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Impact of *Pseudomonas putida* RRF3 on the root transcriptome of rice plants: Insights into defense response, secondary metabolism and root exudation

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Pseudomonas putida is widely used as a biocontrol agent, however, mechanisms by which it initiates the plants' defense response remains obscure. To gain an insight into the molecular changes that occur in plants upon plant growth-promoting rhizobacteria colonization, root transcriptome analysis by using a microarray was performed in rice using *P. putida* RRF3 (a rice rhizosphere isolate). Data analysis revealed a differential regulation of 61 transcripts (48 h post-treatment), of which, majority corresponded to defense response, cell wall modification and secondary metabolism. Seven genes encoding salicylic acid (SA) responsive pathogenesis-related proteins were up-regulated significantly (fold change ranges from 1 to 4), which suggests that RRF3 has a profound impact on a SA-mediated defense signaling mechanism in rice. Investigations performed at later stages of RRF3 colonization by real-time polymerase chain reaction and high-performance liquid chromatography (HPLC) analysis confirmed the above results, demonstrating RRF3 as a potent biocontrol agent. Further, the impact of RRF3 colonization on root exudation, in particular, exudation of SA was investigated by HPLC. However, analysis revealed RRF3 to have a negative impact on root exudation of SA. Overall, this study shows that *P. putida* RRF3 immunizes the rice plants by re-organizing the root transcriptome to stimulate plant defense responses ('priming'), and simultaneously protects itself from the primed plants by altering the rhizosphere chemical constituents.

Keywords. Defense; *Pseudomonas putida*; rice; root exudation; salicylic acid; secondary metabolism

1. Introduction

The use of plant growth-promoting rhizobacteria (PGPR) as biofertilizers has evolved as a promising approach in the recent past to improve crop productivity amidst the environmental stresses. Besides direct growth promotion, by the production of phytohormones, siderophores and phosphate solubilization, PGPR promote the plant growth by acting as biocontrol agents conferring protection against an array of phytopathogens, by the secretion of antimicrobials, competition for nutrients and induction of plants' defense mechanisms (Bakker *et al.* 2007).

The best biocontrol agents among the rhizobacteria are the *Pseudomonas* spp. and the most characterized species are *Pseudomonas fluorescens*, *Pseudomonas putida* and *Pseudomonas aeruginosa*. The exclusive plant growth-promoting traits of these genera include efficient root colonization, induction of systemic responses and production of antimicrobials such as antibiotics (2,4-diacetylphloroglucinol and phenazine), siderophores (pyoverdin and pyochelin) and

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hydrogen cyanide (Dowling and O'Gara 1994; Molina *et al.* 2000). *P. putida* has widely been used in growth promotion of several plants such as wheat, Arabidopsis and maize, in the biocontrol of phytopathogens such as *Fusarium oxysporum* f. sp. *vasinfectum* and in the bioremediation of environmental pollutants (Lemanceau *et al.* 1992; Li *et al.* 2010; Ali *et al.* 2011; Srivastava *et al.* 2012; Planchamp *et al.* 2015).

Research in the past has elucidated the molecular determinants involved in root colonization by PGPR. Flagellins and the O-lipopeptides of the PGPR such as *P. putida* have been reported as the determinants that play a critical role in chemotaxis and colonization onto roots (de Weger *et al.* 1989; Lugtenberg *et al.* 2001). In the reciprocal communication, exopolysaccharides and root exudates (REs) from plants facilitate colonization by the beneficial bacteria (Lugtenberg *et al.* 1999). For instance, exudation of compounds such as organic acids, phenolics and flavonoids through roots has been illustrated to play a key role in bacterial chemotaxis and colonization (Chet *et al.* 1973; Rudrappa *et al.* 2008). However, alteration in the plants' molecular mechanisms induced by PGPR for their establishment evading plant's defense responses and the subsequent changes induced upon the plant signaling cascades for plants' growth promotion are poorly understood.

Plant defense mechanisms are complex phenomena and take place by systemic-acquired resistance (SAR) or induced systemic resistance (ISR) and are regulated by phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). SA regulates SAR, while JA and ET regulate ISR (Pieterse *et al.* 2014). Though similar to phenotypes, the underlying molecular signaling cascades differ. SAR is stimulated during mechanical wounding, herbivory and pathogenic interactions and is characterized by an increase in the synthesis of pathogenesis-related (PR) proteins and production of antimicrobials such as phytoalexins. During pathogen or herbivore attack, these antimicrobials are released out through roots/shoots in the form of either exudates or volatiles to defend themselves against the attackers (Freeman and Beatie 2008). In contrast, ISR is activated during local infection by avirulent bacteria such as PGPR. The presence of PGPR enhances the accumulation of defense-related transcription factors (TFs) in plant cells and during future infection by a pathogen, specific TFs are activated from the previously accumulated TFs, in a shorter duration with higher efficiency, thereby conferring resistance against the pathogen (Van der Ent *et al.* 2009). ISR is mainly characterized by an enhanced expression of JA-responsive genes such as thionin and defensin leading to the synthesis of antimicrobial compounds (van der Ent *et al.* 2009; Vidhyasekaran 2015).

Despite the available phenotypic reports on the effect of PGPR in plant growth promotion, the molecular changes that occur in plants from the time of contact with the PGPR; their recognition and symbiotic association and further alterations in plant metabolism and root exudation are obscure. Studies conducted at the whole-transcriptome level would provide a global insight into the genes regulated in plants in association with PGPR. The aim of this study is to gain an insight into the molecular changes that occur in rice roots in response to colonization by PGPR, such as *P. putida* RRF3 (isolated from rice rhizosphere) using a microarray. Root transcriptome analysis performed using *P. putida* RRF3-treated roots, revealed a differential regulation of 61 transcripts. Among them, those transcripts involved in cell wall modification, defense response and further secondary metabolite production are discussed in this study. In addition, the influence of RRF3 on SA biosynthesis, signaling and root exudation at different stages of root colonization is illustrated by real-time polymerase chain reaction (PCR) and high-performance liquid chromatography (HPLC) analysis.

2. Materials and methods

2.1 *Plant material and bacterial culture*

Rice seeds (TKM 9) used in the present study were obtained from Rice Research Station (RRS), Tirur, Thiruvallur, Tamil Nadu, India. TKM 9 is a stress tolerant, high-yielding variety of rice suitable for all dry and wet conditions. Bacterial culture (RRF3) was isolated from rice rhizospheric soil (Chengalpet, Tamil Nadu, India) and characterized for its plant growth-promoting ability by the following tests using standard protocols: auxin production, biofilm formation, phosphate solubilization and siderophore production (Meyer and Abdallah 1978; Bric *et al.* 1991; Nautiyal 1999; Morikawa *et al.* 2006). Subsequently, the identity of the isolate RRF3 was determined by sequencing using housekeeping genes such as 16S rRNA and gyrase B (*gyrB*). The isolated culture was maintained in King's B agar (KBA) medium and glycerol stock of the culture was stored at −80°C.

2.2 *P. putida RRF3 in plant growth promotion*

The plant growth-promoting ability of *P. putida* RRF3 was tested using rice plants under greenhouse conditions (Srivastava *et al.* 2012). Rice seeds were surface sterilized (Tween 80 for 5 min and 0.1% mercuric chloride for 2–3 min) and sown in pots containing sterile soilrite comprising a mixture of Irish peat moss and horticulture grade expanded perlite in a 75:25 ratio (pH: −5.0 to 6.5). Ten seeds were inoculated into each pot in triplicate and were kept in a greenhouse [light: 12 h daylight (600–700 PAR); temperature: day −28°C, night −25°C; humidity: 50–70%]. The plants were supplied with Hoagland's nutrient solution (100 mL) at weekly intervals and the moisture content in the pots was maintained by routine irrigation with the same volume of sterile distilled water. For treatment of rice plants in the greenhouse, *P. putida* RRF3 was tagged with rifampicin resistance (rif-tag) through repeated sub-cultures in

rifampicin-amended media (Compeau *et al.* 1988) and was used for all further studies. Following rif-tagging, a RRF3 culture suspension was prepared in 0.85% saline with a cell density of 10^7 cells/mL [optical density (OD_{600}): 0.4]. Rice plants grown for 2 weeks in the greenhouse were root-inoculated with this RRF3 culture suspension (1 mL). For control, plantlets were treated with an equal volume of sterile saline. After 10 days of RRF3 treatment, rice plants were harvested to evaluate their vegetative growth.

2.3 *Chemotaxis of RRF3 against rice REs*

For a chemotaxis assay, rice plants were grown aseptically in half-strength Murashige and Skoog (MS) media in a plant growth chamber (photoperiod: 16 h light/8 h dark; light intensity: 150–180 μmol/m^2/s and temperature: 25 ± 2°C). Two weeks post-germination, rice plants were aseptically transferred to glass conical flasks (250 mL) containing sterile deionized water (50 mL) and placed in an orbital shaker at 50 rpm for 48 h. The plants were transferred in such a way that only roots got submerged in water, while the shoots remained above and received adequate light for photosynthesis inside the flask. The flasks were cotton-plugged to avoid contamination and covered at the bottom with aluminum foil to avoid light-induced degradation of compounds in the REs. Each flask contained 25 plants and the REs that are accumulated in the deionized water were collected after 48 h, filtered [to remove the root sheathings and root-border-like cells using 0.4 μm syringe filters (Millex-HV, Merck, MA, USA)] and freeze-dried. From the obtained crude exudate (5 mg/g root fresh weight), 1 mg was resuspended in 1 mL of sterile HPLC water and filtered using 0.2 μm syringe filters (Millex GV, Merck) for chemotaxis assay (Rekha *et al.* 2018).

To examine the chemotactic ability of RRF3 toward rice REs, a chemotaxis assay was performed using the modified capillary method (Mazumder *et al.* 1999; Rekha *et al.* 2018). For the assay, 150 μL of the bacterial culture (OD_{600}: 0.05) was taken in a 200 μL pipette tip and fitted to a 2 mL syringe (with the needle) containing the crude RE (500 μL). The pipette tip serves as a chamber, while the needle acts as a capillary through which the bacteria move toward the chemoattractant (test – RE; control – sterile water). After 30 min of incubation in the laminar air-flow chamber, the contents of the syringe were spread-plated on KBA medium with appropriate dilutions. On overnight incubation, plates were observed for colonies and the colony forming units (CFU) were counted. A relative chemotaxis ratio (RCR), which is the ratio of the bacteria that are chemotactically attracted to the REs to that in the control, was calculated. An RCR of 2 or greater was considered significant. The experiment performed in biological triplicate was statistically analyzed by analysis of variance (ANOVA) using SPSS software (version 16.0, SPSS Inc., Chicago, USA).

2.4 *Analysis of root transcriptome by using a microarray*

For transcriptome analysis, the RRF3 culture suspension was prepared with a cell density of 10^7 cells/mL (OD_{600}: 0.4) in Hoagland's nutrient solution. Thirty-five microliters from this suspension was inoculated into flasks containing 50 mL Hoagland's nutrient solution. Rice seedlings grown aseptically for 14 days in MS media were then transferred to these flasks and placed in an orbital shaker for 48 h in the plant growth chamber. Un-inoculated flasks served as the control. The experiment was performed in triplicate with 25 plants in each treatment. Forty-eight hours post-inoculation (hpi), roots were washed with sterile water, blotted dry and ground in liquid N_2 for total RNA isolation.

Microarrays were obtained from Agilent Technologies (CA, USA) consisting of sequences from the Rice Annotation Project Data Base (RAP-DB). The array contained 42,249 probes (60 bp) comprising 37,141 non-redundant transcripts spotted in sense orientation (Rice_GXP_4X44K AMADID: 064815). Total RNA was isolated from 400 mg root tissues of RRF3-treated plants using the TRIzol reagent (Life Technologies, MA, USA) and was treated with DNAse. The quality of the treated RNA was then analyzed using a bioanalyzer (Agilent; 2100 expert) and a nanodrop spectrophotometer (Thermo Scientific, MA, USA) was used to verify its integrity and quality. First-strand complementary DNA synthesis was then performed using 500 ng of total RNA using a Protoscript II reverse transcriptase (NEB, MA, USA) and oligo dT primer (as per the manufacturer's protocol). From the cDNA, cRNA synthesis and labeling were performed using Cy3 CTP dye according to the manufacturer's instructions (Agilent). The labeled cRNA was subsequently purified (Qiagen RNeasy Mini kit columns, Qiagen, Germany) and quantified using the Nanodrop ND-1000. After purification, 100 ng/μL of the labeled RNA was fragmented and hybridized onto the array (*in situ* hybridization kit, Agilent Technologies). Following hybridization, slides were scanned (Agilent Microarray Scanner, Agilent Technologies – Part Number G2600D) and raw data were extracted using Feature Extraction software. In accordance with the MIAME, data obtained were deposited in the GEO database (*http://www.ncbi.nlm.nih.gov/geo/info/linking.html*) with the accession number GSE99881. Normalization of the data, gene-expression analysis and statistical analysis were performed for the biological triplicate according to the manufacturer's guidelines (GeneSpring GX software from Agilent). From the complete data, differentially expressed genes (DEGs) were filtered and extracted using a volcano plot (threshold fold change 1, p-value ≤ 0.05). Functional categorization of the DEGs based on gene ontology (GO) was performed using a database for annotation, visualization and integrated discovery (DAVID) analysis tool (*http://david.abcc.ncifcrf.gov/*).

2.5 *Real-time PCR analysis*

For validation of microarray data by real-time PCR, seven DEGs related to secondary metabolism and defense response were selected for analysis. Total RNA isolated from the control and bacterized root samples were treated with DNase and reverse transcribed at 42°C for 50 min using Protoscript II reverse transcriptase and oligo(dT). Real-time PCR was performed with the synthesized cDNA (10-fold diluted) using a SYBR green reaction mix (Roche, CA, USA) and gene-specific primers (supplementary table 1). For normalization of target genes, ubiquitin (*OsUBQ5*) was used as the reference gene (Joseph *et al.* 2017). Relative quantification of the genes in the treated samples against the control was analyzed using Light Cycler 480 software (version 1.5.0.39). The experiment performed in technical and biological triplicates was statistically analyzed by ANOVA (Rekha *et al.* 2018).

2.6 *Analysis of defense responses in rice plants at different stages of RRF3 colonization using real-time PCR*

By using root transcriptome analysis, a number of SA-responsive genes were found to be induced by *P. putida* RRF3 (48 hpi). To gain an insight into the impact of RRF3 on the SA-responsive genes at later stages of root colonization, real-time PCR analysis was performed for SA biosynthesis genes and SA-inducible genes at 2nd, 4th and 10th day post-inoculation (dpi). Total RNA was isolated from RRF3-treated rice roots and real-time PCR analysis was performed in triplicate using gene-specific primers as described above.

2.7 *HPLC analysis for SA in root extracts and REs of RRF3-treated plants*

An earlier study in cucumber has reported an increase in SA levels before the onset of systemic resistance (Métraux *et al.* 1990). Hence, to investigate the impact of RRF3 on the endogenous concentrations of SA in rice roots and to corroborate with its corresponding gene expression (as analyzed by real-time PCR), HPLC analysis was performed for SA using the root extracts. Simultaneously, RRF3-induced alterations in the SA content of the REs were analyzed. Rice plants were treated with RRF3 in a hydroponic set-up with sterile deionized water (as described earlier in collection of REs for the chemotaxis assay). After 48 and 96 hpi, the REs (from 75 plants weighing ~ 1 g of fresh root weight) that are accumulated in the deionized water were collected, pooled and filter-sterilized using syringe filters (Rekha *et al.* 2018) for HPLC analysis.

The expression of the SA biosynthesis gene was found to be induced at 96 hpi, hence root samples of the biological triplicate were harvested 96 hpi. The root tissues of the triplicate were pooled together (4 g), cut into fine pieces, dried under shade and extracted twice with 95% ethanol. The samples were then concentrated (using a rotary vacuum evaporator, at 37°C) and resuspended in methanol for analysis. HPLC analysis for the root extracts and REs was performed in a Shimadzu system using a Phenomenex RP C18 column (250 × 4.6 mm, 5 μm, Phenomenex, CA, USA) with a mobile phase consisting of methanol and water (0.1% formic acid) (68:32, v/v). The flow rate and PDA detector were set at 0.8 mL/min and 280 nm, respectively. The presence of SA in the samples was identified by comparing their retention time with that of the standard (SA, HiMedia Laboratories, Mumbai, India). A further confirmation for the presence of SA in the sample was made by injecting a spike sample (standard mixed with the sample). The experiment in triplicate was performed thrice (for consistent results) and was statistically analyzed using ANOVA (SPSS software).

2.8 *SA test*

Based on the HPLC results, the SA test was performed using a spectroscopy method (Warrier *et al.* 2013) to test the capacity of RRF3 to utilize/breakdown SA. The RRF3 culture suspension was prepared with an OD_{600} of 0.4. One percent from this pre-inoculum was inoculated into culture tubes containing 10 mL of sterile nutrient broths supplemented with varying concentrations of SA (0, 0.05, 0.2, 1, 5 and 10 μg/mL). On 24 h incubation, 1 mL from each of the culture broths was centrifuged to obtain the cell-free supernatant. To 1 mL of the culture supernatant, 1 mL of ferric chloride reagent (1 g ferric chloride dissolved in 100 mL of 1% hydrochloric acid) was added and the volume was made up to 10 mL using sterile distilled water. In parallel with the samples, the reaction was set with the standard (SA, HiMedia Laboratories, with concentrations of 5, 10, 15, 20 and 25 μg/mL). After 10 min, the absorbance of the reaction mixtures (sample and standard) was measured at 525 nm. A standard graph was plotted using the absorbance values to estimate the concentration of SA in the samples. The procedure was repeated for the culture supernatants collected at 48 h.

SA being an antimicrobial, spread-plating of the SA-amended culture broths (sampled at 24 and 48 h incubation) was performed simultaneously (with appropriate dilutions) on the nutrient medium to estimate the viability of RRF3 in the presence of SA. After overnight incubation, the number of colonies was counted for the calculation of CFU. The experiments were performed in triplicate and statistical analysis for the same was performed by ANOVA using SPSS software.

3. Results

3.1 *P. putida RRF3 as a PGPR*

The rice rhizosphere isolate, RRF3 was found to possess the abilities to synthesize auxin, form biofilm, solubilize phosphate and produce siderophores. Auxin present in the 48 h

culture supernatant of RRF3 was estimated to be 2.67 μg/mL, while biofilm formed after 48 h had a cell density of 1.607 at OD_{600}. Similarly, RRF3 was able to solubilize 47.51 ± 0.50 μg/mL of phosphate over a 48 h incubation time. In addition, an assay performed using CAS agar medium showed orange coloration around the RRF3 culture indicating its ability to produce siderophores (supplementary figure 1). Molecular characterization of RRF3 by 16S rRNA and *gyrB* sequencing and further BLAST analysis revealed its identity as *P. putida*. The sequences obtained by 16S rRNA and *gyrB* sequencing were then deposited in the NCBI database under the accession nos. KU946985 and MF383338, respectively.

3.2 *Plant growth promotion by P. putida RRF3*

With a plant growth-promotion test, an increase in vegetative growth of rice plants was observed after 10 days of treatment with RRF3 (supplementary figure 2). The shoot length and root length were increased by 5.83 and 10.45%, respectively, as compared with the control. Statistical analysis performed for the replicates using ANOVA showed the plant growth promotion induced by RRF3 to be significant (p-value <0.05).

3.3 *Chemotaxis of RRF3 toward rice REs*

The chemotaxis test performed using the modified capillary method illustrated a positive chemotactic response of *P. putida* RRF3 toward rice REs. The number of bacterial cells (RRF3) attracted toward crude REs was found to be higher (3.86×10^5 cells/mL) than that of the control (sterile water, 7.4×10^4 cells/mL) and gave an RCR of 5.21, which indicates a significant chemotaxis according to Mazumder *et al.* (1999).

3.4 *Transcriptome analysis of rice roots in response to RRF3*

The influence of *P. putida* RRF3 on the root transcriptome of rice plants revealed 61 transcripts to be differentially regulated (with cut-off 1) at 48 hpi. Among the 61 DEGs, 52 transcripts were up-regulated and nine transcripts were down-regulated. Only those transcripts whose relative expression passed the threshold fold change and a p-value ≤ 0.05 among the replicates were taken as DEGs. Functional categorization of the differentially expressed transcripts based on GO using the DAVID tool revealed the following categories of genes to be enriched significantly. Among the up-regulated genes, all the three GO terms namely, 'cellular processes', 'biological process' and 'molecular function' were found to be significantly enriched (figure 1A–C), and the categories include: 'oxidation–reduction' (13.6%), 'response to stress' (11.3%), 'response to stimulus' (11.79%), 'defense response' (5.6%), 'response to oxidative stress' (3.3%), 'secondary metabolic process' (2.35%) and 'polysaccharide catabolic process' (1.88%). Among the down-regulated genes, the GO term 'biological process' was found to be highly enriched (figure 1D). The DEGs with predicted biological function as per GO analysis will be discussed in this paper and are listed in supplementary table 2.

The primary level of interaction between the plant and rhizobacteria begins with changes in root cell wall components. Analysis of root transcriptomic data revealed a significant up-regulation of transcripts encoding cell wall modification enzymes such as xyloglucan galactosyltransferase KATAMARI 1 and peroxidase (2.57- and 1.88-fold, respectively) by *P. putida* RRF3.

Analysis of defense response genes showed SA- and ET-mediated signaling mechanisms to be induced in rice roots by *P. putida* RRF3. There was an up-regulation of a number of transcripts encoding SA-responsive PR proteins such as the PR-1a (Hv-1a) precursor, PR1a protein, PR1c, PR10a, chitinase 8, chitinase 1 precursor and probenazole-inducible proteins (PBZ1) (fold changes range from 1.16 to 4). Transcripts encoding 1-aminocyclopropane-1-carboxylate synthase family protein and 1-aminocyclopropane-1-carboxylic acid oxidase of the ET biosynthesis pathway were also up-regulated significantly (3.39- and 2.22-fold, respectively).

It is demonstrated that colonization by PGPR enhances the biosynthesis of organic acids such as malic acid (Rekha *et al.* 2018). Here, it was observed that most of the transcripts encoding enzymes of the primary metabolism remained unaltered with a few exceptions such as isocitrate lyase (1.45-fold). Isocitrate lyase plays a role in the glyoxylate cycle catalyzing the conversion of isocitrate to glyoxylate and succinic acid. Transcripts of the secondary metabolite pathway such as those encoding sesquiterpene synthase of the terpenoid biosynthesis pathway and chalcone reductase of the flavonoid biosynthesis pathway were up-regulated significantly by 2.68- and 2.78-fold, respectively. Primary and secondary metabolites when exuded through the roots serve as a source of nutrition for the root-colonizing bacteria. Hence, the effect of RRF3 on the transcript encoding transporter proteins was analyzed. Except for a sugar transporter (sugar/inositol transporter domain-containing protein), none of the transcripts coding for transporter proteins were significantly regulated by RRF3 during its interaction with rice roots for 48 h (supplementary table 2).

3.5 *Validation by real-time PCR analysis*

To validate the microarray results, genes involved in defense response and secondary metabolite synthesis were chosen for real-time PCR analysis. The genes include chitinase 8

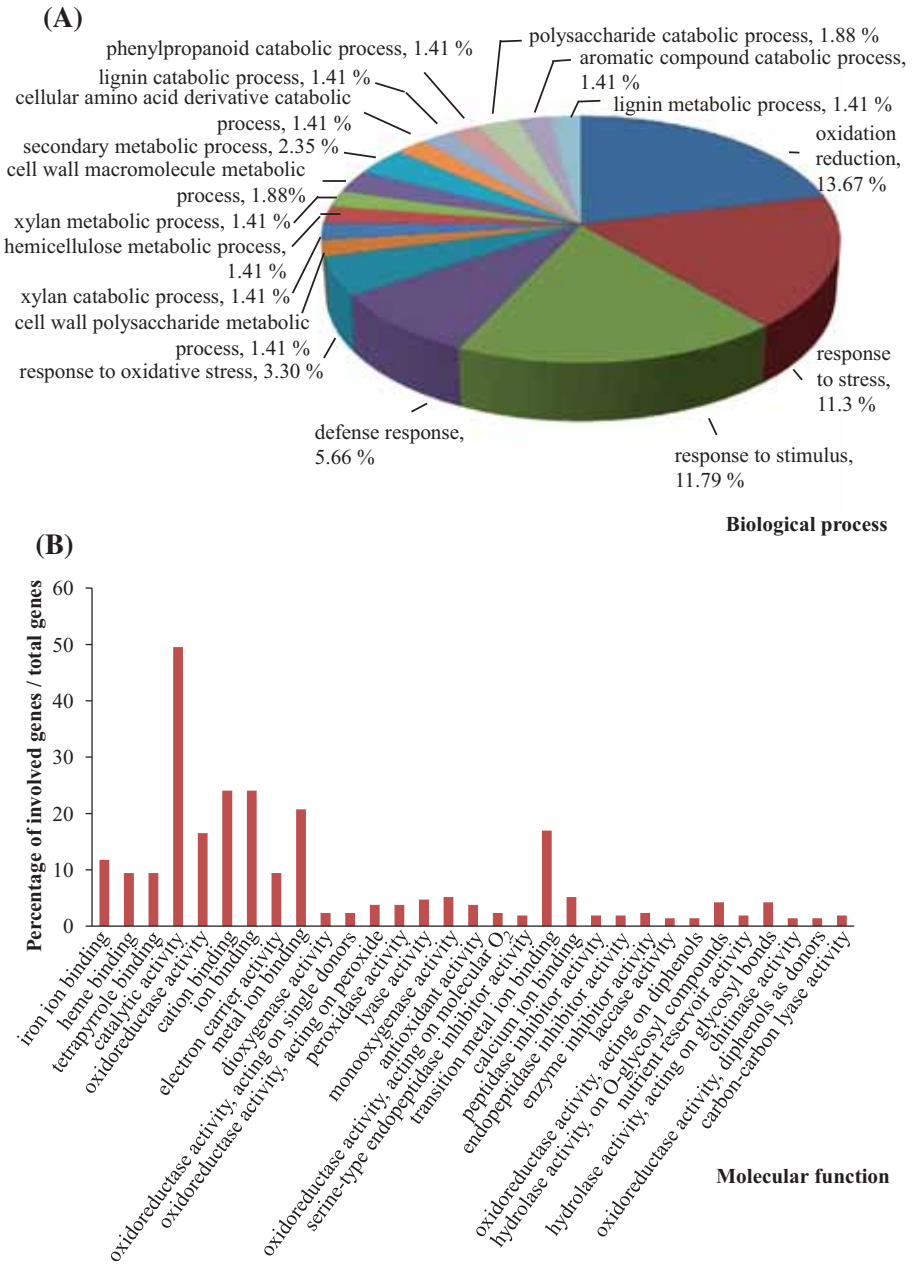

Figure 1. GO analysis of the differentially regulated genes using DAVID. The figures present the GO-based categorization of genes differentially regulated in rice roots by *P. putida* RRF3. (**A–C**) Gene categories enriched among up-regulated genes under the GO terms: 'biological processes', 'molecular function' and 'cellular function', respectively. (**D**) Gene categories enriched among the down-regulated genes. Fisher's exact test was used to analyze the statistical significance of the data (GO terms with a cut-off fold change 1 and *p*-value ≤ 0.05 were considered for analysis).

(*OsCHIA8*), probenazole-inducible protein (*OsPBZ1*), peroxidase (*OsPOX8.1*), lipoxygenase (*OsLOX8*), PR-1a PR protein (Hv-1a) precursor (*OsPR101a*), chalcone reductase (*OsCHR*) and 1-aminocyclopropane-1-carboxylate synthase family protein (*OsACS5*). A comparative analysis of real-time PCR data with that of the microarray data revealed a similar pattern of expression among the genes analyzed (figure 2). Analysis of variance for triplicate showed the data to be significant (*p*-value <0.05).

3.6 *Impact of RRF3 on the defense response of rice plants at different stages of colonization*

Based on the clues obtained by a root transcriptome study for 48 h interaction between rice and *P. putida* RRF3, the relative expression of genes related to SA signaling was analyzed by real-time PCR to gain an insight into the plant defense responses at different stages of colonization (2nd, 4th and 10th dpi). The genes include *OsCHIA8*, *OsPR101a*, *OsPBZ1* and

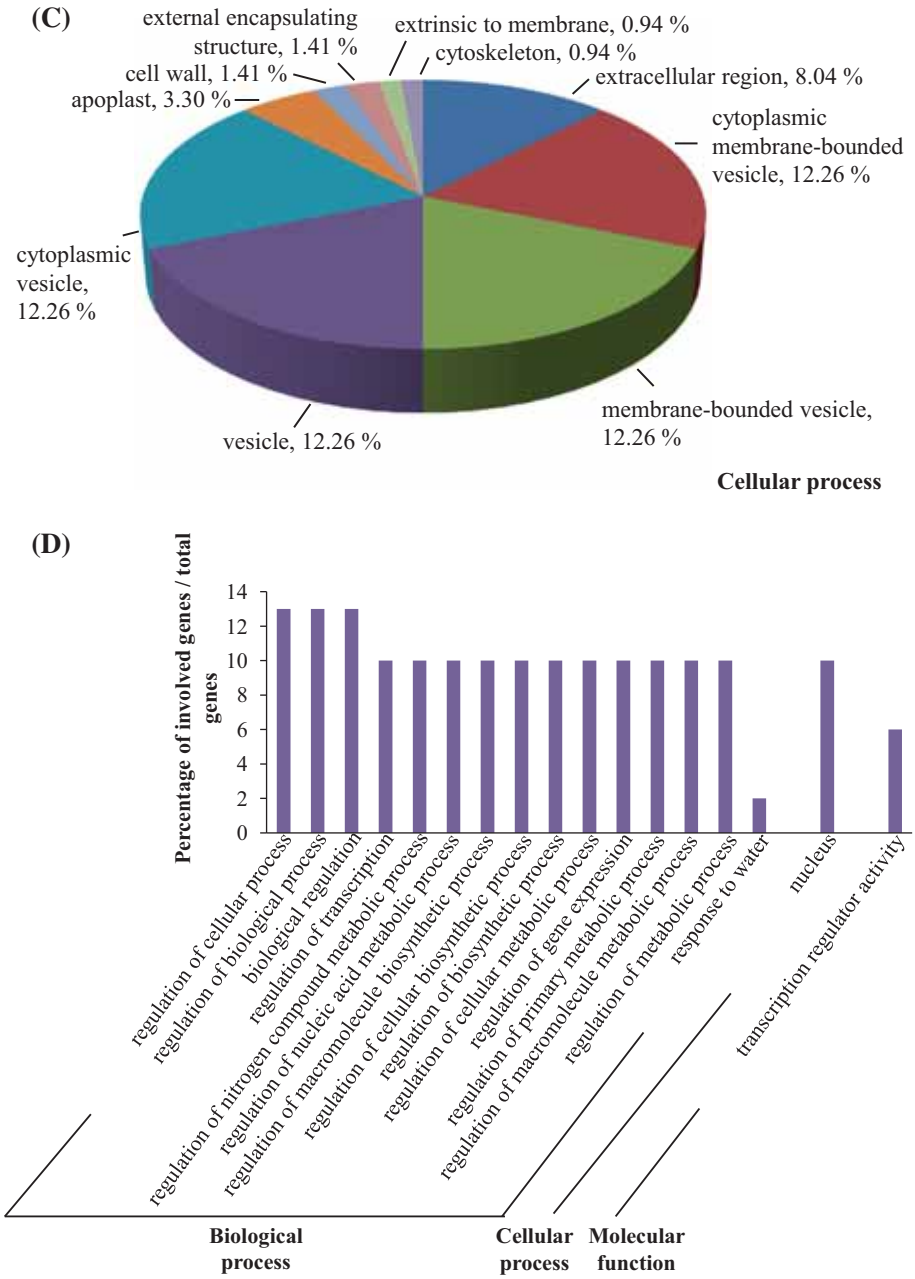

Figure 1. continued

OsPAL (phenylalanine ammonia lyase). *OsPAL* is a major SA-biosynthetic gene, while *OsCHIA8*, *OsPR101a* and *OsPBZ1* are SA-inducible genes (Silverman *et al.* 1995). Real-time PCR analysis showed all the above genes to be up-regulated significantly (p-value <0.05) right from the early stages of RRF3 colonization and decreased gradually (figure 3).

3.7 *Influence of RRF3 on root biosynthesis and root exudation of SA*

HPLC analysis performed using the root extracts demonstrated a significant increase in accumulation of SA in *P. putida* RRF3-treated roots (96 hpi) (8 ± 0.6 µg/g fresh weight of roots) as compared with the control (undetected) (p-value <0.05) (figure 4). A spike (sample + standard) was injected for further confirmation of the peak for SA. This corroborates with the expression of *OsPAL* at 96 hpi. Bacterial cells possess the mechanism to synthesize and secrete SA to the external medium (De Meyer *et al.* 1999); hence for the analysis of REs, the data obtained for RRF3-treated plants were normalized with the plant and the bacterial controls. On normalization, the content of SA was found to be much less in RRF3-treated plant REs at both the time periods tested (−0.58- and −1.86-fold at 48 and 96 hpi, respectively) than the control (figures 5 and 6).

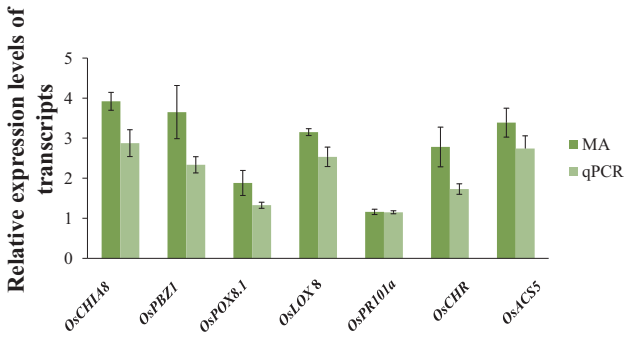

Figure 2. Comparison of the microarray and real-time PCR data for the selected genes. The graph shows the relative expression levels of transcripts of *P. putida* RRF3-treated rice roots (at 48 hpi) from microarray and real-time PCR data. Fold change ratios of treated *vs* control are given as relative expression levels for the genes. MA – microarray; qPCR – quantitative (real-time) PCR. Data are represented as mean ± standard deviation (SD) of the biological and technical triplicates. Analysis of variance among the biological triplicate using SPSS software showed the data to be significant (p-value ≤0.05).

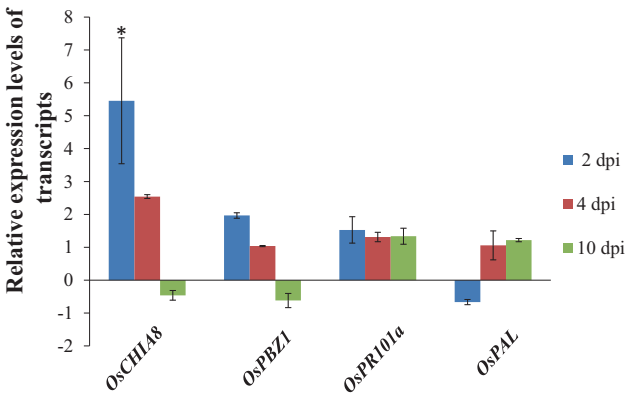

Figure 3. Real-time PCR analysis for relative quantification of SA-related genes. The graph illustrates the relative expression of the SA-biosynthetic and SA-responsive genes in *P. putida* RRF3-treated rice roots at 2nd, 4th and 10th dpi. Fold change ratios of treated *vs* control are given as relative expression levels for the genes. Data are represented as mean ± SD of the biological triplicate. Statistical analysis for the triplicate by ANOVA showed the gene-expression changes in treated samples to be significant (p-value ≤0.05). dpi – days post-inoculation. **OsCHIA8* showed a deviation in fold-change values among the replicates.

3.8 *Effect of SA on the growth and metabolism of RRF3*

Pseudomonas sp. is well known to possess the exclusive trait of feeding on aromatic compounds (De Meyer *et al.* 1999). To investigate if the decrease in the SA (an aromatic acid) content in the REs of RRF3-treated plants is due to breakdown by RRF3, the SA test was performed by spectroscopy. Interestingly, the content of SA was increased in the culture tubes amended with low-SA concentrations (up to 0.5 µg/mL), whereas, at higher concentrations (5 and 10 µg/mL), the contents of SA were decreased drastically from the amended concentrations (p-value <0.05) (figure 7). This indicates that low-SA concentrations enhance the metabolism and proliferation of bacteria, which in turn leads to the bacterial secretion of SA to the external medium and thus could be the probable reason for the increased SA content in the culture broth. While higher concentrations of SA seem to deter the bacterial growth and thus could have caused its rapid breakdown (97–99%) in the culture broths with higher SA concentrations. Corroborating with these results, the CFU of RRF3 showed a gradual increase up to 1 µg/mL SA and decreased thereafter (at 5 and 10 µg/mL of SA) (supplementary table 3).

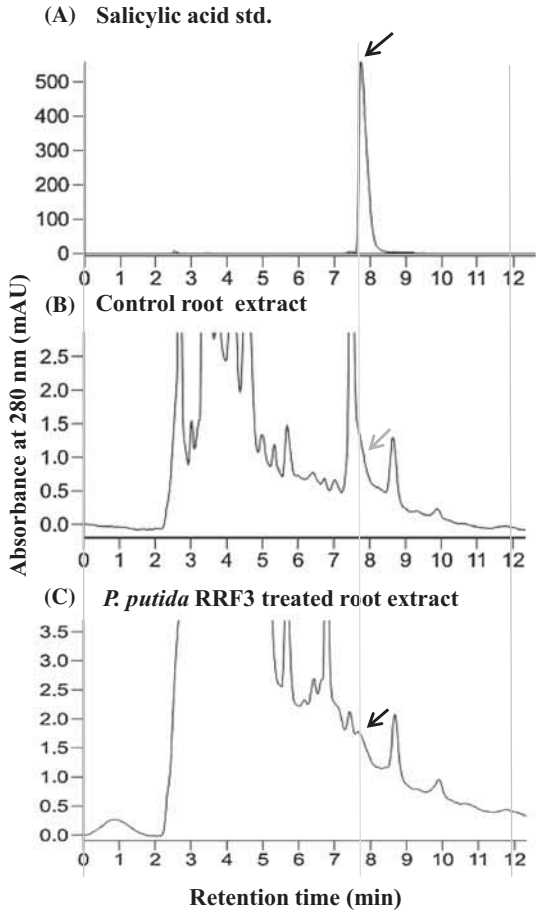

Figure 4. HPLC analysis of *P. putida* RRF3 induced alterations in the levels of total SA in rice roots. Chromatograms illustrate the presence of SA in the root tissues sampled at 96 hpi. (**A**) Standard (SA); (**B**) root extract of control plants and (**C**) root extract of RRF3-treated plants. Black arrows indicate the presence of peak for SA, while a gray arrow indicates its absence. Gray lines are inserted for easier comparison and to obtain a better view of the SA peaks (retention time - 7.6 min). ANOVA analysis showed the data to be significant (p-value <0.05, n = 3).

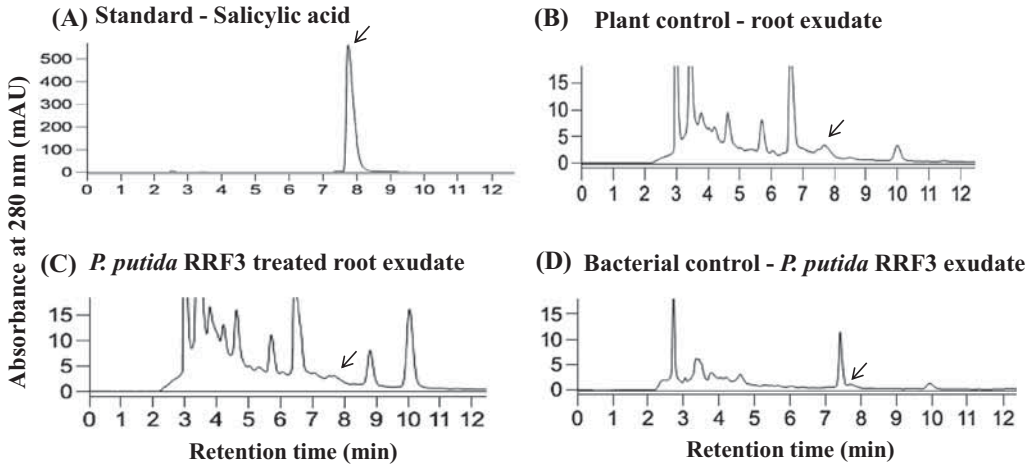

Figure 5. HPLC analysis of RRF3-induced changes in the levels of SA in rice REs sampled at 48 hpi. Chromatograms illustrate the levels of SA in the treated samples (**C**) in comparison with that of the standard (**A**) and the controls (**B** and **D**). The presence of peak for SA is indicated by arrows.

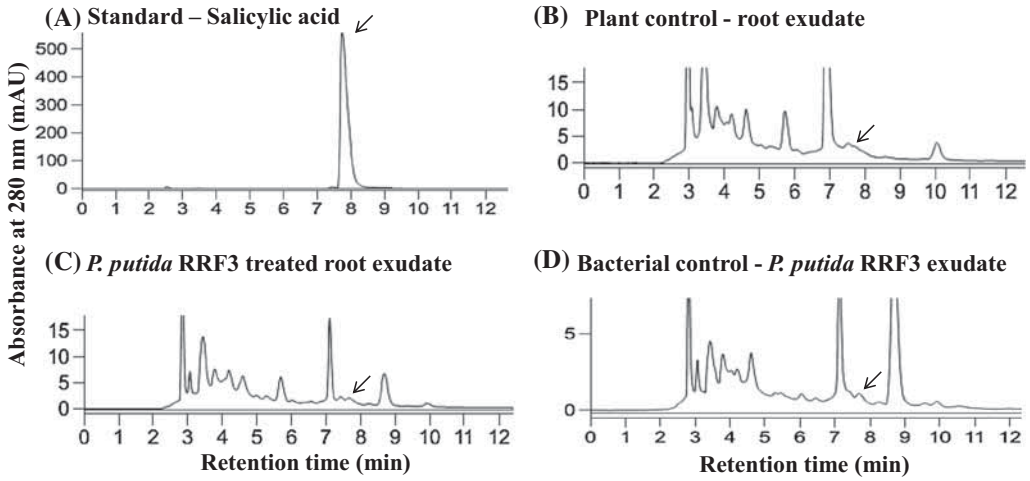

Figure 6. HPLC analysis of RRF3-induced changes in the levels of SA in rice REs sampled at 96 hpi. Chromatograms illustrate the levels of SA in the treated samples (**C**) in comparison with that of the standard (**A**) and the controls (**B** and **D**). The presence of peak for SA is indicated by arrows.

4. Discussion

Plants encounter several biotic and abiotic stresses in their native habitat, due to which productivity of crop plants such as rice has drastically declined (40%) (Oerke and Dehne 2004). Research in the past on plant–PGPR interactions has mainly focused on the PGPR-induced alterations in the plant phenotype (Ali *et al.* 2011). The aim of this study is to analyze the effect of the PGPR, *P. putida* RRF3 on the alterations in the molecular mechanisms in rice roots, particularly, those involved in cell wall modification, defense response, metabolism and root exudation are of interest.

4.1 *P. putida RRF3 – a plant growth promoter*

By using PGPR characterization tests, RRF3 was found to possess the abilities to produce auxin and siderophores, to form biofilms and to solubilize phosphate. The plant growth-promotion test performed under greenhouse conditions showed the positive effect of RRF3 on the enhancement of the growth of rice plants. The results were consistent with previous reports demonstrating the PGPR traits of rhizobacteria (Dowling and O'Gara 1994; Islam *et al.* 2007; Ali *et al.* 2011; Qurashi and Sabri 2012; Ravari and Heidarzadeh 2013). The PGPR traits exhibited by RRF3 were on par with the reported rice PGPR (Thakuria *et al.* 2004; Cui *et al.* 2011). As similar

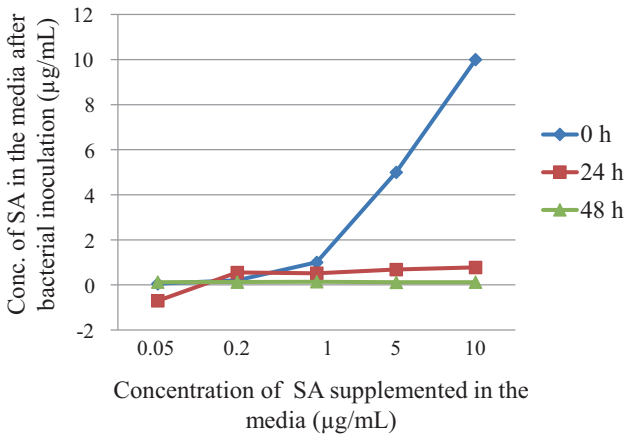

Figure 7. Illustration of the impact of RRF3 on externally supplied SA. The figure shows the impact of RRF3 on the levels of SA (at 24 and 48 h) in SA-amended nutrient broths. Data are mean ± SD. SD – 0.005 to 0.02 µg/mL; *p*-value <0.05, $n = 3$ (ANOVA).

protocols were used in our study, the data reported for PGPR traits in the above studies were taken as a reference in place of the reported positive and negative controls (strains). In addition, RRF3 exhibited positive chemotaxis toward REs of rice plants. A similar result (RCR of 6.15) was obtained with a rice rhizosphere isolate *B. subtilis* RR4 toward a specific compound, malic acid (a major component of REs) (Rekha *et al.* 2018). However, as expected, the RCR obtained for the crude exudate (5.21) in this study was much less than that observed for the specific compound (10 mM malic acid). Phytochemical secretions from roots were reported to serve as chemoattractants to rhizobacteria (Chet *et al.* 1973). An earlier study has reported that the bacterial count of *Pseudomonas* sp. G62 increased in hydroponic medium in the presence of Arabidopsis plants (Schwachtje *et al.* 2011). Positive chemotaxis of RRF3 toward rice REs suggests that it can proliferate and establish colonies on the roots of rice plants.

4.2 *P. putida RRF3 alters the root transcriptome of rice*

Microarray analysis performed to analyze the impact of colonization of *P. putida* RRF3 on the transcriptome of rice roots revealed a differential regulation of 61 transcripts constituting about 0.16% of the transcripts included in the array. Real-time PCR analysis was performed to confirm the validity of the microarray data as shown by a similar pattern of fold change values as observed using the microarray experiment.

4.2.1 *P. putida RRF3 stimulates defense response mechanisms in rice:* Analysis of the transcriptomic data showed that RRF3 activates multiple modes of defense response mechanisms (ET- and SA-mediated signaling) in rice plants as is evident by the enhanced expression of PR proteins and ET biosynthesis genes. Seven transcripts related to SA signaling were induced by RRF3, which suggests that the SA-mediated defense response is predominantly triggered in rice plants by *P. putida* RRF3. As discussed earlier, the plant defense response takes place by JA- and ET-mediated signaling or by SA-mediated signaling. Gene-expression studies on Arabidopsis plants colonized by *P. fluorescens* GM30 demonstrated an activation of abscisic acid (ABA) and ET-dependent pathways (Weston *et al.* 2012), whereas, with *P. fluorescens* SS101, activation of the SA-dependent pathway was observed (Van de Mortel *et al.* 2012). On the other hand, a study on Arabidopsis–*P. fluorescens* FPT9601-T5 interaction showed a down-regulation of ET-responsive genes (Wang *et al.* 2005). At times, multiple defense signaling mechanisms are reported to take part in response to interaction with PGPR (Mur *et al.* 2006). The results obtained in this study are consistent with the above reports and also suggest that SA-mediated signaling is not restricted to pathogen infection or wounding or herbivory. However, studies with different strains such as *P. fluorescens* WCS417r and *P. putida* WCS358r on Arabidopsis demonstrated the defense response to be independent of SA and PR proteins in Arabidopsis and radish, respectively (Hoffland *et al.* 1995). From the above reports, it is observed that, although the interaction involved species of the same genera, a strain-dependent defense response is exhibited by Arabidopsis plants. In another study, JA- and ABA-related genes were shown to be up-regulated by *P. putida* KT2440 in maize plants (Planchamp *et al.* 2015). This suggests that activation of the plant defense response is a strain-specific and a plant-specific mechanism and requires further studies for a deeper understanding.

4.2.2 *P. putida RRF3 induces differentiation of root cell wall components:* In addition to stimulation of defense responses, RRF3 induces re-organization of cell wall components as is evident from the expression of transcripts encoding xyloglucan galactosyltransferase KATAMARI 1 and peroxidase. Xyloglucan galactosyltransferase plays a role in differentiation of cell walls; and peroxidase plays a role in lignification and defense responses. Induction of lignin-forming peroxidases was also observed in a similar study on olive–*P. fluorescens* PICF7 interaction (Schilirò *et al.* 2012). Induction of cell wall components and lignification by PGPR are reported to be parts of the plant defense mechanisms (Pieterse *et al.* 2014), which suggests that RRF3 has a significant effect on plant defense responses.

4.2.3 *RRF3 enhances secondary metabolism in rice roots:* Flavonoids released by plants act as signaling molecules and, activate nodulation genes in the symbiotic bacteria (Peters *et al.* 1986). Here, chalcone reductase, an important gene of the flavonoid biosynthesis pathway was found to be up-regulated by RRF3. This is consistent with the previous studies, which have reported an induction of flavonoid biosynthesis in plants in the presence of *Pseudomonas* sp. (García-Seco *et al.*

2013; Ramos-Solano *et al.* 2014). Moreover, early interaction studies with *Medicago truncatula* and soybean with the mycorrhizal fungi *Glomus intraradices* and *Bradyrhizobium japonicum*, respectively, demonstrated an induction of a similar flavonoid-biosynthetic gene, chalcone synthase (Estabrook and Sengupta-Gopalan 1991; Bonanomi *et al.* 2001). This suggests that PGPR enhance the flavonoid content in the plant roots to facilitate their colonization onto plant roots. Besides flavonoids, a terpenoid biosynthesis gene (sesquiterpene synthase) was found to be up-regulated by RRF3. These genes are known to play important roles in stress and defense responses (Taniguchi *et al.* 2014), which suggests that RRF3 is capable of enhancing the plants' sustainability under stressed environmental conditions.

4.3 *P. putida RRF3 induces priming in rice plants*

From the root transcriptome study, SA-responsive genes were observed to be majorly induced by RRF3 (48 hpi). Real-time PCR analysis performed for the SA-biosynthetic and SA-responsive genes (*OsPAL*, *OsCHIA8*, *OsPR101a* and *OsPBZ1*) at later stages of colonization showed an induction of the above genes in response to RRF3, which suggest that RRF3 maintains the plant immune response to remain in a state of activation. The induction of *OsPAL* and further root accumulation of SA in RRF3-treated roots observed in this study is consistent with previous reports on bean, cucumber and cabbage (Métraux *et al.* 1990; De Meyer *et al.* 1999; Lin *et al.* 2017) and suggests that RRF3 induces systemic resistance in rice plants by enhancing the levels of endogenous SA.

Accumulation of SA in the cells is reported to activate the TF NPR1 (non-pathogenesis-related protein), which then undergoes a conformational change and co-activate defense response genes such as PR1 (Seyfferth and Tsuda 2014). However, by using this root-transcriptome study (48 h interaction of RRF3 with rice roots), SA-responsive genes (*OsPR1* and *OsPBZ1*) were activated, while the corresponding transcripts for the biosynthetic gene (*OsPAL*) and the TF (*OsNPR1*) were not significantly altered. This suggests that TFs other than NPR1 are involved in the activation of SA-responsive genes (PR1 and PBZ). A previous study has shown the role played by bacterial-secreted metabolites (such as SA-derived siderophores) and bacterial cell wall components in stimulating plant defense responses (Meyer *et al.* 1992). Another study had illustrated that the colonization patterns of *Pseudomonas* spp. are similar to those phytopathogens to which they possess an antagonistic effect (Lugtenberg *et al.* 1999). Activation of SA-responsive genes in the absence of *OsPAL* induction, as observed by this transcriptome study, could be attributed to the colonization patterns and secretion of siderophores by RRF3. Comparative analysis of cell wall components and secretions of different PGPR with that of the phytopathogens would provide a deeper understanding of the defense response mechanisms in plant–PGPR interactions.

4.4 *P. putida RRF3 alters the SA content in the REs of rice plants*

In contrast to root extracts, RRF3 showed a negative impact on root exudation of SA. The SA test performed to analyze the possible role of SA in PGPR growth and metabolism showed that low- and high-SA concentrations promote and inhibit the growth of RRF3, respectively. This suggests that root-exuded SA (from rice plants) and bacterial-secreted SA together would have increased the SA concentrations that had eventually led to its breakdown, and decrease in the exudates of RRF3-treated plants as compared with the control. This is in accordance with the previous studies, which have reported an induction of bacterial genes associated with the aromatic compound catabolism and energy generation in response to REs (Lugtenberg and Dekkers 1999; Mark *et al.* 2005). Moreover, it is reported that SA and JA are antagonists to each other and thus the concentrations of SA and JA are always kept under control (Pieterse *et al.* 2012; Derksen *et al.* 2013). Simultaneous activation of multiple defense signaling pathways (SA-, JA- and ET-mediated) by RRF3 observed in this study could be another possibility for the limited exudation of SA from RRF3-treated roots. A study by Meharg and Killham (1995) has illustrated the role played by the bacterial-secreted metabolites in the regulation of root exudation of compounds from plants. Hence as described above, studies on bacterial secretions (of different PGPR) would provide a conclusive understanding of the effect of PGPR in root exudation of compounds.

5. Conclusions

Overall, using our transcriptome study on rice–*P. putida* RRF3 interaction we have reported the effect of RRF3 on the transcriptional regulation of primary metabolism, secondary metabolism and root exudation of metabolites/phytochemicals; in particular, genes regulating defense/stress-related metabolites are discussed. Activation of multiple defense signaling mechanisms in rice plants by RRF3 highlights the potential of RRF3 as an efficient biocontrol agent. Further, the impact of RRF3 on the regulation of plant biosynthesis, signaling and root exudation of SA is demonstrated. With these data together, we have illustrated how PGPR influence plants' defense mechanisms and confer the plant with immunity to protect itself against a future attack by phytopathogens. Moreover, the negative impact of RRF3 on root exudation of SA demonstrates how RRF3, in the inverse communication, protects itself (being a foreign organism) from its priming effect on plants.

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