan metabolism, lignin biosynthesis and biosynthesis of antibiotics [75]
WRKY transcription factor 40	KX305935	Transcription factor activity, sequence-specific DNA binding, regulation of transcription, DNA-templated, transcription factor complex	Relieves repression of ABA signaling [71]
Major pollen allergen Bet v 1-D H	KY286528	Protein phosphatase inhibitor activity, receptor activity, abscisic acid binding, abscisic acid-activated signaling pathway, negative regulation of catalytic activity, regulation of protein serine/threonine phosphatase activity	Shows high sequence similarity to pathogenesis related proteins and plays a role in defense responses [54]
Pathogenesis-related PR-1	KX305936	Protein phosphatase inhibitor activity, receptor activity, abscisic acid binding, abscisic acid-activated signaling pathway, negative regulation of catalytic activity, regulation of protein serine/threonine phosphatase activity	Salicylic acid mediated signaling [42]
Pathogenesis-related PR-4	KX305942	Defense response to bacterium, defense response to fungus	Chitinases with potent antifungal activity [52,53]
Type 2 Metallothionein	KX305941	Metal ion binding	Maintenance of metal levels, ROS scavenging [59]
Heat shock protein 70	KX305944	ATP binding	Molecular chaperones required for folding of nascent proteins and intracellular transportation in addition to stress responses [69]
Glutathione S-transferase	KX305937	Glutathione transferase activity, glutathione metabolic process, obsolete glutathione conjugation reaction	Role in oxidative, chemical stress and disease resistance [64,65]
Catalase	KX305938	Catalase activity, heme binding, response to oxidative stress, oxidation-reduction process, cellular oxidant detoxification, tryptophan metabolic process, obsolete peroxidase reaction, methane metabolic process	ROS scavenging enzymes [55]
Inositol oxygenase	KX305945	Iron ion binding, inositol oxygenase activity, inositol catabolic process, oxidation-reduction process	Myoinositol catabolism, biosynthesis of L-ascorbic acid or supply of precursors for cell wall polymers [63]
dnaJ homolog	KX305946	ATP binding, heat shock protein binding, metal ion binding, unfolded protein binding, protein folding, response to heat	Cochaperones which regulate the functions of heat shock proteins and is involved in stress response [70]
NADPH–cytochrome P450 reductase	KX305947	NADPH-hemoprotein reductase activity, iron ion binding, FMN binding, [methionine synthase] reductase activity, oxidation-reduction process, obsolete electron transport	Plays a role in disease resistance and also contribute for expression of other genes related to defense response [67,68]
Superoxide dismutase [Cu-Zn]	KX305950	Superoxide dismutase activity, metal ion binding, removal of superoxide radicals, oxidation-reduction process	ROS scavenging enzymes [55]
Casein kinase II subunit alpha	KX305954	Protein kinase activity, ATP binding, protein phosphorylation	Key regulator of cell cycle, transcription and translation and also participates in the salicylic acid mediated signal transduction pathway [72,73]
(RS)-norcoclaurine 6-*O*-methyltransferase	KX305952	Methyl transferase activity	Alkaloid biosynthesis [78]

stress induced transcripts and enzymes involved in plant defense metabolism were found to be differentially expressed in black pepper roots.

In this study, PR proteins such as PR-1, PR-4 and Bet v 1 (a class

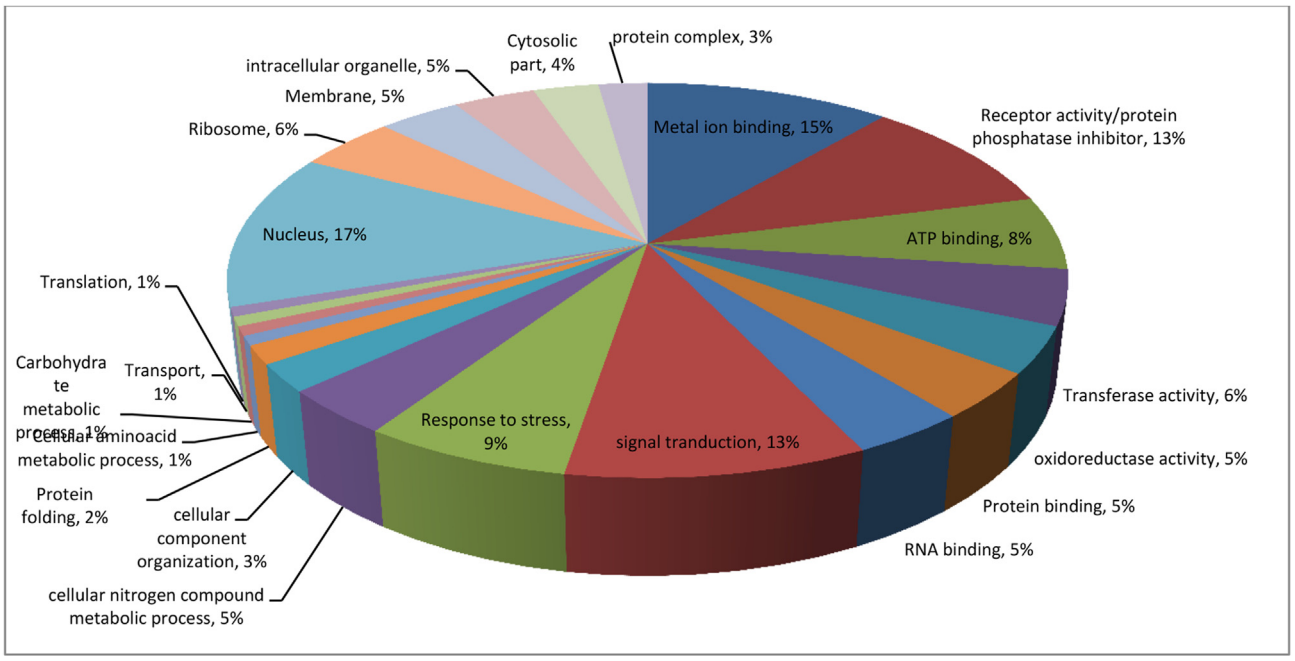

Fig. 2. Functional classification of ESTs induced in black pepper colonized by endophytic *Pseudomonas putida* BP25.

Table 3
Gene Ontology (GO) terms distribution of ESTs induced in black pepper roots colonized by *Pseudomonas putida* BP25.

Biological process	GO terms
Signal transduction	Major pollen allergen Bet v 1-D H-like; pathogenesis-related PR-1
Response to stress	26S protease regulatory subunit 7; Pathogenesis-related PR-4; dnaJ; Catalase; Superoxide dismutase
Sulfur compound metabolic process	Glutathione S transferase
Protein folding	dnaJ
Carbohydrate metabolic process	Inositol oxygenase
Cellular amino acid metabolic process	5-enolpyruvyl shikimate-3-phosphate synthase
Cytoskeleton organization	Actin-depolymerizing factor 7
Transport	Plasma membrane intrinsic protein
Translation	60S ribosomal L5
Cellular component	
Nucleus	Major pollen allergen Bet v 1-D H-like; Pathogenesis-related PR-1; Superoxide dismutase; Histone; Ubiquitin; 26S protease regulatory subunit
Cytosolic part	26S protease regulatory subunit; 60S ribosomal L5; Histone; Ubiquitin
Ribosome	Elongation factor 1 alpha; 60S ribosomal L5; Ubiquitin
Protein complex	WRKY transcription factor; 26S protease regulatory subunit; Histone
Membrane	Pleiotropic drug resistance 7; Plasma membrane intrinsic protein; Cytochrome P450 reductase; Histone; Proline transporter; dnaJ
Molecular function	
Metal ion binding	Type 2 metallothionein; Inositol oxygenase; Superoxide dismutase; NADH cytochrome P450 reductase; dnaJ
Lipid binding/enzyme regulator activity	Major pollen allergen Bet v 1-D H-like; pathogenesis-related PR-1
ATP binding	Heat shock 70
Translation factor activity	Elongation factor 1-alpha
DNA binding	WRKY transcription factor 40; Histone
Unfolded protein binding	dnaJ
rRNA binding/structural constituent of ribosome	60S ribosomal L5
Cytoskeletal protein binding	Actin-depolymerizing factor 7
Oxidoreductase activity	Cytochrome P450 reductase; Inositol oxygenase; Catalase; Superoxide dismutase
Transferase activity	5-enolpyruvylshikimate-3-phosphate synthase; Glutathione S-transferase; Casein kinase II; (RS)-norcoclaurine 6-O-methyltransferase
ATPase activity	Pleiotropic drug resistance 7; 26S protease regulatory subunit 7

of PR-10) were found up-regulated significantly. PR-1 is a well-known component of SA mediated signaling pathway [42] and was commonly found associated with systemic induced resistance (SAR) in response to pathogen infection [43]. Recently we have reported the induction of genes involved in SA mediated pathway in *A. thaliana* upon PpBP25 colonization. It was demonstrated that SA signaling plays a role in regulating the endophytic colonization [13]. Previously, induction of PR-1 proteins and SA was reported in tobacco plants upon root colonization by *P. fluorescens* CHA0 [44]. The ability to induce resistance in tobacco and bean by the plant

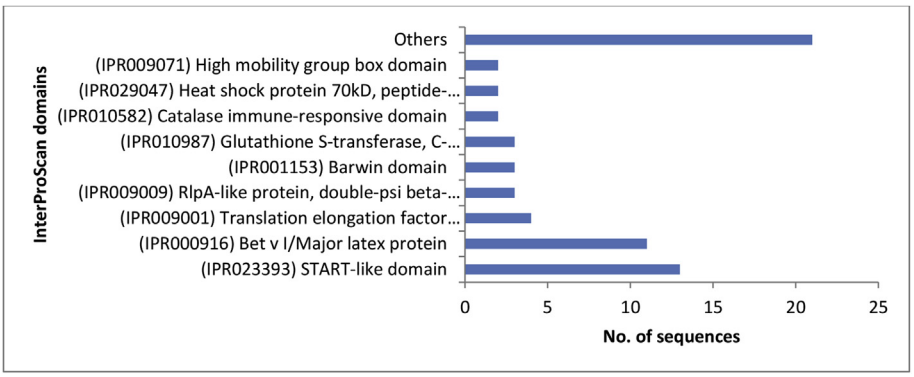

Fig. 3. InterProScan analysis for the major domains in the ESTs induced in black pepper roots colonized by *Pseudomonas putida* BP 25.

Table 4
ESTs related to enzymes in metabolic pathways induced in black pepper roots by *Pseudomonas putida* BP25.

Enzyme	E.C. Number	GO category
5-enolpyruvylshikimate-3-phosphate synthase	2.5.1.19	Phenyl alanine, tyrosine and tryptophan metabolism, biosynthesis of antibiotics
Glutathione S-transferase	2.5.1.18	Glutathione metabolism, metabolism of Xenobiotics
Inositol oxygenase	1.13.99.1	Inositol phosphate metabolism, ascorbate and aldarate metabolism
Catalase	1.11.1.6	Glyoxylate and dicarboxylate metabolism, tryptophan metabolism

Table 5
qPCR analysis of expression of *Pseudomonas putida* BP25 induced defense genes in black pepper.

Time interval	Fold change				
	Catalase	Glutathione S-transferase	Metallothionein	PR-4	WRKY transcription factor
1 d	23.92	1.17	2.34	128.44	0.69
2 d	15.31	0.71	2.40	40.37	0.58
3 d	1.95	0.67	1.71	89.57	2.40
5 d	0.70	3.09	0.29	115.76	0.27
7 d	1.58	0.58	0.77	0.06	1.14
C.D	1.363	0.68	0.21	2.45	0.25
p value	<0.001	<0.001	<0.001	<0.001	<0.001
SE(m)	0.367	0.18	0.05	0.66	0.06
SE(d)	0.519	0.26	0.08	0.93	0.09
C.V	5.965	20.97	5.49	1.24	9.34

root colonizing *Pseudomonas aeruginosa* strain 7NSK2 is also linked to production of SA [45,46]. *Pseudomonas* sp. in chickpea seedlings and *P. fluorescens* 89B, *B. pumilus* and *S. marscescens* in tobacco are also shown to induce the synthesis of SA [12,47]. Similarly, the involvement of SA in local defences was demonstrated in grapevine induced by *B. phytofirmans* PsJN [48]. PR-1 proteins from tomato and tobacco were shown to possess *anti*-oomycetal activity against *Phytophthora infestans* [49]. All these suggest that SA may be produced and play a role in defence responses. Induction of PR-1 can be correlated to the regulation of endophytic population and also antagonistic activity against *P. capsici*. In another study, Yang et al. [50] reported that expression of PR-1 and PR-4 was systemically primed by *Bacillus cereus* BS107 in *Capsicum annuum*. PR-4 proteins are chitinases of which antifungal activity has been widely described [51–53]. It is reported to be induced as a consequence of fungal infection in black pepper [23]. Major pollen allergen, Bet v 1, a class of PR-10 proteins found induced in this study, is reported to show high sequence similarity to pathogenesis related protein PR-1 and plays a key role in defense responses [54]. Previously, induction of PR-4 and Bet v1 protein (PR-10) has been reported in olive roots colonized with *P. fluorescens* PICF7 [41]. Collectively the data confirms that endophytic *P. putida* BP25 triggered expression of PR proteins in black pepper.

Furthermore, induction of reactive oxygen species (ROS) interacting enzymes such as superoxide dismutase and catalase which are involved in the protection of tissues from oxidative damage can be correlated with increased tolerance of the plant against biotic and abiotic stresses [55]. Cell wall mediated resistance is the first line of plant defense against pathogens and the components of plant cell wall are modified by production of ROS such as superoxide anion and H_2O_2 during biotic stress [56,57]. ROS scavenging enzymes prevent its accumulation and protect the plants from toxic effects. It is demonstrated that there is no significant accumulation of H_2O_2 and cell death in cell suspension after challenge with *B. phytofirmans* PsJN [48]. Induction of superoxide dismutase and catalase was reported earlier in olive roots and aerial tissues upon endophytic colonization by *P. fluorescens* PICF7 [41,58]. Metallothioneins, low molecular weight metal-binding proteins probably involved with the maintenance of metal levels in several organisms, are found upregulated upon PpBP25 colonization. It is reported that metallothioneins are also involved in ROS scavenging and its expression is important for defense signaling [59]. In rice, their expression has been reported to be induced in response to abiotic stresses [60], upon infection with the pathogen *Magnaporthe grisea* [61] and upon inoculation with a diazotroph, *Herbaspirillum seropedicae* [62]. It can be suggested that ROS scavenging may be an important mechanism of defense responses in black pepper. Induction of inositol oxygenase involved in cell wall

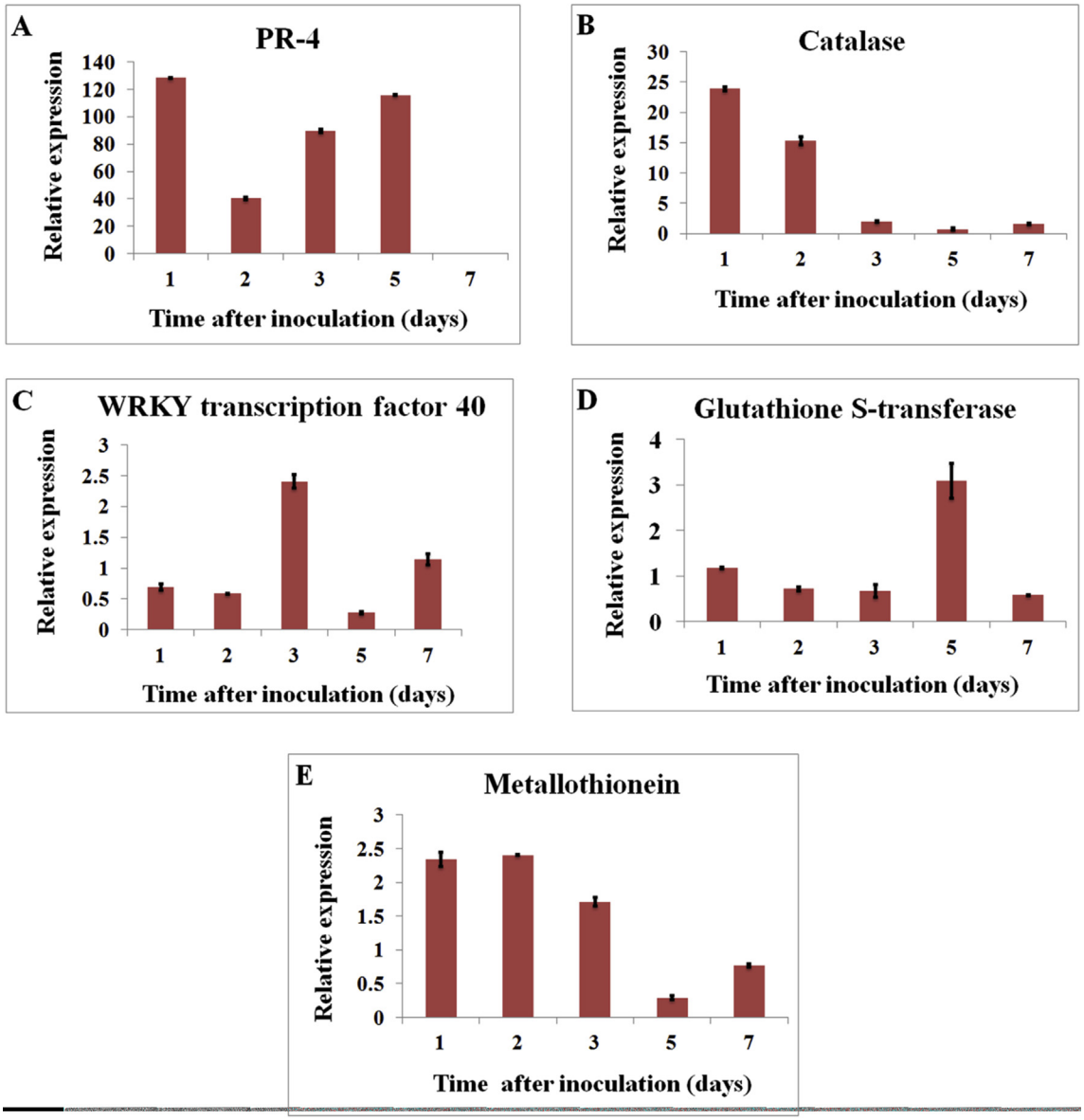

Fig. 4. Relative expression (RE) of selected up-regulated genes in black pepper roots colonized with *Pseudomonas putida* BP25 at 1, 2, 3, 5 and 7 dpi analyzed using quantitative RT-PCR. The transcript levels of the genes A) PR4, B) catalase, C) WRKY transcription factor 40, D) glutathione S transferase and E) metallothionein.

polysaccharide biogenesis can also be linked to increased resistance [63].

Other ESTs upregulated include glutathione S-transferase (GST), cytochrome P450 reductase, heat shock protein (HSP) 70 and dnaJ protein. GST proteins are induced during stress and play a protective role against the oxidative and chemical stress [64]. Dean et al. [65] demonstrated the importance of GST in disease resistance in tobacco upon infection by *Colletotrichum* sp. In addition, coordinated induction of different GST genes in mountain laurel seedlings colonized with *Streptomyces padanus* was associated with protection against infection by *Pestalotiopsis sydowiana* [66]. Cytochrome P450 reductase is also reported to contribute for disease resistance [67,68]. HSP family members along with dnaJ, collectively called molecular chaperones are required for folding of nascent proteins and intracellular transportation in addition to stress responses [69]. HSP70s have a weak ATPase activity that is stimulated by interaction with dnaJ proteins [70]. Induction of cytochrome P450, HSP70 and dnaJ protein was previously reported in rice plants inoculated with *Herbaspirillum* [62]. In our study, induction of all these ESTs may be involved in maintaining a properly functioning environment for other defense responses.

A transcription factor, WRKY40 was found among the subtracted transcripts of bacterized black pepper roots. The role of several WRKY transcription factors have been studied and found associated with resistance against pathogens. It was reported that AtWRKY40 relieves the repression of ABA signaling in the presence of ABA in *Arabidopsis* [71]. In this study, casein kinase 2 was also found differentially expressed. It is involved in transcription and translation and also is a key regulator of the cell cycle [72]. It is reported that casein kinase 2 activity is involved in early events of transcriptional activation induced by SA in tobacco [73]. Therefore, the increase in expression of a casein kinase in our study may be correlated to SA signaling.

The induction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) in bacterized roots indicated the elevated plant defence to pathogens. EPSP synthase participates in the

biosynthesis of the aromatic amino acids phenylalanine, tyrosine and tryptophan, the central molecules in plant metabolism via the shikimate pathway [74]. The increased expression of EPSPS is related to the increase in phenylpropanoid precursors for lignin biosynthesis as well as for chorismate-derived compounds, such as SA or phytoalexins involved in defense responses [75]. The expression of the EPSPS gene is induced in response to infection by the necrotrophic fungal pathogen *Botrytis cinerea* [76]. Using RNAseq analysis, induction of genes related to phenylpropanoid biosynthesis was observed in cassava upon infection with pathogenic and non-pathogenic strains of *Xanthomonas axonopodis* [77]. (RS)-norcoclaurine 6-*O*-methyltransferase, an enzyme involved in alkaloid biosynthesis [78] has also been found upregulated suggesting potential biocontrol activity of PpBP25 upon root colonization in black pepper. This is in accordance with the earlier reports on induction of transcripts involved in phenylpropanoid metabolism and alkaloid biosynthesis on endophytic colonization [41].

qRT PCR assays also validated the induction of all the evaluated ESTs. High level of expression was observed for some of the transcripts at early stages of inoculation. The endophyte may be recognized as non-hostile leading to high level of expression. It is reported that initially induced defense responses are eventually attenuated allowing the establishment of beneficial population [79]. This study enabled the identification of several ESTs associated with defense responses. It is demonstrated that the resistance exerted by PpBP25 involves an array of defense genes as in the case of host-pathogen interaction but without causing any disease symptoms. This is in accordance with the observation of similar defense responses in maize plants upon infection with pathogenic and non-pathogenic strains indicating that disease development is dependent upon the induction/repression of only a few genes [77]. This may also be correlated to the regulation of endophytic population of PpBP25 in *Arabidopsis* as reported in our earlier work [13]. PpBP25 was previously shown to colonize root tissues of black pepper in higher population [18]. The effective biocontrol activity displayed by PpBP25 against *P. capsici* and *R. similis* [17,37] can be explained by the induction of defense related transcripts at the root level in addition to direct antagonism.

All these information suggest that *Pseudomonas putida* PpBP25 provides an enhanced protection level in black pepper roots.

5. Conclusions

Endophytic colonization by PpBP25 in black pepper is governed by many defense related genes identified in this study. Black pepper responds to PpBP25 colonization by deploying several defense responses initially that eventually attenuate allowing their establishment and beneficial effects on host. The information on induction of many genes involved in plant defense system indicates that PpBP25 is able to lessen or prevent pathogen infection. The identified genes and processes can be used to generate information on their role in the interaction of bacteria with plants.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pmpp.2017.07.006.

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