ORIGINAL ARTICLE

Herbaspirillum seropedicae strain HRC54 expression profile in response to sugarcane apoplastic fluid

Daniella Duarte Villarinho Pessoa[1,2] · Carlos Magno Dos-Santos[2] · Marcia Soares Vidal[2] · José Ivo Baldani[2] · Michelle Zibetti Tadra-Sfeir[3] · Emanuel Maltempi de Souza[3] · Jean Luis Simoes-Araujo[2]

Abstract

Bacterial transcriptome profiling in the presence of plant fluids or extracts during microbial growth may provide relevant information on plant–bacteria interactions. Here, RNA sequencing (RNA-Seq) was used to determine the transcriptomic profile of *Herbaspirillum seropedicae* strain HRC54 at the early stages of response to sugarcane apoplastic fluid. Differentially expressed gene (DEG) analysis was performed using the DESeq2 and edgeR packages, followed by functional annotation using Blast2GO and gene ontology enrichment analysis using the COG and KEGG databases. After 2 h of sugarcane apoplastic fluid addition to the *H. seropedicae* HRC54 culture, respectively, 44 and 45 genes were upregulated and downregulated. These genes were enriched in bacterial metabolism (e.g., oxidoreductase and transferase), ABC transporters, motility, secretion systems, and signal transduction. RNA-Seq expression profiles of 12 genes identified in data analyses were verified by RT-qPCR. The results suggested that *H. seropedicae* HRC54 recognized sugarcane apoplastic fluid as the host signal, and some DEGs were closely involved at the early stages of the establishment of plant–bacteria interactions.

Introduction

Sugarcane is an important crop in Brazilian agribusiness because of the high value of its products in various industries, including bioenergy production (Bordonal et al. 2018). Over the last decades, sugarcane yield has decreased slightly but significantly, which is typically attributed to low soil fertility and climate change (Pereira et al. 2013; CONAB de CNA 2020). In this light, need for the application of fertilizers, particularly nitrogen, has increased (Schultz et al. 2015), which can be harmful to the environment and increase agricultural costs (Gomes et al. 2008). An alternative to reduce fertilizer application is the use of inoculants with diazotrophic endophytic bacteria (DEB). DEB can convert N_2 to NH_3 through biological nitrogen fixation (BNF) and can act as plant growth-promoting bacteria (PGPB) (Baldani and Baldani 2005; Oliveira et al. 2006; de Souza et al. 2015; da Fonseca Breda et al. 2019; dos Santos et al. 2020).

Previous studies have shown the positive effects of DEB inoculation on plant growth via BNF, hormone production, defense response, nutrient acquisition, and abiotic and biotic stress tolerance (Oliveira et al. 2002, 2006, 2009; de Souza et al. 2015; Kandel et al. 2017). In sugarcane, the positive effects of inoculating five DEB strains, including *Gluconacetobacter diazotrophicus* PAL5[T] (BR11281), *Herbaspirillum rubrisubalbicans* HCC103 (BR11504), *H. seropedicae* HRC54 (BR11335), *Nitrospirillum* (formerly *Azospirillum*) *amazonense* CBAmC (BR11145), and *Paraburkholderia*

✉ Jean Luis Simoes-Araujo
jean.araujo@embrapa.br

Marcia Soares Vidal
marcia.vidal@embrapa.br

José Ivo Baldani
ivo.baldani@embrapa.br

Emanuel Maltempi de Souza
souzaem@ufpr.br

[1] Programa de Pós-Graduação em Biotecnologia Vegetal, Centro de Ciências da Saúde, UFRJ, Rio de Janeiro, RJ 21941-590, Brazil

[2] Laboratório de Genética e Bioquímica, Embrapa Agrobiologia, Seropédica, RJ 23890-000, Brazil

[3] Departamento de Bioquímica e Biologia Molecular, Centro Politécnico, UFPR, Curitiba, PR 80060-000, Brazil

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(formerly *Burkholderia*) *tropica* PPe8^T (BR11366), have been previously reported (Oliveira et al. 2006; Reis et al. 2009; Renan et al. 2016; dos Santos et al. 2020; Martins et al. 2020; Ramos et al. 2020). However, the beneficial effects attributed to inoculation are highly variable, and their underlying mechanisms remain unclear. Therefore, additional information on the fundamental aspects of plant–microorganism interaction is imperative (Monteiro et al. 2008).

H. seropedicae is a diazotrophic endophytic β-proteobacteria, isolated from crops such as rice, maize, and sugarcane (Baldani et al. 1986; Olivares et al. 1996), and it has also been used as a sugarcane inoculant. This bacterium has been shown to increase plant biomass (Guimarães et al. 2003) through acting on phytohormone signaling (Amaral et al. 2014; Tadra-Sfeir et al. 2015), plant defense responses (Brusamarello-Santos et al. 2012), nitrogen metabolism (Breda et al. 2018), siderophore production, and polyhydroxybutyrate synthesis (Tirapelle et al. 2013; Brader et al. 2014). Furthermore, diverse protein secretion systems help bacteria to successfully interact with plants (Monteiro et al. 2008; Pedrosa et al. 2011). *H. seropedicae* strain HRC54 is a diazotrophic entophyte isolated from sugarcane roots (Baldani et al. 1996), with potential to serve as a PGPB, particularly for gramineuos plants (Junior et al. 2008; Alves et al. 2014; Martins et al. 2020). However, no study has reported the molecular overview of the interaction between HRC54 and sugarcane.

In 2011, the complete genome of *H. seropedicae* strain SmR1 was compiled, which enabled the identification of genes involved in several pathways such as auxin biosynthesis, BNF, siderophore production, protein secretion, and plant–bacteria interactions (e.g., chemotaxis proteins, secretion systems, and flagellar biosynthesis) (Pedrosa et al. 2011). These genes allow the bacteria to recognize plant signals and modulate plant gene expression for endophytic colonization and plant growth promotion (Monteiro et al. 2012). However, the molecular pathways involved at the initial stages of the bacterial endophytic lifestyle have not been well studied.

The aboveground parts of host plants, specifically intercellular spaces, are frequently occupied by endophytic bacteria, and the apoplastic space is a niche for bacterial development, providing nutrients such as sugar, ammonium, nitrite, nitrate, amino acids, and proteins, which are essential for bacterial growth (Asis et al. 2003; Haslam et al. 2003; Tejera et al. 2006; Sattelmacher and Horst 2007; Pechanova et al. 2010). An in vitro system to grow microorganisms in the presence of specific plant fluids or tissues can mimic the endophytic environment and help study the initial stages of plant–microbe interactions and provide information on the expression profiles of genes involved in these processes (Dong et al. 1994). Therefore, the exposure of a bacterial culture to apoplastic fluid during a certain period can provide insight into the signaling cascades that activate the bacterial genes necessary for metabolic adaptations to live within the plant.

Transcriptomics can be applied in such experiments, allowing for the evaluation of microbial gene expression profiles under different scenarios, such as abiotic stress, or in specific pathways and interactions (Vacheron et al. 2013; Chandra et al. 2019; Raju et al. 2020). However, in most plant-associated bacterial transcriptomic studies conducted thus far, bacteria were cultured separately from the host plant to obtain sufficient bacterial mRNA transcripts (Levy et al. 2018). We previously used sugarcane apoplastic fluid in in vitro experiments on *P. tropica* PPe8^T and *N. amazonense* CBAmC and found this approach to be appropriate for evaluating bacterial responses to plant signals (Silva et al. 2018; Terra et al. 2020). The short exposure time to apoplastic fluid set in those experiments allowed us to elucidate initial gene expression changes and their importance at the early stages of plant–bacteria interactions (Pinski et al. 2019; Taulé et al. 2021). To this end, in the present study, we sought to identify the differentially expressed genes (DEGs) of *H. seropedicae* HRC54 in response to the short-term exposure of its culture to sugarcane (RB867515 variety) apoplastic fluid.

Materials and methods

Sugarcane apoplastic fluid collection

The sugarcane variety RB867515 was sampled from the Embrapa Agrobiologia Experimental Field Station. This commercial variety is characterized by its ability to adapt to low-fertility soils, and it is responsive to PGPB inoculation (Schultz et al. 2014; dos Santos et al. 2020). After superficial washing, the stems were peeled, and the internodes were removed and disinfected by flaming (Silva et al. 2018). Sugarcane apoplastic fluid was obtained by centrifuging the stems in 50 mL tubes at $3,000 \times g$ for 20 min at 4 °C (Dong et al. 1994). The extracted apoplastic fluid was passed through a 0.22 μm filter (diameter, 47 mm) and stored at -70 °C until use.

H. seropedicae HRC54 growth in the presence of sugarcane apoplastic fluid

A pre-inoculum of *H. seropedicae* HRC54 was grown in 5 mL of DYGS medium (Baldani et al. 2014) for 12 h at 30 °C while shaking at 100 rpm. Next, the bacteria (10^5 cells mL^{-1}) were inoculated in 100 mL of semi-selective JNFB liquid medium containing nitrogen (Baldani et al. 2014). After 16 h of growth under the same conditions mentioned

above, the culture was divided in half and subjected to the following treatment: addition of sugarcane apoplastic fluid or water to the bacterial culture (at 1:1 proportion). The experiment was performed with three biological replicates for each treatment, resulting in six samples. The treatment lasted for 2 h at 30 °C, while shaking at 100 rpm. From each replicate of both treatments, 5 mL of the culture was collected and centrifuged at 2300×*g* for 10 min (Cordeiro et al. 2013), and the residual medium was discarded.

Cellular viability of *H. seropedicae* HRC54 in the presence of apoplastic fluid

To evaluate the effects of sugarcane apoplastic fluid on *H. seropedicae* HRC54, a *LIVE/DEAD* cell viability assay was performed. At the time of the start of the treatment (T0) and after 2 h (T2), samples (1 mL) were collected and centrifuged at 10,000×*g* for 10 min; the pellet was resuspended in 1 mL of 0.85% saline solution (NaCl, w/v). Then, 1.5 μL each of reagents A and B from the LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen™) was added, and the samples were incubated in the dark for 15 min. An aliquot of 10 μL of the incubated sample was used for microscopy using LSM700 AxioObserver (Zeiss) equipped with the Plan-Apochromat 40x/1.3 Oil DIC M27 and 63x/1.4 Oil DIC M27 objective lenses. For SYTO 9 staining, the excitation wavelength was 555 nm and detection wavelength was 578–800 nm (green fluorescence, live cells). For propidium iodide staining, the excitation wavelength was 488 nm and detection wavelength was 300–578 nm (red fluorescence, dead cells). Transmitted light was used with a T-PMT detector. To confirm the results of microscopy, bacterial colony-forming units were counted (CFU·mL^{-1}) using selective media for *Herbaspirillum* sp. JNFb (Baldani et al. 2014). Briefly, 1 mL of sample from each biological replicate was centrifuged at 10,000×*g* for 10 min, and the resulting pellet was resuspended in 1 mL of 0.9% saline solution (NaCl, w/v). The suspensions were serially diluted to a density of 10^{-10} cells mL^{-1}; then, 10 μL of the diluted suspension was spread on JNFb solid medium containing a nitrogen source and cultured for 24 h at 30 °C. Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). The results were compared using Student's *t* test, considering 0.05 as the level of significance (*p*).

Total RNA extraction, mRNA enrichment, and RNA Sequencing (RNA-Seq)

Total RNA was extracted using the TRIZOL™ reagent (Invitrogen), followed by DNase I (Epicenter) treatment to completely remove genomic DNA. Both procedures were performed according to the manufacturer's protocols. mRNA enrichment was performed using the MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion), following the manufacturer's instructions. Six cDNA libraries were constructed using the Ion PITM Sequencing 200 Kit and sequenced using an ion proton semiconductor sequencer (Life).

Transcriptomic data analysis

The quality of raw reads generated from RNA-Seq was analyzed using FastQC (Andrews 2010). The reads were trimmed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit) and mapped to the genome of *H. seropedicae* HRC54 (unpublished). Read mapping data were used to calculate gene expression levels as reads per kilobase of transcript per million mapped reads (RPKM) using EDGE-pro (Magoc et al. 2013). Only reads aligned at least once with the reference genome of *H. seropedicae* HRC54 were used for the subsequent analyses. The list of DEGs with associated fold changes and significance estimates was generated using the R packages DESeq2 (Love et al. 2014) and edgeR (Robinson et al. 2010), with a cut-off p value of 0.05 and a log2 fold change of 1.5 (fold change of ≤ 1.5 indicated down-regulated genes, and fold change of ≥ 1.5 indicates upregulated genes). The results from both packages were used for DEGS analyses to ensure the reliability of our final data. In the edgeR package, the false discovery rate (FDR) was analyzed at a cut-off of ≤ 0.05. The fold change was calculated as the difference between the strain + apoplast (apoplast) and strain + water (water) treatments. Only genes that were differentially expressed in both packages were selected for functional annotation with Blast2GO (Conesa et al. 2005) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups (COG) databases (Kanehisa and Goto 2000; Tatusov et al. 2000).

RT-qPCR

To validate the results of RNA-Seq analysis, 12 DEGs were randomly selected for RT-qPCR. Primers were designed using Primer3Plus (Untergasser et al. 2007) according to the following criteria: sequence length of 19–22 nucleotides; annealing temperature of 58–62 °C; GC content of 50–80%; and amplicon size of 100–180 base pairs. The primers designed were also checked for dimer and hairpin formation using Oligo Explorer (http://www.uku.fi/~kuulasma/OligoSoftware/) (Table S2). For this assay, a new experiment was designed using the same conditions as above and the same total RNA extraction and DNAse I treatment protocols, followed by cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen). RT-qPCR was performed using the 7500 Fast Real-Time PCR System and QuantiTect SYBR® Green PCR (Qiagen).

The raw fluorescence data were converted to cycle threshold (Ct) using Miner (Zhao and Fernald 2005), and relative expression was calculated using the delta–delta Ct method ($2^{-\Delta\Delta CT}$) and qBase v1.3.5 (Livak and Schmittgen 2001; Hellemans et al. 2007). *rpoA* and *groEL* were used as the reference genes (Pessoa et al. 2016). Statistical analyses were performed as described above (see section *Cellular viability of H. seropedicae HRC54 in the presence of apoplastic fluid*).

Results and discussion

Apoplastic fluid does not negatively affect the viability of *H. seropedicae* HRC54 cells

Confocal microscopy showed that the addition of sugarcane apoplastic fluid to the culture did not negatively affect the viability of *H. seropedicae* HRC54 cells (Fig. 1). However, the result observed in CFU counts was different; as such, the number of cells was not higher following the addition of apoplastic fluid (Fig. 2). Indeed, bacterial growth following the addition of water was higher than that after the addition of apoplastic fluid; however, this analysis was focused on the viability of HRC54 cell following exposure to sugarcane apoplastic fluid. In micrographs D, H, M, and Q, the bacterial morphology was not altered following the addition of sugarcane apoplastic fluid.

Transcriptomic analysis revealed 89 DEGs in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid

After raw data quality analysis, the trimmed reads were mapped against the *H. seropedicae* HRC54 genome, and the RPKM values were calculated (Table 1). Analyses using DESeq2 and edgeR with a *p* value of 0.05 revealed that the expression patterns of 1009 genes were altered. Using a cutoff of log2 fold change of ± 1.5, 89 genes were identified as differentially expressed under the experimental conditions, of which, respectively, 44 and 45 were upregulated and downregulated (Table 2). Of these DEGs, 18% encoded proteins or domains with unknown functions (DUFFs), 16.9% were related to oxidoreductase activity, and 13.5% were involved in transport and transmembrane transport (Tables S4 and S5). In addition, some DEGs encoded proteins related to signal transduction, transferase activity, secretion systems, cell division, and motility (Fig. 3). Finally, few DEGs encoded proteins related to chaperone, dioxygenase, isomerase, ligase, and translation activities ("Others" in Table 2).

Expression of *H. seropedicae* HRC54 genes involved in specific metabolic subsystems was altered in response to sugarcane apoplastic fluid

Oxidoreductase activity

Six downregulated and nine upregulated genes were involved in oxidoreductase activity at the early stages of plant–bacteria interactions, which is associated with response to oxidative stress (Knief et al. 2011; Imlay 2014). Among the six downregulated genes, four were involved in the cytochrome C enzymatic chain (*ccoP*, *coxB*, *coxA*, and *cox11*), which are likely required for bacterial adaptation to different oxygen concentrations (Kulajta et al. 2006). Similarly, in *H. seropedicae* SmR1 inoculated on wheat plants, genes from the cytochrome C enzymatic chain were suppressed after 3 days (Pankievicz et al. 2016). The remaining two downregulated genes encoded glutamate synthase subunits alpha and beta (*gltB* and *gltD*). Six of the nine upregulated genes were related to NADP or NADPH redox activity (*nfnB*, *yahK*, *Hs2057*, *Hs2266*, *Hs3119*, and *Hs3748*), and the remaining three encoded peroxiredoxin (*PRDX2I*), an amino acid dehydrogenase (*glud1_2*), and an aldo/keto reductase (*Hs2082*). *nfnB* encodes NAD(P+)H nitroreductase, which is involved in the metabolism of nitrogen-containing compounds. *yahK* encodes NAD(P+)-dependent alcohol dehydrogenase required for glycolysis. In addition, *Hs3748* encodes NADH dehydrogenase, which is involved in metabolic processes that generally require oxidoreductase activity. The *Hs3119* open reading frame (ORF) was annotated as a transhydrogenase and the *Hs2057* and *Hs2266* ORFs as NADP-dependent oxidoreductases, but no additional functional information was found. Previous experiments with *Paraburkholderia tropica* PPe8[T] revealed the augmentation of genes involved in the oxidoreductase pathway and suppression of genes in the cytochrome C enzymatic chain in response to sugarcane apoplastic fluid (Silva et al. 2018). The differential modulation of these genes could be related to the initial bacterial attempt of adaptation to the presence of sugarcane apoplastic fluid.

Transferases and hydrolases

In *H. seropedicae* HRC54, the expression patterns of nine genes related to transferase activity were altered in response to sugarcane apoplastic fluid (Fig. 3). This modulation of transferase/hydrolase activity may be required for the metabolic adaption and survival of bacteria under stress and may involve compounds necessary for beneficial plant–microbe interactions (Orellana et al. 2017). The *gst*, *puuE*, *tyrB*, *ndk*, *mdoH*, and *Hs799* ORFs were upregulated. *tyrB* encodes an aromatic amino acid transaminase, and *puuE* encodes 4-aminobutyrate aminotransferase,
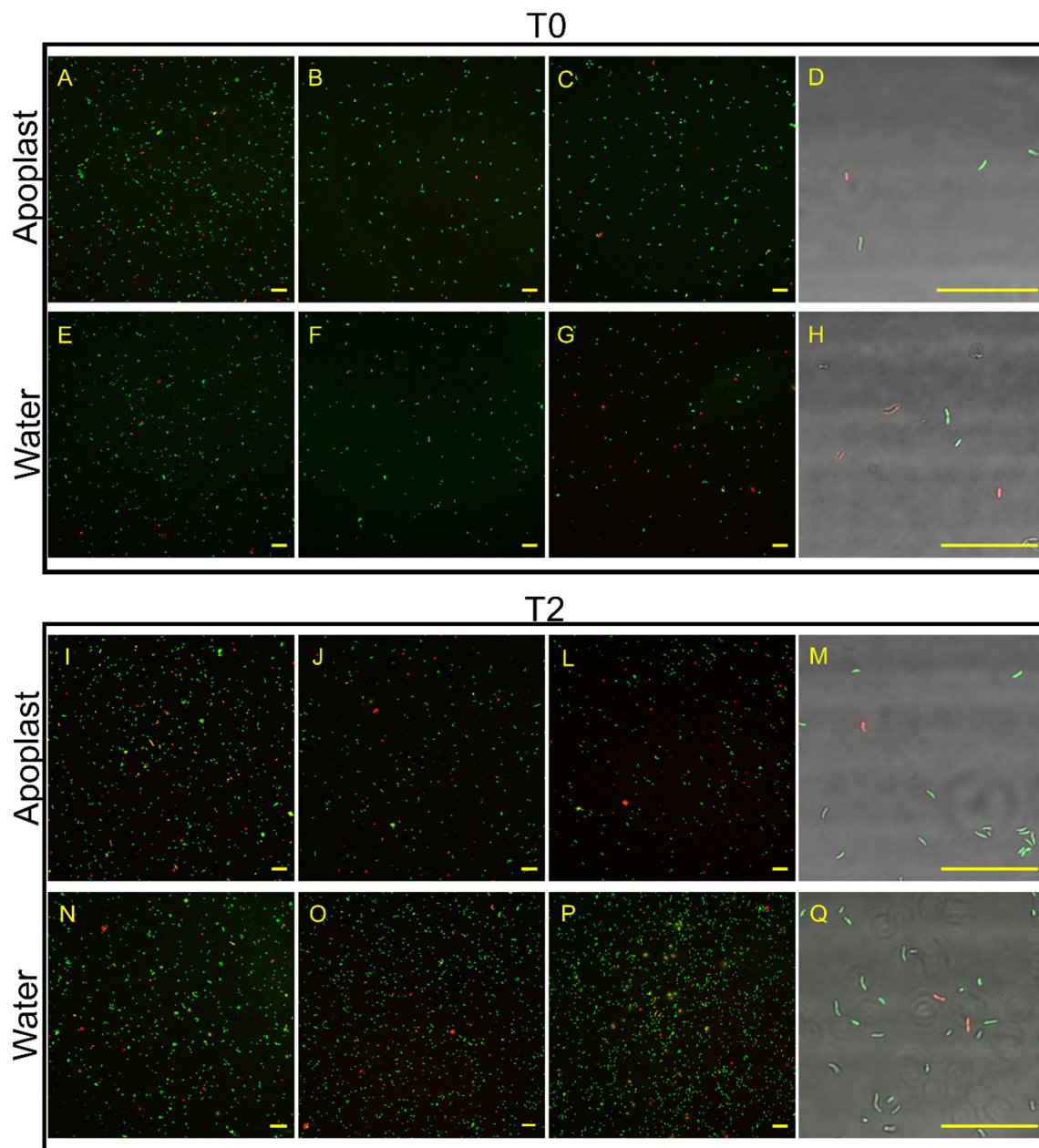
Fig. 1 Confocal microscopy of *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid using LIVE/DEAD BacLight Bacterial Viability Kits (Invitrogen™). Micrographs A, B, C, and D are from sample Apoplast; and micrographs E, F, G and H are from sample Water in the moment of the addition of the treatments (T0). Micrographs I, J, L, and M are from sample Apoplast; and micrographs N, O, P and Q are from sample Water after the two hours exposure of the treatments (T0). Red fluorescence represents dead cells and green fluorescence represents the live cells. Micrographs D, H, M and Q are using the $63\times$ lent, presenting the cell morphology. Scale bars represents 20 μm

both related to amino acid metabolism and nitrogen group transfer. *mdoH* encodes a membrane glycosyltransferase involved in the transfer and metabolism of carbohydrates. *ndK* encodes a nucleoside-diphosphate kinase, a transferase related to ATP binding, and *gst* encodes a protein similar to glutathione S-transferase, a transferase involved in the transfer of aryl and acyl groups in glutathione metabolism.

Glutathione S-transferase overexpression has been observed during *Enterobacter lignolyticus* growth in the presence of lignin (Orellana et al. 2017). The *Hs799* ORF encoded a protein similar to glutamine amidotransferase, but no additional functional information was found. The downregulated genes *thiD*, *selU*, *fadA*, and *tktA* were annotated as hydroxymethylpyrimidine/phosphomethylpyrimidine, tRNA
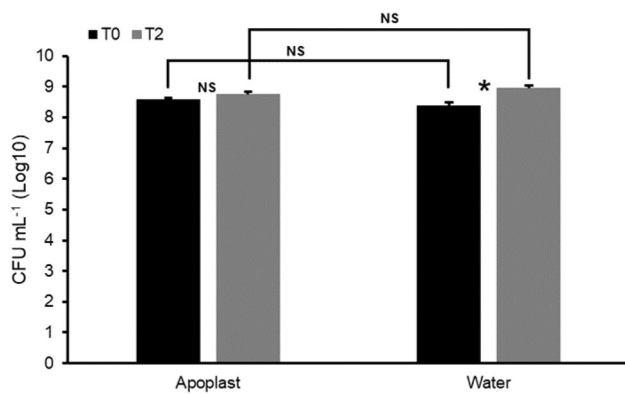
Fig. 2 Count of the UFC/mL from *H. seropedicae* HRC54 from the experiment with apoplastic fluid from sugarcane. Were evaluate for this assay, the moment from addition of the treatments (T0) and after 2 h of exposure (T2). The asterisks represent a significant difference in *t* test ($p < 0.05$), and NS represents a non-significant difference in *t* test ($p > 0.05$)

2-selenouridine synthase, acetyl-CoA C-acyltransferase, and transketolase, respectively. Acetyl-CoA C-acyltransferase suppression was observed in *Burkholderia kururiensis* exposed to rice plant extract (Coutinho et al. 2015). *thiD* is involved in thiamine metabolism, and *fadA* and *tktA* are involved in carbohydrate, specifically fatty acid and pentose phosphate, metabolism. *selU* is involved in transfers during RNA biogenesis. Three genes related to hydrolase activity were differentially expressed, and only *cbiG*, which encodes a cobalamin biosynthesis protein, was downregulated. *cbiG* acts on the carbon–carbon bonds and is involved in porphyrin and chlorophyll metabolism. The *ostB* and *Hs4740* ORFs were upregulated. *ostB* encodes trehalose 6-phosphate phosphatase, which is involved in starch and sucrose metabolism. The *Hs4740* ORF encoded a protein similar to glycoside hydrolase, which is also involved in carbohydrate metabolism, but no additional functional information was found. *H. seropedicae* SmR1 associated with maize has been reported to exhibit differential expression patterns of hydrolases (Balsanelli et al. 2016). Overall, the overexpression of genes related to transferase and hydrolase activities suggests that

Table 2 Total number of differentially expressed genes from *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid identified using DESeq2 and edgeR packages (with cut-off *p* value of 0.05 and log2 fold change of 1.5, in both packages) and their main functions

Function	Up-regulated	Down-regulated	Total genes
Oxireductase	9	6	15
Transferase	6	4	10
Hydrolase	2	1	3
Signal transduction	1	8	9
Transport	2	6	8
Transmembrane transport	3	1	4
Binding	1	3	4
Cell division	1	1	2
Motility	–	2	2
Secretion system	3	–	3
Chaperone	2	–	2
Hemagglutinin	–	2	2
Others	5	4	9
Unknown	9	7	16
Differentially expressed	44	45	89

the bacteria altered their gene expression pattern to adapt to and metabolize nutrients in sugarcane apoplastic fluid.

Signal transduction

Nine genes involved in signal transduction were differentially expressed, of which eight were downregulated and one was upregulated (Fig. 3). During plant–bacteria interactions, chemotaxis and signal transduction systems are key to other processes such as host adhesion and recognition (Falke et al. 1997; Batista et al. 2018). The upregulated gene *glrR* is a response regulator that enables bacteria to adapt and respond to the environment. The signal transduction genes *aer*; *mcp*; and the *Hs2330*, *Hs3454*, *Hs3539*, *Hs4087*, *Hs505*, and *Hs707* ORFs were downregulated. *mcp* encodes a methyl-accepting chemotaxis protein, which serves as an aerotaxis receptor. Similar expression patterns have been

Table 1 Reads mapped against the genome of *Herbaspirillum seropedicae* strain HRC54 using EDGE-pro software

| Sample	Raw data reads	Total reads after quality analysis	Total reads mapped against HRC54 genome	% Reads mapped against genome	% Unique reads mapped against genome
Apoplast + HRC54 1	4,660,273	3,201,458	2,354,893	79.07	5.51
Apoplast + HRC54 2	4,370,557	3,007,949	2,407,667	83.28	3.24
Apoplast + HRC54 3	7,181,611	6,032,121	5,261,784	88.72	1.49
Water + HRC54 1	2,801,113	1,339,224	1,085,216	86.98	5.94
Water + HRC54 2	3,618,072	1,895,847	1,390,060	77.66	4.34
Water + HRC54 3	4,364,421	3,128,588	2,676,803	87.78	2.23
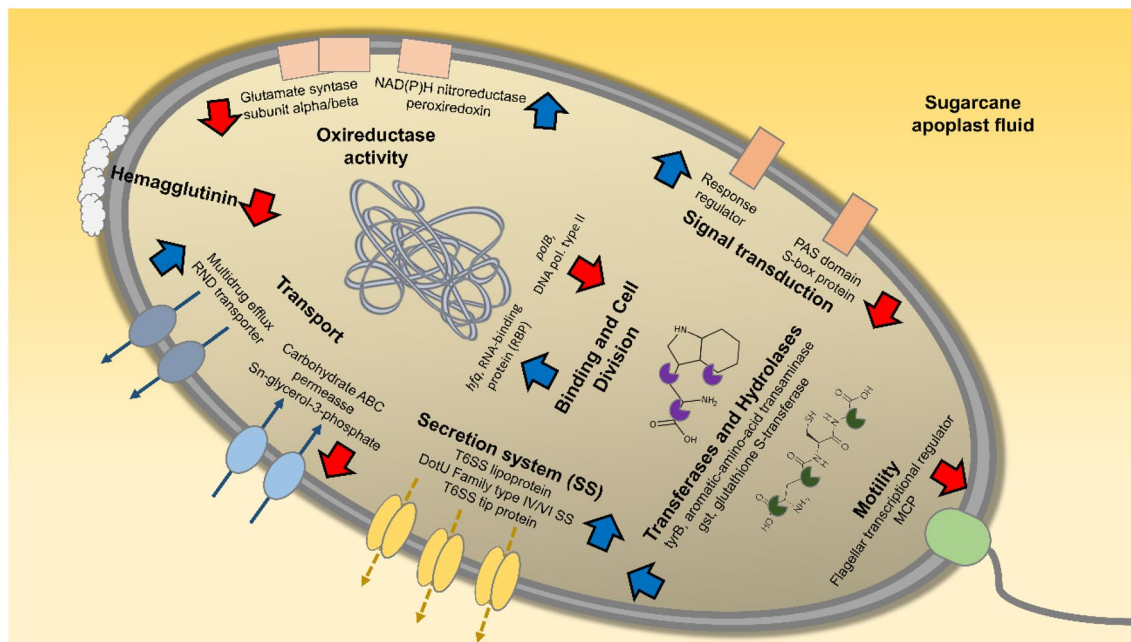
Fig. 3 Overview of differential expressed genes (DEGs) from *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid, highlighting predominant functions with up and down-regulation such as transport, oxidoreductase activity and signal transduction; and unique regulations as motility, secretion system and hemagglutinin. The blue arrows represent upregulation and red arrows represent down-regulation

reported during the growth of *H. seropedicae* SmR1 associated with maize and in the presence of the flavonoid naringenin (Tadra-Sfeir et al. 2015; Balsanelli et al. 2016) as well as in *Nitrospirillum amazonense* CBAmC in response to sugarcane apoplastic fluid (Terra et al. 2020). The *Hs3454*, *Hs3539*, *Hs2330*, and *Hs505* ORFs were also annotated as chemotaxis proteins, but no additional functional information was found. These DEGs involved in signal transduction may be related to bacterial adaptation, signaling, and recognition. However, the annotation of these genes was automatic and no specific function was found; therefore, additional experiments are warranted to elucidate their biological function and involvement in the interaction process.

Transport system

Several genes encoding sorbitol, mannitol, and branched-chain amino acids of the ABC transport system and saccharide transporters were suppressed. ABC transporters are extremely important for bacterial metabolism because of their function in nutrient uptake and exchange (Fatht and Kolter 1993). Several genes related to ABC transporters (Fig. 3), such as *smog* and *ugpC* involved in saccharide transport as well as *livH* and *livM* involved in amino acid transport, were suppressed. Similarity analysis did not identify the orthologous of the *Hs1900* OFR, but its location in the *H. seropedicae* HRC54 genome was very close to

that of the *Hs1901* and *Hs1902* ORFs (*livH* and *livM*), suggesting that *Hs1900* is related to the *liv* enzymatic chain. However, additional experiments are required to confirm this hypothesis. Similar expression profiles of genes related to transport systems were found in *H. seropedicae* SmR1 associated with wheat plants (Pankievicz et al. 2016) as well as in *P. tropica* PPe8$^{\mathrm{T}}$ and *N. amazonense* CBAmC in response to sugarcane apoplastic fluid (Silva et al. 2018; Terra et al. 2020). The *Hs80* ORF, annotated as a tripartite tricarboxylate transporter, was downregulated, but its function remains unknown. Only two genes involved in this pathway were upregulated in the presence of sugarcane apoplastic fluid. *ctpV* (copper transport) and the *Hs2747* ORF were annotated as ABC transporter substrate-binding proteins. Copper is involved in homeostatic processes that regulate plant defense mechanisms, allowing infection and colonization during interaction (Thiebaut et al. 2014). Suppression of these genes suggests that the bacteria specifically modulated carbohydrate metabolism, probably in response to sugars present in the apoplastic fluid.

Four genes related to transmembrane transport, a more specific pathway within the transport system, were differentially expressed in *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid. *acrA* and *acrB*, which are part of the multi-drug efflux system in Gram-negative bacteria, were upregulated. This family participates in the antimicrobial resistance pathway, specifically the surface adhesion chain.

The resistance nodulation and cell division family (RND) proteins are responsible for bacterial surface adhesion. Genes encoding these proteins were are also upregulated in *Azospirillum* sp. associated with wheat plants (Camilios-neto et al. 2014) and *Burkholderia kururiensis* exposed to rice plant extract (Coutinho et al. 2015). During competition with other bacteria in the presence of *Mimosa* plant extract, genes of the RND family were induced in *Burkholderia phymatum* (Klonowska et al. 2018). The upregulated expression of the RND family genes in different plant–bacteria interaction experiments highlights the importance of this family in the interaction process. The *Hs4729* ORF, a putative porin, was also upregulated. This protein is part of small exchange channels in the membrane and is involved in the membrane exchange and metabolism of poly-beta-hydroxybutyrate, an important compound in interaction (Tirapelle et al. 2013). The upregulation of this gene has also been reported in *H. seropedicae* SmR1 associated with wheat and maize (Balsanelli et al. 2016; Pankievicz et al. 2016). The bile acid gene *Hs3295*, a sodium transporter, was the only downregulated gene among those belonging to the transmembrane transport system. This gene may be related to photorespiratory metabolism in plants, but its function in bacteria remains unknown. Changes in the expression patterns of these genes highlight the importance of transport systems, which can help the bacteria to metabolize host-derived nutrients. However, further experiments are required to confirm this hypothesis.

Binding and cell division

Four genes related to binding were differentially expressed (Fig. 3). Meanwhile, *polB* and the *Hs2304* and *Hs2300* ORFs were downregulated. *polB* encodes a type II DNA polymerase responsible for DNA repair during DNA synthesis. The *Hs2304* and *Hs2300* ORFs were annotated as a DNA-binding response regulator and tetratricopeptide repeat protein, respectively. The DNA-binding response regulator is a part of the output domain of response regulators related to cellular processes, while the tetratricopeptide repeat protein is a motif with no specific function reported to date. *hfq*, an RNA-binding protein (RBP), was upregulated. RBPs are responsible for RNA regulation and metabolism and play crucial roles in cellular function, transport, and localization.

Regarding cell division, only one gene was upregulated and the others were downregulated. *pal*, a peptidoglycan-associated lipoprotein involved in bacterial survival under endophytic conditions (Godlewska et al. 2009), was upregulated. Although its role in virulence or interaction remains unclear, *pal* upregulation indicates the attempt of *H. seropedicae* HRC54 to identify sugarcane apoplastic fluid as a possible host plant signal. Cell division proteins are important during the initial stages of plant–bacteria interactions

(dos Santos et al. 2010; Knief et al. 2011). *ftsZ*, required for the formation of a contractile ring structure (Z ring) at the future cell division and bacterial multiplication site, was downregulated. The ring assembly regulates the timing and site of cell division. In contrast, upregulation of this protein was reported in *Gluconactobacter diazotrophicus* associated with sugarcane (Lery et al. 2011) and *Rhizobium tropici* in response to heat stress (Gomes et al. 2012). Additional experiments with prolonged exposure to sugarcane apoplastic fluid may provide more details of genes related to cell division and their roles in plant–bacteria interaction.

Motility and secretion system

The flagelin and flagellar transcription genes *fliC* and *flhC*, which are important primary genes in bacterial flagellar assembly, were downregulated. This suppression of the motility system can be related to the bacterial switch from free swimming to motile habit for attaching to the host surface. Regarding the secretion system, three genes were upregulated at only 2 h after the addition of apoplastic fluid. *Lip*, *dotU*, and *vgrG* are members of the type VI secretion system (T6SS), involved in bacterial symbiosis and other processes of the plant–bacteria interaction. T6SS comprises nine genes, of which one-third were upregulated in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid. The motility system is important for bacterial interaction and is typically related to chemotaxis (Pedrosa et al. 2011; Monteiro et al. 2012), which is an initial step during interaction (Pankievicz et al. 2016).

Suppression of the motility system-related genes has also been reported in *H. seropedicae* SmR1 associated with maize and wheat plants as well as in response to naringenin and sugarcane extract (Cordeiro et al. 2013; Tadra-Sfeir et al. 2015; Balsanelli et al. 2016; Pankievicz et al. 2016). Similar suppression of these genes has also been reported in other species such as *P. tropica* PPe8[T] and *N. amazonense* CBAmC in response to sugarcane apoplastic fluid (Silva et al. 2018; Terra et al. 2020). In the present experiment, *H. seropedicae* HRC54 genes related to flagelin and flagellar transcription were suppressed in response to sugarcane apoplastic fluid, suggesting that the bacteria recognized the fluid as part of the host plant and initiated changes in the motility system essential to the endophytic life.

T6SS is believed to be exclusive to pathogenic bacteria (Jani and Cotter 2010). Nevertheless, recent studies have shown the importance of this secretion system in competition with other bacteria and further successful interaction with plants (Filloux 2009; Jani and Cotter 2010). Interestingly, three members of the T6SS (*Lip*, *dotU*, and *vgrG*) were overexpressed in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid. T6SS was also upregulated in *B. kururiensis* in the presence of rice plant extract as well

as in *P. tropica* in response to sugarcane apoplastic fluid (Coutinho et al. 2015; Silva et al. 2018). These results highlight the importance of T6SS at the initial stages of beneficial plant–microbe interactions and the need for the comprehensive investigation of this system.

Hemagglutinins and chaperones

fhaB and the *Hs2067* ORF, which encode filamentous hemagglutinin N-terminal domain proteins, were downregulated. Hemagglutinins may be related to bacterial virulence, but more recent data suggest that in some Gram-negative bacteria, these proteins are involved in adhesion and surface attachment as well as biofilm formation—aspects that are often related to bacterial invasion; endophytic bacteria may use similar mechanisms for interaction with plants (Bernal et al. 2018). Genes related to the chaperone activity, including *trxA* (thioredoxin) and *hsIU* (ATP-dependent protease), were upregulated in *H. seropedicae* HRC54 in response to sugarcane apoplastic fluid (Fig. 3). ATP-dependent proteases are required for the degradation of specific intracellular molecules. Thioredoxin is part of the cellular antioxidant system, which is important for the protection of bacteria from oxidative damage caused by reactive oxygen species (ROS). With the addition of sugarcane apoplastic fluid, the bacteria were exposed to a "new" environment, with a high probability of the presence of ROS. However, identification of only one gene is not sufficient to support this hypothesis.

Genes encoding hemagglutinin are relevant to plant–microbe interactions. Such genes have been identified in the genome of *H. seropedicae* SmR1 (Pedrosa et al. 2011). In the present experiment, two genes encoding hemagglutinin (*fhaB* and *Hs2067*) were suppressed in *H. seropedicae* HRC54 in response to apoplastic fluid. Although hemagglutinin has generally been believed to be related to the virulence of pathogenic bacteria, more recent data have shown its importance in surface attachment and biofilm formation (Ariyakumar and Nishiguchi 2009; Gottig et al. 2009; Bernal et al. 2018; Taulé et al. 2021). Interestingly, in the present experiment, genes related to another system believed to be exclusive to pathogenic bacteria (T6SS) were also differently expressed in HRC54 in response to sugarcane apoplastic fluid. Perhaps, these systems are related to plant–bacteria interactions and warrant further exploration.

Several ORFs annotated with general functions such as lyase activity, transcription, and protein activation in bacterial metabolism, including *Hs245*, *Hs947*, and *Hs430*, were downregulated. *trpS*, *bpeT*, *pmm-pgm*, *RP-L31*, and the *Hs4167* ORF were upregulated in response to sugarcane apoplastic fluid. *trpS* and *RP-L31* are related to the translation and metabolism of tryptophan, which is an important amino acid for bacterial growth (Glick 2015). *pmm-pgm* is a phosphomannomutase/phosphoglucomutase, which is

related to glycolysis and involved in virulence factor synthesis in *Pseudomonas aeruginosa* (Regni et al. 2006). The *Hs4167* ORF was annotated as a Tim44 domain-containing protein, which is responsible for the translocation of nuclear-encoded proteins across the mitochondrial inner membrane. These processes could be relevant to plant–bacteria interaction process and warrant further research.

Unknown and other functions

Several metabolic functions were affected by at least one of the altered genes (Fig. 3). *Hs38*, a putative membrane protein; *Hs245*, a glyoxalase/bleomycin resistance/extradiol dioxygenase; *Hs430*, a class I SAM-dependent methyltransferase; and *Hs947*, related to the TetR family transcriptional regulators, were downregulated. Meanwhile, *trpS*, *bpeT*, *pmm-pgm*, *RP-L31*, and the *Hs4167* ORF were upregulated. *TrpS* is a tryptophan–tRNA ligase and *RP-L31* is a type B 50S ribosomal protein, both related to translation. They are also members of the LysR transcriptional regulator family, a diverse family of genes related to virulence, motility, and quorum sensing. Similarity analysis showed that some DEGs were annotated as unknown functions in the COG and KEGG databases (Table S1). However, based on the position of their ORFs in the *H. seropedicae* HRC54 genome, it is reasonable to assume that they serve some specific functions hitherto unknown. For instance, the *Hs3922* and *Hs3926* ORFs were identified as proteins with domains of unknown function (DUFFs), whereas *Hs3924* and *Hs3925* are related to cytochrome C oxidase. Nevertheless, more detailed studies are warranted to confirm this hypothesis. Such domains or putative proteins in *H. seropedicae* strains have not been previously reported; therefore, future comprehensive studies of some of these differentially expressed proteins are essential to identify their functions at the initial stages of plant–bacteria interactions.

Expression patterns of 12 genes were validated with RT-qPCR

To confirm the expression patterns of *H. seropedicae* HRC54 genes in response to sugarcane apoplastic fluid, 12 genes were randomly selected for validation using RT-qPCR, of which 6 each were upregulated and downregulated (Table S3). The upregulated genes showed higher relative expression levels and the downregulated genes showed lower relative expression levels in samples exposed to sugarcane apoplastic fluid. The expression patterns of 9 of the 12 genes were confirmed by the assay (Fig. 4). The *Hs4308* ORF showed a lower relative expression level in samples exposed to sugarcane apoplastic fluid, which was contradictory to the results of RNA-Seq analysis, in which this gene was upregulated. Similarly,
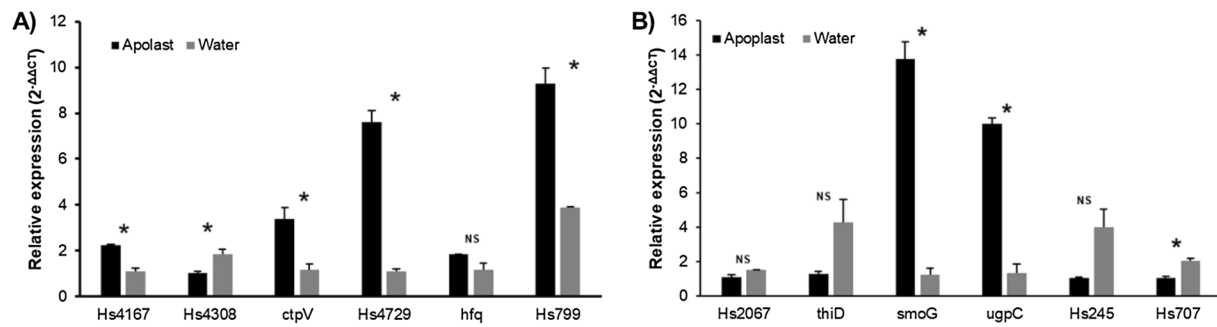
Fig. 4 Relative expression of genes from *H. seropedicae* HRC54 in the presence of sugarcane apoplastic fluid using RT-qPCR. Validation was performed using up (A) and downregulated genes (B). The expression level was calculated by qBASE software using Delta CT method ($2^{-\Delta\Delta CT}$). The symbols represents a significant difference in *t* test ($p < 0.05$), and NS represents a non-significant difference in *t* test ($p > 0.05$)

smoG and *ugpC* showed higher relative expression levels in samples exposed to sugarcane apoplastic fluid, which was contradictory to the results of transcriptomic analysis (Fig. 4). This small variation in the results can be attributed to the fact that RT-qPCR is a more sensitive method. However, given that the expression patterns of majority of the genes were confirmed by the RT-qPCR assay, our transcriptomic data derived from in silico analyses can be considered reliable.

Conclusions

DEGs explored in the present study constitute the early response of *H. seropedicae* HRC54 to compounds present in the apoplastic fluid of the sugarcane variety RB867515. The transcriptomic profile of *H. seropedicae* HRC54 during 2 h in the presence of sugarcane apoplast fluid showed the modulation of genes related to carbohydrate and amino acid metabolism, motility, secretion, oxidoreductase activity, and signal transduction. This expression pattern is consistent with bacterial response to host plant signals and adaptation to and survival under endophytic life conditions, suggesting that the bacteria recognized sugarcane apoplast fluid as the host plant environment. Additionally, in *H. seropedicae* HRC54, metabolism was altered to use compounds in the apoplastic fluid as a nutrient source and genes related to motility, secretion, transferase activity, and signal transduction necessary to establish interaction with the host plant were modulated. Furthermore, many putative proteins or proteins with DUFFs were differentially expressed in the transcriptomic data. Therefore, additional experiments are warranted to determine the importance of such proteins in the sugarcane–*H. seropedicae* interaction.

Accession numbers

The raw nucleotide sequences data for the six libraries were deposited in the National Center for Biotechnology Information (NCBI), in Sequence Read Archive (Leinonen et al. 2011) and are available under the BioProject accession number PRJNA694965 and BioSamples accession numbers SAMN17587759, SAMN17587760, SAMN17587761, SAMN17587762, SAMN17587763, SAMN17587764.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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